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Abdeslem El Idrissi
William J. L'Amoreaux *Editors*

Taurine 8

Volume 2: Nutrition and Metabolism, Protective Role, and Role in Reproduction, Development, and Differentiation

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Volume 2: Nutrition and Metabolism, Protective Role, and Role in Reproduction, Development, and Differentiation

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Editors
Abdeslem El Idrissi
City University of New York
Center for Developmental Neuroscience
College of Staten Island
Staten Island, NY, USA

William J. L'Amoreaux
City University of New York
Department of Biology
College of Staten Island
Staten Island, NY, USA

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Preface

The organizing committee wishes to thank all attendees of the 18th International Taurine Meeting that took place in Marrakesh, Morocco, from April 7th to 13th. This year, the conference highlighted the "*Mystique of Taurine*." Taurine investigators have had the privilege of attending these scientific meetings on three continents: Asia, Europe, and North America. This marked the first time that our conference was held in Africa. As a result, we present here the data from investigators from five of the six continents (sadly taurine research has yet to hit Antarctica). With this geographical expansion, the interest in taurine research has exponentially grown. This international meeting was attended by approximately 120 scientists. We present here information on the roles of taurine in a variety of organ systems, from the brain to the reproductive system and every system in between. As you are keenly aware, there is certainly a mystique to taurine. Is it beneficial or harmful? Does it protect cells or induce cell death? Can it be used in conjunction with another molecule to benefit health or cause death? The answer (or at least a hint to the answer) to these and other questions lies within this body of works. Of course, not all questions were answered but there were many discussions that generated numerous new ideas that will be taken home and tested in the laboratory.

This meeting was also unique in that many undergraduate and graduate students from the College of Staten Island/CUNY attended and presented their research as part of a study abroad program. This opportunity represented the first time that most of these students attended an international conference. More importantly, it served to stimulate interest in taurine research and recruit future taurine researchers. We are greatly appreciative for the overwhelming support of the College of Staten Island's administration, particularly Dr. Deborah Vess, Associate Provost for Undergraduate Studies and Academic Programs; Dr. William Fritz, the provost; Renee Cassidy, study abroad advisory from The Center for International Service;

Debra Evans-Greene, Director of the Office of Access and Success Programs; and Dr. Claude Braithwaite of the City College of New York and the Louis Stokes Alliance for Minority Participation.

The abstracts of the conference were published in the journal "Amino Acids" (Vol. 42, Issue 4). We thank Drs. Lubec and Panuschka for making this possible.

Because of the success of this meeting, the organizing committee wishes to gratefully acknowledge the following:

- Taisho Pharmaceutical Co., Ltd., Tokyo Japan for their generous financial support.
- Professor Dr. Gert Lubec, FRSC (UK), Medical University of Vienna and Editor in Chief of AMINO ACIDS.
- Dr. Claudia Panuschka, Springer Wien, New York, Senior Editor Biomedicine/ Life Sciences.
- Dr. Portia E. Formento, Editor, Biomedicine, Springer US.
- Dr. Melanie Tucker (Wilichinsky) Editor, Genetics and Systems Biology, Springer US.

On behalf of the organizing committee, I thank all the attendees of the 18th international Taurine Meeting and the sponsors that made this meeting possible.

Staten Island, NY, USA Abdeslem El Idrissi

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Part I
Taurine in Nutrition and Metabolism

Chapter 1
Taurine, Glutathione and Bioenergetics

Svend Høime Hansen and Niels Grunnet

Abstract Biochemistry textbook presentations of bioenergetics and mitochondrial function normally focus on the chemiosmotic theory with introduction of the tricarboxylic acid cycle and the electron transport chain, the proton and electrical gradients and subsequent oxidative phosphorylation and ATP-production by ATP synthase. The compound glutathione (GSH) is often mentioned in relation to mitochondrial function, primarily for a role as redox scavenger. Here we argue that its role as redox pair with oxidised glutathione (GSSG) is pivotal with regard to controlling the electrical or redox gradient across the mitochondrial inner-membrane. The very high concentration of taurine in oxidative tissue has recently led to discussions on the role of taurine in the mitochondria, e.g. with taurine acting as a pH buffer in the mitochondrial matrix. A very important consequence of the slightly alkaline pH is the fact that the NADH/NAD^+ redox pair can be brought in redox equilibrium with the GSH redox pair GSH/GSSG.

An additional consequence of having GSH as redox buffer is the fact that from the pH dependence of its redox potential, it becomes possible to explain that the mitochondrial membrane potential has been observed to be independent of the matrix pH. Finally a simplified model for mitochondrial oxidation is presented with introduction of GSH as redox buffer to stabilise the electrical gradient, and taurine as pH buffer stabilising the pH gradient, but simultaneously establishing the equilibrium between the NADH/NAD^+ redox pair and the redox buffer pair GSH/GSSG.

S.H. Hansen (✉)
Department of Clinical Biochemistry, 3-01-1, Rigshospitalet,
Copenhagen University Hospital, København, Denmark
e-mail: shhansen@rh.dk

N. Grunnet
Department of Biomedical Sciences, University of Copenhagen,
København, Denmark

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Abbreviations

GSH	Glutathione
GSSG	Oxidised glutathione
ROS	Reactive oxygen species

1.1 Introduction

The chemiosmotic theory proposed by Peter Mitchell in the 1960s is today accepted as the basis for the understanding of the oxidative phosphorylation and subsequent ATP production in the bioenergetic processes in the mitochondria (Mitchell 1966, 1968; Nicholls and Ferguson 2002). The presentation in most biochemical textbooks focuses on the pH and the electrical gradients across the mitochondrial membranes. The gradients combine to form an electrical potential ΔE_{Total} for moving protons across the inner-membrane:

$$\Delta E_{\text{Total}} = \Delta\Psi - \log(10)\frac{\text{RT}}{\text{F}}\Delta\text{pH}. \tag{1.1}$$

This potential, often referred to as the proton-motive force, drives by use of proton movement the ATP production through the ATP synthase protein complex localised in the mitochondrial inner-membrane.

A series of arguments based on experimental observation can be given that the pH in the cytosol is about 7.0–7.4, and in the mitochondrial matrix pH is most likely in the range 7.8–8.5. In order to stabilise the ATP production, it seems evident that localisation of a pH buffer in the mitochondrial matrix is necessary (Hansen et al. 2010).

Furthermore, it is generally accepted that the proton-motive force can be considered as constant about 200 mV. It is generally accepted that no appreciable dependence on the matrix pH is observed [e.g. Fig. 4.5 in Bioenergetics 3 (Nicholls and Ferguson 2002)]. However, such constancy of two apparently independent contributions needs explanation from a theoretical argument (see later in Sect. 1.2.6 and Fig. 1.1).

1.2 Mitochondria: pH and Redox Buffering

1.2.1 Taurine: pH Buffer

Taurine has previously (Hansen et al. 2006, 2010) been presented as a compound that possesses the optimal characteristics to be a pH buffer in the mitochondrial matrix. Taurine is found ubiquitously in animal tissue with concentrations in the millimolar range. Notably high concentrations of taurine in oxidative tissue lead to

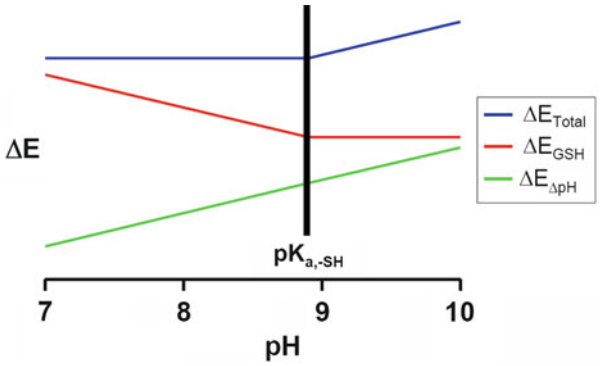


Fig. 1.1 Expected pH dependence of the total mitochondrial membrane potential ΔE_{Total} based on the assumption that glutathione controls the contribution from the electrical (or redox) gradient as the term ΔE_{GSH}. The ionisation constant for the thiol group in glutathione (see Table 1.3) is indicated with the vertical bar. Notice that the pH dependence of the GSH redox potential balances the potential contribution $\Delta E_{\Delta pH}$ from the pH difference, when pH is below the glutathione thiol ionisation constant $pK_{a,-SH}$

a compartmental argument on mitochondrial localisation (Hansen et al. 2006), which has later been confirmed by analytical determinations of taurine in isolated mitochondria (Jong et al. 2010). Ideally, the taurine concentration should be determined inside the mitochondrial matrix, but such analytical methods are currently not available.

Slightly alkaline pH is an ideal environment for beta-oxidation of fatty acids, and as the oxidation is very pH dependent, the existence of a pH buffer seems necessary to stabilise the oxidation (Hansen et al. 2010).

Furthermore, the original presentations of the chemiosmotic theory by Mitchell clearly emphasise the importance of the mitochondrial buffering capacities. The presence of a low-molecular-mass pH buffer in the matrix will thus increase the energy storage capacity (Mitchell 1966, 1968; Mitchell and Moyle 1967). The mitochondrial matrix-buffering should be remembered when evaluating mitochondrial function and dysfunction.

1.2.2 Redox Pairs NADH/NAD⁺, NADPH/NADP⁺, GSH/GSSG

Traditionally, when presenting the mitochondrial redox systems the major focus is given to the redox pair NADH/NAD $^{+}$, probably as a consequence of the fact that NADH is the product of the tricarboxylic acid cycle and subsequent substrate of the electron transport chain complexes. In addition, the NADPH/NADP⁺ is traditionally presented, as NADPH is a product of matrix-localised oxidation.

Although NADH/NAD⁺ and NADPH/NADP⁺ without any doubt are directly involved in matrix-localised oxidation, the concentration of these compounds is not

sufficient to control the redox potential of the redox environment in the mitochondrial matrix. A detailed discussion on the redox environment of the cell has been reviewed (Schafer and Buettner 2001) with focus on the glutathione (GSH) and thioredoxin systems. In order to control the redox potential, it is concluded that in the mitochondrial matrix the only redox couple of compounds found in sufficient amount is the GSH/oxidised glutathione (GSSG) couple. Besides, when discussing oxidative stress and redox state the GSH system is accepted as the most important system (Jones 2006, Jones and Go 2010).

However, these three sets of redox pairs are interconnected through two simple enzyme pathways as follows:

(a) Transhydrogenase can interconvert the two sets of nicotinamide nucleotides:

$$\mathrm{NADP^+ + NADH \rightleftharpoons NADPH + NAD^+}.$$

(b) GSH reductase can subsequently convert NADPH to GSH:

$$\mathrm{NADPH + H^+ + GSSG \rightleftharpoons NADP^+ + 2GSH}.$$

1.2.3 pH Dependence of Redox Potentials

The redox potentials for the redox pairs can now be compared in order to evaluate the related equilibria for the redox pairs.

The redox equations for the redox pairs are as follows:

$$\mathrm{NAD^+ + H^+ + 2e^- \rightleftharpoons NADH}$$

$$\mathrm{NADP^+ + H^+ + 2e^- \rightleftharpoons NADPH}$$

$$\mathrm{GSSG + 2H^+ + 2e^- \rightleftharpoons 2\,GSH}.$$

As all three equations involve protons, the associated redox potentials become pH dependent according to the Nernst equations as follows:

$$E = E^0 - \frac{60\ \mathrm{mV}}{2} \log\left(\frac{[\mathrm{GSH}]^2}{[\mathrm{GSSG}]}\right) - 60\ \mathrm{mV}\cdot\mathrm{pH} \tag{1.2}$$

$$E = E^0 - \frac{60\ \mathrm{mV}}{2} \log\left(\frac{[\mathrm{NADH}]}{[\mathrm{NAD^+}]}\right) - \frac{60\ \mathrm{mV}}{2}\cdot\mathrm{pH} \tag{1.3}$$

$$E = E^0 - \frac{60\ \mathrm{mV}}{2} \log\left(\frac{[\mathrm{NADPH}]}{[\mathrm{NADP^+}]}\right) - \frac{60\ \mathrm{mV}}{2}\cdot\mathrm{pH}. \tag{1.4}$$

Table 1.1 Approximate midpoint redox potentials $E_{m,pH}$ (mV) at different pH values

Redox pair	pH = 7.0	pH = 7.5	pH = 8.0	pH = 8.5
NADH/NAD$^+$	−320	−335	−350	−365
NADPH/NADP$^+$	−320	−335	−350	−365
GSH/GSSG	−172	−202	−232	−262

Based directly on the values given in Table 3.2 in Bioenergetics 3 (Nicholls and Ferguson 2002). In their calculations the factor 2.303 RT/F from the Nernst equation was set to 60 mV (corresponding to $T = 302$ K or 29.2°C)

Please notice that in all the equations and calculations the factor log(10) RT/F from the Nernst equation has been set to 60 mV as in Bioenergetics 3 (Nicholls and Ferguson 2002). This approximation corresponds to a temperature of $T = 302$ K or 29.2°C.

The redox potentials for the redox pairs are pH dependent, but the Nernst equations show that $\Delta E/\mathrm{pH} \approx -60$ mV/pH for GSH/GSSG, but NADH/NAD$^+$ and NADPH/NADP$^+$ has only a dependence of $\Delta E/\mathrm{pH} \approx -30$ mV/pH.

The thiol group of GSH becomes deprotonated in alkaline pH above the pK_a for the thiol group, and thus leading to a simplified redox equilibrium

$$\mathrm{GSSG} + 2\mathrm{e}^- \rightleftharpoons 2\,\mathrm{GS}^-$$

and an associated Nernst equation without any pH dependence as follows:

$$E = E^0 - \frac{60\ \mathrm{mV}}{2} \log\left(\frac{[\mathrm{GS}^-]^2}{[\mathrm{GSSG}]}\right). \tag{1.5}$$

The p$K_a = 9.0$ (25°C) for GSH can be found in Table 1.3, and a more detailed discussion can be found elsewhere, e.g. (Schafer and Buettner 2001). The pH dependence for the GSH redox potential is indicated as the ΔE_{GSH} curve in Fig. 1.1.

1.2.4 Redox Equilibrium NADH/NAD$^+$ and GSH/GSSG

From the Nernst equations above, the midpoint potentials can be calculated at relevant pH values in the range from 7.0 to 8.5 as presented in Table 1.1.

However, the midpoint potentials do not take into account the actual concentrations of the individual species. A better representation is obtained by the use of typical values for the redox pair concentrations and GSH pool concentration of 10 mM (Nicholls and Ferguson 2002; Wahllander et al. 1979). If these actual concentrations found in the mitochondrial matrix are inserted into Nernst's equation, the actual redox potentials as presented in Table 1.2 and Fig. 1.2 are obtained.

A surprising consequence when increasing the pH to slightly alkaline conditions at about pH 8.5 is now the result that the actual redox potentials for the NADH/NAD$^+$

Table 1.2 Approximate actual redox point potentials $E_{actual,pH}$ (mV) at different pH values

Redox pair	Typical ox/red ratio	pH = 7.0	pH = 7.5	pH = 8.0	pH = 8.5
NADH/NAD$^+$	10	−290	−305	−320	−335
NADPH/NADP$^+$	0.01	−380	−395	−410	−425
GSH/GSSG(when 10 mM)	0.01	−240	−270	−300	−330

Based directly on the values given in Table 3.2 in Bioenergetics 3 (Nicholls and Ferguson 2002). In their calculations the factor 2.303 RT/F from the Nernst equation was set to 60 mV (corresponding to $T = 302$ K or 29.2°C)

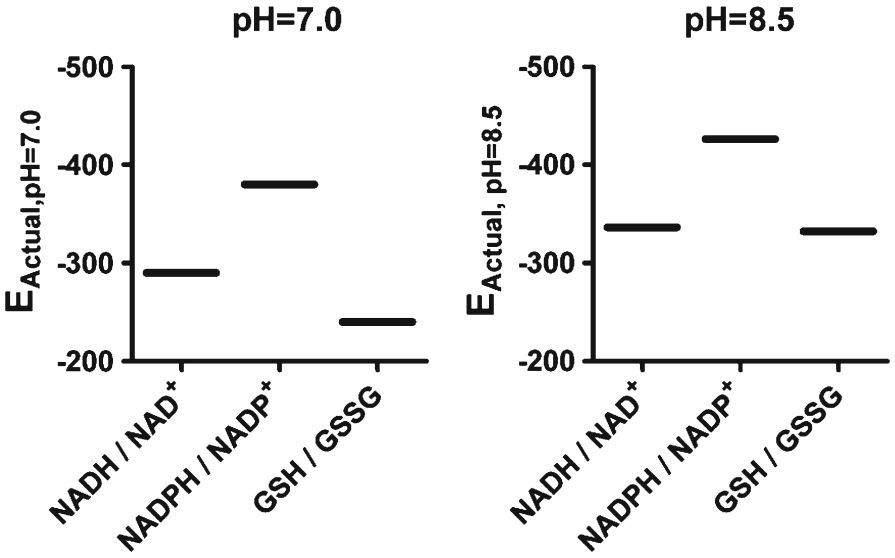
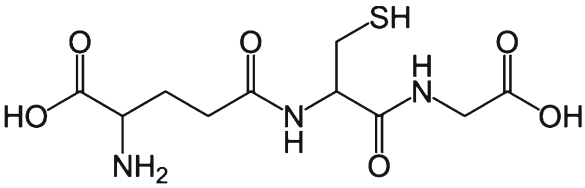
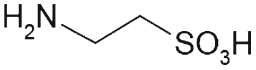


Fig. 1.2 Estimation of actual redox potentials $E_{actual,pH}$ (mV) for the redox pairs NADH/NAD$^+$, NADPH/NADP$^+$, and GSH/GSSG at pH = 7.0 and pH = 8.5, respectively. The concentrations of the compounds are based on the values given in Table 3.2 in Bioenergetics 3 (Nicholls and Ferguson 2002). At the slightly alkaline pH = 8.5, it is possible to obtain redox equilibrium between NADH/NAD$^+$ and GSH/GSSG

and the GSH/GSSG redox pairs become almost identical. That is in the pH range of 8.0–8.5, where taurine acts as a pH buffer, it is possible to obtain thermodynamic equilibrium between the NADH/NAD$^+$ and GSH/GSSG, whereas NADPH/NADP$^+$ acts as a sort of cofactor for the involved enzymes! Consequently, taurine can suddenly be given a very important supporting role to GSH in the mitochondrial matrix.

In a recent advanced theoretical and numerical calculation model for mitochondrial energetics only the NADH/NAD$^+$-redox couple was included (Wei et al. 2011), although the GSH/GSSG was included in a previous unifying hypothesis focusing on reactive oxygen species (ROS) and oxidative stress (Aon et al. 2010). Now, it seems that taurine also should be included in such calculation models due to its contribution to the mitochondrial pH buffering capacity.

Table 1.3 Chemical structures and ionisation constants for glutathione and taurine

Compound	Ionisation constants (25°C)
 Glutathione (L-γ-glutamyl-L-cysteinylglycine)	$pK_a(-NH_3^+) \approx 9.2$ $pK_a(-SH) \approx 9.0$ The values depend on the protonation form of glutathione. (See Table 1.1 in (Rabenstein 1973) for further details)
 Taurine (2-amino-ethane sulfonic acid)	$pK_a(-NH_3^+) = 9.0$ (Hansen et al. 2010)

1.2.5 Glutathione: Redox Buffer

Not only protons but also electrons need to be buffered during the metabolic processes in the mitochondria. The oxidative processes obviously involve electron transfers, requiring the redox buffers to provide the available electrons and thus ensure a stable redox environment. An excellent background presentation on the concept of cellular redox environments and redox buffer can be found elsewhere (Schafer and Buettner 2001).

In order to obtain redox buffering in a biochemical environment, thiol groups need to be involved through redox equilibria with disulphide formation. In general, available thiol groups for redox balanced are normally considered to be found in either thioredoxin proteins or in the tripeptide GSH (L-γ-glutamyl-L-cysteinylglycine), which is found in millimolar concentrations in the cytosol and mitochondria (Schafer and Buettner 2001; Wahllander et al. 1979). The thiol group in GSH accounts for the action as redox buffer of GSH. However, other proteins could be involved in the redox buffering, as a recent determination of exposed thiol groups in the mitochondrial matrix found an unexpected high concentration of free protein thiol groups (Requejo et al. 2010).

Focusing on the role of GSH as redox buffer (or actually any thiol group), it must be remembered that the redox potential of a thiol group is strongly pH dependent, as the thiol group has to be considered as a weak acid. However, GSH also contains an amino group (see Table 1.3). Actually, the ionisation constants, pK_a, for the amino and thiol groups in GSH have been reported to be similar, but slightly higher for the amino group as shown in Table 1.3 (Rabenstein 1973). Comparing with the ionisation constant of the amino group of taurine (Hansen et al. 2006, 2010), this value seems to be almost identical with the ionisation constant of the thiol group in GSH (see Table 1.3).

1.2.6 Controlling the Mitochondrial Inner-Membrane Potential

When accepting the hypothesis that GSH (or perhaps thiol groups in general) acts as redox buffer for the mitochondrial matrix it also means that the thiol/disulphide redox equilibrium is responsible for the electrical redox gradient across the mitochondrial inner-membrane corresponding to a potential difference ΔE_{GSH}. If it is also assumed that the redox potential in the cytosol can be considered as constant E_0, the electrical gradient contribution in equation (1.1) can be expressed as $\Delta\Psi = \Delta E_{GSH} + E_0$. When adding a potential contribution $\Delta E_{\Delta pH}$ from the pH difference, it means that the potential difference ΔE_{Total} (or proton-motive force) across the mitochondrial inner-membrane can be expressed as follows:

$$\Delta E_{Total} = \Delta E_{GSH} + \Delta E_{\Delta pH} + E_0. \tag{1.6}$$

As shown in Fig. 1.1 this equation makes it possible to explain that the mitochondrial membrane potential has been reported to be independent on the matrix pH (see Fig. 4.5 in Bioenergetics 3, Nicholls and Ferguson 2002; Nicholls 1974). The higher concentration of taurine in mitochondria means that taurine acts as the primary pH buffer in the matrix, and the thiol group in GSH becomes protected from deprotonation and to focus on being a redox buffer in the oxidative matrix environment. With taurine as pH buffer, the matrix pH must be expected to be below the ionisation constant for the thiol group in GSH. Consequently, the mitochondrial membrane potential will be kept almost constant (see Fig. 1.1).

1.3 Model for Mitochondrial Bioenergetics

A simplified model for mitochondrial bioenergetics can now be presented as in Fig. 1.3. The basic substrate acetyl-CoA is provided either from pyruvate oxidation by pyruvate dehydrogenase or from beta-oxidation of fatty acids. Subsequently, acetyl-CoA is oxidised to CO_2 with the reduction of NAD^+ to NADH by the tricarboxylic acid cycle. NADH is used by the electron transport chain to pump protons and thus creating a mitochondrial inner-membrane proton gradient. Besides the proton pumping, NADH is also used for setting up the GSH redox equilibrium. Several of the processes are stabilised through pH buffering by taurine.

1.4 Perspectives and Future Developments

The presented model for the mitochondrial function needs obviously to be extended with incorporation of the complexes from the electron transport chain, and with the formation of free radicals and ROS, which are well-known by-products from the electron transport chain. However, although such an extension of the model is in progress, it is by no means easily formulated. An initial observation to be included

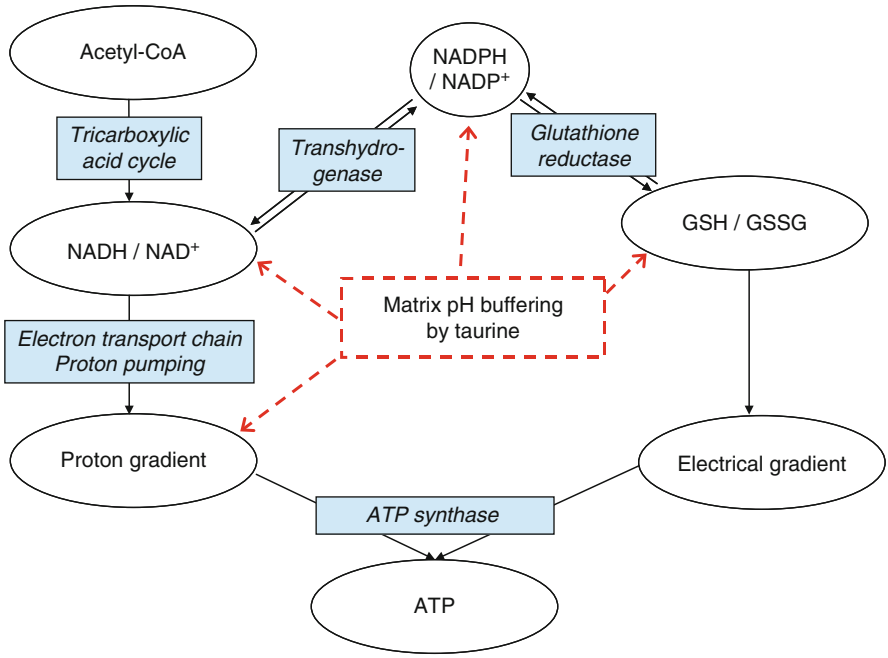


Fig. 1.3 Simplified model for mitochondrial function. The proton gradient is built up by the tricarboxylic acid cycle and subsequent electron transport chain. Simultaneously, the electrical gradient is established through a redox equilibrium between NADH/NAD$^+$ and the redox buffer GSH/GSSG. Several of these processes are stabilised by taurine as matrix pH buffer. The proton and electrical gradients drive together the ATP production by ATP synthase and associated proton movement

is the pH dependence of free radical and ROS formation (Selivanov et al. 2008). But also the roles of mitochondrial thiols in antioxidant scavenging and redox signalling have to be dealt with (Murphy 2012).

Further perspectives on mitochondrial function could be based on the increased reactivity of thiols and GSH with increasing pH. Consequently, the reported opening of mitochondrial ion channels or opening of the uncoupling proteins (UCP) due to GSH redox status or glutathionylation (Aon et al. 2007; Slodzinski et al. 2008; Mailloux et al. 2011) could be a direct response to increased pH in the matrix. That is the increased tendency to glutathionylation protects the matrix from excessive alkalinisation by opening the UCPs for incoming protons.

The consequence of taurine depletion on mitochondrial function has been discussed elsewhere (Hansen et al. 2010; Jong et al. 2012), as well as the results of GSH deficiency or depletion (Meister 1995; Aon et al. 2007). However, the elements in the presented model with focus on the interplay between taurine and GSH need to be included when analysing mitochondrial function and dysfunction, e.g. in diabetes (Hansen 2001; Hansen et al. 2010). Currently, the two compounds are hardly introduced in even advanced presentations or calculation models of mitochondrial function and bioenergetics.

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Chapter 2
Molybdenum Cofactor Deficiency: Metabolic Link Between Taurine and S-Sulfocysteine

Abdel Ali Belaidi and Guenter Schwarz

Abstract Molybdenum cofactor deficiency (MoCD) is a rare inherited metabolic disorder characterized by severe and progressive neurologic damage mainly caused by the loss of sulfite oxidase activity. Elevated urinary levels of sulfite, thiosulfate, and S-sulfocysteine (SSC) are hallmarks in the diagnosis of both MoCD and sulfite oxidase deficiency. Sulfite is generated throughout the catabolism of sulfur-containing amino acids cysteine and methionine. Accumulated sulfite reacts with cystine, thus leading to the formation of SSC, a glutamate analogue, which is assumed to cause *N*-methyl-D-aspartate receptor-mediated neurodegeneration in MoCD patients. Recently, we described a fast and sensitive HPLC method for diagnostic and treatment monitoring of MoCD patients based on SSC quantification. In this study, we extend the HPLC method to the analysis of hypotaurine and taurine in urine samples and no interference with other compounds was found. Besides the known elevation of SSC and taurine, also hypotaurine shows strong accumulation in MoCD patients, for which the molecular basis is not understood. SSC, hypotaurine, and taurine urinary excretion values from control individuals as well as MoCD patients are reported and over 20-fold increase in taurine urinary excretion was determined for MoCD patients demonstrating a direct link between sulfite toxicity and taurine biosynthesis in MoCD.

Abbreviations

Moco	Molybdenum cofactor
MoCD	Molybdenum cofactor deficiency
SOD	Sulfite oxidase deficiency

A.A. Belaidi (✉) • G. Schwarz
Department of Chemistry and Center for Molecular Medicine Cologne, Institute of Biochemistry, University of Cologne, Cologne, Germany
e-mail: belaidia@uni-koeln.de

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_2,

SSC S-sulfocysteine
HPLC High-performance liquid chromatography

2.1 Introduction

Molybdenum cofactor deficiency (MoCD) is a rare inherited metabolic disorder (Johnson et al. 1980; Johnson and Duran 2001) caused by defects in the biosynthesis of the molybdenum cofactor (Moco) leading to the simultaneous loss of activities of all molybdenum-dependent enzymes: sulfite oxidase, xanthine dehydrogenase, aldehyde oxidase, and the mitochondrial amidoxime-reducing component (Schwarz et al. 2009). Affected patients exhibit severe neurological abnormalities, such as microcephaly and seizures, and they usually die in early childhood (Johnson and Duran 2001). Sulfite oxidase deficiency (SOD) is less frequent but clinically indistinguishable from MoCD, which renders sulfite oxidase as the most important Moco enzyme in humans (Tan et al. 2005). Sulfite oxidase catalyzes the oxidation of sulfite, which is generated throughout the catabolism of sulfur-containing amino acids, to sulfate (Griffith 1987; Johnson and Duran 2001). Deficiencies of Moco and sulfite oxidase result in the accumulation of sulfite, a highly toxic molecule that breaks disulfide bridges in proteins and cystine, thereby affecting many protein and cellular functions (Zhang et al. 2004). Sulfite accumulation is accompanied by the formation of secondary metabolites such as thiosulfate and S-sulfocysteine (SSC) (Johnson and Duran 2001), which together with reduced homocysteine levels (Sass et al. 2004) are common biochemical indicators for MoCD and SOD.

Sulfite is generated throughout the catabolism of sulfur-containing amino acids in two steps. First, the cytosolic enzyme cysteine dioxygenase catalyzes the formation of cysteine sulfinic acid (CSA). Second, either CSA undergoes a transamination in mitochondria, which leads to the formation of sulfite, or it is decarboxylated in the cytosol leading to the formation of hypotaurine, which is further oxidized to taurine. In MoCD sulfite first accumulates in liver, where most of the catabolism of sulfur-containing amino acids takes place. Subsequently, accumulation of sulfite in plasma is detectable and finally sulfite crosses the blood–brain barrier triggering a devastating and progressive neuronal damage (Schwarz et al. 2009).

Using a knockout animal model for MoCD (Lee et al. 2002) a substitution therapy with cyclic pyranopterin monophosphate has been established (Schwarz et al. 2004) and recently a first successful treatment for an MoCD (type A) patient has been reported (Veldman et al. 2010). Before treatment was initiated, a manifested rapid increase of urinary sulfite, thiosulfate, and SSC values was recorded. However, within few days after treatment was initiated, a remarkable normalization of all MoCD biomarkers as well as a significant clinical improvement of the patient were observed. Recently, we reported the development of a new HPLC method for diagnosis and treatment monitoring of MoCD patients, which enables an accurate and sensitive measurement of urinary as well as serum SSC levels and is being currently used to diagnose the disease to monitor treated patients (Belaidi et al. 2011).

2.2 Methods

2.2.1 Creatinine Analysis

Creatinine determination was based on the Jaffe method and carried out as previously described (Belaidi et al. 2011). Briefly, 50 µl of diluted urine samples were mixed with 150 µl alkaline picrate solution (1.2% picric acid in 0.75 M sodium hydroxide) and the formation of an orange–red complex between creatinine and alkaline picrate was quantified by measuring the absorbance at 490 nm.

2.2.2 HPLC

HPLC analyses were carried out on an Agilent 1200 SL system (Agilent Technologies GmbH, Boeblingen, Germany). The chromatographic conditions were identical to the previously reported SSC quantification method (Belaidi et al. 2011). Automated pre-column derivatization with O-phthaldialdehyde (OPA) was used and the analyzed compounds were separated on a reversed-phase C18 column: XBridge (150×4.6 mm, 3.5 µm, Waters GmbH, Eschborn, Germany). For detection the UV absorbance at 338 nm was recorded and compound identification was achieved by comparing the retention time with that obtained for a standard. Peak area was used for calibration. SSC, hypotaurine, and taurine amounts were determined by standard addition and normalized to creatinine concentration.

2.3 Results

2.3.1 HPLC Determination of Hypotaurine and Taurine in Urine Samples

HPLC analysis of amino acids with OPA derivatization is one of the most sensitive methods for amino acid quantification with detection limits in the femtomole range. We previously developed a method for SSC determination in urine samples using pre-column derivatization with OPA, which resulted in fast and accurate measurement (Belaidi et al. 2011). In this study we extend the method to the measurement of hypotaurine and taurine in addition to SSC. Under the chromatographic conditions described above, separation was completed within 15 min using isocratic elution. Hypotaurine and taurine yielded sharp peaks eluting at 13.8 and 14.3 min, respectively, whereas SSC eluted at 8 min (Fig. 2.1a). Urine analysis in a sample derived from a healthy individual revealed the presence of very low amounts of SSC (4 mmol/mol creatinine), whereas hypotaurine and taurine levels were 25 and 30 mmol/mol creatinine, respectively (Fig. 2.1a). In contrast, analysis of a urine

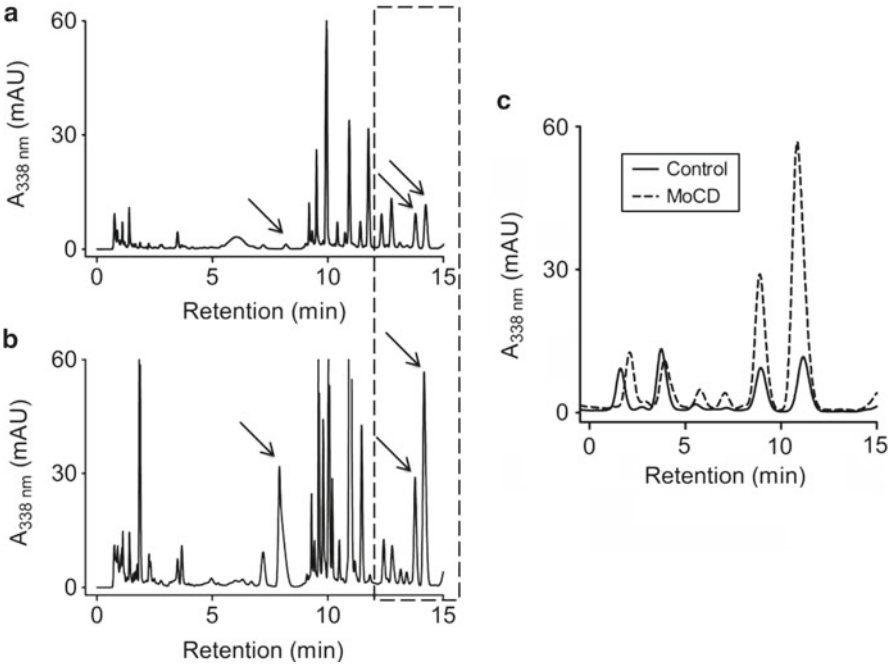

Fig. 2.1 HPLC analysis of hypotaurine, taurine, and SSC in a control individual (**a**) and an MoCD patient (**b**). An overlay of both chromatograms—control individual (*solid line*) and MoCD patient (*dashed line*)—highlighting hypotaurine and taurine peaks is shown in (**c**). SSC, hypotaurine, and taurine are highlighted in panels (**a**) and (**b**) by *arrows*

sample derived from an MoCD patient showed—in addition to an accumulated SSC peak—a clear accumulation of both hypotaurine and taurine (Fig. 2.1b). Comparison of the chromatograms derived from a healthy control sample (Fig. 2.1c, solid line) and an MoCD patient sample (Fig. 2.1c, dashed line) revealed a tenfold increase in the urinary excretion levels of hypotaurine and taurine in the MoCD patient (Fig. 2.1c).

2.3.2 Determination of Taurine Excretion Levels in Healthy and MoCD Patients

After confirming that hypotaurine and taurine excretion levels are up-regulated in an MoCD patient, the method was applied to the analysis of nine urine samples derived from MoCD patients as well as urine samples from control individuals. As expected, the SSC values were very low in control samples (1–9 mmol/mol creatinine), while hypotaurine and taurine levels ranged from 10 to 70 (median 21) and 30 to 100 (median 67) mmol/mol creatinine, respectively (Fig. 2.2a). In contrast, samples derived from MoCD patients showed, in addition to SSC accumulation

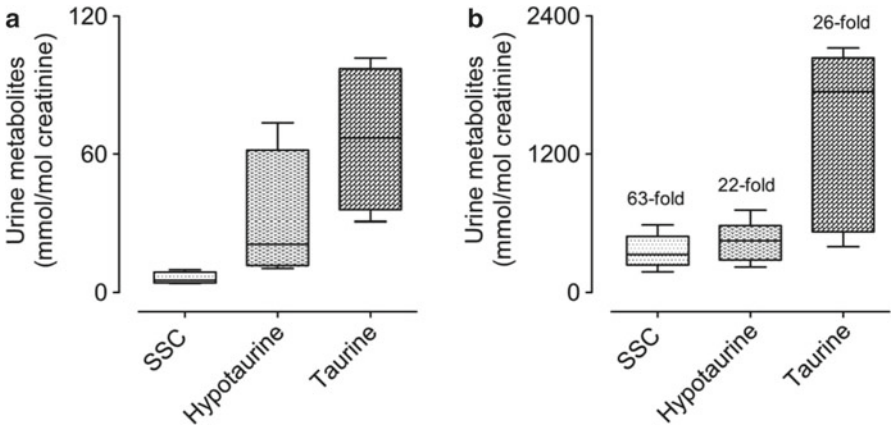

Fig. 2.2 Hypotaurine, taurine, and SSC urinary levels in control individuals and MoCD patients. Urine samples derived from control individuals and nine MoCD patients are shown in panel (**a**) and (**b**), respectively. In addition, numbers over the bars in panel (**b**) indicate the fold increase in the median value in MoCD patients for each metabolite. Metabolites in urine were normalized to creatinine concentration and horizontal bars show the median values

(180–600 mmol/mol creatinine, Fig. 2.2b), very high levels of both hypotaurine and taurine. The excretion levels of taurine were in the millimolar range and reached over 25-fold increase in the median value of healthy individuals, while a 21-fold increase in the median value of hypotaurine was found (Fig. 2.2b).

2.4 Discussion

MoCD is a rare metabolic disorder characterized by a severe and massive neurodegeneration leading to death in early childhood. SSC which is present at very low levels in healthy individuals (Johnson and Duran 2001; Belaidi et al. 2011) is one of the most elevated metabolites in MoCD patients and due to its structural similarity to glutamate, it is believed to act on NMDA receptors (Olney et al. 1975). In the past, many reports showed an important role of taurine in modulating glutamate and GABA signaling (El Idrissi and Trenkner 1999, 2004). Furthermore, taurine has been shown to prevent excitotoxicity through modulation of intracellular calcium homeostasis (El Idrissi and Trenkner 1999). Knowing the importance of calcium signaling in the glutamate-induced neurotoxicity and the fact that taurine and sulfite are both formed directly from CSA, we asked to which extent taurine is also affected in MoCD. We developed an HPLC method for the simultaneous detection of SSC, hypotaurine, and taurine in urine samples aiming to determine the excretion levels of those compounds in control and MoCD patients. Our results showed over 20-fold higher excretion values for hypotaurine and taurine in MoCD patients as compared

to control individuals. The fact that not only taurine but also hypotaurine, the direct precursor for taurine synthesis, are excreted in high levels in urine of MoCD patients provides evidence for an up-regulation of the entire taurine biosynthesis pathway from CSA via hypotaurine to taurine. Thus, an exclusive contribution of taurine transport is not the sole explanation. As a 64-fold increase in SSC levels was measured in MoCD patients, while only a 20-fold increase in both hypotaurine and taurine was found, we assume that sulfite-mediated SSC formation precedes the accumulation of taurine and hypotaurine, pointing to a more distal metabolic relationship. In summary, it remains unclear how sulfite and/or SSC contributes to this up-regulation. Due to the previously reported important role of taurine in preventing neurotoxicity (El Idrissi and Trenkner 1999), we speculate that taurine up-regulation may result from a compensatory effect to overcome the toxicity caused by SSC in the brain or a feedback inhibition of the sulfite branch in cysteine catabolism, thus leading to an increased taurine formation. Additional experiments are required to elucidate the effect of taurine, especially on the SSC-induced neurotoxicity.

2.5 Conclusion

Here we confirm the link between MoCD and taurine biosynthesis using a novel method for the simultaneous detection of SSC, taurine, and hypotaurine in healthy control individuals and MoCD patients. Interestingly, the analysis of urine samples derived from MoCD patients revealed over 20-fold increase in both hypotaurine and taurine levels as compared to control individuals, thus providing evidence for an up-regulation of the hypotaurine and taurine pathway and demonstrating a link between sulfite toxicity and taurine biosynthesis in MoCD patients. However, it remains unclear by which mechanisms taurine and hypotaurine are up-regulated in MoCD.

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Chapter 3
Taurine and Chinese Traditional Medicine Accelerate Alcohol Metabolism in Mice

Gaofeng Wu*, Jiancheng Yang*, Shumei Lin, Ying Feng, Qunhui Yang, Qiufeng Lv, and Jianmin Hu

Abstract Excessive alcohol consumption is dangerous and causes serious damage to health. The main organ capable of alcohol oxidizing is liver which is also the main organ synthesizing taurine, a sulfur-containing β-amino acid, which is the major free intracellular amino acid presenting in many tissues of human and animals and exerting many physiologic and pharmacologic functions. To investigate the effect of taurine and Chinese traditional medicine on alcohol metabolism after acute alcoholic intake, male Kunming mice were administered with 60% alcohol (0.4 ml) intragastrically. Water, taurine, or taurine coadministration with Chinese traditional medicine was intragastrically administered to mice 30 min before or after alcohol intake. The disappearance of body-righting reflex was used to determine the intoxication of mice. Durations between alcohol intake and intoxication (tolerance time), intoxication and recovery (maintenance time) were recorded. The concentration of blood alcohol, levels of hepatic alcohol dehydrogenase (ADH), and acetaldehyde dehydrogenase (ALDH) were detected at 20, 50, 90, 120, and 150 min after alcohol intake. The results showed that taurine administered alone or together with Chinese traditional medicine could both significantly reduce the number of intoxicated mice, postpone the tolerance time, shorten the maintenance time, and could obvisouly decrease blood level of alcohol, increase hepatic levels of ADH and ALDH. The results indicated that taurine administered alone or

*These authors contributed equally to the work.

G. Wu • J. Yang • S. Lin • Y. Feng • Q. Yang • Q. Lv • J. Hu (✉)
College of Animal Science and Veterinary Medicine, University of Shenyang Agricultural University, Shenyang, Liaoning Province, Shenyang 110866, P.R.China
e-mail: hujianmin59@163.com

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_3,

together with traditional Chinese medicine could significantly accelerate the metabolism of alcohol, reduce the toxicity of alcohol, and coadministration of taurine and traditional Chinese medicine had better effects.

Abbreviations

ADH Alcohol dehydrogenase
ALDH Acetaldehyde dehydrogenase

3.1 Introduction

Alcohol consumption is customary in most cultures and alcohol abuse is common worldwide. Globally, alcohol causes 3.2% (or 1.8 million) of all deaths annually and accounts for 4% of disease burden (WHO 1999). China is the most populated country in the world, with 22% of the world's population. Beverage brewing has a long history dating back to the Shang Dynasty (1600–1110 BC). Alcohol is considered as an important aspect of Chinese culture and drinking is socially accepted as a significant part of major events in daily life (Hao and Young 2000). Excessive alcohol consumption is a major risk factor for morbidity. In addition to the potentially lethal effects of alcohol poisoning to the brain, the liver, which is the main organ for alcohol oxidation, can also be acutely affected.

Taurine (2-aminoethanesulfonic acid), a sulfur-containing amino acid, is one of the most abundant free amino acids in many tissues. Although taurine does not involve in the synthesis of protein, it exerts widespread physiological and pharmacological functions including detoxification, antioxidation, membrane stabilization, osmoregulation and neuromodulation, as well as brain and retina developments (Lee et al. 2004). Liver is the main organ capable of synthesizing taurine. Many protective functions of taurine on the liver damage have been reported. Taurine has been found to have preventive effects on the development of hepatic steatosis induced by a high-fat dietary habit in hamster (Yuan-Yen Chang et al. 2011). It also have the effects of curtailing oxidative stress in iron-potentiated alcoholic liver fibrosis, producing inflammatory and fibrogenic mediators and activating stellate cells (Shanmugam Lakshmi Devi et al. 2010). As taurine was first isolated from ox bile in 1827 by German scientists Friedrich Tiedemann and Leopold Gmelin (Tiedemann and Gmelin 1827), it is also an important constituent of Bezoar Bovis which is a famous Chinese traditional medicine. Besides taurine, many Chinese traditional medicines have also been reported to have hepatoprotective effects (Liu et al. 1994; Ching-Yi Liu et al. 2010). The present study aimed to investigate the effects of taurine and Chinese traditional medicine on alcohol metabolism after acute alcohol intake, in order to prevent and accelerate recovery from intoxication induced by alcohol.

Table 3.1 Experimental design

Group	Alcohol intake	Treatment (i.g.)
I	30 min before	Water
II	30 min before	2% taurine
III	30 min before	2% T+CTM
IV	30 min after	Water
V	30 min after	2% taurine
VI	30 min after	2% T+CTM

3.2 Methods

3.2.1 Antialcohol Intoxication Effects of Taurine and Chinese Traditional Medicine on Mice

60 Kunming mice were divided into six groups randomly. Mice in each group were intragastrically (i.g.) administered with alcohol (i.g., 60%, 0.4 ml) 30 min before or after i.g. with water (C), 2% taurine (T), or 2% taurine coadministration with Chinese traditional medicine (T+CTM), the volume of which were all 0.4 ml. The disappearance of the body-righting reflex which was used to determine the intoxication of mice was recorded when the mice were kept in a supine position without turned over for more than 30 s. Durations between alcohol intake and intoxication (tolerance time), intoxication and recovery (maintenance time) were recorded. The experimental design is shown in Table 3.1.

3.2.2 Determination of Alcohol Concentration in Blood After Alcohol Intake

60 Kunming mice were divided into three groups and were treated as group I, II, and III as shown in Table 3.1. Blood were collected from each group 20, 30, 90 and 150 min after alcohol intake. Alcohol concentrations were detected by way of gas chromatography.

3.2.3 Determination of Hepatic Activities of ADH and ALDH

Liver were collected at the same time of blood collection. Hepatic activities of alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) were detected by chromatometry using spectrophotometer.

3.2.4 Statistical Analysis

Values are mean ± SEM. Comparisons were performed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Differences were considered statistically significant when the calculated P value was less than 0.05.

3.3 Results

3.3.1 Antialcohol Intoxication Effects of Taurine and Chinese Traditional Medicine

In this study, we examined the effects of taurine and Chinese traditional medicine supplementation on alcohol intoxication. There were 10 mice in each group at the beginning of the experiment, while after alcohol intake, 10 mice were intoxicated in group I and IV, 9 mice were intoxicated in group II and V, only 5 mice were intoxicated in group III. Tolerance time in group II and III was significantly longer than group I ($P<0.05$, $P<0.01$), while maintenance time in group II and III was significantly shorter than group I ($P<0.05$, $P<0.01$); there were no significant differences between group II and III ($P>0.05$). Tolerance time in group V and VI was extremely longer than group IV ($P<0.01$); maintenance time in group V and VI was obviously shorter compared with group IV ($P<0.01$) (Fig. 3.1).

3.3.2 Taurine and Chinese Traditional Medicine Decreased Blood Concentration of Alcohol

As shown in Fig. 3.2, there were no significant differences in blood concentrations of alcohol among all the groups 20 min after alcohol intake ($P>0.05$). From the 50 min point, the values in T+CTM group decreased very significantly at each time point ($P<0.01$), while those in the 2% Tau group declined obviously at 50, 120, and 150 min, extremely significant at 90 min compared with the values in the control group ($P<0.01$). The peak points of alcohol concentrations in each group were 90, 50, and 20 min, respectively.

3.3.3 Taurine and Chinese Traditional Medicine Increased Activities of ADH and ALDH in the Liver

As shown in Fig. 3.3, hepatic activities of ADH and ALDH increased with time changing, especially in T+CTM and 2% taurine groups, the values of which were significantly higher compared with the control group ($P<0.05$). The activities of ADH and ALDH both reached the peak at 150 min point.

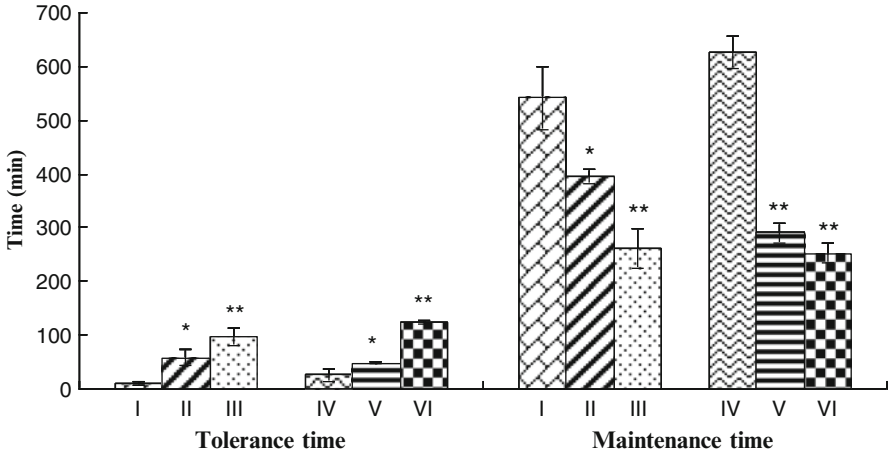

Fig. 3.1 Results of antialcohol intoxication experiment. Mice in group I, II, and III were intragastrically administered with alcohol 30 min before medicine administration. Mice in group IV, V, and VI were intragastrically administered with alcohol 30 min after medicine intake. Tolerance time and maintenance time were recorded. There were ten mice in each group. Taurine administered alone or together with Chinese traditional medicine could significantly extend the tolerance time and shorten maintenance time after alcohol intake. Each column and vertical bar represents the mean ± SEM. Values with asterisk are significantly different ($P < 0.05$), values with double asterisks are very significantly different ($P < 0.01$ ANOVA followed by the Tukey's post hoc test)

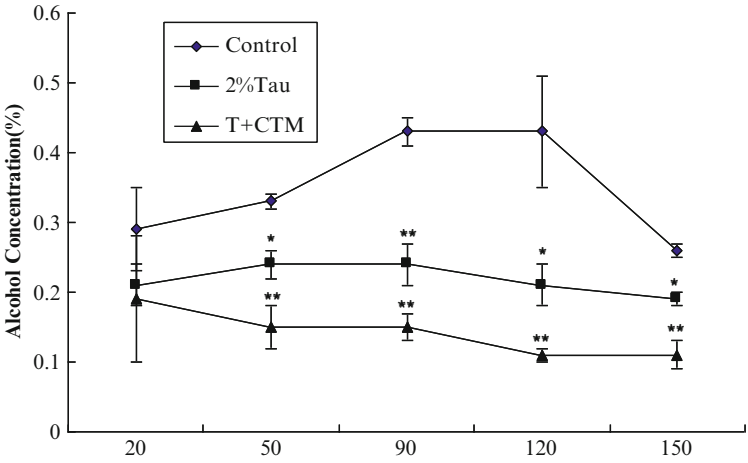

Fig. 3.2 Blood concentration of alcohol. Mice in the control, 2% taurine, and T+CTM groups were administered with water (control), 2% taurine (2% Tau), or 2% taurine coadministration with Chinese traditional medicine (T+CTM), respectively, 30 min before alcohol intake. Blood were collected 20, 50, 90, 120 and 150 min after alcohol intake. Each column and vertical bar represents the mean ± SEM. Values with asterisk are significantly different ($P < 0.05$), values with double asterisks are very significantly different ($P < 0.01$ ANOVA followed by the Tukey's post hoc test)

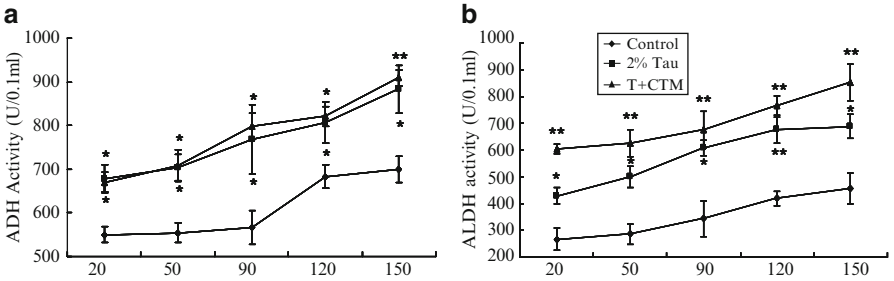

Fig. 3.3 (**a**) Hepatic activity of ADH. (**b**) Hepatic activity of ALDH. Mice in the control, 2% Tau, and T+CTM groups were administered with water, 2% taurine, or 2% taurine coadministration with Chinese traditional medicine, respectively, 30 min before alcohol intake. Liver were collected 20, 50, 90, 120, and 150 min after alcohol intake. Each column and vertical bar represents the mean ± SEM. Values with asterisk are significantly different ($P < 0.05$), values with double asterisks are very significantly different ($P < 0.01$ ANOVA followed by the Tukey's post hoc test)

3.4 Discussion

It is now clear that acute alcohol binges is not only toxic to the liver but also can contribute to the chronicity of ALD. Potential mechanisms by which acute alcohol causes damage include steatosis, dysregulated immunity and inflammation, and altered gut permeability (Massey and Arteel 2012). Ethanol is mainly metabolized in the liver by two oxidative pathways. In the first pathway, ethanol is oxidized to acetaldehyde by the cytoplasmic ADH enzyme, and acetaldehyde is then oxidized to acetic acid by the mitochondrial ALDH. The second pathway is inducible and involves the microsomal ethanol-oxidizing system (MEOS), in which the oxidation of ethanol to acetaldehyde and acetic acid also leads to generation of reactive oxygen species (ROS). Chronic or acute ethanol consumption significantly inhibits mitochondrial ADH and ALDH activity, resulting in a striking increase of tissue and blood alcohol and acetaldehyde levels (Lieber 1997).

The main enzyme for taurine synthesis is mainly distributed in the liver, which is considered to be the main organ capable of taurine synthesis, suggesting that taurine may play vital roles in the liver protection. We have also previously found that taurine had significant protective effects on alcoholic liver disease (Wu et al. 2009). Many Chinese traditional medicines which were used in the present research have been reported to have hepatoprotective effects such as radix bupleuri, fructus aurantii, and Salvia and Chrysanthemum flowers (MingHong Yen et al. 2005, Lihua Liu et al. 2009, Tetuya Sugavara and Kiharu Igarashi 2009). Moreover, flower of kudzuvine has been reported to accelerate the metabolism of alcohol (Ping Li and Gansheng Zhong 2009).

In the present study, blood alcohol concentration increased obviously in the control group, and arrived at the highest point 90 min after alcohol intake. Taurine group showed a slightly increase and the highest point was at 50 min after alcohol intake. While in T+CTM group, blood concentration began to decrease 20 min after

alcohol intake. These results suggested that taurine administered alone or together with Chinese traditional medicine could significantly decrease the blood concentration of alcohol, while taurine coadministration with Chinese traditional medicine had better effects than taurine alone.

Because the metabolism processes of alcohol were activated in the liver, especially the ADH pathway, hepatic activities of ADH and ALDH increased after alcohol intake. In the present study, both ADH and ALDH activities in 2% taurine and T+CTM groups were significantly increased, suggesting that taurine added alone or together with Chinese traditional medicine could accelerate the metabolism of alcohol by way of increasing the activities of ADH and ALDH in the liver, so alcohol could be quickly oxidized into acetaldehyde which could also be catalyzed by ALDH into acetic acid, and finally changed into CO_2 and H_2O_2. In this way, taurine and Chinese traditional medicine could obviously reduce the concentrations of alcohol and its metabolic products in the liver as well as in the blood so as to reduce the injury to the liver and the brain caused by these chemicals, which could be obviously seen from the antialcohol intoxication results in this study, and taurine coadministered with Chinese traditional medicine had better effects.

3.5 Conclusion

In summary, this study showed that supplementation of taurine could accelerate the metabolism of alcohol and reduce the toxicity of alcohol, and taurine coadministration with Chinese traditional medicine had better effects.

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Chapter 4
Lethality of Taurine and Alcohol Coadministration in Mice

Andrey G. Taranukhin, Pirjo Saransaari, and Simo S. Oja

Abstract Alcohol consumption by mothers during pregnancy causes a fetal alcohol syndrome associated with massive neuronal apoptosis. We have recently shown that taurine at a dose of 2 g/kg saves about 50% of dying cerebellar neurons from ethanol-induced apoptosis in 7-day-old mice. However, a further increase in the taurine dose to ethanol-treated mice had a toxic and in some cases lethal effect. In the present work we studied the toxic effects of taurine and ethanol coadministration in three age groups: 7-day-old, adult (5 to 6 months old), and old (12 to 13 months old) mice. Taurine and ethanol were injected in two half-doses: taurine at 0 and 4 h and ethanol at 1 and 3 h. The minimal 100% lethal doses in coadministration of taurine and ethanol were the following: 7-day-old mice—6 g/kg taurine + 5 g/kg ethanol, adult mice—10 g/kg of taurine + 8 g/kg of ethanol, and old mice—above 6 g/kg of taurine + 6 g/kg of ethanol. All mice treated with taurine or ethanol alone survived. The adult and old mice dying from the combined toxicity of taurine and ethanol showed a marked fall in blood glucose, which may be one reason for lethality. A comparison of the lethal doses of taurine and ethanol coadministration in different age groups allows us to conclude that the adverse effect of the combined toxicity of taurine and ethanol is age dependent.

A.G. Taranukhin (✉)
University of Tampere Medical School,
Tampere, Finland

Laboratory of Comparative Somnology and Neuroendocrinology, Sechenov Institute of Evolutionary Physiology and Biochemistry, St.-Petersburg, Russia
e-mail: andrey.taranukhin@uta.fi

P. Saransaari
University of Tampere Medical School, Tampere, Finland

S.S. Oja
Department of Pediatrics, Tampere University Hospital, Tampere, Finland

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_4,

4.1 Introduction

Maternal alcohol consumption during pregnancy may give rise to a set of serious childhood health problems known as fetal alcohol syndrome (Eustace et al. 2003) and to alcohol-related birth defects (Warren and Bast 1988), among them growth retardation, structural brain abnormalities, behavioral and cognitive problems, and learning difficulties (Chudley et al. 2005). In rodents (Ikonomidou et al. 2000; Dikranian et al. 2005) and nonhuman primates (Farber et al. 2010) in vivo and in humans in vitro (Hao et al. 2003) alcohol induces massive death of neurons in the developing brain by apoptosis, a kind of cell death (Hotchkiss et al. 2009), this possibly accounting for the morphological and functional disorders in children prenatally exposed to alcohol. Prevention of ethanol-induced apoptosis can save many neurons and significantly reduce sequences of alcohol intoxication. Among possible drugs for apoptosis prevention taurine seems particularly attractive, since it is a compound naturally present in abundance in the nervous system (Huxtable 1992; Sturman 1993), involved in apoptosis regulation (Takatani et al. 2004; Wu et al. 2009) and protective to many types of cells under different pathological conditions (Ulrich-Merzenich et al. 2007; Taranukhin et al. 2008; Das et al. 2009; Zhang et al. 2010). We have previously shown that taurine at a dose of 2 g/kg saves about 50% of dying neurons from ethanol-induced apoptosis in the internal (Taranukhin et al. 2010) and external (Taranukhin et al. 2012) layers of the developing cerebellum of 7-day-old mice. However, a further increase in taurine doses with an eye to protecting more neurons becomes dangerous for the whole organism and may even kill treated animals. In the present work we describe the new phenomenon of combined toxicity of taurine and ethanol and seek to establish its possible mechanisms.

4.2 Materials and Methods

4.2.1 Animals and Experimental Protocols

Adult NMRI mice for experiments and breeding were purchased from Harlan, the Netherlands. In the experiments male and female mice of three age groups, 7 days old (day of birth is day 0), adult (5 to 6 months old), and old (12 to 13 months old), were used.

The mice were divided into four experimental groups: control, ethanol treated, taurine treated, and ethanol+taurine treated. Ethanol (20% w/v solution diluted in saline) and taurine (7% w/v solution diluted in saline) were administered subcutaneously to 7-day-old mice and intraperitoneally to adult and old mice. The different doses of ethanol tested ranged from 0 to 12 g/kg and of taurine from 0 to 12 g/kg together with their combinations, the objective being to find a minimal 100% lethal dose at each age. Taurine and ethanol were injected in two half-doses: taurine at 0 and 4 h, and ethanol at 1 and 3 h. The control animals were given saline injections equal to those given to the ethanol+taurine-treated group. The animals were monitored for 14 days to detect any signs of toxicity or lethality.

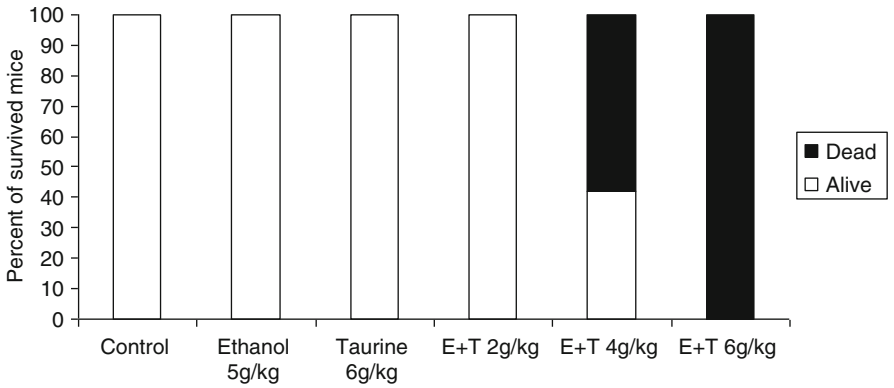

Fig. 4.1 Lethality among 7-day-old mice co-administered with taurine and ethanol. Control, $n=6$; ethanol 5 g/kg, $n=6$; taurine 6 g/kg, $n=6$; E+T 2 g/kg, $n=6$; E+T 4 g/kg, $n=12$; E+T 6 g/kg, $n=12$

4.2.2 Measurements of Blood Glucose

A part of the adult and old mice were used for measurement of blood glucose levels. Deaths of mice used for the glucose assays were not included in calculation of lethality to avoid any possible influence on the rate of lethality arising from the procedure of blood collection and hemorrhage. Blood samples were taken from the tail vein of each animal at two time-points—in adult mice 2 h before the first taurine injection to measure the baseline level of glucose and then 0.5 h after the last taurine injection, and in old mice also 2 h before the first taurine injection and then 1, 2, or 3 h after the last taurine injection to assess the effect of ethanol and taurine on blood glucose. This difference in time-points of blood sampling in adult and old mice was adapted to obtain samples maximally close to and immediately prior to death. At each time-point duplicate blood samples (5 μl) were collected into HemoCue Glucose cuvettes and immediately analyzed in a HemoCue B-Glucose Analyzer (HemoCue AB, Ängelholm, Sweden).

4.2.3 Statistical Analyses

Data on mouse mortality are expressed in percentages (Figs. 4.1–4.3). Each value for blood glucose is expressed as the mean ± SD. Statistical significance was determined by Student's *t*-test (for adult mice) and by one-way ANOVA with Tukey–Kramer post hoc test (for old mice). Statistical comparison was made between "before" and other time points for each experimental group separately. Differences were considered statistically significant at a P value <0.05.

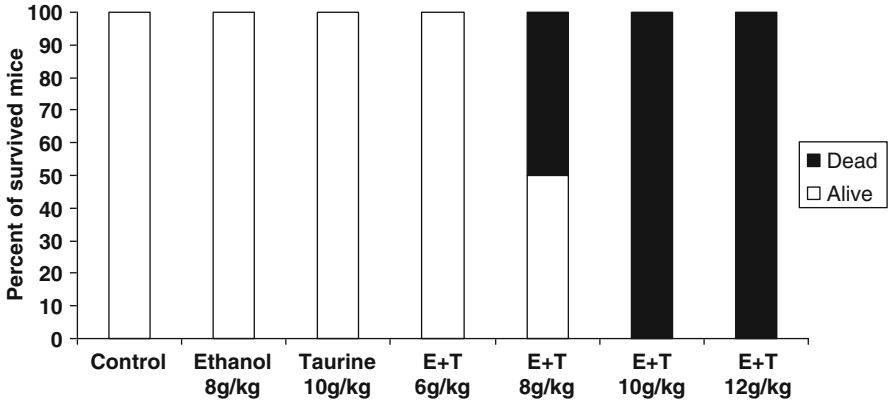

Fig. 4.2 Lethality among 5- to 6-month-old mice co-administered with taurine and ethanol. Control, $n=2$; ethanol 8 g/kg, $n=2$; taurine 10 g/kg, $n=2$; E+T 6 g/kg, $n=2$; E+T 8 g/kg, $n=2$; E+T 10 g/kg, $n=5$, E+T 12 g/kg, $n=2$

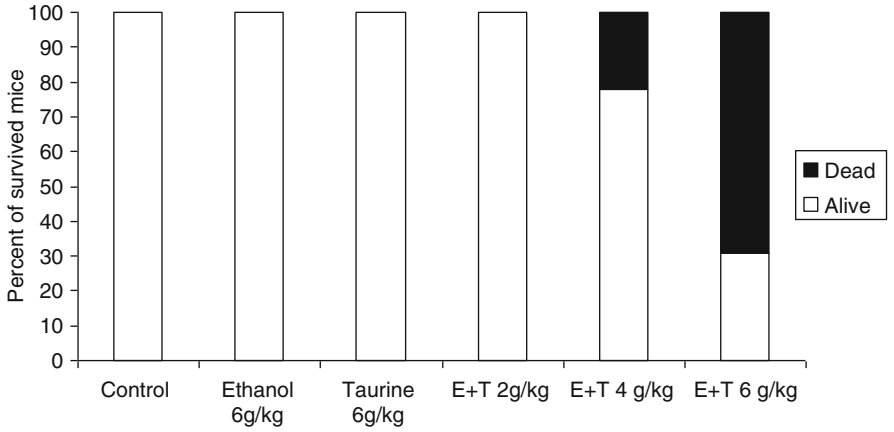

Fig. 4.3 Lethality among 12- to 13-month-old mice co-administered with taurine and ethanol. Control, $n=2$; ethanol 6 g/kg, $n=5$; taurine 6 g/kg, $n=4$; E+T 2 g/kg, $n=3$; E+T 4 g/kg, $n=9$; E+T 6 g/kg, $n=13$

4.3 Results

4.3.1 Combined Toxicity of Taurine and Ethanol in 7-Day-Old Mice

To reduce the possible influence of individual hereditary characteristics on experimental results the mice from each litter were divided into different experimental groups. Each group consisted of an equal number of males and females. As there

Table 4.1 Mouse mortality data

	Death/number in a group	
Ethanol, g/kg	Adult mice	Old mice
12	2/2	–
10	2/2	4/4
8	0/2	3/4
6	–	0/4
0	0/2	0/4

was no difference in lethality between males and females, we show total data on lethality in both sexes. Taurine at a total dose of 4 g/kg co-administered with ethanol at a total dose of 5 g/kg killed 58% of 7-day-old mice. An increase in taurine dose to 6 g/kg administered to ethanol-treated mice induced 100% mortality (Fig. 4.1). Though we monitored the mice for 14 days after treatment to detect any signs of toxicity, they usually died during the first hours after the last taurine injection. All mice exposed to ethanol or taurine alone and mice treated with 2 g/kg taurine co-administered with 5 g/kg ethanol survived.

4.3.2 Combined Toxicity of Taurine and Ethanol in Adult Mice

In the first set of experiments on adult (5 to 6 months old) mice we established that the total dose of ethanol 8 g/kg administered in two half-doses with a 2-h interval was the maximal dose not lethal for mice of this age (Table 4.1). Coadministration of taurine at a dose of 8 g/kg with ethanol (8 g/kg) killed 50% of the adult mice. One hundred % lethality was observed at a taurine dose of 10 g/kg administered to ethanol-treated mice (Fig. 4.2). All mice treated with taurine or ethanol alone at the doses used survived for the 14 days of observation. All those treated with 6 g/kg taurine co-administered with 8 g/kg ethanol likewise survived.

4.3.3 Combined Toxicity of Taurine and Ethanol in Old Mice

In the preliminary experiments on old (12 to 13 months old) mice we found the total dose of ethanol 6 g/kg administered in two half-doses with a 2-h interval to be the maximal dose not lethal for mice of this age (Table 4.1). Testing different taurine doses co-administered with 6 g/kg of ethanol we found taurine at a dose of 4 g/kg administered to the ethanol-exposed mice to kill 22% of treated mice. Taurine at a total dose of 6 g/kg combined with 6 g/kg ethanol resulted in 69% mortality (Fig. 4.3). All mice treated with taurine or ethanol alone survived the whole 14-day period of observation.

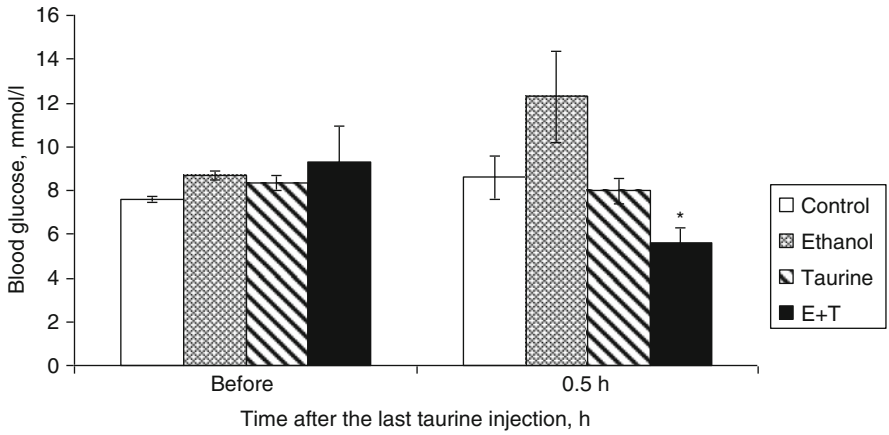

Fig. 4.4 Drop in blood glucose in 5- to 6-month-old mice after taurine and ethanol coadministration. Control, $n=3$; ethanol 8 g/kg, $n=3$; taurine 10 g/kg, $n=3$; E 8 g/kg +T 10 g/kg, $n=3$

4.3.4 Changes in Blood Glucose Level After Taurine and Ethanol Coadministration in Adult Mice

Adult mice treated with 10 g/kg taurine combined with 8 g/kg ethanol usually died within 1 h after the last taurine injection. To measure the blood glucose level maximally close to death we assayed it 0.5 h after the last taurine injection. Two hours before the treatment we measured the basic level of glucose. It was similar in all experimental groups (8.5 ± 0.9 mmol/l), with individual variations from 7.5 to 11.1 mmol/l. Coadministration of taurine and ethanol significantly reduced the glucose level from 9.3 ± 1.6 mmol/l to 5.6 ± 0.7 mmol/l (Fig. 4.4). Ethanol alone tended to increase blood glucose, but the difference from 8.6 ± 0.2 mmol/l to 12.3 ± 2.1 mmol/l was not statistically significant by reason of wide variation. Taurine alone did not alter the blood glucose level.

4.3.5 Changes in Blood Glucose Level After Taurine and Ethanol Coadministration in Old Mice

Old mice treated with 6 g/kg of taurine and 6 g/kg of ethanol died within 3–4 h after the last taurine injection. We measured blood glucose at 1, 2, and 3 h after the last taurine injection to monitor changes in blood glucose prior to death. Two hours before the treatment the basic level of glucose was similar in all experimental groups (8.5 ± 1.2 mmol/l), with individual variations ranging between 6.9 and 11.2 mmol/l. Taurine and ethanol coadministration significantly lowered the glucose level from

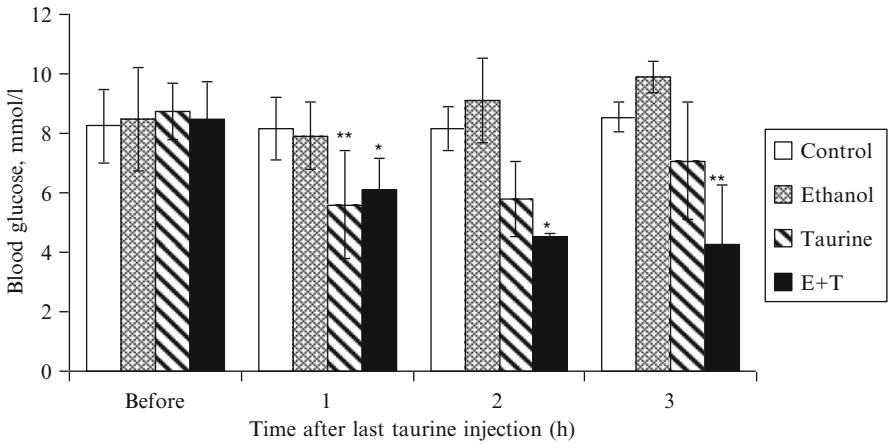

Fig. 4.5 Drop in blood glucose level in 12- to 13-month-old mice after taurine and ethanol coadministration. Control, $n=3$; ethanol 6 g/kg, $n=4$; taurine 6 g/kg, $n=5$; E 6 g/kg +T 6 g/kg, $n=5$

8.5 ± 1.3 mmol/l to 6.1 ± 1.0 mmol/l at 1 h after the last taurine injection. This decrease was preserved for the next 2 h, being 4.3 ± 2.0 mmol/l before the death of the animals (Fig. 4.5). The high SD (2.0 mmol/l) at this last time-point results from substantial differences in blood glucose levels in mice treated with ethanol and taurine. The individual differences varied between 2.2 and 6.0 mmol/l. Taurine alone lowered blood glucose in the old mice from 8.8 ± 1.0 mmol/l to 5.6 ± 1.8 mmol/l in 1 h after the last taurine treatment. The blood glucose level increased slightly during the next 2 h to reach almost to the level before experiments. Ethanol alone did not affect the blood glucose level.

4.4 Discussion

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid abundant in electrically excitable tissues such as the brain, retina, heart, and skeletal muscles (Lourenco and Camilo 2002). It is involved in a wide range of physiological processes such as osmoregulation, lipid metabolism, intracellular calcium regulation, neuronal development, neuromodulation, and cell protection (Huxtable 1992; Saransaari and Oja 2000; Anderzhanova et al. 2006; Oja and Saransaari 2007). Growing evidence indicates that taurine is also involved in the regulation of apoptosis (Takatani et al. 2004; Ulrich-Merzenich et al. 2007; Taranukhin et al. 2008; Wu et al. 2009). Clinically taurine has been tested for the treatment of a variety of diseases, including for example alcoholism (Birdsall 1998). Previously, we successfully used taurine at a dose 2 g/kg to save about 50% of dying neurons from ethanol-induced apoptosis in the developing cerebellum of 7-day-old mice (Taranukhin et al. 2009, 2010, 2012). We now increased the taurine dosage two- and threefold in an attempt to obtain more significant benefits

in cell protection against alcohol-induced apoptosis. However, contrary to our expectations taurine at a dose of 4 g/kg co-administered with 5 g/kg ethanol killed about 60% of treated mice and 6 g/kg combined with 5 g/kg ethanol 100%. Interestingly, 6 g/kg taurine and 5 g/kg ethanol alone were safe for the animals. It would thus appear that taurine potentiates the adverse effects of ethanol.

It is well known that ethanol at high doses can induce death, but little is known of taurine toxicity or of the combination of taurine with ethanol or other drugs. In experiments on dogs taurine at a single intravenous dose of 2 g/kg has proved safe for the animals and during a 14-day period of observation there were no dead animals or any signs of toxicity (Nishizawa et al. 1991). Experiments on Wistar rats have shown that taurine in a single dose of 7 g/kg (intravenously) or 5 g/kg (orally) has no toxic effect on the animals, which also survived for 14 days without any sign of toxicity or abnormality upon autopsy (Kihara et al. 1991). All these studies show that taurine is minimally toxic. We found only one study in which taurine at doses of 7.5 and 6.0 g/kg involved 100 and 50% lethality, respectively, after a single subcutaneous injection (Goldberg and Jefferies 1946). In our experiments taurine alone administered at a dose of 6 g/kg was not toxic to the animals, possibly because we injected it in two half-doses 4 h apart. When the animals received first only 3 g/kg taurine and after 4 h again 3 g/kg, taurine from the first injection was partly incorporated into the bile or secreted into the urine, as shown by the decrease in taurine levels in the blood (Lallemand and De Witte 2004; Taranukhin et al. 2010).

To test the hypothesis that the lethality of combined taurine and ethanol not only pertains for developing 7-day-old mice, which at this age are highly sensitive to ethanol toxicity (Ikonomidou et al. 2000), we repeated our experiments on adult (5 to 6 months old) and old (12 to 13 months old) mice. We thus established the maximal doses of ethanol for adult (8 g/kg) and old (6 g/kg) mice which do not induce mortality. Our present data (8 g/kg) differ slightly from the results of some other authors (Schechter and Meehan 1995) with regard to the ethanol dose which does not induce death in young adult mice (6 g/kg), probably by reason of differences in ethanol sensitivity between the two strains of mice tested. Our data on differences in the toxicity of different doses of ethanol in adult and old mice are similar to those obtained on rats, in which the 50% lethal dose for 3–4-month-old rats was significantly higher than that for 10–12-month-old rats (Wiberg et al. 1970). In adult and old mice the combined toxicity of taurine and ethanol sufficient to induce 100% mortality was significantly higher than in 7-day-old mice. Sensitivity to the combined toxicity of taurine and ethanol is thus age dependent.

Taurine treatment significantly attenuates the stress-induced elevation in blood glucose in rats (Nakagawa and Kuriyama 1975). Taurine exerts a hypoglycemic action in glucose supplementation-induced hyperglycemia (Kulakowski and Maturo 1984; Kaplan et al. 2004). These findings on the ability of taurine to reduce blood glucose and the observation that ethanol can also lower it (Huang and Sjöholm 2008) allow us to assume that the lethality of taurine and ethanol coadministration can be induced by hypoglycemia. Blood glucose after taurine and ethanol coadministration was significantly decreased in adult and old mice. In adult mice the decrease in glucose concentration was still far from the dangerous level. The old mice could

be divided into two subgroups, one of which had blood glucose about 6 mmol/l and the other 2.2 mmol/l, which is very low and may be the reason for death. We would suggest that these two subgroups could explain why only 70% of the taurine- and ethanol-treated mice died. The remaining 30% may have had a less dangerous level of blood glucose (6 mmol/l).

4.5 Conclusion

In summary, our finding would indicate that taurine alone administered even at high doses is safe for mice. In contrast, a combination of high doses of taurine with alcohol has a toxic effect and will kill the animals. This lethal effect of taurine and ethanol coadministration may be related to a dramatic drop in blood glucose. Comparison of the lethal doses of taurine and ethanol coadministration in 7-day-old adult and old mice allows us to conclude that the adverse effects of this combined toxicity is age dependent. The present findings constitute a serious warning of the toxic interactions of taurine and ethanol particularly for young people mixing taurine-containing energy drinks with alcohol.

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Chapter 5
The Effect of Long-Term Taurine Supplementation and Fructose Feeding on Glucose and Lipid Homeostasis in Wistar Rats

Lea Hüche Larsen, Laura Kofoed Hvidsten Ørstrup, Svend Høime Hansen, Niels Grunnet, Bjørn Quistorff, and Ole Hartvig Mortensen

Abstract The nonprotein amino acid taurine has been shown to counteract the negative effects of a high-fructose diet in rats with regard to insulin resistance and dyslipidemia. Here we examined the long-term (26 weeks) effects of oral taurine supplementation (2% in the drinking water) in fructose-fed Wistar rats.

The combination of fructose and taurine caused a significant increase in fasting glucose compared to the control diet without changing hepatic phosphoenol pyruvate carboxykinase mRNA levels. The combination of fructose and taurine also improved glucose tolerance compared to control. Neither a high-fructose diet nor taurine supplementation induced significant changes in body weight, body fat or total calorie intake, fasting insulin levels, HOMA-IR, or insulin-induced Akt phosphorylation in skeletal muscle.

Fructose alone caused a decrease in liver triglyceride content, with taurine supplementation preventing this. There was no effect of long-term fructose diet and/or taurine supplementation on plasma triglycerides, plasma nonesterified fatty acids, as well as plasma HDL, LDL, and total cholesterol.

In conclusion, the study suggests that long-term taurine supplementation improves glucose tolerance and normalize hepatic triglyceride content following long-term fructose feeding. However, as the combination of taurine and fructose also increased fasting glucose levels, the beneficial effect of taurine supplementation towards amelioration of glucose intolerance and insulin resistance may be questionable.

L.H. Larsen • L.K.H. Ørstrup • N. Grunnet • B. Quistorff • O.H. Mortensen (✉)
Department of Biomedical Sciences, Cellular and Metabolic Research Section, University of Copenhagen, Copenhagen, Denmark
e-mail: ole@hartvig.org

S.H. Hansen
Department of Clinical Biochemistry, Rigshospitalet and Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark

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Abbreviations

Fru	Fructose
HDL	High-density lipoprotein cholesterol
HOMA-IR	Homeostasis model assessment of insulin resistance
LDL	Low-density lipoprotein cholesterol
OGTT	Oral glucose tolerance test
Tau	Taurine

5.1 Introduction

Fructose has recently been hypothesized to be one of the main effectors driving the increased incidence of the metabolic syndrome in countries that have adopted the Western diet (Lustig et al. 2012; Stanhope 2012).

Taurine (2-aminoethanesulfonic acid) is a nonprotein semi-essential amino acid found in large amounts in both tissues (5–50 mM) and plasma (50–100 μM) and known to have a number of physiological functions, such as conjugation with bile acids, intracellular osmolyte for volume regulation, and antioxidant properties (Hansen 2001).

In 1935 it was demonstrated by Ackermann et al. that taurine had a beneficial effect upon glucose homeostasis in diabetic patients (Ackermann and Heinsen 1935). Taurine stimulates glycolysis and glycogenesis, thus presenting insulin-like activity (Dokshina et al. 1976). Furthermore, it is evident that plasma and tissue taurine levels change in a variety of metabolic pathophysiological conditions (Hansen 2001) and taurine concentration in plasma has been demonstrated to decrease in type 2 diabetic patients (De Luca et al. 2001). This reduction in plasma taurine levels has also been seen in diabetic animal models (Franconi et al. 1995, 1996). An increase in taurine intake has been shown to decrease plasma glucose in type 1 diabetic patients (Elizarova and Nedosugova 1996). However, in studies examining prediabetic and diabetic complications, differences in fasting plasma glucose and insulin after taurine supplementation were not reported (Franconi et al. 1996; Nishimura et al. 2002). Together these data indicate that taurine may have a protective role in hyperglycemia, something which is corroborated by recent literature reviews showing an interaction between taurine and diabetes (Franconi et al. 2006; Kim et al. 2007).

Several animal studies have shown that taurine prevents fructose-induced insulin resistance, as judged by both oral glucose tolerance tests and a normalization of total insulin-induced kinase activity. Furthermore, a high-fructose diet in rats has been reported to decrease plasma and liver taurine levels (El Mesallamy et al. 2010; Nandhini et al. 2004, 2005; Nandhini and Anuradha 2002). In a rat model of non-insulin-dependent diabetes, the Otsuka Long Evans Tokushima Fatty (OLETF) rat (Kawano et al. 1992) taurine supplementation caused a decrease in serum triglycerides and cholesterol, with no effect on body weight and abdominal fat mass (Harada et al. 2004; Nakaya et al. 2000).

It has been speculated that the effect of taurine on glucose homeostasis could involve a lipid lowering effect (El Mesallamy et al. 2010) and/or enhancement of insulin signaling (Nandhini et al. 2005). However, the mechanism(s) by which taurine affects glucose homeostasis is largely unknown and no long-term studies of this effect have been performed. Thus, we performed a long-term study in fructose-fed Wistar rats examining fasting glucose and lipid parameters as well as glucose tolerance with or without taurine supplementation.

5.2 Methods

5.2.1 Animals, Study Design, and Diet

All experimental procedures complied with guidelines laid down by The Danish Animal Experiments Inspectorate and by the local animal facility at the University of Copenhagen, Denmark. Male Wistar Hannover GALAS (HanTac:WH) rats (Taconic, Ejby, Denmark) were fed ad libitum, housed two rats per cage, and kept at a 12-h light/dark cycle. Animals as well as food and water intake were measured biweekly.

Twenty-four 5-week-old rats were fed four different diet regimes for 26 weeks. The rats were divided randomly into four groups of six, ensuring a similar start weight of each group, and fed a control diet or a high-fructose diet with 10% (w/v) fructose (Sigma-Aldrich, St. Louis, MO, USA) in the drinking water either with or without 2% (w/v) taurine supplementation. The carbohydrate control diet contained 68% energy from glucose (corn starch or maltodextrin), 21% casein, and 12% corn oil (Research Diets, New Brunswick, NJ, USA). The fructose-rich diet contained 50% energy from fructose, 18% energy from glucose (corn starch or maltodextrin), 21% casein, and 12% energy from corn oil (Research Diets). As the animals receiving the fructose diet also obtained fructose from the drinking water, approximately 62% of their energy intake was derived from fructose (data not shown).

Fructose and taurine were supplemented in drinking water by dissolving fructose and/or taurine directly in the water used in the animal facility. The taurine used for supplementation was of the chemically synthesized variety (Sigma-Aldrich).

An Echo MRI 4-in-1 for Small Animals (EchoMRI, Houston, TX, USA) was used to monitor total body fat mass of unanesthetized animals. Values are averages of duplicate measurements.

5.2.2 Oral Glucose Tolerance Test

Animals were fasted for 16–18 h before an oral glucose tolerance test (OGTT) was carried out. The required amount of glucose (2 g per kg) was given as a solution (45% w/v) by gavage. Blood glucose levels were determined before glucose

administration (time −30 min) in blood from the tail vein followed by a blood sample of 100 μl for plasma fasting insulin. Blood glucose levels were furthermore determined in blood from tail vein at 0, 30, 60, 90, and 120 min after glucose administration using two different automated Accu-Check Glucometers (Roche, Basal, Switzerland), thus measuring blood glucose at all time points in duplicate. The area under the curve (AUC) of the glucose tolerance test measurements was calculated from between 0 and 120 min (following glucose administration) from baseline (before glucose bolus).

5.2.3 Insulin Signaling

Overnight fasted rats were sedated with a mixture of Hypnorm (active ingredients fentanyl and fluanisone at a concentration of 0.079 and 2.5 mg/ml, respectively) and Dormicum (active ingredient midazolam at a concentration of 1.25 mg/ml) in water given as 0.3 ml per 100 g of body weight. Quadriceps muscle was dissected from one leg, and the rat was cut open. A liver lobe was removed by disconnecting the blood supply using suture. An 18 G catheter was inserted into vena portae for injection of 4.2 nmol/kg of insulin in a 2 ml volume of Krebs–Henseleit bicarbonate buffer (KHB) (an aqueous solution at pH 7.4 of 4.74 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 118.5 mM NaCl, 24.7 mM $NaHCO_3$, 2.5 mM $CaCl_2$) with 0.1% BSA (free from free fatty acids, Sigma) to directly measure insulin signaling in vivo. After 5 min of insulin stimulation, another liver lobe and the other quadriceps muscle were dissected. All tissues were quick frozen in liquid nitrogen and stored at −80°C for further analysis.

5.2.4 Quantitative Real-Time PCR

RNA was extracted from liver tissue using Qiazol (Qiagen, Valencia, CA, USA) as described by the manufacturer. In short, samples were homogenized in Qiazol with 5 mm steal beads, using a Qiagen Tissuelyzer (Qiagen) three times for 1 min at 30 Hz. Upon lysis, RNA was extracted with chloroform (Sigma) and precipitated using isopropanol (Sigma). Finally, precipitated RNA was washed with 75% ethanol and dissolved in 30 μl diethyl pyrocarbonate-treated water (DEPC water) (Sigma). Quality of RNA was assured using a Bioanalyzer (Agilent, Santa Clara, CA, USA) as described by the manufacturer. RNA concentrations were measured at a Saveen Werner Nanodrop Spectrophometer ND-1000 (Saveen Werner, Limhamn, Sweden).

Total RNA was mixed (at a concentration >0.15 μg/μl for a total of 2 μg RNA in 20 μl volume) with reverse transcriptase, random hexamer primers and nucleotides, and cDNA synthesis performed using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instruction employing an Eppendorf Thermo cycler (Applied

Biosystems). A non-reverse transcription and a non-template control reaction were included in all cases and the cDNA product was diluted to a volume of 200 µl and stored at −20°C until further analysis.

Amplification mixtures were amplified using a SYBR Green mastermix (Applied Biosystems) according to standard conditions ((95°C 10 min)×1, (95°C 15 s, 60°C 1 min, 95°C 15 s, 60°C 15 s, 95°C 15 s)×50 cycles in a total volume of 10 µl with a melting curve from 60 to 100°C) in 384-well plates in triplicate on an ABI PRISM 7900 sequence detector (Applied Biosystems). Target gene mRNA levels were normalized to TATA-binding protein (TBP) mRNA levels.

Primers: PCK1-forward: 5′-TGGAGACCACAGGATGAGGAACCG-3′, PCK1-reverse:5′-AATGGGACATTGGCTGGCAGGG-3′,TBP-forward:5′-CCCACCAGCAGTTCAGTA-3′, TBP-reverse: 5′-CAATTCTGGGTTTGATCATTC-3′.

5.2.5 Western Blot

Muscle biopsies were mixed with a modified RIPA lysis buffer with protease and phosphatase inhibitors (an aqueous solution at pH 7.4 of 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.25% deoxycholate, 1% Triton X-100, 1 µg/ml pepstatin A, 1 mM Na_3VO_4, 1 mM NaF, phosphatase inhibitor 1 and 2 (Sigma), and a complete protease inhibitor cocktail (Roche)). Tissue samples were mechanically homogenized using the Tissuelyzer (Qiagen) for 1 min at 30 Hz and 15-min incubation on ice, repeated three times. Samples were rotated end over for 1 h at 4°C before being centrifuged for 30 min at 20,000×*g* at 4°C and the supernatant transferred to a new tube. Protein concentration was determined using the Bio-Rad DC kit (Bio-Rad) with BSA used as standard. All determinations were done in triplicate.

Twenty-five micrograms of protein lysate per lane were boiled in NuPAGE LDS Sample Buffer (Invitrogen), separated on 4–12% Bis-Tris NuPAGE gels (Invitrogen), and transferred to PVDF membranes (Hybond-P, GE Healthcare, Little Chalfont, United Kingdom). Membranes were then blocked for 1 h at room temperature in blocking buffer (Tris-buffered saline with 0.1% Tween-20 and 5% Skim milk (Sigma-Aldrich, St. Louis, MO, USA)). The membranes were then incubated overnight at 4°C in 5% BSA in Tris-buffered saline with 0.1% Tween-20 containing a primary antibody against phospho-AKT (#4060, Cell Signaling Technology, Inc., Danvers, MA) at a 1:1,000 dilution. The membranes were then washed three times for 5 min in wash buffer (Tris-buffered saline with 0.1% Tween-20) and incubated for 1 h at room temperature with secondary antibody (Goat Anti-rabbit HRP, P0448, Dako, Glostrup, Denmark) at a 1:10,000 dilution in blocking buffer, followed by three-times 5-min washes in wash buffer. The protein bands were detected using ECL (Pierce ECL substrate, Pierce) and quantified using a CCD image sensor (Syngene G:BOX Cemi XR5, VWR, Copenhagen, Denmark) and ImageJ software (Abramoff et al. 2004) was used to estimate the optical density of the bands. The above method was also used to quantify total AKT(pan) (#4691, Cell Signaling Technology). The amount of p-AKT was expressed as arbitrary units relative to total AKT content.

5.2.6 Biochemical Analysis

Fasting plasma insulin concentrations were measured using the ultrasensitive rat insulin ELISA kit as described by the manufacturer (Mercodia, Uppsala, Sweden). Tissue insulin sensitivity was evaluated by the previously validated (Bonora et al. 2000) homeostasis model assessment (HOMA) using the HOMA index of insulin resistance (HOMA-IR) = fasting insulin (mU/L) × fasting glucose (mM)/22.5 (Matthews et al. 1985).

Plasma nonesterified fatty acids (NEFA) were measured at 546 nm using NEFA-HR (2) Kit according to instructions from the manufacturer (WAKO, Richmond, VA, USA) at 37°C.

Triglycerides were measured in 10 μl plasma or 50 mg liver tissue, hydrolyzed in 0.5 M KOH/85% ethanol at 60°C for 30 min (Kates 1986). After cooling, $MgSO_4$ was added to 0.1 M and samples were vortexed and centrifuged at 16,000 × *g* for 20 min at 4°C. Glycerol was measured spectrophotometrically at 340 nm as described (Wieland 1984).

High-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol in rat plasma were measured at 450 nm using an ELISA Kit according to instructions from the manufacturer (Novatein Biosciences, Cambridge, MA, USA) at 37°C. Total cholesterol was calculated as HDL + LDL.

5.2.7 Statistical Analysis

Data are presented as means ± standard error of the mean (SEM). Statistical analyses were carried out using Bonferroni corrected student's *t*-tests. The mRNA data were log-transformed before statistical analysis in order to obtain a normal distribution. All statistical analyses were performed using SAS 9.2 (The SAS Institute, Cary, NC, USA). A p value less than 0.05 was considered significant.

5.3 Results

5.3.1 Body Weight, Body Fat, Food Intake, and Water Intake

All animal groups demonstrated a steady weight gain rate (data not shown). No significant differences in weight gain or total body fat were observed (Table 5.1). Interestingly, the fructose-fed groups had a decreased food intake, but also an increase in water intake, making the total calorie intake equal due to the 10% fructose in the water (Table 5.1). Due to the difference in water intake, the fructose-fed animals obtained double the amount of taurine throughout the 26-week study period compared to control. No effect of taurine upon food intake, weight gain, or body fat was observed in either study.

Table 5.1 Physiological, glucose, and lipid metabolism parameters

	Group			
Parameter	Con	Con + Tau	Fru	Fru + Tau
Body weight (g)	513.3 ± 24.6	463.3 ± 21.6	443.3 ± 20.9	457.2 ± 17.3
Body fat %	20.1 ± 1.5	17.1 ± 1.4	19.4 ± 1.7	20.3 ± 1.8
Food intake (kcal)	12,662 ± 433	11,538 ± 479	9,589 ± 209[a,b]	9,666 ± 343[a,b]
Calorie intake (kcal)	12,662 ± 433	11,538 ± 479	12,682 ± 272	12,874 ± 55
Water intake (ml)	3,946 ± 95	3,674 ± 240	7,618 ± 53[a,b]	7,902 ± 850[a,b]
Taurine intake (g)		73.5 ± 4.8		158.0 ± 17[b]
Fasting glucose (mM)	4.65 ± 0.13	4.95 ± 0.09	4.93 ± 0.15	5.29 ± 0.15[a]
Fasting insulin (ng/ml)	1.24 ± 0.19	0.62 ± 0.14	1.01 ± 0.32	0.82 ± 0.28
HOMA-IR (mM·mU/L)	7.38 ± 1.20	3.86 ± 0.82	6.46 ± 2.12	5.70 ± 1.99
OGTT AUC (min·mM)	252.0 ± 15.0	225.1 ± 18.7	299.1 ± 15.6	235.1 ± 10.5[c]
Muscle basal p-AKT/AKT	0.54 ± 0.09	0.65 ± 0.12	0.58 ± 0.07	0.74 ± 0.09
Muscle ins. stim. p-AKT/AKT	2.14 ± 0.22[d]	2.20 ± 0.26[d]	2.43 ± 0.37[d]	3.13 ± 0.48[d]
TG liver (mmol/g)	0.087 ± 0.007	0.077 ± 0.003	0.050 ± 0.010[a]	0.078 ± 0.012
TG plasma (mM)	1.62 ± 0.22	1.26 ± 0.12	1.35 ± 0.12	1.73 ± 0.36
NEFA plasma (mM)	1.10 ± 0.05	1.05 ± 0.04	1.36 ± 0.09	1.30 ± 0.14
HDL (mg/dl)	25.0 ± 1.9	29.0 ± 2.7	28.3 ± 1.2	33.9 ± 5.4
LDL (mg/dl)	19.8 ± 1.1	20.4 ± 1.6	22.6 ± 1.4	22.5 ± 1.1
Total cholesterol (mg/dl)	44.8 ± 2.6	49.3 ± 3.3	50.9 ± 1.2	56.4 ± 5.0
Liver PCK1 mRNA (a.u)	1.23 ± 0.08	1.10 ± 0.13	1.07 ± 0.10	1.04 ± 0.03

[a] Different from con
[b] Different from con + tau
[c] Different from Fru
[d] Different from basal. a.u.; arbitrary units
Male Wistar rats, $N = 6$ per group, were subjected to four different diet regimes for 26 weeks as described in materials and methods. *HOMA-IR* homeostatic model assessment of insulin resistance, *Con* control, *Tau* taurine, *Fru* fructose, *OGTT* oral glucose tolerance test, *AUC* area under the curve, *TG* triglycerides, *NEFA* nonesterified fatty acids, *HDL* high-density cholesterol, *LDL* low-density cholesterol, *PCK1* phosphoenolpyruvate carboxykinase 1

5.3.2 *Glucose Homeostasis, Glucose Tolerance, and Insulin Signaling*

The combination of a high-fructose diet and taurine supplementation caused a significant increase in fasting glucose compared to the control diet alone, whereas taurine or fructose on their own had no significant effect on fasting glucose levels (Table 5.1). Neither a high-fructose diet nor taurine supplementation or the combination induced any change in fasting insulin levels or HOMA-IR (Table 5.1). Taurine supplementation of the fructose-fed animals caused a significant improvement in glucose tolerance, whereas this effect was not significant when animals were fed the control diet. We examined if this was due to an increase in insulin sensitivity in skeletal muscle by measuring insulin-induced Akt phosphorylation in quadriceps, but despite a higher value in the taurine-supplemented fructose group, we did not find a significant difference (Table 5.1). We also examined if the increase in fasting glucose could be due to an increased expression of the rate-limiting

enzyme of gluconeogenesis, phosphoenol pyruvate carboxykinase (PCK1), but found no difference in mRNA levels between groups (Table 5.1).

5.3.3 Lipid Parameters

Fructose caused a significant decrease in hepatic triglyceride content, with taurine supplementation rescuing this effect. No effect of taurine alone on hepatic triglyceride content was observed (Table 5.1). No difference between groups was observed with regard to plasma triglycerides, nonesterified free fatty acids, HDL, LDL, or total cholesterol (Table 5.1).

5.4 Discussion

The current study set out to examine the effect of long-term taurine supplementation in rats receiving a high-fructose diet. Surprisingly we saw both beneficial and detrimental effects of taurine upon glucose metabolism.

5.4.1 Taurine and Glucose Metabolism

Taurine has in studies from two different laboratories been shown to counteract the effects of a high-fructose diet on glucose metabolism in Wistar rats, with no observable negative side effects of taurine supplementation (El Mesallamy et al. 2010; Nandhini et al. 2004, 2005; Nandhini and Anuradha 2002).

In the current study, taurine seems to have a beneficial effect on glucose tolerance in fructose-fed rats, but not when given together with a control diet. As taurine has previously been shown to improve insulin signaling (Nakaya et al. 2000; Nandhini et al. 2005) we examined whether or not this could be due to increased insulin-induced skeletal muscle signaling through AKT. However, although we did see an increase in AKT phosphorylation in the group receiving fructose and taurine, the increase was not significant compared to the other groups, ruling out this mechanism of action as an explanation of the improvement in glucose tolerance, although further studies are needed to confirm this. Taurine has been shown to potentiate insulin release (Carneiro et al. 2009), and although some disagree (Kulakowski and Maturo 1984), one might speculate that this could be one possible explanation for the improved glucose tolerance. Unfortunately we did not measure insulin levels during the glucose tolerance tests and although we found no difference in fasting insulin levels, this question remains unanswered.

We examined whether or not the increase in fasting glucose could be due to an increase in hepatic gluconeogenesis by measuring the mRNA level of the rate-determining gluconeogenic enzyme PCK1 (Pilkis and Granner 1992), but found no evidence of PCK1 being increased. Increased glycogenolysis could also explain the

increase in fasting glucose, but this is contradicted by previous findings that taurine can attenuate glycogenolysis (Lau-Cam and Patel 2006; Patel and Lau-Cam 2006). Taurine has been shown to potentiate insulin release (Carneiro et al. 2009), but that would, if anything, cause a decrease and not an increase in fasting glucose. As such we are at present unable to explain the mechanism by which taurine might increase fasting glucose levels.

The discrepancy between our results and previous studies, in terms of increased fasting glucose when taurine is given together with fructose, may relate to the duration of the experiments. In reports using the same general strain of rats and the same concentration of taurine in the drinking water, the duration of the experiments was about 30 days (El Mesallamy et al. 2010; Nandhini et al. 2004, 2005; Nandhini and Anuradha 2002) making the duration of this study six times longer than previous studies. Furthermore, substrain differences in the outbred Wistar rat strain might also be a possible explanation as some rat strains seem to respond differently to fructose (Stark et al. 2000). The Wistar substrain used in the current study seems surprisingly tolerant towards fructose and displays only small diabetogenic effects when fed fructose compared to other Wistar substrains that show extreme increases in HOMA-IR and become severely glucose intolerant after being fructose fed (El Mesallamy et al. 2010; Nandhini et al. 2005; Perret et al. 2007).

5.4.2 Taurine and Lipid Metabolism

Earlier studies have shown an improvement in hepatic and plasma lipid parameters such as plasma triglycerides and free fatty acids as well as hepatic triglyceride content following taurine administration (Nardelli et al. 2011; Yan et al. 1993). In the current study we found no effect of taurine upon plasma lipid parameters in direct contradiction with earlier shorter studies (El Mesallamy et al. 2010; Nardelli et al. 2011). However, taurine did seem to normalize the hepatic triglyceride content following a high-fructose diet indicating an interaction between taurine and liver lipid metabolism or lipid storage. The differences between this and previous studies are most likely due to the Wistar rat substrain used in the current study that displayed no fructose-induced changes in plasma lipids, indicating a large degree of tolerance towards fructose. As such, any normalization by taurine upon plasma lipid parameters would not have been noticed in the current study.

5.4.3 Taurine and Fructose Perspective

Dietary fructose is thought, at elevated concentrations, to be a contributing factor to the marked increase in insulin resistance, obesity, and type 2 diabetes seen in the last few decades (Lustig et al. 2012; Stanhope 2012; Tappy et al. 2010; Tappy and Lê 2010) and many studies have reported that a high-fructose diet in rodents can cause insulin resistance, dyslipidemia, and type 2 diabetes (Basciano et al. 2005; Samuel 2011;

Tappy et al. 2010). However, several human and animal studies have found none or only a small effect of a fructose diet on glucose metabolism (Abdullah et al. 2009; Tappy and Lê 2010). Thus, in some rat strains, long-term high-fructose diets cause an adaptation to the diet, completely abolishing the effect of a high-fructose diet on glucose and lipid homeostasis and glucose tolerance (Stark et al. 2000). Furthermore, fructose effects may depend on the composition of the control diet, i.e., effects of fructose have been reported to be significant only when compared to chow but not if compared to a diet with fructose substituted by starch (Kim et al. 1999).

An increase in fasting blood glucose has been linked to overall mortality and development of metabolic and cardiovascular disease (Seshasai et al. 2011; Singh et al. 2010). The results of the current study are particularly worrying, as taurine is an often used ingredient in energy drinks, and thereby automatically consumed together with a large amount of fructose (from either sucrose or high-fructose corn syrup used as sweeteners). There is a good case from epidemiological studies for fructose ingestion as a precipitator of insulin resistance albeit age, obesity, study duration, and other dietary factors are possible confounding factors (Stanhope 2012). Thus, the exact circumstances in which increased fructose consumption may be detrimental in humans are currently not well established. However, this study points towards a possible link between glucose homeostasis and a combined intake of fructose and taurine. As such further studies are needed to examine this interaction between taurine and fructose.

5.5 Conclusion

In summary, the current study indicates that long-term taurine supplementation may be beneficial with regard to glucose tolerance when given together with fructose. However, taurine also increases fasting glucose when given together with fructose. Thus, this study did not corroborate earlier findings showing that taurine has a solely beneficial effect on glucose and lipid homeostasis. Despite these negative findings, when taking into account earlier studies and the current study, it seems quite clear that there is a connection between taurine and glucose homeostasis and possibly a connection to dietary fructose, but also that a number of unanswered questions remain.

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Chapter 6
Effect of Taurine Feeding on Bone Mineral Density and Bone Markers in Rats

Mi-Ja Choi and Ji-Na Seo

Abstract The purpose of this study was to investigate the effect of dietary taurine supplementation on bone mineral density (BMD) and bone mineral content (BMC) in rats. Twenty Sprague–Dawley male rats (body weight 200 ± 10 g) were divided into two groups, control and taurine group (2% taurine-supplemented diet). All rats were fed on experimental diet and deionized water and libitum for 6 weeks. Serum alkaline phosphatase (ALP) activity, osteocalcin, PTH, and urinary deoxypyridinoline cross-links value were measured as markers of bone formation and resorption. BMD and BMC were measured using PIXImus (GE Lunar Co., Wisconsin) in spine and femur. The effect of diet on ALP, osteocalcine, and PTH was not significant. There were no significant differences in ALP, osteocalcine, and PTH concentration. Urinary calcium excretion was lower in taurine group than in control group. Femur BMC/weight of taurine group was significantly higher than control group. The results of this study showed the possible role of taurine in bone metabolism in male rats.

Abbreviations

ALP	Alkaline phosphatase
DPD	Deoxypyridinoline
Tau	Taurine
DPD/Cr	Creatinine excretion
BMD	Bone mineral density
BMC	Bone mineral content
FER	Food efficiency ratio
SBMD	Spine bone mineral density

M.-J. Choi (✉) • J.-N. Seo
Department of Food and Nutrition, Keimyung University,
1000 Sindangdong, Dalseogu, Daegu 704-701, South Korea
e-mail: choimj@kmu.ac.kr

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SBMC Spine bone mineral content
FBMD Femur bone mineral density
FBMC Femur bone mineral content

6.1 Introduction

Taurine (2-aminoethanesulphonic acid) is a sulfur-containing amino acid that is present in the diet. Taurine, a free amino acid, is found in millimolar concentrations in most mammalian tissues. Mammals are able to synthesize taurine endogenously, but some species such as humans are more dependent on dietary sources of taurine. Taurine, a sulfur-containing amino acid, has been termed a functional nutrient (Bouckenooghe et al. 2006). Although it is not incorporated into proteins, taurine has been shown and proposed to have a number of essential biological functions (Huxtable 1992). Recent and past studies suggested that taurine might be a pertinent candidate for use as a nutritional supplement to protect against oxidative stress, diabetes mellitus, neurodegenerative diseases, or atherosclerosis (Bouckenooghe et al. 2006). It is best known for its role in lipid metabolism, where taurine enhances the absorption of fat in the intestine by stimulating bile acid synthesis, and the degradation of cholesterol (Huxtable 1992). Also, in animals, dietary taurine improved high blood pressure (Anuradha and Balakrishnan 1999) and hypercholesterolemia (Murakami et al. 2002). The effects of taurine in mammals are numerous and varied. Taurine is a powerful agent in regulating and reducing the intracellular calcium levels. Two specific targets of taurine action are reported to be Na^{+}–Ca^{2+} exchangers and metabotropic receptors mediating phospholipase-C (PLC) in central nervous system (Foos and Wu 2002). However, its effect on bone is not clear. Increased bone mineral density (BMD) and bone mineral content (BMC) in growth period has been the subject of numerous epidemiological studies. Taurine could act either directly or indirectly by enhancing growth factor production. Although evidence from animal study indicates taurine effect on bone (Lubec et al. 1997; Koide et al. 1999), there are no published reports evaluating the role of taurine supplementation on bone in male growing rats. The purpose of this study was to investigate the effect of dietary taurine supplementation on BMD and BMC in male rats.

The purpose of this study was to determine the effect of taurine supplementation with appropriate calcium level on bone in growing rat.

6.2 Methods

6.2.1 Materials

Twenty Sprague-Dawley male rats (body weight 200 ± 10 g) were divided into two groups, control and taurine group (2% taurine-supplemented diet). The animals had free access to their experimental diet and deionized water during the entire experiment,

Table 6.1 Composition of experimental diets (g/1 kg diet)

	Dietary group	
	Control	Taurine
Corn starch	531	531
Sucrose	100	100
Casein[a]	200	200
Soybean oil	70	70
Cellulose[b]	50	50
Mineral-Mix[c]	35	35
Vitamin-Mix[d]	10	10
L-Cystein[e]	1.8	1.8
Choline[f]	2.5	2.5
TBHQ[g]	0.008	0.008
Taurine[h]	0	20

[a] Casein, Maeil Dairy industry Co. Ltd. 480 Gagok-Ri, JinwiMyun, Pyungtaek-City, Kyunggi-Do, Korea
[b] α-Cellulose, Sigma Chemical Co., St. Louis, Mo, USA
[c] Mineral-mix, AIN-93 G-Mx, Teklad Test Diets, Medison, Wisconsin, USA
[d] Vitamin-mix, AIN-93-VM, Teklad Test Diets, Medison, Wisconsin, USA
[e] L-Cystine, Sigma Chemical Co., St. Louis, MO, USA
[f] Choline bitartate, Sigma Chemical Co., St. Louis, MO, USA
[g] Tert-bultyl hydroquione, Sigma-Aldrich Inc., St. Louis, MO, USA
[h] Taurine, Dong-A Pharm Co. Ltd. 434–4 Moknae-dong. Ansan-City

and their food intake and body weights were measured every other day and once a week, respectively. The rats were fed a AIN-96 control diet and control plus taurine diet (2% wt/wt) for 6 weeks. Compositions of the diets were as described in Table 6.1. Blood samples were collected from the abdominal aorta and serums were separated at 3,000 rpm for 20 min. Serums were stored at −70°C until analysis. Serum concentrations of alkaline phosphatase (ALP) and osteocalcin were measured. Serum calcium and phosphate were also measured. Serum ALP and osteocalcin and urinary deoxypyridinoline (DPD) cross-link values were measured as markers of bone formation and resorption. ALP activity was reported as units per liter (U/L). The concentration of urine DPD was measured with an enzyme immunoassay that preferentially recognizes the free form of DPD (CLIA, Pyrilinks-D DPC, USA). DPD was corrected for creatinine excretion (DPD/Cr). The concentration of serum osteocalcin was measured with an osteocalcin kit (IRMA, OSTEORIACT, Cis Bio, Saclay, France), which recognizes the intact form of osteocalcin. Serum ALP activity was measured with a kit from Enzymatic assay (Prueauto S ALP) following the manufacturer's instructions.

BMD and BMC were measured using PIXImus (GE Lunar Co, Wisconsin, USA) in spine and femur on 6 weeks after feeding. The experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC) in Keimyung University and conformed to the Guide for the Care and Use of Laboratory Animals.

Table 6.2 Body weight change of experimental rats

Variables	Control	Taurine	Significance
Initial body weight (g)	237.12 ± 4.88[1]	242.00 ± 2.44	NS[2]
Final body weight (g)	392.30 ± 28.11	380.78 ± 25.44	
Weight gain (g)	158.50 ± 27.78	138.90 ± 31.35	

[1]Mean ± SD
[2]*NS* not significantly different at $p < 0.05$

Table 6.3 Food intake and FER of rats fed during experimental period

Variables	Control	Taurine	Significance
Food intake (g/day)	18.87 ± 5.28[1]	21.79 ± 1.00	NS[2]
FER[3]	0.25 ± 0.16	0.15 ± 0.03	

[1]Mean ± SD
[2] *NS* not significantly different at $p < 0.05$
[3] *NS*

6.2.2 Statistics Analysis

Differences between two groups were analyzed by Student's unpaired *t*-test. All results are presented as mean ± standard deviation (SD). A *p* value of 0.05 or less was considered statistically significant.

6.3 Results and Discussion

6.3.1 Weight Gain, Food Intake, and Food Efficiency Ratio

Table 6.2 showed the weight at beginning, weight at sacrifice, and weight gain of experimental rats. At beginning, there were no significant differences in weight between groups with and without taurine supplementation. Taurine group was approximately 12.4% lower in weight gain compared with control group; however, this difference did not reach statistical significance. Taurine supplementation did not affect food intake and food efficiency ratio (FER) in experimental rats (Table 6.3).

6.3.2 Serum Ca and P Concentrations

The concentrations of serum Ca and P were not significantly different between the experimental group (Table 6.4). The mean concentrations of serum Ca were 10.34 ± 0.16 and 9.71 ± 0.30 (mg/dl) for the control and taurine group, respectively.

Table 6.4 Serum calcium and phosphorus of rats fed experimental diets

Variables	Control	Taurine	Significance
Ca (mg/dl)	10.34 ± 0.16[1]	9.71 ± 0.30	NS[2]
P (mg/dl)	7.04 ± 0.21	7.92 ± 0.51	

[1]Mean ± SD
[2]*NS* not significantly different at $p < 0.05$

Table 6.5 Urinary calcium and phosphorus excretion of rats fed experimental diets

Variables	Control	Taurine	Significance
Ca (mg/day)	0.18 ± 0.07[1]	0.08 ± 0.03	**
P (mg/day)	20.56 ± 8.05	15.97 ± 9.96	NS[2]

**$p < 0.01$
[1]Mean ± SD
[2]*NS* not significantly different at $p < 0.05$

Table 6.6 Creatinine, DPD, and cross-link value of rats fed experimental diets

Variables	Control	Taurine	Significance
Creatinine (mM)	2.96 ± 1.01[1]	4.60 ± 2.06	NS[2]
DPD (nM)	599.80 ± 239.91	872.01 ± 286.45	
Cross-link value (nM/mM)	200.60 ± 31.98	189.56 ± 46.27	

[1]Mean ± SD
[2]*NS* not significantly different at $p < 0.05$

6.3.3 *Urine Calcium, Phosphorus, Deoxypyridinoline, Creatinine, and Cross-Link Value*

The levels of urinary calcium excretion were 0.18 ± 0.07 mg/day in the control group and 0.08 ± 0.03 mg/day in the taurine group. The urinary calcium excretion of taurine group was significantly lower than in control group. However, urinary phosphorus excretion was not significantly different (Table 6.5).

Cross-link values were 200.60 ± 31.98 and 189.56 ± 46.27 (nM/mM) for the control and taurine groups, respectively. Although urinary cross-link value was generally higher in the control group than in the taurine group, the difference was not statistically significant (Table 6.6).

6.3.4 *Bone Markers*

Table 6.7 showed the serum ALP and osteocalcin concentration of experimental rats. The concentrations of serum ALP were 278.0 ± 45.4 IU/L in the control group and 260.1 ± 36.4 IU/L in the taurine group. And the concentrations of serum

Table 6.7 Serum ALP and osteocalcin concentration of rats fed experimental diets

Variables	Control	Taurine	Significance
ALP (IU/L)	278.0 ± 45.4[1]	260.1 ± 36.4	NS[2]
Osteocalcin (ng/ml)	5.7 ± 0.69	3.2 ± 0.09	

[1]Mean ± SD
[2]*NS* not significantly different at $p < 0.05$

Table 6.8 PTH of rats fed experimental diets

Variables	Control	Taurine	Significance
PTH (pg/ml)	15.28 ± 12.10[1]	23.42 ± 15.46	NS[2]

[1]Mean ± SD
[2]*NS* not significantly different at $p < 0.05$

Table 6.9 Spine BMD and BMC of rats fed experimental diets

Variables	Control	Taurine	Significance
SBMD (g/cm^2)	0.152 ± 0.008[1]	0.153 ± 0.009	NS[2]
SBMC (g)	0.544 ± 0.050	0.576 ± 0.056	NS
SBMD/wt (kg)	0.38 ± 0.03	0.40 ± 0.02	NS
SBMC/wt (kg)	1.38 ± 0.08	1.51 ± 0.12	NS

[1]Mean ± SD
[2]*NS* not significantly different at $p < 0.05$

osteocalcin were 5.7 ± 0.69 ng/ml in the control group and 3.2 ± 0.09 ng/ml in the taurine group. No differences were observed in serum ALP and osteocalcin between the experimental groups.

Serum PTH were 15.28 ± 12.10 pg/ml and 23.42 ± 15.46 pg/ml for the control and taurine groups, respectively (Table 6.8). The concentration of serum PTH was not significantly different between the experimental groups. Several studies indicated that diurnal variations in serum PTH concentrations influence bone metabolism and that nocturnal increases in PTH secretion favorably affect bone mass (McKee 1999; Chen et al. 2003).

6.3.5 Bone Mineral Density and Bone Mineral Content

The results of spine BMD and spine BMC were shown in Table 6.9. Supplementation of taurine to the diet did not affect spine BMD and BMC in comparison with control group.

Table 6.10 showed the femur BMD and BMC in the experimental male rats. Femur BMD, BMC, and femur BMD per weight (FBMD/wt) were not significantly different between experimental groups. However, femur BMC per weight (FBMC/wt) was significantly higher in the rat-fed taurine-supplemented diet than in the rat-fed control diet (1.16 ± 0.05 g/wt vs. 1.27 ± 0.10 g/wt).

Table 6.10 Femur BMD and BMC of rats fed experimental diets

Variables	Control	Taurine	Significance
FBMD (g/cm^2)	0.218 ± 0.007[1]	0.230 ± 0.017	NS[2]
FBMC (g)	0.454 ± 0.033	0.48 ± 0.041	NS
FBMD/wt (kg)	0.55 ± 0.02	0.60 ± 0.05	NS
FBMC(g)/wt (kg)	1.16 ± 0.05	1.27 ± 0.10	*

*$p < 0.05$
[1]Mean ± SD
[2]*NS* not significantly different at $p < 0.05$

Taurine supplementation did not significantly affect the spine BMD and femur BMD. But these data suggested that FBMC per weight was significantly increased, without bone markers, in the taurine-supplemented diet. Therefore, it is possible that dietary taurine might be used to reduce the burden of osteoporosis.

6.4 Discussion

In the present study, taurine supplementation to the growing male rats increased femur BMC per weight and had marked effects on early growth stage. Compared with control group, rats given taurine supplementation diet had higher values for both femur and femur per weight BMC, as suggested previously by Choi and DiMarco (Choi and DiMarco 2009). Also, taurine supplementation has been shown to have a positive effect on bone in OVX rats with appropriate calcium (Choi and DiMarco 2009) but no positive effects in the ovariectomized rats fed a calcium-deficient diet (Choi 2009). Taurine supplementation may have an effect on GH/IGF-1 axis, and taurine has been shown to stimulate GH secretion in rats (Ikuyama et al. 1988) and in the human (Mantovani and DeVivo 1979). In animal studies, a resistance to IGF-I at the cellular level has been proposed as a possible mechanism causing bone loss during immobilization (Sakata et al. 2004). In summary, dietary taurine supplemented at 2% of the diet by weight offers potential to increase BMC. Because taurine is also known to reduce atherosclerosis and inflammation in experimental animal models (Belury 2002), it could prove beneficial in the overall management of chronic disease (Hayes and Stuiman 1981). In another study, Ji et al. (2012) reported that taurine was proved having the proliferation-promoting and anti-replicative senescence effect on rat bone marrow-derived multipotent stromal cells (BMSCs), providing a new view angle to understand the protecting and nourishing effect of taurine.

These results indicate that taurine supplementation might have a beneficial effect on bone in growing rats. In conclusion, we show that the addition of 2% taurine in control diet increase femur BMC per weight in growing male rats.

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Chapter 7
The Effects of Bisphosphonates on Taurine Transport in Retinal Capillary Endothelial Cells Under High Glucose Conditions

Na-Young Lee and Young-Sook Kang

Abstract Diabetic retinopathy (DR) is a major cause of blindness in diabetic patients. Elevated glucose and vascular endothelial growth factor (VEGF) in retina can trigger many of the retinal vascular changes caused by diabetes and DR. Recently, bisphosphonates, antiosteoporosis drugs, have been reported to have anti-angiogenic effect by decreasing VEGF. Taurine has several biological processes such as osmoregulation and antioxidation in retina. Therefore, the purpose of this study is to clarify the regulation of taurine transport activity by high glucose concentration and the effect of inhibitors for VEGF function, bisphosphonates, on taurine transport under high glucose condition using TR-iBRB cell lines as an in vitro model of inner blood-retinal barrier (iBRB). As a result, by exposing TR-iBRB cells to high glucose for 48 h, [^{3}H]taurine uptake was decreased continuously. [^{3}H] Taurine uptake was increased significantly by pretreatment of alendronate and pamidronate compared with the values for high glucose. Increased [^{3}H]taurine uptake by pretreatment of alendronate and pamidronate was significantly reduced by mevalonate pathway intermediates, geranylgeraniol (GGOH). In conclusion, taurine transport through the iBRB under high glucose condition can be regulated by bisphosphonates via mevalonate pathway. Therefore, we suggest that bisphosphonates could have the beneficial effects on DR by regulation of taurine contents in retina.

Abbreviations

iBRB	Inner blood-retinal barrier
DR	Diabetic retinopathy

N.-Y. Lee • Y.-S. Kang, Ph.D (✉)
College of Pharmacy and Research Center for Cell Fate Control,
Sookmyung Women's University, Seoul, South Korea
e-mail: yskang@sookmyung.ac.kr

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VEGF Vascular endothelial growth factor
GGOH Geranylgeraniol
TAUT Taurine transporter

7.1 Introduction

Taurine (2-aminoethanesulfonic acid) is the most abundant free amino acid in the retina (12 mM in rat) where its concentration is 100 times greater than that in the serum (100–300 μM) (Jacobsen and Smith 1968; Pasantes-Morales et al. 1972; Ando et al. 2012). In the retina, taurine deficiency causes a retinal abnormality and visual impairment in humans and rodents (Geggel et al. 1985; Heller-Stilb et al. 2002). The taurine supply to the retina from the circulating blood is mediated by the taurine transporter TAUT in retinal capillary endothelial cells (inner blood-retinal barrier, iBRB) and retinal pigment epithelial cells (outer BRB) (Törnquist and Alm 1986; El-Sherbeny et al. 2004; Tomi et al. 2007). Diabetic retinopathy (DR) remains a prevalent complication of diabetes and one of the leading causes of blindness among working-age adults (Frank 2004). Elevated glucose is believed to contribute to loss of microvascular barrier integrity (Frank 1984, 2004). Especially, vascular endothelial growth factor (VEGF) can trigger many of the retinal vascular changes caused by diabetes, including leukocyte adhesion to retinal capillaries and vascular leakage (Miyamoto et al. 2000). Accordingly, transport activity of taurine may be also changed at the iBRB under high glucose condition, and this change could intensely affect the neuroprotective effect of taurine by influencing taurine concentration in the retina. Therefore, the purpose of this study is to clarify the regulation of taurine transport activity by high glucose concentration and the effect of VEGF inhibitors, bisphosphonates, in taurine transport under high glucose condition using TR-iBRB cell lines as an in vitro model of iBRB.

7.2 Methods

7.2.1 Cell Culture

The TR-iBRB cells were grown routinely in rat tail collagen type 1-coated tissue culture dishes (Iwaki, Tokyo, Japan) at 33°C and cultured in a humidified atmosphere of 5% CO_2/air. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA), 15 μg/L endothelial cell growth factor (Roche, Mannheim, Germany), and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Grand Island, NY, USA).

7.2.2 [^{3}H]Taurine Uptake Study in TR-iBRB Cells

TR-iBRB cells were cultured at 33°C on rat tail collagen type 1-coated 24-well plates (Iwaki, Tokyo, Japan) for 2 days and washed with 1 mL extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose, and 10 mM Hepes (pH 7.4) at 37°C. Uptake was initiated by addition of 200 μL ECF buffer containing [^{3}H] taurine at 37°C. After appropriate time periods, uptake was terminated by removing the solution and washed with 1 mL ice-cold ECF buffer. To investigate the change of taurine uptake under high glucose condition, the TR-iBRB cells were pretreated with 25 mM glucose for 2, 4, 8, 12, 24, and 48 h and the uptake study was performed as described above. To test the effect of bisphosphonates on the change of taurine uptake under high glucose condition, TR-iBRB cells were exposed to 10 μM alendronate and 10 μM pamidronate in the presence or the absence of 10 μM geranylgeraniol (GGOH) for 0.5, 4, and 6 h under exposing TR-iBRB cells to 25 mM glucose for 48 h. Then, the cells were dissolved in 1 N NaOH overnight at room temperature. An aliquot (50 μL) was taken for protein assay using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. The remaining solution (500 μL) was mixed with 4.5 mL of scintillation cocktail (Hionic-fluor, Packard, Meriden, CT, USA) for the measurement of radioactivity using a liquid scintillation counter (LS6500, Beckman Instruments Inc. Fullerton, CA, USA).

7.2.3 Statistical Analysis

Statistical significance was determined by one-way ANOVA with Dunnett's post hoc test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

7.3 Results

7.3.1 Effect of High Glucose on the [^{3}H]Taurine Uptake in TR-iBRB Cells

To investigate the effect of excess glucose on taurine transport at the iBRB, [^{3}H] taurine uptake activity was examined in TR-iBRB cells under 25 mM glucose pretreatment conditions. By exposing TR-iBRB cells to high glucose for 48 h, [^{3}H]taurine uptake was decreased continuously (Fig. 7.1).

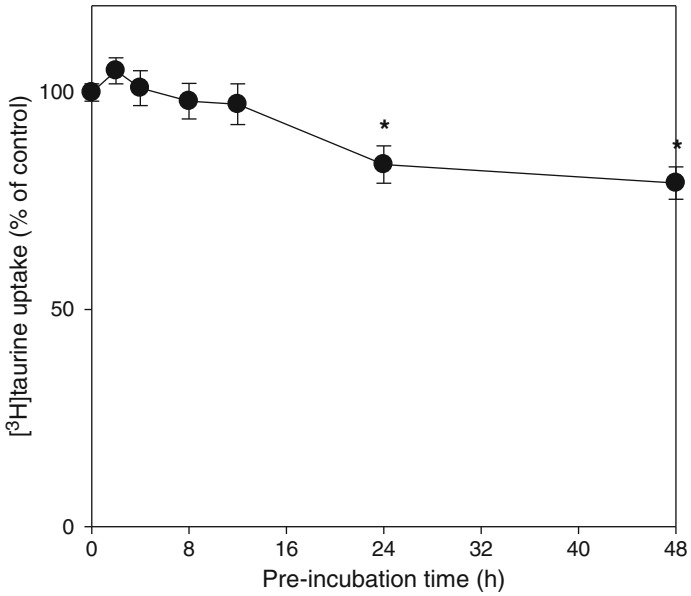

Fig. 7.1 Time-course of the effect of high glucose on [^{3}H]taurine uptake by TR-iBRB cells. 25 mM glucose was pre-incubated for the time period in the figure. The cells were incubated for 5 min at 37°C with ECF buffer containing [^{3}H]taurine (28 nM). Each point represents the mean ± SEM ($n = 3$–4). * $p < 0.05$, significantly different from time 0

7.3.2 Effect of Bisphosphonates on the [^{3}H]Taurine Uptake in TR-iBRB Cells Under High Glucose Condition

It has been suggested that bisphosphonates such as alendronate and pamidronate have anti-angiogenic effects by inhibiting upregulation of VEGF in the diabetic retina (Yokota et al. 2007). We investigated the change of [^{3}H]taurine uptake by bisphosphonates in TR-iBRB cells under high glucose condition. Under exposing TR-iBRB cells to 25 mM glucose for 48 h, TR-iBRB cells were exposed to 10 μM alendronate and 10 μM pamidronate for 0.5, 4, and 6 h. As shown in Fig. 7.2, [^{3}H] taurine uptake was increased significantly by pretreatment of alendronate and pamidronate compared with the values for 25 mM glucose.

7.3.3 Effect of Mevalonate Pathway Intermediate, Geranylgeraniol on the [^{3}H]Taurine Uptake in TR-iBRB Cells Under High Glucose Condition

Increased [^{3}H]taurine uptake by pretreatment of alendronate and pamidronate was significantly reduced by mevalonate pathway intermediates, GGOH (10 μM) (Fig. 7.3).

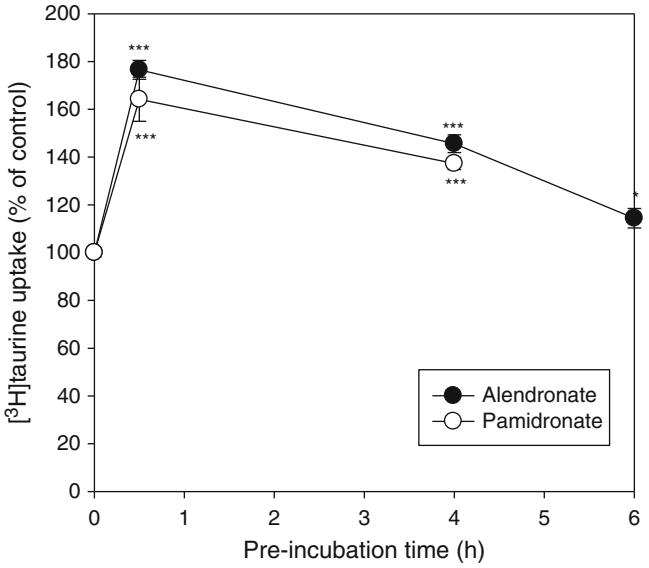

Fig. 7.2 Per-incubation time dependency of bisphosphonates on [^{3}H]taurine uptake by TR-iBRB cells in high glucose condition (25 mM). The cells were pretreated with 10 μM alendronate or pamidronate for 0.5, 4, 6 h. [^{3}H]taurine uptake was performed with ECF buffer containing [^{3}H] taurine (28 nM) for 5 min at 37°C. Each point represents the mean ± SEM ($n = 4$). *$p < 0.05$; ***$p < 0.001$, significantly different from control

7.4 Discussion

In the present study, we used TR-iBRB cells as an in vitro iBRB model. TR-iBRB cell lines, conditionally immortalized rat retinal capillary endothelial cell lines, have been established by transfecting the retrovirus vector-encoded temperature-sensitive (ts) simian virus (SV) 40 large T-antigen (Hosoya et al. 2001). These cell lines are good in vitro model for various endogenous and exogenous compound transport to the retina and as a screening tool for drugs which might be capable of delivery to the retina (Terasaki and Hosoya 2001). RT-PCR and immunoblot analysis showed that TauT is expressed in rat retina and TR-iBRB cells (Tomi et al. 2007).

Taurine is well known to act as an antioxidant and osmolyte. In diabetic state, elevated glucose level will obviously disturb cellular osmoregulation. Actually, the changes in taurine transporter activities have been reported in streptozotocin (STZ)-induced diabetic rats, human retinal pigment epithelial cells by high glucose, and diabetic neuropathy models (Stevens et al. 1999). Our results also revealed suppressed [^{3}H]taurine uptake in TR-iBRB cells under high glucose condition for 48 h (Fig. 7.1). In vitro exposure to high glucose has been shown to rapidly increase VEGF expression in various cell types and tissues (Natarajan et al. 1997), which may account for the elevated VEGF in the retina of humans and animals with diabetes (Mathews et al. 1997; Gilbert et al. 1998). Schrufer et al. have recently found that hyperglycemia increases expression of VEGF in retinal Müller cells in vitro

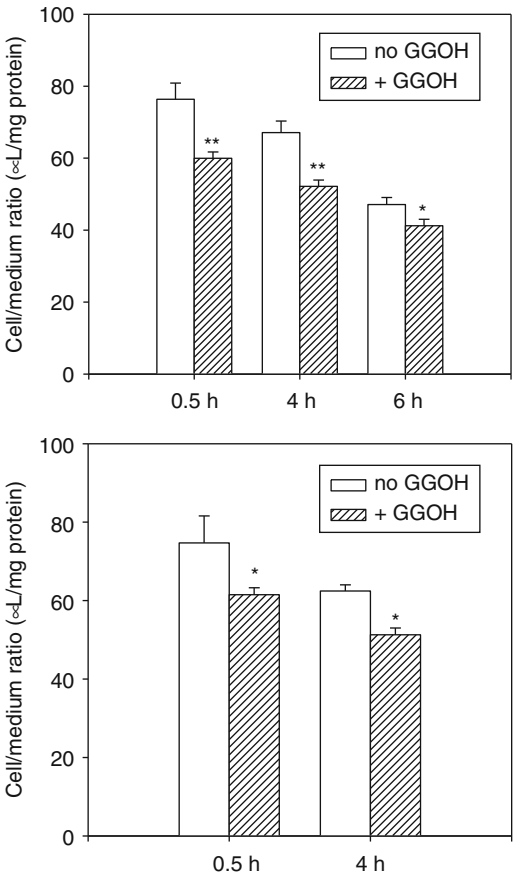

Fig. 7.3 Effect of GGOH on increased [^{3}H]taurine uptake by bisphosphonates in TR-iBRB cells under high glucose condition (25 mM). The cells were pretreated with 10 μM alendronate (**a**) and pamidronate (**b**) in the absence (□) or the presence (▨) of GGOH for 0.5, 4, 6 h. [^{3}H]Taurine uptake was performed with ECF buffer containing [^{3}H]taurine (28 nM) for 5 min at 37°C. Each point represents the mean ± SEM ($n = 4$). *$p < 0.05$; **$p < 0.01$, significantly different from the absence of GGOH

and in vivo through translational control (Schrufer et al. 2010). A number of evidences suggest that VEGF, and consequently angiogenesis, is involved in the pathogenesis of DR (Nicholson and Schachat 2010). Therefore, we hypothesized that VEGF inhibitors can recover the reduced taurine uptake at iBRB. Nitrogen-containing bisphosphonate drugs are the primary nonsurgical treatment for decreasing osteoclast action in various bone diseases such as Paget's disease, osteoporosis, and osteogenesis imperfecta. Bisphosphonate drugs are also used extensively to reduce circulating VEGF, thereby reducing metastasis for many forms of cancer (Santini et al. 2002). As a result, alendronate or pamidronate significantly increased [^{3}H]taurine uptake which was reduced by high glucose in TR-iBRB cells (Fig. 7.2). The main mechanism by which bisphosphonates alter cellular function is through inhibition of various enzymes of the mevalonate pathway of isoprenoid lipid synthesis (Luckman et al. 2005). One of isoprenoid intermediates, geranylgeranyl pyrophosphate, is essential for the posttranslational modifications necessary for membrane anchoring and activation of the small GTP-associated proteins involved in intracellular signaling. Provision of mevalonate pathway intermediate, GGOH,

has been shown to rescue the effects of bisphosphonate-mediated inhibition on cellular signaling (Andela et al. 2003), thereby providing a mechanism to demonstrate whether the bisphosphonates utilized the mevalonate pathway in the inhibition of secreted VEGF in retina. In our result, increased [^{3}H]taurine uptake by pretreatment of alendronate and pamidronate was significantly reduced by GGOH in TR-iBRB cells under high glucose condition (Fig. 7.3).

7.5 Conclusion

In conclusion, taurine transport through the iBRB under high glucose condition can be regulated by bisphosphonates via mevalonate pathway. Therefore, we suggest that bisphosphonates could have the beneficial effects on DR by regulation of taurine contents in retina.

Acknowledgements This work was supported by the Research Grant of Sookmyung Women's University.

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Chapter 8
Perinatal Taurine Imbalance Alters the Interplay of Renin–Angiotensin System and Estrogen on Glucose–Insulin Regulation in Adult Female Rats

Sanya Roysommuti, Atcharaporn Thaeomor, Sawita Khimsuksri, Wichaporn Lerdweeraphon, and J. Michael Wyss

Abstract Perinatal taurine depletion followed by high sugar intake (postweaning) alters the renin–angiotensin system (RAS) and glucose regulation in adult female rats. This study tests the hypothesis that in adult female rats, RAS and estrogen contribute to insulin resistance resulting from perinatal taurine imbalance. Female Sprague–Dawley rats were fed normal rat chow with 3% β-alanine (taurine depletion, TD), 3% taurine (taurine supplementation, TS), or water alone (control, C) from conception to weaning. Their female offspring were fed normal rat chow with 5% glucose in water (TDG, TSG, CG) or water alone (TDW, TSW, CW) throughout the experiment. At 7–8 weeks of age, animals were studied with or without captopril inhibition of the RAS and with or without estrogen receptor inhibition by tamoxifen. Compared to CW and CG groups, perinatal taurine depletion but not supplementation slightly increased plasma insulin levels. High sugar intake slightly increased plasma insulin only in TSG. Captopril treatment significantly increased plasma insulin in all groups except CG (the greatest increase was in TDG). Changes in insulin resistance and insulin secretion paralleled the changes in plasma insulin levels. In contrast, tamoxifen treatment increased insulin resistance and decreased

S. Roysommuti (✉) • W. Lerdweeraphon
Department of Physiology, Faculty of Medicine, Khon Kaen University,
Khon Kaen 40002, Thailand
e-mail: sanya@kku.ac.th

A. Thaeomor
School of Physiology, Institute of Science, Suranaree University of Technology,
Nakhonratchasima 30000, Thailand

S. Khimsuksri
Department of Oral Biology, Faculty of Dentistry, Khon Kaen University,
Khon Kaen 40002, Thailand

J.M. Wyss
Department of Cell, Developmental and Integrative Biology, School of Medicine,
University of Alabama at Birmingham, Birmingham, AL 35294, USA

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insulin secretion only in TDG and this group displayed hyperglycemia and glucose intolerance. These data indicate that perinatal taurine imbalance alters the interplay of RAS and estrogen on glucose–insulin regulation in adult female rats.

Abbreviations

CW	Control with water intake alone
CW + Cap	CW plus captopril treatment
CW + Tam	CW plus tamoxifen treatment
CG	Control with high sugar intake
CG + Cap	CG plus captopril treatment
CG + Tam	CG plus tamoxifen treatment
TDW	Perinatal taurine depletion with water intake alone
TDW + Cap	TDW plus captopril treatment
TDW + Tam	TDW plus tamoxifen treatment
TDG	Perinatal taurine depletion with high sugar intake
TDG + Cap	TDG plus captopril treatment
TDG + Tam	TDG plus tamoxifen treatment
TSW	Perinatal taurine supplementation with water intake alone
TSW + Cap	TSW plus captopril treatment
TSW + Tam	TSW plus tamoxifen treatment
TSG	Perinatal taurine supplementation with high sugar intake
TSG + Cap	TSG plus captopril treatment
TSG + Tam	TSG plus tamoxifen treatment
RAS	Renin–angiotensin system
SD	Sprague–Dawley

8.1 Introduction

A beta-amino acid taurine plays many physiological roles in humans and animals throughout life. Several lines of evidence indicate that taurine supplementation can reduce hyperglycemia in animal models of diabetes mellitus (Kim et al. 2007) and prevents sugar-induced hypertension (Harada et al. 2004; Nandhini and Anuradha 2004; Rahman et al. 2011). Perinatal protein malnutrition induces pancreatic damage in mature offspring, the effect that is prevented by taurine supplementation (Boujendar et al. 2002; Merezak et al. 2001). In addition, prenatal taurine supplementation delays the onset of diabetes mellitus in non-obese diabetic mice (Arany et al. 2004). Conversely, perinatal taurine depletion induces low birth weights in animals, and these animals develop obesity, insulin resistance, glucose intolerance, diabetes mellitus, and hypertension in mature life (Aerts and Van Assche 2002). In addition, this apparently epigenetic modification can be transferred to the next generation. Although

taurine has been added to many supplemented diets and energy drinks, the advantage of taurine to treat diabetic patients has not been conclusively demonstrated.

Our previous experiments indicate that perinatal taurine depletion induces mild hyperinsulinemia without hyperglycemia or glucose intolerance in adult female Sprague–Dawley (SD) rats (Thaeomor et al. 2010), and this hyperinsulinemia is exacerbated by an acute inhibition of renin–angiotensin system (RAS). In these animals, a blunted baroreceptor reflex does not underlie these changes since the baroreflex effect is abolished by an angiotensin-converting enzyme inhibitor captopril. Some experiments suggest that the perinatal taurine supplementation induces obesity and insulin resistance in adult rats (Hultman et al. 2007). Whether perinatal taurine supplementation influences the insulin–glucose regulation through the RAS has not been tested. In addition, many lines of evidence indicate that perinatal taurine exposure programs adult function and disease in a sex-dependent manner (Roysommuti et al. 2009b). Thus, estrogen may contribute significantly in this phenomenon. This study tests the hypothesis that in adult female rats, the RAS and estrogen contribute to insulin resistance resulting from perinatal taurine imbalance.

8.2 Methods

8.2.1 Animal Preparation

SD rats were bred at the animal unit of Faculty of Medicine, Khon Kaen University and maintained at constant humidity (60 ± 5%), temperature (24 ± 1°C), and light cycle (06.00–18.00 h). Female SD rats were fed normal rat chow with 3% beta-alanine (taurine depletion, TD), 3% taurine (taurine supplementation, TS), or water alone (control, C) from conception to weaning (Fig. 8.1). Their female offspring were fed with the normal rat chow with either 5% glucose in tap water (TD with glucose, TDG; TS with glucose, TSG; C with glucose, CG) or tap water alone (TDW, TSW, CW) throughout the experiment.

To test the possible role of RAS, another six separated groups were treated with captopril in drinking water (an angiotensin-converting enzyme inhibitor, 400 mg/l) from 7 days before parameter measurements until the end of experiment (CW + Cap, CG + Cap, TDW + Cap, TDG + Cap, TSW + Cap, TSG + Cap). In addition to test the possible role of estrogen, six separated groups were treated with an estrogen receptor antagonist (tamoxifen, 10 mg/kg/day, oral) since 7 days before the study (CW + Tam, CG + Tam, TDW + Tam, TDG + Tam, TSW + Tam, TSG + Tam). Blood chemistry and cardiovascular parameters were studied between 7 and 8 weeks of age in conscious rats.

All experimental procedures were approved by the Animal Ethics Committee of Khon Kaen University (Khon Kaen, Thailand) and were conducted in accordance with the US National Institutes of Health guidelines.

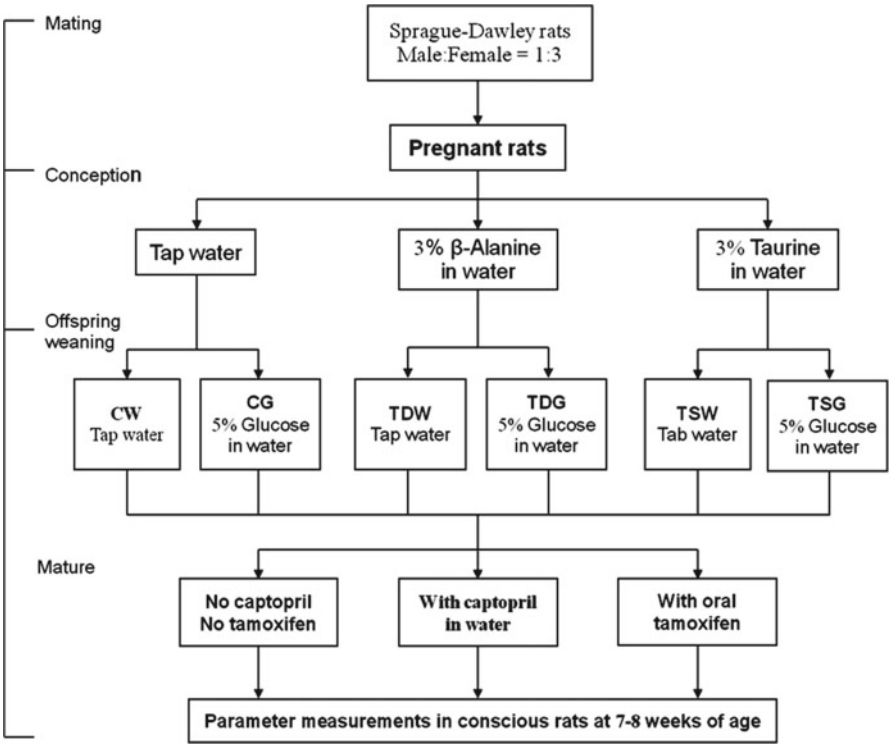

Fig. 8.1 Animal treatment protocol (*CW* control with water intake alone, *CW* + *Cap* CW plus captopril treatment, *CW* + *Tam* CW plus tamoxifen treatment, *CG* control with high sugar intake, *CG* + *Cap* CG plus captopril treatment, *CG* + *Tam* CG plus tamoxifen treatment, *TDW* perinatal taurine depletion with water intake alone, *TDW* + *Cap* TDW plus captopril treatment, *TDW* + *Tam* TDW plus tamoxifen treatment, *TDG* perinatal taurine depletion with high sugar intake, *TDG* + *Cap* TDG plus captopril treatment, *TDG* + *Tam* TDG plus tamoxifen treatment, *TSW* perinatal taurine supplementation with water intake alone, *TSW* + *Cap* TSW plus captopril treatment, *TSW* + *Tam* TSW plus tamoxifen treatment, *TSG* perinatal taurine supplementation with high sugar intake, *TSG* + *Cap* TSG plus captopril treatment, *TSG* + *Tam* TSG plus tamoxifen treatment)

8.2.2 Experimental Protocol

At 7–8 weeks of age, under thiopental anesthesia (50 mg/kg body weight, i.p.), all female rats were implanted with femoral arterial and venous catheters. Two days later and after an overnight fasting, arterial blood samples (0.2 ml each) were collected for plasma insulin and fasting blood sugar and replaced with normal saline. Thereafter, glucose tolerance testing (GTT) was initiated by intravenous injection of glucose (2 g/kg in saline), and blood glucose levels were immediately measured at 30, 60, and 120 min. At the end of the experiment, all animals were sacrificed by a lethal dose of thiopental and heart and kidney weights were collected.

All blood glucose samples were immediately measured by a glucometer (Accu-Check Advantage II, Roche, Indianapolis, Indiana, USA) while plasma insulin concentrations were measured by the Srinagarind Hospital Chemical Analysis Unit (Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand).

8.2.3 Statistical Analyses

All data are expressed as mean ± SEM and were statistically analyzed using one-way ANOVA and a post hoc Duncan's multiple range test. Statistically significant difference is defined as p-values of less than 0.05.

8.3 Results

At 7–8 weeks of age, body, heart, and kidney weights were not significantly different among groups, as previously reported (Thaeomor et al. 2010). Although perinatal taurine imbalance (with or without high sugar intake after weaning) did not affect adult fasting blood glucose concentration, estrogen receptor blockade (but not angiotensin-converting enzyme inhibition) significantly increased the fasting blood glucose concentration (Fig. 8.2) and induced significant glucose intolerance (Fig. 8.3) in TDG but not in any other group.

In control (normal perinatal taurine) animals, high sugar intake did not affect plasma insulin levels and captopril (but not tamoxifen treatment) slightly and significantly increased the plasma insulin levels in CW (but not in CG) rats (Fig. 8.4). Compared to CW, perinatal taurine depletion slightly and significantly increased plasma insulin, and this effect was moderately exacerbated by captopril (but not tamoxifen) treatment. Although high sugar intake did not further significantly increase plasma insulin in TDG, captopril (but not tamoxifen) treatment greatly increased plasma insulin in TDG compared to all other groups. Plasma insulin status in TSW rats (with or without captopril or tamoxifen treatment) appeared similar to that in CW rats, but high sugar intake significantly increased plasma insulin. This effect was further elevated by captopril (but not tamoxifen) treatment in both TS groups.

The differences between groups in estimated beta cell insulin secretion (estimated by HOMA1-%B; HOMA1-%B = (plasma insulin × 20)/(blood sugar—3.5); Fig. 8.5) essentially mirror the differences in plasma insulin levels (Fig. 8.4), except for the effect of perinatal taurine depletion, which is somewhat different. Compared to CW, perinatal taurine depletion significantly increased insulin secretion, and this effect was significantly decreased by tamoxifen (but not by captopril) treatment. Although high sugar intake did not further increase the insulin secretion in TDG rats, insulin secretion was markedly and significantly increased in response to captopril but markedly decreased to tamoxifen treatment in the TDG animals. TSW insulin responses

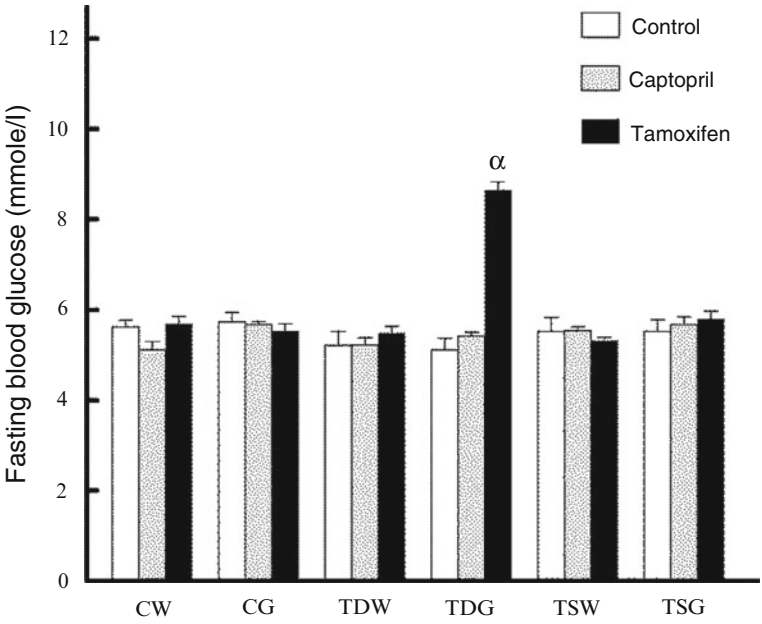

Fig. 8.2 Comparison of fasting blood glucose concentrations among groups (*CW* control with water intake alone, *CW* + *Cap* CW plus captopril treatment, *CW* + *Tam* CW plus tamoxifen treatment, *CG* control with high sugar intake, *CG* + *Cap* CG plus captopril treatment, *CG* + *Tam* CG plus tamoxifen treatment, *TDW* perinatal taurine depletion with water intake alone, *TDW* + *Cap* TDW plus captopril treatment, *TDW* + *Tam* TDW plus tamoxifen treatment, *TDG* perinatal taurine depletion with high sugar intake, *TDG* + *Cap* TDG plus captopril treatment, *TDG* + *Tam* TDG plus tamoxifen treatment, *TSW* perinatal taurine supplementation with water intake alone, *TSW* + *Cap* TSW plus captopril treatment, *TSW* + *Tam* TSW plus tamoxifen treatment, *TSG* perinatal taurine supplementation with high sugar intake, *TSG* + *Cap* TSG plus captopril treatment, *TSG* + *Tam* TSG plus tamoxifen treatment; $^{\alpha}p < 0.05$ compared to all other groups)

were similar to those of CW of same treatment. High sugar intake in TSG did not significantly increased insulin secretion, but it was slightly and significantly elevated by captopril (but not tamoxifen) treatment. The rise in insulin secretion in response to RAS inhibition was much higher in TDG compared to all other groups.

The patterns of estimated insulin resistance (estimated by HOMA1-IR; HOMA1-IR = blood sugar × plasma insulin/22.5; Fig. 8.6) paralleled the plasma insulin data (Fig. 8.4), except in the TDG group in which insulin resistance was significantly increased by tamoxifen when compared to control TDG, TDW + tam, and CG + tam groups.

8.4 Discussion

Perinatal taurine depletion leads to low birth weight of the pups which subsequently develop several disorders in adulthood, including diabetes mellitus (Aerts and Van Assche 2002). Conversely, taurine supplementation can prevent

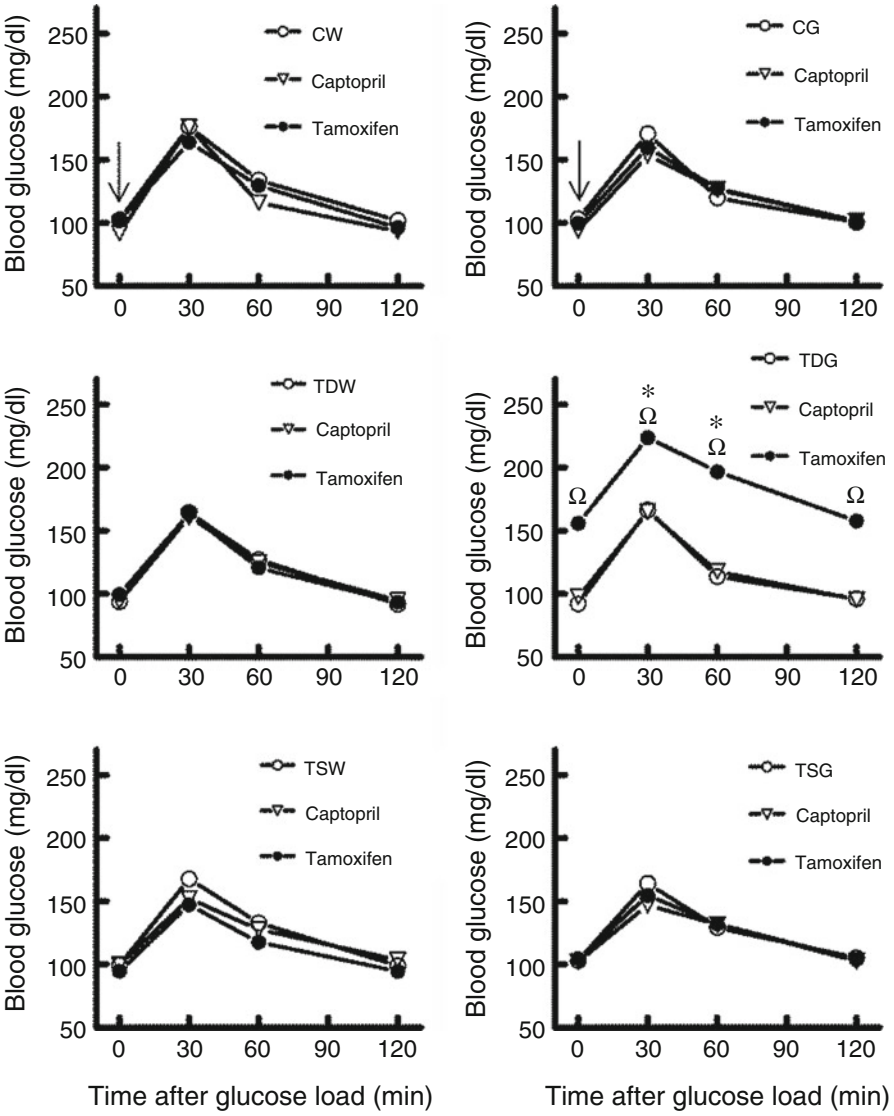

Fig. 8.3 Changes in blood glucose concentrations after intravenous injection of a bolus glucose solution (note that the glucose-supplemented groups are depicted in the three right panels; *CW* control with water intake alone, *CW* + *Cap* CW plus captopril treatment, *CW* + *Tam* CW plus tamoxifen treatment, *CG* control with high sugar intake, *CG* + *Cap* CG plus captopril treatment, *CG* + *Tam* CG plus tamoxifen treatment, *TDW* perinatal taurine depletion with water intake alone, *TDW* + *Cap* TDW plus captopril treatment, *TDW* + *Tam* TDW plus tamoxifen treatment, *TDG* perinatal taurine depletion with high sugar intake, *TDG* + *Cap* TDG plus captopril treatment, *TDG* + *Tam* TDG plus tamoxifen treatment, *TSW* perinatal taurine supplementation with water intake alone, *TSW* + *Cap*, TSW plus captopril treatment, *TSW* + *Tam* TSW plus tamoxifen treatment, *TSG* perinatal taurine supplementation with high sugar intake, *TSG* + *Cap* TSG plus captopril treatment, *TSG* + *Tam* TSG plus tamoxifen treatment; $*p < 0.05$ compared to baseline of same group; $^{\Omega}p < 0.05$ compared to all other groups)

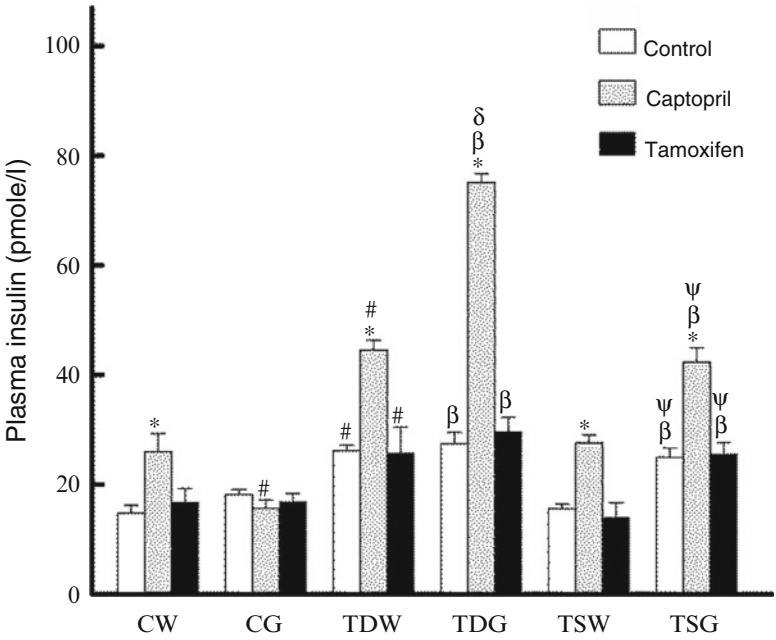

Fig. 8.4 Comparison of plasma insulin concentrations among groups (*CW* control with water intake alone, *CW* + *Cap* CW plus captopril treatment, *CW* + *Tam* CW plus tamoxifen treatment, *CG* control with high sugar intake, *CG* + *Cap* CG plus captopril treatment, *CG* + *Tam* CG plus tamoxifen treatment, *TDW* perinatal taurine depletion with water intake alone, *TDW* + *Cap* TDW plus captopril treatment, *TDW* + *Tam* TDW plus tamoxifen treatment, *TDG* perinatal taurine depletion with high sugar intake, *TDG* + *Cap* TDG plus captopril treatment, *TDG* + *Tam* TDG plus tamoxifen treatment, *TSW* perinatal taurine supplementation with water intake alone, *TSW* + *Cap* TSW plus captopril treatment, *TSW* + *Tam* TSW plus tamoxifen treatment, *TSG* perinatal taurine supplementation with high sugar intake, *TSG* + *Cap* TSG plus captopril treatment, *TSG* + *Tam* TSG plus tamoxifen treatment; $^{*}p < 0.05$ compared to untreated control of same taurine status; $^{\#}p < 0.05$ compared to CW of same treatment; $^{\beta}p < 0.05$ compared to CG of same treatment; $^{\delta}p < 0.05$ compared to TDW of same treatment; $^{\psi}p < 0.05$ compared to TSW of same treatment)

pancreatic damage induced by gestational protein malnutrition (Boujendar et al. 2002; Merezak et al. 2001) and can delay the onset of diabetes mellitus in non-obese diabetic mice (Arany et al. 2004). The present study indicates that perinatal taurine depletion induces mild insulin resistance without hyperglycemia or glucose intolerance in adult female rats, an effect that is not heightened by high sugar intake after weaning. However, short-term inhibition of RAS greatly increases insulin resistance in rats that are perinatally taurine depleted and receive a high sugar diet. Rats that receive perinatally taurine excess followed by the high sugar intake show mild insulin resistance and moderate increase in insulin resistance following RAS treatment in adult rats, and this increase is similar in magnitude to that seen in control rats in response to RAS inhibition. Estrogen inhibition causes moderate to no change in insulin resistance in rats that receive perinatal taurine depletion or excess.

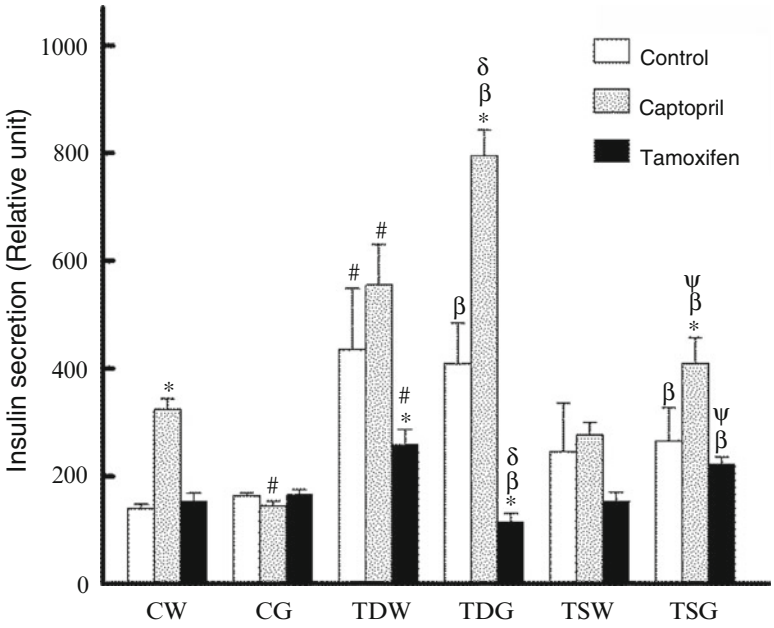

Fig. 8.5 Comparison of pancreatic insulin secretion among groups (*CW* control with water intake alone, *CW* + *Cap* CW plus captopril treatment, *CW* + *Tam* CW plus tamoxifen treatment, *CG* control with high sugar intake, *CG* + *Cap* CG plus captopril treatment, *CG* + *Tam* CG plus tamoxifen treatment, *TDW* perinatal taurine depletion with water intake alone, *TDW* + *Cap* TDW plus captopril treatment, *TDW* + *Tam* TDW plus tamoxifen treatment, *TDG* perinatal taurine depletion with high sugar intake, *TDG* + *Cap* TDG plus captopril treatment, *TDG* + *Tam* TDG plus tamoxifen treatment, *TSW* perinatal taurine supplementation with water intake alone, *TSW* + *Cap* TSW plus captopril treatment, *TSW* + *Tam* TSW plus tamoxifen treatment, *TSG* perinatal taurine supplementation with high sugar intake, *TSG* + *Cap* TSG plus captopril treatment, *TSG* + *Tam* TSG plus tamoxifen treatment; $^{*}p < 0.05$ compared to untreated control of same taurine status; $^{\#}p < 0.05$ compared to CW of same treatment; $^{\beta}p < 0.05$ compared to CG of same treatment; $^{\delta}p < 0.05$ compared to TDW of same treatment; $^{\psi}p < 0.05$ compared to TSW of same treatment)

RAS contributes importantly to the pathogenesis of many disorders including diabetes mellitus and hypertension. Long-term inhibition of the RAS by angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists improves insulin sensitivity and insulin secretion in type 2 diabetes mellitus (Henriksen 2007). Its initial effect is to increase insulin secretion rather than increase tissue insulin sensitivity (Rodriguez et al. 2012; Suzuki et al. 2008), but the long-term effect of the RAS treatment is to improve glucose–insulin regulation. Similarly, in humans and animal models, acute angiotensin II infusion increases insulin sensitivity in basal state or during euglycemic insulin clamp (Buchanan et al. 1993; Fliser et al. 2000; Jonk et al. 2010; Patiag et al. 2000; Townsend and DiPette 1993). This may be the result of redistribution of blood flow to muscles since angiotensin II has a potent and immediate vasoconstrictor action but little immediate metabolic effect. This mechanism may explain why short-term captopril treatment increases insulin secretion

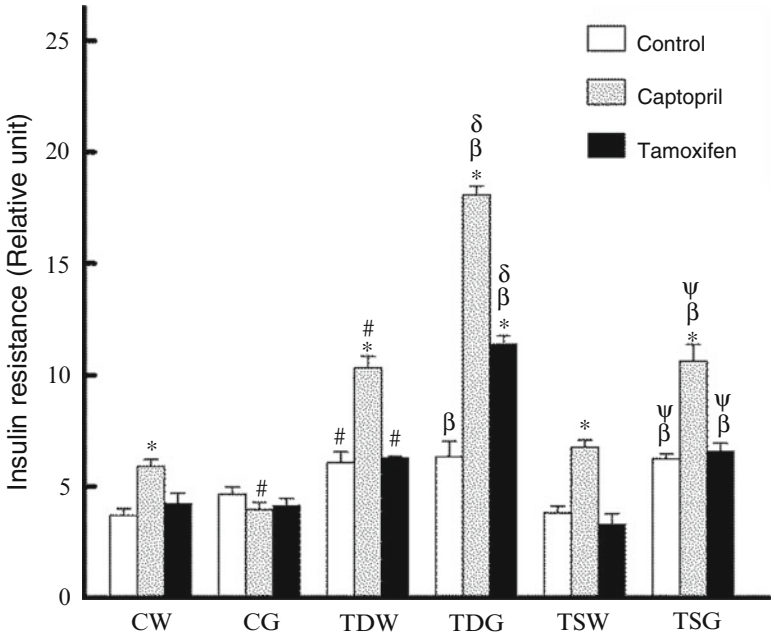

Fig. 8.6 Comparison of insulin resistance among groups (*CW* control with water intake alone, *CW* + *Cap* CW plus captopril treatment, *CW* + *Tam* CW plus tamoxifen treatment, *CG* control with high sugar intake, *CG* + *Cap* CG plus captopril treatment, *CG* + *Tam* CG plus tamoxifen treatment, *TDW* perinatal taurine depletion with water intake alone, *TDW* + *Cap* TDW plus captopril treatment, *TDW* + *Tam* TDW plus tamoxifen treatment, *TDG* perinatal taurine depletion with high sugar intake, *TDG* + *Cap* TDG plus captopril treatment, *TDG* + *Tam* TDG plus tamoxifen treatment, *TSW* perinatal taurine supplementation with water intake alone, *TSW* + *Cap* TSW plus captopril treatment, *TSW* + *Tam* TSW plus tamoxifen treatment, *TSG* perinatal taurine supplementation with high sugar intake, *TSG* + *Cap* TSG plus captopril treatment, *TSG* + *Tam* TSG plus tamoxifen treatment; $^{*}p < 0.05$ compared to untreated control of same taurine status; $^{\#}p < 0.05$ compared to CW of same treatment; $^{\beta}p < 0.05$ compared to CG of same treatment; $^{\delta}p < 0.05$ compared to TDW of same treatment; $^{\psi}p < 0.05$ compared to TSW of same treatment)

and insulin resistance in the present study in all groups except in CG animals. Whether some diabetogenic factors including plasma catecholamine, cortisol, glucagon, and growth hormone play a significant role in this case has to be further investigated.

Perinatal protein malnutrition or taurine depletion induces glucose intolerance, insulin resistance, and diabetes mellitus in adult offspring (Aerts and Van Assche 2002). These changes coincide with a high risk of many cardiovascular disorders particularly obesity, hypertension, and coronary vascular disease. The present experiment confirms this finding. Perinatal taurine depletion induces mild hyperinsulinemia and insulin resistance without hyperglycemia or glucose intolerance. Acute inhibition of the RAS further increases insulin resistance in TDW while RAS inhibition increases insulin resistance much more in TDG rats, suggesting

that the combination of perinatal taurine depletion followed by high sugar intake after weaning amplifies the beneficial chronic effects of RAS on glucose–insulin regulation. In this case, RAS affects both insulin sensitivity and insulin secretion, thus protecting the rats from hyperglycemia and glucose intolerance.

We previously reported that perinatal taurine depletion slightly and significantly blunts baroreceptor reflex sensitivity of heart rate in adult female rats, and this effect was exacerbated by high sugar intake after weaning (Roysommuti et al. 2009a). Acute inhibition of RAS by captopril completely abolishes sugar-induced baroreflex dysregulation and sympathetic nerve overactivity, suggesting that the RAS overactivity underlies these effects (Thaeomor et al. 2010). In contrast, in the present study, a similar RAS inhibition worsened insulin resistance in TDG rats. These data imply that RAS-mediated changes in baroreflex and/or sympathetic nerve overactivity may not contribute to insulin resistance in perinatal taurine-depleted rats. These findings together suggest that the effects of RAS on glucose–insulin regulation may be age (or time post taurine exposure) sensitive.

Angiotensin II can decrease insulin secretion by directly binding to AT_1 receptors on beta cells. Further, angiotensin II may act in conjunction with an aldosterone-receptor complex and alters cell glucose metabolism, ATP-sensitive potassium channels, glucose transporter translocation, and insulin granule exocytosis (Luther and Brown 2011). Recent evidence indicates that pancreatic islet cells contain local RAS that can generate both angiotensin II and angiotensin (1–7). While both local and systemic angiotensin II have similar actions on beta cells, angiotensin (1–7) acts on AT_{1-7} receptor, typically antagonizing angiotensin II's action (Cheng and Leung 2011; Hayden et al. 2011). Other than its hemodynamic action, chronic angiotensin II can induce insulin resistance by acting on AT_1 receptors on insulin-sensitive cells, increasing oxidative stress, and disturbing several cellular mediators of glucose uptake (Luther and Brown 2011).

Perinatal taurine supplementation is commonly taken by pregnant and lactating women and given to newborn children as supplements. Although adverse effects of taurine excess have not been definitively demonstrated in humans, taurine supplementation in late pregnant rats stimulates postnatal growth and induces obesity and insulin resistance in 12-week-old offspring (Hultman et al. 2007). The present study demonstrates that perinatal taurine supplementation without high sugar intake after weaning does not alter body weight, insulin secretion, insulin sensitivity, and glucose tolerance in adult offspring (TSW), and that the actions of RAS and estrogen on glucose–insulin regulation are preserved in these rats. However, when the taurine-supplemented rats are placed on a high sugar diet after weaning (TSG), these parameters are slightly impaired, but remain much better than those of TDG rats. While 7–8-week-old TSG rats do not display insulin resistance severe enough to produce hyperglycemia and glucose intolerance, the effect may amplify with advancing age, as previously reported (Hultman et al. 2007).

The incidence of diabetes mellitus and cardiovascular diseases increases in menopausal women and hormonal therapy can prevent or improve these abnormalities (Kim 2012; Meyer et al. 2011; Kim 2012; Meyer et al. 2011). Estrogen plays a key role in fat distribution and energy expenditure in females. Thus, estrogen

receptor alpha knockout mice display increased adipose tissue, increased weight, insulin resistance, impaired glucose tolerance, hyperglycemia, and decreased energy expenditure (Heine et al. 2000). The present study indicates that estrogen plays a very minor role on glucose–insulin regulation in young adult female rats that receive normal or excess taurine during the perinatal period. In contrast, in rats receiving perinatal taurine depletion followed by the high sugar intake after weaning, estrogen plays a relatively important role in preventing hyperglycemia and glucose intolerance. This very specific effect of estrogen is only seen in the TD group on a high sugar diet. Several lines of evidence indicate that estrogen may directly or indirectly inhibit RAS and sympathetic nervous system (Ashraf and Vongpatanasin 2006); however, inhibition of the RAS in the TD group inhibits the sympathetic nervous system (Thaeomor et al. 2010) and increases both insulin secretion and insulin resistance without hyperglycemia and glucose intolerance.

We reported previously that the high sugar intake (similar to the present protocol) induces renal dysfunction before hyperglycemia and glucose intolerance, an effect that is reversed by captopril treatment (Roysommuti et al. 2002). In rats receiving normal perinatal taurine, high sugar intake after weaning abolishes the effect of RAS inhibition on insulin resistance and insulin secretion, a situation not observed in other groups. While the mechanism underlying this phenomenon cannot presently be resolved, this suggests that perinatal taurine exposure alters RAS in response to a high sugar diet in the adult.

8.5 Conclusion

In summary, perinatal taurine exposure affects adult glucose–insulin regulation and affects the RAS and estrogen regulation of glucose/insulin homeostasis. This effect may involve epigenetic programming of RAS and estrogen function, a phenomenon that can be altered or amplified by a high sugar diet after weaning. While both perinatal taurine excess and depletion can alter the effects of RAS and estrogen on glucose–insulin regulation, perinatal taurine depletion appears to have a much greater effect. Further, in contrast to the chronic effects of RAS and estrogen inhibition, short-term RAS and estrogen inhibition cause rather different responses. The short-term responses found in this study suggest that endogenous estrogen protects against glucose intolerance, while endogenous RAS protects against insulin resistance. How these effects relate to the effects of long-term treatment with RAS or estrogen blockers remains to be determined.

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Chapter 9
Reduced Placental Taurine Transporter (TauT) Activity in Pregnancies Complicated by Pre-eclampsia and Maternal Obesity

Michelle Desforges, Andrea Ditchfield, Chloe R. Hirst, Claire Pegorie, Kathryn Martyn-Smith, Colin P. Sibley, and Susan L. Greenwood

Abstract Taurine is an important nutrient in intrauterine life, being required for fetal organ development and cellular renewal of syncytiotrophoblast (STB), the nutrient transport epithelium of the placenta. As taurine is conditionally essential in human pregnancy, the fetal and placental demand for taurine is met by uptake from maternal blood into STB through the activity of TauT. Pre-eclampsia (PE) and maternal obesity are serious complications of pregnancy, associated with fetal growth restriction (FGR) and abnormal renewal of STB, and maternal obesity is a major risk factor for PE. Here we test the hypothesis that STB TauT activity is reduced in maternal obesity and PE compared to normal pregnancy.

STB TauT activity, measured in fragments of placental tissue, was negatively related to maternal BMI over the range 18–46 kg/m^2 in both the first trimester (7–12 weeks gestation) and at term ($p<0.01$; linear regression). Neither TauT activity nor expression in the first trimester differed to normal pregnancy at term. STB TauT activity was significantly lower in PE than normal pregnancy ($p<0.01$). Neuropeptide Y (NPY), a protein kinase C (PKC) activator which is elevated in PE and obesity, reduced STB TauT activity by 20% (50 pM–50 nM: 2 h) ($p<0.03$). Activation of PKC by phorbol 12-myristate-13-acetate (1 μM) reduced TauT activity by 18% ($p<0.05$). As TauT activity is inhibited by phosphorylation, we propose that NPY activates PKC in the STB which phosphorylates TauT in PE and maternal obesity.

Reduced TauT activity could contribute to dysregulated renewal of STB and FGR that are common to PE and maternal obesity.

M. Desforges • A. Ditchfield • C.R. Hirst • C. Pegorie
K. Martyn-Smith • C.P. Sibley • S.L. Greenwood (✉)
Maternal and Fetal Health Research Centre, Institute of Human Development, Manchester Academic Health Sciences Centre, University of Manchester, Manchester M13 9WL, UK
e-mail: susan.greenwood@manchester.ac.uk

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Abbreviations

TauT	Taurine transporter
STB	Syncytiotrophoblast
MVM	Microvillous membrane
BM	Basal membrane
PE	Pre-eclampsia
FGR	Fetal growth restriction
BMI	Body mass index
NPY	Neuropeptide Y
PKC	Protein kinase C
PMA	Phorbol12-myristate-13-acetate
QPCR	Quantitative polymerase chain reaction

9.1 Introduction

Taurine is a vital nutrient for fetal well-being and animal studies demonstrate a key role for this amino acid in promoting the development of fetal brain, heart, kidney, pancreas, retina, and skeletal muscle (Sturman 1988; Han et al. 2000; Heller-Stilb et al. 2002). In human pregnancy taurine is conditionally essential, as the fetus and placenta lack the enzyme required for taurine synthesis (Gaull et al. 1972), and the fetal demand for taurine must be met by placental transfer from maternal blood. Nutrients are transported across the human placenta via the syncytiotrophoblast (STB), a highly specialised multinucleate epithelium with a microvillous plasma membrane (MVM) in direct contact with maternal blood and a basal membrane (BM) in close apposition to the fetal capillary. Taurine is transported into STB by the Na^+-dependent amino acid transporter TauT, which is expressed on the MVM (Roos et al. 2004). TauT accumulates taurine in the cell (STB concentration 10 mM, maternal and fetal plasma 60 and 120 μM, respectively) such that taurine is the most abundant free amino acid in STB (Philipps et al. 1978). This high intracellular taurine provides a driving force for taurine efflux to the fetus, thought to occur through taurine-permeable anion channels (Shennan and McNeillie 1995; Vallejos and Riquelme 2007).

Studies of fetal growth restriction (FGR) suggest that taurine is important for human fetal growth and development. Idiopathic FGR is a condition in which the fetus fails to achieve its growth potential in the absence of genetic or environmental abnormalities and the growth-restricted fetus is at increased risk of neonatal mortality and morbidity (McCormick 1985) and development of metabolic and cardiovascular disease in later life (Calkins and Devaskar 2011; Barker 1999). Plasma taurine concentration is lower in FGR compared to the normally grown fetus (Economides et al. 1989; Cetin et al. 1990) and this is associated with a significantly lower TauT activity in the STB MVM compared to normal pregnancy (Norberg et al. 1998).

Pre-eclampsia (PE) is a serious condition affecting 5% of pregnancies worldwide and is the leading cause of maternal and fetal mortality (Hibbard and Milner 1994; CESDI 1998). Those fetuses that survive are at increased risk of FGR and associated morbidities. The aetiology of the disease is complex but its origin lies in abnormal placental development and function (Roberts and Gammill 2005) and the only treatment for PE is premature delivery of the placenta and baby. The incidence of PE rises with increasing maternal body mass index (BMI) and is four times higher in morbidly obese women compared to their ideal weight counterparts (Mbah et al. 2010). The reason that maternal obesity is a major risk factor for developing PE is not understood but as obesity, and in particular morbid obesity, is increasing in women of reproductive age (Heslehurst et al. 2010; Mbah et al. 2010) the incidence of PE is likely to rise in parallel. Maternal obesity is itself associated with abnormal fetal growth, increasing the risk of stillbirth with FGR fivefold compared to mothers of ideal weight (Nohr et al. 2005).

As normal fetal growth and development depend on the appropriate supply of taurine by the placenta we hypothesised that, in common with idiopathic FGR, a reduction in STB TauT activity in maternal obesity and PE could contribute to the increased risk of FGR evident in these conditions. We determined STB TauT activity in placental villous tissue isolated from first trimester and term pregnancies and related activity to maternal BMI at booking. In separate studies we compared STB TauT activity in PE with normal pregnancy. As TauT activity in renal cells is inhibited by protein kinase C (PKC)-induced phosphorylation (Han et al. 2006), we explored the possibility that TauT activity is modulated by neuropeptide Y (NPY), a hypothalamic peptide that activates PKC in STB (Robidoux et al. 1998), is elevated in obese individuals (Baltazi et al. 2011), and is higher in maternal plasma in PE compared to normal pregnancy (Khatun et al. 2000).

9.2 Methods

9.2.1 Tissue Acquisition and Ethical Approval

Placentas were obtained with written informed consent as approved by the Central Manchester Research Ethics Committee. First trimester placentas (7–13 weeks gestation) were obtained following elective medical or surgical termination of pregnancy. Gestational age was estimated from the date of last menstrual period and confirmed by ultrasound dating. Term placentas (38–40 weeks gestation) were collected following caesarean section or vaginal delivery from uncomplicated singleton pregnancies. Maternal BMI was determined either at admission (studies of first trimester placentas) or at booking (~12 weeks: studies of term placentas) and women defined as ideal weight (BMI 18.5–24.9), overweight (25–29.9), or obese (>30). Placentas were also collected from women (BMI <30) with PE (defined as hypertension >140/90 mmHg in previously normotensive women plus proteinuria >300 mg/L in a 24-h urine collection after 20 weeks gestation).

9.2.2 Quantitative PCR Analysis of TauT mRNA Expression

Placental tissue was lysed and total RNA extracted using Absolutely RNA Miniprep Kit (Stratagene, USA). RNA was quantified using Quant-iT Ribogreen kit (Molecular Probes) and 100 ng of total RNA from each sample reverse transcribed using AffinityScript cDNA synthesis kit with random primers (Stratagene, USA). mRNA for TauT (SLC6A6) and β-actin were quantified by QPCR using Stratagene's MX3000P real-time PCR machine and Brilliant SYBR Green I QPCR mastermix (Stratagene, USA) as described previously (Desforges et al. 2006). Primers (MWG-Biotech) for SLC6A6 (forward: 5′ CGTACCCCTGACCTACAACAAA 3′, reverse: 5′ CAGAGGCGGATGACGATGAC 3′) and β-actin (Lacey et al. 2005) were used at a final concentration of 200 nM. QPCR data are presented as median values of percentage expression relative to a 40-week placental sample, designated the calibrator, which was included in each QPCR run as an internal standard (Lacey et al. 2005). The data were analysed by Kruskal–Wallis and Dunn's post hoc tests and $p<0.05$ was considered significant.

9.2.3 Western Blot Analysis

Protein was extracted from placental villous homogenates and Western blot analysis of TauT and β-actin protein expression carried out as described previously (Champion et al. 2004; Desforges et al. 2006) using a rabbit anti-TauT affinity-purified polyclonal antibody (Alpha Diagnostics. 1:400 dilution; 2.5 μg/ml). For a negative control, the purified TauT antigenic peptide was used in 5× excess to pre-absorb the antibody. Primary and horseradish peroxidase-conjugated secondary antibody incubations were performed for 1 h at room temperature. Positive signals were detected using ECL and the density of the immunoreactive species was assessed using a GS 700 Imaging Densitometer (Bio-Rad Laboratories, Hemel Hempstead, UK) with Molecular Analyst software. Data were analysed using a Mann Whitney test and $p<0.05$ was considered significant.

9.2.4 TauT Activity Measurements and Effect of Neuropeptide Y

Placental villous fragments were dissected and rinsed in a 1:1 or 1:3 mix of Dulbecco modified Eagle medium (DMEM)/control Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 5.6 mM glucose, pH 7.4). Uptake of ^{3}H-taurine (5/10 μM; 0.5/1 μCi/ml) into villous fragments was measured in control and Na^+-free Tyrode's buffer (135 mM choline chloride replaced NaCl, pH 7.4) as previously described (Greenwood and Sibley 2006). The Na^+-dependent component of ^{3}H-taurine uptake, representing TauT activity, was calculated and expressed per mg fragment protein. The uptake of ^{3}H-taurine at initial rate was considered to

be a measure of TauT activity in STB. To study the effect of NPY/PMA on TauT activity, placental fragments were incubated for 2 h (37°C) in NPY (5pM-5nM) or PMA (1 μM) prior to measurement of ^{3}H-taurine uptake. TauT activity in treated tissue was expressed relative to the corresponding untreated control and analysed using a Wilcoxon Signed Rank test where $p<0.05$ was considered significant.

9.3 Results

9.3.1 Placental TauT Expression and Activity in Normal Pregnancy

There were no significant differences in SLC6A6 mRNA expression between 6–9 weeks and 10–13 weeks gestation, or between these gestations and term (Fig. 9.1a). Western blot analysis of first trimester and term placental homogenates (Fig. 9.1b) revealed a single immunoreactive signal at ~70 kDa in all samples, which corresponds to the predicted size of TauT (Ramamoorthy et al. 1994). Pre-absorption of the antibody with 5× peptide abolished this signal (data not shown), confirming antibody specificity for TauT. Densitometric analysis revealed no differences in either TauT (Fig. 9.1b) or β-actin (used to indicate protein loading; data not shown) expression in placentas from the first trimester compared to term. Na^{+}-dependent ^{3}H-taurine uptake (Fig. 9.1c), representing TauT-specific activity, by first trimester and term placental villous fragments was linear over 5–30 min ($p<0.005$ for both; least squares linear regression) indicating that uptake was at initial rate. TauT activity in the first trimester did not differ to that at term (Fig. 9.1c).

9.3.2 Placental STB TauT Activity in Maternal Obesity and PE

In both the first trimester and at term, there was significant negative relationship between STB TauT activity and maternal BMI (Fig. 9.2a). Comparison of placental TauT activity in obese women (BMI >30) and ideal weight women (BMI 18–24.9) revealed a significant difference at both gestations ($p<0.05$, MannWhitney-*U* test). TauT activity was also significantly lower in PE (ideal weight) than normal pregnancy (Fig. 9.2b).

9.3.3 Placental TauT Activity Following Exposure to Neuropeptide Y

Figure 9.3a shows concentration-dependent inhibition of TauT activity (30 min) in term villous fragments by NPY (50 pM–50 nM; 2 h). TauT activity was reduced to a similar extent by the PKC activator PMA (1 μM) (Fig. 9.3b).

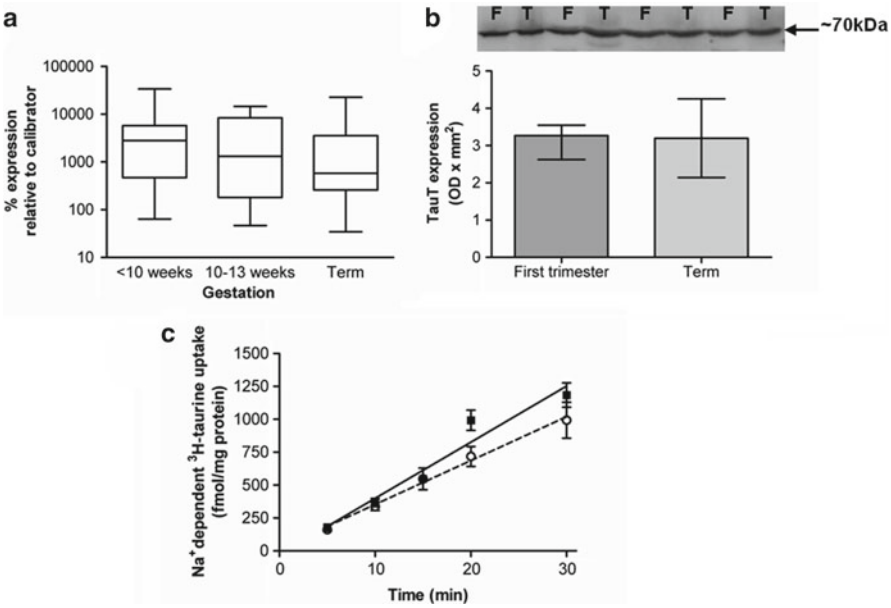

Fig. 9.1 Placental TauT expression and activity are similar in first trimester and at term. (**a**) TauT mRNA expression relative to a 40-week placental sample, designated the calibrator, included in each run as an internal standard. The groups are early first trimester (6–9 weeks, $n=23$), late first trimester (10–13 weeks, $n=12$), and term (38–40 weeks, $n=21$). (**b**) Western blot of first trimester (F) and term placental (T) samples probed for TauT. A single immunoreactive species was detected at the expected molecular weight of 70 kDa. Bar chart displays densitometric analysis of signal intensity ($n=4$ for each group, median and interquartile range). (**c**) Na^+-dependent ^{3}H-taurine uptake by first trimester (*open circles*) and term (*closed squares*) placental villous fragments ($n=6$ in each group; mean ± SE). At both gestations, ^{3}H-taurine uptake significantly increased with time ($p<0.005$; least square linear regression). There was no difference in ^{3}H-taurine uptake between first trimester and term placenta (2-way ANOVA)

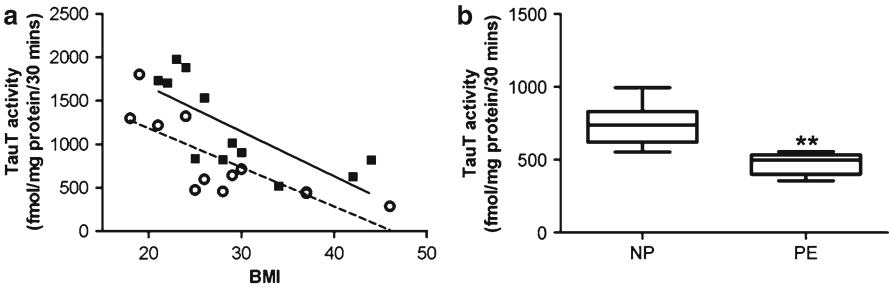

Fig. 9.2 Placental STB TauT activity is negatively related to maternal BMI and reduced in pre-eclampsia (PE). (**a**) Negative relationship between maternal BMI and TauT activity in first trimester (*open circles*, $n=12$) and term (*closed squares*, $n=12$) placental villous fragments. Least square linear regression; $r^2=0.60$ and 0.54, respectively, $p<0.01$ for each. (**b**) TauT activity in normal pregnancy (NP, $n=9$) and PE (PE, $n=5$) in women with BMI <30. $p<0.01$: Mann Whitney-*U* test

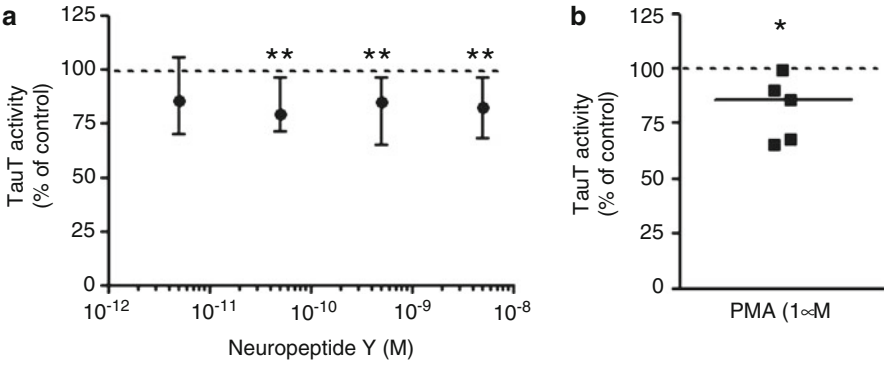

Fig. 9.3 Placental STB TauT activity is inhibited by neuropeptide Y (NPY) and PMA. Effect of short-term exposure (2 h) to (**a**) NPY (median and interquartile range; $n = 10$) and (**b**) PMA (line at median) on TauT activity in placental fragments (fmol/mg protein/30 min) expressed as % of control. $^{**}p < 0.03$; $^{*}p < 0.05$ vs. 100% (control): Wilcoxon signed-rank test

9.4 Discussion

The activity of TauT in the MVM of the human placental STB is important to achieve a high intracellular taurine concentration and maintain a gradient that favours taurine efflux towards the fetus. The finding that STB TauT activity and expression are similar in placentas from the first trimester and at term implies that taurine delivery to the fetus is important for fetal growth and development throughout pregnancy.

Maternal obesity and PE are associated with increased risk of poor fetal outcome, including stillbirth and FGR, which might be related to inadequate transfer of taurine across the placenta. In support of this we found that STB TauT activity was inversely related to maternal BMI, recorded at the first antenatal visit, in both the first trimester and at term. Average TauT activity in women with a BMI >30 was 60–70% lower than their ideal weight (BMI 18.5–24.9) counterparts at both gestations. Furthermore, STB TauT activity was ~35% lower in placentas of women with PE (BMI < 30) compared to women having normal pregnancy. This reduction in TauT activity could predispose to FGR in these pregnancy conditions but is unlikely to be singularly responsible, as most of the babies born to the women studied were appropriately grown for gestational age.

A reduction in MVM STB TauT activity will lower intracellular taurine in the absence of compensatory changes in taurine efflux. Placental taurine, measured by chromatography, was found to be lower in infants with low birth weight compared to normal (Ghisolfi et al. 1989) and in preliminary studies (Hirst et al. 2012) we showed that the accumulation of radiolabelled taurine in STB at steady state was lower in PE than in normal pregnancy. In addition to restricting taurine efflux to the fetus, a reduction in intracellular taurine has implications for the maintenance of STB. STB is a unique epithelium, being renewed during pregnancy by a process of cellular turnover involving proliferation of the underlying

cytotrophoblast cells followed by differentiation, fusion, and incorporation of their nuclei into the STB (Huppertz et al. 2006; Heazell and Crocker 2008). STB volume is maintained by the deportation of aged nuclei into maternal blood. Coordination of STB renewal is critical for normal pregnancy and cellular turnover is dysregulated in PE (Crocker et al. 2003; Heazell and Crocker 2008) and maternal obesity (Higgins et al. 2011). This dysregulation causes the release of toxic material from STB into maternal blood which can initiate a widespread inflammatory response in the mother and trigger PE (Roberts and Gammill 2005). Using cytotrophoblast cells in vitro, we showed that knocking down TauT expression with siRNA inhibited TauT activity, reduced intracellular taurine, and inhibited the differentiation and fusion of cells to form multinucleate syncytia (Parsons et al. 2009). In addition, TauT knockdown increased apoptosis in response to TNFα (Desforges et al. 2010), a cytokine that is elevated in PE (Tosun et al. 2010) and maternal obesity (Challier et al. 2008). Thus, the reduction in TauT activity and intracellular taurine in PE could impair STB renewal and lower cytoprotection to damaging cytokines, leading to reduced nutrient delivery to the fetus and the release of necrotic material to the mother. The fall in STB TauT activity with increasing maternal BMI, particularly in the first trimester, could predispose to abnormal STB renewal later in pregnancy and might in part explain the increased incidence of PE in obese mothers.

The reduction in STB TauT activity in FGR is not associated with a change in expression (Roos et al. 2004) and our preliminary data show that TauT protein expression is also unaffected by PE (Hirst et al. 2012) or morbid obesity (unpublished observation). This suggests that the low STB TauT activity in these conditions of pregnancy is due to post-translational down-regulation of transporter activity. Regulation of TauT activity has not been studied extensively in placenta but in common with other tissues, activity in trophoblast-derived choriocarcinoma cells (JAr) shows adaptive regulation in response to altered taurine concentration (Jayanthi et al. 1995). As maternal plasma taurine concentration is higher in the first trimester of pregnancies that subsequently develop FGR, compared to those that proceed normally (Di Giulio et al. 2004), adaptive down-regulation could contribute to the reduced STB TauT activity in FGR. However, the reduction in STB TauT activity in FGR has been demonstrated at 32–39 weeks (Norberg et al. 1998) and at this later stage of gestation maternal plasma taurine concentration is reported to be unchanged (Economides et al. 1989) or significantly lower (Cetin et al. 1990) in FGR than normal pregnancy. In PE without FGR, maternal plasma taurine does not differ from normal (Evans et al. 2003) and, although it has yet to be measured in obese mothers in late pregnancy, plasma taurine levels are lower in obese individuals in the general population compared to their ideal weight counterparts (Zhang et al. 2004). Therefore it is unlikely that the reduced STB TauT activity in PE and obesity is caused by adaptive down-regulation. It is of interest that, following labour, the taurine concentration in umbilical blood of normally grown fetuses is reported to be higher in PE than normal pregnancy (Evans et al. 2003). Bearing in mind that STB TauT activity was shown to be reduced in PE in the current study, it is possible that higher umbilical plasma taurine arises from altered fetal metabolism and/or reduced uptake of taurine

from fetus to placenta in PE. STB basal membrane expresses TauT (Roos et al. 2004) and vesicle studies show that the activity of TauT in this membrane is not affected by FGR (Norberg et al. 1998). In situ, TauT on the BM would transport taurine from the fetus into STB but the influence of BM TauT activity on net maternal-fetal taurine flux has not been determined in normal or compromised pregnancy. Studies of maternal, placental, and fetal taurine levels in PE and maternal obesity following caesarian section delivery, as well as taurine uptake and efflux mechanisms on STB MVM and BM, are required to gain a better understanding of the relationships beween taurine concentration, TauT activity, and delivery of taurine to the fetus.

In renal epithelial cells, TauT activity is down-regulated by PKC-induced phosphorylation of the transporter (Han et al. 2006) and activation of PKC in JAr cells inhibits TauT activity (Kulanthaivel et al. 1991). STB expresses several PKC isoforms (Tertrin-Clary et al. 1990; Ruzycky et al. 1996), and these can be activated in cytotrophoblast cells in vitro by NPY, a hyopthalamic peptide that is also produced by STB, through activation of Y1 and Y3 receptors on the MVM (Robidoux et al. 1998). As NPY levels in maternal serum are higher in PE than normal pregnancy (Khatun et al. 2000), and elevated in obese compared to ideal-weight individuals in the general population (Baltazi et al. 2011), we investigated whether NPY could downregulate TauT activity in STB. NPY treatment of villous tissue (2 h) induced a small but significant reduction in TauT activity at pathophysiologically relevant concentrations (Petraglia et al. 1989). We also confirmed previous reports that the PKC activator PMA reduces STB TauT activity (Roos et al. 2004). It is possible that NPY is a modulator of TauT activity that is common to both PE and obesity and future work will address whether PCK is activated in PE and obesity and whether there is an increase in phosphorylated TauT compared to normal pregnancy.

9.5 Conclusion

STB TauT activity is lower in placentas of obese mothers compared to mothers of ideal weight and inversely related to maternal BMI in both the first trimester and at term. STB TauT activity is also lower in PE compared to normal pregnancy. We propose that this reduction in TauT activity lowers STB taurine concentration which impairs STB renewal and reduces taurine transfer to the fetus, contributing to the increased risk of FGR in these conditions. The fall in STB TauT activity with increasing maternal BMI in the first trimester could predispose to abnormal STB renewal and development of PE later in pregnancy. The PKC activator, NPY, caused a small but significant reduction in TauT activity and could downregulate TauT in both PE and obesity. Determining the reasons for, and consequences of, reduced placental TauT activity could lead to strategies to improve pregnancy outcome and fetal growth in obesity and PE.

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Chapter 10
Effects of Taurine Supplementation Upon Food Intake and Central Insulin Signaling in Malnourished Mice Fed on a High-Fat Diet

Rafael L. Camargo, Thiago M. Batista, Rosane A. Ribeiro, Lício A. Velloso, Antônio C. Boschero, and Everardo M. Carneiro

Abstract Feeding behavior is a major determinant of body composition, adiposity, and glucose homeostasis. Both obesity and malnutrition are risk factors for the metabolic syndrome and are associated with altered food intake. Here we assessed the effects of taurine (TAU) supplementation upon adiposity, food intake, and central insulin signaling in malnourished mice fed on a high-fat diet (HFD). Weaned male C57BL/6 mice were fed a control (14% protein-C) or a protein-restricted (6% protein-R) diet. After 6 weeks, both groups received or not HFD for 8 weeks (CH and RH). Half of the HFD groups were supplemented with 5% TAU (CHT and RHT). Both HFD groups were overweight and showed increased perigonadal and retroperitoneal fat pads. TAU supplementation attenuated obesity in CHT but not in RHT mice. HFD induced hypercholesterolemia and glucose intolerance, although only CH group presented fasting hyperglycemia. TAU supplementation also improved glucose homeostasis only in CHT mice. Western blot analysis showed a reduction of 55% in CH hypothalamic content of phosphorylated IRS-1 (pIRS-1) at basal condition compared with C. TAU treatment increased 35% Akt phosphorylation levels in CHT without modification in RHT hypothalamus. However, TAU supplementation did not alter hypothalamic pIRS-1 amount. CH and RH mice presented increased calorie intake that was normalized in CHT but not in RHT. In conclusion, mice fed on an HFD developed obesity, hypercholesterolemia, glucose intolerance, and

R.L. Camargo (✉) • T.M. Batista • A.C. Boschero • E.M. Carneiro
Departamento de Biologia Funcional e Estrutural, Instituto de Biologia,
Universidade Estadual de Campinas, SP, Brazil
e-mail: rlcamargo.bio@gmail.com

R.A. Ribeiro
Núcleo em Ecologia e Desenvolvimento Sócio-Ambiental de Macaé (NUPEM),
Universidade Federal do Rio de Janeiro (UFRJ), Macaé, RJ, Brazil

L.A. Velloso
Departamento de Clínica Médicas, Faculdade de Ciências Médicas,
Universidade Estadual de Campinas, SP, Brazil

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_10,

increased calorie intake. TAU promoted increased hypothalamic insulin action only in CH mice which was linked to prevention of overfeeding, obesity, and glucose intolerance. Protein-restriction promoted metabolic damages that were not prevented by TAU supplementation.

Abbreviations

TAU	Taurine
ARC	Arcuate nuclei
T2DM	Type 2 diabetes mellitus
CNS	Central nervous system
ICV	Intracerebroventricular
TNF	Tumor necrosis factor

10.1 Introduction

The hypothalamic Arcuate nuclei (ARC) control feeding behavior and sympathetic/parasympathetic tones modulating body metabolism (Plum et al. 2006; Schwartz et al. 2000). Insulin crosses the blood–brain barrier and binds to its receptor (IR) located in the ARC. IR activated phosphorylates itself and its substrates (IRS) and leads to phosphatidylinositol-3 kinase (PI3K) activation and increased Akt phosphorylation (pAkt) (Tsai et al. 2003). Central activation of this pathway inhibits the synthesis of orexigenic neuropeptides, such as the neuropeptide Y (NPY) and the agouti peptide (AgRP), and stimulates the secretion of anorexigenic neuropeptides, proopiomelanocortin (POMC), and the cocaine-and-amphetamine-regulated transcript (CART) (Morton et al. 2006; Badman and Flier, 2005). Therefore, the hypothalamic activation of insulin signaling regulates food intake in order to maintain an adequate body weight.

Obesity is a factor linked to peripheral and central insulin resistance leading to type 2 diabetes (T2DM) (Kahn et al. 2006). Impaired central action of insulin in the obesity results in a vicious circle which provides an increase in weight gain (Schwartz and Porte 2005).

Protein-malnutrition during gestation is a frequent cause of low birth weight, and is associated with increased susceptibility for developing chronic diseases (Barker et al. 1993). Previous reports showed no alteration in IRS1/2 and Akt protein content in the cerebral cortex from calorie-restricted rats (Mollinedo et al. 2010). Disrupted insulin signaling in the central nervous system (CNS) reflects on food intake and may link malnutrition in early life and development of T2DM in adulthood (Orozco-Solís et al. 2010).

Taurine (TAU) is a sulfur-containing amino acid present in high concentration in newborn mice brain. This amino acid is responsible for the maintenance of intracellular osmotic balance (L'Amoreaux et al. 2010). TAU treatment during mice development causes increase of size and number of pancreatic islets without altering exocrine

function (El Idrissi et al. 2009). Central administration of TAU exerts an anorexigenic effect, decreasing the expression of orexigenic neuropeptides and reducing food intake (Solon et al. 2012). As occurs in peripheral tissues (Carneiro et al. 2009), TAU also potentiates insulin action in hypothalamus (Solon et al. 2012).

In this way, how little is known about TAU effects upon malnutrition and obesity in hypothalamic insulin action and food intake regulation. Here we show that TAU supplementation improved body glucose control and enhanced central insulin sensitivity, resulting in decreased caloric intake that reduces body fat deposition in obese mice.

10.2 Methods

10.2.1 Animals and Diets

All experiments were approved by the ethics committee at UNICAMP. The studies were carried out on weaned 30-day-old male C57Bl/6J mice obtained from the breeding colony at UNICAMP and maintained at 22 ± 1°C, on a 12-h light–dark cycle, with free access to food and water intake. The mice were fed a control (14% protein-C) or a protein-restricted (6% protein-R) diet. After 6 weeks, both groups received or not high-fat diet (HFD) for 8 weeks (CH and RH). Half of the HFD groups were supplemented with 5% TAU in their drinking water since the weaning time until the end of the experiment (CHT and RHT groups).

10.2.2 General Nutritional Parameters

Body weight (BW) was measured at the end of the experimental period. In the last week of HFD treatment both mice groups were placed in metabolic cages and had their food intake monitored, as previously reported (Morrison & Campbell, 1960). At the end of the experimental period (14 weeks), fasted mice were decapitated, had their blood collected, and plasma was stored at −20°C. Commercial kits were used according to the manufacturer's instructions for quantification of total plasma cholesterol (Roche/Hitachi; Indianapolis, USA). Plasma glucose was measured using a glucose analyzer (Accu-Chek Advantage, Roche Diagnostic, Switzerland).

10.2.3 Food Intake

Food intake was monitored during the last week of treatment (8th week) using metabolic cages as previously reported (Morrison & Campbell, 1960).

10.2.4 Intraperitoneal Glucose Tolerance Tests

For intraperitoneal glucose tolerance tests (ipGTT), blood glucose levels (time 0) were measured in overnight fasted mice using a glucose analyzer (Accu-Chek Advantage, Roche Diagnostic, Switzerland). A glucose load of 2 g/Kg body weight was then administered by ip injection and additional blood samples were collected at 15, 30, 60, and 120 min.

10.2.5 Western Blot

For protein expression experiments, after 12h of fasting the hypothalamus from all mice groups were removed and immediately homogenized in buffer containing 100 mmol/L Tris pH 7.5, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L PMSF, and 1% Triton X-100. The extracts were then centrifuged at 12,000 rpm at 4°C for 40 min to remove insoluble material. The protein concentration was assayed using the Bradford dye method (Bradford, 1976), using BSA as a standard curve and Bradford reagent (Bio-Agency Lab., São Paulo, SP, BRA). For SDS gel electrophoresis and Western blot analysis, the samples were treated with a Laemmli sample buffer containing dithiothreitol. After heating to 95°C for 5 min, the proteins were separated by electrophoresis (70 μg protein/lane, 10% gels). Following electrophoresis, proteins were transferred to nitrocellulose membranes. The nitrocellulose filters were treated overnight with a blocking buffer (5% nonfat dried milk, 10 mmol/L Tris, 150 mmol/L NaCl, and 0.02% Tween 20) and were subsequently incubated with a polyclonal antibody against pAkt (1:1,000, cat. sc-7985R, Santa Cruz Biotechnology), Akt (1:1000, cat. sc-8313, Santa Cruz Biotechnology), pIRS1 (1:1,000, cat abcam-4888), and IRS-1 (1:100, cat abcam-653-200). Detection was performed after 2-h incubation with a horseradish peroxidase-conjugated secondary antibody (1:10,000, Invitrogen, São 5 Paulo, SP, BRA). The band intensities were quantified by optical densitometry using the free software, Image Tool (http://ddsdx.uthscsa.edu/dig/itdesc.html). Densitometry values obtained from phosphorylated proteins (pAkt and pIRS-1) were normalized by total protein expression (Akt and IRS-1), as previously described (Batista et al. 2012; Ribeiro et al. 2012).

10.2.6 Statistical Analysis

Results are presented as means + SEM for the number of determinations (n) indicated. The statistical analyses were carried out using a one-way analysis of variance (ANOVA) followed by the Newman–Keuls post hoc test ($P < 0.05$) and performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

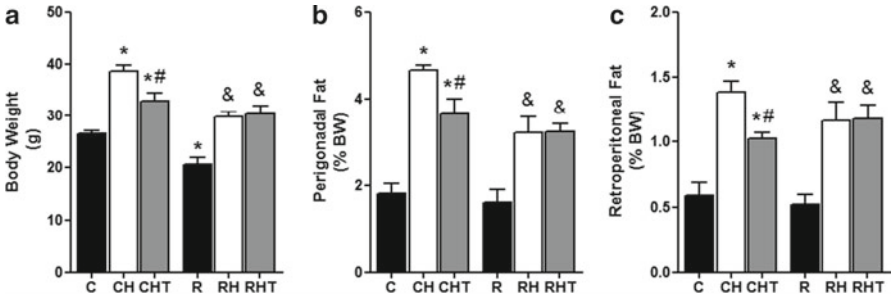

Fig. 10.1 (**a**) Body weight; (**b**) retroperitoneal and (**c**) perigonadal fat pads in C, CH, CHT, R, RH, and RHT mice. Values are means ± SEM. *$P < 0.05$ compared to C, # compared to CH, and compared to R. $N = 6–12$

10.3 Results

10.3.1 Mice Features

The low-protein diet promoted lower body weight in R mice compared to C ($P < 0.05$; Fig. 10.1a). HFD increased BW as well as perigonadal and retroperitoneal fat pads ($P < 0.05$; Fig. 10.1a–c). TAU supplementation prevented obesity and adiposity in CHT but not in RHT group. HFD treatment also increased fasting plasma glucose and cholesterol which were reduced in CHT but not in RHT group (Tab.1).

Both CH and RH groups were glucose intolerant as indicated by a higher area under glycemic curve (AUC) during the ipGTT (Fig. 10.2c). Blood glucose levels peaked at 30 min for all experimental groups but its decay was impaired in CH and RH mice. TAU supplementation improved glucose tolerance in CHT but not in RHT group as indicated by the AUC (Fig. 10.2c). Both groups treated with HFD increased energy intake and TAU supplementation decreased the caloric intake only in CHT group (Fig. 10.3).

10.3.2 Insulin-Signaling Proteins in Hypothalamus

Malnourished mice showed a 38% lower pIRS-1 hypothalamic amount compared to C mice ($P < 0.05$; Fig. 10.4a). HFD reduced basal hypothalamic pIRS-1 in CH and CHT mice compared to C ($P < 0.05$). In RH mice, despite a decrease of 17% in pIRS-1 protein content in the hypothalamus, no statistical significant difference was observed when compared to R mice (Fig. 10.4a). Akt phosphorylation did not differ in both types of mice groups fed on an HFD compared with their respective controls (Fig. 10.4b). However, TAU supplementation increased 35% pAkt hypothalamic content in CHT mice compared to CH ($P < 0.05$: Fig. 10.4b).

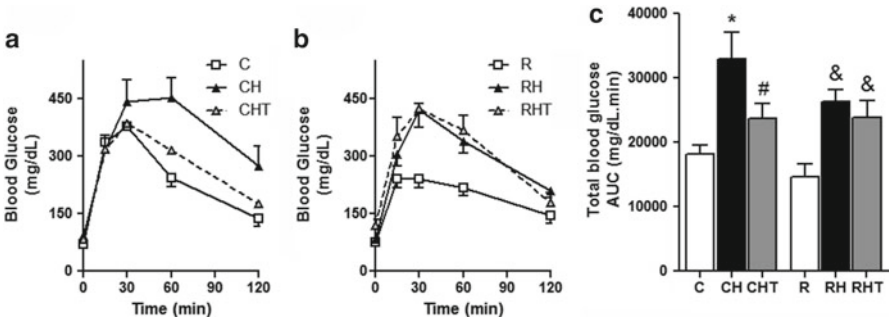

Fig. 10.2 Glucose tolerance test (GTT). (**a**) Groups C, CH, and CHT; (**b**) groups R, RH, and RHT; (**c**) area under curve. *Bars* represent means ± SEM of the area under curve. *$p < 0.05$ compared to C, # compared to CH, and compared to R. $N = 6$

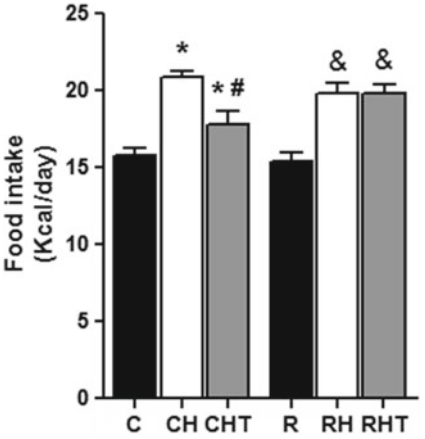

Fig. 10.3 Food intake (Kcal/dia) of C, CH, CHT, R, RH, and RHT mice. Values are mean ± SEM. *$p < 0.05$ compared to C, # compared to CH, and compared to R groups. $N = 7$

10.4 Discussion

Fetus that underwent a protein restriction in utero showed poor islet vascularization as well as increased apoptosis (Snoeck et al. 1990; Boujendar et al. 2002). Rats submitted to protein restriction during gestation become diabetic at 17 months of age (Petry et al. 2001). In addition these rodents showed neuroendocrine and sympathetic action alterations, changing growth hormone (GH)–insulin-like growth factor I (IGF-I) axis (Heilbronn and Ravussin, 2003). Our results indicate an increase of adiposity and glucose intolerance in malnourished mice submitted to HFD. These evidences demonstrate that fetal and early life stages are critical periods where nutrient deprivation may provoke long-lasting effects favoring diseases in adult life.

Previous reports showed that TAU plasma concentrations are reduced in different types of experimental obese rodents. TAU supplementation prevented body weight gain and adiposity induced by HFD (Tsuboyama-Kasaoka et al. 2006).

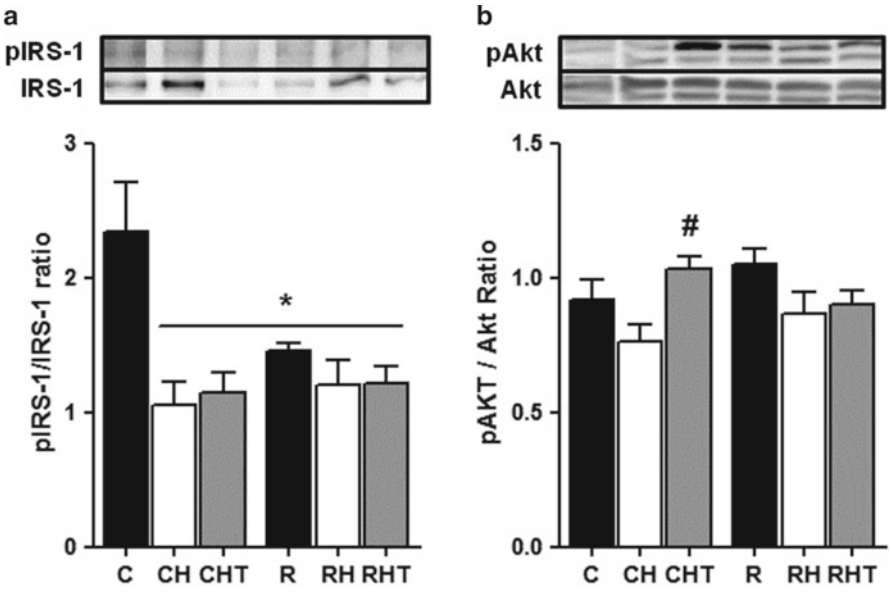

Fig. 10.4 Protein expression. (**a**) Ratio of pIRS-1 by IRS-1 total content [pIRS-1/IRS-1]; (**b**) ratio of pAKT by AKT total content [pAKT/AKT]. Data represents protein expression in the hypothalamus of animals C, CH, CHT, R, RH, and RHT. *Bars* represent means ± SEM. $*p < 0.05$ compared to C. $N = 4–8$

Here, we also show that TAU supplementation prevented body fat accumulation, hypercholesterolemia, and hyperglycemia caused by HFD in normal-protein mice (Table 10.1 and Fig. 10.1). Nardelli et al. (2011) also showed reduction in body adiposity as well as lower lipids in plasma and liver of obese rats supplemented with TAU. Thus, lower lipid storage may contribute to the improved plasma lipid profile observed in our study.

In addition, TAU improved glucose tolerance in obese normal-protein mice (Fig. 10.2). This effect may be due to a possible interaction of TAU with the IR (Maturo and Kulakowski, 1988; Carneiro et al. 2009). Also, TAU treatment normalizes islet-cell proliferation and insulin secretion in fetal protein-restricted rodents (Kalbe et al. 2005). Restoration of insulin secretory capacity was observed in rats submitted to protein restriction started at weaning and treated with TAU (Batista et al. 2012).

Recently, Solon et al. (2012) showed that TAU dose dependently had an anorexigenic effect upon hypothalamus. Intracerebroventricular (icv) administration of TAU reduces NPY expression without modifying the amount of POMC, decreasing food intake in rats. This TAU effect was accompanied by enhanced hypothalamic content of pAkt. In addition, TAU increases insulin hypothalamic sensitivity via Akt/FOXO1, JAK2, STAT3, and mTOR activation (Solon et al. 2012), suggesting a direct interaction of the amino acid with this pathway in CNS as already described in peripheral tissues (Carneiro et al. 2009). Our study is in accordance with the

Table 10.1 Plasma cholesterol and blood glucose of fasted C, CH, CHT, R, RH, and RHT mice

	C	CH	CHT	R	RH	RHT
Glucose (mg/dL)	68.75 ± 6.51	98.50 ± 11.18*	80.43 ± 3.84*,#	78.67 ± 6.73	86.67 ± 5.47&	93.86 ± 5.14&
Cholesterol (mg/dL)	108.2 ± 11.64	146.7 ± 6.5*	117.2 ± 11.24#	98.27 ± 10.61	142.4 ± 9.19&	130.9 ± 8.28&

Values are means ± SEM. *$P < 0.05$ compared to C, # compared to CH, & compared to R. $N = 6–12$

observations above since TAU supplementation also decreased food intake and increased hypothalamic pAkt in control mice fed on an HFD.

Few studies reported CNS insulin action in malnutrition. Mollinedo et al. (2010) described no significant difference in the IRS-1/2 and Akt protein content in the cerebral cortex of rats submitted to caloric restriction. On the other hand, microarray analysis in the hypothalamus of rats born to protein-restricted dams revealed increased expression of several genes involved in insulin signaling such as IRS-1, PI3K, and Akt (Orozco-Solís et al. 2010). However, in our study we observed lower pIRS-1 without modification on pAkt hypothalamic content in protein-restricted mice (Fig. 10.4).

It is known that rats fed on an HFD presented high levels of tumor necrosis factor (TNF)-α, interleukin-1β, and interleukin-6 in the hypothalamus (De Souza et al. 2005). Hypothalamic TNF-α impairs the anorexigenic action induced by insulin and leptin, via serine phosphorylation of IRS-1, inactivating and inhibiting subsequent events of hypothalamic insulin signaling (Romanatto et al. 2007; Amaral et al. 2006; De Souza et al. 2005). Moreover, other proteins such as SOCS3 and PTP-1B are activated and promote degradation or dephosphorylation of the IRS (Pirola et al. 2004; Zabolotny et al. 2008; Bence et al. 2006). In accordance with these studies, our model of obesity induced by HFD also showed a decreased hypothalamic content of pIRS-1 indicating central insulin action impairment. TAU supplementation enhanced pAkt and decreased food intake only in CHT mice (Figs. 10.3 and 10.4), showing that malnutrition leads to hypothalamic dysfunction that was not prevented by TAU.

10.5 Conclusion

In conclusion our results show that mice fed an HFD developed obesity, hypercholesterolemia, glucose intolerance, and calorie intake disturbances, both in control and malnourished mice. TAU promoted increased hypothalamic insulin action which prevented overfeeding and obesity. Protein-restriction promoted metabolic disturbances that were not restored by TAU supplementation showing enhanced risk to develop metabolic syndrome.

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Chapter 11
Positive Correlation Between Serum Taurine and Adiponectin Levels in High-Fat Diet-Induced Obesity Rats

Jeong Soon You, Xu Zhao, Sung Hoon Kim, and Kyung Ja Chang

Abstract The purpose of this study was to investigate the relationship between serum taurine level and serum adiponectin or leptin levels in high-fat diet-induced obesity rats. Five-week-old male Sprague–Dawley rats were randomly divided into three groups for a period of 8 weeks (normal diet, N group; high-fat diet, HF group; high-fat diet + taurine, HFT group). Taurine was supplemented by dissolving in feed water (3% w/v), and the same amount of distilled water was orally administrated to N and HF groups. In serum, adiponectin level was higher in HFT group compared to HF group. The serum taurine level was negatively correlated with serum total cholesterol (TC) level and positively correlated with serum adiponectin level. These results suggest that dietary taurine supplementation has beneficial effects on total cholesterol and adiponectin levels in high-fat diet-induced obesity rats.

Abbreviations

N	Normal diet
HF	High-fat diet
HFT	High-fat diet + taurine
E-fat	Epididymal fat
R-fat	Retroperitoneal fat
TG	Triglyceride
TC	Total cholesterol
HDL-C	High-density lipoprotein-cholesterol
BW	Body weight

J.S. You • X. Zhao • K.J. Chang (✉)
Deptartment of Food and Nutrition, Inha University, Incheon, South Korea
e-mail: kjchang@inha.ac.kr

S.H. Kim
Department of Chemistry, Konkuk University, Seoul, South Korea

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11.1 Introduction

Excess of intra-abdominal fat is associated with metabolic syndrome (Maury and Brichard 2010) and cardiovascular disease (Fasshauer et al. 2004). Adipose tissue is the biggest storage site for excess energy (Large et al. 2004); however, recent studies suggest that adipocytes are not merely energy-storing cells but also they secrete a variety of adipokines (Fantuzzi 2005). Most adipokines like leptin with pro-inflammatory properties are overproduced while some adipokines like adiponectin with anti-inflammatory properties are decreased in obesity (Wozniak et al. 2009). These dysregulations of adipokine production may promote obesity-linked metabolic disorders and cardiovascular disease (Maury and Brichard 2010). Taurine (2-aminoethan sulfonic acid) is the most abundant amino acid in various mammalian tissues. Taurine benefits dyslipidemia (Yang et al. 2010) and obesity in rats (Du et al. 2010) and humans (Brons et al. 2004) but the relationship between dietary taurine supplementation and serum adipokine level has rarely been investigated. The purpose of this study was to investigate the relationship between serum taurine level and serum adiponectin and leptin levels with taurine supplementation in high-fat diet-induced obesity rats.

11.2 Methods

11.2.1 Animals and Diet

Five-week-old male Sprague–Dawley rats were supplied from DBL (Eumseong, Korea) and were kept at laboratory animal housing at Inha University following the recommendation of the Guide for the Care and Use of Laboratory Animals (Research 1996) with a constant 12-h light and dark cycle (a.m. 09:00–p.m. 09:00), controlled temperature (23 ± 1°C), and humidity (55 ± 10%). Following 1 week of acclimatization with a commercial diet, rats were randomly divided into three groups for a period of 8 weeks (normal diet group, N group; high-fat diet group, HF group; high-fat diet + taurine group, HFT group). Taurine was supplemented by dissolving in feed water (3% w/v). Food and water were provided *ad libitum.* The composition of the experimental diet was based on AIN76 as shown in Table 11.1.

11.2.2 Sampling and Chemical Analysis

After 8 weeks of feeding the experimental diets, the animals were fasted for 12 h before sacrifice. Blood was collected from the heart and serum was separated by centrifugation at 3,000 rpm for 20 min. The weights of the liver, kidney, spleen,

Table 11.1 Composition of experimental diet (g/100 g diet)

	Normal diet (g)	High-fat diet (g)
Casein	20	20
Corn starch	15	10
Sucrose	50	40
Cellulose	5	5
Bean oil	5	5
Lard	0	15
Vitamin mixture[a]	1	1
Mineral mixture[b]	3.5	3.5
DL-Methione	0.3	0.3
Choline bitartrate	0.2	0.2

[a]AIN-76 vitamin mixture (g/kg); thiamin hydrochloride 600 mg, riboflavin 600 mg, pyridoxine hydrochloride 700 mg, nicotinic acid 3 g, D-calcium pantothenate 1.6 g, folic acid 200 mg, D-biotin 20 mg, cyanocobalamin 1 mg, retinyl palmitate premix (250,000 IU/g) 1.6 g, DL-alpha-tocopherol acetate (250 IU/g) 20 g, cholecalciferol (400,000 IU/g) 250 mg, menaquinone 5 mg, sucrose, finely powdered 972.9 g

[b]AIN-76 mineral mixture (g/kg); calcium phosphate dibasic 500 g, sodium chloride 74 g, potassium citrate monohydrate 220 g, potassium sulfate 52 g, magnesium oxide 24 g, manganous carbonate (43 ~ 48%Mn) 3.5 g, ferric citrate (16 ~ 17%Fe) 6 g, zinc carbonate (70% ZnO) 1.6 g, cupric carbonate (53 ~ 55% Cu) 0.3 g, potassium iodate 0.01 g, sodium selenite 0.01 g, chromium potassium sulfate 0.55 g, sucrose finely powdered 118 g

epididymal fat (E-fat), and retroperitoneal fat (R-fat) were measured. The serums were immediately frozen in liquid nitrogen, and then stored at −70°C until analysis.

Level of serum taurine was analyzed using HPLC system (McMahon et al. 1996). Serum samples were mixed with acetonitrile and the supernatant was adjusted to pH 9 by borate buffer. These solutions mixed with fluorescamine were analyzed on the HPLC (Agilent Technologies 1200 series HPLC) and Waters C_{18} reverse-phase column (250 × 4.6 mm i.d) at 385 nm. The mobile phases tetrahydrofuran–acetonitrile–phosphate buffer (pH 3.5) (4:25:71, v/v/v, solvent A) and tetrahydrofuran–acetonitrile–phosphate buffer (pH 3.5) (4:24:72, v/v/v, solvent B) were used to gradient elution with flow rate at 1 ml/min.

Levels of serum TG and total TC were analyzed using automatic analyzer (BPC Biosed srl., Italy). HDL-C was obtained from the whole serum with high-density lipoprotein precipitation reagent (AM204-1, Asan Pharmaceutical, Korea) after precipitation of low-density lipoprotein and very-low-density lipoprotein for 10 min at 3,000 rpm (Hettich Mikro 200R, Tuttlingen, Germany) (Warnick and Albers 1978) and then analyzed for HDL-C using the same method as with TC. Standard serum (Asan Pharmaceutical, Korea) was used for calibration before every parameter was analyzed. All of the results were expressed as mg/dl serum.

Serum adiponectin and leptin levels were determined by enzyme-linked immunosorbent assay (ELISA) kit and radio immune assay (RIA) kit in Samkwang Medical laboratories (Seoul, Korea), respectively.

Table 11.2 Final body weight, organ weight, and adipose tissue weight

Variables	N	HF	HFT
Final BW	535.3 ± 24.0[a]	568.4 ± 12.3	541.4 ± 8.3
Relative liver weight (g/100 g BW)	2.7 ± 0.1	2.6 ± 0.1	2.4 ± 0.1
Relative epididymal fat weight (g/100 g BW)	2.2 ± 0.1*,[b]	3.0 ± 0.1**	3.1 ± 0.2**
Relative retroperitoneal fat weight (g/100 g BW)	2.4 ± 0.3*	3.6 ± 0.2**	3.6 ± 0.1**

[a]Mean ± SE

[b]Values with different superscripts (* and **) within the row are significantly different at $p < 0.05$ by Duncan's multiple range test

11.2.3 Statistical Analysis

All analyses were performed using SPSS 17.0 program. Each value was expressed as the mean ± SE. Data were analyzed for significant difference by one-way analysis of variance followed by Duncan's multiple range tests at a $p < 0.05$. The correlation between serum taurine and adipokine levels was analyzed using Spearman's correlation coefficient.

11.3 Results and Discussion

11.3.1 Final Body Weight, Organ Weight, and Adipose Tissue Weight

After 8 weeks, there was no significant difference in final body weight among three groups (Table 11.2). The adipose tissue weights (E-fat and R-fat) of HF group and HFT group were significantly higher compared to N group. However, there was no significant difference in the relative liver weight among three groups.

11.3.2 Serum Lipid Profiles

Serum lipid profiles are shown in Table 11.3. The level of serum TG was significantly lower in N group compared to other groups. The level of serum TC was significantly lower in HFT group compared to other groups. Taurine supplementation may have the effect of decreasing the level of serum TC. The previous study reported that various amounts of taurine supplementation in rats fed a high-cholesterol diet significantly decreased serum total cholesterol in a dose-dependent manner (Yokogoshi et al. 1999).

Table 11.3 Serum lipid profiles

Variables	N	HF	HFT
TG (mg/dl)	110.2 ± 12.5[a,*,b]	136.8 ± 6.9**	141.5 ± 7.0**
TC (mg/dl)	65.5 ± 2.5*	71.0 ± 4.8*	50.5 ± 1.7**
HDL-C (mg/dl)	23.1 ± 1.7*	19.3 ± 0.9*,**	15.3 ± 0.6**,***

[a]Mean ± SE
[b]Values with different superscripts (*, **, and ***) within the row are significantly different at $p < 0.05$ by Duncan's multiple range test

Table 11.4 Serum taurine, adiponectin, and leptin levels

	N	HF	HFT
Taurine (μg/ml)	27.0 ± 0.8[a,*,b]	25.8 ± 0.5*	40.5 ± 2.3**
Adiponectin (μg/ml)	17.2 ± 1.6	12.5 ± 1.1	16.6 ± 1.8
Leptin (ng/ml)	3.2 ± 0.7	5.0 ± 0.6	5.0 ± 0.5

[a]Mean ± SE
[b]Values with different superscripts within the row (* and **) are significantly different at $p < 0.05$ by Duncan's multiple range test

11.3.3 Serum Taurine, Adiponectin, and Leptin Levels

The serum taurine level was significantly higher in HFT group compared to other groups ($p < 0.05$) (Table 11.4). The serum adiponectin level in HF group tends to be lower compared to normal group and HFT group and serum leptin levels in HF and HFT groups tend to be higher compared to normal group, but not significantly.

11.3.4 Correlation Between Serum Taurine and Adiponectin or Leptin Levels

The correlation between serum taurine and adiponectin or leptin levels is shown in Table 11.5. There was positive correlation between serum taurine and serum adiponectin levels ($p < 0.01$), while there was no significant correlation between serum taurine and serum leptin levels.

11.3.5 Correlations Between Serum Taurine Level and Adipose Tissue Weight or Lipid Profiles

The correlations between serum taurine level and adipose tissues or lipid profiles are shown in Table 11.6. There was negative correlation between serum taurine

Table 11.5 Correlation between serum taurine and adiponectin or leptin levels

	Taurine	Adiponectin	Leptin
Taurine	1		
Adiponectin	0.782**[a]	1	
Leptin	0.022	−0.156	1

[a]Significant correlation was assessed by the Spearman correlation (**$p<0.01$)

Table 11.6 Correlation between serum taurine level and adipose tissue weight or lipid profiles

	Taurine	TG	TC	Relative liver weight	Relative E-fat weight	Relative R-fat weight
Taurine	1					
TG	0.318	1				
TC	−0.642*[a]	−0.049	1			
Relative liver weight	−0.762*	−0.370	0.415	1		
Relative E-fat weight	0.267	0.627*	−0.288	−0.467	1	
Relative R-fat weight	0.267	0.818**	−0.097	−0.450	0.830**	1

[a]Significant correlation was assessed by the Spearman correlation (*$p<0.05$, **$p<0.01$)

level and serum TC level ($p<0.05$) and relative liver weight ($p<0.05$), while there was no significant correlation between serum taurine level and serum TG level or relative E-fat and R-fat.

11.4 Conclusion

These results suggest that dietary taurine supplementation has beneficial effects on serum total cholesterol and adiponectin levels in high-fat diet-induced obesity rats.

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Chapter 12
Relationship Among Serum Taurine, Serum Adipokines, and Body Composition During 8-Week Human Body Weight Control Program

Jeong Soon You, Ji Yeon Park, Xu Zhao, Jin Seok Jeong, Mi Ja Choi, and Kyung Ja Chang

Abstract Human adipose tissue is not only a storage organ but also an active endocrine organ to release adipokines. This study was conducted to investigate the relationship among serum taurine and adipokine levels, and body composition during 8-week human body weight control program in obese female college students. The program consisted of diet therapy, exercise, and behavior modification. After the program, body weight, body fat mass, percent body fat, and body mass index (BMI) were significantly decreased. Serum triglyceride (TG), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C) levels were significantly decreased. Also serum adiponectin level was significantly increased and serum leptin level was significantly decreased. There were no differences in serum taurine and homocysteine levels. The change of serum adiponectin level was positively correlated with change of body fat mass and percent body fat. These results may suggest that body fat loss by human body weight control program is associated with an increase in serum adiponectin in obese female college students. Therefore, further study such as taurine intervention study is needed to know more exact correlation between dietary taurine intake and serum adipokines or body composition.

J.S. You • J.Y. Park • X. Zhao • K.J. Chang (✉)
Department of Food and Nutrition, Inha University, Incheon, South Korea
e-mail: kjchang@inha.ac.kr

J.S. Jeong
Research Laboratories of Dong-A Pharmaceutical Co. Ltd,
Youngin Gyeonggi-do, South Korea

M.J. Choi
Department of Food and Nutrition, Keiomyung University,
Daegu, South Korea

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_12,

Abbreviations

BMI	Body mass index
TG	Triglyceride
TC	Total cholesterol
LDL-C	Low-density lipoprotein-cholesterol
HDL-C	High-density lipoprotein-cholesterol
HPLC	High-performance liquid chromatography
ELISA	Enzyme-linked immunosorbent assay
RIA	Radio immune assay

12.1 Introduction

Obesity is an international health problem with major cause of morbidity, related with an increased risk of metabolic syndrome and cardiovascular disease, and various cancers. Excessive accumulation of intra-abdominal fat contributes to the impairment of lipid metabolism in human obesity (Fujioka et al. 1987) and is fundamental to the pathogenesis of the metabolic syndrome with deregulating adipokines such as adiponectin and leptin. Adiponectin acts as an anti-atherogenic plasma protein and an endogenous modulator in vascular remodeling (Ouchi et al. 2003). Leptin inhibits food intake and decreases body weight and fat store. Recent study suggests that taurine (2-aminoethane sulfonic acid) supplementation interrupts vicious circle promoting obesity and may prevent obesity(Tsuboyama-Kasaoka et al. 2006). The purpose of this study was to investigate the relationship among serum taurine level, serum adipokine level, and body composition during 8-week human body weight control program in Korean obese female college students.

12.2 Methods

12.2.1 Study Subjects

Study subjects (22 female college students aged between 19 and 24 years) were recruited from a university located in Incheon, Korea. All of the subjects submitted to researcher written informed consent to take part in an 8-week body weight control program. The subjects were free-living and they ate self-selected food. No medicine or other nutritional supplements were taken. The study was conducted from May to July in 2010. Result analysis was conducted for the 18 students who completed the 8-week program (drop rate: 18.2%). None of the women had any chronic disease and all were free of any medication.

12.2.2 Human Body Weight Control Program

The body weight control program consisted of diet therapy, exercise, and behavioral modification. All subjects were recommended an individualized low-calorie diet by a dietitian at an introductory class. Also each subject of exercise group was made to perform treadmill exercise three times a week during the 8-week program with intensity corresponding to 70% of anaerobic threshold. So subjects can consume 200 kcal during exercise. To learn behavioral modification, subjects were provided an online lecture and were asked to submit a self-monitoring diet and exercise diary to the researcher each week. In addition, subjects were counseled through the face-to-face meeting every week and e-mails.

12.2.3 Body Composition Assessment

Body composition assessment was conducted from each subject. The heights of subjects were measured with a stadiometer. Body composition such as body weight, soft lean mass, body fat mass, percent body fat, and body mass index (BMI) were assessed at least once per week using bioelectrical impedance (In body 3.0, Biospace, Korea).

12.2.4 Serum Taurine Level

Level of serum taurine was analyzed using high-performance liquid chromatography (HPLC) system (McMahon et al. 1996). Serum samples were mixed with acetonitrile and the supernatant was adjusted to pH 9 by borate buffer. These solutions mixed with fluorescamine were analyzed on the HPLC (Agilent Technologies 1200 series HPLC) and Waters C_{18} reverse-phase column (250×4.6 mm i.d) at 385 nm. The mobile phases tetrahydrofuran–acetonitrile–phosphate buffer (pH 3.5) (4:25:71, v/v/v, solvent A) and tetrahydrofuran–acetonitrile–phosphate buffer (pH 3.5) (4:24:72, v/v/v, solvent B) were used in gradient elution with flow rate at 1 ml/min.

12.2.5 Serum Lipid Profiles and Serum Adipokine Levels

Blood was collected after a fasting overnight at the same time in the morning before and after the human body weight control program. The collected blood was centrifuged at 3,000 rpm for 15 min. The supernatant serum was separated in microtubes and stored under −70°C until the determination of serum lipid levels. Serum total

cholesterol (TC), high-density lipoprotein (HDL)-cholesterol, and triglyceride (TG) levels were determined by using an automatic clinical analyzer (BPC Biosed srl., Italy). Serum low-density lipoprotein (LDL)-cholesterol level was calculated from serum TC, HDL, and TG levels (Friedewald et al. 1972).

Serum adiponectin, leptin, and homocysteine levels were determined by enzyme-linked immunosorbent assay (ELISA) kit, radio immune assay (RIA) kit, and enzymatic method in Samkwang Medical laboratories (Seoul, Korea), respectively.

12.2.6 Statistical Analysis

Experimental data are expressed as mean ± SEM. Statistical Package for the Social Sciences (SPSS) for windows version 17.0 was used for the analysis. Differences between groups were compared with the Mann–Whitney test. The correlation among serum taurine and adipokine levels, and body compositions was analyzed using Spearman's correlation coefficient. Differences were considered statistically significant when the calculated p value was less than 0.05.

12.3 Results and Discussion

12.3.1 General Characteristics

General characteristics of the subjects are shown in Table 12.1. The average of age was 20.8 years. Eleven subjects (61.1%) lived with family, and 10 subjects (55.6%) spent 210,000–400,000 Korean won (approximately US$180–$343) for pocket money per month.

12.3.2 Body Composition

Changes in body composition of the subjects between before and after human body weight control program are presented in Table 12.2. Average body weight of the subjects was 60.3 kg and average BMI was 23.5 kg/m^2 before they joined the program. According to the fourth Korea National Health and Nutrition Examination Survey, average weight of 19–29-year-old women was 55.5 kg. Because our subjects wanted to lose weight, the average weight of our subjects was higher than that of the similar age Korean women.

After the 8-week body weight control program, the average weight and BMI were reduced to 57.3 kg ($p < 0.001$) and 22.5 kg/m^2 ($p < 0.001$). Body fat mass and

Table 12.1 General characteristics of the subjects

Variables	Subjects ($n=18$)
Age (years)	20.8 ± 0.4[a]
Residence type	
Living with family	11 (61.1)[b]
Preparation of own meals	4 (22.2)
Dormitory	3 (16.7)
Pocket money (1,000won/month)	
≤200	4 (22.2)
210–400	10 (55.6)
≥400	4 (22.2)

[a]Values are mean ± SEM
[b]Values are N(%)

Table 12.2 Changes in body composition of the subjects

Variables	Before	After	Change
Body weight (kg)	60.3 ± 2.0[a]	57.3 ± 1.7	−2.2 ± 0.4***[b]
BMI (kg/m^2)	23.5 ± 0.6	22.5 ± 0.6	−0.8 ± 0.2***
Soft lean mass (kg)	38.7 ± 0.9	39.6 ± 0.9	0.9 ± 0.3***
Body fat mass (kg)	21.3 ± 0.7	17.8 ± 0.7	−3.1 ± 0.3***
Percent body fat (%)	34.6 ± 0.6	30.3 ± 0.7	−3.8 ± 0.3***

[a]Values are mean ± SEM
[b]Values with * superscripts are significantly different between before and after the body weight control program by paired *t*-test (***$p<0.001$)

percent body fat were reduced from 21.3 to 17.8 kg ($p<0.001$) and 34.6 to 30.3% ($p<0.001$), respectively. Soft lean mass was increased from 38.7 to 39.6 kg ($p<0.001$). According to the previous researches about short-term weight control program for female college students, an average 1.1 kg of body weight was decreased (Kang et al. 2004) and 0.6 kg of body weight was decreased (Lee et al. 2007). The reduced average body weight of this program tends to be more compared to other body weight control programs.

12.3.3 Serum Lipid Profiles

Serum lipid profile is one of the effective parameters of obesity. Differences in serum lipid profiles between before and after the 8-week human body weight control program are shown in Table 12.3. The serum TC ($p< 0.001$), LDL-C ($p< 0.001$), and TG levels ($p< 0.001$) were reduced significantly during the human body weight control program. It was similarly reported that human body weight control program improved serum lipid profiles (Lee et al. 2005, 2007; Seo 2005).

Table 12.3 Changes in serum lipid profiles of the subjects

Serum lipid level	Before	After	Change
TC (mg/dl)	181.8 ± 3.6[a]	171.0 ± 3.3	−10.2 ± 2.1***[b]
HDL-C (mg/dl)	39.5 ± 1.0	40.1 ± 0.9	0.6 ± 0.9
LDL-C (mg/dl)	118.6 ± 3.2	106.9 ± 4.2	−10.8 ± 2.2***
TG (mg/dl)	93.2 ± 5.8	74.8 ± 4.0	−20.9 ± 3.5***

[a]Mean ± SEM
[b]Values with * superscripts are significantly different between before and after the human body weight control program by paired *t*-test (***$p < 0.001$)

Table 12.4 Changes in serum taurine and adipokine levels of the subjects

	Before	After	Change
Taurine (μg/ml)	10.3 ± 1.0[a]	8.1 ± 0.9	−2.2 ± 1.5
Adiponectin (μg/ml)	15.2 ± 1.7	19.6 ± 2.4	4.4 ± 1.3**[b]
Leptin (ng/ml)	14.8 ± 1.3	10.0 ± 0.8	−4.8 ± 1.2**
Homocysteine (ng/ml)	8.1 ± 0.5	8.5 ± 2.1	−0.3 ± 0.3

[a]Mean ± SEM
[b]Values with * superscripts are significantly different between before and after the body weight control program by paired *t*-test (*$p < 0.05$, **$p < 0.01$)

12.3.4 Serum Adipokine Level

Differences in serum taurine and adipokine levels between before and after the 8-week body weight control program are shown in Table 12.4. There was no significant difference in serum taurine and homocysteine levels between before and after the body weight control program. The serum adiponectin level was increased significantly ($p < 0.01$) and serum leptin level was reduced significantly ($p < 0.01$) during the body weight control program. Similar results have been reported that plasma adiponectin level was reduced in obese subjects (Hoffstedt et al. 2004; Ouchi et al. 2003) and adiponectin levels in obese children were negatively correlated to body fat (Reinehr et al. 2004). Also it was reported that leptin level was decreased in obese children during a short-term weight reduction program (Reiterer et al. 2011).

12.3.5 Correlation Between Change of Body Composition and Serum Adipokine Level

The correlation between change of body composition and serum adipokine levels of the subjects is shown in Table 12.5. There was positive correlation between change of serum adiponectin level and change of body fat mass ($p < 0.05$) and percent body

Table 12.5 Correlation between changes of body composition and serum adipokine level of the subjects

	Change of serum adiponectin level	Change of serum leptin level	Change of serum homocysteine level
Change of body weight	0.470	0.196	0.457
Change of soft lean mass weight	–0.137	0.053	0.404
Change of body fat mass weight	0.667*[a]	–0.039	0.274
Change of percent body fat	0.791**	–0.028	0.011

[a]Significant correlation was assessed by the Spearman's correlation (*$p<0.05$, **$p<0.01$)

fat ($p< 0.01$). There was no significant correlation between change of serum leptin and homocysteine levels and body composition.

12.4 Conclusion

These results may suggest that body fat loss by human body weight control program is associated with an increase of serum adiponectin level in obese female college students. Therefore, further study such as taurine intervention study is needed to know more exact correlation between dietary taurine intake and serum adipokines or body composition.

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Chapter 13
Dietary Taurine and Nutrient Intake and Dietary Quality by Alcohol Consumption Level in Korean Male College Students

Jeong Soon You, So Young Kim, So Yoon Park, and Kyung Ja Chang

Abstract Heavy alcohol consumption is related to various negative healthy consequences. To investigate difference of taurine intake according to the alcohol consumption level, we studied body composition, intake of dietary nutrients including taurine, and dietary quality in Korean male college students that were divided according to their alcohol consumption level. Surveys were conducted using a questionnaire and a 3-day recall method for assessing dietary intake in 220 male college students residing in Incheon, Korea. The subjects were divided into two groups by alcohol consumption level: heavy drinking group (average drinking over 5 cans (355 ml) of beer or 7 shots (45 ml) of soju) and light drinking group (average drinking less than 5 cans of beer or 7 shots of soju or not drinking any alcohol at all at one time during the previous month). The average body mass index (BMI) in the heavy drinking group was significantly higher compared to the light drinking group ($p<0.05$). There was no significant difference in dietary taurine intake between heavy and light drinking group. With regard to the dietary quality evaluation of the subjects, the nutrient densities (ND) of carbohydrate, niacin, vitamin C, and zinc in the heavy drinking group were significantly lower than those of the light drinking group. Therefore, continuous nutrition education for heavy drinking Korean male college students may be needed to improve balanced nutritional status and further studies such as case–control study or taurine intervention study are required to know the relationship between dietary taurine intake and alcohol consumption.

J.S. You • S.Y. Kim • S.Y. Park • K.J. Chang (✉)
Department of Food and Nutrition, Inha University,
Incheon, South Korea
e-mail: kjchang@inha.ac.kr

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_13,

Abbreviations

BMI Body mass index
NAR Nutrient adequacy ratio
MAR Mean adequacy ratio
ND Nutrient density

13.1 Introduction

A light alcohol consumption may not be harmful but heavy alcohol consumption is related to various negative consequences such as the reduced brain mass, neuronal loss, and impairment of cognitive and physical functions (Ham and Hope 2003).

Dietary taurine (2-aminoethane sulfonic acid) intake may play an important role in physical and psychological aspect. Recent findings also imply the protective effect of taurine related to alcohol-induced problems. Taurine supplementation was neuroprotective against ethanol-induced apoptosis in cells (Taranukhin et al. 2010) and had a preventive role by antagonizing the effects induced by alcohol (Rosemberg et al. 2012).

There are many previous reports about the relationship between alcohol consumption and nutrient intake (Rittmueller et al. 2012; Rosemberg et al. 2012; Sieri et al. 2009). However, there exists only a limited amount of studies about the relationship between taurine intake and alcohol consumption. The purpose of this study is to investigate difference of dietary intake of nutrients including taurine, and the dietary quality in Korean male college students that were divided according to their alcohol consumption level.

13.2 Methods

13.2.1 Subjects

The subjects were male college students residing in the Incheon area and attending a nutrition-related nonmajor class via the Internet. The subjects were divided into two groups by alcohol consumption level: heavy drinking group (average drinking over 5 cans (355 ml) of beer or 7 shots (45 ml) of soju) and light drinking group (average drinking less than 5 cans of beer or 7 shots of soju or not drinking any alcohol at all at one time during the previous month).

13.2.2 General Characteristics and Body Composition

Questions for general characteristics included three items such as age, type of residence, and pocket money. The height of subjects was measured with a stadiometer.

Body weight and body composition such as percent body fat and body mass index (BMI) were determined with an InBody 3.0 Body Composition Analyzer (InBody 3.0, Biospace, Korea).

13.2.3 Dietary Taurine and Nutrient Intake Assessment

Surveys were conducted using a questionnaire and a 3-day recall method for assessing dietary intake in 220 male college students residing in Incheon area. Dietary intake was assessed by a 3-day recall method (2 weekdays and 1 weekend day). Dietary taurine and nutrient intakes were estimated using the computer-aided nutrition program (CAN-pro 3.0, The Korean Nutrition Society, Korea) inputted with a taurine content database for 17 food groups and 321 commonly used food items.

13.2.4 Dietary Quality

The nutrient adequacy ratio (NAR) is the ratio of an individual's mean daily intake to the age-specific recommended dietary allowance. The mean adequacy ratio (MAR) was calculated by the sum of NAR for nutrients divided by the number of nutrients ($n=9$) (Madden and Yoder 1972). NAR were truncated at 1 for calculation of the MAR to prevent an excess intake of one nutrient from compensating for inadequate intakes. To adjust for energy intake, we used the nutrient density (ND) method. We divided several nutrient intakes by total energy intake (per 1,000 kcal).

13.2.5 Statistic Analysis

The Statistical Package for Social Sciences (SPSS) 17.0 program was used. The frequency and percentage, and the mean and SEM for each survey question, were calculated. We used the chi-square test and Student *t*-test to compare. Differences were considered statistically significant when the calculated p value was less than 0.05.

13.3 Results

13.3.1 General Characteristics

General characteristics of the subjects are shown in Table 13.1. The average ages of light drinking and heavy drinking group were 22.8 and 23.1 years, respectively. Forty subjects of light drinking group (45.5%) and 80 subjects of heavy drinking group (60.6%) lived with their families and 47 subjects of light drinking group

Table 13.1 General characteristics of the subjects

Variables	Light drinking group ($n=88$)	Heavy drinking group ($n=132$)	χ^2 value or t-value
Age (years)	22.8 ± 0.2[a]	23.1 ± 0.2	−1.043
Type of Residence			
Living with family	40 (45.5)[b]	80 (60.6)	8.339*[c]
Preparation of own meal	31 (35.2)	42 (31.8)	
Boarding or dormitory	17 (19.3)	10 (7.6)	
Pocket money (1,000 won/month)			
≤200	22 (25.0)	20 (15.2)	3.316*
210–400	47 (53.4)	80 (60.6)	
≥400	19 (21.6)	32 (24.2)	

[a]Mean ± SEM; [b]N (%); [c]Values with * superscript are significantly different between light and heavy drinking group by chi-squre test (*: $p<0.05$)

Table 13.2 Body compositions of the subjects

Variables	Light drinking group ($n=88$)	Heavy drinking group ($n=132$)	t-value
Height	174.6 ± 0.6[a]	174.9 ± 0.5	−0.334
Weight	70.1 ± 1.0	72.6 ± 0.9	−1.765
Percent body fat	17.9 ± 0.6	18.3 ± 0.4	−0.502
BMI	22.8 ± 0.3	23.6 ± 0.3	−2.159*[b]

[a]Mean ± SEM; [b]Value with * superscript is significantly different between light and heavy drinking group by Student t-test (*: $p<0.05$)

(53.4%) and 80 subjects of heavy drinking group (60.6%) spent 210,000–400,000 Korean won (approximately US$180–$343) per month for pocket money. There are significant differences in type of residence ($p<0.05$) and pocket money ($p<0.05$) according to alcohol consumption level.

13.3.2 Body Composition

The average height, weight, percent body fat, and BMI were 174.6 cm, 70.1 kg, 17.9%, and 22.8 kg/m^2 for light drinking group and those of heavy drinking group were 174.9 cm, 72.6 kg, 18.3%, and 23.6 kg/m^2, respectively (Table 13.2). There was significant difference in BMI between light and heavy drinking groups. There was a similar result that male higher level drinkers had a higher BMI than abstainers in research about alcohol consumption pattern, diet, and body weight in 10 European countries (Sieri et al. 2009). According to the 4th Korea National Health and Nutrition Examination Survey, the average height, weight, body fat percentage, and BMI of 19–29-year-old men were 175.0 cm, 72.3 kg, 22.3%, and 23.6 kg/m^2, respectively. The average height, weight, and BMI of our subjects were similar to those of the same age group of Korean men, while percent body fat of our subjects was lower than that of the similar age Korean men.

Table 13.3 Dietary taurine intake of the subjects

Variable	Light drinking ($n=88$)	Heavy drinking ($n=132$)	t-value
Taurine (mg/day)	126.4 ± 6.9[a]	120.9 ± 5.3	0.630

[a]Mean ± SEM

Table 13.4 Dietary nutrient intakes of the subjects

Variables	Light drinking ($n=88$)	Heavy drinking ($n=132$)	t-value
Energy (kcal/day)	2,101.1 ± 51.7[a]	2,039.5 ± 40.9	0.945
Carbohydrate (g/day)	271.1 ± 6.5	257.7 ± 4.9	1.668
Total protein (g/day)	88.9 ± 2.5	85.8 ± 1.9	0.985
Total fat (g/day)	67.9 ± 2.3	67.0 ± 2.0	0.273
Fiber (g/day)	18.1 ± 0.6	17.4 ± 0.4	0.914
Total Ca (mg/day)	500.4 ± 20.2	489.0 ± 16.3	0.446
Total Fe (mg/day)	14.7 ± 0.8	13.6 ± 0.4	1.303
P (mg/day)	1,145.9 ± 31.7	1,093.3 ± 24.1	1.343
Na (mg/day)	4,336.2 ± 132.6	4,432.9 ± 136.2	−0.492
Zn (mg/day)	10.3 ± 0.3	9.5 ± 0.2	2.167*[b]
Vit A (μg RE/day)	751.0 ± 34.6	744.9 ± 25.9	0.142
Vit B_1 (mg/day)	1.5 ± 0.1	1.5 ± 0.04	−0.066
Vit B_2 (mg/day)	1.3 ± 0.1	1.3 ± 0.03	0.035
Vit B_6 (mg/day)	2.2 ± 0.1	2.1 ± 0.05	1.458
Niacin (mg/day)	21.2 ± 0.6	20.0 ± 0.6	1.469
Vit C (mg/day)	73.7 ± 2.7	69.5 ± 2.3	1.209
Folic acid (mg/day)	224.4 ± 7.6	216.8 ± 5.3	0.849
Cholesterol (mg/day)	424.5 ± 17.5	438.9 ± 15.7	−0.604

[a]Mean ± SEM; [b]Values with * superscripts are significantly different between light and heavy drinking group by Student t-test (*: $p<0.05$)

13.3.3 Dietary Taurine and Nutrient Intakes

Average dietary taurine intake is shown in Table 13.3. Average dietary taurine intakes in light and heavy drinking groups were 126.4 and 120.9 mg/day, respectively. There was no significant difference in dietary taurine intake between two groups. The dietary taurine intake of the Korean male college student in 2008 was 126.8 mg/day (functional constipation patients) and 105.1 mg/day (control) in the same area where this study was conducted (You et al. 2010) and in 2006 was 124.1 mg/day (Sung and Chang 2008).

Average energy intakes in light and heavy drinking groups were 2,101.1 and 2,039.5 kcal/day, respectively (Table 13.4). The intake of zinc was significantly lower in the heavy drinking group compared to the light drinking group ($p<0.05$). It was previously reported from the US Department of Agriculture's Nationwide Food Consumption survey in 1977–1978 that energy levels were higher than for

Table 13.5 Nutrient adequacy ratio (NAR) and mean adequacy ratio (MAR) of the subjects

Variables	Light drinking group ($n=88$)	Heavy drinking group ($n=132$)	t-value
Protein	0.998 ± 0.001[a]	0.997 ± 0.002	0.493
Vit A	0.860 ± 0.018	0.860 ± 0.019	0.098
Vit B_1	0.985 ± 0.005	0.991 ± 0.003	–1.101
Vit B_2	0.820 ± 0.020	0.840 ± 0.021	–0.847
Niacin	0.987 ± 0.005	0.982 ± 0.005	0.786
Vit C	0.730 ± 0.019	0.690 ± 0.018	1.267
Ca	0.660 ± 0.017	0.650 ± 0.020	0.237
P	1.000 ± 0.000	1.000 ± 0.000	0.237
Fe	0.989 ± 0.004	0.991 ± 0.003	–0.377
MAR	0.896 ± 0.008	0.895 ± 0.006	0.098

[a]Mean ± SEM

Table 13.6 Nutrient density (ND) of the subjects

Variables	Light drinking group ($n=88$)	Heavy drinking group ($n=132$)	t-value
Protein (g/1,000 kcal)	42.0 ± 0.5[a]	41.8 ± 0.5	0.269
Carbohydrate (g/1,000 kcal)	133.1 ± 1.4	128.1 ± 1.5	2.317*[b]
VitA (μg RE/1,000 kcal)	337.7 ± 12.5	366.3 ± 12.5	–1.568
VitB_1 (mg/1,000 kcal)	0.7 ± 0.02	0.7 ± 0.01	–0.817
VitB_2 (mg/1,000 kcal)	0.6 ± 0.02	0.6 ± 0.01	–0.376
Niacin (mg/1,000 kcal)	10.3 ± 0.2	9.8 ± 0.2	1.996*
VitB_6 (mg/1,000 kcal)	1.1 ± 0.02	1.1 ± 0.02	0.950
Vit C (mg/1,000 kcal)	37.4 ± 1.5	32.5 ± 0.9	2.967**[b]
Ca (mg/1,000 kcal)	234.2 ± 8.2	235.2 ± 7.2	–0.089
Na (mg/1,000 kcal)	2128.2 ± 63.7	2168.6 ± 61.6	–0.447
Fe (mg/1,000 kcal)	6.5 ± 0.1	6.4 ± 0.1	0.687
Zn (mg/1,000 kcal)	4.8 ± 0.7	4.6 ± 0.1	2.152*

[a]Mean ± SEM; [b]Values with * superscripts are significantly different between light and heavy drinking group by Student t-test (*: $p<0.05$, **: $p<0.01$)

non-alcohol drinkers while average daily nutrient intake for most nutrients was similar for drinkers and nondrinkers (Windham et al. 1983). In addition, it was reported in the Northwest Territories, Canada, that energy intake was higher among all alcohol consumers (Rittmueller et al. 2012).

13.3.4 Dietary Quality

With regard to the dietary quality evaluation of the subjects, there was no significant difference in NAR of 9 nutrients and MAR between heavy and light drinking groups (Table 13.5), while the nutrient densities of carbohydrate, niacin, vitamin C,

and zinc in the heavy drinking group were significantly lower than those of the light drinking group (Table 13.6). It was previously reported from the US Department of Agriculture's Nationwide Food Consumption survey in 1977–1978 that the nutrient density of diets of drinkers was significantly lower than that of nondrinkers with respect to protein, fat, carbohydrate, calcium, iron, phosphorus, vitamin A, and thiamin (Windham et al. 1983).

13.4 Conclusion

In this study, there was no significant difference in dietary taurine intake between heavy and light drinking groups and heavy drinkers have low dietary quality. Therefore, continuous nutrition education for heavy drinking Korean male college students may be needed to improve balanced nutritional status and further studies such as case–control study or taurine administration study are required to know the relationship between dietary taurine intake and alcohol consumption.

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Chapter 14
Taurine Supplementation Restores Insulin Secretion and Reduces ER Stress Markers in Protein-Malnourished Mice

Thiago Martins Batista, Priscilla Muniz Ribeiro da Silva, Andressa Godoy Amaral, Rosane Aparecida Ribeiro, Antonio Carlos Boschero, and Everardo Magalhães Carneiro

Abstract Endoplasmic reticulum (ER) stress is a cellular response to increased intra-reticular protein accumulation or poor ER function. Chronic activation of this pathway may lead to beta cell death and metabolic syndrome (MS). Poor nutrition during perinatal period, especially protein malnutrition, is associated with increased risk for MS in later life. Here, we analyzed the effects of taurine (TAU) supplementation upon insulin secretion and ER stress marker expression in pancreatic islets and in the liver from mice fed a low-protein diet. Malnourished mice had lower body weight and plasma insulin. Their islets secreted less insulin in response to stimulatory concentrations of glucose. TAU supplementation increased insulin secretion in both normal protein and malnourished mice. Western blot analysis revealed lower expression of the ER stress markers CHOP and ATF4 and increased phosphorylation of the survival protein Akt in pancreatic islets of TAU-supplemented mice. The phosphorylation of the mitogenic protein extracellular signal-regulated kinase (ERK1/2) was increased after acute incubation with TAU. Finally, the ER stress markers p-PERK and BIP were increased in the liver of malnourished mice and TAU supplementation normalized these parameters.

T.M. Batista (✉) • P.M.R. da Silva • A.C. Boschero • E.M. Carneiro
Departamento de Biologia Funcional e Estrutural, Instituto de Biologia,
Universidade Estadual de Campinas, SP, Brazil
e-mail: thiagombatista@yahoo.com.br

A.G. Amaral
Divisão de Nefrologia e Medicina Molecular,
Universidade de São Paulo, São Paulo, SP, Brazil

R.A. Ribeiro
Núcleo em Ecologia e Desenvolvimento Sócio-Ambiental de Macaé (NUPEM),
Universidade Federal do Rio de Janeiro (UFRJ), Macaé, RJ, Brazil

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In conclusion, malnutrition leads to impaired islet function which is restored with TAU supplementation possibly by increasing survival signals and lowering ER stress proteins. Lower ER stress markers in the liver may also contribute to the improvement of insulin action on peripheral organs.

Abbreviations

ATF4	Activating transcription factor 4
BIP	Binding immunoglobulin protein
CHOP	*C/EBP* homologous protein
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase
IRE-1	Inositol-requiring enzyme-1
PERK	PKR-like ER kinase
SERCA	Sarco(endo)plasmic reticulum Ca^{2+}-ATPase
TAU	Taurine

14.1 Introduction

The ER is a highly specialized organelle where newly synthesized proteins are folded into their tridimensional structure which is crucial for their biological activity (Hotamisligil 2010). ER stress is a cellular response activated by the intra-reticular accumulation of misfolded proteins due to increased protein synthesis or poor ER function. Adequate protein folding is dependent on ER Ca^{2+} stores that are maintained by the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump that actively transports Ca^{2+} from the cytoplasm to the ER lumen (Eizirik et al. 2008). In fact, ER Ca^{2+} depletion using SERCA pump inhibitors such as thapsigargin leads to impaired protein folding capacity and activation of ER stress response initiated by the ER membrane-residing proteins PKR-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE1), and activating transcription factor-6 (ATF6) (Lytton et al. 1991; Lin et al. 2008). In addition, ER stress immediately inhibits protein synthesis that occurs through the PERK/eIF2/ATF4 branch of this pathway. Persistence of the stressing conditions increases the expression of the transcription factor C/EBP homologous protein (CHOP) leading to cell death via apoptosis (Hotamisligil 2010).

Poor nutrition during gestation and early life can predispose to the development of metabolic disturbances in adulthood such as hypertension, obesity, and type 2 diabetes mellitus (Remacle et al. 2007). Previous studies showed that low-birth-weight children had increased risk for becoming insulin resistant in adulthood (Hales and Barker 1992; Jaquet et al. 2000). Increased ER stress was already reported in protein malnutrition (Sparre et al. 2003; Vo and Hardy 2012) as well as

type 2 diabetes (Ozcan et al. 2004; Cnop et al. 2012) and may be the molecular link between these conditions.

Taurine (TAU) is a sulfur-containing amino acid know to exert positive effects upon beta cell function and glucose homeostasis (Nakaya et al. 2000; Tsuboyama-Kasaoka et al. 2006; Carneiro et al. 2009; Ribeiro et al. 2009; Ribeiro et al. 2012). Plasma TAU levels are reduced in plasma of protein-restricted dams and their fetuses (Cherif et al. 1998). It was previously reported by our group that TAU supplementation to malnourished rats normalized insulin secretion and glucose tolerance and increased protein expression of SERCA3 in pancreatic islets (Batista et al. 2012), suggesting improved islet ER function. Here we assessed the effects of TAU supplementation upon insulin secretion and ER stress markers in pancreatic islets and in the liver from malnourished mice.

14.2 Methods

14.2.1 Animals and Groups

All experiments were approved by the ethics committee at UNICAMP. The studies were carried out on 21-day-old male Swiss mice obtained from the breeding colony at UNICAMP and maintained at 22 ± 1°C, on a 12-h light–dark cycle, with free access to food and water. The mice were distributed into four groups: mice that received a diet containing 17% of protein without (NP) or with 2.5% of TAU in their drinking water (NPT), or mice submitted to an isocaloric diet containing 6% of protein (low-protein diet) without (LP) or with TAU supplementation (LPT). During experimental period, body weight was monitored weekly. Diet composition was previously reported (Filiputti et al. 2008).

14.2.2 Plasma Insulin

At the end of the diet and supplementation period, anesthetized fed mice were decapitated and their blood was collected and centrifuged at 10,000 rpm for 5 min at 4°C. Plasma was collected and stored at −20°C. Plasma insulin was measured by radioimmunoassay (RIA; as previously reported by Ribeiro et al. 2010).

14.2.3 Islet Isolation and Static Insulin Secretion

Islets were isolated by collagenase digestion of the pancreas. For static incubations, five islets from each group were first incubated for 30 min at 37°C in Krebs–bicarbonate (KBR) buffer with the following composition: NaCl 115 mmol/L, KCl 5 mmol/L, CaCl2 2.56 mmol/L, MgCl2 1 mmol/L, NaHCO3 10 mmol/L, and

HEPES 15 mmol/L, supplemented with 5.6 mmol/L glucose and 3 g of BSA/L, and equilibrated with a mixture of 95% O_2/5% CO_2 to give pH 7.4. This medium was then replaced with fresh buffer and the islets were incubated for 1 h with 2.8, 11.1, 16.7, or 22.2 mmol/L glucose. At the end of the incubation, the supernatant was collected and insulin of the medium was measured by RIA.

14.2.4 Western Blot

Liver fragments and pancreatic islets were homogenized in extraction buffer containing 100 mmol/L Tris pH 7.5, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L PMSF, and 1% Triton X-100. The extracts were then centrifuged at 12,000 rpm at 4°C for 40 min to remove insoluble material. The protein concentration in the supernatants was assayed using the Bradford dye method (Bradford 1976). Next, samples were treated with a Laemmli sample buffer containing dithiothreitol. After heating at 95°C for 5 min, the proteins were separated by electrophoresis (30–70 μg protein/lane, 10% gels). Following electrophoresis, proteins were transferred to nitrocellulose membranes. The membranes were treated overnight with a blocking buffer (5% nonfat dried milk, 10 mmol/L Tris, 150 mmol/L NaCl, and 0.02% Tween 20) and were subsequently incubated with specific antibodies against p-Akt, Akt, p-ERK, ERK, ATF4, CHOP, α-tubulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and p-PERK, BIP (Cell Signaling Inc. Danvers, MA, USA). Detection was performed after 2-h incubation with a horseradish peroxidase-conjugated secondary antibody (1:10,000, Invitrogen, São Paulo, SP, BRA). The band intensities were quantified by optical densitometry using the free software, Image J Tool (http://ddsdx.uthscsa.edu/dig/itdesc.html).

14.2.5 Statistical Analysis

Results are presented as means ± SEM for the number of determinations (n) indicated. The statistical analyses were carried out using two-way analysis of variance (ANOVA) followed by the Newman–Keuls post hoc test ($P \leq 0.05$) and performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

14.3 Results

14.3.1 Growth Analysis

Body weight (BW) was recorded weekly as illustrated in Fig. 14.1a. Total BW, calculated by the area under curve (AUC), was reduced in LP compared with NP mice ($P < 0.001$; Fig. 14.1b). TAU supplementation had no effect on BW of either group.

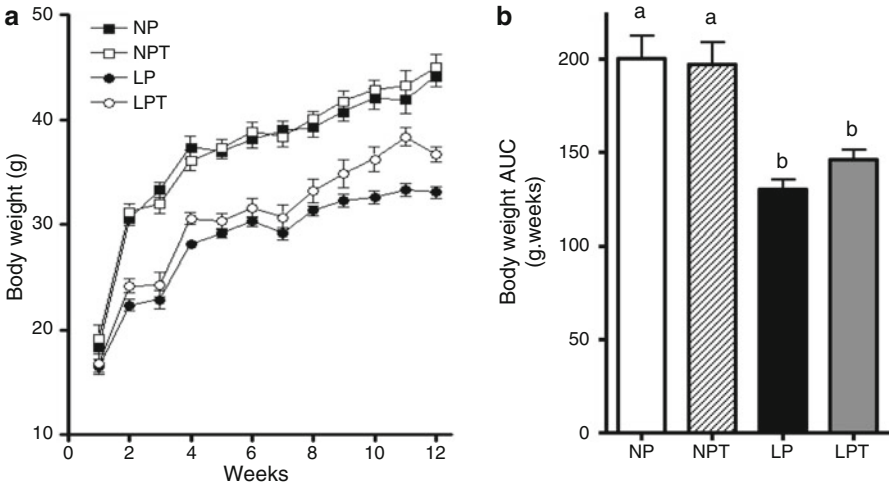

Fig. 14.1 (**a**) Body weight and (**b**) area under growth curve (AUC) of NP, NPT, LP, and LPT mice. Values are mean ± SEM ($n = 8$); different letters over bars indicate statistical difference; $P < 0.05$ (two-way ANOVA, Newman–Keuls post hoc test)

14.3.2 Plasma Insulin and Insulin Secretion

Fed plasma insulin levels were reduced in LP mice compared with NP ($P < 0.05$, Fig. 14.2a). TAU supplementation had no effect on this parameter.

Insulin release by isolated islets from LP mice was reduced at all stimulatory glucose concentrations (11.1–22.2 mmol/L) when compared to NP group (Fig. 14.2b, $P < 0.05$). TAU supplementation increased insulin secretion in NPT islets in the presence of 16.7 and 22.2 mmol/L glucose ($P < 0.03$) and at all glucose concentrations LPT islets showed a similar insulin secretion to that observed of NP islets (Fig. 14.2b).

14.3.3 ER Stress Marker Protein Expression

Isolated islets from LP mice showed a similar expression of ER stress markers compared with NP (Fig. 14.3). TAU supplementation significantly reduced CHOP protein expression in both NPT and LPT islets compared with NP ($P < 0.05$ and $P < 0.01$, respectively, Fig. 14.3a) and lowered islet ATF4 protein content only in NPT group ($P < 0.05$, Fig. 14.3b). PERK phosphorylation (p-PERK) and BIP expression were not altered between groups (Fig. 14.3c, d). The phosphorylated form of the pro-survival protein Akt (p-Akt) was increased in NPT compared with NP islets ($P < 0.05$; Fig. 14.3e). Also, NP pancreatic islets incubated with 3 mmol/L TAU presented higher ERK1/2 phosphorylation (p-ERK1/2) after 90 s ($P < 0.001$) and

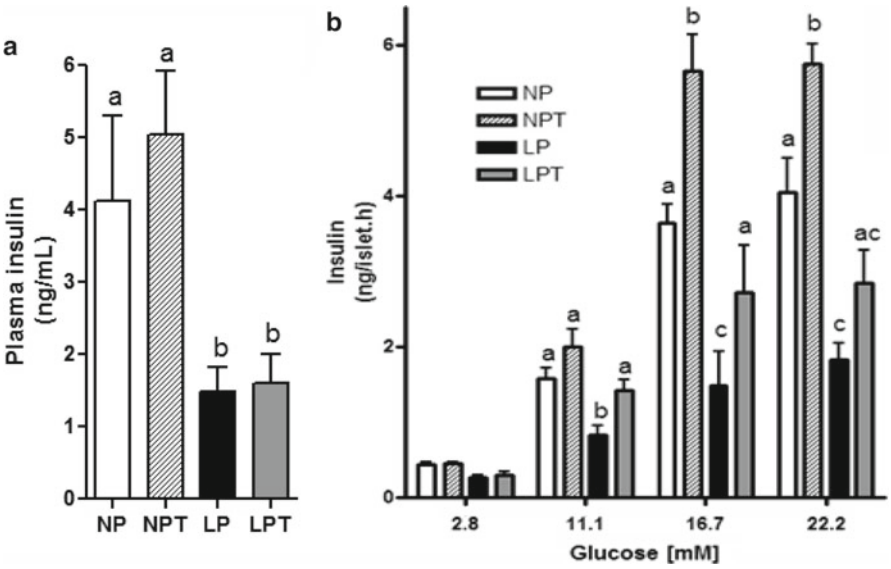

Fig. 14.2 (**a**) Fed plasma insulin ($n = 5$–16) and (**b**) glucose-induced insulin secretion in isolated pancreatic islets ($n = 8$) from NP, NPT, LP, and LPT mice. Values are mean ± SEM; different letters over bars indicate statistical difference; $P < 0.05$ (two-way ANOVA, Newman–Keuls post hoc test)

15 min ($P < 0.05$) and returned to normal levels after 1 h (Fig. 14.3f). Akt phosphorylation was not altered by acute incubation with TAU (Fig. 14.3g).

Despite no modification in islet protein ER stress maker profile in LP islets, p-PERK and BIP protein expression in the liver of LP mice was higher than in NP mice ($P < 0.05$; Fig. 14.4a, b). Increased liver ER stress marker expression in LPT mice was prevented by TAU supplementation.

14.4 Discussion

Here, we describe that mice fed on a low-protein diet have lower BW and plasma insulin and isolated islets from these mice secrete less insulin in response to glucose (Figs. 14.1 and 14.2). These findings are in accordance with previous studies from our group (Amaral et al. 2010; Filiputti et al. 2010; da Silva et al. 2012) and others (Chen et al. 2009; Theys et al. 2009).

In this study, TAU supplementation enhanced glucose-stimulated insulin secretion (GSIS) in isolated islets from control and malnourished mice (Fig. 14.2b). TAU supplementation was already reported to enhance beta cell responsiveness to nutrients and other stimuli (Carneiro et al. 2009; Ribeiro et al. 2009). These effects of TAU were mainly due to the improvement upon beta cell

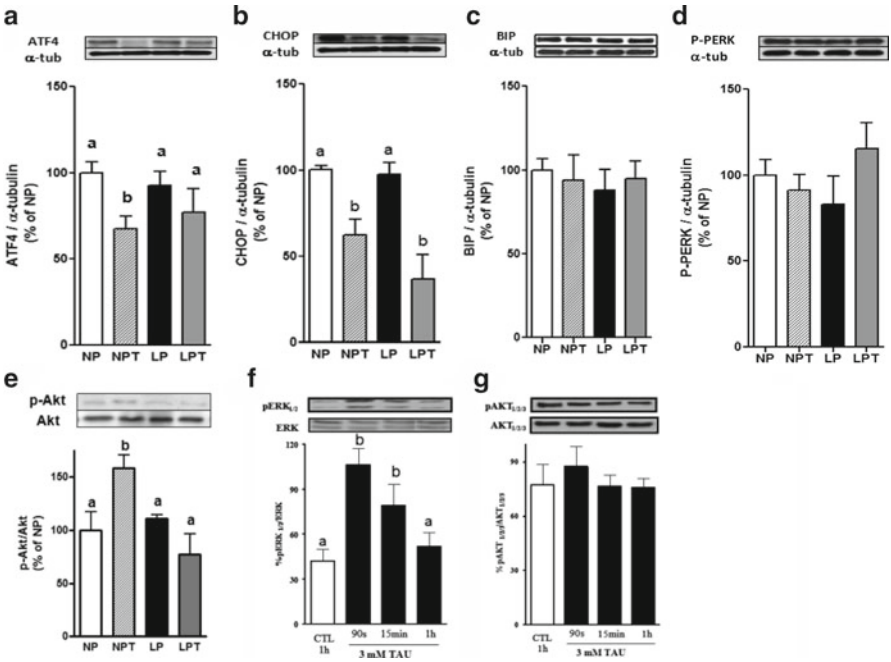

Fig. 14.3 Protein expression of (**a**) CHOP, (**b**) ATF4, (**c**) BIP, (**d**) p-PERK, and (**e**) p-Akt and α-tubulin (internal control) in islets from NP, NPT, LP, and LPT mice ($n = 4–7$). Groups of fresh isolated islets from NP mice were incubated with 3 mmol/L TAU for evaluation of (**f**) p-$ERK_{1/2}$/$ERK_{1/2}$ and (**g**) pAkt/Akt ratio. Values are mean ± SEM ($n = 5$); different letters *over bars* indicate statistical difference; $P < 0.05$ (two-way ANOVA, Newman–Keuls post hoc test)

Ca^{2+} handling, since enhanced Ca^{2+} uptake and the β2 subunit of the voltage-sensitive Ca^{2+} channel protein expression were observed in islets from TAU-treated mice (Ribeiro et al. 2009). Another finding is that TAU supplementation enhanced intracellular Ca^{2+} mobilization using the cholinergic agonist, carbachol (Ribeiro et al. 2010), suggesting an increased compartmentalization of the cation into the ER that may be maintained by SERCA3, since isolated islets from malnourished and control TAU-supplemented rats presented higher expression of this protein (Batista et al. 2012).

Considering these actions of TAU upon intra-reticular Ca^{2+} stores and that maternal protein-restriction leads to increased ER stress marker expression in the offspring (Sparre et al. 2003; Vo and Hardy 2012), we decided to evaluate the expression of these proteins in pancreatic islets and in the liver from malnourished mice supplemented with TAU. Western blot analysis revealed that malnutrition did not alter the expression of ER stress markers in pancreatic islets but PERK phosphorylation and BIP expression were increased in the liver from malnourished mice (Figs. 14.3 and 14.4). It was reported that young malnourished rats display increased glucose tolerance and insulin sensitivity (Reis et al. 1997; da Silva et al. 2012), but at 15

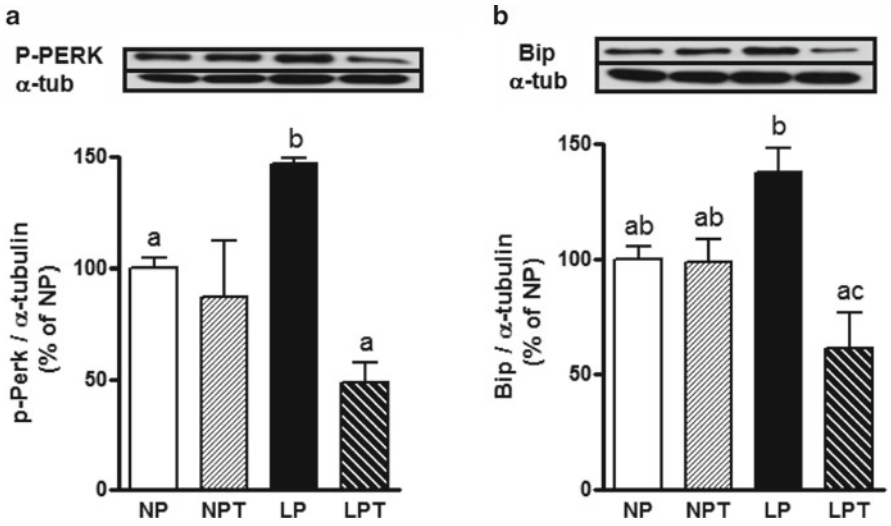

Fig. 14.4 Protein expression of (**a**) p-PERK and (**b**) BIP in liver of NP, NPT, LP, and LPT mice. Values are mean ± SEM ($n = 5$); different letters over bars indicate statistical difference; $P < 0.05$ (two-way ANOVA, Newman–Keuls post hoc test)

months of age insulin signaling in adipocytes was impaired (Ozanne et al. 2001) and at 17 months, these rats become diabetic (Petry et al. 2001). Since ER stress impairs insulin signaling and is associated to the pathogenesis of obesity and type 2 diabetes (Ozcan et al. 2004; Zhou et al. 2011), we believe that this pathway may link the transition from increased sensitivity to insulin resistance that occurs throughout the life span in malnourished rodents.

Finally, we observed that TAU supplementation normalized ER stress markers in the liver from malnourished mice and lowered their expression in pancreatic islets from both supplemented groups (Figs. 14.3 and 14.4). TAU was reported to reduce ER stress induced by several agents in different tissues and cell types. In primary neuron cultures, TAU treatment reduces hypoxia and glutamate-induced ER stress (Pan et al. 2012). TAU protects H4IIE liver cells from palmitate-induced cell death and caspase-3 activation and prevents hepatic steatosis in high sucrose-fed rats through suppression of the PERK/eIF2/ATF4 branch of the ER stress pathway (Gentile et al. 2011). The proper mechanisms by which TAU reduces ER stress are still not clear. Here we show for the first time that TAU supplementation increases p-Akt in pancreatic islets and acute incubation with this amino acid increased islet p-ERK1/2 content (Fig. 14.3e, f). These findings could be explained by a direct interaction of TAU with the insulin receptor leading to its activation (Maturo & Kulakowski 1988; Carneiro et al. 2009). Transgenic mice overexpressing Akt in the heart showed prevention of contractile dysfunction provoked by tunicamycin, a chemical that induces ER stress (Zhang et al. 2011). Thus, TAU-induced increase in Akt activation (Fig. 14.3e) could contribute to decreased ER stress protein expression in pancreatic islets.

14.5 Conclusion

In conclusion, our data indicate that ER stress is present in the liver from protein-malnourished mice and this could be a risk factor for the development of insulin resistance and type 2 diabetes in later life. TAU supplementation increases insulin secretion capacity and reduces ER stress proteins in pancreatic islets and in the liver possibly through increased Akt and ERK 1/2 activation.

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Chapter 15
Taurine as a Marker for the Identification of Natural *Calculus Bovis* and Its Substitutes

Kayoko Shimada, Yuko Azuma, Masaya Kawase, Toshiharu Takahashi, Stephen W. Schaffer, and Kyoko Takahashi

Abstract *Calculus Bovis* (*C. Bovis*) is a commonly used animal-derived therapeutic preparation. To meet the increasing clinical demand for the preparation, two artificial substitutes for *Bos Taurus* have been introduced in China: artificial *C. Bovis* and in vitro cultured *C. Bovis*. However, information on their efficacy and safety is inadequate. Therefore, we investigated the biological differences between the commonly used natural preparation and its two substitutes, with the aim of not only identifying the differences but also providing a procedure to distinguish between the different preparations.

In the study, we prepared 9 natural *C. Bovis*, 2 artificial *C. Bovis*, and 2 in vitro cultured *C. Bovis* preparations for evaluation. Differences were noted between the three preparations relative to their effect on viability of cardiac fibroblasts from 1-day-old Wistar rats. Although natural *C. Bovis* had no effect on cell viability, 1-h treatment of the cells with 0.25 mg/ml of the substitutes significantly reduced cell viability, as

K. Shimada (✉) • Y. Azuma
Department of Applied Pharmacognosy, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
e-mail: k-simada@phs.osaka-u.ac.jp

M. Kawase
Nagahama Institute of Bio-Science and Technology, Shiga, Japan

T. Takahashi
Kyoto University Research Reactor Institute, Kyoto University, Osaka, Japan

S.W. Schaffer
School of Medicine, University of South Alabama, Mobile, AL, USA

K. Takahashi
Department of Applied Pharmacognosy, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

The Museum of Osaka University, Osaka, Japan

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detected by the MTS assay. Based on liquid chromatography and inductively coupled plasma mass spectrometry, the preparations also differed in composition. Indeed, the substitutes contained more taurine, cholic acid, iron, magnesium, and calcium than the natural preparations. They also differed spectroscopically.

The present results reveal significant biological differences between natural *C. Bovis* and two of its substitutes. Since the substitutes appear to contain more taurine, cholic acid, and elements, these constituents may serve as markers to distinguish between natural *C. Bovis* and its substitutes.

Abbreviations

ICP-MS	Inductively coupled plasma mass spectrometry
THz	Terahertz
LINAC	Linear accelerator
KURRI	Kyoto University Research Reactor Institute
PCA	Principal components analysis

15.1 Introduction

Calculus Bovis (*C. Bovis*: *Bezoar Bovis*) is an important animal-derived therapeutic agent that originates from gallstones formed in the gall sac of *Bos Taurus* Linné var. *domesticus* Gmelin (Bovidae) in the Japanese pharmacopoeia 16th edition (Ministry of Health, Labour and Welfare 2011). Only one out of every 1,000 cattle contains gallstones for *C. Bovis*, making the preparation quite expensive. However, there is increased therapeutic demand for it.

Many studies have been carried out to find substitutes for natural *C. Bovis*. There are two kinds of substitutes of *C. Bovis* in Chinese pharmacopoeia that are available for clinical practice: one is artificial *C. Bovis* (Bovis Calculus Artifactus), a mixture of bile salts, bilirubin, taurine, and some undetermined ingredients. The other is in vitro cultured *C. Bovis* (Bovis Calculus Sativus), produced in vitro under conditions mimicking in vivo gallstone formation (Qin 2008; Chinese Pharmacopoeia Committee of People's Republic of China 2010). Previously we showed that the substitutes are distinguishable by multiple element analysis from natural *C. Bovis* (Takahashi et al. 2010). Moreover, differences in organic composition (Yan et al. 2007; Kong et al. 2010) and morphology (Yamaguchi et al. 2008) between natural *C. Bovis* and its substitutes have been observed. However, further information is required to properly use the substitutes in clinical practice.

Therefore, the aim of the present study is to identify differences between natural *C. Bovis* and its substitutes relative to biological activity and composition. The intention of the report is to (1) establish the importance of these differences and (2) introduce methodology to distinguish between natural and artificial preparations.

Table 15.1 Summary of the materials used in the study

No.	Species	Properties	Collection site	Collection year	Sample name
1	Natural *C. Bovis*	Powder	Australia	2006	Aus-1
2	Natural *C. Bovis*	Powder	Australia	2001	Aus-2
3	Natural *C. Bovis*	Powder	Argentine	2001	Argentine
4	Natural *C. Bovis*	Powder	Brazil	2001	Brazil
5	Natural *C. Bovis*	Powder	Guatemala	2001	Guatemala
6	Natural *C. Bovis*	Powder	Mexico	2001	Mexico
7	Natural *C. Bovis*	Powder	Kenya	2001	Kenya
8	Natural *C. Bovis*	Clod	China	2007	China
9	Natural *C. Bovis*	Clod	India	1978	India
10	Artificial *C. Bovis*	Powder	China	2007	Art-1
11	Artificial *C. Bovis*	Powder	China	1971	Art-2
12	In vitro cultured *C. Bovis*	Clod	China, Anhui	2009	Cul-1
13	In vitro cultured *C. Bovis*	Clod	China, Hubei	2009	Cul-2

15.2 Methods

15.2.1 Sample Preparation

Thirteen samples of *C. Bovis* were collected from various locations as shown in Table 15.1. Dr. K. Takahashi purchased the samples named "China" and "Art-1" from local stores in Shenyang, while "India" and "Art-2" are historical specimens stored in the Museum of Osaka University, Japan. The rest of the specimens were purchased from Tochimoto Tenkai-Do (Osaka, Japan). Seventy-five milligram of each sample was extracted with 1 ml DMSO and then 0.5 ml water was added. Samples were vortexed, and sonicated for 5 min before centrifugation at 12,000 × *g* for 20 min at 4°C. The supernatant was collected and stored at −20°C.

15.2.2 Evaluation of Cell Viability

Primary cardiac fibroblast cultures were prepared from 1-day-old Wistar rats as described previously (Takahashi et al. 2002). The cells were seeded in a 96-well plate after a few days' incubation in serum-containing culture medium, Dulbecco's modified Eagle's medium/F-12 (Dainippon Pharmaceutical Co., Ltd.) (1:1 vol/vol) supplemented with newborn calf serum (GIBCO; 10%, heat-inactivated). After 24-h incubation in serum-containing culture medium, cells were transferred to serum-free medium. After 24-h incubation, cells were treated with 0.25 mg/ml of Aus-1, Art-1, Cul-1, and Cul-2 for 1 h. Following removal of the sample solution, the cells were then incubated in serum-free medium (100 μl) with CellTiter 96® aqueous one solution reagent (Promega Corporation) (20 μl) for 3 h. Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells at 490 nm.

15.2.3 LC Detection

Ten mg of samples were extracted with 5 ml of water for 0.5 h at room temperature. The suspension was vortexed and centrifuged at 12,000×*g* for 20 min at 4°C. LC analysis was performed on a LaChromUltra System (HITACHI) as shown previously (Hitachi High-Technologies Corporation) and LCMS-2020 System (SHIMADZU). LC-MS separations were carried out at 40°C with a Shim-pack XR-ODS column, 2.2 μm, 2.0 mm×75 mm (SHIMADZU). Solvent A was 5 mM $HCOONH_4$ and 0.1% HOOH mixture and solvent B was CH_3CN. The initial composition of the binary solvent was 100% of B from 0 to 10 min. Solvent B was decreased from 100 to 20% in 0.01 min and it remained for 5 min. A flow rate was set at 0.4 ml/min, and ESI-MS was used to detect the bile acids.

15.2.4 ICP-MS Measurement

Ten milligram of samples were added to 1 ml of HNO_3 (Nacalai Tesque), vortexed, and allowed to stand overnight at room temperature. Then, 100 μl of samples were diluted with 9.9 ml of water and filtered through 0.45 μm pore size hydrophilic PTFE membrane filter (Millipore). Inductively coupled plasma mass spectrometry (ICP-MS) analysis was performed on the Agilent 7500 Series ICP-MS (Agilent Technologies).

Among the attained data, 9 elements exhibited concentrations exceeding 1 μg/l on average. Each data in the chart was shown as relative concentration by setting the maximum concentration of each element in the samples as 1.

15.2.5 Spectroscopic Analysis

The dried and grounded samples were analyzed using coherent transition radiation millimeter wave, coherent terahertz (THz) wave. Coherent THz wave was generated by L-band electron linear accelerator (L-band LINAC) at Kyoto University Research Reactor Institute (KURRI). The spectra were detected as shown by Okuda and Takahashi (2008), with Martin-Puplett-type interferometer and liquid He-cooled silicon bolometer.

15.2.6 Statistical Analysis

Statistical significance was determined by the Student's *t*-test and Kruskal–Wallis test. Each value was expressed as the mean ± S.E. Differences were considered statistically significant when the calculated P value was less than 0.05.

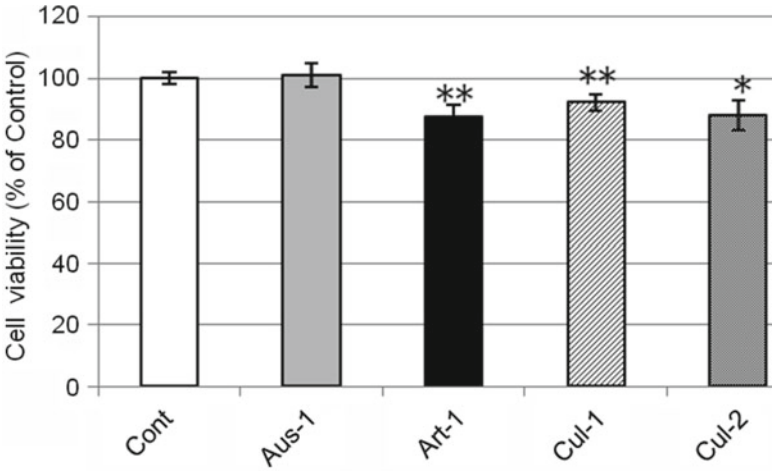

Fig. 15.1 Influence of natural *C. Bovis* and its substitutes on cardiac fibroblast viability. Cells were incubated for 1 h with medium containing 0.25 mg/ml sample extracts. Values are expressed as percent of control, in which the control cells were untreated. Data represent means ± S.E. obtained from 15 to 29 samples from triplicate experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with control

15.3 Results and Discussion

15.3.1 The Biological Effects of Natural C. Bovis and Its Substitutes on the Morphology of Cultured Fibroblasts

C. Bovis is a common animal-derived therapeutic preparation often used as a cardiotonic, an antipyretic, an antispasmodic, an antidote, and a child sedative. As we reported previously, of the 230 cardioactive OTC drugs, 228 (98.7%) contain *C. Bovis* in Japan (Takahashi et al. 2008). In order to ascertain the biological differences of natural *C. Bovis* from its substitutes, we investigated their effects on cultured cardiac fibroblasts using the MTS assay. As shown in Fig. 15.1, although 0.25 mg/ml of natural *C. Bovis* (Aus-1) did not affect fibroblast viability, substitutes (Art-1, Cul-1, Cul-2) decreased cell viability approximately 10% after 1-h incubation.

15.3.2 The Organic Composition of Natural C. Bovis and Its Substitutes

In order to investigate differences in organic composition between *C. Bovis* and its substitutes, bile acids, such as cholic acid, taurocholic acid, and deoxycholic acid, were measured. Although cholic acid accounts for less than 40% of bile acid content in natural *C. Bovis* samples (Aus-1, Aus-2, Brazil, Mexico, Kenya, and China),

about 50–80% of bile acid content consisted of cholic acid in the substitutes. These values are in agreement with Yan et al. (2007), who previously reported that substitutes contained much higher levels of cholic acid than the natural preparations. Based on principal components analysis (PCA), Kong et al. (2010) concluded that natural *C. Bovis* and its substitutes underwent different manufacturing procedures, leading to significant differences in cholic acid content. We suggest that cholic acid is likely added intentionally during the manufacturing process of the substitutes; therefore, cholic acid serves as a predictable marker of the substitutes. Moreover, the high content of cholic acid likely contributes to the toxicity of the substitutes towards cardiac myocytes and fibroblasts (Takahashi et al. 2010).

Although most natural *C. Bovis* preparations (Aus-1, Aus-2, Brazil, Mexico, and Kenya) contain less than 5 μmol/g taurine, Cul-2 and Art-1 contain more than 200 μmol/g of the amino acid. Taurine has been identified as a characteristic amino acid component of *C. Bovis*. It has been previously shown that the ratio of taurine to free amino acids and total amino acids of *C. Bovis* is significantly higher than in other crude animal drugs. According to Hashimoto et al. (1994), the taurine content of *C. Bovis* is about 3.6–11.6 μmol/g, a range consistent with the value reported in this study. On the other hand, some substitutes contain more than 200 μmol/g of the cardioprotective amino acid (Wójcik et al. 2012; Yamori et al. 2009, 2010). It also appears to be added to the preparation of the substitutes and could serve as a marker to distinguish natural samples from their substitutes.

15.3.3 The Inorganic Composition of Natural C. Bovis and Its Substitutes

We also focused on the levels of certain inorganic ingredients using ICP-MS. The relative concentration of 9 elements that were present in the sample extracts at a concentration exceeding 1 μg/l are shown in Fig. 15.2. Natural *C. Bovis* contained more copper, manganese, and zinc than the substitutes. On the other hand, the substitutes were characterized by high levels of iron, magnesium, and calcium. According to Okasaka (2008), in vitro cultured *C. Bovis* is made from bilirubin, some bile acids, ferrous sulfate, magnesium sulfate, cattle bile, water, and other additives. Thus, the elements added during the manufacturing process could also serve as markers to distinguish the substitutes from natural *C. Bovis*.

15.3.4 The Spectroscopic Analysis of Natural C. Bovis and Its Substitutes

We have previously reported differences between natural *C. Bovis* and its substitutes using Mössbauer analysis (Azuma et al. submitted). In the present study, we analyzed the spectroscopic characteristics of the extract samples using KURRI-LINAC

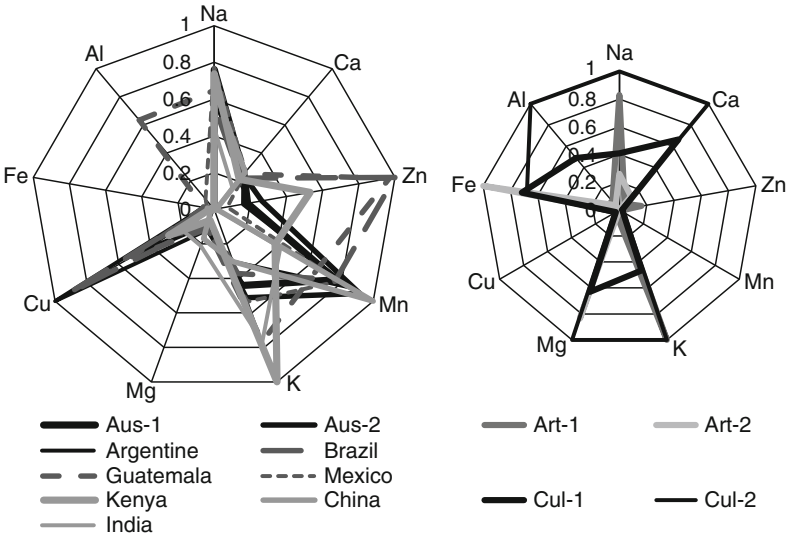

Fig. 15.2 Typical chart of ICP-MS of natural *C. Bovis* and its substitutes. Shown are the concentrations of various elements found in sample extracts at concentrations exceeding 1 μg/l. The data represent relative concentrations, with the maximum concentration of each element in the extract set at 1 ($n=3$)

(Okuda and Takahashi 2008; Takahashi and Takami 2008) as a coherent transition radiation millimeter wave. Typical spectra between 0 and 30 cm^{-1} are shown in Fig. 15.3. While the spectrum of Art-1 is comparatively complicated [Fig. 15.3b], the spectrum of Cul-1 [Fig. 15.3c] is quite distinct and reveals differences between the natural and artificial preparations [Fig. 15.3a].

Coherent radiation is a popular light source that spans the millimeter- and submillimeter-wave region. In this region, vibration and rotation characteristics of various molecules determine the nature of the absorption bands; therefore, one would expect the spectrum to be quite simple. Remarkably, this is the first report of the spectra of natural samples attained using this method. In this study, the spectra attained from Aus-1, Art-1, and Cul-1 were different, which reflects the physical properties of the samples. In order to establish the utility of this novel method, further analysis of the spectra is necessary.

This study identifies potential markers to distinguish natural *C. Bovis* from its substitutes, including differences in organic composition (taurine and cholic acid), inorganic ingredients (iron, magnesium, calcium), and spectroscopic properties. It is possible that the higher organic and inorganic content of the substitutes could affect the clinical actions of the substitutes relative to natural *C. Bovis*.

The clinical demand for natural *C. Bovis* is increasing at a rate that renders the current supply inadequate. This has led to the development of active substitutes. According to Yan et al. (2007), there is a high batch-to-batch uniformity of the substitutes and in vitro cultured *C. Bovis*, a property that facilitates proper clinical use.

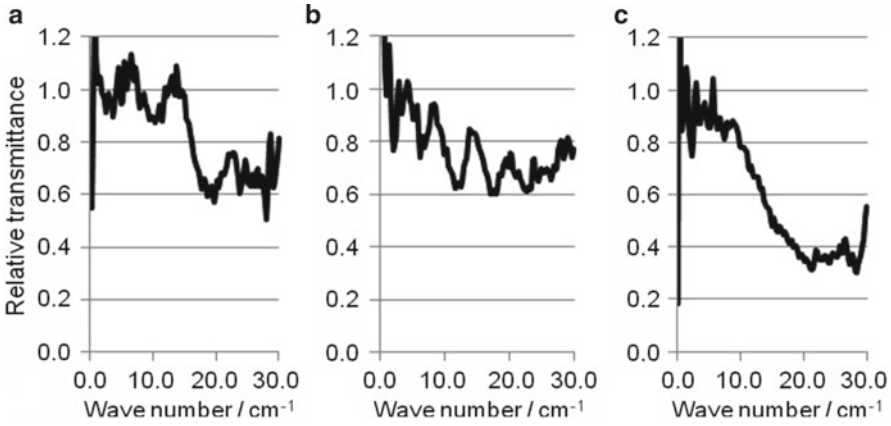

Fig. 15.3 Typical light spectrum measured using coherent transition radiation millimeter wave light source for (**a**) Aus-1, (**b**) Art-1, (**c**) Cul-1

Yan et al. (2007) also noted the high concentration of bioactive compounds among various substitutes. It is noteworthy, however, that despite the high content of the cardioprotective agent, taurine, the substitutes, but not natural *C. Bovis*, decreased cardiac fibroblast viability. Moreover, although natural *C. Bovis* has no effect on morphology, viability, and beating pattern of cardiac cells in culture, the equivalent concentration of cholic acid and deoxycholic acid causes cell injury (Takahashi et al. 2010). This result suggests that the adequate proportion of components is important for *C. Bovis* to exert its medicinal effect. The efficacy of in vitro cultured *C. Bovis* and artificial *C. Bovis* has been examined using in vivo and in vitro models with the aim of attaining equal efficacy for the various preparations (Li et al. 2010; Zang et al. 2011). The present studies suggest methodology to distinguish natural *C. Bovis* and its substitutes and provide clues for improvement of the substitutes.

15.4 Conclusion

In summary, this study shows significant biological differences between natural *C. Bovis* and two of its substitutes. The substitutes were revealed to contain more taurine, cholic acid, and specific elements than the natural preparations. These components may serve as markers to distinguish natural *C. Bovis* from its substitutes.

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Part II
Protective Role of Taurine

Chapter 16
Taurine Deficiency and MELAS Are Closely Related Syndromes

Stephen W. Schaffer, Chian Ju Jong, Danielle Warner, Takashi Ito, and Junichi Azuma

Abstract MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) is a mitochondrial disease caused by one or more mutations of $tRNA^{Leu(UUR)}$. These mutations reduce both the aminoacylation of $tRNA^{Leu(UUR)}$ and a posttranslational modification in the wobble position of $tRNA^{Leu(UUR)}$. Both changes result in reduced transcription of mitochondria-encoded proteins; however, reduced aminoacylation affects the decoding of both UUG and UUA while the wobble defect specifically diminishes UUG decoding. Because 12 out of the 13 mitochondria-encoded proteins are more dependent on UUA decoding than UUG decoding, the aminoacylation defect should have a more profound effect on protein synthesis than the wobble defect, which more specifically alters the expression of one mitochondria-encoded protein, ND6. Taurine serves as a substrate in the formation of 5-taurinomethyluridine-$tRNA^{Leu(UUR)}$; therefore, taurine deficiency should mimic 5-taurinomethyluridine-$tRNA^{Leu(UUR)}$ deficiency. Hence, the wobble hypothesis predicts that the symptoms of MELAS mimic those of taurine deficiency, provided that the dominant defect in MELAS is wobble modification deficiency. On the other hand, if the aminoacylation defect dominates, significant differences should exist between taurine deficiency and MELAS. The present review tests this hypothesis by comparing the symptoms of MELAS and taurine deficiency.

S.W. Schaffer (✉) • C.J. Jong • D. Warner
Department of Pharmacology, School of Medicine, University of South Alabama, Mobile, AL, USA
e-mail: sschaffe@jaguar1.usouthal.edu

T. Ito • J. Azuma
Hyogo University of Health Sciences, School of Pharmacy, Kobe, Japan

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_16,

Abbreviation

MELAS Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes

16.1 Introduction

The ubiquitous β-amino acid taurine is considered nutritionally essential for cats, fox, and some monkeys; nonessential for rodents; and conditionally essential for humans (Gaull 1986). The animals that exhibit an absolute dependence on a nutritional source of taurine develop a host of pathological abnormalities when fed a taurine-deficient diet, including cardiovascular defects, metabolic and endocrine alterations, renal insufficiency, muscle weakness, neurological abnormalities, retinal disorganization, and hearing loss. A major cause of these pathologies is impaired electron transport chain function, with normal respiratory activity relying on unimpeded biosynthesis of mitochondria-encoded protein subunits of the respiratory chain complexes. The biosynthesis of these proteins is determined by several posttranslational reactions, including the formation of 5-taurinomethyluridine in the wobble position of $tRNA^{Leu(UUR)}$. Reductions in 5-taurinomethyluridine-$tRNA^{Leu(UUR)}$ content reduce the decoding of UUG codons (Kirino et al. 2004). Taurine deficiency deprives the mitochondria of substrate (taurine) for 5-taurinomethyluridine-$tRNA^{Leu(UUR)}$ biosynthesis, which in turn diminishes the cellular levels of UUG-dependent mitochondria-encoded proteins (Jong et al. 2012). Moreover, UUG decoding is also impaired in MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes), a mitochondrial disease caused by one or more mutations of $tRNA^{Leu(UUR)}$ that reduce the formation of 5-taurinomethyluridine-$tRNA^{Leu(UUR)}$ (Yasukawa et al. 2000).

Another major defect associated with MELAS-linked mutations is diminished aminoacylation of $tRNA^{Leu(UUR)}$, a reaction required for efficient decoding of both UUG and UUA. Because the mRNAs of 12 out of the 13 mitochondria-encoded proteins contain more UUA codons than UUG codons, diminished aminoacylation should theoretically decrease the biosynthesis of the 12 affected mitochondria-encoded proteins. On the other hand, mRNA of the remaining mitochondria-encoded protein, namely, ND6, contains 8 UUG codons, rendering its translation highly sensitive to the wobble defect (Jong et al. 2012). Because ND6 is a mitochondria-encoded protein subunit of complex I, taurine deficiency should only dramatically impact the activity of complex I. By comparison, an aminoacylation defect should diminish the activities of complexes I and III–V. Thus, a condition in which the taurine-linked posttranslational modification of $tRNA^{Leu(UUR)}$ becomes the key pathophysiological defect of MELAS should yield symptoms nearly indistinguishable from those of taurine deficiency. Thus, a comparison between the symptoms of taurine deficiency and MELAS provides key information on the importance of the wobble defect in the development of the MELAS syndrome.

The major pathway of taurine biosynthesis involves the oxidation of cysteine to cysteine sulfinic acid, the decarboxylation of cysteine sulfinic acid to hypotaurine, and the oxidation of hypotaurine to taurine (Jacobsen and Smith 1968). The amount of taurine generated by this hepatic pathway depends upon the activity of the rate-limiting enzyme, cysteine sulfinic acid decarboxylase, whose activity is nonexistent in cats, low in humans, and high in rodents (Bagley and Stipanuk 1995; Gaull 1986; Jacobsen and Smith 1968). Thus, cats can be nutritionally depleted of taurine but a taurine-deficient state can only be achieved in rodents by either inhibiting or knocking out the taurine transporter, which supplies extrahepatic cells with taurine from nutritional and biosynthetic sources. In the present review, the primary symptoms of MELAS (in humans) and taurine deficiency (in nutritionally depleted cats, taurine transporter knockout mice, and taurine transporter-inhibited rats) are compared.

16.2 Link Between MELAS Syndrome and Taurine Deficiency

16.2.1 Cardiovascular Defects of MELAS and Taurine Deficiency

Comorbidity of MELAS and cardiac disease is relatively common (Anan et al. 1995). This is not surprising because the heart requires high rates of ATP biosynthesis for the maintenance of normal contractile function. Interestingly, both hypertrophic and dilated cardiomyopathies have been observed in patients suffering from MELAS, suggesting that the pathophysiology of the mitochondrial disease is complex and may involve multiple factors. In fact, MELAS is also associated with conduction defects, including a high incidence of Wolff–Parkinson–White syndrome.

A cardiomyopathy and conductance defects are common in taurine-deficient animals. The taurine-deficient cardiomyopathy in cats exhibits both diastolic and systolic dysfunction (Novotny et al. 1991, 1994) although the majority of cats presenting with taurine-deficient cardiomyopathy suffer from only systolic dysfunction. Pion et al. (1987, 1992) found that cats with taurine deficiency-mediated dilated cardiomyopathy are successfully treated with taurine (Pion et al. 1987, 1992). However, prior to the discovery of taurine's role in the disease, many cats died with signs of cardiogenic shock and congestive heart failure (Pion et al. 1992). Although there are no reports of taurine deficiency causing Wolff–Parkinson–White-like syndrome, taurine depletion leads to prolongation of the action potential and the QT interval, which also increase the risk of arrhythmias (Lake et al. 1987). Thus, there are numerous similarities between the cardiovascular defects of taurine deficiency and MELAS. The possibility that taurine therapy might benefit MELAS, as it does congestive heart failure, is worthy of consideration (Azuma et al. 1983).

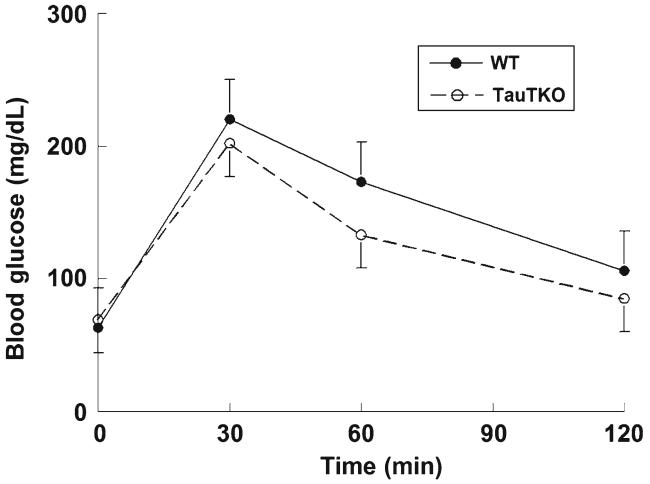

Fig. 16.1 Effect of taurine deficiency on glucose tolerance test

16.2.2 Endocrine and Metabolic Defects of MELAS and Taurine Deficiency

The most important link between the endocrine system and MELAS is diabetes. According to Vionnet et al. (1993), 2% of type 2 diabetic patients carry the primary MELAS-linked $tRNA^{Leu(UUR)}$ mutation. Although enhanced fatty acid metabolism contributes to the development of glucose intolerance in type 2 diabetes, a defect in glucose-induced insulin secretion by the pancreas can also cause glucose intolerance (Blondel et al. 1990; Maassen et al. 2006; Schaffer and Wilson 1993). A likely mechanism underlying the development of type 2 diabetes in patients with MELAS is a defect in insulin secretion related to oxidative damage of the pancreatic β-cell (Blondel et al. 1990; Maassen et al. 2004). When diabetes is superimposed on MELAS, the metabolic state of several tissues is altered. While diabetes is associated with reduced glucose metabolism and elevated fatty acid metabolism, in the nondiabetic MELAS patient energy production is dominated by anaerobic metabolism (Arakawa et al. 2010). Besides the decline in respiratory chain flux, MELAS is also associated with declines in citric acid cycle flux and fatty acid oxidation, as well as an increase in the $NADH/NAD^+$ ratio that results in enhanced lactate production culminating in lactic acidosis.

Hansen (2001) proposed that taurine depletion might potentiate the complications of diabetes through elevations in protein glycation, reduced osmotic control, endothelial dysfunction, impaired neutrophil function, and enhanced platelet aggregation. Because diabetes is considered a disease of oxidative stress, one would predict that reductions in taurine, which is an antioxidant, should potentiate the severity of diabetic complications. Taurine depletion might also increase the risk of pancreatic damage (Chang 2000). However, based on glucose tolerance tests of 3-month-old female taurine transporter knockout mice, there is no evidence of diabetes (Fig. 16.1). Nonetheless,

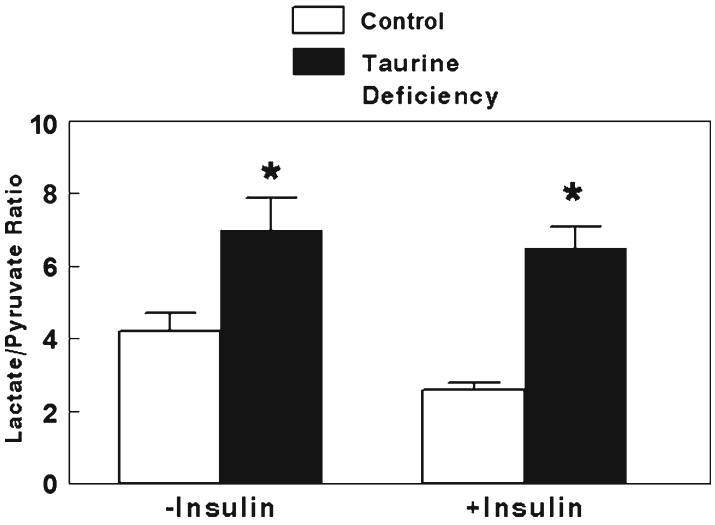

Fig. 16.2 Effect of taurine deficiency on lactate/pyruvate ratio. Hearts of taurine-deficient rats were perfused with Krebs–Hanseleit buffer containing or lacking insulin. The lactate/pyruvate ratio of the perfusate is shown. *Asterisks* indicate significant difference between taurine-deficient and control groups ($p < 0.05$)

the metabolic pattern of taurine deficiency favors the metabolism of glucose to lactate (anaerobic) at the expense of glucose oxidation (aerobic) (Fig. 16.2). This occurs because the decline in respiratory chain activity leads to an elevation in the NADH/NAD^+ ratio, which reduces the entry of pyruvate into the citric acid cycle and promotes the conversion of pyruvate to lactate (Mozaffari et al. 1986). This pattern is nearly identical to that seen in nondiabetic MELAS patients that present with lactic acidosis arising from inhibition of aerobic metabolism.

16.2.3 Growth Failure in MELAS and Taurine Deficiency

Growth failure is a widely recognized manifestation of MELAS that is often associated with migraine headaches, learning disabilities, and exercise intolerance (Sproule and Kaufmann 2008). Although it has been proposed that growth failure might involve deficiencies in growth hormone, inadequate conjugation of taurine with $tRNA^{Leu(UUR)}$ may be involved.

Hayes et al. (1980) found that taurine depletion of infant monkeys led to a striking depression in growth. Although the mechanism underlying this effect has not been determined, it would be attractive to suggest that growth retardation is related to impaired mitochondrial protein synthesis and respiratory chain dysfunction (Jong et al. 2012; Kirino et al. 2004). Further studies are warranted to investigate the mechanism underlying the development of this phenomenon.

16.2.4 Gastrointestinal Defects in MELAS and Taurine Deficiency

Common gastrointestinal manifestations of MELAS include abdominal discomfort and constipation, with vomiting, dysmotility, gastroparesis, malabsorption, and pseudo-obstruction being less common (Sproule and Kaufmann 2008). Although the mechanism underlying these complications has not been established, it is interesting that gastrointestinal defects are sometimes accompanied by hepatic dysfunction, which is found in taurine deficiency.

There have been no studies on the effect of taurine deficiency on the development of gastrointestinal defects. Nonetheless, taurine deficiency leads to chronic liver disease, associated with chronic hepatitis and liver fibrosis (Warskulat et al. 2006). The liver defect is caused in part by the loss of hepatocytes through apoptosis. However, in contrast to other examples of hepatocyte loss, the cell loss in taurine-deficient mice does not lead to a stimulation in hepatocyte production, an effect possibly related to the regulation of checkpoint genes by taurine (Golubnitschaja et al. 2003). Clearly the study of the gastrointestinal effects of taurine deficiency warrants further investigation.

16.2.5 Hearing Loss in MELAS and Taurine Deficiency

A maternally inherited family pedigree combining type 2 diabetes and sensorineural hearing loss has been described (van den Ouweland et al. 1992). The mutation causing this defect was ultimately shown to be identical to that causing MELAS. Overall, the frequency of hearing impairment in MELAS is about 0.1% at birth but greater than 50% by age 80 (Sproule and Kaufmann 2008).

Abnormal auditory brainstem-evoked potential has been detected in taurine-deficient kittens and preterm infants (Tyson et al. 1989; Vallecalle-Sandoval et al. 1991). Although these findings implicate taurine deficiency in the development of the auditory system, there is no firm evidence that taurine deficiency leads to overt hearing loss (Davies et al. 1994). However, it is interesting to note that type II cochlear afferents are stimulated by ATP (Weiss et al. 2009). Whether the decrease in ATP production by neurons of MELAS patients or taurine-deficient animals affects the function of type II cochlear afferents remains to be determined.

16.2.6 Muscle Weakness and Taurine Deficiency

Myopathy is one of the hallmark symptoms of MELAS (Sproule and Kaufmann 2008). In a study by Hirano and Pavlakis (1994), more than 85% of 110 MELAS patients examined were exercise intolerant; in a related study 73% of MELAS

patients exhibited exercise intolerance (Sproule et al. 2007). Not surprisingly, many MELAS patients show an unusual increase in lactic acid production upon exercise, an effect consistent with reduced respiratory chain activity (Hilton 1995).

Taurine treatment enhances physical endurance (Yatabe et al. 2003) while taurine deficiency leads to exercise intolerance (Ito et al. 2008; Warskulat et al. 2004). The symptom of muscle weakness in taurine transporter knockout mice has been attributed to the loss of muscle mass, diminished muscle size, and an increase in intracellular lactate content, effects that are comparable to those observed in MELAS patients.

16.2.7 Neurologic Defects in MELAS and Taurine Deficiency

MELAS is associated with several neurological and visceral manifestations. The term "stroke-like episodes" was adopted to distinguish the nonischemic origin of the MELAS symptom from the ischemic nature of thrombotic strokes. Possible causes of the "stroke-like episodes" are vascular dysfunction (Silbert et al. 1996) and neuronal hyperexcitability that progresses to epileptic activity in the surrounding cortex, which in turn mediates capillary damage and triggers the spread of cerebral lesions (Iizuka and Sakai 2005). Inability of cortical cells to cope with mitochondrial dysfunction also leads to neurological deficits, which are made worse by mitochondria-mediated oxidative stress and angiopathy (Sue et al. 1998). As the neuropathy progresses, cortical atrophy, linked to extensive neuronal loss, is observed (Turnbull et al. 2010). Ultimately, an increase in the frequency of seizures can culminate in fatal status epilepticus (Chiang et al. 1995).

Taurine-deficient animals are also susceptible to the development of seizures (Pasantes-Morales et al. 1987; Trachtman et al. 1988), a phenomenon initially attributed to the "inhibitory neurotransmitter" activity of taurine (Trachtman et al. 1988). Taurine deficiency also alters the density of $GABA_A$ and the glutamatergic system in specific brain regions, leading to changes in synaptic activity (Oermann et al. 2005; Sergeeva et al. 2007). The encephalopathy that develops in taurine deficiency exhibits changes in the $GABA_A$ system similar to those described for hepatic encephalopathy (Sergeeva et al. 2007). However, the possibility that abnormalities of the taurine-deficient brain might arise from the deficiency of energy supply and oxidative stress has not been considered. Also unclear is the role of taurine deficiency in the progression of cerebral cortex atrophy (Harris et al. 1997).

16.2.8 Opthalmic Defects of MELAS and Taurine Deficiency

The prevalence of opthalmic abnormalities in MELAS is modest, with the incidence in one study ranging from 20% for optic atrophy to 16% for pigmentary retinopathy (Thambisetty et al. 2002). Interestingly, abnormalities in retinal pigmentation vary

from patchy hyperpigmentation in the macular region to complete loss of retinal pigmentation (Rummelt et al. 1993; Smith et al. 1999). Atrophic photoreceptor outer segments are commonly detected in MELAS but normal photoreceptor cells dominate in the periphery (Rummelt et al. 1993). The electroretinogram of one MELAS patient was characterized by markedly reduced scotopic and photopic b-wave amplitudes (Latkany et al. 1999).

One of the characteristic features of taurine-deficient cats is a retinopathy. Indeed, the retinopathy was the first reported pathological symptom of taurine deficiency (Hayes et al. 1975). Like MELAS, the amplitude of the electroretinogram b-wave of the taurine transporter knockout mouse and the taurine-depleted cat is severely diminished (Heller-Stilb et al. 2002; Schmidt et al. 1977). However, abnormal electroretinograms have also been seen in patients undergoing long-term total parenteral nutrition, an effect normalized by taurine treatment but may be more closely tied to changes in zinc and vitamin E levels (Geggel et al. 1985; Vinton et al. 1990). Ultrastructural changes of the photoreceptor outer segments vary from mild to severe disorganization, with cones affected more than rods (Heller-Stilb et al. 2002; Imaki et al. 1987). Severe disruption of the tapetum, a unique feline cell involved in the reflection of light, is also a characteristic feature of taurine deficiency in cats (Wen et al. 1979). Heller-Stilb et al. (2002) proposed that apoptosis is a likely contributor to retinal degeneration, although no apoptotic cells have been detected in deficient cats. Also contributing to opthalmic dysfunction are the loss of optic nerve fibers (Lake et al. 1988) and atrophy of the olfactory bulb (Anderson et al. 1979). While changes in the index of visual function have been attributed in part to oxidative stress, further investigation of the causes of the taurine deficiency-mediated retinopathy is warranted.

16.2.9 Renal Defects in MELAS and Taurine Deficiency

The most common renal defects reported in MELAS patients are proximal tubulopathy, proteinuria, and focal segment glomerulosclerosis (Hirano et al. 2002; Sproule and Kaufmann 2008). According to a study by Hirano et al. (2002), proteinuria was present in all cases of MELAS studied. Focal segment glomerulosclerosis was also present in most cases of MELAS, particularly at an early stage of the nephropathy. At later stages of the nephropathy, diffuse global glomerulosclerosis with marked tubulointerstitial changes is observed (Hirano et al. 2002). To date, the mechanism linking mitochondrial dysfunction to renal defects has not been established, indicating the need for further research.

Taurine functions as a free radical scavenger, a cation transport modulator, and an osmoregulator in the kidney (Han and Chesney 2012). In accordance with taurine's role as an osmolyte, excretion of water and salt is restricted in the taurine-deficient rat, with the excretion of a hypotonic NaCl load being particularly diminished by taurine deficiency. In the uninephrectomized adult rat and the fetal/prenatal rat, taurine depletion accelerates the onset of hypertension (Mozaffari et al.

2006; Roysommuti et al. 2009). However, there is no evidence that taurine deficiency is associated with the development of global glomerulosclerosis although one would predict that the loss of its antioxidant and anti-inflammatory activities may render the kidney susceptible to glomerulosclerosis. According to Trachtman et al. (1992), taurine treatment has been shown to decrease the degree of renal injury in chronic puromycin aminonucleoside proteinuric renal disease, which is a model of focal segmental glomerulosclerosis.

16.3 Conclusions

16.3.1 Are the MELAS Symptoms and Taurine Deficiency Defects Identical?

The MELAS syndrome and taurine deficiency are remarkably similar. Both conditions lead to cardiomyopathies, metabolic abnormalities, growth failure, hearing defects, muscle weakness and exercise intolerance, retinopathies, and renal defects. These findings suggest that the pathophysiology of the two conditions is probably similar. Although there is no experimental evidence that taurine deficiency leads to reduced levels of 5-taurinomethyluridine-tRNA$^{Leu(UUR)}$, there is evidence that taurine serves as the substrate for the posttranslational modification of the wobble uridine base (unpublished data). There is also evidence that taurine deficiency, like deficiency of 5-taurinomethyluridine-tRNA$^{Leu(UUR)}$, leads to impaired UUG decoding, decreased respiratory chain activity, enhanced oxidative stress, and stimulation of anaerobic metabolism (Jong et al. 2012; Mozaffari et al. 1986). Because the generation of ATP and the regulation of superoxide production are basic functions required for maintenance and viability of all cells, it is not surprising that taurine deficiency and MELAS affect the function of virtually all organ systems. The observation that the effects of taurine deficiency are so biochemically similar to the pathophysiology of MELAS unconditionally links the two conditions.

16.3.2 Unique Taurine-Deficient Actions

Historically, taurine serves as a neuromodulator, regulator of the immune system, membrane stabilizer, osmoregulator, and modulator of cation transport (Huxtable 1992). These effects are unrelated to MELAS because they do not involve changes in 5-taurinomethyluridine-tRNA$^{Leu(UUR)}$ levels. Rather, these effects involve a distinct conjugation reaction (formation of taurine chloramine), a change in protein or enzyme activity (membrane stabilization, neuromodulation), an alteration in tissue osmolyte load (osmoregulation, cation transport modulation), or a change in cation transport (via taurine transporter). Although the regulation of mitochondrial reactive

oxygen species generation and ATP production are essential cellular functions, the other actions of taurine are also very important. Based on the impressive list of mitochondrial and extra-mitochondrial actions, it is clear that taurine is a basic nutrient required for normal life.

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Chapter 17
Antioxidant and DNA Protection Effects of Taurine by Electron Spin Resonance Spectroscopy

Sun Hee Cheong, Sang Ho Moon, Seung Jae Lee, Sung Hoon Kim, and Kyung Ja Chang

Abstract Taurine may play an important role in protecting cells against toxic injury by an antioxidant. However, there is a lack of evidence to support this hypothesis. The objective of this study was to examine the in vitro antioxidant properties of taurine against different reactive species at various concentrations. The radical scavenging effects of taurine on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, hydroxyl radical, superoxide radical, and alkyl radical were investigated using a spin-trapping electron method and compared with the electron spin resonance (ESR) signal intensity. ESR assays showed that DPPH radical scavenging activity of taurine at various concentrations (0.0625 ~ 1 mg/mL) was elevated with a decrease of ESR signals in a dose-dependent manner. Moreover, taurine exhibited the radical scavenging activities against

Sun Hee Cheong and Sang Ho Moon contributed equally to this work.

S.H. Cheong (✉)
Department of Applied Biological Chemistry,
Tokyo University of Agriculture and Technology,
Fuchu, Tokyo 183-8509, Japan

Department of Food and Nutrition, Inha University,
Incheon 402-751, South Korea
e-mail: sunheecheong@gmail.com

S.H. Moon
Department of Food Bio Science, College of Natural Sciences,
Konkuk University, Chungju, Chungchongbuk-do 380-701, South Korea

S.J. Lee
Department of Biotechnology, Division of Life Science, Konkuk University, College of Biomedical and Health Science, Chungju, Chungchongbuk-do 380-701, South Korea

S.H. Kim
Department of Chemistry, Konkuk University, Seoul 143-701, South Korea

K.J. Chang
Department of Food and Nutrition, Inha University, Incheon 402-751, South Korea
e-mail: kjchang@inha.ac.kr

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hydroxyl radicals, superoxide radicals, and alkyl radicals. Findings from this study suggest that taurine may be a useful radical scavenger and a potential supplement for the food, pharmaceutical, and cosmetic industries as well as feed and/or antibiotic because of its potent antioxidant capacities against various reactive radicals.

Abbreviations

DPPH 1,1-Diphenyl-2-picrylhydrazyl
ESR Electron spin resonance

17.1 Introduction

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing β-amino acid which is found in all animal cells at millimolar concentration (Oliveira et al. 2010). Previous studies have shown that taurine possesses several biological functions, such as bile acid conjugation, maintenance of calcium homeostasis, osmoregulation, membrane stabilization, and antidiabetic, antihypertensive, antioxidant, and hepatoprotective activities (Huxtable 1992; Pushpakiran et al. 2005; Liu et al. 2008). One of the possible mechanisms of its pharmacological action involves the free radical scavenging activity (Tabassum et al. 2006). Taurine has been widely used to rescue oxidative stress and as a neuroprotective agent in disparate models (Shuaib 2003; El Idrissi 2006). Taurine is also known to react with and detoxify hypochlorous acid (HOCl) generated by activated neutrophils from myeloperoxidase (MPO), hydrogen peroxide, and chloride during the oxidative burst, and this protective action involves the formation of stable taurine chloramines (Cunningham et al. 1998). Moreover, it has been reported that taurine has potential free radical scavenging activities against many oxygen-derived radicals (Aruoma et al. 1988; Shi et al. 1997). Nonetheless, there is no conclusive evidence that taurine regulates the levels of the antioxidant defense system and the mechanism on the antioxidant activity of taurine. The objective of the present study was to examine the in vitro DNA protective activity and antioxidant properties of taurine against different reactive species at various concentrations using a spin-trapping electron method.

17.2 Methods

17.2.1 Reagents

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and *alpha*-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest grade available commercially.

17.2.2 Measurement of Free Radical Scavenging Activity

17.2.2.1 DPPH Radical Scavenging Activity

DPPH radical scavenging activity was measured using the method described by Nanjo et al. (1996). Briefly, to a methanol solution of DPPH (to 60 μL, 60 μM) was added various concentration of taurine sample (60 μL). After mixing vigorously for 10 s, the solution was transferred into a 100 μL Teflon capillary tube, and the scavenging activity of each sample for the DPPH radical was measured using an ESR spectrometer. A spin adduct was measured on an electron spin resonance (ESR) spectrometer exactly 2 min later. Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5; and temperature, 298 K.

17.2.2.2 Hydroxyl Radical Scavenging Activity

Hydroxyl radicals were generated by iron-catalyzed Haber–Weiss reaction (Fenton-driven Haber–Weiss reaction), and the generated hydroxyl radicals rapidly reacted with nitrone spin-trap DMPO. The resultant DMPO-OH adduct was detectable with an ESR spectrometer. Briefly, 0.2 mL of taurine sample was mixed with 0.2 mL of DMPO (0.3 M), 0.2 mL of $FeSO_4$ (10 mM), and 0.2 mL of H_2O_2 (10 mM) in a phosphate buffer solution (pH 7.2), and then transferred to a 100 μL Teflon capillary tube. After 2.5 min, an ESR spectrum was recorded using a JES-FA ESR spectrometer (JEOL Ltd., Tokyo, Japan). Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5; and temperature, 298 K.

17.2.2.3 Superoxide Radical Scavenging Activity

Superoxide radicals were generated by UV irradiation of a riboflavin/ethylenediaminetetraacetic acid solution. The reaction mixtures containing 0.1 mL of 0.8 mM riboflavin, 0.1 mL of 1.6 mM EDTA, 0.1 mL of 800 mM DMPO, and 0.1 mL sample were irradiated for 1 min under a UV lamp at 365 nm. The mixtures were transferred to a 100 μL quartz capillary tube and placed in an ESR spectrometer for measurement. Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 10 mW; gain, 6.3×10^5; and temperature, 298 K.

17.2.2.4 Alkyl Radical Scavenging Activity

Alkyl radicals were generated by AAPH. The phosphate-buffered saline (pH 7.4) reaction mixtures containing 10 mM AAPH, 10 mM 4-POBN, and the indicated concentrations of tested samples were incubated at 37°C in a water bath for 30 min

and then transferred to a 100 μL quartz capillary tube. The spin adduct was recorded on an ESR spectrometer. Measurement conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5; and temperature, 298 K.

17.2.3 DNA Nicking Assay

The method of hydroxyl radical-induced DNA breakage in plasmid pBR322 was modified from Kitts and others (2000). Briefly, 2 μL of taurine (1 mg/mL) was mixed with 2 μL of EDTA-Na_2 (10.09 g/L), KH_2PO_4 buffer (0.05 mol/L, pH 7.4), H_2O_2 (1.02 g/L), $FeSO_4$ (2.42 g/L), and 0.1 μg/mL of pBR322 plasmid DNA in a 500 μL micro-centrifuge tube. The molar ratio of $FeSO_4$/EDTA was kept at 0.53. The final volume of the reaction mixture was brought to 12 μL with deionized distilled water and incubated for 1 h at 37°C. Following incubation, 3 μL of autoclaved distilled water and 3 μL of 6 × DNA loading dye (Fermentas) was loaded onto a 1.2 g/100 mL of agarose gel. Electrophoresis was conducted at 80 V in a Tris–acetate–EDTA.Na_2 (TAE) buffer (7.25 g/L of Tris–acetate and 0.29 g/L of EDTA, pH 7.4) using a horizontal submarine gel electrophoresis apparatus (Atto Corp., Yushima, Bunkyo, Tokyo, Japan). After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/mL in deionized distilled water) for 10–15 min with gentle shaking followed by destaining with distilled water until the background was clear. DNA bands were visualized under illumination of UV light and photographed with Gel Doc system (Ultra-Violet Products [UVP] Ltd., Cambridge, UK).

17.2.4 Statistical Analyses

All data were expressed as the mean ± SEM, and statistical analyses were performed using Statistical Analysis System version 8.0 (SAS Institute, Cary, NC, USA). The differences between means were assessed by the Student's *t*-test, and statistical significance was defined at $P < 0.05$.

17.3 Results

17.3.1 Free Radical Scavenging Activity

In the present study, the radical scavenging activities of taurine at various concentrations were investigated using DPPH, hydroxyl, alkyl, and superoxide radical scavenging properties measured using an ESR spectrometer. DPPH is a stable radical that is used to

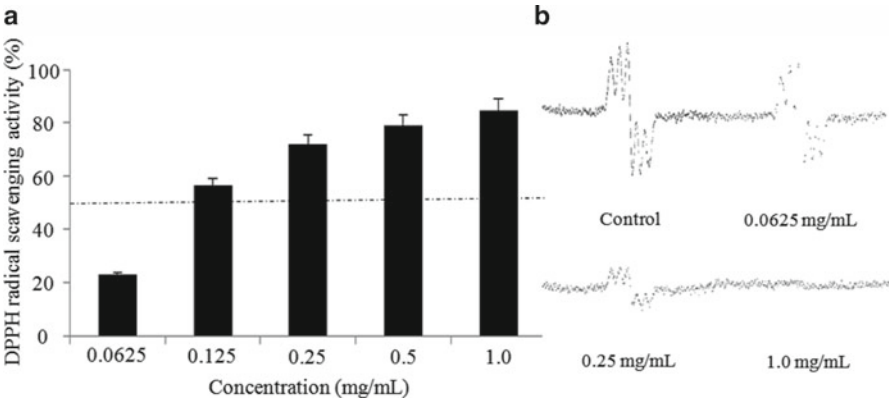

Fig. 17.1 DPPH radical scavenging activity (%) (**a**) and ESR spectra (**b**) according to the taurine treatment at various concentrations (0.0625–1 mg/mL). Each bar represents the mean ± SEM of three independent experiments. The dashed horizontal line indicates the value at the concentration inhibiting 50% of free radical generation (IC_{50})

screen the free radical scavenging ability of compounds or antioxidant activity of plant extracts. The effect of taurine on DPPH radical scavenging activity is shown in Fig. 17.1. DPPH radical scavenging activity, at the concentration inhibiting 50% of free radical generation (IC_{50}), was 0.144 mg/mL as shown in Fig. 17.1a. Additionally, a decrease of ESR signals was observed with the dose increment of taurine compared to the control (Fig. 17.1b).

Figure 17.2 shows the hydroxyl radical scavenging activity and ESR spectra of the taurine. As shown in Fig. 17.2a, the IC_{50} of hydroxyl radical scavenging activity was 0.241 mg/mL. Hydroxyl radicals generated in the Fe^{2+}/H_2O_2 system were trapped by DMPO, forming a spin adduct detected by ESR spectrometry, and the typical 1:2:2:1 ESR signal of the DMPO-OH adduct was observed; see Fig. 17.2b. In the present study, ESR assays showed that hydroxyl radical scavenging activity of taurine at various concentrations (0.0625 ~ 1 mg/mL) was elevated with a decrease of ESR signals in a dose-dependent manner.

Figure 17.3 shows the superoxide radical scavenging activity and ESR spectra of the taurine. In our present study, ESR assays showed that superoxide radical scavenging activity of taurine at various concentrations (0.25 ~ 1 mg/mL) was elevated with a decrease of ESR signals in a dose-dependent manner. However, the superoxide radical scavenging activity by taurine was weaker than for DPPH and hydroxyl radical scavenging.

In the present study, the IC_{50} of alkyl radical scavenging activity was 0.206 mg/mL. In addition, the decrease of ESR signal was observed with the dose increment of the taurine (Fig. 17.4). These results indicate that the taurine effectively scavenged various reactive radicals, including DPPH, hydroxyl, superoxide, and alkyl radicals.

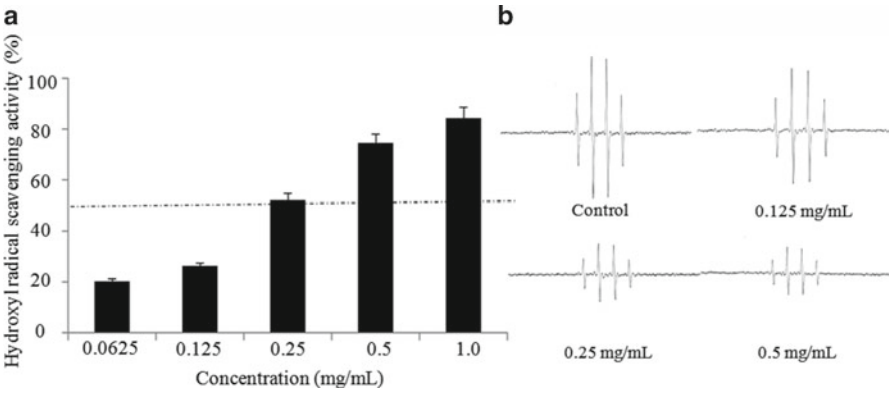

Fig. 17.2 Hydroxyl radical scavenging activity (%) (**a**) and ESR spectra (**b**) according to the taurine treatment at various concentrations (0.0625–1 mg/mL). Each bar represents the mean ± SEM of three independent experiments. The dashed horizontal line indicates the value at the concentration inhibiting 50% of free radical generation (IC_{50})

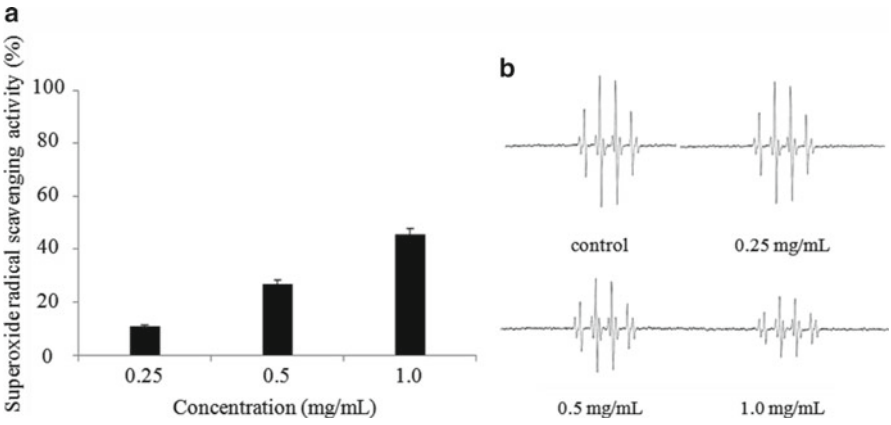

Fig. 17.3 Superoxide radical scavenging activity (%) (**a**) and ESR spectra (**b**) according to the taurine treatment at various concentrations (0.0625–1 mg/mL). Each bar represents the mean ± SEM of three independent experiments

17.3.2 DNA Protective Activity

The free radical scavenging effect of taurine was studied on plasmid DNA damage (Fig. 17.5). The taurine at the concentration of 0.5 mg/mL and 1.0 mg/mL showed significant reduction in the formation of nicked DNA and increased native form of DNA (supercoiled DNA). These results indicate that the taurine is a DNA protector, which provides antioxidant activity to prevent DNA damage. Therefore, the presence of taurine may contribute to the protective effect against free radical-induced DNA damage.

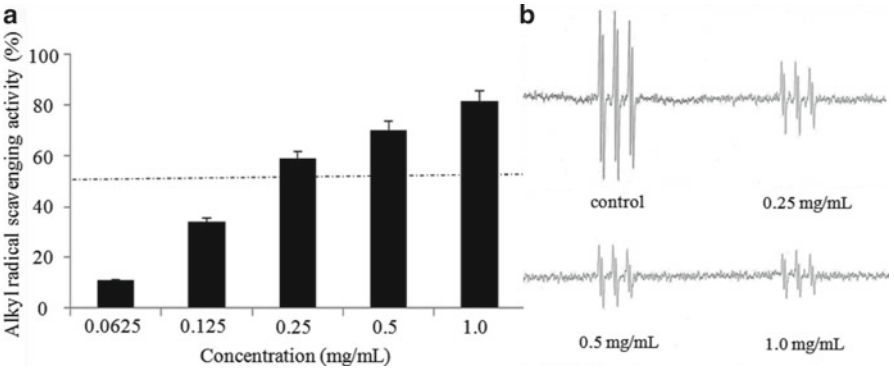

Fig. 17.4 Alkyl radical scavenging activity (%) (**a**) and ESR spectra (**b**) according to the taurine treatment at various concentrations (0.0625–1 mg/mL). Each bar represents the mean ± SEM of three independent experiments. The dashed horizontal line indicates the value at the concentration inhibiting 50% of free radical generation (IC_{50})

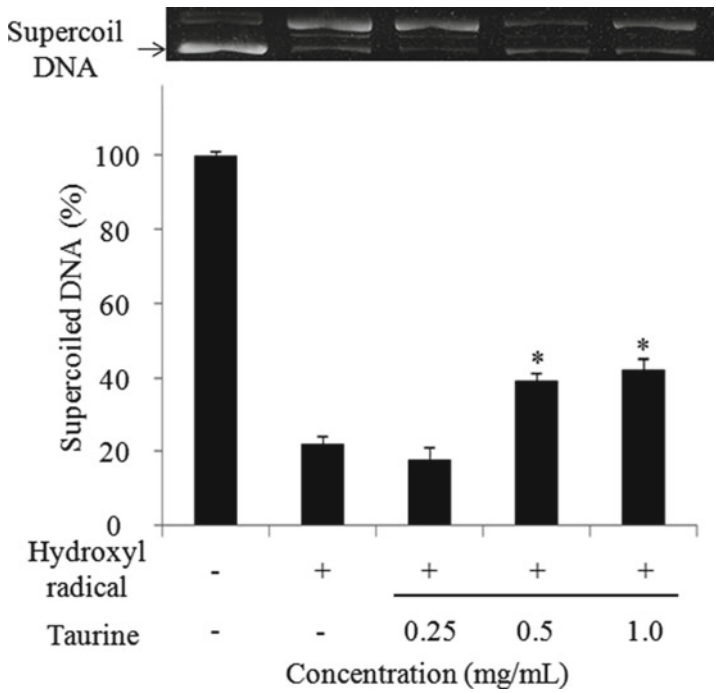

Fig. 17.5 Inhibitory effects of taurine at various concentrations (0.25–1 mg/mL) on DNA nicking caused by hydroxyl radicals

17.4 Discussion

In general, reactive oxygen species (ROS) are highly reactive molecules or intermediates that are continuously produced by all aerobic organisms, primarily as a consequence of aerobic respiration. Accumulation of ROS results in oxidative damage to important molecules such as protein, lipids, and DNA, which may result in cellular dysfunction and mutation, thus promoting the development of disease (Halliwell and Aruoma 1991).

On the other hand, taurine and its metabolic precursor, hypotaurine, have been proposed to participate in the cellular mechanisms involved in the protection against oxidative damage such as inhibition of lipid peroxidation. Especially hypotaurine has an excellent ability to scavenge hydroxyl radical, the most potent oxidant that can be formed from oxygen considered, the likely initiator of lipid peroxidation (Huxtable 1992). By contrast, the molecular mechanisms of taurine have still to be defined as this amino acid reacts poorly with superoxide, hydrogen peroxide, and hydroxyl radicals (Aruoma et al. 1988). Therefore, the objective of the present study was to examine the in vitro antioxidant properties of taurine against several reactive species such as DPPH, hydroxyl, superoxide, and alkyl radical at various concentrations using a spin-trapping electron method.

In the present study, DPPH radical scavenging activity of taurine at various concentrations, from 0.0625 to 1 mg/mL, was elevated with a decrease of ESR signals in a dose-dependent manner compared to the control. These results indicated that the taurine possessed strong scavenging activity against DPPH radicals, and that the ability of the taurine may be attributed to its hydrogen donating ability. The hydroxyl radical is one of the most reactive radicals generated from biologic molecules and can damage living cells (Bergamini et al. 2004). In our present study, the taurine exhibited stronger scavenging activity against hydroxyl radical, and the radical scavenging activity occurred in a dose-dependent manner. Recently, it was reported that taurine acts as a good in vitro scavenger of reactive oxygen such as peroxyl radical and anion superoxide and nitrogen species including nitric oxide and peroxynitrate (Oliveira et al. 2010). However, Navneet et al. (2008) reported that taurine did not influence the level of hydroxyl radical production caused by 1-methyl-4-phenylpyridinium (MPP^+), which is the oxidized metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mitochondria in mice. In contrast, it has been reported that the intraperitoneal injection of 200 mg/kg taurine is sufficient to prevent hypoxia-induced lactate accumulation and lipid peroxidation in rat brain (Mankovskaya et al. 2000). Other previous studies reported that taurine deficiency increased malondialdehyde (MDA) levels, which is a relatively stable end product of lipid peroxidation that decreased in taurine-supplemented animals (Kaplan et al. 1993; Öz et al. 1999). These previous results showed that taurine has been considered a potent free radical scavenger and antioxidant. In general, free radical generator, AAPH, is an azo compound that undergoes thermal decomposition to produce molecular nitrogen and two carbon radicals, which rapidly react with oxygen to give peroxyl radicals (Krasowska et al. 2000). Our present study showed that taurine exhibited stronger scavenging activity against alkyl radical. It has been well known that superoxide radical can cause cellular damage by

itself or react with other compounds to yield radicals that are more reactive. Also, it is reported that taurine prevents the diversion of electrons into superoxide generation by improving the function of the electron transport chain in vivo (Turrens 2007). In our present study, the superoxide radical scavenging activity of taurine at the concentrations from 0.25 to 1 mg/mL was elevated with a decrease of ESR signals in a dose-dependent manner, although the superoxide radical scavenging activity by taurine was weaker than for DPPH, hydroxyl, and alkyl radical scavenging activities. Oliveira et al. (2010) reported that taurine concentrations above 15 mM may prevent the superoxide radical generation. Another previous study indicates that taurine can serve as a general scavenger of ROS, such as superoxide and peroxide (Babior et al. 1983). Also, it was reported that the superoxide scavenging by taurine was larger than for hydroxyl radical scavenging by ESR spin trapping (Kilic et al. 1999). In our previous study, however, the IC_{50} values of taurine against DPPH radical, alkyl radical, and hydroxyl radical scavenging activity were above 4 mg/mL, although taurine has very weak radical scavenging activities (Du et al. 2010).

In the present study, we examined the free radical scavenging effect on plasmid DNA damage of taurine additionally. In general, DNA damage may cause perturbations in inner mitochondrial membrane permeability, the so-called mitochondrial permeability transition (MPT). A sudden increase in MPT is a central coordination event in the apoptotic process (Gottlieb 2000; Tada-Oikawa et al. 2000). In the present investigation involving plasmid nicking assay, it was seen that the taurine provided protection against the damage caused by hydroxyl radical. In a previous study, it was reported that taurine at concentrations normally found in cells can inhibit oxidative damage to DNA (Messina and Dawson 2000). Recently, Das et al. (2011) reported that taurine suppressed doxorubicin-triggered oxidative stress and cardiac apoptosis in rat. Similarly, it was reported that administration of taurine alleviated pathological changes and DNA damage caused by arsenic through the RNS signal pathway in vivo (Ma et al. 2010). In another previous study, it was reported that taurine (100 mg/kg) prevents tamoxifen-induced mitochondrial oxidative damage in mice (Parvez et al. 2008).

Although the antioxidant acts of taurine remains a controversial topic, our present in vitro data suggests that taurine may protect DNA damage and oxidative stress by acting as an efficient scavenger against several free radicals including DPPH, hydroxyl, superoxide, and alkyl radicals in vitro system. It is worthy to further investigate the potential effectiveness of taurine in preventing the diseases caused by overproduction of radicals.

17.5 Conclusion

In summary, our present study shows that taurine has protective effect on the DNA damage and oxidative stress by acting as an efficient scavenger against several free radicals. These protective effects of taurine may contribute to prevent several diseases caused by overproduction of radicals.

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Chapter 18
Additional Effects of Taurine on the Benefits of BCAA Intake for the Delayed-Onset Muscle Soreness and Muscle Damage Induced by High-Intensity Eccentric Exercise

Song-Gyu Ra, Teruo Miyazaki, Keisuke Ishikura, Hisashi Nagayama, Takafumi Suzuki, Seiji Maeda, Masaharu Ito, Yasushi Matsuzaki, and Hajime Ohmori

Abstract Taurine (TAU) has a lot of the biological, physiological, and pharmocological functions including anti-inflammatory and anti-oxidative stress. Although previous studies have appreciated the effectiveness of branched-chain amino acids (BCAA) on the delayed-onset muscle soreness (DOMS), consistent finding has not still convinced. The aim of this study was to examine the additional effect of TAU with BCAA on the DOMS and muscle damages after eccentric exercise. Thirty-six untrained male volunteers were equally divided into four groups, and ingested a combination with 2.0 g TAU (or placebo) and 3.2 g BCAA (or placebo), thrice a day, 2 weeks prior to and 4 days after elbow flexion eccentric exercise. Following the period after eccentric exercise, the physiological and blood biochemical markers for DOMS and muscle damage showed improvement in the combination of TAU and BCAA supplementation rather than in the single or placebo supplementations. In conclusion, additional supplement of TAU with BCAA would be a useful way to attenuate DOMS and muscle damages induced by high-intensity exercise.

S.-G. Ra (✉) • T. Suzuki • S. Maeda • H. Ohmori
Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan
e-mail: ra.song.gyu@gmail.com

T. Miyazaki
Joint Research Center, Tokyo Medical University Ibaraki Medical Center, Ami, Japan

K. Ishikura
Sports Research and Development Core, University of Tsukuba, Tsukuba, Japan

H. Nagayama
School of Health and Physical Education, University of Tsukuba, Tsukuba, Japan

M. Ito
Livence Co. Inc., Chuo, Tokyo, Japan

Y. Matsuzaki
Division of Gastroenterology and Hepatology, Department of Internal Medicine, Tokyo Medical University Ibaraki Medical Center, Ami, Japan

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18.1 Introduction

Unaccustomed and intense exercise induces delayed-onset muscle soreness (DOMS) and muscle damage. Furthermore, eccentric exercise (ECC) induces more severe DOMS and muscle damages than concentric exercise (Fridén et al. 1984). Suffering from the DOMS is one of the major reasons to let beginner avoid the habitual exercise, and also influences the athletic performances and daily training.

Several studies have previously investigated the effectiveness of oral branched-chain amino acid (BCAA) (valine, isoleucine, leucine) administration on the DOMS. Shimomura et al. reported that BCAA supplementation prior to squat exercise in human decreased DOMS occurring for a few days after exercise (Shimomura et al. 2006, 2010). On the other hand, the study reported by Jackman et al. could not find the significant differences on DOMS between placebo and BCAA supplementations during recovery from higher intense ECC induced by repetitional knee extension (Jackman et al. 2010). These results imply that BCAA supplementation does not always attenuate DOMS and muscle damages such as high-intensity exercise.

Taurine (TAU), 2-aminoethanesulfonic acid, is abundantly found in the skeletal muscle as well as most tissues (Jacobsen and Smith 1968). We have shown the reductions of exercise- and drug-induced oxidative stress by oral TAU administration to rats (Miyazaki et al. 2004a, 2004b, 2005). Because the increased oxidative stress and intracellular Ca^{2+} concentration caused by ECC, TAU supplementation has possible effect in human to attenuate DOMS and muscle damage after ECC.

In the present study, we investigated the hypothesis in a randomized, placebo-controlled, double-blind study that addition of TAU to BCAA supplementations might be efficient nutritional strategy to attenuate the DOMS and muscle damages.

18.2 Methods

18.2.1 Grouping of Subjects and Amino Acid Supplementation

Thirty-six male volunteers (22.5 ± 3.8 years) without any musculoskeletal disorders and regular resistance training were recruited. Subjects were randomly and equally divided into four groups ($N = 9$ per group), placebo control (PLCB), BCAA, TAU, and additional taurine with BCAA (AdTB). There were no statistical differences in all physical characteristic parameters (age, height, body weight, body fat, muscle weight, maximal voluntary strength of isometric elbow contraction) between the groups before experiment. Subjects were orally administered with two packages of combination with TAU (or placebo) and BCAA (or placebo) powders with water after every meal for 2 weeks prior to exercise (Fig. 18.1). TAU supplement was 3.0 g powder composed by 2.0 g of TAU or starch degradation in the active or placebo, respectively, and 1.0 g of artificial sweetener with flavor. BCAA supplement was 4.0 g powder composed by 3.2 g of BCAA mixture (Ile:Leu:Val = 1:2:1) or starch degradation in the active or placebo, respectively, and 0.8 g of artificial sweetener with flavor.

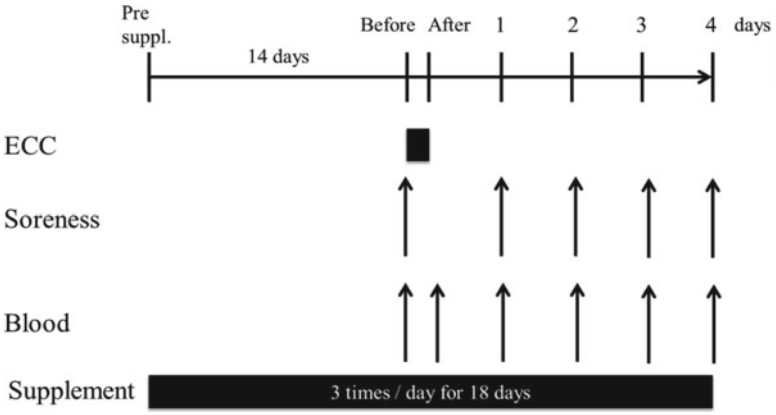

Fig. 18.1 Schematic showing of the protocol of the present study. Subjects were supplemented (BCAA or/and taurine) for 14 days before the exercise experiment, exercise on day, and following 4 days. *Abbreviations*: *Pre suppl*. pre-supplementation, *Before* before the exercises, *After* immediately after the exercise, *1 ~ 4* following 4 days, *ECC* eccentric exercise, *Soreness* muscle soreness evaluated by VAS, *Blood* blood sampling, *Supplement* supplements were amino acid consumptions three times per day for 18 days by each condition

The present study was carried out in accordance with the Declaration of Helsinki and was approved by the Human Subjects Committee of the University of Tsukuba. All subjects provided informed written consent.

18.2.2 Exercise Protocol

On the exercise day, the subjects assembled to laboratory at 7 a.m. after an overnight fasting, and ingested the amino acid supplement before 15 min of the ECC. In the present study, the exercise protocol to induce the DOMS and muscle damages in the biceps brachii muscle was modified from the method of ECC reported by Lavender et al. (Lavender and Nosaka 2008). Before at least a week of the supplementations, maximal voluntary strength of isometric contraction (MVC) in the nondominant arm was measured at 1.57 rad (90°) elbow flexion in each subject. In the exercise day, subjects were loaded to repetitive dumbbell lowering by 90% MVC of individuals (11.7 ± 0.4 kg) as voluntary eccentric muscle contraction (Fig. 18.2). Subjects were seated on a bench with the arm positioned in front of the body resting on a padded support so that the shoulder was secured at an angle of 0.79 rad (45°) flexion, and the forearm was kept supinated throughout exercise. ECC protocol consisted of six sets of five actions from an elbow flexed at 90° to an extended position in 5 s keeping the velocity as constant as possible by following the investigator's counting. After each muscle action, the investigator returned the dumbbell to the starting position to prevent any muscle actions by the weight. Rests were taken 3 s and 2 min between muscle actions and sets, respectively. This protocol using 90% MVC induced natural muscle damage by eccentric

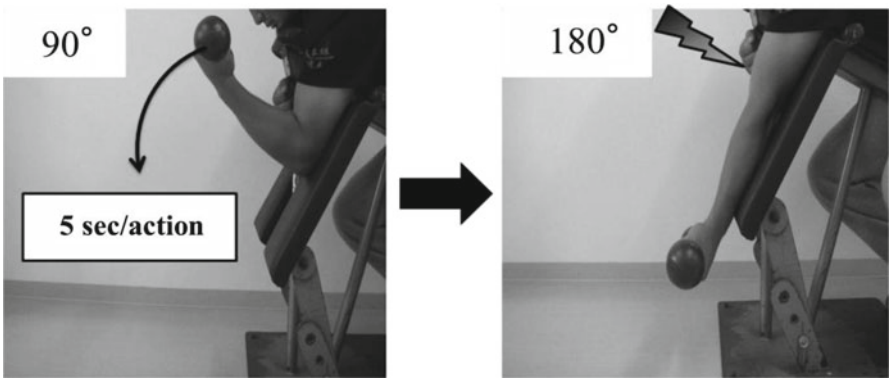

Fig. 18.2 Schematic designs of eccentric exercise procedures. Nondominant arm positioned in front of the body resting on a padded support so that the shoulder was secured at an angle of 1.57 rad (90°) flexion, and the forearm was kept supinated throughout exercise. Subjects performed six sets of five lengthening (eccentric) muscle actions by lowering the dumbbell from an elbow flexed (1.57 rad, 90°) to an extended position (3.14 rad, 180°) in 5 s keeping the velocity as constant as possible by following the investigator's counting "0" for the beginning and "1, 2, 3, 4, and 5" for the movement. After each eccentric action, the investigator returned the dumbbell to the starting position so that subjects performed no concentric actions with the weight. The rest between each eccentric action was approximately 3 s, and 2-min rest was given between sets

contractions because subjects were hard to keep the constant lowering the dumbbell as instructed in later sets due to fatigue. In the later exercise sets, the investigator encouraged verbally and assisted subjects to keep the constant velocity.

18.2.3 Evaluation of DOMS

Subjective evaluation of muscle soreness was surveyed using a visual analogue scale (VAS) consisting of a 100 mm line with "no pain" at one end and "extremely sore" at another (Nosaka et al. 2006). The VAS was carried out in cases of the elbow joint extension before and following 4 days of the exercise (Fig. 18.1). The investigator passively extended the elbow joint to test each subject perception of soreness.

18.2.4 Blood Parameter of Muscle Damage

Blood sample was collected from an antecubital vein at 6 points; immediately before and after the exercise, and following 4 days before breakfast (Fig. 18.1). Serum was immediately separated after collection, and serum activities of lactate dehydrogenase (LDH) were analyzed as parameter of muscle damages.

18.2.5 Statistic Analysis

Data are expressed as the mean ± S.E. Throughout the experimental period, significant differences within each group were compared via a repeated-measures ANOVA followed by the Dunnett's multiple comparison post hoc tests. At each point, significant differences in the parameters between the groups were analyzed via a one-way analysis of variance (ANOVA) multiple comparison followed by the Dunnett's multiple comparison post hoc test or nonparametric Wilcoxon tests. Significant differences (two-tailed) were set at $P < 0.05$. Statistical analyses were conducted with the SPSS software version 18.0 for Windows (Chicago, Illinois, USA).

18.3 Results

18.3.1 DOMS After ECC

DOMS was subjectively evaluated using VAS in conditions of forcible extension of elbow joint (Fig. 18.3). VAS scores in all groups significantly increased on the 1st day, and then, declined from the 2nd to 4th days (Fig. 18.3a). On the 2nd day, the increased VAS score in the AdTB group decreased compared to that on the 1st day, while the scores in the PLCB and TAU groups further increased. In the AdTB group, VAS score on the 2nd day was significantly lower than in the PLCB group. AUC values of the VAS score in the extension condition were 28 and 37% lower in the BCAA and AdTB groups, respectively, than the PLCB group (Fig. 18.3b).

18.3.2 Serum Marker of Muscle Damage

Serum activity of LDH in the progress and AUC during the experimental period was shown as a blood marker of muscle damages (Fig. 18.4). Serum LDH activity was unchanged in all groups until immediately after exercise, but increased from the 1st to 4th day. In the PLCB, BCAA, and TAU groups, serum LDH activity was significantly higher on the 3rd and 4th days than before (Fig. 18.4a). In the AdTB group, there was no significant difference in LDH activity between before and 3rd day, but significantly between before and 4th day. In the AdTB group, LDH activity from the 1st to 3rd days and AUC was significantly lower in the AdTB group than before (Fig. 18.4b).

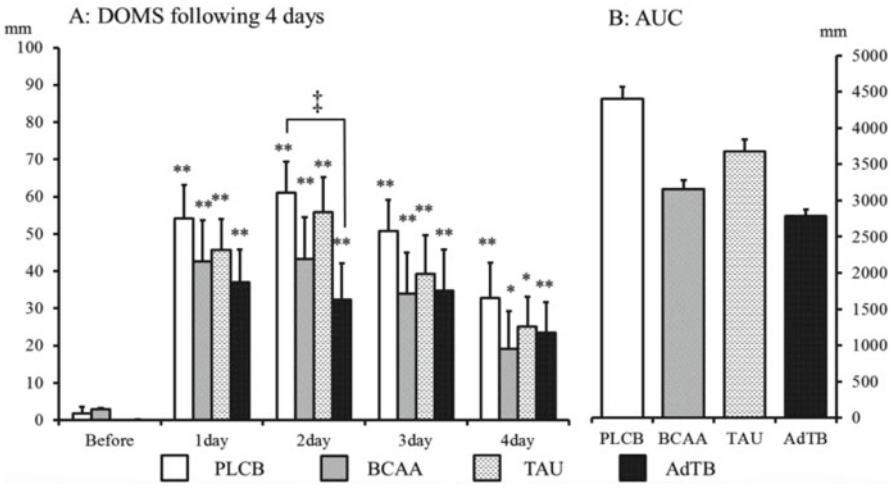

Fig. 18.3 Muscle soreness and areas under the curve (AUC) of VAS in the period from before exercise to following 4 days. DOMS was subjectively determined from VAS scores following the extension of the exercised arm. *Abbreviations*: *DOSS* delayed-onset muscle soreness, *AUC* area under the curve of VAS during the experimental period, *Before* before exercise, *PLCB* placebo supplement group, *BCAA* BCAA supplement group, *TAU* taurine supplement group, *AdTB* addition of taurine with BCAA supplement group. Values are expressed in mean ± S.E.*$P < 0.05$ and **$P < 0.01$ vs. Before (repeated measures ANOVA followed by the Dunnett's multiple comparison *post hoc test*). ‡$P < 0.05$ vs. PLCB (one-way ANOVA multiple comparison and nonparametric Wilcoxon tests)

18.4 Discussion

In the present study, the additional effect of TAU with BCAA supplementations on the DOMS and muscle damages induced by ECC was investigated in a randomized, placebo-controlled, double-blind study. Our findings were that the combination of TAU (6 g/day) and BCAA (9.6 g/day) supplementation throughout from 2 weeks before and following 4 days after exercise attenuated DOMS and muscle damages after high-intensity ECC.

Although the previous studies have already shown that BCAA intake attenuated DOMS, the present study suggests that supplementation of TAU and BCAA would be a more active ingredient for DOMS attenuation. Interestingly, the combination of BCAA with other amino acids, but not TAU, has been reported to have no effect on the DOMS (Nosaka et al. 2006; White et al. 2008). Because of many physiological and pharmacological roles including anti-oxidation (Silva et al. 2011), TAU might have many effects to enhance the BCAA action for attenuation of DOMS.

In addition, the supplemental amount of BCAA per muscle mass might be a crucial factor for DOMS attenuation. In the present study, single supplementation of BCAA for 18 days decreased the DOMS and muscle damages, but not significantly. Similarly, some previous studies (Nosaka et al. 2006; White et al. 2008) also reported that the BCAA supplements with only one or two occasions showed no significant

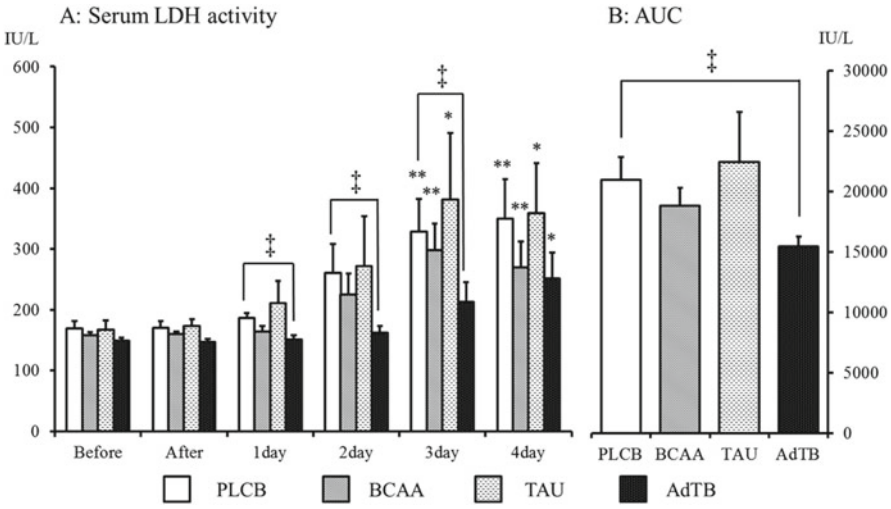

Fig. 18.4 Serum lactate dehydrogenase (LDH) activity level and areas under the curve (AUC) throughout the experimental period. *Abbreviations*: *LDH* lactate dehydrogenase, *AUC* area under the curve of VAS during the experimental period, *Before* before exercise, *After* after exercise, *PLCB* placebo supplement group, *BCAA* BCAA supplement group, *TAU* taurine supplement group, *AdTb* addition of taurine with BCAA supplement group. Values are expressed in mean ± S.E. $*P < 0.05$ and $**P < 0.01$ vs. Before (repeated measures ANOVA followed by the Dunnett's multiple comparison *post hoc test*). ‡$P < 0.05$ vs. PLCB (one-way ANOVA multiple comparison and nonparametric Wilcoxon tests)

effect on DOMS. However, Shimomura et al. (2006) demonstrated that DOMS after squat exercise was significantly reduced by only a single supplement of BCAA (77 mg/kg) in females but not in males. This result attributed the difference to the fact that females ingested a larger amount of BCAA relative to their muscle mass compared with males. In the present study, the total amounts of BCAA intake per body weight were smaller than the aforementioned study (Shimomura et al. 2006): 45 and 51 mg/kg in the BCAA and AdTB groups, respectively. Therefore, the dose of BCAA in each ingestion relative to individual muscle mass may be a key point for attenuation of DOMS rather than the supplemental duration.

In this study, the duration of oral TAU administration as 6.0 g/day prior to exercise was two weeks, and consequently, plasma TAU concentration was significantly increased in the TAU and AdTB groups (data not shown). Previously, we have already confirmed in human that oral TAU administration as 6.0 g/day for a week significantly increased plasma TAU concentration (Ishikura et al. 2008). Furthermore, our previous data have shown that skeletal muscle TAU content was enhanced when blood TAU level was increased by TAU administration (Ishikura et al. 2011; Miyazaki et al. 2004a; Yatabe et al. 2003). Thereby, it is suggested that the present protocol of oral TAU supplementation might lead to increased TAU content in the skeletal muscles. In contrast to BCAA supplementation, a certain

period of TAU supplementation should be needed to prevent from the DOMS and muscle damages.

18.5 Conclusion

In the present placebo-controlled double-blind study, we confirmed the additional effect of TAU with BCAA supplementation on the attenuation of the DOMS and muscle damages induced by ECC in elbow flexor with higher intensity that is unable to be suppressed by single supplementation of either TAU or BCAA. Therefore, the additional effect of TAU with BCAA supplementation would be the useful strategy for attenuation of DOMS and muscle damage after high-intensity ECC.

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Chapter 19
Taurine Enhances Anticancer Activity of Cisplatin in Human Cervical Cancer Cells

Taehee Kim and An Keun Kim

Abstract Taurine is a nonessential amino acid and has a variety of physiological and pharmacological effects. Recently, protective effects of taurine against anticancer drugs on normal cells were investigated. But anticancer effects of taurine on cancer cells remain poorly understood. Therefore, we investigated the anticancer effects of taurine alone and combination of cisplatin with taurine in human cervical cancer cells. Single treatment of taurine decreased cell proliferation in a time- and dose-dependent manner. In co-treatment of cisplatin with taurine, cell proliferation was more decreased than single treatment of cisplatin. Reduced cell proliferation was caused by apoptosis induction. Thus, after treatment of cisplatin with taurine, apoptotic cells were investigated. Apoptotic cells were increased more than taurine or cisplatin alone. Induction of apoptosis was related to p53 expression and activation of caspase-3, caspase-6, caspase-7, and caspase-9. In present study, the results indicated that co-treatment of cisplatin with taurine was more effective than single treatment of cisplatin.

19.1 Introduction

Cisplatin is an effective chemotherapeutic agent that is widely used to treat a variety of solid cancers. (Go and Adjei 1999; Boulikas and Vougiouka 2004). However, cisplatin affects not only the cancer cells but also normal cells. For that reason, cisplatin induces side effects such as ototoxicity, renal damage, gastrointestinal toxicity, and hematopoietic toxicity. (Gottlieb and Drewinko 1975; McKeage 1995). Therefore, in order to increase the effectiveness and decrease the side effects of

T. Kim • A.K. Kim, Ph.D. (✉)
College of Pharmacy, Sookmyung Women's University,
Chungpa-Dong, Yongsan-Ku, 140-742 Seoul, South Korea
e-mail: akkim@sookmyung.ac.kr

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_19,

chemotherapy, combined treatment of natural resources was investigated (Saif et al. 2009; Ghavami et al. 2011; Suganuma et al. 2011).

Taurine is a sulfur-containing amino acid. It is a low molecular weight amino acid and forms zwitterions. Taurine is one of the most abundant free amino acids in animal tissues and biological fluids (Barbeau 1975). 20 to 30 years ago, taurine has not been known to any other physiological activity except for conjugation of bile acids (Danielsson and Sjövall 1975). Recently several studies reported that taurine has a variety of biological functions including antioxidant activity, brain development, activation of retinal photoreceptor, contraction of the heart muscle, regulation of osmotic pressure, and maintenance of the immune system (Huxtable 1992).

Some recent researches show the protective effects of taurine against anticancer drugs in vivo. Taurine prevents cardiotoxicity induced by doxorubicin in chick and mice (Hamaguchi et al. 1988; Hamaguchi et al. 1989, Ito et al. 2009). In addition, taurine has protective effects against cisplatin-induced renal interstitial fibrosis and nephrotoxicity (Saad and Al-Rikabi 2002; Sato et al. 2002). But antitumor effect of taurine has rarely been investigated. Therefore, the present study was conducted to investigate the anticancer effects and mechanisms of single treatment of taurine or co-treatment of cisplatin with taurine in HeLa cells.

19.2 Methods

19.2.1 Cell Culture

Human cervical cancer HeLa cells were purchased from KCLB (Korea Cell Line Bank; Seoul, South Korea). Cells were cultured in RPMI1640 (Welgene, Daegu, South Korea) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Welgene, Daegu, South Korea), and heat-inactivated 10%FBS (fetal bovine serum; Welgene, Daegu, South Korea) in 5% CO_2 atmosphere at 37°C. Cells were routinely subcultured when about 80% confluent.

19.2.2 Measurement of Cell Viability

Cell viability was measured by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated into 96-well plates at 3,000 cells per well. Cisplatin and taurine were treated with different concentrations for 24, 48, and 72 h. At the end of the treatment, 20 μl MTT solution (5 mg/ml in PBS; Sigma, St. Louis, MO, USA) was added in each well and incubated at 37°C for 4 h. Formed formazan crystals were dissolved in 200 μl dimethyl sulfoxide (DMSO) and measured by a microplate reader (EL800, BioTEK Instruments Inc., Winooski, VT, USA) at a wavelength of 540 nm.

19.2.3 Western Blot Analysis

Cisplatin and taurine were treated for 72 h. Cells were lysed in 200 μl RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). Equal amount of proteins was separated by 12% SDS-polyacrylamide gel electrophoresis and electrotransferred nitrocellulose membrane. The membranes were blocked with Tris-buffered saline (20 mM Tris-HCl and 140 mM NaCl, pH 7.6) containing 0.3% Tween 20 (TBS-T) and 5% nonfat dry milk at room temperature for 2 h. The membranes were incubated overnight with primary antibodies at 4°C. After being washed, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h. All antibodies were purchased from Cell Signaling (Cell Signaling Technology, Inc., Danvers, MA, USA). The signal was detected using ECL advanced detection kit (GE Healthcare Bio-Sciences Corp., NJ, USA).

19.2.4 Microscopic Images of DAPI Staining

Cells were plated at 0.3×10^6 cells in 6-well plate. The next day, cells were treated with various concentrations of taurine and cisplatin for 72 h. After treatments, cells were washed with cold PBS and then fixed in methanol/DMSO (4:1) solution for 24 h at 4°C. Cells were washed with PBS three times and then stained with DAPI for 10 min and observed under the fluorescence microscope.

19.2.5 Statistical Analysis

Statistical significance was determined by Tukey multiple comparisons (SigmaStat; Jandel, San Rafael, CA, USA). Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

19.3 Results

19.3.1 Co-treatment of Cisplatin with Taurine Decreases Cell Proliferations

Cells were treated with taurine and cisplatin for 48 and 72 h, and cell proliferation was investigated by MTT assay. In single treatment of taurine, cell proliferation was

decreased in a dose-dependent manner. After 48 and 72 h incubation with 20 mM taurine, cell proliferations were decreased to 28.9% and 37.5%, respectively, compared to control. Single treatment of cisplatin (low concentration) has little decreased cell proliferations (11.8% at 48 h and 21.3% at 72 h). Furthermore, co-treatment of cisplatin with taurine has more decreased cell proliferations than single treatment of cisplatin or taurine. In co-treatment of cisplatin with taurine for 48 h and 72 h, cell proliferations were decreased to 42.4% and 53.8% compared with the control. Because of higher effects of 72 h than 48 h, the following experiments were performed for 72 h (Fig. 19.1).

19.3.2 Co-treatment of Cisplatin with Taurine Increases Apoptotic Cells

Reduced cell proliferations were caused by induction of apoptosis. For investigation of apoptotic cells, cells were imaged using DAPI as fluorescent probes. DAPI binds to DNA in the cell nucleus, so that chromatin agglomeration and fragmentation occurring by apoptosis were observed by fluorescence microscopy. The results indicated that single treatment of taurine or cisplatin induced apoptosis. In co-treatment of cisplatin with taurine, the incidence of apoptosis was more increased than single treatment of taurine or cisplatin (Fig. 19.2).

19.3.3 The p53 Was Increased in Single and Co-treatment of Cisplatin with Taurine

When apoptosis occurred, expression of p53 was investigated by western blotting. Taurine or cisplatin increased p53 level in a dose-dependent manner. In co-treatment of cisplatin with taurine, expression of p53 was increased compared to control (Fig. 19.3).

19.3.4 Taurine Increases Cleavage of Caspases in a Dose-Dependent Manner

Apoptosis is mostly mediated by apoptosis-related enzymes known as caspases. In this study, we investigated caspase-9, caspase-7, and caspase-6 by western blotting. Caspase-9 was increased by single treatment of cisplatin or taurine compared to control, but co-treatment of cisplatin with taurine does not enhance the effects. In single treatment of taurine, caspase-7 was increased in a dose-dependent manner. Taurine more affected the expression of caspase-7 than cisplatin, whereas caspase-7

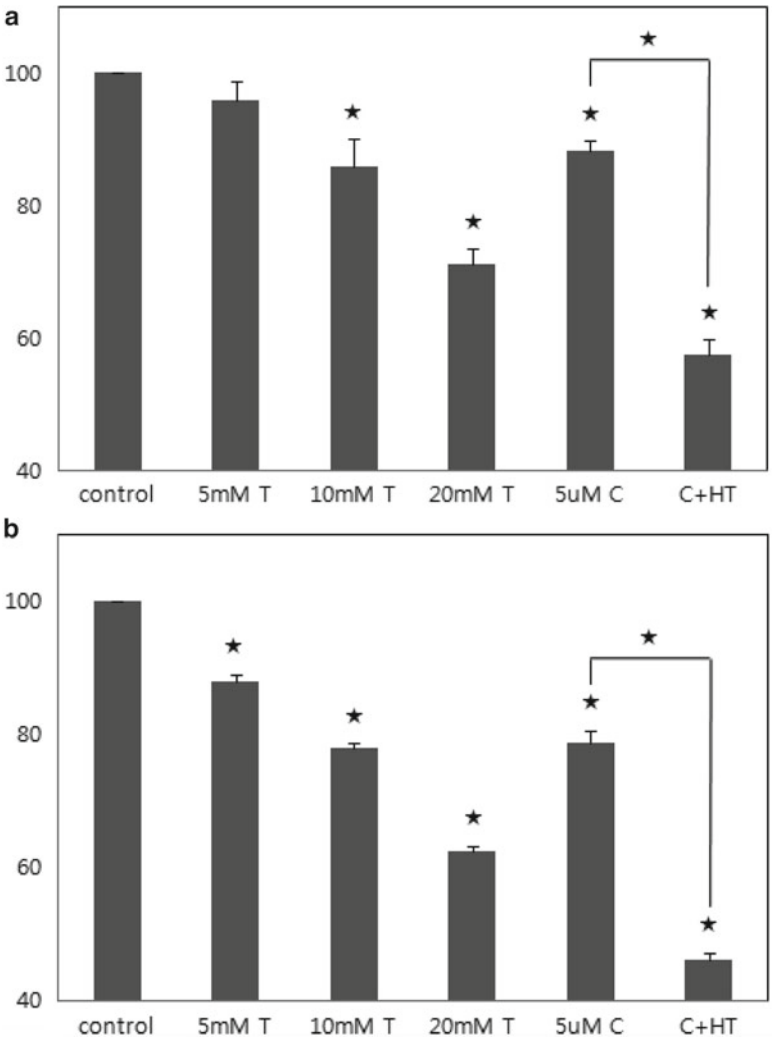

Fig. 19.1 Effects of low concentration of cisplatin and taurine on cell proliferations. Cell proliferation was determined using the MTT assay. Cells were treated with different concentration of cisplatin (5 μM) and taurine (5, 10, and 20 mM) for 48 (**a**) and 72 h (**b**). ★ $P<0.001$, significantly different from the control group

was slightly decreased in co-treatment of cisplatin with taurine. The expression of caspase-6 was shown as similar with caspase-7. Single treatment of taurine or cisplatin increased caspase-6, but co-treatment of cisplatin with taurine slightly decreased caspase-6 (Fig. 19.4).

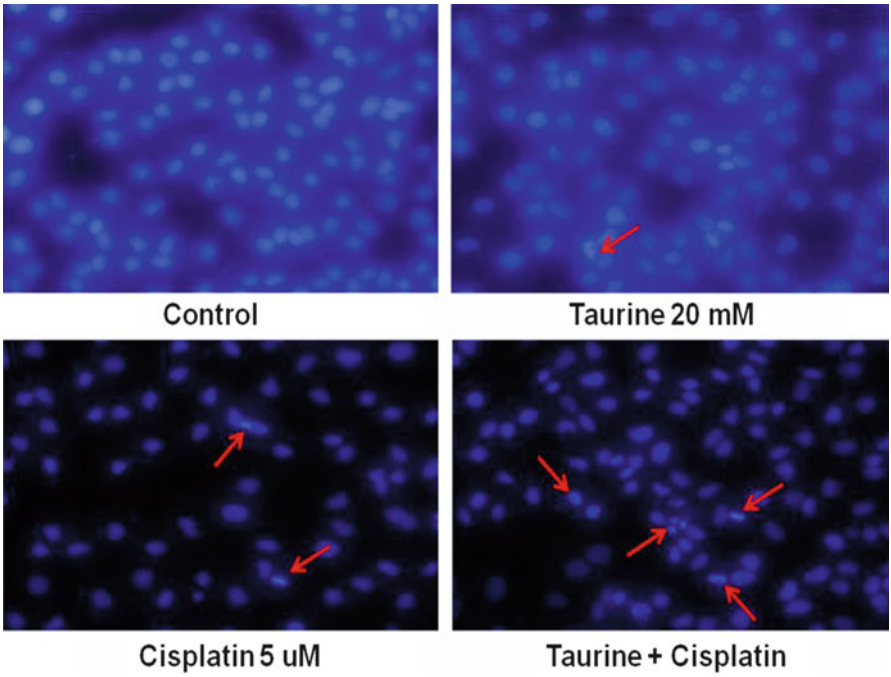

Fig. 19.2 Co-treatment of cisplatin with taurine increases apoptotic cells. Apoptotic cells were imaged using DAPI as fluorescent probes. Cells were treated with 0 and 20 mM of taurine and 5 μM of cisplatin for 72 h. *Arrows* indicated the apoptotic cells

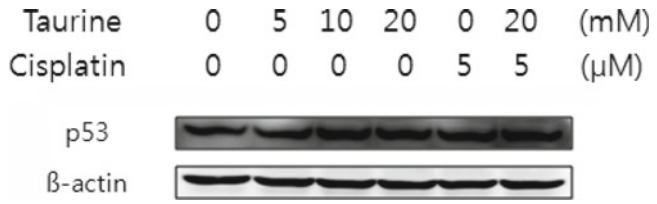

Fig. 19.3 Effects of cisplatin and taurine on p53 proteins. Protein expressions were measured using western blotting. Cells were treated with cisplatin (5 μM) and taurine (5, 10, and 20 mM) for 72 h

19.3.5 In Co-treatment of Cisplatin with Taurine, Caspase-3 Was Increased, Whereas PARP Was Unchanged

Caspase-3 and PARP were detected by western blotting. Caspase-3 is an apoptosis-regulating enzyme, and it leads to apoptosis. In single treatment of taurine, caspase-3 was increased in a dose-dependent manner, and combination treatment strongly activated caspase-3. PARP is assisting in the repair of single-strand DNA nicks, so it inhibits

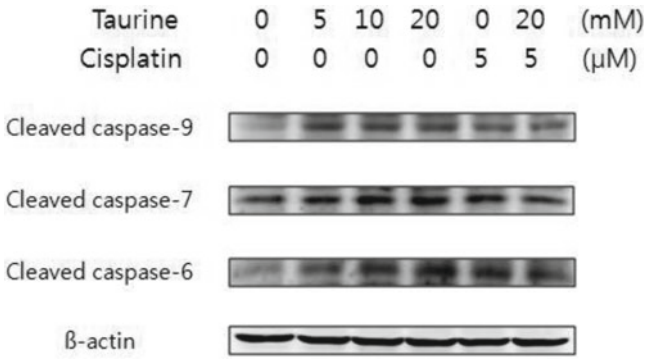

Fig. 19.4 Effects of cisplatin and taurine on caspase-6, caspase-7, and caspase-9 proteins. Protein expressions were measured using western blotting. Cells were treated with cisplatin (5 μM) and taurine (5, 10, and 20 mM) for 72 h

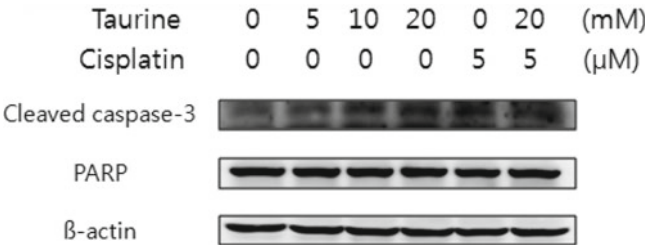

Fig. 19.5 Effects of cisplatin and taurine on apoptosis-related proteins, caspase-3, and PARP. Protein expressions were measured using western blotting. Cells were treated with cisplatin (5 μM) and taurine (5, 10, and 20 mM) for 72 h

apoptosis. The results indicated that single treatment of taurine or cisplatin did not affect PARP expression. Similarly, expression of PARP was not increased in co-treatment of cisplatin with taurine (Fig. 19.5).

19.4 Discussion

In the present study, we investigated the anticancer effects and mechanisms of single treatment of taurine or co-treatment of cisplatin with taurine in human cervical cancer HeLa cells in order to enhance anticancer effects and reduce side effects of cisplatin.

After treatment of taurine for 48 h or 72 h, cell proliferation was significantly decreased in a time- and dose-dependent manner. In previous studies, taurine is known to promote cell proliferation in normal cell line (Chen et al. 1998; Lima and Cubillos 1998; Hernández-Benítez et al. 2012). However, according to a recent

study, taurine reduced cell proliferation in hepatic stellate cells (Chen et al. 2004). Co-treatment of cisplatin with taurine has more decreased cell proliferation than single treatment of cisplatin or taurine despite the low concentration of cisplatin. The results of this experiment indicated that the combination of cisplatin with taurine effectively inhibits cell proliferation.

To verify that taurine and/or cisplatin treatment induced apoptosis, we investigated apoptotic cells by DAPI staining. Increase of apoptosis in cancer cells is one of the aims of anticancer treatment (Nimmanapalli and Bhalla 2003; Bremer et al. 2006). Anticancer drugs have the effects via induction of apoptosis in various cancer cells (Yu and Zhang 2004). In the present study, in cells treated with taurine and/or cisplatin for 72 h, apoptotic cells were observed. In co-treatment of cisplatin with taurine, the incidence of apoptosis was more increased than single treatment of taurine or cisplatin.

The p53 tumor suppressor protein is closely related to apoptosis. The p53 protein plays a major role in cellular response to DNA damage and other genomic aberrations. The main mechanisms of p53 are repair of DNA damage, inhibition of cell cycle via expression of cell cycle-related proteins, and induction of apoptosis through regulation of apoptosis-related proteins (Maclaine and Hupp 2009).The p53 is mutated in many cancer cells, and absence of p53 in cancer cells is known to stimulate the growth of cancer cells (Chène 2001). For those reasons, induction of p53 is important in anticancer activities. Cells were treated with taurine or cisplatin for 72 h, and expression of p53 was increased compared with control. Similarly, p53 expression was increased in co-treatment of cisplatin with taurine compared with control. According to the results of this study, induction of apoptosis caused by treatment of taurine and/or cisplatin was related to p53 expression.

After treatment of taurine and/or cisplatin for 72 h, caspase-9, caspase-7, and caspase-6 were observed by western blotting. Caspases are a family of cysteine proteases. Caspase-cascade system plays essential roles in apoptosis, necrosis, and inflammation (Budihardjo et al. 1999; Fan et al. 2005). The results indicated that the treatment of taurine increased caspase-7 and caspase-6 in a dose-dependent manner. Especially, caspase-6 was highly increased at 20 mM of taurine compared with the single treatment of cisplatin. But co-treatment of cisplatin with taurine slightly decreased caspase-7 and caspase-6 compared to single treatment of taurine. In treatment of taurine, caspase-9 was increased regardless of the taurine concentration compared to control. But co-treatment of cisplatin with taurine does not enhance the effects. Through this experiment, taurine treatment alone and combination of cisplatin with taurine caused the processing of procaspases to cleave, and activation of caspases leads to the induction of apoptosis.

Cisplatin and taurine were treated for 72 h, and expressions of caspase-3 and PARP were investigated. Caspase-3 is a key factor in apoptosis execution (Porter and Jänicke 1999). The results indicated that caspase-3 was increased after treatment of taurine in a dose-dependent manner. Combined treatment also increased caspase-3. PARP also induces apoptosis directly (Konopleva et al. 1999, Hong et al. 2004). However, PARP expressions were not changed. Treatment of taurine and/or cisplatin had no effects on PARP expression. According to the results of this study,

single treatment of taurine or cisplatin and co-treatment of cisplatin with taurine induced apoptosis via expression of caspase-3 rather than PARP expression.

This study suggests that combination treatment of cisplatin with taurine enhanced anticancer activity and reduced side effects of cisplatin by lowering the dose of anticancer drugs.

19.5 Conclusion

In this study, we investigated anticancer effects of taurine and synergy effects of taurine and cisplatin in human cervical cancer HeLa cells. Cell proliferations were decreased in a dose-dependent manner, and co-treatment of cisplatin with taurine was more effective than single treatment. Apoptotic cells were increased by treatments of cisplatin with taurine. Induction of apoptosis was related to p53 expression and caspase-3, caspase-6, caspase-7, and caspase-9. The present study suggests that co-treatment of cisplatin with taurine enhanced anticancer effects of cisplatin through apoptosis induction in HeLa cells.

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Chapter 20
Comparative Evaluation of the Effects of Taurine and Thiotaurine on Alterations of the Cellular Redox Status and Activities of Antioxidant and Glutathione-Related Enzymes by Acetaminophen in the Rat

Miteshkumar Acharya and Cesar A. Lau-Cam

Abstract The present study was carried out to ascertain the impact of replacing the sulfonate group of TAU with thiosulfonate, as present in thiotaurine (TTAU), on the protective actions of TAU against hepatocellular damage and biochemical alterations related to oxidative stress and glutathione redox cycling, synthesis, and utilization caused by a high dose of acetaminophen (APAP). To this end, male Sprague-Dawley rats, 225–250 g, were intraperitoneally treated with a 2.4 mmol/kg dose of TAU (or TTAU), followed 30 min later by 800 mg/kg of APAP. A reference group received 2.4 mmol/kg of *N*-acetylcysteine (NAC) prior to APAP. Naive rats served as controls. The animals were sacrificed 6 h after receiving APAP and their blood and livers were collected. Plasma and liver homogenates were analyzed for indices of cell damage (plasma transaminases, plasma lactate dehydrogenase), oxidative stress (malondialdehyde = MDA, reduced glutathione = GSH, glutathione disulfide = GSSG, catalase, glutathione peroxidase, superoxide dismutase), glutathione cycling (glutathione reductase), utilization (glutathione S-transferase), and synthesis (γ-glutamylcysteine synthetase) activities. APAP increased MDA formation and lowered the GSH/GSSG ratio and all enzyme activities, especially those of antioxidant enzymes. In general, TTAU was equipotent with NAC and more potent than TAU in protecting the liver. Taken into account the results of a previous study comparing the actions of TAU and hypotaurine (HTAU), the sulfinate analog of TAU, it appears that the sulfinate and thiosulfonate analogs are somewhat more effective than the parent sulfonate TAU in counteracting APAP-induced hepatic alterations in the liver and plasma.

M. Acharya • C.A. Lau-Cam (✉)
Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, Jamaica, NY 11439, USA
e-mail: claucam@usa.net

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Abbreviations

APAP	Acetaminophen
TAU	Taurine
TTAU	Thiotaurine
NAC	*N*-acetylcysteine
ALT	Alanine transaminase
AST	Aspartate transaminase
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
GSH	Reduced glutathione
GSSG	Glutathione disulfide
CAT	Catalase
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
GS	γ-Glutamylcysteine synthetase
GR	Glutathione reductase
GST	Glutathione S-transferase

20.1 Introduction

Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP) is a synthetic centrally acting compound widely used for its analgesic and antipyretic effects in infants, children, and adults. At therapeutic doses this over-the-counter medication is well tolerated, but in overdoses it is one of the most common causes of both intentional and unintentional poisoning and toxicity, often culminating in hepatic failure and even death (Chung et al. 2009; Lee 2007).

The toxicity of APAP is closely linked to its hepatic metabolism, which may involve the depletion of protein and glutathione thiol groups, the elevation of the cytoplasmic Ca^{2+} (Moore et al. 1985), and the development of oxidative/nitrosative stress (Jaeschke et al. 2003; van de Straat et al. 1987).

After the ingestion of a standard dose of APAP, up to 90% of this phenolic compound enters into hepatic conjugation at the *meta*-position to yield innocuous glucuronide (~55%) and sulfate (35%) conjugates, with only a small fraction (about 5–10%) undergoing metabolism to the toxic electrophile *N*-acetyl-*para*-benzoquinoimine (NAPQI) by hepatic cytochrome P450 isoforms, mainly CYP 2E1, CYP 1A2, and CYP 3A2 (Bessems and Vermeulen 2001; Patten et al. 1993; Smilkstein et al. 1988). At low doses of APAP, NAPQI is conjugated with glutathione and subsequently excreted in the urine as the mercapturic acid and cysteine derivatives (Patten et al. 1993). At high doses of APAP, however, the normal conjugating pathways for this compound become rapidly saturated and a greater proportion of the drug is available for conversion to NAPQI, which then depletes the

hepatic stores of glutathione. As a result, NAPQI is left unopposed to effect cell injury by covalently binding to hepatic proteins, primarily though not exclusively, to cysteine residues to generate stable 3-(cystein-S-yl) APAP adducts (Nelson and Bruschi 2003). While this mechanism may account for APAP-related cell injury, the fraction of the dose of APAP entering into covalent binding to hepatic proteins is small. Hence, additional mechanisms have been postulated to account for the bulk of liver injury by APAP. One such proposal considers lipid peroxidation (LPO) as a mechanism of cell death based on the increased formation of reactive oxygen (ROS) and nitrogen (RNS) species observed in hepatocytes undergoing necrotic changes (Hinson et al. 2010). In this case, the binding of NAPQI to mitochondrial proteins of the respiratory chain will decrease mitochondrial respiration, oxidative phosphorylation, and ATP formation, and the flow of electrons will be diverted towards oxygen to generate superoxide anion (Jaeschke et al. 2003). In turn, superoxide anion arising from mitochondrial stress will propagate and amplify the liver injury upon reacting with nitric oxide to form peroxynitrite, a powerful oxidant and nitrating agent with the ability to modify cellular macromolecules and to aggravate mitochondrial dysfunction and ATP depletion (Jaeschke et al. 2003). Furthermore, mitochondria oxidative stress will alter calcium homeostasis and calcium-controlled cellular processes and will stimulate signaling pathways for the activation of transduction responses ending in mitochondrial permeability transition and the loss of membrane potential, events that further contribute to centrilobular hepatic necrosis and acute liver failure (Hinson et al. 2010).

In parallel with its depleting action of the intrahepatic glutathione, APAP is also capable of exerting varying effects on the levels of glutathione disulfide and of lowering the activities of antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase) as well as of enzymes participating in glutathione redox cycling (glutathione reductase), utilization (glutathione S-transferase), and synthesis (γ-glutamylcysteine synthetase) (Acharya and Lau-Cam 2010).

In general, the treatment of APAP poisoning has been directed at inhibiting its activation by the cytochrome P450 enzyme system or at restoring hepatic glutathione reserves to sustain conjugation with glutathione. Since protection of the liver against APAP toxicity by decreasing the formation of NAPQI through inhibition of the cytochrome P450 enzyme system with cysteamine, the descarboxy analog of L-cysteine, was not high enough (Miller and Jollow 1986), most antidotal approaches for APAP poisoning have been targeted at restoring the levels of intracellular glutathione. In spite of the numerous attempts to develop prodrugs of L-cysteine for GSH synthesis, only *N*-acetylcysteine (NAC) has received recognition as a first-line treatment for APAP poisoning. In addition to reversing APAP-induced depletion of glutathione and insuring the excretion of both APAP and NAPQ in the bile as glutathione conjugates (Lauterburg et al. 1983), NAC is also an effective antioxidant (Cotter et al. 2007; Ozaras et al. 2003; Sathish et al. 2011; Victor et al. 2003).

In a previous study we compared the effects of NAC with those of the sulfonic ($-SO_3H$) and sulfinic ($-SO_2H$) analogs of 2-aminoethane, namely, taurine (TAU) and hypotaurine (HYTAU), as protection against APAP-mediated LPO, changes in glutathione redox state, and declines in the activities of enzymes involved in

glutathione redox cycling, transfer, and synthesis in the rat (Acharya and Lau-Cam 2010). The rationale for testing these sulfur-containing compounds as protection against APAP hepatotoxicity stemmed from previous recognition that TAU was capable of attenuating LPO, apoptosis, necrosis, and DNA fragmentation in hepatocytes from rats treated with a toxic dose of APAP (Waters et al. 2001) and that TAU and HYTAU effectively lowered LPO, the fall of the GSH/GSSG ratio, and the loss of antioxidant enzyme activities in erythrocytes from diabetic rats (Gossai and Lau-Cam 2009). Since these results pointed to a determining role for the sulfur-containing functionality in the potency differences noted in erythrocytes between TAU and HYTAU, the present study was undertaken to further validate this assumption by comparing the actions of TAU in APAP-related hepatotoxicity against those of thiotaurine (TTAU), the thiosulfonate (-SO_2-SH) analog thiotaurine which has also been found to possess antioxidant actions (Egawa et al. 1999; Yoshiyuki 1998; Yoshiyuki and Yoshiki 2000).To better define the degree of protective action of TAU and TTAU in APAP-induced hepatotoxicity, their activities were compared against those of NAC.

20.2 Methods

20.2.1 Chemicals

The chemicals used in the present study were obtained from commercial sources in the USA. NAC, APAP, and chemicals used in the preparation of the biological samples and in the biochemical assays were purchased from Sigma-Aldrich, St. Louis, MO. TTAU was from Wako Chemicals USA, Inc., Richmond, VA.

20.2.2 Animals, Treatments, and Sample Collections

Male Sprague-Dawley rats, weighing 200–250 g, were obtained from Taconic, Germantown, New York, USA. The study received the approval of the Institutional Animal Care and Use Committee of St. John's University, Jamaica, New York, and the animals were cared in accordance with guidelines established by the United States Department of Agriculture. The experimental groups consisted of six rats each and they were used in the nonfasted state. The treatment solutions were prepared just prior to an experiment. The APAP solution was made in warm 50% polyethylene glycol (PEG) 400 and allowed to cool to ambient temperature before its administration. The test compounds (NAC, TAU, TTAU) were dissolved in distilled water. A treatment compound was administered as a single, 2.4 mmol/kg/2 mL, dose 30 min before a hepatotoxic, 800 mg/kg/2 mL, dose of APAP. Animals serving as controls only received 50% PEG 400 in a volume equal to 2 mL. All treatments

were carried out by the intraperitoneal route. At 6 h after the administration of APAP or 50% PEG 400, the animals were sacrificed by decapitation and their blood collected in heparinized tubes. Immediately thereafter the livers were removed using the freeze-clamp technique of Wollenberger et al. (1960). From each blood sample, a portion was set aside for the assay of reduced (GSH) and disulfide (GSSG) glutathione, and the remaining portion was centrifuged at 3,000 rpm (500 × *g*) and 4°C for 10 min to obtain the corresponding plasma fraction. For each liver, a 500 mg portion, kept cold on an ice bath, was mixed in a 1:20 (w/v) ratio with Tris buffer pH 7.0 containing 1 mg of phenylmethylsulfonyl fluoride and homogenized using a handheld electric blender. The resulting suspension was centrifuged at 12,000 rpm (8000 × *g*) and 4°C for 30 min to isolate the supernatant, which was kept on ice until needed.

20.2.3 Assay of Liver Malondialdehyde

Malondialdehyde (MDA) was determined as thiobarbituric acid-reactive substances (TBARS) by the endpoint assay method of Buege and Aust (1978). The amount of TBARS in the sample was derived from a calibration curve of MDA prepared from serial dilutions of a stock solution of 1,1,3,3-tetraethoxypropane which had been treated in the same manner as the sample preparation, and the results were reported as nM of MDA/mg of tissue.

20.2.4 Assay of the Plasma and Hepatic Levels of GSH and GSSG

The concentration of GSH in plasma and liver samples was measured by the method of Hissin and Hilf (1976), after reaction with *ortho*-phthalaldehyde to form a highly fluorescent product. The concentration of GSSG was measured in another aliquot of the same sample, following removal of any preexisting GSH upon reaction with *N*-ethylmaleimide. The concentrations of GSH and GSSG in the sample preparation were determined by reference to standard curves of these compounds prepared on the day of the assay and were reported either as nM/mL of plasma or as μM/g of tissue.

20.2.5 Assay of Plasma and Liver Antioxidant Enzymes

The catalase (CAT) activity was measured by the spectrophotometric method of Aebi (1984), the glutathione peroxidase (GPX) activity was measured as described by Flohé and Günzler (1984), and the activity of CuZn superoxide dismutase (SOD) was measured using the spectrophotometric method of Misra (1985). These activities were expressed as U/min/mg of protein.

20.2.6 Assay of the Plasma and Liver γ-Glutamylcysteine Synthetase (GCS), Glutathione S-Transferase (GST) and Glutathione Reductase (GR) Activities

The GCS activity was measured according to the method of Zhou and Freed (2005), the GST activity was measured as described by Habig et al. (1974), and the GR activity was measured by the spectrophotometric method of Wheeler et al. (1990). The results were expressed in U/min/mg of protein.

20.2.7 Statistical Analysis of the Data

The experimental results are reported as mean ± SEM for $n = 6$. They were analyzed for statistical significance using unpaired Student's *t*-test and a commercial computer software (JMP 7, JMP® Statistical Discovery Software, Cary, NC 27513) followed by one-way analysis of variance and Newman-Keuls post hoc test. Intergroup differences were considered to be statistically significant at $p \leq 0.05$.

20.3 Results and Discussion

The protective effects of TAU and TTAU against APAP-induced hepatotoxicity were assessed by measuring biochemical parameters consonant with cellular injury and oxidative stress. To more accurately define their respective potencies, they were further compared with an equidose (2.4 mmol/kg) of NAC, the current antidote of choice for APAP overdoses.

The degree of injury to the hepatocytes by a high dose of APAP was investigated by measuring the plasma ALT, AST, and LDH, three abundant intrahepatic enzymes whose release into the circulation is taken as evidence of liver injury (Duong and Loh 2006). As shown in Table 20.1, the plasma activities of all three enzymes were significantly elevated ($p < 0.001$), with the values decreasing in the order LDH (+292%) > AST (+135%) > ALT (+64%) probably in direct proportion to their intracellular abundance and location (Moss et al. 1986). TAU and TTAU were highly protective and about equipotent, with the increases amounting to only ~104% ($p < 0.001$), ~37% ($p < 0.01$), and ~33% ($p < 0.01$) of corresponding control values, respectively. NAC was more potent than either TAU or TTAU since the enzyme activities of LDH, AST, and ALT were only 60% ($p < 0.001$), 28% ($p < 0.01$), and 21% ($p < 0.05$), respectively, above control values.

Hepatic LPO is a common consequence of acute APAP intoxication which is typically manifested in rodents by increased ethane inhalation, increased hepatic MDA, and GSH depletion (Wendel 1983). The detection of MDA accumulation is a rather sensitive indicator of hepatotoxicity since it is increased before the appearance of necrosis (Nakae et al. 1990). The results presented in Fig. 20.1 indicate that

Table 20.1 Effects of NAC, TAU, and TTAU on plasma activities of ALT, AST, and LDH of rats when given in the absence or presence of a hepatotoxic (800 mg/kg i.p.) dose of APAP

Group	ALT, U/L	AST, U/L	LDH, U/L
Control	40.55 ± 0.89	69.23 ± 4.91	507.82 ± 11.95
NAC	40.04 ± 1.06	71.39 ± 3.50	510.38 ± 5.06
TAU	39.95 ± 1.80	68.13 ± 4.62	480.38 ± 5.49
TTAU	40.13 ± 1.61	70.11 ± 4.12	465.17 ± 6.85
APAP	66.50 ± 1.53***	162.40 ± 5.13***	1992.76 ± 89.54***
NAC + APAP	48.98 ± 1.06*,†	88.35 ± 6.23*,†††	810.85 ± 115.75***,†††
TAU + APAP	54.78 ± 1.34*,†	96.22 ± 4.28*,†††	1030.71 ± 58.1***,††
TTAU + APAP	53.30 ± 2.04**,†	92.43 ± 4.87**,††	1036.43 ± 54.21***,†††

Values are the mean ± SEM for $n = 6$. Differences were significant from control at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ and from APAP at $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$

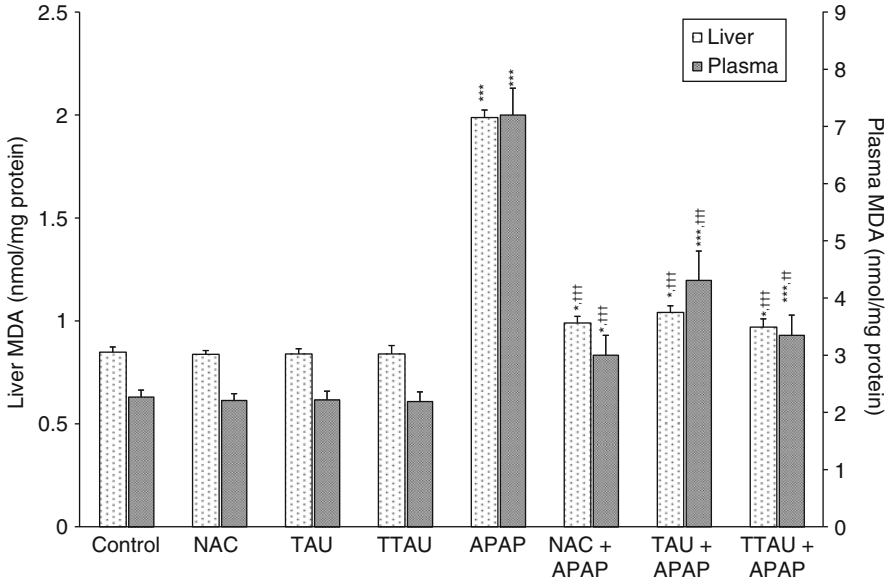

Fig. 20.1 The effects of NAC, TAU, and TTAU on liver and plasma MDA levels of rats treated with a hepatotoxic (800 mg/kg i.p.) dose of APAP. Differences were significant from control at $^{*}p < 0.05$ and $^{***}p < 0.001$ and from APAP at $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$. Values are shown as mean ± SEM for $n = 6$

APAP markedly raised the MDA level in the plasma (by >200%) and liver (by >130%). Both TAU and TTAU were able to attenuate these increases, with TTAU appearing more potent (+48%, $p < 0.01$, and +14%, respectively) than TAU (+91%, $p < 0.001$, and +22%, $p < 0.05$, respectively). On the other hand, NAC was more potent than either TAU or TTAU especially in the plasma (32%, $p < 0.01$, and 16%, $p < 0.05$, respectively).

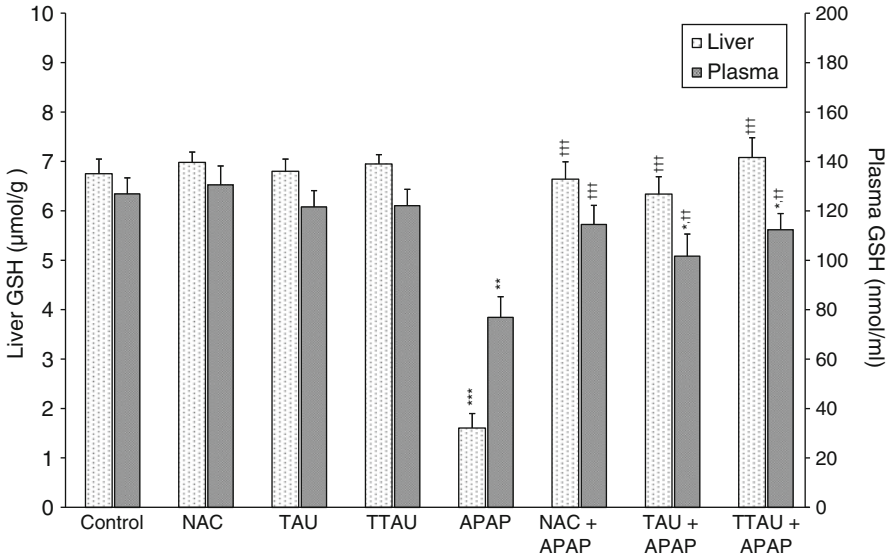

Fig. 20.2 The effects of NAC, TAU, and TTAU on liver and plasma GSH levels of rats treated with a hepatotoxic (800 mg/kg i.p.) dose of APAP. Differences were significant from control at $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ and from APAP at $^{\dagger\dagger}p<0.01$ and $^{\dagger\dagger\dagger}p<0.001$. Values are shown as mean ± SEM for $n=6$

Experiments in rodents have demonstrated that an acute high dose of APAP can lower the hepatic GSH levels as early as 15 min after its administration (Lores Arnaiz et al. 1995; Lauterburg et al. 1983). From the results presented in Fig. 20.2, it is apparent that APAP depleted GSH both in the liver (by 76%, $p<0.001$) and plasma (by 39%, $p<0.01$), changes that were much less pronounced in the presence of TTAU (+5% and −11%, respectively). By comparison, protection was less in the presence of TAU (−20%, $p<0.01$, and −46%, $p<0.001$, respectively) and about equal following a treatment with NAC (−10% and −2%, respectively) relative to TTAU.

The loss of GSH upon the administration of APAP has occurred concomitantly with a proportional decrease in arterial plasma GSH concentration but not with a corresponding increase in GSSG, findings that are consistent with the hypothesis that the liver is a major source of plasma GSH (Adams et al. 1983). In the present study, APAP (800 mg/kg) lowered the GSSG levels in both plasma (−25%, $p<0.01$) and liver (−47%, $p<0.001$) (Fig. 20.3), effects that were counteracted by TAU (−19% and −22%, $p<0.05$, respectively) and TTAU (−15%, $p<0.05$, and +66%, $p<0.001$) relative to control values. NAC, on the other hand, was about equipotent with TTAU (−14% and +46%, $p<0.001$, respectively). In parallel with a decrease in hepatic GSH and GSSG, there was also a corresponding decrease in the GSH/GSSG ratio both in the plasma (−19%, $p<0.05$) and liver (−54%, $p<0.001$) (Fig. 20.4). These changes were attenuated by TAU (−23% and −29%, respectively, both at $p<0.05$) and reversed by TTAU (+10% and 66%, $p<0.001$, respectively) and NAC (+4 and +46%, $p<0.001$, respectively).

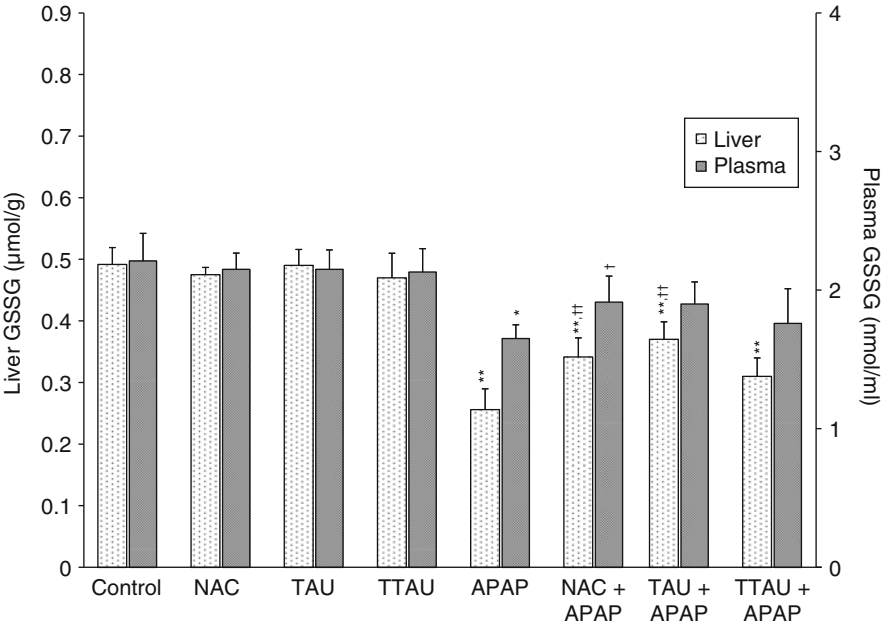

Fig. 20.3 The effects of NAC, TAU, and TTAU on liver and plasma GSSG levels of rats treated with a hepatotoxic (800 mg/kg i.p.) dose of APAP. Differences were significant from control at $*p<0.05$ and $**p<0.01$ and from APAP at $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. Values are shown as mean ± SEM for $n=6$

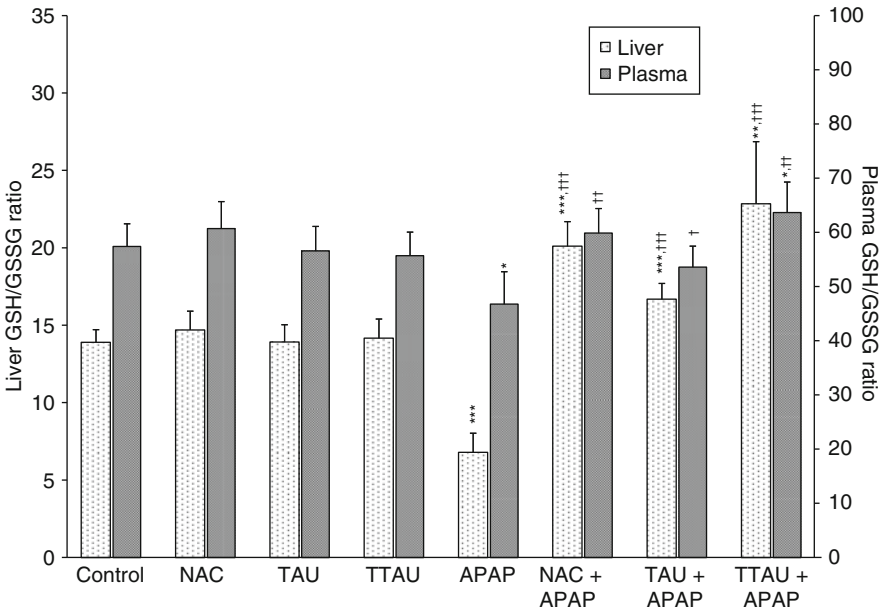

Fig. 20.4 The effects of NAC, TAU, and TTAU on the liver and plasma GSH/GSSG ratios of rats treated with a hepatotoxic (800 mg/kg i.p.) dose of APAP. Differences were significant from control at $*p<0.05$, $**p<0.01$, and $***p<0.001$ and from APAP at $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, and $^{\dagger\dagger\dagger}p<0.001$. Values are shown as mean ± SEM for $n=6$

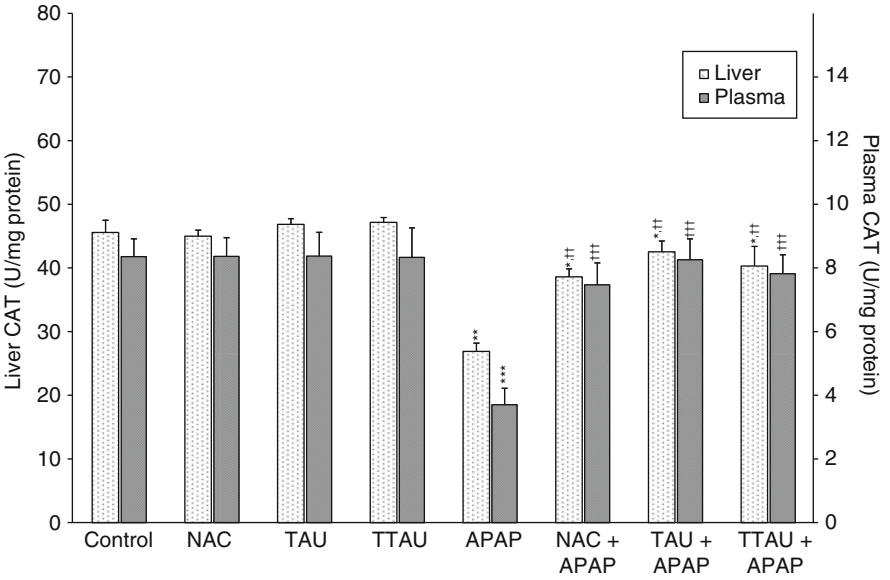

Fig. 20.5 The effects of NAC, TAU, and TTAU on the activities of liver and plasma CAT of rats treated with a hepatotoxic (800 mg/kg i.p.) dose of APAP. Differences were significant from control at $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ and from APAP at $^{\dagger\dagger}p<0.01$ and $^{\dagger\dagger\dagger}p<0.001$. Values are shown as mean ± SEM for $n=6$

At high doses APAP can make the liver more vulnerable to the deleterious effects of oxidative stress through its inhibitory interaction with antioxidant enzymes known to interact and destroy specific ROS (Matés 2000). In agreement with the results reported by other investigators in rats (Sabina et al. 2009) and mice (Olaleye and Rocha 2008), this work verified that the activity of CAT and SOD was decreased by a high dose of APAP both in the plasma and liver. This situation will certainly add to the susceptibility of liver cells to oxidative stress inasmuch as each of these enzymes is involved in ROS detoxification (hydrogen peroxide by CAT, hydrogen peroxide and other peroxides by GPx, superoxide anion by SOD). As seen in Figs. 20.5 and 20.6, the plasma activities of these enzymes were reduced by 56% and 29%, respectively, relative to control values ($p \leq 0.01$) and by 41% and 58%, respectively ($p \leq 0.01$), in the liver. All the pretreatment compounds were able to prevent the losses in antioxidant enzyme activities to a significant extent in the plasma (CAT by only 1–11%, SOD by ~11%) and liver (CAT 7–15%, SOD by 26–35%, $p<0.01$), with TAU providing a nonsignificantly greater protection than either TTAU or NAC. While the activity of CAT may protect against LPO by preventing iron-catalyzed generation of ROS, an increase in SOD activity may curtail the accumulation of MDA in parallel with liver necrosis (Nakae et al. 1990).

In vitro studies in which isolated rat hepatocytes were exposed to an oxidant in the presence and absence of inhibitors of GPx and GR have suggested that the GPx/GR system can protect hepatocytes from damage by oxidative stress and can decrease

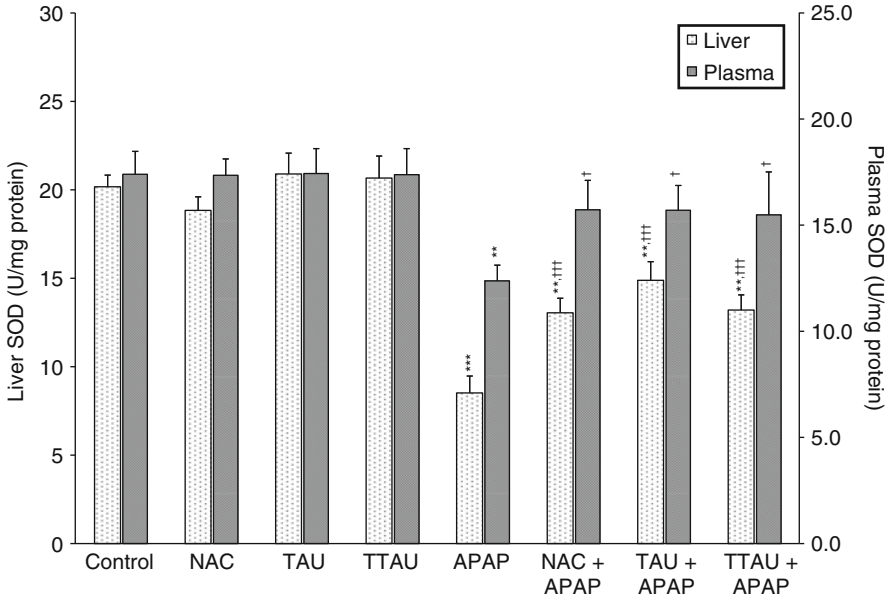

Fig. 20.6 The effects of NAC, TAU, and TTAU on the activities of liver and plasma SOD of rats treated with a hepatotoxic (800 mg/kg i.p.) dose of APAP. Differences were significant from control at $^{**}p<0.01$ and $^{***}p<0.001$ and from APAP at $^{\dagger}p<0.01$ and $^{\dagger\dagger\dagger}p<0.001$. Values are shown as mean ± SEM for $n=6$

cell susceptibility to APAP toxicity in response to oxidative stress initiated by ROS, particularly by peroxides normally handled by the GPx/GR system (Adamson and Harman 1989). Overdoses of APAP may negatively affect the activity of GPx by lowering the availability of its cofactor GSH, by promoting the formation of ROS capable of oxidizing critical thiol groups, and through arylation by NAPQI (Lores Arnaiz et al. 1995; Tirmenstein and Nelson 1990). On the other hand, GR, an enzyme that converts GSSG back to GSH, may be inhibited by APAP-GSH conjugate (Roušar et al. 2010). Not surprisingly, in the present study the activities of GPX and GR were significantly decreased by APAP in the plasma (−57% and −34%, $p<0.01$, respectively) and liver (−69%, $p<0.001$, and −23%, $p<0.05$, respectively) (Figs. 20.7 and 20.8). A pretreatment with a sulfur-containing compound ameliorated these losses to rather similar extents in both the plasma (36–43% for GPx, $p<0.01$; 3–22% for GR) and liver (40–52%, $p\leq0.01$, for GPX; ≤12% for GR).

γ-Glutamylcysteine synthetase (γ-GCS) catalyzes the rate-limiting step in de novo synthesis of GSH in liver cells and, consequently, plays a major role in the antioxidant capacity of these cells. The protective role of this enzyme was demonstrated by knocking down the heavy chain mRNA of γ-GCS in a rat model to induce a protracted GSH depletion and to potentiate APAP toxicity when compared with normal rats (Akai et al. 2007). For this reason, individuals who are heterozygous for γ-GCS deficiency may have a limited capacity for detoxifying

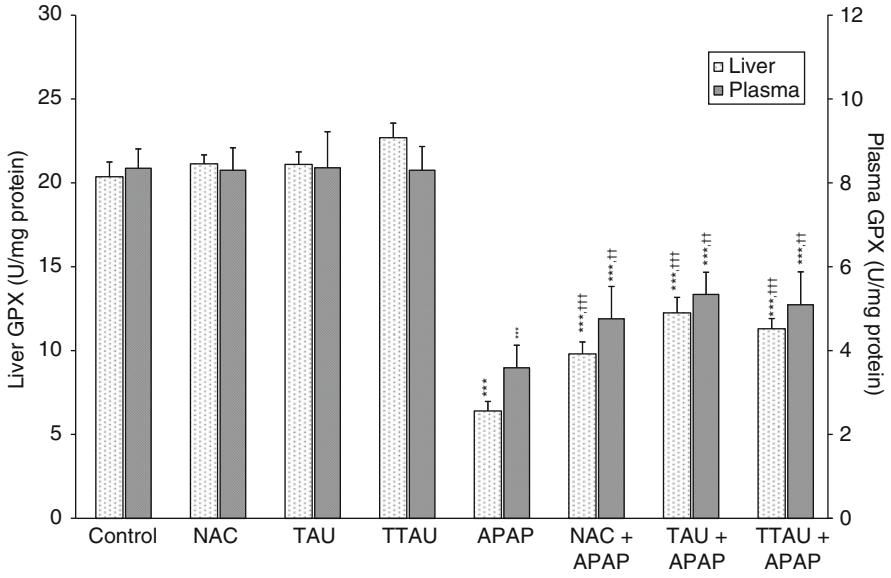

Fig. 20.7 The effects of NAC, TAU, and TTAU on the liver and plasma activities of GPx of rats treated with a hepatotoxic (800 mg/kg i.p.) dose of APAP. Differences were significant from control at $^{***}p<0.001$ and from APAP at $^{\dagger\dagger}p<0.01$ and $^{\dagger\dagger\dagger}p<0.001$. Values are shown as mean ± SEM for $n=6$

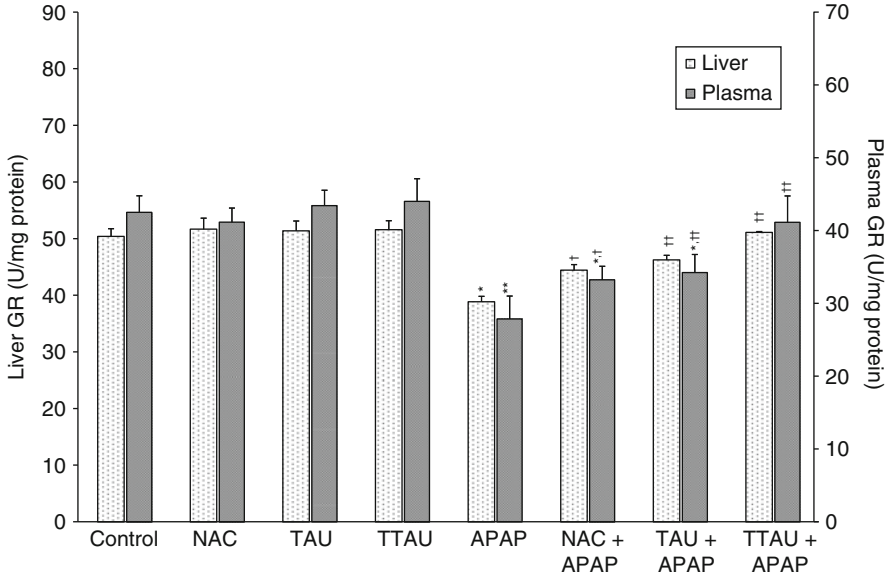

Fig. 20.8 The effects of NAC, TAU, and TTAU on the activities of liver and plasma GR of rats treated with a hepatotoxic (800 mg/kg i.p.) dose of APAP. Differences were significant from control at $^{*}p<0.05$ and $^{**}p<0.01$ and from APAP at $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. Values are shown as mean ± SEM for $n=6$

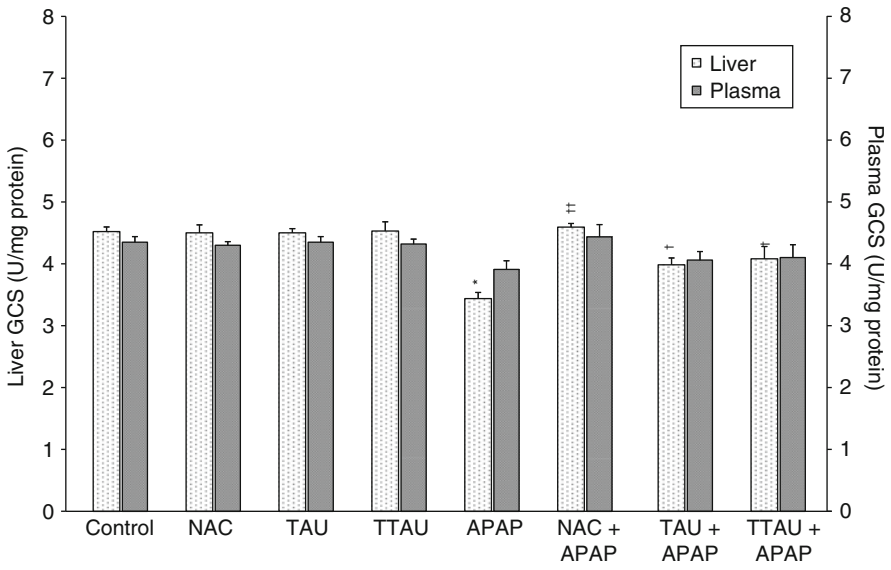

Fig. 20.9 The effects of NAC, TAU, and TTAU on the liver and plasma activities of GCS of rats treated with a hepatotoxic (800 mg/kg i.p.) dose of APAP. Differences were significant from control at $^{*}p<0.05$ and from APAP at $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. Values are shown as mean ± SEM for $n=6$

NAPQI through conjugation with GSH (Spielberg 1985). Relative to control values, APAP drastically lowered the plasma and hepatic activity of γ-GCS by 61% and 70% ($p<0.001$), respectively (Fig. 20.9). A pretreatment with TTAU virtually reversed the effects (≤3% decrease) and one with TAU was markedly protective (−19%, $p<0.05$, and −8%, respectively). NAC was equipotent with TTAU.

GSTs are a group of cytosolic and membrane-associated isoenzymes with the ability to catalyze the nucleophilic addition of the thiol of reduced glutathione to a variety of electrophiles (Hayes and Strange 1995; Rushmore and Pickett 1993). At low doses of APAP, GST is responsible for the bulk of the detoxification of NAPQI through conjugation with GSH (Henderson et al. 2000; Ketterer et al. 1983). However, a protective role for GST in APAP overdoses has been questioned after experiments showing that mice nulled for GST became resistant to the hepatotoxicity of APAP and that wild and nulled animals showed no difference in APAP metabolism and the same degree of APAP-reactive metabolites binding to cellular proteins, thus indicating that GST does not contribute in vivo to the formation of GSH conjugates of APAP but instead plays an unexpected role in the toxicity of this compound (Henderson et al. 2000).

Under the present experimental conditions, the plasma activity of GST was found to be reduced by APAP significantly ($p<0.001$) in the plasma (by 70%) and liver (by 61%) when compared to control values (Fig. 20.10). These decreases were more than halved in the plasma (28–31% decreases, $p<0.01$) and nearly halved (34–42% decreases, $p<0.01$) in the liver by a pretreatment

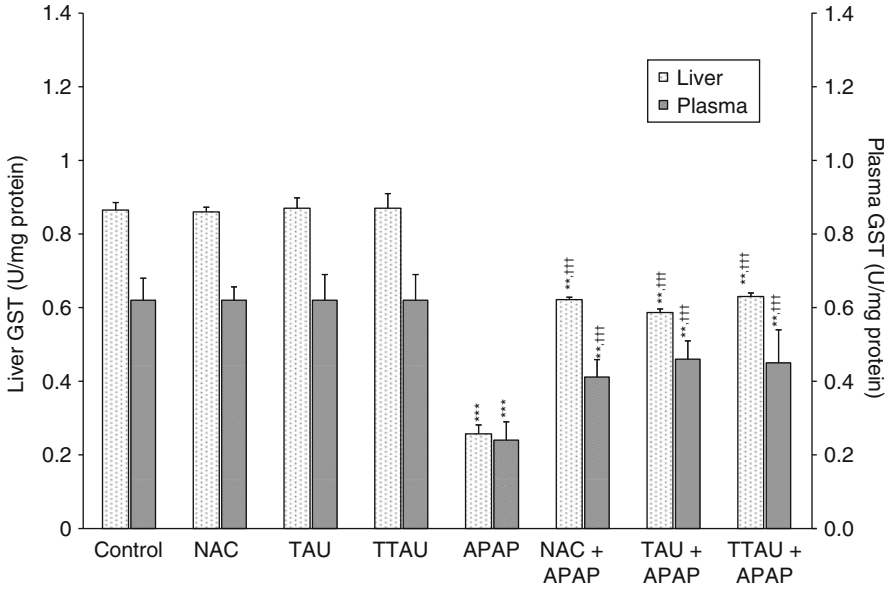

Fig. 20.10 The effects of NAC, TAU, and TTAU on the activities of liver and plasma GST of rats treated with a hepatotoxic (800 mg/kg i.p.) dose of APAP. Differences were significant from control at $^{**}p<0.01$ and $^{***}p<0.001$ and from APAP at $^{\dagger\dagger\dagger}p<0.001$. Values are shown as mean ± SEM for $n=6$

with TAU, TTAU, or INS compared to controls. These results contrast with those reported by Polaniak et al. (2011) in rats chronically treated with a daily 2.4 g/kg intraesophageal dose of APAP for periods up to 12 weeks and who found the activities of GST and GR to be elevated while that of GPx was decreased. The existence of a wide variability in the type of effect exerted by APAP on GSH-related enzymes is exemplified by the results of two studies conducted in rodents. In one study conducted in rats, treatment with a single 300 mg/kg dose of APAP lowered the activities of GPx, GR, and GST to below control values; in the other, the treatment of mice with a single 90–150 mg/kg dose of APAP enhanced the activity of GST in the serum but had the opposite effect on that associated with liver microsomes and homogenate (Wang and Peng 1993).

20.4 Conclusion

In short, this work has verified that the protective actions of the TAU molecule are maintained upon its conversion to the thiosulfonate analog TTAU. Even though the pattern of protective actions derived from these 2-aminoethane derivatives is identical and comparable to that expressed by NAC, TTAU appears to be more potent than TAU in terms of preserving the glutathione redox status of the cell. From the

present results and those gathered in an early study with TAU and HTAU, it is apparent that the protection rendered by these sulfur-containing compounds is highly dependent on the sulfur-containing functionality and that their protective effect against APAP hepatotoxicity is the result of an antioxidant action that translates into reduced LPO, preservation of antioxidant defenses, and maintenance of enzymatic mechanisms needed for GSH redox cycling, synthesis, and utilization. When ranked according to their relative protective potencies, TTAU was more akin to NAC and somewhat more effective than TAU.

Overall, the sulfinate analog of 2-aminoethane HYTAU appears to be a better antioxidant than either the thiosulfonate or sulfonate analogs.

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Chapter 21
Effects of Taurine on Myocardial cGMP/cAMP Ratio, Antioxidant Ability, and Ultrastructure in Cardiac Hypertrophy Rats Induced by Isoproterenol

Qunhui Yang, Jiancheng Yang, Gaofeng Wu, Ying Feng, Qiufeng Lv, Shumei Lin, and Jianmin Hu

Abstract Taurine is the most abundant free amino acid in the human body and accounts for more than 50% of the total amino acid pool in the mammalian heart. To investigate the preventive effects of taurine on cardiac hypertrophy in rats, myocardial injury was established by hypodermic injection of isoprenaline (ISO) (10 mg/kg d) for 7 days. The preventive effects of taurine (100 mg/kg d, 200 mg/kg d, and 300 mg/kg d, i.p) on heart coefficient; ultrastructure of cardiac muscle; the levels of creatine kinase heart isoenzyme (CK-MB), cAMP, and cGMP; and antioxidant ability were investigated. The results showed that taurine could significantly prevent the increase of heart coefficient induced by ISO. Compared with the model group, 100 mg/kg and 200 mg/kg taurine significantly decrease the levels of cAMP and cGMP, while 300 mg/kg taurine could significantly decrease the levels of cAMP in myocardium, and all the three concentrations of taurine could significantly increase the ratio of cGMP/cAMP. The level of serum CK-MB was significantly increased by ISO; 200 mg/kg taurine could significantly decrease it, but 100 mg/kg and 300 mg/kg taurine had no significant effect. As for the antioxidant ability, ISO administration could significantly increase the myocardial level of MDA but had no significant effects on the myocardial levels of SOD, GSH, GSH-Px, and T-AOC. However, taurine administration could significantly decrease the myocardial level of MDA and increase the levels of GSH and T-AOC compared with the model group. The serum levels of SOD, GSH-Px, GSH, and T-AOC were significantly reduced by ISO administration, but the level of MDA showed no significant changes compared with the control group. Taurine administration could significantly increase the serum levels of SOD, GSH-Px, GSH, and T-AOC and decrease the level of MDA compared with the model group. All the results indicated that 200 mg/kg taurine had better effects. The ultrastructure of cardiomyocytes showed that taurine administration could significantly reverse the injury caused by ISO. In conclusion,

Q. Yang • J. Yang • G. Wu • Y. Feng • Q. Lv • S. Lin • J. Hu (✉)
College of Animal Science and Veterinary Medicine, Shenyang Agricultural University, Shenyang 110866, P.R. China
e-mail: hujianmin59@163.com

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_21,

the present study demonstrated that taurine could inhibit the injury induced by ISO by increasing myocardial negative inotropic effect and antioxidant ability, decreasing the hypertrophic response to isoproterenol and protecting the integrity of myocardial ultrastructure, decreasing myocardial leak of CK-MB.

Abbreviations

ISO	Isoprenaline
CK-MB	Creatine kinase heart isoenzyme
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
MDA	Malondialdehyde
SOD	Superoxide dismutase
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
T-AOC	Total antioxidation capacity

21.1 Introduction

Taurine(2-aminoethanessulphonic acid), a sulfur-containing amino acid, is the most abundant free amino acid in the human body and accounts for more than 50% of the total amino acid pool in the mammalian heart (Lombardini 1996). Although it can be synthesized endogenously, the major source of taurine is from the diet. Earlier studies demonstrated that nutritional depletion of taurine leads to the development of a dilated cardiomyopathy in cat and fox (Pion et al. 1987; Moise et al. 1991). Accumulating clinical and experimental studies has shown that taurine deficiency is associated with structural and functional disorders in heart (Yamori et al. 2001; Shiny et al. 2005; Ito et al. 2008); exogenous administration of taurine possesses a preventive and therapeutic effect on a variety of cardiovascular diseases (Shiny et al. 2005; Allard et al. 2006; YJ et al. 2008; Li et al. 2009; Sahin et al. 2011).

Cardiac hypertrophy is an adaptive response of the heart muscle to a variety of extrinsic and intrinsic stimuli; it represents a typical feature of various cardiomyopathies including ischemic heart disease and hypertension. Cardiac hypertrophy is initially compensatory for an increased workload; however, prolongation of this process eventually leads to heart failure (Lorell and Carabello 2000; Tardiff 2006; Hill and Olson 2008). It has been reported that taurine reduced angiotensin II (Ang II)-induced cardiac hypertrophy by inhibiting the activation of protein kinase C and mitogen-associated protein kinase (Azuma et al. 2000). Li et al. (2009) have shown taurine prevents cardiomyocyte apoptosis by inhibiting NADPH oxidase-mediated calpain activation in norepinephrine (NE)-induced cardiomyocytes (Li et al. 2009). Although studies in animal and humans suggest that taurine plays an important

protective roles in heart under various pathophysiological conditions (Allard et al. 2006; YJ et al. 2008), the mechanism remains to be unclear.

In the present study, the preventive effects of taurine on heart coefficient; ultrastructure of cardiac muscle; the levels of CK-MB, cAMP, and cGMP; and antioxidant ability in cardiac hypertrophy rats induced by isoproterenol were investigated.

21.2 Methods

21.2.1 Experimental Animals and Treatments

Male Wistar rats (weighted 250 g ± 30 g) were obtained from the animal center of development zone in Changchun, China (Certificate No 2003-0004). They were maintained in controlled light (14 h light, 10 h dark) and temperature (22 ± 2°C) and were given free access to rat chow diet and tap water. After acclimatizing for 3 days, 45 rats with normal electrocardiogram were randomly divided into five groups, nine in each group. In control group, rats were administered with physiological saline by subcutaneous (s.c.) injections. Isoprenaline (ISO) at a dosage of 10 mg (dissolved in physiological saline)/kg d was s.c. administered to rats of the model group for 7 days. The other groups were the taurine and ISO co-treated; rats were administered with 100 mg/kg d (preventive group I, PI), 200 mg/kg d (preventive group II, PII), and 300 mg/kg d (preventive group III, PIII) taurine, respectively, by intraperitoneal (i.p.) injection 30 min before injection of ISO. The experiment was lasted for 7 days.

21.2.2 Chemicals

Taurine and isoprenaline were purchased from Sigma Chemical Company (St. Louis, MO, USA). Reagent kits of total protein (TP), reduced glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GSH-Px), total antioxidation capacity (T-AOC), a creatine kinase MB (CK-MB) Elisa (ADL, USA), cyclic adenosine monophosphate (cAMP) Elisa (ADL, USA), and cyclic guanosine monophosphate (cGMP) Elisa (ADL, USA) were purchased from Nanjing Jiancheng Bioengineering Institute (CHINA).

21.2.3 Heart Coefficient

On the 8th day, rats were sacrificed after being fasted overnight; the hearts were quickly excised; atrium cordis and connective tissue were removed, rinsed with

ice-cold physiological saline, and weighted; then the heart coefficient (the ratio of heart weight to body weight) was calculated.

21.2.4 Sample Collection and Biochemical Analysis

Blood samples were collected from jugular vein; sera were separated from the blood immediately and frozen at −80°C until assayed. Serum CK-MB level was measured with a commercial kit according to the manufacture's recommendations. The optical density at 450 vnm was recorded by using enzyme-labelled meter (Thermo, USA). Serum antioxidant enzymes (GSH, SOD, GSH-Px, and T-AOC) activities and MDA level were determined by colorimetry using kits on spectrophotometry according to the procedure provided, respectively.

After weighing, the ventricular myocardium was separated from the heart, frozen in liquid nitrogen, and stored at −80°C for further biochemical analysis. Frozen myocardial tissue samples were homogenated in cold physiological saline; myocardial antioxidant enzymes (GSH, SOD, GSH-Px, and T-AOC) activities and MDA level were determined by colorimetry using kits on spectrophotometry. Myocardial cGMP and cAMP levels were measured by ELISA technique based on the introduction of reagent kits. Myocardial content of TP was also determined by colorimetry using Coomassie brilliant blue as the visualization reagent.

21.2.5 Transmission Electron Microscope Analysis

Hearts were excised rapidly; the apex of the heart was quickly cut into $1mm^3$ or 1 cubic millimeter pieces on a dry ice-cooled sample plate, then transferred to vials containing 2% glutaraldehyde, and stored at 4°C for further ultrastructure analysis. Seven days later, the tissue samples were postosmicated with 2% osmium tetroxide solution and dehydrated in ethanol series and embedded in epoxy resin. Sections were viewed by transmission electron microscope, JEM-1200EX (JEOL, Japan), at a magnification of ×8,000–15,000.

21.2.6 Statistical Analysis

Data were presented as the mean ± SEM and significant differences were determined by Duncan's multiple range test using SPSS 16.0 statistical analysis software. *P* value less than 0.05 was considered significant.

21.3 Results

During the experimental period, the overall mortality was two rats in ISO group; no death occurred in the other group's rats.

21.3.1 Taurine Decreases the Heart Coefficient and Serum CK-MB Level in Cardiac Hypertrophy Rats

The effects of taurine on heart coefficient and serum CK-MB level were presented in Fig. 21.1. The heart coefficient was significantly increased when treated with isoproterenol compared with control group ($p<0.01$) while three concentrations of taurine obviously prevented the isoproterenol-induced increase in the heart coefficient ($p<0.05$, $p<0.05$, $p<0.01$), but not in a dose-dependent manner. The level of serum CK-MB was increased significantly by isoproterenol, 200 mg/kg taurine decreased significantly the serum CK-MB compared with model group ($p<0.05$), but 100 mg/kg and 300 mg/kg taurine had no significant effect.

21.3.2 Taurine Decreases Myocardial cAMP and cGMP Levels and Increases the Ratio of Myocardial cGMP/cAMP in Cardiac Hypertrophy Rats

The effects of taurine on myocardial cAMP and cGMP levels and cGMP/cAMP ratio were presented in Fig. 21.2. The levels of cAMP and cGMP in myocardium were increased extremely by isoproterenol compared with control group ($p<0.01$); 100 mg/kg and 200 mg/kg taurine significantly decrease the levels of cAMP and cGMP compared with model group ($p<0.01$, $p<0.01$). However 300 mg/kg taurine could significantly decrease the level of cAMP ($p<0.01$) but has no effects on the level of cGMP compared with model group. After analyzing the data of cGMP/cAMP ratio, we can see that isoproterenol caused the ratio of cGMP/cAMP to decline significantly compared to control rats ($p<0.05$), while all the three concentrations of taurine obviously prevented the isoproterenol-induced diminution in the ratio of cGMP/cAMP in myocardium ($p<0.01$, $p<0.01$, $p<0.01$), but there was not dose-dependent manner.

21.3.3 Taurine Increases Myocardial Antioxidant Ability in Cardiac Hypertrophy Rats

As shown in Fig. 21.3, the level of MDA was significantly increased by isoproterenol administration ($p<0.05$). In model group the levels of SOD and GSH were

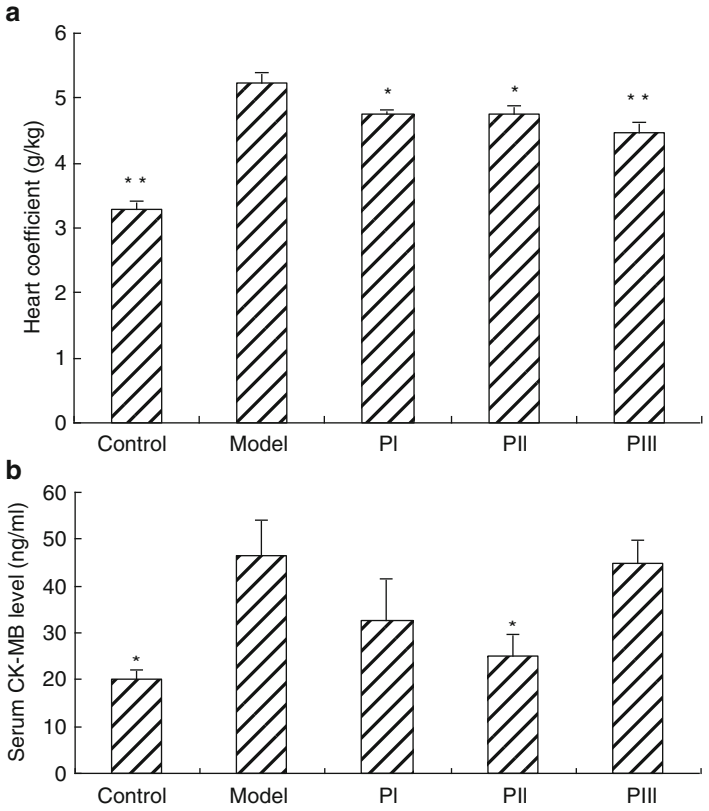

Fig. 21.1 Effects of taurine on heart coefficient, serum CK-MB level. Results are presented as mean ± SEM ($n = 9$ in heart coefficient; $n = 5$ in CK-MB). *: significantly different from the model group ($p < 0.05$), **: significantly different from the model group ($p < 0.01$)

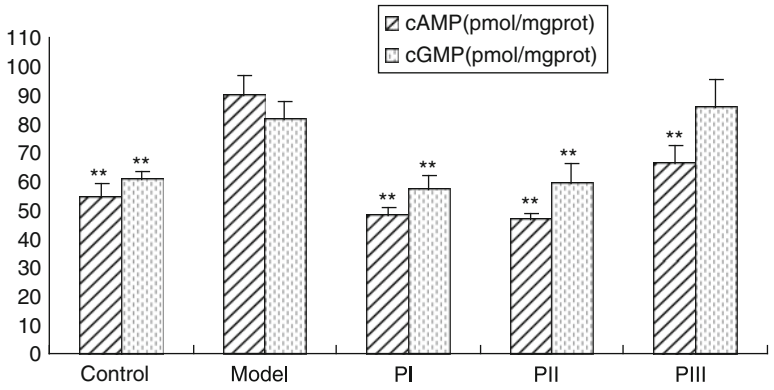

Fig. 21.2 Effects of taurine on myocardial cGMP and cAMP levels and myocardial cGMP/cAMP. Results are presented as mean ± SEM ($n = 5$ in cAMP, cGMP, and cGMP/cAMP). *: significantly different from the model group ($p < 0.05$), **: significantly different from the model group ($p < 0.01$)

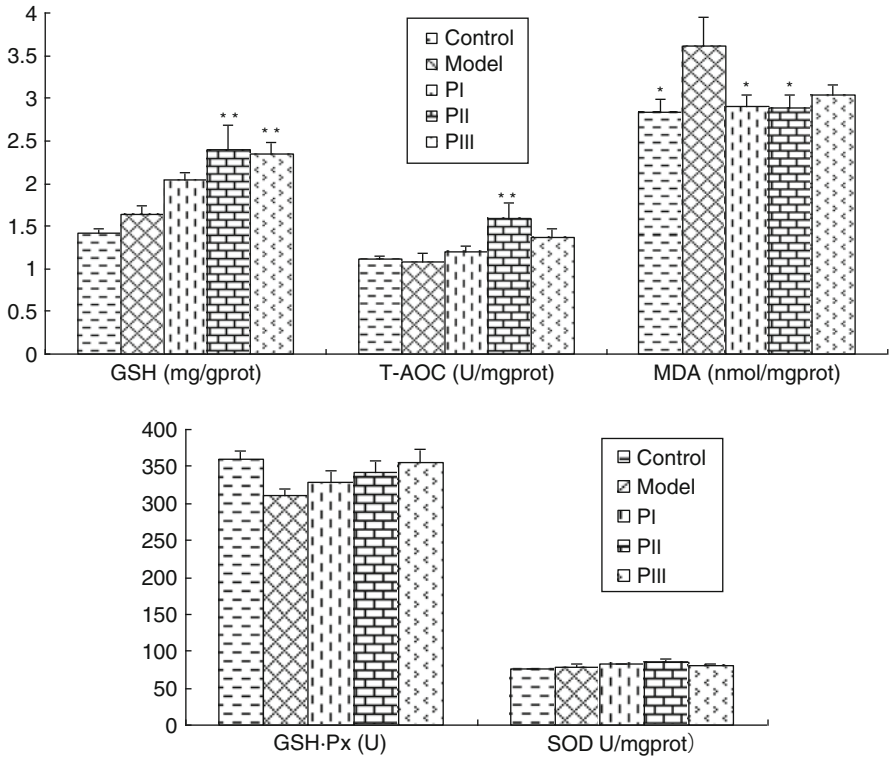

Fig. 21.3 Effects of taurine on myocardium levels of GSH and MDA and activities of GSH.Px, SOD, and T-AOC in rats. Results are presented as mean ± SE ($n = 5$). *: significantly different from the model group ($p < 0.05$), **: significantly different from the model group ($p < 0.01$)

increased by 3.68% and 15.78%, respectively; the levels of GSH-Px and T-AOC were decreased, but showed no significant changes compared with control group. Whereas taurine administration could significantly decrease the level of MDA and obviously increase the levels of GSH and T-AOC, but had no effects on the levels of SOD and GSH-Px compared with the model group.

21.3.4 Taurine Obviously Increases Serum Antioxidant Ability in Cardiac Hypertrophy Rats

As shown in Fig. 21.4, the levels of SOD, GSH-Px, and T-AOC were significantly reduced by isoproterenol administration ($p < 0.01$, $p < 0.05$, $p < 0.01$), but the levels of GSH and MDA showed no significant changes compared with control group. However, administration of taurine could significantly increase the levels of SOD, GSH-Px, GSH, and T-AOC and significantly decrease the level of MDA compared with the model group.

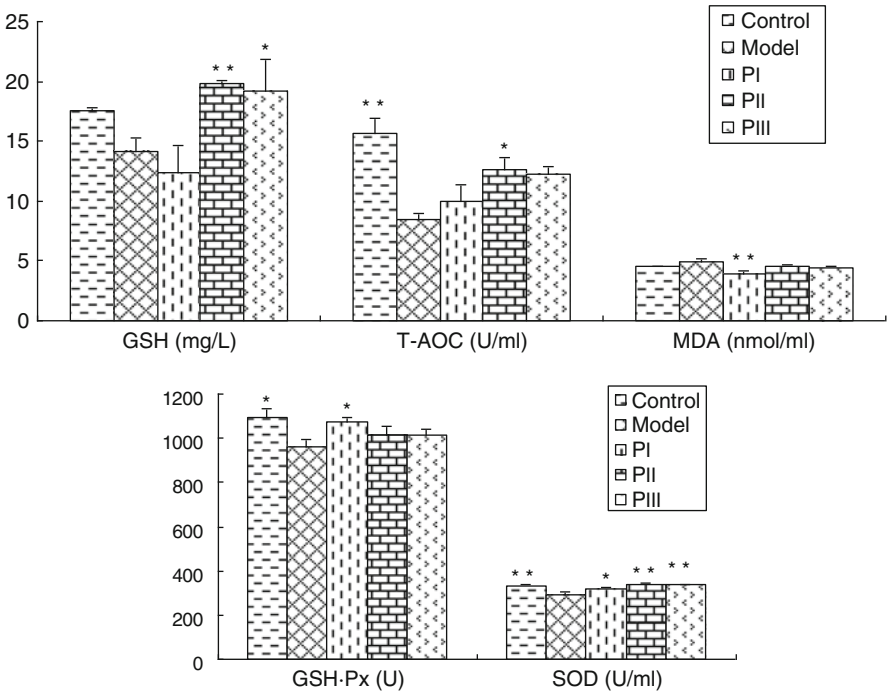

Fig. 21.4 Effects of taurine on serum levels of GSH and MDA and activities of GSH.Px, SOD, and T-AOC in rats. Results are presented as mean ± SE ($n = 5$). *: significantly different from the model group ($p < 0.05$), **: significantly different from the model group ($p < 0.01$)

21.3.5 Taurine Reverses the Ultrastructural Injury of Cardiomyocytes Caused by ISO

The ultrastructure of cardiomyocytes from the left ventricular at the level of the apex image analysis is shown in Fig. 21.5. In control group, myocardial tissue showed a typical structure and slightly contracted myofibrils (Fig. 21.5 a, b). All myofibrils and mitochondria were well arranged. The sarcolemma was attached to the underlying myofibrils at each Z-line. The size of mitochondria was equal and the cristae in mitochondria were tightly packed. The electron microscopy revealed the ultrastructure changes that occurred in model group (Fig. 21.5 c, d): myofibrils were disorderly with obvious fragmentation and dissolution, Z-line was curved, and mitochondria were severely swollen with some cristae vanishing and disruption of mitochondrial membranes. Sarcolemma was ruptured accompanied by an edema of sarcoplasm. The mitochondria were markedly aggregated near the nucleus. The cardiomyocytes in group PI demonstrated less injury (Fig. 21.5e, f). Compared with model group, there was no evidence of myofibrils disruption and interstitial edema, and mitochondria were slightly swollen with some cristae vanishing. The cardiomyocytes in group PII (Fig. 21.5g, h) were similar to those

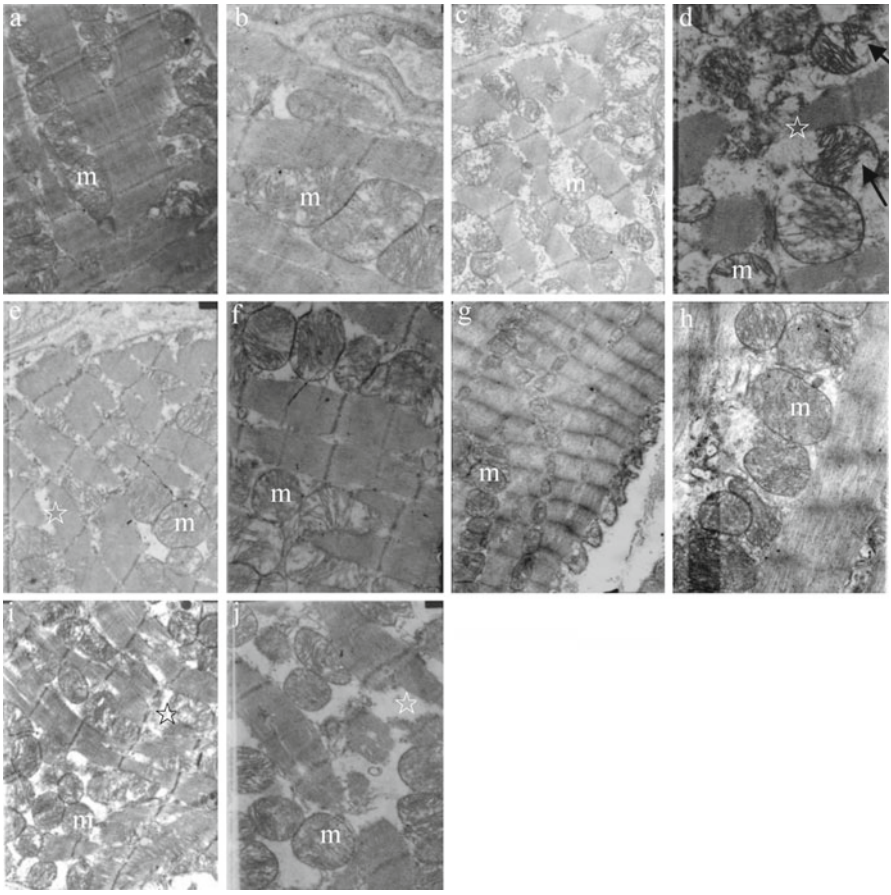

Fig. 21.5 The ultrastructural changes of cardiomyocytes from the left ventricular at the level of the near apex. (**a**, **b**) Group C showed fine dispersed nuclear chromatin, slightly contracted myofibrils, well-arranged Z disk, mitochondria with tightly packed cristae, and an intact sarcolemma. (**c**, **d**) Model group showed disarrayed myofibrils with obvious fragmentation and dissolution; curved Z-line; swollen mitochondria with obvious amorphous matrix densities, some cristae vanishing, and disruption of mitochondrial membranes; ruptured sarcolemma; and edema of sarcoplasm. (**e**, **f**) Group PI showed a lesser extent injury than the model group. (**g**, **h**) Group PII showed results similar to those described in control group. (**i**, **j**) Group P III showed the structure of mitochondria was preserved by taurine administration compared with model group. Magnification (as indicated): (**a**, **c**, **e**, **g**, **i**) ×8,000; (**b**, **d**, **f**, **h**, **j**) ×15,000. Scale bars = 1.25 μm (×8,000) and 0.67 μm (×15,000). *M* mitochondrion, *arrows* mitochondria breakdown, *asterisk* myofilament breakdown

described in control group. The cardiomyocytes in group PIII (Fig. 21.5i, j) also showed obvious edema of sarcoplasm, disarrayed mitochondria that are slightly swollen, and myofibrils fragmentation and dissolution compared with control group. However the structure of mitochondria was preserved by taurine compared with model group. The results indicated 200 mg/kg taurine had better protective effect.

21.4 Discussion

In recent years, various functions have been attributed to taurine including osmoregulation, antioxidation, and modulation of both calcium fluxes and protein phosphorylation. Taurine supplementation of the diet has been used in many clinical studies to treat various pathological conditions. The results indicated that heart coefficient, serum CK-MB, and myocardial cGMP and cAMP levels significantly increased while the myocardial cGMP/cAMP ratio was significantly decreased in model group by ISO-mediated myocardial cell hypertrophy, membrane damage, and phosphorylation. The creatine kinase system plays an important role in myocardial energy metabolism by maintaining ADP levels high at the mitochondria and low at sites of ATP utilization (Wallimann et al. 1992). Increase in the activity of CK-MB in serum is a marker of myocardial cell injury (Adams et al. 1993). It is well recognized that, in the mammalian heart, adrenergic stimulation results in increases in the rates of force development and relaxation (Grazyna et al. 1999). Most effects of β-adrenergic stimulation are mediated by cAMP-dependent mechanisms. Increased cytosolic cAMP activates cAMP-dependent protein kinases, which induce the phosphorylation of membrane and contractile proteins. cAMP has a positive inotropic effect through an increase in L-type calcium current (Balligand 1999). Therefore cAMP-dependent control of cardiac relaxation function is probably compromised in the hypertrophied heart. In non-stimulated hearts, cGMP has been suggested to augment contractile function at low concentrations, likely via cross talk with cAMP-dependent signaling, inhibiting phosphodiesterase-3(PDE-3) and degradation of cAMP (Vila-Petroff et al. 1999). At higher concentrations, cGMP has a negative inotropic effect by antagonizing cAMP via protein kinase G (in mammals) or PDE-2 stimulation (in amphibians) (Vila-Petroff et al. 1999). With β-adrenergic activation, both cAMP and cGMP synthesis increase, with the net effect of cGMP being negative on the inotropic response (a brake) (Balligand 1999). Our results are in-line with the earlier study. It has been reported that reducing cGMP level by NOS inhibition enhances β-adrenergic responsiveness (Hare et al. 1998). In addition to the negative inotropic action of cGMP, it reduces oxygen consumption and offsets the development of cardiac hypertrophy (Calderone et al. 1998; Straznicka et al. 1999). However, taurine administration would appear to attenuate the increase of heart coefficient, reduce CK-MB leaked into the blood, inhibit the synthesis of cGMP and cAMP, and significantly increase the cGMP/cAMP ratio in hypertrophied ventricular tissue. These indicated that taurine could prevent cardiac hypertrophy by blocking the β-adrenergic stimulation mediated by cAMP-/cGMP-dependent mechanisms and enhancing the negative inotropic effect of cGMP against cardiac hypertrophy in vivo.

Reactive oxygen species (ROS) have emerged as key mediators of hypertrophic remodeling and cardiac dysfunction. Recent years have witnessed accumulating evidences for the involvement of oxidative stress in cardiac diseases including cardiac hypertrophy (Sawyer et al. 2002; Itoh et al. 2008). Isoproterenol leads to oxidative stress which contributes significantly to the pathophysiology of the myocardial disease (Chattopadhyay et al. 2003). In the present study, the serum levels of SOD, GSH-Px,

GSH, and T-AOC were significantly decreased by isoproterenol administration, while myocardial MDA was significantly increased. Besides results presented in Fig. 21.3 also denote that isoproterenol administration increased slightly activities of antioxidant enzymes in myocardium such as SOD and GSH, which indicate that oxidative stress caused by isoproterenol may upregulate the activity of antioxidant enzymes in myocardium to facilitate rapid removal of the accumulated reactive oxygen species. GSH is one of the most important compounds, which helps in the detoxification and excretion of oxygen radicals. SOD is the first line of defense against ROS; as a metalloprotein, its antioxidant functions were exhibited by enzymatically detoxifying the peroxides and superoxide anion. MDA as a breakdown product from oxidation reactions of polyunsaturated fatty acids, the level of MDA can be taken as an indicator for the state of lipid peroxidation. Gan et al. (2006) have suggested that MDA can be as the one of the major indicators of free radical-mediated tissue injury. T-AOC which is an indicator of body antioxidant ability could indicate the compensatory ability of antioxidase system and nonenzymatic system to stimuli.

The present results showed the levels of GSH and T-AOC in myocardium, and the levels of serum SOD, GSH-Px, GSH, and T-AOC were significantly increased, but the MDA in myocardium and serum was significantly decreased by administration of taurine compared with model group. Taurine is an important endogenous antioxidant; many studies have demonstrated that taurine exhibits a protective action under oxidative stress condition (Oudit et al. 2004; Chang et al. 2004; Shiny et al. 2005; Schaffer et al. 2009; Yang et al. 2010). The possible mechanisms for taurine protective actions include a direct effect as a free radical scavenger, or indirectly, preventing generation of reactive oxygen species and/or protecting cell membranes from ROS damage (Schaffer et al. 2009). In the study, we also found that taurine was apparently beneficial for the ISO-induced myocardial injury in rats, decreasing MDA formation and augmenting the antioxidative enzymes activities. This was consistent with Shi et al. (2002).

The results indicated that taurine can not only improve the myocardium oxidative stability but also obviously improve serum oxidative stability, decrease lipid peroxidation, and strengthen the myocardial membrane. Previous research has suggested that except fox and cat, the size of the intracellular taurine pool of most animal species remains fairly constant even with significant reductions in dietary taurine content, which was caused by the decline in plasma taurine levels accompanied by enhanced cellular retention of taurine (Hamaguchi et al. 1991). On the other hand, research has suggested taurine levels were increased under some conditions, such as failing and hypertrophic heart (Huxtable and Bressler 1974). This is the reason why the taurine increases the serum oxidative stability more obviously.

Myocardial ultrastructure observations showed that taurine is capable of not only obviously inhibiting myofibrillar disruption but also enhancing the integrity of mitochondrial membranes and cristae; the results indicated administration of 200 mg/kg taurine has better protective effect. It had been reported that downregulation of the taurine gene leads to lost cell volume, develops mitochondrial defects, and undergoes myofibrillar disruption (Ito et al. 2008). The results are consistent with the action of taurine as an osmoregulator, mitochondrial modulator, and a cytoprotective agent.

21.5 Conclusion

In conclusion, the present study demonstrated that taurine could inhibit the injury induced by ISO by increasing myocardial negative inotropic effect and antioxidant ability, decreasing the hypertrophic response to isoproterenol and protecting the integrity of myocardial ultrastructure, decreasing myocardial leak of CK-MB.

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Chapter 22
Protective Effect of Taurine on Tri*ortho*cresyl Phosphate (TOCP)-Induced Cytotoxicity in C6 Glioma Cells

Yachen Li, Fengyuan Piao, and Xiaohui Liu

Abstract Tri*ortho*cresyl phosphate (TOCP) an organophosphorus ester can cause neurotoxicity via oxidative stress pathway. Taurine is an antioxidant. The objective of this study was to investigate the protective effect of taurine on TOCP-induced cytotoxicity in C6 glioma cell. The C6 glioma cells were pretreated with 0, 1, 3, and 9 mM of taurine for 30 min prior to 1 mM TOCP treatment. After 48 h, cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) release. The content of glutathione (GSH) and the activity of glutathione peroxidase (GPx) were also analyzed by kits. Our results showed that survival of the glioma cells decreased in the group treated with TOCP alone and increased significantly in the groups pretreated with taurine in a concentration-dependent manner. TOCP induced decrease in the activity of GPx and the content of GSH. However, taurine prevented these decreases. Our results suggested that taurine has protective effect on TOCP-induced toxicity to glioma cells via elevating antioxidant capacity.

Abbreviations

TOCP	Tri*ortho*cresyl phosphate
LDH	Lactate dehydrogenase
GPx	Glutathione peroxidase
GSH	Glutathione

Y. Li • F. Piao (✉) • X. Liu
Department of Occupational and Environmental Health,
Dalian Medical University,
Dalian, Liaoning 116044, China
e-mail: piaofy_dy@yahoo.com

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22.1 Introduction

TOCP, one of organophosphorus compounds (OPs), has been widely used as a plasticizer, a flame-retardant, and a gasoline additive in industry (Craig and Barth 1999; Winder and Balouet 2002). It has been known that TOCP induced neurotoxicity in humans and animal species (Barrett and Oehme 1994; Zhang et al. 2007). Studies on TOCP neurotoxicity also focused mainly on neurons, which are considered to be the primary target for neurotoxicity. Recent studies show that astrocytes are also an important target for many OPs neurotoxicity. However, there have no reports of TOCP induced astrocyte toxicity. Thus, one major question concerning the neurotoxicity of TOCP is whether TOCP exerts astrocyte toxicity.

As the most abundant glial cell types in the brain, astrocytes have a number of roles in modulation of synaptic transmission and plasticity, secretion of growth factors, uptake of neurotransmitters, and regulation of extracellular ion concentrations and metabolic support of neurons (Hertz and Zielke 2004; Nedergaard et al. 2003; Newman 2003). In addition, they have high concentrations of antioxidants and antioxidant enzymes, for example, GSH and GPx, and play prominent role in the brain's antioxidant defense (Desagher et al. 1996; Dringen et al. 2000). Garcia et al. (2001) reported that chlorpyrifos enhanced reactive oxygen species (ROS) formation in C6 glioma cells. Qiao et al. (2005) also reported that oxidative mechanisms contribute to the developmental neurotoxicity of chlorpyrifos. Thus, does the neurotoxicity of TOCP involve in disruption the antioxidant defense of astrocyte?

Taurine, 2-aminoethanesulfonic acid is a simple aminosulfonic acid which presents in large amounts in most cell types and tissues as a free amino acid. Taurine has a function as neuromodulator or neurotransmitter in CNS which could maintain the structural integrity of the membrane, regulate calcium binding and transport (Wu et al. 2005; Wu and Prentice 2010), and exhibit a protective effect in a variety of detrimental situations, often acting as antioxidant (Schaffer et al. 2009). However, there are no reports about the protective effects of taurine on TOCP-induced neurotoxicity.

In the present study, we investigated the effects of TOCP exposure and taurine pretreatment on cell viability, LDH leakage, morphological changes, and the activity of GPx and the content of GSH in C6 glioma cells, in order to evaluate the potential role of taurine on TOCP-induced cytotoxicity.

22.2 Methods

22.2.1 Cell Culture

Rat C6 glioma cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, in an incubator aerated with 5% CO_2 at 37°C.

22.2.2 MTT Assay

C6 glioma cells viability was determined by assaying the degree of MTT reduction. 1×10^4 cells/well was seeded in 96-well plate. Twenty-four hours later, cells were treated with various concentrations of taurine (0, 1, 3, 9 mM) for 30 min prior to treatment with 1 mM TOCP (dissolved in DMSO, the final concentration of DMSO in the culture medium was 0.1% (v/v)). After exposure to TOCP for 48 h, 10 μl MTT solution (5 mg/ml in phosphate buffered saline, PBS) was added into each well, and the cells were further incubated for 3 h at 37°C. Afterwards, the media were aspirated and 100 μl of dimethyl sulfoxide (DMSO) was added for the dissolution of formazan crystals. The absorbance of each well was read at 570 nm using an ELISA plate reader. Cell viability was expressed as a percentage of the control culture.

22.2.3 LDH Release Assay

Cells were treated as described above. After various treatments, the amount of LDH released into the medium was determined using a diagnostic kit (Nanjing Jiancheng Bioengineering Institute, China). In brief, NADH and pyruvate (0.1%, w/v) were added and the samples were incubated at 37°C for 15 min. Next, the samples were incubated with the coloring reagent for 15 min. The reaction was stopped by adding 0.4 mol/l NaOH, and the activity (U/ml) of LDH in each sample were calculated from formula. The LDH release was expressed as a percentage of the control culture.

22.2.4 Morphological Observation

To examine the cellular morphology of C6 glioma cells, cells were seeded into 24-well plates. After various treatments as described above, cells were observed by phase-contrast microscope.

22.2.5 Measurement of GSH Content and GPx Activities

The content of GSH and the activities of GPx were all determined by using assay kits (Nanjing Jiancheng Bioengineering Institute, China). GSH was measured based on that 5,50-dithiobis (2-nitrobenzoic acid) (DTNB) reacts with GSH to generate 2-nitro-5-thiobenzoic acid and GSSG. The concentration of GSH was calculated from formula and expressed as nmol per milligram protein. The assay for GPx activity was assayed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H_2O_2 catalyzed by GPx. One unit of GPx was defined

as the amount that reduced the level of GSH by 1 mmol in 1 min per milligram protein. Cells were plated into 6-well plates. After various treatments, cultures were washed twice in ice-cold PBS (pH 7.4) and homogenized. The homogenate was centrifuged for 10 min at 10,000 rpm at 4°C and supernatant was used for GSH assays and GPx activities according to the manufacturer's instructions. Protein content was measured by Coomassie blue protein-binding method using bovine serum albumin as standard.

22.2.6 Statistical Analysis

All the experiments were performed in triplicate and data are shown as mean ± S.D. based on three separate experiments. Statistical analysis was performed according to the Student's *t*-test and one-way ANOVA analysis. The probability values of $P < 0.05$ were considered as significant.

22.3 Results

22.3.1 Effects of Taurine Pretreatment on Cell Viability

Exposure to 1 mM TOCP for 48 h, the cell viability decreased to 50.7 ± 4.9% comparing with the control group. When compared to 1 mM TOCP group, pretreatment with taurine significantly increased cell viability to 61.4 ± 4.0%, 64.8 ± 3.9%, and 79.5 ± 3.2% at 1 mM, 3 mM, and 9 mM, respectively (Fig. 22.1).

22.3.2 Effects of Taurine Pretreatment on LDH Release

C6 glioma cells exposed to 1 mM TOCP alone, LDH release was 183.9 ± 4.7% of control. However, treated with taurine, LDH release decreased to 160.9 ± 6.7% at 1 mM, 152.1 ± 3.2% at 3 mM, and 124.2 ± 5.8% at 9 mM (Fig. 22.2). The results similar to MTT assay indicate that adding taurine prior to TOCP exposure could reduce cell death and elevate cell viability.

22.3.3 Effects of Taurine Pretreatment on Morphological Changes

The protective effects of taurine were also shown through morphological observation. Visual inspection by phase-contrast microscopy demonstrated that those

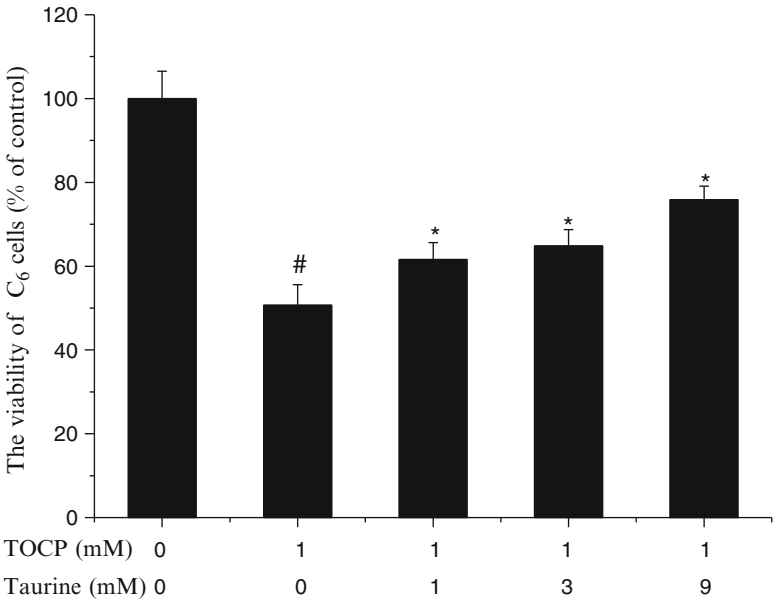

Fig. 22.1 Effects of taurine on the viability of C6 glioma cells exposed to TOCP. C6 glioma cells were treated with 0, 1, 3, and 9 mM of taurine for 30 min followed by exposure to 1 mM TOCP for 48 h. Cell viability was measured by MTT assay. Data are shown as mean ± S.D. from three separate experiments; the symbol (#), $P<0.05$ as compared with control; the symbol (*) $P<0.05$ as compared with TOCP exposure alone

normal C6 glioma cells had clear edges and nucleus. Exposed to TOCP, C6 glioma cells displayed obvious cell body shrinkage, nuclear condensation, and loss in cell membrane integrity (Fig. 22.3). The addition of taurine, however, dramatically prevented cell morphological deterioration. Most of C6 glioma cells showed normal cell morphology with normal nuclear size and integrity (Fig. 22.3).

22.3.4 Effects of Taurine Pretreatment on the Content of GSH and the Activity of GPx

The average GSH content was 8.6 ± 1.0 mg/g protein, and the activity of GPx was 78.6 ± 4.9 U/mg protein in C6 glioma cells, respectively. After exposure to 1 mM TOCP, C6 glioma cells antioxidant system was disturbed, namely, the content of GSH and the activity GPx decreased (Fig. 22.4). Pretreatment with taurine significantly prevented the depletion of GSH (Fig. 22.4a) and preserved the activity of GPx (Fig. 22.4b).

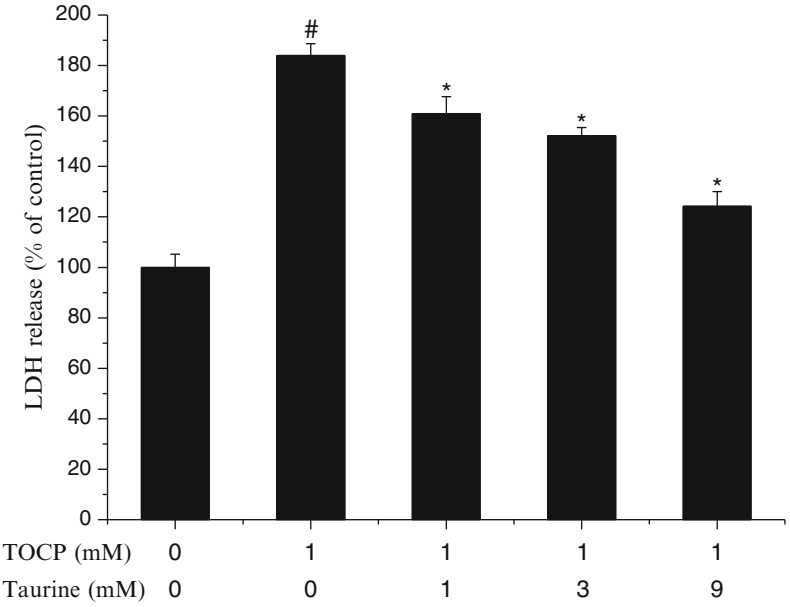

Fig. 22.2 Effects of taurine on LDH release of C6 glioma cells exposed to TOCP. C6 glioma cells were treated with 0, 1, 3, and 9 mM of taurine for 30 min followed by exposure to 1 mM TOCP for 48 h. LDH release was measured. Data are shown as mean ± S.D. from three separate experiments; the symbol (#), $P<0.05$ as compared with control; the symbol (*) $P<0.05$ as compared with TOCP exposure alone

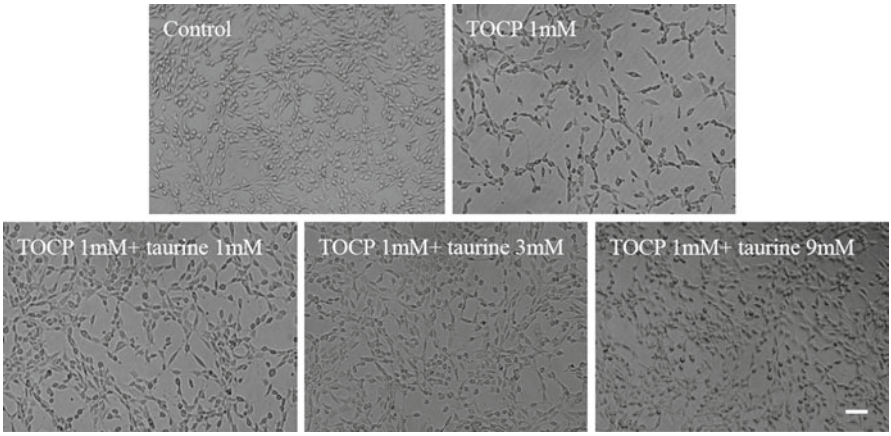

Fig. 22.3 Effects of taurine on morphological changes of C6 glioma cells exposed to TOCP. C6 glioma cells were treated with 0, 1, 3, and 9 mM of taurine for 30 min followed by exposure to 1 mM TOCP for 48 h. Cellular morphology was observed with light microscope. Scale bars, 20 μm

22.4 Discussion

It has been known that TOCP exert neurotoxicity in humans and sensitive animal species (Craig and Barth 1999; Zhang et al. 2007).Studies on the mechanism and protection of TOCP neurotoxicity mainly focused on neurons. However, recent research indicates that astrocytes are affected in, and contribute to, the neurotoxicity of OPs. Zurich et al. (2004) reported that the neurotoxicity of two OPs, chlorpyrifos and parathion, are involved in astrocytes. Guizzetti et al. (2005) also reported an inhibitory effect on astrocyte proliferation using chlorpyrifos in vitro. In the present study, we used C6 glioma cells pretreatment with taurine and then exposure to TOCP to assess the protective effects of taurine on C6 glioma cells against TOCP-induced injury. The experimental results demonstrated that when C6 glioma cells are exposed to TOCP, there is a significant decrease in cell viability by MTT assay and increase in LDH release. Pretreatment with taurine significantly increases cell viability and decreases LDH release in a concentration-dependent manner. Our results are consistent with the above studies also suggest that TOCP exerts toxic effects on astrocyte and taurine has protective role against TOCP-induced injury.

To support the conclusion of TOCP cytotoxicity on the cultured astrocytes, the morphology changes were observed by the inverted phase-contrast microscope. When compared with the untreated astrocytes, the density of normal cells was consciously decreased. C6 glioma cells displayed obvious cell body shrinkage, nuclear condensation, and loss in cell membrane integrity. Taurine dramatically prevented cell morphological deterioration in a concentration-dependent manner.

Oxidative stress is one of the major mechanisms of cell death in a variety of disease and injury (Floyd 1999; Love 1999; Qiao et al. 2005). It is characterized by an imbalance between the intracellular productions of reactive oxygen species (ROS) and the cellular defense mechanisms, which is an excess of ROS accompanied by a reduced capability of the natural antioxidant systems. Astrocyte is prominent antioxidant defense system in the brain and they have high concentrations of GSH and GPx (Desagher et al. 1996; Dringen et al. 2000). It was reported that astrocyte GSH system responds to many toxic substances in time- and concentration-dependent manners. Zhang et al. (2007) reported that TOCP caused changes of lipid peroxidation and antioxidative status in nerve tissues of hens. Garcia et al. (2001) reported that chlorpyrifos enhanced reactive oxygen species (ROS) formation in C6 glioma cells. In this study, we found that TOCP decreased the content of GSH and the activity GPx in C6 glioma cells (Fig. 22.4). This finding suggests that TOCP may disturb astrocyte antioxidant system which leads to cell injury.

Taurine is an important molecule able to modulate glutamatergic signaling, preventing excitotoxicity and oxidative stress (Junyent et al. 2011; Saransaari and Oja 2010). In particular, taurine exerts cytoprotective effects against various types of brain injury (Gao et al. 2011; Ricci et al. 2009; Taranukhin et al. 2012).In the current study we also found that taurine has protection against TOCP-induced injury and significantly prevented the depletion of GSH (Fig. 22.4a) and preserved the activity of GPx (Fig. 22.4b).

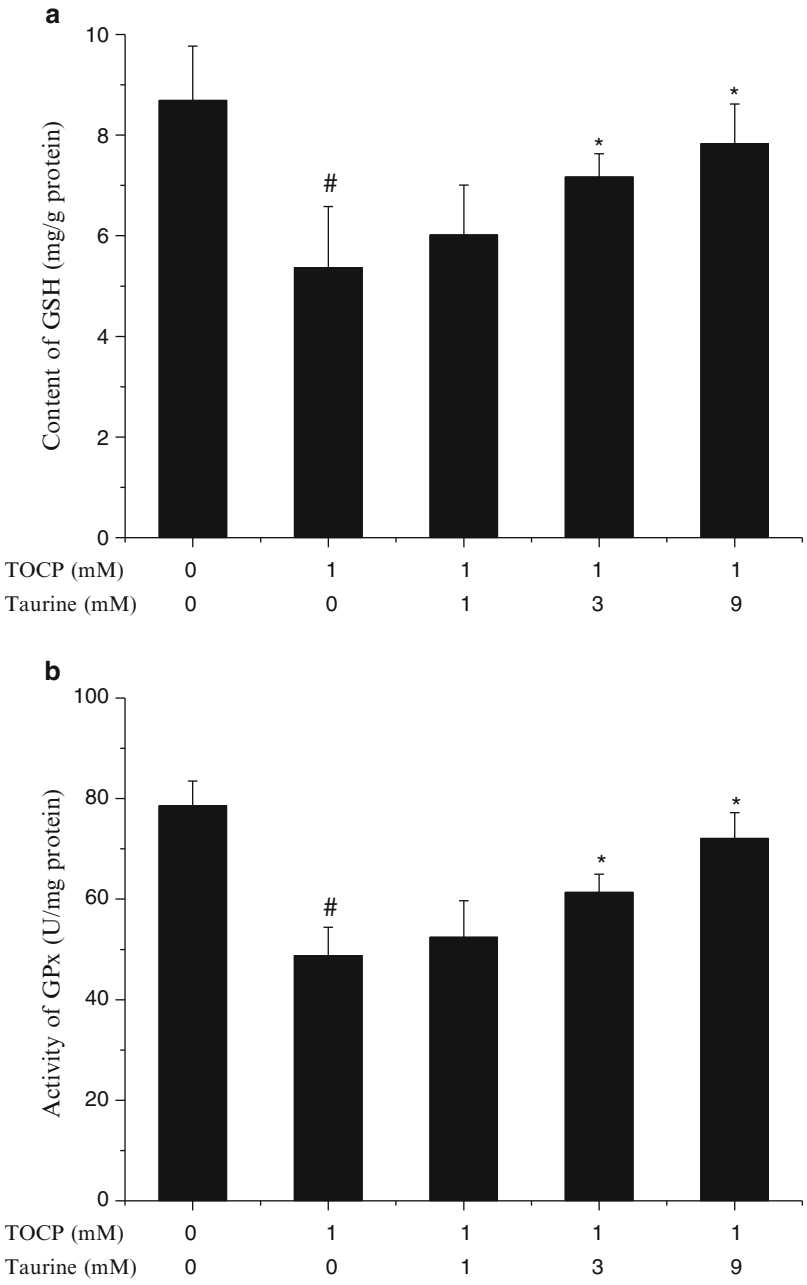

Fig. 22.4 Effects of taurine on the content of GSH and the activity of GPx in C6 cells exposed to TOCP. C6 glioma cells were treated with 0, 1, 3, and 9 mM of taurine for 30 min followed by exposure to 1 mM TOCP for 48 h. The content of GSH (**a**) and the activities of GPx (**b**) were determined. Data are shown as mean ± S.D. from three separate experiments; the symbol (#), $P < 0.05$ as compared with control; the symbol (*) $P < 0.05$ as compared with TOCP exposure alone

22.5 Conclusion

The data generated in the present study suggest that taurine has protective effect on TOCP-induced toxicity to glioma cells via elevating antioxidant capacity.

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Chapter 23
The Mechanism of Taurine Protection Against Endoplasmic Reticulum Stress in an Animal Stroke Model of Cerebral Artery Occlusion and Stroke-Related Conditions in Primary Neuronal Cell Culture

Payam Mohammad Gharibani, Jigar Modi, Chunliu Pan, Janet Menzie, Zhiyuan Ma, Po-Chih Chen, Rui Tao, Howard Prentice, and Jang-Yen Wu

Abstract Taurine is an inhibitory neurotransmitter and is one of the most abundant amino acids present in the mammalian nervous system. Taurine has been shown to provide protection against neurological diseases, such as Huntington's disease, Alzheimer's disease, and stroke. Ischemic stroke is one of the leading causes of death and disability in the world. It is generally believed that ischemia-induced brain injury is largely due to excessive release of glutamate resulting in excitotoxicity and cell death. Despite extensive research, there are still no effective interventions for stroke. Recently, we have shown that taurine can provide effective protection against endoplasmic reticulum (ER) stress induced by excitotoxicity or oxidative stress in PC12 cell line or primary neuronal cell cultures. In this study, we employed hypoxia/reoxygenation conditions for primary cortical neuronal cell cultures as an in vitro model of stroke as well as the in vivo model of rat focal middle cerebral artery occlusion (MCAO). Our data showed that when primary neuronal cultures were first subjected

P.M. Gharibani (✉) • C. Pan • J. Menzie • Z. Ma
Department of Biomedical Sciences, Charles E. Schmidt College of Medicine, Florida Atlantic University, 777 Glades Road, Boca Raton, FL 33431, USA
e-mail: pgharibani@gmail.com; pgharibani@yahoo.com; cpan@fau.edu; jmenzi@fau.edu; zma@fau.edu

J. Modi
Department of Biomedical Sciences, Charles E. Schmidt College of Medicine, Florida Atlantic University, 777 Glades Road, Boca Raton, FL 33431, USA

Centre of Complex Systems and Brain Sciences, Florida Atlantic University, Boca Raton, FL 33431, USA
e-mail: jmodi@fau.edu

P.-C. Chen
Department of Biomedical Sciences, Charles E. Schmidt College of Medicine, Florida Atlantic University, 777 Glades Road, Boca Raton, FL 33431, USA

Department of Neurology and Sleep Center, Shung Ho Hospital, Taipei Medical University, Taipei, Taiwan
e-mail: chenpochih1979@gmail.com

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to hypoxic conditions (0.3%, 24 h) followed by reoxygenation (21%, 24–48 h), the cell viability was greatly reduced. In the animal model of stroke (MCAO), we found that 2 h ischemia followed by 4 days reperfusion resulted in an infarct of 47.42 ± 9.86% in sections 6 mm from the frontal pole. Using taurine greatly increased cell viability in primary neuronal cell culture and decreased the infarct area of sections at 6 mm to 26.76 ± 6.91% in the MCAO model. Furthermore, levels of the ER stress protein markers GRP78, caspase-12, CHOP, and p-IRE-1 which were markedly increased in both the in vitro and in vivo models significantly declined after taurine administration, suggesting that taurine may exert neuroprotection functions in both models. Moreover, taurine could downregulate the ratio of cleaved ATF6 and full-length ATF6 in both models. In the animal model of stroke, taurine induced an upregulation of the Bcl-2/Bax ratio and downregulation of caspase-3 protein activity indicating that it attenuates apoptosis in the core of the ischemic infarct. Our results show not only taurine elicits neuroprotection through the activation of the ATF6 and the IRE1 pathways, but also it can reduce apoptosis in these models.

Abbreviations

ER	Endoplasmic reticulum
MCAO	Middle cerebral artery occlusion
GRP78	Glucose-regulated protein 78

23.1 Introduction

The endoplasmic reticulum (ER) is an important subcellular organelle that is responsible for intracellular calcium homeostasis, protein secretion, and lipid biosynthesis (Ma and Hendershot 2004; Anelli and Sitia 2008). ER stress plays a

R. Tao (✉)
Department of Biomedical Sciences, Charles E. Schmidt College of Medicine, Florida Atlantic University, 777 Glades Road, Boca Raton, FL 33431, USA

Program in Integrative Biology, Florida Atlantic University, Boca Raton, FL 33431, USA
e-mail: rtao@fau.edu

H. Prentice (✉) • J.-Y. Wu (✉)
Department of Biomedical Sciences, Charles E. Schmidt College of Medicine, Florida Atlantic University, 777 Glades Road, Boca Raton, FL 33431, USA

Program in Integrative Biology, Florida Atlantic University, Boca Raton, FL 33431, USA

Centre of Complex Systems and Brain Sciences, Florida Atlantic University, Boca Raton, FL 33431, USA
e-mail: hprentic@fau.edu; jwu@fau.edu

crucial role in hypoxia/ischemia-induced cell dysfunction (Azfer et al. 2006; DeGracia and Montie 2004). Cerebral hypoxia or ischemia leads to a decrease of oxygen and glucose availability which in turn induces the release of glutamate at the presynaptic level. The high levels of glutamate and the subsequent excessive activation of glutamatergic postsynaptic receptors are the main cause of the death of neurons (Choi and Rothman 1990; Nicholls and Attwell 1990). Overstimulation of glutamate receptors in neuronal injury has been observed in several neurodegenerative disorders and in acute insults, and this leads to massive brain cell death related to excitatory imbalance, which occurs in stroke and epilepsy (Lipton and Paul 1994; Mattson 2003). Hypoxia triggers the accumulation of unfolded proteins in the ER, leading to the unfolded protein response (UPR) (Kaufman 1999). Pathways that are initiated in response to the UPR include activation of PKR-like endoplasmic reticulum kinase (PERK), transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1), which in turn activate distinct signaling cascades mediating the ER stress response (Wang et al. 1998; Harding et al. 2000a). In normal neuronal homeostasis, PERK, ATF6, and IRE1 activities are inhibited by binding to glucose-regulated protein 78 (GRP78), an ER chaperone. In ER dysfunction, GRP78 dissociates from PERK, ATF6, and IRE1, inducing the dimerization and phosphorylation of PERK and IRE1, and cleavage of ATF6 (P90) to ATF6 (P50). Finally these components cause more apoptosis through the action of the CHOP protein. Taurine, 2-aminoethanesulfonic acid, is a free amino acid and the most abundant amino acid present in mammalian nervous system (Wu and Prentice 2010). It has been shown that taurine can provide protection against neurological diseases, including Huntington's disease, Alzheimer's disease, and stroke (Lousada 2004; Tadros et al. 2005; Takahashi et al. 2003). It has been proposed not only that taurine can protect neurons against glutamate-induced neurotoxicity by preventing glutamate-induced membrane depolarization and calpain activation due to elevation of intracellular [Ca^{2+}] but also that it can upregulate Bcl-2 and prevent apoptosis (Wu et al. 2009). Membrane integrity, intracellular calcium homeostasis, osmoregulation, and antioxidant actions are also important functions of taurine in the brain (Balkan et al. 2002; Chen et al. 2001; Moran et al. 1987; Wade et al. 1988). It has been shown that not only does taurine have its own specific receptors on the cell membrane, but also it can elicit hyperpolarization by the inward movement of chloride through GABA and glycine receptors to reduce neuronal excitability (Hussy et al. 1997; Wang et al. 2007; Wu et al. 1992). Recently, it has been shown that taurine can reduce rat neurological deficits, brain infarct volume, and also caspase-3 activities in the ischemic penumbra 24 h after middle cerebral occlusion (MCAO) (Sun and Xu 2008). Stroke and especially the ischemic stroke is one of the leading causes of serious disability and death; there has been little progress toward the development of treatments to improve its prognosis (Weant and Baker 2012). Therefore, novel therapeutic strategies may be beneficial for improving clinical outcomes. In this study, we showed that taurine can exert a protective function against hypoxia by increasing the cell viability, decreasing infarct volume, and reducing ER stress both in vitro and in vivo.

23.2 Material and Methods

All animal procedures were carried out in accordance with the Animal Use and Care Guidelines issued by the National Institutes of Health using a protocol approved by the Florida Atlantic University, Boca Raton, Animal Use and Care Committee.

23.2.1 In Vitro Study

23.2.1.1 Primary Neuronal Cell Culture

According to the method of Hartung et al., pregnant rats were sacrificed after isoflurane exposure, and embryos at 16–18 days were removed. Brains were isolated from the fetuses and placed in Basal Media Eagle (BME) supplemented with 2 mM glutamine, 26.8 mM glucose, and 20% heat-inactivated fetal bovine serum. This medium is referred to as growth media eagle (GME). The cortices were then dissociated by passing the tissue through a 14-G cannula. Cells were centrifuged at 300 g/min for 5 min at room temperature. The resulting pellet was resuspended in GME and plated on appropriate tissue culture plates pre-coated with 5 μg/ml of poly-D-lysine. Cells were maintained for 1 h in a humidified incubator (37°C, 99% humidity, and 5% CO_2). Incubation medium was replaced with serum-free neurobasal medium supplemented glutamine, and the cells were then maintained in an incubator for 12–18 days until they were ready for handling (Hartung 1998).

23.2.1.2 Hypoxia and Reoxygenation

To generate hypoxic conditions, 14-day-cultured neurons in 6- or 96-well plates were placed in a hypoxia chamber with oxygen levels maintained at 0.3–0.4%. The level of oxygen was continuously monitored using an oxygen electrode. Primary cortical neuronal cultures in the absence or presence of taurine were subjected to 20 h of hypoxia. Reoxygenation was performed by removing cultured plates from the hypoxic chamber and transferring them into normal culture incubator remaining for another 20 h.

23.2.1.3 ATP Assay

Primary cortical neuronal cells in 96-well plates were treated with or without taurine (1, 5, and 10 mM) for 1 h, and then cells were subjected to hypoxia–reoxygenation conditions for 20 h to induce cell death. ATP solution (Promega) was added to each

well, and cells were incubated for 10 min after which the amount of ATP was quantified through a luciferase reaction. The luminescence intensity was determined using a luminometer with lysates in a standard opaque-walled multi-well plate. The ATP content was determined by running an internal standard and expressed as a percentage of untreated cells (control).

23.2.2 In Vivo Study

23.2.2.1 Transient Focal Middle Cerebral Artery Occlusion (MCAO)

Male adult Sprague–Dawley rats (weighing 260–300 g, Harlem Chicago, IL) were given access to food and water ad libitum. Before surgery, rats were fasted overnight with free access to water prior to surgery, and the following day they were weighed and anesthetized by IP injection with ketamine hydrochloride (80 mg/kg body weight IP; Putney) and xylazine hydrochloride (20 mg/kg body weight IP; Vedco) (McCollum et al. 2010). During the experiment, core temperature was maintained at 37°C by a thermostatically controlled heating pad regulated via a rectal temperature probe (CMA 450). Local cerebral blood flow (LCBF) was monitored in the cerebral cortex of left hemisphere in the supply territory of the middle cerebral artery (MCA) by laser Doppler flowmeter (LDF) (Perimed Inc., OH, USA). Transient focal cerebral ischemia of the middle cerebral artery (MCA) for 2 h was induced by the suture occlusion technique (Longa et al. 1989; Sun et al. 2011). Briefly, the left common carotid artery and the left external carotid artery were exposed through a midline neck incision. A 4-0 monofilament nylon suture coated with silicon (Doccol Co., NM, USA) was inserted through an arteriectomy in the external carotid artery, gently advanced into the internal carotid artery, and positioned approximately 17 mm from the carotid bifurcation. LCBF was monitored continuously during the MCAO surgery. With the use of this technique, the tip of the suture occludes the origins of the MCA, the proximal anterior cerebral artery, and the posterior communicating artery. Reperfusion was accomplished by withdrawing the filament 2 h after MCAO (Longa et al. 1989; Sun et al. 2011).

23.2.2.2 Rat Treatment Schedules

After surgery, animals were allowed to recover from the anesthesia and given food and water ad libitum. Fifteen rats were randomly assigned as controls (MCAO rats which received only the vehicle, saline 0.9%), experimental (MCAO rats which received taurine, 40 mg/kg), and sham-operated (received the same surgical procedure without insertion of the silicon filament). Taurine was delivered subcutaneously to the experimental group 24 h after the reperfusion for 4 days.

23.2.2.3 Determination of Infarct Volume

Animals were sacrificed by isoflurane (Phoenix), and brains were removed for 2,3,5-triphenyltetrazolium chloride (TTC) staining and collecting samples for Western blot (Kramer et al. 2010). Using an adult rat brain slicer (Matrix, Zivic Instruments), brains were sectioned coronally into six 2 mm coronal slices (2, 4, 6, 8, 10, and 12 mm from the frontal pole) and incubated for 5 min in a 2% (wt/vol) solution of TTC (J.T. Baker, India) at 37°C. TTC, a water soluble salt, is reduced by mitochondrial dehydrogenases to formazan, which turns normal tissue deep red (Bederson et al. 1986; Rich et al. 2001). Thus, reduced TTC staining identifies regions of diminished mitochondrial function in the ischemic tissue. To assess lesion volume, TTC-stained slices were scanned using an HP ScanJet 5530 and analyzed by Image J analysis software (public domain software developed at NIH (http://rsbweb.nih.gov/ij/)). Lesion volume was determined as the percent of the total ipsilateral hemispheric volume as described previously (Swanson et al. 1990; O'Donnell et al. 2006). Briefly, to eliminate the effect of brain edema, the corrected infarct volume was calculated as follows: $[(V_R - V_{Ln})/V_R]100$ in which V_R is the volume of right hemisphere and V_{Ln} is the volume of nonlesioned tissue in left hemisphere (Schäbitz et al. 1999, 2000; O'Donnell et al. 2006). After the TTC experiment, while the sections were on ice, the ischemic parts of the left hemisphere (core and penumbra) and the right hemisphere (identical regions) were quickly dissected (Fig. 23.1a) (Ashwal et al. 1998).

23.2.2.4 Western Blot Analysis

Primary cortical neuronal cultures and rat brain samples were lysed in RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 1% (v/v) mammalian protease inhibitor cocktail and 1% (v/v) phosphatase inhibitor cocktail from Sigma and Thermo Scientific, respectively. Proteins in cell lysates were separated on a SDS-PAGE. After proteins were transferred to a nitrocellulose membrane, the membrane was then blocked in blocking buffer (20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, 5% milk) for 1.5 h at room temperature. After blocking, membranes were incubated overnight with following antibodies: GRP78 and p-IRE1(1:2,000; abcam), CHOP and p-Perk (1:1,000; Cell Signaling) and caspase-12 (1:500; Santa Cruz Biotechnology). Membranes were then incubated with ECL horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:3,000; GE Healthcare, UK) for 90 min in room temperature. GAPDH (1:3,000; Cell Signaling) and β-actin (1:2,000; Santa Cruz Biotechnology) were used as internal controls. Extensive washes with blocking buffer were performed between each step. The protein immunocomplex was visualized using ECL detection reagents purchased from Thermo Scientific. Quantitative Western blot results were obtained by densitometric analysis using Image Processing and Analysis in Java (Image J).

23.2.3 Data Expression and Statistical Analysis

All data were expressed as the mean ± SEM. The statistical significance of the data was determined with *t*-test or one-way ANOVA combined with Dunnett post hoc or Tukey test for comparison between groups.

23.3 Results

23.3.1 Primary Neuronal Culture Viability and Percent Area of Ischemic Injury in Rat Brain

Our data showed that different concentrations of taurine can attenuate cell death in hypoxia/reoxygenation. In order to determine the appropriate concentration of taurine in cultures, cortical neurons were exposed to hypoxia and reoxygenation in the presence of 1, 5, and 10 mM taurine as shown in Fig. 23.1b. After hypoxia and reoxygenation, viability of neurons without taurine treatment dropped to approximately 49% of control. Taurine treatment dramatically increased the cell viability. The presence of 1 mM taurine substantially increased the cell viability to greater than 70% of the control level. When taurine concentration was increased to 10 mM, cell viability was further enhanced to 85% of the control.

In the rat MCAO stroke experiments, representative coronal brain sections from the control group (MCAO vehicle-treated) and experimental group (MCAO taurine-treated) stained with 1% TTC are shown in Fig. 23.1c. Four days of reperfusion following 2 h of ischemia resulted in an infarct of 47.42 ± 9.86% in the control group. Although in all sections the infarct volume was decreased in the taurine-treated group versus vehicle-treated group, only in sections 6 mm from the anterior pole (infarct volume of 26.76 ± 6.91%) was the difference significant ($p < 0.05$). The sham-operated group showed no ischemic injury as determined by TTC staining.

23.3.2 The ATF6 and IRE1 Pathways Were Inhibited by Taurine, But There Was No Effect on the PERK Pathway

PERK, ATF6, and IRE1 are the three major ER stress-induced signaling pathways. Since taurine can downregulate GRP78 in hypoxic conditions in cell culture and in a stroke model (data not shown), we aimed to further identify which signaling pathway is involved in the protection. The phosphorylation of eIF2α, a downstream PERK pathway component, specifically regulates the translation of the transcription factor ATF4, leading to translational attenuation (Szegezdi et al. 2006). ATF4 is highly expressed after hypoxia/reoxygenation and increased by approximately

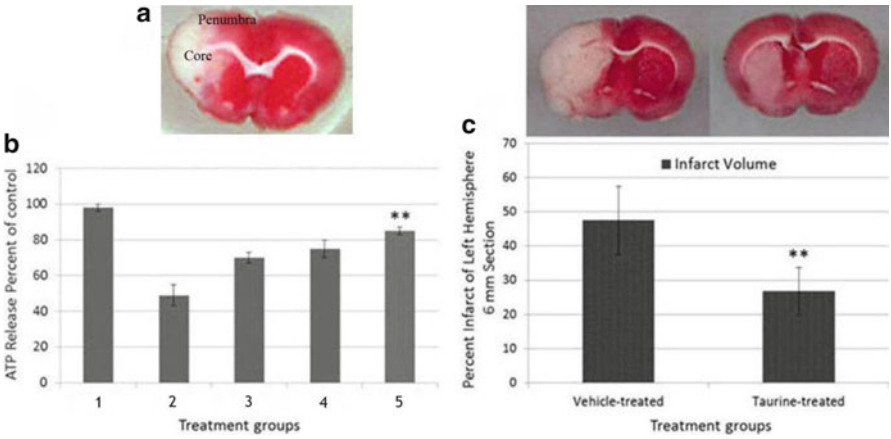

Fig. 23.1 Effect of Taurine on cell viability in primary neuronal cell culture and TTC on MCAO model of stroke. (**a**) Core and penumbra of the lesion part of the left hemisphere. (**b**) Dose-dependent neuroprotection of taurine against hypoxia/reoxygenation. (1) Control; (2) hypoxia; (3) hypoxia + 1 mM taurine; (4) hypoxia + 5 mM taurine; (5) hypoxia + 10 mM taurine. Cell viability was measured by ATP assay. Control values were fixed at 100%. The values for Hyp and Tau + Hyp were normalized relative to the control values and represent mean ± SEM of five preparations (**$p < 0.01$ versus hypoxia). (**c**) Effects of taurine on infarct volume of 6 mm section on day 4 of reperfusion after 2 h of focal cerebral ischemia. Vehicle or taurine was injected subcutaneously 24 h after ischemia. The infarct zone was displayed by TTC staining in treated rats. Sham-operated group showed no infarct zone. Representative images are slices of 6 mm section from the frontal pole. Data were presented as mean ± SD, $n = 16$ (**$p < 0.05$ vs. vehicle)

threefold over control cultures. After treatment with taurine, followed by hypoxia/reoxygenation, however, the levels of ATF4 in cortical neurons is similar to that of hypoxia/reoxygenation alone (Fig. 23.2a), indicating that taurine does not inhibit the initiation of the PERK pathway under this condition. Similarly, the expression of ATF4 in the MCAO model does not change with taurine treatment in the core of the infarct by comparison with the vehicle-treated group (Fig. 23.2b). These results indicate that taurine has no observable effects on PERK pathway activation in either cortical neurons or in the MCAO stroke model.

We next examined the effect of taurine on the ATF6 pathway in cortical neurons subjected to hypoxia/reoxygenation and in the brain of rats subjected to MCAO occlusion. After dissociation from GRP78, ATF6 translocates from the ER to the Golgi apparatus where it is cleaved to its active form (Chen et al. 2002). Treatment with taurine considerably reduced the level of cleaved ATF6 in both primary neuronal cultures and in the core of the infarct of MCAO rats. Interestingly, the ratio of cleaved ATF6 to ATF6 in neurons and MCAO rats treated with taurine dramatically declined by approximately 50% relative to neurons under hypoxia/reoxygenation or MCAO rats, respectively, in the absence of taurine as shown in Fig. 23.3a, b. These results demonstrate that taurine can prevent the activation of the ATF6 pathway in vitro and in vivo. To determine if taurine can affect the IRE1 pathway, we tested the expression of p-IRE1 in rat primary cortical neurons under hypoxia/reoxygenation

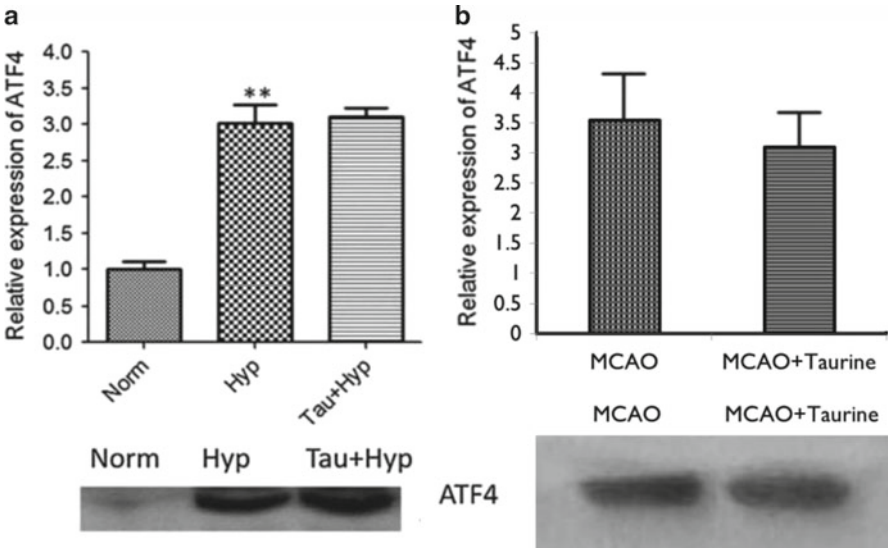

Fig. 23.2 Effect of taurine on ATF4 (from the PERK pathway) in neuronal cell cultures under hypoxia/reoxygenation condition and in rat MCAO stroke model. Taurine does not alter activity of the PERK pathway in primary neuronal cell culture and MCAO model. *Norm* normoxia, *Hyp* hypoxia (0.3% O_2) for 20 h, reoxygenation for 20 h, *Tau+Hyp* neurons were treated with 10 mM taurine for 1 h, then hypoxia for 20 h, reoxygenation for 20 h, *MCAO* middle cerebral artery occlusion for 2 h followed by 4 days reperfusion, *MCAO+taurine* middle cerebral artery occlusion for 2 h followed by 4 days reperfusion, taurine was injected 24 h after reperfusion subcutaneously and injection continued for 4 days. (**a**) ATF4 expression analyzed by Western blot in primary neuronal cell culture. The *bar graphs* reflect the densitometric data from the experiment of ATF4 Western blot results with arbitrary units. (**b**) ATF4 expression analyzed by Western blot in MCAO rats in the core area. The values in bar graph represent mean ± SEM, ($n=3$, $^{**}p<0.01$ versus norm)

conditions and in the core of the infarct of MCAO rats with and without taurine treatment by Western blot analysis (Fig. 23.3). The results showed that phosphorylated IRE1 is highly expressed in cortical neurons under hypoxia/reoxygenation and in the core of the infarct in MCAO rat brain. Taurine decreases the expression of p-IRE1 to normal condition both in primary neuronal culture under hypoxia/reoxygenation levels (Fig. 23.3c) and in the core of the infarct of MCAO rats (Fig. 23.3d), demonstrating that taurine significantly inhibits the IRE1 pathway in ER stress.

23.3.3 Taurine Can Decrease Apoptosis by Downregulation of Apoptotic Markers and Caspase-12 in Primary Neuronal Culture Induced by Hypoxia/Reoxygenation and in the Core of the Brain of MCAO

The Bcl-2 family plays crucial roles in the regulation of the mitochondrial pathways of apoptosis during experimental stroke. Bax is a member of the Bcl-2 family which

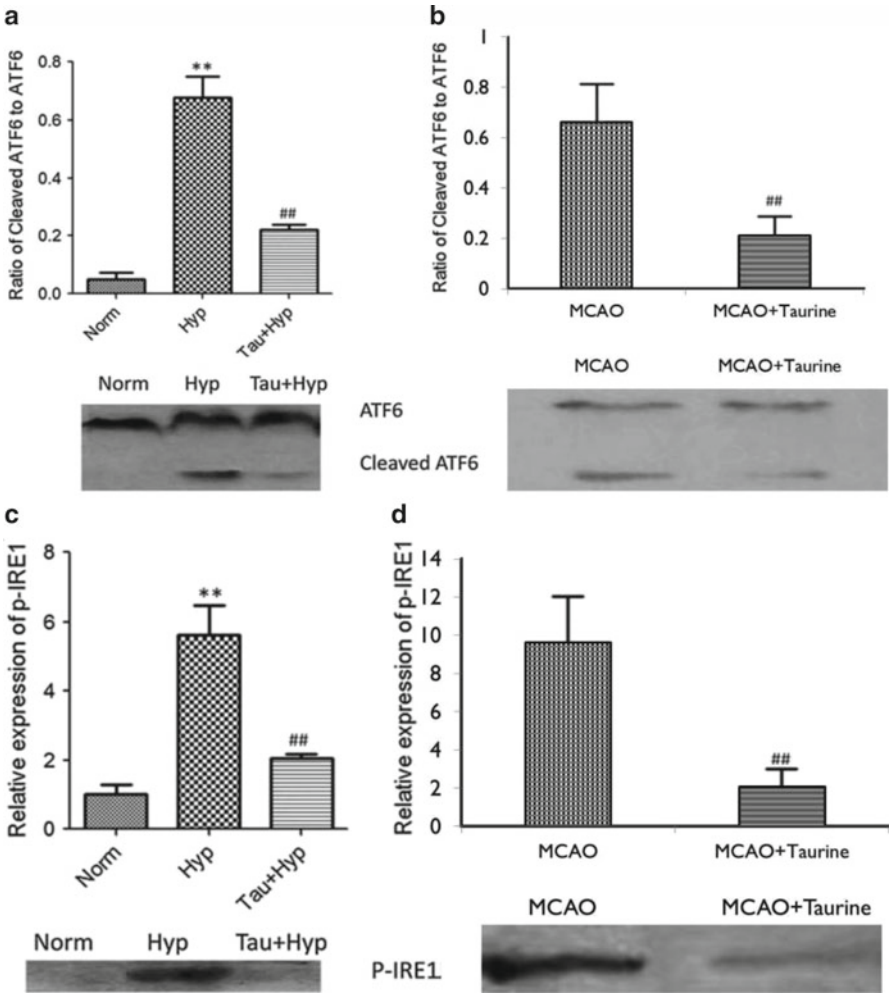

Fig. 23.3 Effect of taurine on ATF6 and p-IRE1 pathway in neuronal cell cultures under hypoxia/reoxygenation condition and in rat MCAO stroke model. Taurine does not alter activity of the PERK pathway, but inhibits the ATF6 and IRE1 pathway after hypoxia/reoxygenation and stroke. *Norm* normoxia, *Hyp* hypoxia (0.3% O_2) for 20 h, reoxygenation for 20 h, *Tau + Hyp* neurons were treated with 10 mM taurine for 1 h, then hypoxia for 20 h, reoxygenation for 20 h, *MCAO* middle cerebral artery occlusion for 2 h followed by 4 days reperfusion, *MCAO + taurine* middle cerebral artery occlusion for 2 h followed by 4 days reperfusion, taurine was injected 24 h after reperfusion subcutaneously and injection continued for 4 days. (**a**) ATF6 expression in primary neuronal culture analyzed by Western blot. (**b**) ATF6 expression in the core of MCAO brain analyzed by Western blot. The *bar graphs* represent the ratio of cleaved ATF6 to ATF6 using the densitometric data from the experiment of ATF6 Western blot results with arbitrary units. (**c**) P-IRE1 expression in primary neuronal culture analyzed by Western blot. (**d**) P-IRE1 expression in the core of MCAO brain analyzed by Western blot. The *bar graphs* reflect the densitometric data from the experiment of P-IRE1 Western blot results with arbitrary units. The values in bar graph represent mean ± SEM, $n = 3$, $^{**}p < 0.01$ versus norm; $\#\#p < 0.01$ versus hyp or MCAO

translocates from the cytosol to the mitochondria after brain ischemia and causes release of cytochrome C which in turn activates caspase-3 (Sun et al. 2011). Caspase-3 is believed to be at the final stage of apoptosis. These results demonstrate that taurine can prevent the activation of caspase-3 by increasing the ratio of Bcl-2 to Bax in the core of the infarct in MCAO rats by more than fourfold (Fig. 23.4a, b). To determine the effect of taurine on apoptosis induced by ER stress, we measured the expression of CHOP by Western blot analysis in primary neuronal cultures after hypoxia/reoxygenation and in the MCAO stroke model. As shown in Fig. 23.4c, the expression of CHOP was upregulated after exposure to hypoxia/reoxygenation.

Western blot analysis showed that taurine can decrease the levels of CHOP both in vitro in primary neuronal culture and in vivo in the MCAO stroke model (Fig. 23.4c, d). Taurine also significantly reduced the expression of caspase-12 and cleaved caspase-12 in vitro, demonstrating that taurine has the ability to inhibit the apoptosis induced by ER stress in hypoxia/reoxygenation (Fig. 23.4e).

23.4 Discussion

In the present study, the potential neuroprotective effects of taurine in an in vitro experimental model of brain ischemia/reperfusion and an in vivo model of MCAO stroke in rat were investigated. The main goal of this study was to investigate the effects of taurine on ER stress pathways in both the core of the brain infarct after MCAO and in primary neuronal cell culture after hypoxia/reoxygenation. We showed that taurine can not only protect primary neuronal cultures under hypoxia/reoxygenation conditions in a dose-dependent manner but also downregulate some ER stress and apoptotic markers in the brain in vivo after MCAO. Taurine as a neurotransmitter, neuromodulator, membrane stabilizer, and major intracellular free amino acid is employed in experimental therapies against neuronal damage, hypoxia, and epilepsy (Birdsall 1998). It has been shown that during cerebral ischemia, taurine may exert its neuroprotective function through both extracellular mechanisms by inhibiting calcium influx and intracellular mechanisms by protecting the mitochondrion through preventing mitochondrial dysfunction resulting from cytoplasmic calcium overload (El Idrissi and Trenkner 2004; Foos and Wu 2002; El Idrissi 2008; Huxtable 1992). Other functions of taurine, such as its role as an antioxidant, an osmoregulator, or an anti-inflammatory, contribute to its neuroprotective action (Huxtable 1992). During stroke, the levels of taurine in the extracellular fluid increases (Lo et al. 1998). The increases in the extracellular taurine levels under brain ischemia may constitute an important endogenous protective mechanism against neuronal damage (Saransaari and Oja 2000). However, intracellular taurine may be depleted resulting a disruption of intracellular homeostasis, leading to neuronal damage (Michalk et al. 1997; Huxtable 1992). Therefore, exogenous administration of taurine after brain ischemia may contribute to the recovery from ischemic damage by reducing the release of taurine, thus contributing to the restoration of intracellular homeostasis and the reduction of ischemic damage through both extracellular and intracellular mechanisms.

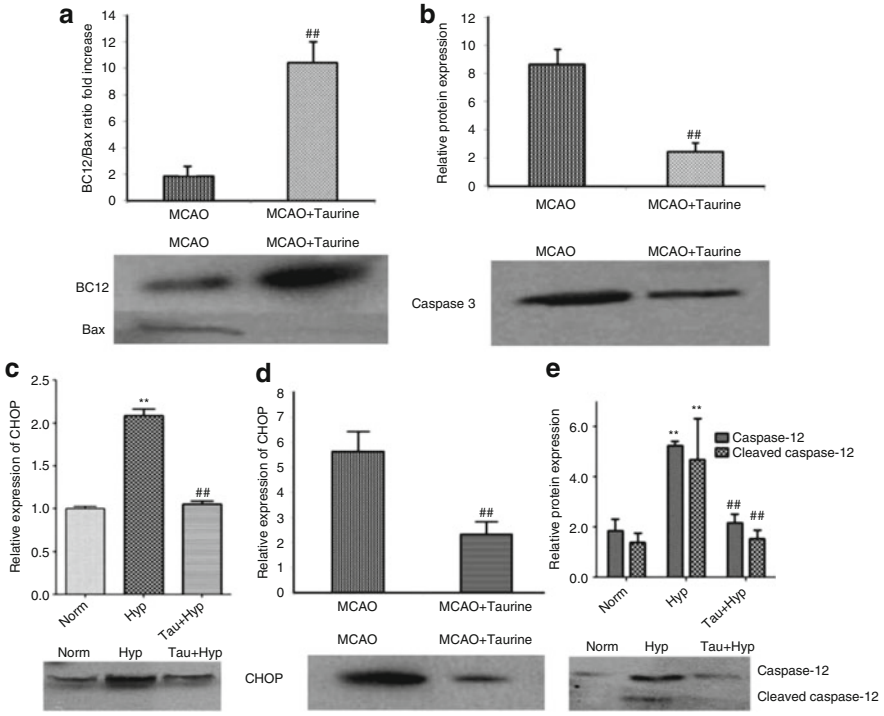

Fig. 23.4 Effect of taurine on expression of Bcl2, Bax, caspase-3, CHOP and, caspase-12 in rat MCAO stroke model and CHOP and caspase-12 in neuronal cell cultures under hypoxia/reoxygenation conditions. (**a**) Bax and Bl2 expression in the core of MCAO brain analyzed by Western blot, the *graph* shows the ratio of BCl2 to Bax in the core of MCAO brain in MCAO (middle cerebral artery occlusion for 2 h followed by 4 days reperfusion) and MCAO + taurine (middle cerebral artery occlusion for 2 h followed by 4 days reperfusion, taurine was injected 24 h after reperfusion subcutaneously and injection continued for 4 days). (**b**) Caspase-3 expression in the core of MCAO brain analyzed by Western blot in MCAO and MCAO + taurine. (**c**) CHOP expression analyzed by Western blot in primary neuronal culture. *Norm* normoxia, *Hyp* hypoxia (0.3% O_2) for 20 h, reoxygenation for 20 h, *Tau + Hyp* neurons were treated with 10 mM taurine for 1 h, then hypoxia for 20 h, reoxygenation for 20 h. The *bar graphs* reflect the densitometric data from the experiment of CHOP Western blot results with arbitrary units. (**d**) CHOP expression analyzed by Western blot in the core of the MCAO rat brain. (**e**) Caspase-12 expression analyzed by Western blot. The *bar graphs* reflect the densitometric data from the experiment of caspase-12 and cleaved caspase-12 Western blot results with arbitrary units. The values in bar graph represent mean ± SEM, $n = 3$, $^{**}p < 0.01$ versus norm and $^{\#\#}p < 0.01$ versus hyp or MCAO.1.3.3 Effect of TonEBP and dominant-negative TonEBP

Several protective mechanisms of taurine such as improvement in osmotic status and calcium homeostasis in cell damage caused by hypoxia or glutamate excitotoxicity have been suggested (Chang et al. 2004; El Idrissi and Trenkner 1999; Michalk et al. 1997). We recently showed that ER stress inhibition may also be involved in taurine's protective mechanisms under conditions of glutamate excitotoxicity and hypoxia (Pan et al. 2010, 2011). However, details of the relevant signaling pathways remain to be elucidated.

In this chapter, levels of cell viability as measured by the ATP assay were significantly enhanced by taurine after hypoxia/reoxygenation treatment, confirming the protective role of taurine. On the other hand, TTC results showed that posttreatment with taurine after MCAO could decrease the volume of cerebral damage, although this effect was not as strong as that of taurine pretreatment (Sun and Xu 2008; Sun et al. 2011). TTC staining clearly shows the stroke region which allows one to determine the exact size of cerebral infarction as well as to distinguish between the core of the infarct area, the penumbra, and healthy brain tissue (Benedek et al. 2006). Our data showed that using taurine (40 mg/kg), 24 h after reperfusion can still decrease lesion volume after 4 days. The colorless TTC is enzymatically reduced to a red formazan product by endogenous dehydrogenase enzyme complexes which are most abundant in mitochondria. Our ATP assay and TTC data confirm previous reports showing that taurine can regulate mitochondrial protein synthesis, enhance electron transport chain activity, and thereby increase the ATP levels and protect against excessive toxic superoxide generation (Schaffer et al. 2009; Jong et al. 2011). As a neuroprotective agent, taurine must pass through the blood–brain barrier (BBB) and enter into the brain under neuropathological conditions. On one hand, there are some reports of increases in radioactive taurine uptake in brain after systemic administration of radiolabeled taurine (Pasantes-Morales and Arzate 1981; Urquhart et al. 1974); on the other hand, in neuropathological conditions, the BBB may be ruptured and drugs can pass more freely. Moreover, taurine has been used with varying degrees of success in clinical therapy for epilepsy and other seizure disorders, and these data provide supporting evidence that taurine will cross the BBB and reach the damaged area when it is administrated subcutaneously after MCAO.

It is believed that brain ischemia followed by glutamate excitotoxicity leads to intracellular calcium overload and initiates a series of intracellular events, such as the release of apoptotic proteins leading to necrotic and apoptotic cell death (Nakka et al. 2008; Lipton 1999). Some reports have demonstrated that taurine can regulate intracellular calcium homeostasis through enhancing mitochondrial function, reducing the release of calcium from intracellular storage pools, and increasing the capacity of mitochondria to sequester calcium (Foos and Wu 2002; El Idrissi 2008; El Idrissi and Trenkner 2004). These data suggest that inhibiting intracellular calcium overload may be essential for the protection of taurine against MCAO. Taurine may block caspase-3 by regulating the release of mitochondrial cytochrome C. Cytochrome C release is regulated by the BCL-2 protein family of apoptotic regulators (Juin 1998). During brain ischemia, Bax expression is increased, and Bax protein translocates to mitochondria to induce cytochrome C release (Schäbitz et al. 2003; Gao and Dou 2000). We showed that 4 days after MCAO, taurine could decrease Bax protein expression, while Bcl-2 protein expression increased. Thus regulation of Bcl-2 and Bax has been demonstrated in our results, although the effect of taurine on intracellular calcium has not been directly investigated in this study. A high ratio of Bcl-2 to Bax can prevent release cytochrome C from mitochondria which results in decreased caspase-3 activity. As we showed in vitro in primary neuronal cultures, the proapoptotic factor CHOP is expressed at low levels

under physiological conditions but is strongly induced in ER stress under hypoxic conditions (Nemetski and Gardner 2007; Oyadomari and Mori 2004; Paschen et al. 1998). We showed that an increase in CHOP was prevented by administration of taurine both in primary neuronal cultures and in the MCAO stroke model. Taurine can upregulate Akt phosphorylation to prevent ischemia-induced apoptosis (Taranukhin et al. 2008) and to attenuate ER stress (Yung et al. 2007). Taurine has also been shown to affect the pathways related to ER stress (Pan et al. 2010, 2011). Our current study demonstrated that taurine has beneficial effects on the protection against ER stress in the core of the MCAO infarct and on cortical neurons under hypoxia/reoxygenation. It has been shown that caspase-12, the first ER-associated member of the caspase family, is activated by ER stress (Yoneda et al. 2001; Nakagawa et al. 2000). We analyzed the expression of caspase-12 in the presence or in the absence of taurine in both in vivo and in vitro models. Our data demonstrated that the caspase-12 or cleaved caspase-12 expression was clearly reduced by taurine following hypoxia/reoxygenation in primary neuronal culture, but no change was seen in the MCAO stroke model. PERK, ATF6, and IRE1, three ER-resident transmembrane proteins, serve as the main proximal sensors of the ER stress response. In this chapter, we tried to identify which particular ER stress-induced pathway can be affected by taurine treatment in the brain of MCAO model and also in the cortical neuronal culture model under hypoxia/reoxygenation. Under ER stress conditions, PERK has proved to be responsible for repressing global protein synthesis via phosphorylation of a subunit of eIF2a (Kumar et al. 2001; Harding et al. 2000b). Phosphorylation of eIF2a, on the other hand, can also indirectly control gene transcription by positively regulating the translation of transcription factors as has been shown for mammalian ATF4 (Szegezdi et al. 2006). Since p-eIF2a and ATF4 are two downstream proteins in the PERK pathway of ER stress, it is appropriate to measure expression levels of these two proteins in order to determine the extent of PERK pathway response in the presence or in the absence of taurine treatment. We found that in the MCAO model of stroke and after hypoxia/reoxygenation in primary cell culture, there was a strong increase in ATF4 expression, indicating that the PERK pathway is activated in both models. However, there were no significant alterations of ATF4 protein levels in taurine-treated groups both in vitro and in vivo. These results suggest that taurine may have neither suppressed nor facilitated the activation of the PERK pathway, which plays an important role in attenuating protein translation to restore neuronal homeostasis during ER stress. After dissociation of GRP78, ATF6 translocates from the ER to the Golgi apparatus where it is cleaved to its active form (cleaved ATF6) (Chen et al. 2002). The ratio of cleaved ATF6 to full-length ATF6 demonstrates that taurine clearly inhibits ATF6 cleavage in both MCAO stroke model and in primary neuronal cultures under hypoxia/reoxygenation. The levels of p-IRE1 in both the MCAO stroke model and in the hypoxia/reoxygenation model of primary neuronal cultures were measured to test whether taurine has an effect on the IRE1 pathway. The results indicate that the elevation of p-IRE1 is strongly suppressed by taurine treatment, either using in vivo or in vitro experiments. These findings provide strong evidence that activation of the IRE1 pathway can be inhibited by taurine. Furthermore, the results indicating suppression

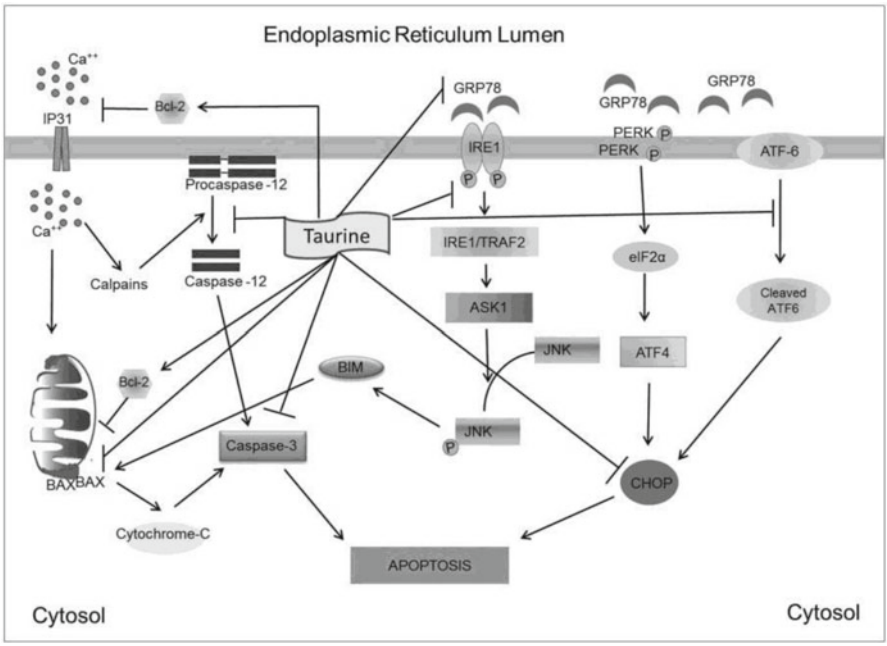

Fig. 23.5 Schematic diagram showing the mode of action of taurine in alleviating the apoptosis induced by ER stress and mitochondrial dysfunction. Taurine can exert its regulation by decreasing GRP78 release, caspase-12 activity, Bax, caspase-3, p-IRE1, ATF6 cleavage, CHOP, and increasing Bcl-2

of both CHOP and caspase-12 by taurine treatment provide substantial evidence that taurine can contribute to an effective inhibition of ER stress induced by hypoxia/reoxygenation.

23.5 Conclusion

In summary, as it is shown in Fig. 23.5, we demonstrated that taurine can exert its protective effect on CNS neurons both in the in vitro model of hypoxia/reoxygenation and in vivo model of the MCAO through suppression of ER stress. Moreover, the effect of taurine treatment on the three ER stress-induced signaling pathways showed that taurine significantly inhibited apoptosis by activation of the ATF6 and the IRE1 pathway, but not the PERK pathway.

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Chapter 24
Relationship Among Self-Reported Fatigue, Dietary Taurine Intake, and Dietary Habits in Korean College Students

So Yoon Park, Jeong Soon You, and Kyung Ja Chang

Abstract The purpose of this study was to investigate the relationship among self-reported fatigue, dietary taurine intake, and dietary habits in Korean college students. The subjects were 239 college students (142 male and 97 female) residing in the Incheon, Korea. Self-reported fatigue score was determined using a questionnaire of "Subjective Symptoms of Fatigue Test." The average physical fatigue score ($p < 0.001$), mental fatigue score ($p < 0.01$), nervous fatigue score ($p < 0.001$), and total fatigue score ($p < 0.001$) of female students were significantly higher compared to male students. Average dietary taurine intake in male and female was 102.5 mg/day and 98.0 mg/day, respectively. There was no significant correlation between self-reported fatigue score and dietary taurine intake. However, there was significantly negative correlation between self-reported fatigue scores and dietary habits such as "eating meals at regular times" ($p < 0.05$), "eating foods such as meat, fish, eggs, and beans more than two times a day" ($p < 0.05$), "eating greenish yellow vegetable every meal" ($p < 0.05$), and "avoiding eating sweet foods everyday" ($p < 0.05$). Therefore, in order to reduce self-reported fatigue, it is necessary to provide nutrition education and counseling for better dietary habit in Korean college students, and a further large-scale study is needed about relationship of self-reported fatigue and dietary taurine intake.

Abbreviations

BMI Body mass index
BFP Body fat percentage

S.Y. Park • J.S. You • K.J. Chang (✉)
Department of Food and Nutrition, Inha University, Incheon, South Korea
e-mail: kjchang@inha.ac.kr

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24.1 Introduction

Fatigue is a feeling of extreme physical or mental tiredness. The fatigue can aggravate depression or stress. Fatigue is "a subjectively reported symptom for which there are currently no tests or objective signs allowing the clinician to quantify its severity" (Morrow et al. 2009). Taurine intake may play a significant role in physical and mental well-being (Stapleton et al. 1997). In addition, taurine has the effect of antifatigue in rat (Zhang and Chen 1999) and has beneficial effect on exercise-induced fatigue in human (Wei et al. 2001).

College students are a group especially prone to fatigue (D'Zurilla and Sheedy 1991). They accomplish competing academic and social goals as well as their sensitive reactions to both disappointment and success (Michelle et al. 2009). Therefore, the purpose of this study was to investigate the relationship among self-reported fatigue, dietary taurine intake, and dietary habits in Korean college students.

24.2 Methods

24.2.1 Subjects

The subjects were 239 college students (142 male and 97 female) residing in Incheon, Korea. A cross-sectional study was carried out using a self-administered questionnaire.

24.2.2 Anthropometric Measurement

The subject's height was measured using a stadiometer. Measurements of body weight, body mass index (BMI), and body fat percentage (BFP) were taken with the InBody 3.0 Body Composition Analyzer (InBody 3.0, Biospace, Seoul, Korea).

24.2.3 Self-Reported Fatigue Measurement

Self-reported fatigue score was determined using a questionnaire of "Subjective Symptoms of Fatigue Test." The higher fatigue scores indicate heavier fatigue. The fatigue score scale contained 30 items about their experience frequency of fatigue.

Table 24.1 Age and anthropometric data of the subjects

Variables	Male ($n=142$)	Female ($n=97$)
Age (years)	23.6 ± 0.2[a]	20.9 ± 0.1***[b]
Height (cm)	173.5 ± 1.3	161.9 ± 0.5***
Weight (kg)	74.0 ± 1.0	54.5 ± 0.7***
BMI (kg/m^2)	24.2 ± 0.3	20.8 ± 0.3***
BFP (%)	22.9 ± 0.6	30.8 ± 0.5

[a] Values are mean ± SE
[b] ** $P < 0.01$; *** $P < 0.001$ (by Student's t-test)

24.2.4 Dietary Taurine and Nutrients Intakes Assessment

A 3-day recall method was used for dietary assessment (2 weekdays and 1 weekend day). Dietary taurine and nutrients intakes were estimated using the computer-aided nutrition program (CAN-pro 3.0, The Korean Nutrition Society Korea) imputed with a taurine content database for17 food groups, commonly used 321 food items (Kim et al. 1999; Park 2000).

24.2.5 Dietary Habit Score Measurement

The dietary habit questionnaire included 18 items which were based on previous study (Park et al. 2010). Each item responses ranged from 1 to 5.

24.2.6 Statistical Analysis

The statistical analysis was conducted using the SPSS 17.0 program. Mean and standard errors were calculated for all variables and analyzed by analysis of the Student's t-test. The correlation among self-reported fatigue, dietary taurine intake, and dietary habits was analyzed using Pearson's correlation coefficient.

24.3 Results and Discussion

24.3.1 Anthropometric Data

Anthropometric data of the subjects are shown in Table 24.1. The average age was 23.6 ± 0.2 years and 20.9 ± 0.1 years in male and female subjects, respectively.

Table 24.2 Self-reported fatigue scores of the subjects

Variables	Male ($n=142$)	Female ($n=97$)
Physical fatigue	26.3 ± 0.7[a]	30.6 ± 0.8[***b]
Mental fatigue	22.3 ± 0.6	24.9 ± 0.7**
Nervous fatigue	21.0 ± 0.6	25.6 ± 0.7***
Total fatigue	69.7 ± 1.7	80.9 ± 1.8***

[a]Values are mean ± SE
[b]**$p < 0.01$; ***$p < 0.001$ (by Student's t-test)

Table 24.3 Dietary taurine intake and major nutrients of the subjects

Variables	Male ($n=142$)	Female ($n=97$)
Energy (kcal/day)	1,904.6 ± 44.0[a]	1,702.6 ± 35.4**[b]
Carbohydrate (g/day)	268.1 ± 6.5	238.4 ± 5.1**
Total fat (g/day)	57.8 ± 1.8	53.2 ± 1.6
Total protein (g/day)	71.3 ± 2.1	64.8 ± 1.6*
Taurine (mg/day)	102.5 ± 5.0	98.0 ± 6.8

[a]Values are mean ± SE
[b]*$p < 0.05$; **$p < 0.01$ (by Student's t-test)

24.3.2 Self-Reported Fatigue Scores

Self-reported fatigue scores of the subjects are shown in Table 24.2. The average physical fatigue score ($p < 0.001$), mental fatigue score ($p < 0.01$), nervous fatigue score ($p < 0.001$), and total fatigue score ($p < 0.001$) of female students were significantly higher compared to male students. Self-reported fatigue score of female was significantly higher than that of male. It was similarly reported that female college students had high fatigue scores (Yoon and Hwang 1994).

24.3.3 Dietary Taurine Intake and Major Nutrients

The average dietary taurine intake is shown in Table 24.3, along with data on the energy and major nutrients intakes. The average energy intake in the male and female subjects was 1,904.6 ± 44.0 kcal/day and 1,702.6 ± 35.4 kcal/day, respectively. The average dietary taurine intake among the male and female subjects was 102.5 ± 5.0 mg/day and 98.0 ± 6.8 mg/day, respectively. There was no significant difference in dietary taurine intake between male and female students.

24.3.4 Dietary Habit Scores

Table 24.4 shows the average dietary habit scores of male and female. The average dietary habit scores of "eating meals at regular times" ($p < 0.05$), "eating three meals

Table 24.4 Dietary habit scores of the subjects

Variables	Male ($n=142$)	Female ($n=97$)
Eating breakfast regularly	3.1 ± 0.1[a]	3.2 ± 0.1
Eating meals at regular times	3.2 ± 0.1	2.7 ± 0.1*[b]
Eating adequate amount of meals	3.0 ± 0.1	3.2 ± 0.1
Eating three meals a day	3.1 ± 0.1	2.7 ± 0.1*
Having meals with diverse foods	2.8 ± 0.1	2.5 ± 0.1*
Avoiding eating spicy foods	2.8 ± 0.1	2.7 ± 0.1
Not eat salty foods	3.1 ± 0.1	3.1 ± 0.1
Eating foods such as meat, fish, eggs, and beans more than two times a day	3.1 ± 0.1	2.9 ± 0.1
Avoiding eating foods containing oil or mayonnaise more than two times a day	2.6 ± 0.1	2.6 ± 0.1
Eating greenish yellow vegetable every meal	3.3 ± 0.1	3.2 ± 0.1
Eat dairy foods(milk, yogurt, etc.) everyday	3.0 ± 0.1	2.9 ± 0.1
Eat fruits everyday	2.6 ± 0.1	2.8 ± 0.1
Not eat sweet foods(snack, chocolate, etc.) everyday	3.5 ± 0.1	3.3 ± 0.1
Eat natural foods mostly	2.7 ± 0.1	2.5 ± 0.1
Avoiding eating harmful foods	3.3 ± 0.1	2.9 ± 0.1*
Check nutrition facts	2.5 ± 0.1	2.6 ± 0.1
Drink 2 L water everyday	3.1 ± 0.1	2.6 ± 0.1
Apply nutrition knowledge to daily life	3.2 ± 0.1	3.2 ± 0.1
Total score	51.3 ± 0.9	47.2 ± 0.9**

[a]Values are mean ± SE
[b]*$p < 0.05$; **$p < 0.01$ (by Student's t-test)

a day" ($p < 0.05$), "having meals with diverse foods" ($p < 0.05$), and "avoiding eating harmful foods" ($p < 0.05$) were significantly lower in female students compared to male students. Total dietary habit scores of males were significantly higher than total dietary habit scores of females. It was similarly reported that female college students had poor dietary habits (Ko 2007).

24.4 Correlation Between Self-Reported Fatigue Scores and Dietary Habit Score

There was no significant correlation between self-reported fatigue scores and dietary taurine intake (Table 24.5). However, there was significant negative correlation between self-reported fatigue scores and dietary habit score such as "eating meals at regular times" ($p < 0.05$), "eating foods such as meat, fish, eggs, and beans more than two times a day" ($p < 0.05$), "eating greenish yellow vegetable every meal" ($p < 0.05$), and "avoiding eating sweet foods everyday" ($p < 0.05$). Self-reported fatigue was associated with poor dietary habits.

Table 24.5 Correlation between self-reported fatigue scores and dietary habit score

	Physical fatigue	Mental fatigue	Nervous fatigue	Total
Eating breakfast regularly	−0.048	−0.11	−0.143*[a]	−0.112
Eating meals at constant times	−0.106	−0.1	−0.197**	−0.153*
Eating three meals a day	−0.067	−0.114	−0.148*	−0.122
Eating foods such as meat, fish, eggs, and beans more than two times a day	−0.154*	−0.101	−0.146*	−0.151*
Eating greenish yellow vegetable every meal	−0.114	−0.133*	−0.102	−0.135*
Not eat sweet foods (snack, chocolate, etc.) everyday	−0.092	−0.143*	−0.092	−0.123
Avoiding eating harmful foods	−0.136*	−0.024	−0.201**	−0.138*
Total	−0.129*	−0.096	−0.109	−0.128*

[a]*$p < 0.05$; **$p < 0.01$ (by Pearson's correlation)

24.5 Conclusion

Our study investigated the relationship among self-reported fatigue, dietary taurine intake, and dietary habits in Korean college students. There was no significant correlation between dietary taurine intake and self-reported fatigue scores. However, these results show that female students have higher self-reported fatigue scores and lower dietary habit score compared to male students and there was significant relationship between dietary habit score and self-reported fatigue scores. Therefore, in order to reduce self-reported fatigue, it is necessary to provide nutrition education and counseling for better dietary habit in Korean college students and a further large-scale study is needed about relationship of self-reported fatigue and dietary taurine intake.

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Chapter 25
Simulative Evaluation of Taurine Against Alopecia Caused by Stress in *Caenorhabditis elegans*

Hyemin Kim, Hyunsook Chang, and Dong-Hee Lee

Abstract Hair loss or alopecia has been portrayed as a modern malady which is aggravated by stressful conditions. Major cases of alopecia were found among individuals of 40s–50s, nowadays, even among the 20s–30s. This study characterized taurine's potential against alopecia caused by chemical stress agents based on the comparison with other commercially available anti-alopecia agents using *Caenorhabditis elegans*. The criteria used are their effects on the expression of stress markers and measurements of vital signs: lifespan comparison, progeny number, and mobility. *C. elegans* showed the typical stress symptoms under treatment with tunicamycin, endoplasmic reticulum stress agent. Hsp-70 protein expression increased, while worm's lifespan and per capita progeny number significantly decreased along with an unusually retarded movement. A positive response was shown when worms were treated with taurine along with astressin-B and finasteride. Between the treatments, finasteride showed better outcomes in terms of stress-reducing effects. Taurine helped worms recover more effectively from adverse influence of stress. In conclusion, there is strong evidence that taurine has a great potential as anti-alopecia effect especially against the one caused by the chemical stress. The present study implies that taurine might strongly work against hair loss when used in combination with other commercially available anti-alopecia agents.

H. Kim • D.-H. Lee (✉)
Department of Life Sciences, University of Seoul, Seoul 130-743, South Korea
e-mail: leedh@uos.ac.kr

H. Chang
Department of Child Studies, Korea Nazarene University,
Cheonan 331-718, South Korea

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_25,

Abbreviations

Tau	Taurine
Tun	Tunicamycin
NGM	Nematode growth medium
ER	Endoplasmic reticulum
RR	Rescue rate

25.1 Introduction

Hair is a characteristic feature of mammals and has significant biological usages. Hairs grow out of the epidermis to develop a shield against potential damages from ultraviolet light, heat, and external harmful debris. Humans have shown a trend to eliminate hairs during the course of evolution from their ancestors. In hair, living part exists only at the root part; however, its functional integrity depends on the whole structure. Thus, hair can be considered as continuous functional structure from root to end and the strength of hair root greatly affects the entirety of hair.

Alopecia refers to the general sense of hair loss from the head and baldness is usually the typical outcome of alopecia. Earlier alopecia exerts a significant sense of frustration especially to younger generations. The cause of alopecia is not well understood to date; however, modern stressful living conditions appear to coincide with alopecia for humans. There are three major categories in terms of cause and development of alopecia (Fig. 25.1). Alopecia results from extended cellular stress in hair root cells along with individual genetic background. Combined with individual genetic background, alopecia can occur at early stage of life and intensifies with stressful circumstances. The chance for alopecia increases with age and males are more susceptible to alopecia than females (Table 25.1, compiled from Dior 2011).

Extreme physical stress is believed to worsen the health of hair (Novak and Meyer 2009). Among adolescents, alopecia is triggered by unusual hair care maintenance, such as twisting hair treatments, bleaching, and behavioral pulling of hair. Recent studies indicate that alopecia is also caused by a type of organelle stress such as endoplasmic reticulum stress (Gupta et al. 2009; Sarkar et al. 2011). According to the theory, various stresses affect the cellular conditions within stem cells in hair follicles and the eventual apoptosis depreciates the stem cells of hair follicles. Under circumstances, anti-ER stress compounds may reduce the occurrence of alopecia and even avoid the symptom when its application is implemented beforehand at high-risk groups.

A number of medicines are available to treat the symptoms related to alopecia. Majority of the medicine has been developed to strengthen the integrity of hair at its

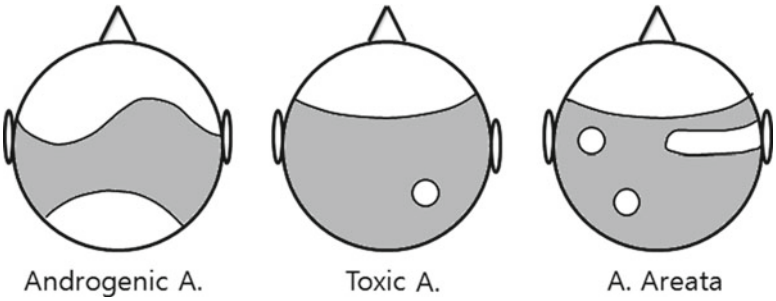

Fig. 25.1 Top-down view of different alopecias. Alopecia (A.) varies in shape and pattern of development. Androgenic A. and A. areata are affected mostly by individual genetic backgrounds. The areas of hair loss, caused by androgenic A. and A. areata, expand with age. Toxic A. is affected by environmental stresses and can be cured with relevant treatments

Table 25.1 Alopecia statistics

Sex	Characterization	(%)
Male	–Androgenic in all baldness	95
	–Showing signs of balding by 50s	67
	–With male pattern baldness by 50s	50
	–Balding by 30s	25
Female	–Suffering hair loss by 50s	25

root. Recently, astressin-B has been approved as anti-alopecia medicine by the FDA of the United States as a nonselective antagonist to corticotropin-releasing hormone. It prompted copious hair growth in mice which was subjected to an alopecia treatment (Wang et al. 2011). Finasteride is used to treat alopecia especially in the form of male pattern baldness as well as benign prostatic hyperplasia. Finasteride inhibits the conversion of testosterone to dihydrotestosterone (Evers et al. 2010).

Hair growth and regeneration test model is available in vitro and in vivo. Mice are frequently used as model systems in evaluating anti-alopecia candidate agents (Porter 2003). This study, however, utilizes *Caenorhabditis elegans* as the model system considering they correctly represent cellular stress leading to ER stress at the individual level (Link et al. 2001; Silverman et al. 2009). Under treatment of candidate agents including taurine, their response to the ER stress will be very comprehensive and less laborious. In this study, the two commercially available anti-alopecia agents were evaluated in terms of ER stress level along with taurine: The extent of anti-ER stress effect was evaluated according to their effect on the stress protein expression and, functionally, on the basis of life length, mobility, and offspring numbers which constitute very useful indicators for physiological stress (Estes et al. 2005; Ayyadevara et al. 2007; Boyd et al. 2007; Davies and Hart 2008).

25.2 Methods

25.2.1 Synchronous Culture Conditions and Induction of ER Stress

Wild type of *C. elegans* N2 was cultured at 25°C on the nematode growth medium (NGM) according to the standard method (Stiernagle 2006; Szewczyk et al. 2003). To induce ER stress, worms were grown for 3 h on media containing tunicamycin at 10 μg/ml. They were further incubated with various concentrations of anti-alopecia agents. The anti-alopecia agents used in this experiment were astressin-B, finasteride, and taurine which were all purchased from Sigma (St. Louis, USA). The anti-alopecia agents were delivered into the media as the final concentration of 10 or 100 μg/ml. The stress conditions were assessed by examining the stress protein marker expression. The expression of hsp-70 was detected according to the standard immunoblotting procedures. The antisera against hsp-70 were purchased from Santa Cruz Biotechnology (Santa Cruz, USA) and diluted as 1,000 times for immunoblotting in the present experiments.

25.2.2 Measurement of Worm's Vital Signs: Lifespan Extension, Progeny Number, and Mobility Restoration

The effect on life length extension was estimated using the method of Hyun et al. (2008). As a brief description, worms were initially destroyed except the eggs by bleaching and ten eggs were incubated on NGM supplemented with OP50 at 25°C until worms reached the young adult stage. Fifty worms were then transferred onto plates containing 10 μg/ml of tunicamycin, where they were incubated for 3 h. They were subsequently transferred to media containing 10 or 100 μg/ml of astressin-B, finasteride, or taurine. Live worms were counted daily. Worms were excluded from counting if they failed to react to a stimulus with a platinum wire. The lifespan extension effect was calculated as in a rate of rescuing worms from the chemical stress. The rescue rate (RR, %) was calculated according to the following formula: RR(%)=(A−B)/(C−B) X 100(%), where A refers to offspring number with each drug treatment after Tun, B to offspring number with Tun-only treatment, and C to offspring number with drug-free treatment.

In order to evaluate the effect on offspring number, the number of eggs was counted daily and standardized per number of adult after the anti-alopecia treatment under the ER stress conditions. The adults were selected for visual consistency and removed to a fresh plate with 10 μg/ml of tunicamycin. After subsequent 3 h incubation on the Tun media, they were further treated with anti-alopecia agents at 10 or 100 μg/ml. The number of fertilized eggs and larvae was counted and standardized as a rate of increase on 2, 3, and 4 days after the beginning of the culture.

The mobility of worm was assessed by monitoring the total moving length of the worms in the presence of anti-alopecia agents. As in the life duration experiment above, worms were treated with 10 μg/ml of tunicamycin and relocated to the anti-alopecia media with their usual content at 10 or 100 μg/ml. After the repositioning into the anti-alopecia media, the extent of movement was determined according to the turbidity of the NGM media. Their turbidity was visually compared against anti-alopecia-free.

25.3 Results

In order to characterize taurine's potential as anti-alopecia agent against the hair loss caused by stress, this study focused on whether it reduces the level of stress caused by tunicamycin in *C. elegans* along with other leading anti-alopecia agents: astressin-B and finasteride. Two categories of assay were performed. Firstly, the expression of hsp-70, ER stress marker, was measured and the overall level of hsp-70 increased after the tunicamycin treatment but significantly diminished under treatment with taurine. As a second line of approach, measurements were made focusing on the level of vital signs: survivorship, offspring number, and mobility of the worms. In terms of these functional markers, taurine and the two anti-alopecia agents caused a reduction in ER stress by restoring the vital signs toward those of the no stress controls.

25.3.1 Reduction of Hsp-70 Under Tau Treatment After Tun

The expression of hsp-70 significantly increased compared to no stress control when the worms were treated with tunicamycin. In the presence of taurine, however, the level of hsp-70 expression was significantly affected. The extent of hsp-70 expression showed a dose-dependent manner, along with the amount of the tunicamycin added (Fig. 25.2). When incubated with astressin-B or finasteride, the worms failed to show any significant variation in terms of hsp-70 protein expression (data not shown). The data imply that taurine may lower the level of ER stress caused by tunicamycin, since the reduced expression of hsp-70 represents the decreased level of ER stress in the worms.

25.3.2 Higher Rescue Rate Under Taurine Treatment

The treatment with tunicamycin curtailed the lifespan of worms. Beginning two days after the start of the culture, tunicamycin was shown to adversely affect the growth of *C. elegans* according to the significant reduction of worms in number

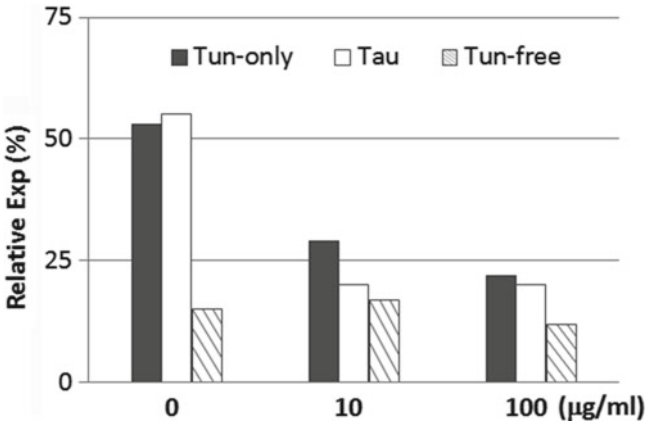

Fig. 25.2 Effect of taurine on hsp-70 expression in ER-stressed *C. elegans*. The expression of hsp-70 was compared at the three concentrations of taurine. Tun-treated worms showed a higher expression of hsp-70 compared to no Tun (or no stress) treatment. Along the increase of taurine concentration, however, the level of hsp-70 expression decreased significantly

when they were treated singularly with tunicamycin. A significant worm fraction perished within 20 days after the start of the treatment. When the worms were treated with the three candidate agents, their rescue rate increased in comparison to Tun-only treatment (Fig. 25.3). Among the three candidate chemicals, taurine notably enhanced the rescue rate. Both astressin-B and finasteride exerted positive effects on the rescue rate although taurine surpassed the two anti-alopecia agents considerably. These data indicate that the agents against alopecia may help the worms recover from the chemical stress by Tun. Also, the enhanced rescue rate strongly implies that the agents may help the extension of worm's lifespan which has been significantly shortened under chemical stress by Tun.

25.3.3 Progeny Number Recovered Under Treatments with Anti-Alopecia Agent

Each offspring number was recorded daily up to 4 days following the application of the three candidate molecules to the tunicamycin-treated worms. When worms were treated with Tun, they produced much less offspring compared to the Tun-free treatment. Nevertheless, the worms appeared to recover from the effect of Tun when they were transferred to the media containing the respective candidate chemicals (Fig. 25.4). Both finasteride and astressin-B show a positive effect on the number of progeny. Compared to the two chemicals, taurine showed a more positive effect on the ER-stressed worms. Throughout the 2–4 day count, taurine-treated worms produce at least 20% more offspring than Tun-only-treated worms on the per capita basis. These results indicate that the three anti-alopecia agents help the worms to

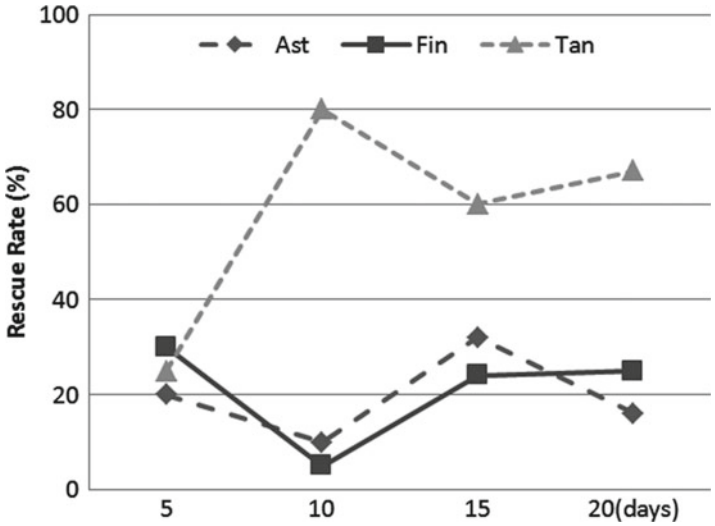

Fig. 25.3 Effect of anti-alopecia agents on lifespan extension. The effect of anti-alopecia agents on lifespan was calculated as rescue rate according to the description in Sect. 25.2. Taurine significantly prolonged worm's lifespan; however, astressin-B and finasteride slightly helped worms to recover from the stress compared to taurine

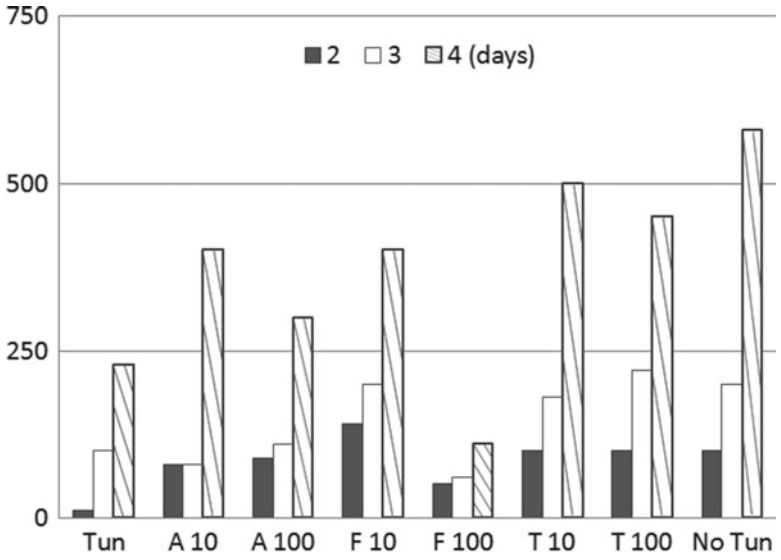

Fig. 25.4 Progeny number under treatments with anti-alopecia agents. Progeny numbers were scored daily after worms were treated with astressin-B (A), finasteride (F), and taurine (T) at 10 or 100 μg/ml for 2–4 days. Under taurine treatment, the offspring number was consistently greater than those of A and F

Table 25.2 Comparison of mobility after drug treatment

	Conc.(μg/ml)		
Drug	0	10	100
Astressin-B	+	++	+
Finasteride	+	+	++
Tau	+	++	+++
Tun-only	+	+	+

recover from the ER stress at the level of organism. The increased number of offspring may originate from the anti-ER stress effect of the three candidate molecules.

25.3.4 Restoration of Mobility Among Worms Treated with Anti-Alopecia Agents

To evaluate the effect of anti-alopecia agents on the mobility of the stressed worms, their moving distances were figured out according to the turbidity which was caused by the worm's path. The turbidity was visually compared at three different concentrations of the candidate chemicals. When treated with Tun, worms showed a considerable decline in mobility. The reduced movement of worms, however, apparently recovered to reach the level of ER stress-free control when worms were treated with taurine or two other alopecia agents following taurine application. Table 25.2 summarizes the mobility of worms cultured at two different concentrations of the three drugs, as expressed in the turbidity of the media. Along with astressin-B and finasteride, taurine facilitates the worms to recover from the stress. This result strongly implies that taurine helped the worms recuperate from the adverse influence of ER stress on their mobility.

25.4 Discussion

A considerable percentage of adults and youths suffer severe emotional stress from alopecia. Alopecia occurs in adolescents even at the age of 14. Astressin-B and finasteride cannot be applied to the adolescents less than 18 years old and hair implant is not a feasible alternative at this age, and development of safe anti-alopecia agents is very important under the circumstances. The present study utilized *C. elegans* to develop an assay system for anti-alopecia drugs which are especially effective against stresses.

C. elegans displayed usual ER stress signs under tunicamycin with treatment such as augmented expression of hsp-70, heat shock proteins. The tunicamycin

treatment caused many physiological stress symptoms: decreased survivorship, stunted movement, and reduced number of offsprings. When the worms were treated with the commercial anti-alopecia agents and taurine, however, they showed positive responses against ER stress conditions. Although there is a variation among the three agents, they showed positive rescue rates and increased mobility and progeny number.

Although astressin-B and finasteride are two prominent drugs to treat alopecia, the data in this study suggest that taurine may work better than the two anti-alopecia agents against hair loss caused particularly by stresses. These results strongly implicate that taurine might alleviate the chemical stress to help hair root cells to sustain their integrity.

Future study may be meaningful to characterize whether a synergistic effect may exist among the agents used in this study. Also, a new study should employ a mouse hair loss system to verify the results achieved from the present *C. elegans* model study.

25.5 Conclusion

Considering hair loss causes adolescent to suffer devastating emotional stress, the significance of safe, effective anti-alopecia agent cannot be overstated. Development of screening systems for anti-alopecia agents is very important. This study used *C. elegans* to evaluate astressin-B, finasteride, and taurine in terms of anti-stress potential. All of them helped with restoring the affected vital signs in *C. elegans* which was subjected to ER stress. Among the three agents, taurine exerts better results and may be used as an anti-alopecia agent especially against the one caused by stress.

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Chapter 26
Protective Effect of Taurine on the Decreased Biogenic Amine Neurotransmitter Levels in the Brain of Mice Exposed to Arsenic

Xiaohui Liu, Fengyuan Piao, and Yachen Li

Abstract Arsenic (As) exposure has a toxic effect on the central nervous system, especially on learning and memory. Norepinephrine (NE), dopamine (DA), and serotonin (5-HT) play an important role in learning and memory function of the brain. In the present study, the protective effect of taurine on the disturbed biogenic amine neurotransmitter levels in the mouse brain induced by arsenic was examined. Sixty SPF mice were divided into three groups. The As exposure group was administered with 4 ppm As_2O_3 through drinking water for 60 days. The protective group was treated with both 4 ppm As_2O_3 and 150 mg/kg taurine. The control group was given drinking water alone. The levels of NE, DA, and 5-HT were determined by HPLC in the cerebrum and cerebellum of mice. Ultrastructure of synapses in brain tissue of mice was observed in these groups by transmission electron microscopy. The mRNA expressions of dopamine beta hydroxylase (DBH), tyrosine hydroxylase (TH), and tryptophan hydroxylase (TPH) as NE, DA, and 5-HT synzymes were also analyzed by real-time RT-PCR. The results showed that the concentrations of NE, DA, and 5-HT; the number of synaptic vesicles; and the expressions of TH, TPH, and DBH genes in the brains of mice exposed to As alone were significantly decreased. However, administration of taurine significantly alleviated the toxic effect on biochemicals detected in the experiment, compared with that in the brain of mice exposed to As alone. These results indicated that taurine was effective in counteracting the decreased biogenic amine neurotransmitter level and the mRNA expressions of their synzymes induced by arsenic.

X. Liu • F. Piao (✉) • Y. Li
Department of Occupational and Environmental of Health,
Dalian Medical University, Dalian, Liaoning 116044, China
e-mail: Piaofy_dy@yahoo.com.cn

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_26,

Abbreviations

As	Arsenic
DBH	Dopamine beta hydroxylase
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase
NE	Norepinephrine
DA	Dopamine
5-HT	Serotonin

26.1 Introduction

Taurine (2-aminoethanesulfonic acid), the end product of L-cysteine metabolism, is highly expressed in a variety of organs of most mammals, such as the brain, heart, and kidneys (Flora et al. 2007). The biological occurrence of taurine had been documented for more than a 100 years and was found to take part in many physiological functions, including neuromodulation, regulation of calcium-dependent processes, osmoregulation, thermoregulation, membrane stabilization, neurotransmission, and neuroprotection, specially detoxication in the organisms destroyed by pollutants (Pan et al. 2010). Recently, taurine was reported to be widely involved in protecting the central nervous system against the toxic effect induced by arsenic (As) (Das et al. 2009).

As is a ubiquitous element distributed in environment and millions of people are exposed to As worldwide (Abernathy et al. 1999). Chronic exposure to As has been associated with a wide range of illnesses, including gastrointestinal, respiratory, cardiovascular, neurological, genitourinary, endocrine, hematopoietic, and skin (Jomova and Valko 2011; Rios et al. 2009; Zarazua et al. 2010). The central nervous system is considered as one of the major targets by the fact that As exposure resulted in the deficits in the central nervous system not only in experimental animals but also in human (Wasserman et al. 2004; Wright et al. 2006). Our previous study also found that memory function was decreased in mice subchronic exposed to As (Wang et al. 2009). In recent years, the abnormal function in the brains of animals exposed to As were attributed to the disordered concentration of norepinephrine (NE), dopamine (DA), and serotonin (5-HT) disturbed by As (Tripathi et al. 1997; Delgado et al. 2000). NE, DA, and 5-HT were crucially involved in the control of behavioral processes related to exploration, anxiety, learning, and memory (Barnes and Sharp 1999; Myhrer 2003). However, whether the application of taurine could mediate the abnormal concentration of NE, DA, and 5-HT in the brains induced by As is still unclear. In the present study, the potential role of taurine in As that disturbed the concentration of NE, DA, and 5-HT in the brains of mice was explored.

26.2 Methods

26.2.1 Chemicals and Reagents

As_2O_3, NE, DA, and 5-HT were purchased from Sigma Chemical Company (St. Louis, USA). HPLC-grade methanol was supplied by Sinopharm Chemical Reagent Co., Ltd. Analytical grade sodium acetate was bought from Shanghai Chemical Agent Cooperation (Shanghai, China). PrimeScriptTM RT reagent Kit and SYBR® Premix Ex TaqTM kit were supplied by TaKaRa (Dalian, China). Other reagents were commercially available.

26.2.2 Animals and Treatment

Sixty SPF mice (7 weeks old, 26.3–30.9 g) were supplied by Experimental Animal Center, Dalian Medical University in China. The animals were caged under a 12 h dark–light cycle in standard conditions of temperature (18–22°C) and humidity (50%). The mice were randomly divided into three groups after 7 days acclimatization. Groups 1 and 2 were administrated with drinking water alone as control or 4 ppm As_2O_3 ad libitum for 60 consecutive days, respectively. The protective group was treated with both 4 ppm As_2O_3 and 150 mg/kg taurine. The protocol was performed in accordance with the Animal Guideline of Dalian Medical University and in agreement with the Ethical Committee of Dalian Medical University.

26.2.3 Ultrastructure of Synapses

For the synapses ultrastructure, brain tissues of mice treated with drugs were prepared after the final treatment. Then the tissues were immediately fixed in a 3% glutaraldehyde solution in PBS (pH 7.2) overnight at 4°C and washed in 0.15 M PBS for 30 min. Then the samples were postfixed for 2 h in 0.1 M PBS osmium tetroxide (1%) solution at 4°C, dehydrated in graded EtOH, and embedded in Araldite (Fluka, Buchs, Switzerland). The ultrathin sections were contrasted using both lead citrate plus uranyl acetate. And the samples were observed by transmission electron microscopy (TEM) (JEM-2000EX, Olympus, Japan).

26.2.4 Quantification of Monoamine Neurotransmitter Levels by HPLC

The brains of mice in the experiment were removed and stored at −80°C after the final treatment. Then the tissues were weighed before homogenizing with

perchloric acid (0.1 mol/L) and centrifuging twice at 10,000×r/min for 10 min at 4°C. Before detection, the supernatant was collected and filtered through 0.45 μm Acrodisc filter. Each sample (20 μl) was injected into an HPLC system (Waters 1525) with fluorescence detector (EICOM, Kyoto, Japan) with an ODS column (EICOMPAC SC-5 3.0 mm×150 mm; EICOM Inc., Kyoto, Japan). The mobile phase was sodium acetate 0.1 mm/L EDTA·2Na (pH 5.0)/methanol (95%/5%). The flow rate was kept constant at 1.0 ml/min and the temperature of the column was 25°C. The levels of NE, DA, and 5-HT were measured. Each of the standard solutions of the three monoamine neurotransmitters was prepared at a concentration of 0.1 ng/ml. Each standard (20 μl) was analyzed by HPLC, and the standard chromatographic peaks per 1 ng for each sample were obtained. The amount of each monoamine was determined with peak-area ratios using HPLC chromatogram analysis software, eDAQ Power Chrom (eDAQ, New South Wales, Australia).

26.2.5 Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the brain parts of each mouse in each group using RNAiso Plus reagent (TaKaRa) according to the instructions of the manufacture. The yield of total RNA was determined by measuring the absorption at 260 and 280 nm separately, and 500 ng of which was in reverse transcribed to first strand cDNA in a 10 μl reaction volume. Reverse transcription was performed using the PrimeScript® RT Master Mix Perfect Real Time bought from TaKaRa (Dalian, China) according to the instructions. Briefly, 2 μl 5× PrimeScript® RT Master Mix with 3 μl RNase-free water and 5 μl total RNA was added in the reaction system. And the mixture was incubated at 37°C for 15 min. Then the reaction was stopped by heating at 85°C for 5 s. The solution for cDNA was stored at 4°C until use.

26.2.6 Real-Time Reverse Transcription-Polymerase ChainReaction

Real-time reverse transcription-polymerase chain reaction (Real-Time RT-PCR) was performed according to the SYBR® Premix Ex TaqTM kit bought from TaKaRa (Dalian, China). The primers are based on the sequences from NCBI database. The primers of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH), and dopamine-β-hydroxylase (DBH) are designed by TaKaRa Biotechnology Company (Japan), and the related important information of these primers is presented in Table 26.1. The PCR programmer consisted of an initial denaturation at 95°C for 30 s, followed by 40 PCR cycles: 95°C for 5 s and 60°C for 30 s with Thermal Cycler Dice® Real Time System

Table 26.1 Primer sequence for the real-time transcription-polymerase chain reaction used in the experiment

Gene name	Accession number	Sequence of the primer (5′-3′)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM 008084	F:TGTGTCCGTCGTGGATCTGA R:TTGCTGTTGAAGTCGCAGGAG
Tyrosine hydroxylase (TH)	NM 009377	F:TCTCAGAGCAGGATACCAAGCA R:GCATCCTCGATGAGACTCTGC
Tryptophan hydroxylase (TPH)	NM 009414	F:TTGGGCTGTGCAAACAAGATG R:TGTTTACAGGCAATCTTGGGATCA
Dopamine-β-hydroxylase (DBH)	NM 138942	F:TCCATCTGGATTCCCAGCAAG R:ATGCAGGCCTGAGGTGTTGA

(TaKaRa Code:TP800). At the end of the cycles, melting temperatures of the PCR products were determined to be between 57 and 95°C. GAPDH mRNA was used as an internal control to measure the relative quantitation of the expression of the target genes. Data were analyzed by comparative threshold cycle method (ΔΔCT). Then, the relative expression level of tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH), and dopamine-β-hydroxylase (DBH) were normalized against the expression of GAPDH.

26.2.7 Statistical Analysis

Data were analyzed using SPSS program version 10.0 (SPSS Inc., Chicago, IL). Values are presented as means ± SD. The analysis of variance (ANOVA) test was used to compare the means of different groups of data, followed by LSD and Dunnett's T3 test. $P < 0.05$ was considered to be significant.

26.3 Results

26.3.1 Effect of Taurine on Synaptic Ultrastructure in the Brains of Mice Exposed to As

The ultrastructure of synapse in the brain in the experiments was observed in Fig. 26.1. Abundant synaptic vesicles in synapse of mouse brains were shown in the controls (Fig. 26.1a), while the less synaptic vesicles were observed in the brain of mice exposed to 4 ppm As_2O_3 (Fig. 26.1b). The synaptic vesicles in the synapse of mouse brains exposed to 4 ppm As_2O_3 and 150 mg/kg taurine were more than that in the group exposed to 4 ppm As_2O_3 alone (Fig. 26.1c).

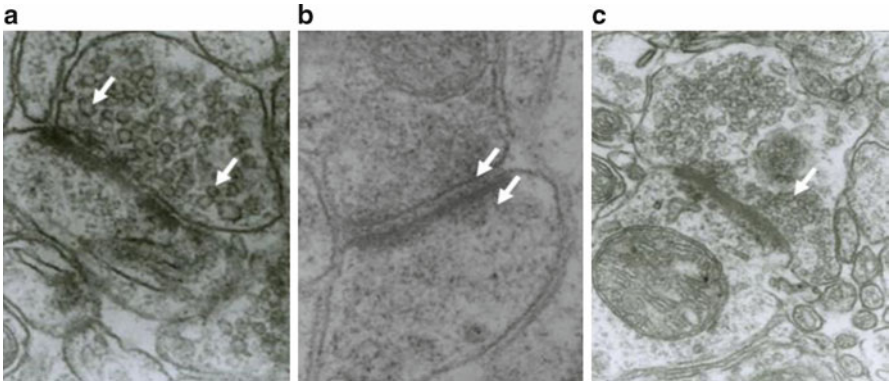

Fig. 26.1 The representative picture of ultrastructure changes of the brain by transmission electron microscope after arsenic or arsenic and taurine exposure for 60 days. The magnification for the picture is 10^5×. The *arrows* are the place of synaptic vesicle. (**a**) Normal structure of synapse in the brain. (**b**) Ultrastructure of synapse in the brain of mice exposed to 4 ppm arsenic. (**c**) Ultrastructure of synapse in the brain of mice exposed to 4 ppm arsenic and 150 mg/kg taurine

26.3.2 Effect of Taurine on Concentrations of Monoamine Neurotransmitters in the Brains of Mice Exposed to As

The effect of taurine on concentrations of monoamine neurotransmitters in the brain of mice exposed to As was shown in Table 26.2. The concentrations of NE, DA, and 5-HT both in the cerebrum and cerebellum of mice were significantly lower in As-treatment groups than those in the controls ($P<0.05$). However, the concentrations of NE, DA, and 5-HT in the cerebellum of mice received 4 ppm As_2O_3 and 150 mg/kg taurine were significantly increased than that in group exposed to 4 ppm As_2O_3 alone ($P<0.05$). The similar results were also found in the cerebrum, except the concentration of 5-HT.

26.3.3 Effect of Taurine on the mRNA Expressions of DBH, TH, and TPH in the Brains of Mice Exposed to As

The mRNA expressions of NE, DA, and 5-HT synzymes in the brains of mice were shown in Figs. 26.2 and 26.3. The mRNA expressions of DBH, TH, and TPH in the cerebrum of mice received 4 ppm As_2O_3 were significant lower than those in control in Fig. 26.2 ($P<0.01$), which were decreased about 52, 33, and 50% respectively, compared to controls. And the mRNA expressions of DBH and TH in the cerebrum of mice that received 4 ppm As_2O_3 and 150 mg/kg taurine were significantly increased than that in group exposed to 4 ppm As_2O_3 alone ($P<0.01$).

In Fig. 26.3, the expressions of TPH genes in the cerebellum of mice were significant lower in the group exposed to 4 ppm As_2O_3 than those in controls

Table 26.2 Content of three monoamine neurotransmitters in the brain of mice administered with As or As and taurine for 60 days

		Cerebrum			Cerebellum		
Groups	*n*	NE	DA	5-HT	NE	DA	5-HT
Control	6	169.90 ± 34.40	153.54 ± 24.79	78.67 ± 28.90	113.38 ± 16.36	27.18 ± 7.31	89.05 ± 10.60
4 ppm	6	37.57 ± 9.36[a]	62.68 ± 18.08[a]	57.73 ± 15.75[a]	1.88 ± 0.56[a]	4.44 ± 0.30[a]	39.35 ± 8.62[a]
4 ppm + Taurine	6	74.01 ± 15.73[b]	135.47 ± 13.93[b]	67.58 ± 8.81	37.75 ± 6.98 [b]	12.54 ± 0.89[b]	61.41 ± 14.09[b]

Values are mean ± SD as ng/g of tissue (wet weight). *NE* norepinephrine, *DA* dopamine, *5-HT* serotonin. Different from control group, [a]$P < 0.05$; Different from 4 ppm group, [b]$P < 0.05$

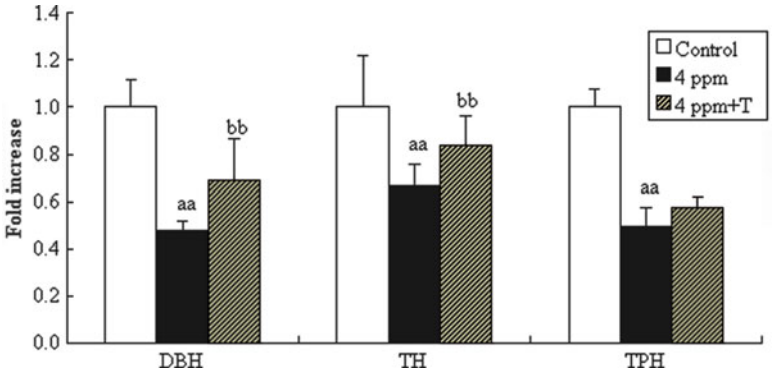

Fig. 26.2 Fold changes in DBH, TH, and TPH mRNA expressions in mice's cerebrum exposed to 4 ppm arsenic or 4 ppm arsenic and 150 mg/kg taurine for 60 days. The number for each experiment was 6. *T* is short for taurine. Data are presented as mean ± SD. Different from control group, $^{a}P<0.05$, $^{aa}P<0.01$; Different from 4 ppm group, $^{b}P<0.05$, $^{bb}P<0.01$

($P<0.05$), specially for the expression of DBH and TH ($P<0.01$). The mRNA expressions of TH, TPH, and DBH in the group received 4 ppm As_2O_3 decreased about 90, 74, and 79% respectively, compared to controls. And the mRNA expressions of DBH, TH, and TPH in the cerebellum of mice that received 4 ppm As_2O_3 and 150 mg/kg taurine were significantly increased than that in group exposed to 4 ppm As_2O_3 alone ($P<0.05$).

26.4 Discussion

The paper exhibited the protective effect of taurine on the decreased biogenic amine neurotransmitter levels in the brains of mice exposed to As through reducing the mRNA expressions of their synzymes.

Being a detoxicant, taurine was proved to protect the central nervous system against the toxic effect induced by a number of pollutants, such as Pb, Mn, and bilirubin (Fan et al. 2009; Gao et al. 2011; Zhang and Huang 2008). Although the protection of taurine against As-induced neurotoxicity was also investigated, the underlying mechanism was still poorly understood. Ma et al. reported that administration of arsenic with taurine could alleviate DNA damage of neurons in the brain caused by arsenic through the RNS signal pathway (Ma et al. 2010). The result was according with the findings by other labs. Das et al. also found that oral administration of taurine was very effective in the prevention of As-induced oxidative impairment in the brain tissue of the experimental rats (Das et al. 2010). These results mentioned above indicated that the protection role of taurine was as an antioxidant in the oxidative stress induced by As. However, the discussed protection mechanisms of taurine included not only counteracting oxidative stress but also the promotion of neuron, enhancement of the expression of c-fos, activating the calcium

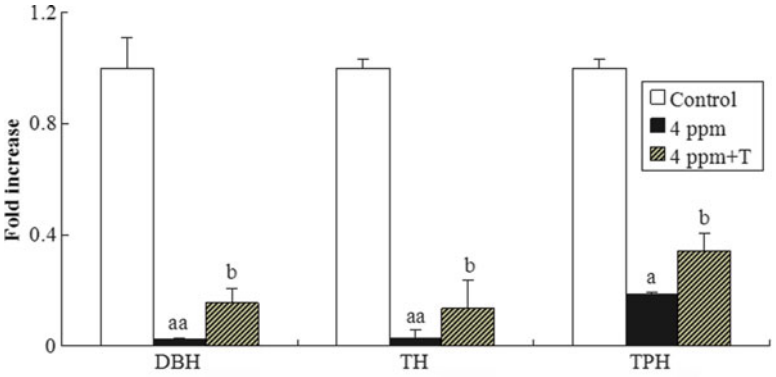

Fig. 26.3 Fold changes in DBH, TH, and TPH mRNA expressions in mice's cerebellum exposed to 4 ppm arsenic or 4 ppm arsenic and 150 mg/kg taurine for 60 days. The number for each experiment was 6. *T* is short for taurine. Data are presented as mean ± SD. Different from control group, $^{a}P<0.05$, $^{aa}P<0.01$; Different from 4 ppm group, $^{b}P<0.05$, $^{bb}P<0.01$

signaling, and specially neurotransmission (Iio et al. 2012; Junyent et al. 2009; Ozan et al. 2012; Pan et al. 2010). It was reported that the deficient in behavior induced by As might be closely related to the disordered concentration of NE, DA, and 5-HT in the central nervous system (Delgado et al. 2000). Whether the application of taurine could also alleviate the disturbed concentration of NE, DA, and 5-HT induced by As, and the potential mechanism for taurine intervening the concentration of NE, DA, and 5-HT induced by As are still unknown.

The results in the present study showed that the concentration of NE, DA, and 5-HT in the cerebellum exposed to As and taurine were significantly increased, compared with that in the brains exposed to As alone. The similar results could also be found in the cerebrum of mice coadministered As with taurine subchronically, except that the tendency for the increased concentration of 5-HT wasn't significant. Neurotransmitters are mainly stored in the synaptic vesicles, and the ultrastructure change of synapse was also observed in the present study. The results showed that the number of synaptic vesicles was also increased in the brains exposed to As and taurine together. These results indicated that taurine also had a protective effect on the decreased concentration of NE, DA, and 5-HT in the brains induced by As. It was reported that treatment of realgar, the main element is As, could decrease the amino acid neurotransmitters in the brains of rats, whereas the concentration of taurine wasn't affected (Huo et al. 2012). And extracellular taurine addition could activate the glycine receptor and the specific taurine transporter, increase the efflux of DA, following the release of other neurotransmitters in the central nervous system (Chepkova et al. 2002; Chepkova et al. 2005). These might be the reasons that taurine had a protective effect on the As induced the decreased concentration of neurotransmitters in the brains in the present study.

It was reported that dopamine beta hydroxylase (DBH), tyrosine hydroxylase (TH), and tryptophan hydroxylase (TPH) are the rate-limiting enzymes for the synthesis of NE, DA, and 5-HT, respectively (Szot et al. 1996). In order to further

disclose the mechanism underlying the protective role of taurine against the decreased level of neurotransmitter induced by As, the key synzymes in the synthesis of neurotransmitters were examined. And the results in the present study showed that the expression of DBH, TH, and TPH mRNA were significantly increased in the cerebellum of mice exposed to As and taurine together, except that the changed in TPH mRNA in the cerebrum wasn't significant. These results indicated that taurine also had a protective effect on the decreased biogenic amine neurotransmitter levels in the brain of mice exposed to As through reducing the expression of DBH, TH, and TPH mRNA. In fact, the protect effect of taurine on the moleculars at the translation level disturbed by As in the central nervous system had also been reported. Hong et al. showed that the decreased expression of Sdha mRNA in the brains of mice exposed to As could be partially rescued by coadministered with taurine (Hong et al. 2009). However, the expression of Camk4 mRNA in the brains of mice exposed to As wasn't affected with the intervention of taurine in the same experiment (Wang et al. 2009). Anyway, the exact mechanism about the effect on the translation level induced by taurine needs to be further explored.

26.5 Conclusion

In summary, the present study showed that subchronic As exposure decreased the concentration of NE, DA, and 5-HT; the number of synaptic vesicles; and the expressions of TH, TPH, and DBH genes in the brains of mice. And the toxic effect induced by As could be intervened by the coadministration of taurine. These results indicated that taurine might have the protective effect on the decreased biogenic amine neurotransmitter levels and the downregulated gene expressions of their synzymes in the brain of mice exposed to As.

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Part III
Roles of Taurine in Reproduction, Development and Differentiation

Chapter 27
Differential Regulation of *TauT* by Calcitriol and Retinoic Acid via VDR/RXR in LLC-PK1 and MCF-7 Cells

Russell W. Chesney and Xiaobin Han

Abstract The interaction between taurine and the absorption of fat-soluble vitamins, such as vitamin A and D, has been an interesting topic in the field of nutrition science, because taurine-conjugated bile acid optimizes fat and fat-soluble vitamin absorption. However, whether the hormone calcitriol (1,25-dihydroxyvitamin D_3) and retinoic acid regulate the expression of the *TauT* gene is unknown. In this study, we test the hypothesis that the *TauT* gene is regulated by vitamin D_3 (VD_3) and retinoic acid (RA) via activation of the vitamin D receptor (VDR) and retinoic acid receptor (RXR). Taurine uptake, Western blotting, gene reporter assay, and immunohistochemical analysis of TauT, VDR, and RXR were used in VD_3- and/or RA-treated LLC-PK1 and MCF-7 cells. We demonstrated that VD_3 alone had little effect on *TauT* expression in both LLC-PK1 and MCF-7 cells. Expression of *TauT* was significantly increased by RA, which was synergized by the addition of VD_3 after RXR activation in LLC-PK1 cells. In contrast, expression of *TauT* was significantly decreased by the combination of VD_3 and RA in MCF-7 cells. Regulation of *TauT* by VD_3/RA appears to occur at the transcriptional level, as determined by a reporter gene assay of the *TauT* promoter. Immunohistochemical study showed that VDR and RXR were activated by VD_3 and RA, respectively, in both LLC-PK1 and MCF-7 cells. The activated VDR and RXR also colocated in nuclei of both cells, suggesting that a VDR/RXR complex is involved in the transcriptional regulation of *TauT*. Our results show that expression of TauT is differentially regulated by VD_3 and RA via formation of VDR and RXR complexes in the nuclei in a cell type-dependent manner.

R.W. Chesney, M.D. (✉) • X. Han, M.D., Ph.D.
Division of Nephrology, Department of Pediatrics,
University of Tennessee Health Science Center,
Le Bonheur Children's Hospital, Children's Foundation Research Tower,
Rm 476R, 50 North Dunlap,
38103 Memphis, TN, USA
e-mail: rchesney@uthsc.edu; xhan@uthsc.edu

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_27,

Abbreviations

TauT	Taurine transporter gene
VDR	Vitamin D receptor
RXR	Retinoic acid receptor

27.1 Introduction

Taurine is necessary to enhance the intestinal absorption of fat-soluble vitamins, including vitamins A and D (Zamboni et al. 1993). Taurine, along with glycine, is used to conjugate bile acids and promote lipid solubility across intestinal epithelial surfaces (Hepner et al. 1973). We have recently shown that a contributing factor in the pathogenesis of rickets in hand-reared polar bear cubs is a reduction in dietary intake of taurine when an artificial cow milk-based formula is fed to the pups as a milk replacer (Chesney et al. 2009). The milk of free-ranging, lactating polar bear sows contains up to 80-fold higher taurine content than cow milk formula, probably because of the taurine-rich marine seal and whale diet of the pregnant sow (Hedberg et al. 2011). This interaction between intracellular taurine and the fat-soluble vitamins led us to speculate that these vitamins might be critical for taurine handling. Accordingly, we asked whether these vitamins influenced the synthesis or activity of the taurine transporter protein (TauT). This article focuses upon vitamin D and its possible role in taurine transport.

Vitamin D deficiency in man is associated with aminoaciduria, including taurinuria (Brodehl et al. 1971; Fraser et al. 1967; Scriver 1974). This aminoaciduria has been ascribed to the effect of secondary hyperparathyroidism (Chesney and Harrison 1975; Scriver 1974) because vitamin D deficiency is associated with elevated serum values of parathyroid hormone (PTH). Urinary cAMP is also increased in vitamin D deficiency and has been felt to possibly influence renal amino acid reabsorption, potentially by interacting with renal proximal tubule cell membranes (Harrison 1979). However, when isolated renal brush border membranes were co-incubated with varying concentrations of cAMP, there was no influence on taurine uptake by vesicles (Dabbagh et al. 1989). In rats made vitamin D deficient, the uptake of taurine into these brush border membrane vesicles, representing the uptake phase of taurine accumulation by the renal proximal tubule, was diminished (Dabbagh et al. 1990). This vitamin D deficiency-induced reduction in taurine uptake was evident despite the calcium and/or phosphate content of the diet and at quite different values of PTH in rat serum. Put differently, reduced taurine uptake was independent of PTH secretion and its ambient circulating levels. These results also suggest a role for vitamin D in renal taurine handling.

What is vitamin D and how could it influence gene action and protein/peptide synthesis? Vitamin D is a secosteroid compound that is present in certain foods (bony fish, yeast) or that is formed within the skin (DeLuca 2008). Regardless of

origin, vitamin D must undergo further metabolism before it is active (DeLuca 2004). The vitamin compound undergoes a hepatic 25-hydroxylation at carbon 25 to yield $25(OH)D_3$, and then, through the action of 1α-hydroxylase (Cyp 27B1) in the kidney, it is changed to the most biologically active form, $1,25(OH)_2D_3$ (calcitriol). This compound, sometimes termed the hormonal form of vitamin D, acts by means of a nuclear receptor (VDR) to carry out its biologic functions. Among these are the regulation of body calcium homeostasis, a role in the immune system, and a protective function in some types of cancer (DeLuca 2008).

Following binding of $1,25(OH)_2D_3$ to a VDR, the complex undergoes heterodimerization with either the retinoic acid receptor (RAR) or the retinoid X receptor (RXR) to enter into the nucleus of the cell. This RXR/VDR complex, which contains RA, or all-*trans* retinoic acid (atRA) and $1,25(OH)_2D_3$ bound to their respective receptors, then binds to specific sites on the promoter regions of genes that code for at least 200–300 proteins and peptides (Adams and Hewison 2010; Walker and Modlin 2009). The details of the vitamin D response element involving an RNA polymerase II mechanism and its interaction with captivators (CoA) and transcriptional factor IIb (B) are reviewed elsewhere (Dusso et al. 2004). This process is responsible for the transcriptional regulation of proteins/peptides involved in calcium and phosphate homeostasis, cell proliferation, cell differentiation, and immune function; in other words, vitamin D functions (DeLuca 2008).

Examples of the role of vitamin D and its most active metabolite, $1,25(OH)_2D_3$, are its pivotal place in bone mineralization and its role in innate immunity. The calcium–vitamin D–parathyroid hormone–endocrine axis is a feedback loop controlled system in which $1,25(OH)_2D_3$ produced in the kidney enhances calcium and phosphate absorption by the intestine, calcium mobilization in bone, and renal distal tubular calcium reabsorption. All of these normalize the extracellular concentrations of calcium and phosphate and allow appropriate mineralization of bone (DeLuca 2004). With regard to innate immunity, macrophages take up $25(OH)D_3$ by endocytosis, and when Toll-like receptors in the plasma membrane are stimulated by an appropriate ligand, $1,25(OH)_2D_3$ is synthesized locally by a Cyp 27B1 enzyme that is not under the classical feedback regulation that exists in the kidney. This $1,25(OH)_2D_3$ produced in the macrophage then binds to its receptor and increases the synthesis of the antimicrobial peptide cathelicidin (LL-37), which is a rapidly acting agent that affects microbial plasma membranes such as *Mycobacterium tuberculosis* or *Escherichia coli*.

Our group has examined many transcriptional factors that either up- or downregulate the *TauT* gene (Han and Chesney 2010). Among these factors are p53, WT1, c-Jun, E2, tonicity response binding protein, and factors that act through AP1 and taurine per se (Chesney et al. 2011). The promoter region of *TauT* also contains several important binding sites for these ligands, including p53, c-Myb, AP1, WT1, TonE, and SP1. Certain of these signals interact with appropriate binding sites and upregulate TauT protein synthesis such that more taurine is accumulated within the cell by an active process. Because intracellular taurine abundance may be a factor in optimal conjugation of bile acids to promote vitamin A and D absorption by the intestine, we sought evidence for a vitamin D response element in the *TauT* gene.

27.2 Materials and Methods

27.2.1 Cell Lines and Cell Culture

HK293, MDCK, LLC-PK1 renal cells, and MCF-7 human breast cancer cells were obtained from the American Type Culture Collection. The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 50 units/ml penicillin, and 50 μg/ml streptomycin. All cells were maintained in a humidified 37°C incubator with 5% $CO_{2,}$ fed every 3 days with a complete medium, and subcultured when confluence was reached.

27.2.2 Measurement of Taurine Transport

Taurine transport studies were performed on confluent monolayers 3 days after seeding cells. The cells were rinsed with Earle's balanced salt solution (EBSS) at 37°C. Uptake was initiated by the addition of an uptake buffer (2 mM KCl, 1 mM $MgCl_2$, 96 mM NaCl, 1.8 mM $CaCl_2$, 5 mM Hepes, pH 7.6) to which 50 μM unlabelled taurine and 0.5 μCi/ml ^{14}C-taurine (Perkin Elmer, Boston, MA) were added. After a 30-min period of incubation at room temperature, uptake was terminated by the removal of uptake buffer followed by three rapid washes with cold EBSS. Cells were dissolved in 1% SDS in 0.2 N NaOH and radioactivity was counted in a Packard 2000-CA Liquid Scintillation Analyzer.

27.2.3 Western Blot Analysis

Cells were lysed in 50 μl M-PER mammalian protein extraction reagent (Pierce, Inc., Rockford, IL) supplemented with a protease inhibitor cocktail for use with mammalian cell and tissue extracts (Sigma). The lysates were cleared by centrifugation at 14,000 × *g* for 2 min, and the supernatants were transferred to clean tubes. Equal amounts of protein (50 μg) were separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore, Bedford, MA) using a semi-dry electrophoretic transfer system (Bio-Rad). Membranes were incubated in 5% nonfat dry milk in Tris base/sodium chloride (TBS) buffer with 0.2% Tween 20 (TBST) at 4°C overnight. The membranes were incubated with primary antibodies for 1 h at room temperature. Blots were washed with TBST and incubated with horseradish peroxidase-linked secondary antibody (Sigma) for another hour, and then proteins of interest were detected using a chemiluminescence detection kit (Pierce, Inc.).

27.2.4 Immunohistochemistry

Cells were cultured directly on sterile cover slips (Sigma) that were placed into 6-well tissue culture plates for 2 days. The medium was removed from cells, which were rinsed once with 1× PBS at room temperature. The cells were fixed for 10 min at room temperature in 3.7% buffered formaldehyde and washed once in 1× PBS. Fixed cells were dehydrated by immersing in 70, 95, and 100% ethanol for 5 min each followed by air drying for 10 min each. Samples were rehydrated in decreasing ethanol series (100, 95, and 70%) for 5 min each. Then samples were immersed in 1× PBS for 5 min at room temperature. The slides were immersed in quenching solution (3% H_2O_2 in methanol) for 5 min and then washed twice in dH_2O for 10 min. Slides were blocked for 20 min with the blocking buffer. Primary antibodies (antibody against VDR, RXR) were applied to slides and incubated for 1 h. Slides were washed for 10 min with PBS, treated with a secondary antibody, and incubated for 1 h. Afterwards the slides were rinsed with PBS for 10 min.

27.2.5 Statistical Analysis

All experiments using tissue cultures were performed in triplicate. Statistical comparisons were made using one-way ANOVA and Student's *t*-test to determine significant differences in the means between experimental groups.

27.3 Results

27.3.1 Regulation of Expression of *TauT* by 1, 25(OH)$_2$D$_3$ and Retinoic Acid (RA) in Renal Cells

To investigate whether 1,25(OH)$_2$ D$_3$ regulates *TauT* expression, LLC-PK1, MDCK, and 293 renal cells were transfected with a full-length *TauT* promoter–reporter gene (p923) and cultured for 24 h in the presence or absence of 1,25(OH)$_2$D$_3$ (10 nM). As shown in Fig. 27.1a, LLC-PK1 and 293 cells showed higher reporter gene expression than did MDCK cells. Treatment with 1,25(OH)$_2$D$_3$ had no influence on the activity of the *TauT* promoter in all three lines of renal cells.

We then used LLC-PK1 cells to test the dose response of the *TauT* promoter to 1,25(OH)$_2$D$_3$, because it is known that LLC-PK1 cells express vitamin D receptors (VDRs) (Barletta et al. 2004). Again, we found that there was no dose–response of the *TauT* promoter to the 1,25(OH)$_2$D$_3$ (0–20 nM) treatment (Fig. 27.1b). However, taurine uptake was elevated after 9-*cis* RA and all-*trans* retinoic acid (atRA) addition

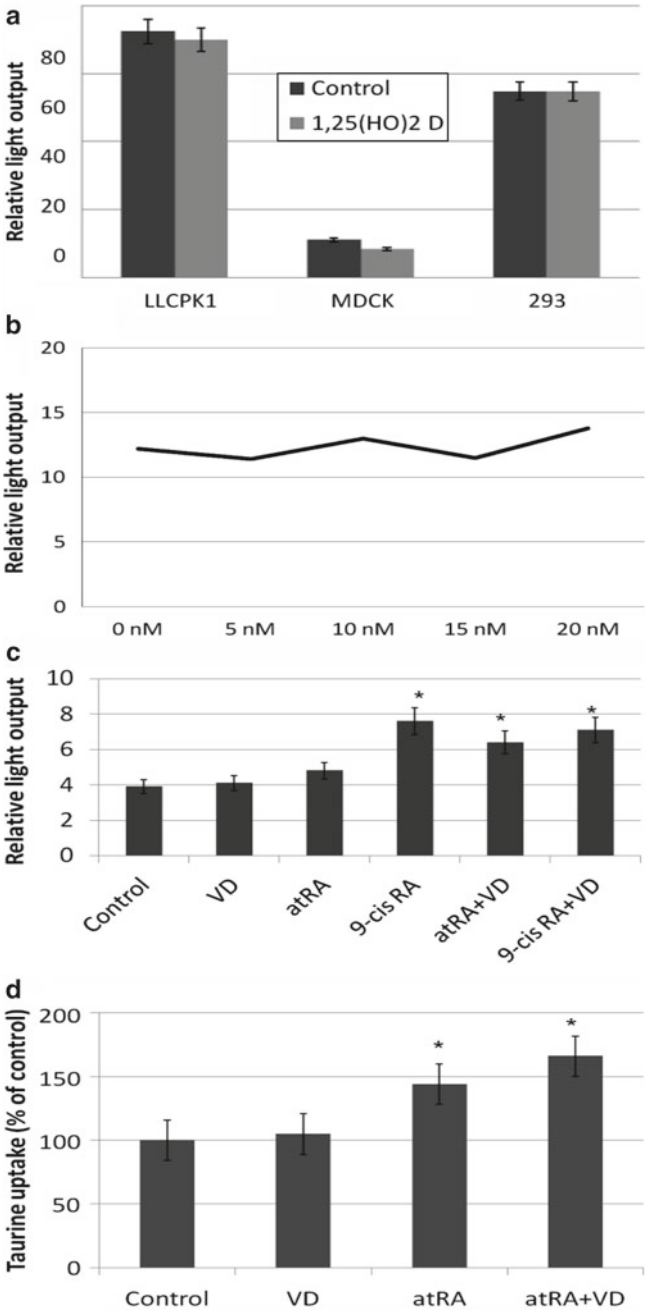

Fig. 27.1 Effect of $1,25(OH)_2D_3$ and RA on TauT expression in renal cells. Cells were cultured in the presence of $1,25(OH)_2D3$ (VD) and retinoic acid (RA) for 1–3 days. (**a**) Reporter gene assay in LLC-PK1, MDCK, and 293 cells. (**b**) Reporter gene assay in LLC-PK1 cells with different doses of VD. (**c**) Synergetic effect of VD/RA on *TauT* promoter activity in LLC-PK1 cells. (**d**) Taurine uptake by LLC-PK1 cells treated with VD, atRA, or both. (**e**) Western blot analysis of *TauT* expression. (**f**) Relative density of Western blot

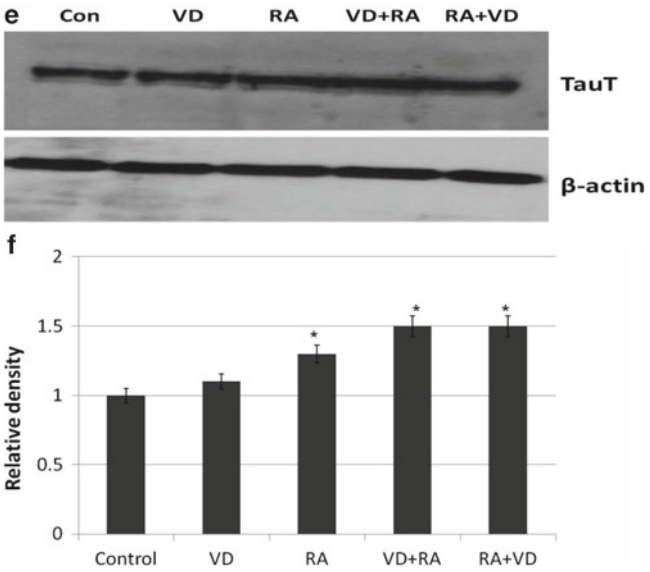

Fig. 27.1 (continued)

to LLC-PK1 cells, and the effect was synergized by addition of $1,25(OH)_2D_3$ after treatment with atRA for 3 days. This synergetic effect was not observed in the cells treated with 9-*cis* RA and $1,25(OH)_2D_3$ (Fig. 27.1c). These observations were confirmed by enhanced taurine uptake and Western blot analysis in the LLC-PK1 cells treated with $1,25(OH)_2D_3$ with or without RA (Fig. 27.1d, e, f).

27.3.2 Activation of Retinoic Acid and Vitamin D Receptors by RA and/or Vitamin D in LLC-PK1 Kidney Cells

To study if RA and/or $1,25(OH)_2D_3$ activate RXR and/or VDR, which in turn regulate *TauT* expression in LLC-PK1 renal cells, we treated the cells with RA and/or $1,25(OH)_2D_3$ as described above. As shown in Fig. 27.2a, LLC-PK1 cells express both RXR and VDR, which are upregulated by RA and/or $1,25(OH)_2D_3$. Expression of *TauT* was enhanced after activation of RXR or VDR, respectively. Expression of *TauT*, RXR, and VDR was further elevated in the cells treated with RA and $1,25(OH)_2D3$ (Fig. 27.2a, b).

Western blot analysis showed that expression of RXR was increased by RA, and addition of $1,25(OH)_2D_3$ after 3 days of pretreatment with RA induced expression of a 35 kDa RXR subunit in LLC-PK1 cells. Addition of $1,25(OH)_2D_3$ alone or pretreatment of cells with $1,25(OH)_2D_3$ had a slight effect on RXR expression.

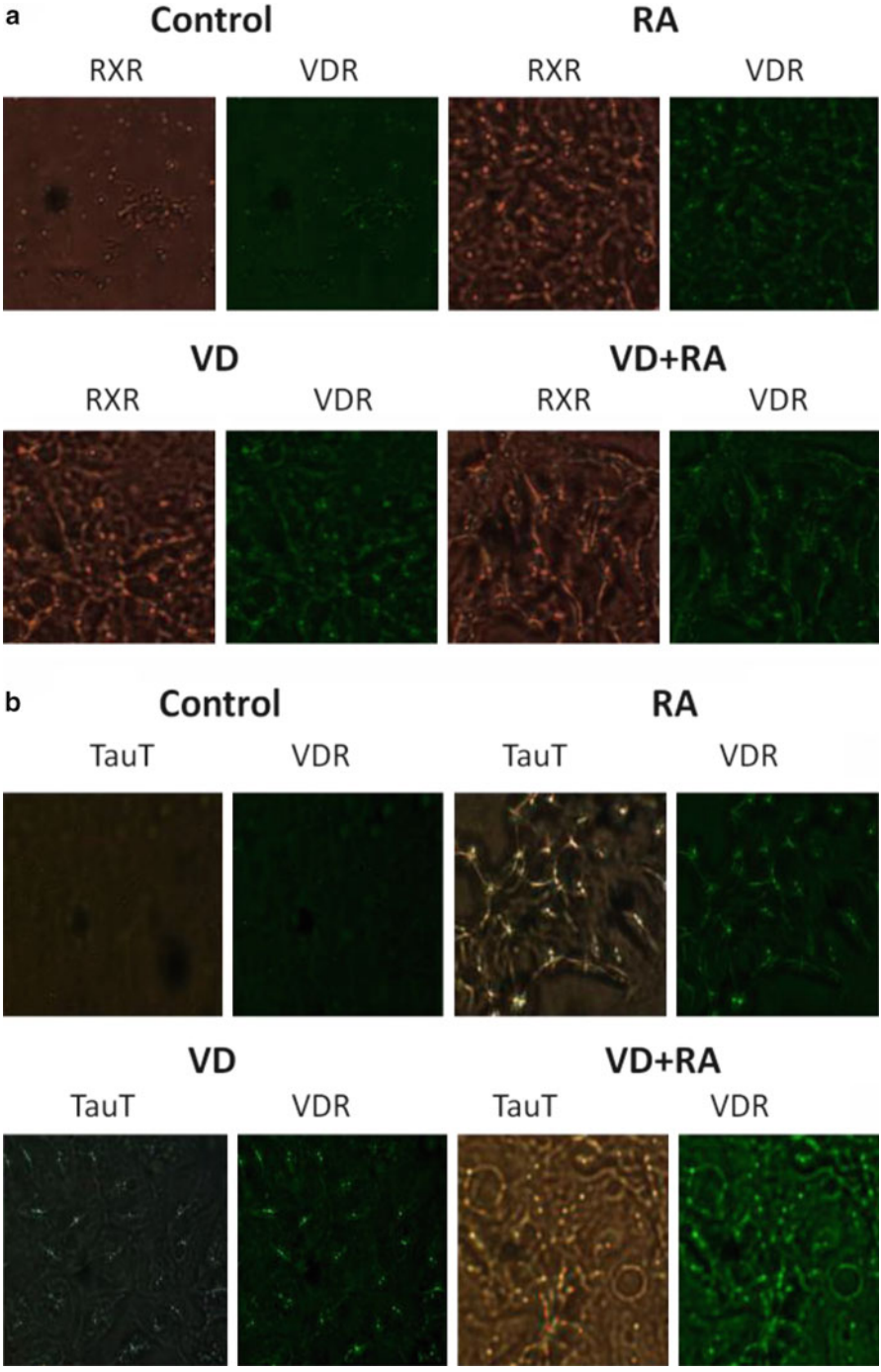

Fig. 27.2 Regulation of *TauT* by VDR and RXR activation in LLC-PK1 cells. Cells were treated with VD, RA, or VD plus RA to activate VDR or RXR. (**a**) Immunohistochemistry of RXR and VDR after treatment of LLC-PK1 cells with VD, RA, or VD plus RA, respectively. (**b**) Upregulation of *TauT* by VD, RA, or VD plus RA in LLC-PK1 cells. (**c**) Activation of RXR by RA or RA plus VD. (**d**) Transcription regulation of *TauT* by VDR/RXR complex

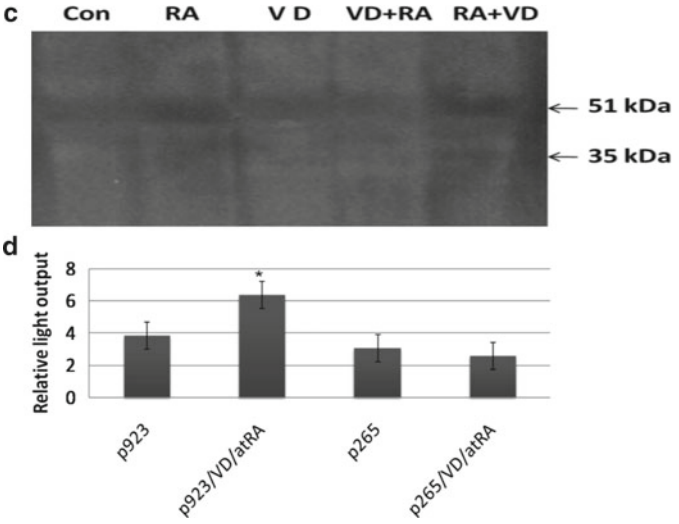

Fig. 27.2 (continued)

Deletion of regulatory region of the *TauT* promoter, which contains several transcription factors (including two AP1 sites), abolished the effect of RA/1,25(OH)$_2$D$_3$ on *TauT* expression. These findings suggest that the synergetic regulation of *TauT* expression by 1,25(OH)$_2$D$_3$ and RA may require the formation of the RXR/VDR complex, which in turn binds to the *TauT* promoter region to regulate *TauT* expression. Activation of RXR by RA appears to be critical for upregulation of *TauT* in LLC-PK1 cells.

27.3.3 *Regulation of TauT Expression by 1,25(OH)$_2$D$_3$ and RA in MCF-7 Cells*

To study whether 1,25(OH)$_2$D$_3$ and/or RA plays a role in *TauT* regulation in MCF-7 cells, the approaches used were similar to those described for the LLC-PK1 cells experiments. As shown in Fig. 27.3, 1,25(OH)$_2$D$_3$ or atRA alone showed only a slight effect on the expression of *TauT*. However, a combination of 1,25(OH)$_2$D$_3$ and atRA significantly decreased expression of *TauT* (as determined by reporter gene assay; Fig. 27.3a), taurine uptake (Fig. 27.3b), and Western blot analysis (Fig. 27.3c, d). Interestingly, 9-cis RA showed little effect on *TauT* expression with or without 1,25(OH)$_2$D$_3$ in MCF-7 cells (Fig. 27.3f), suggesting that regulation of *TauT* by 9-*cis* RA or atRA occurs via different mechanisms in MCF-7 cells compared to LLC-PK1 cells. A summary of regulation of the *TauT* gene by 1,25(OH)$_2$D$_3$ and RA in LLC-PK1 and MCF-7 cells is shown in Table 27.1 and further depicted in Fig. 27.4.

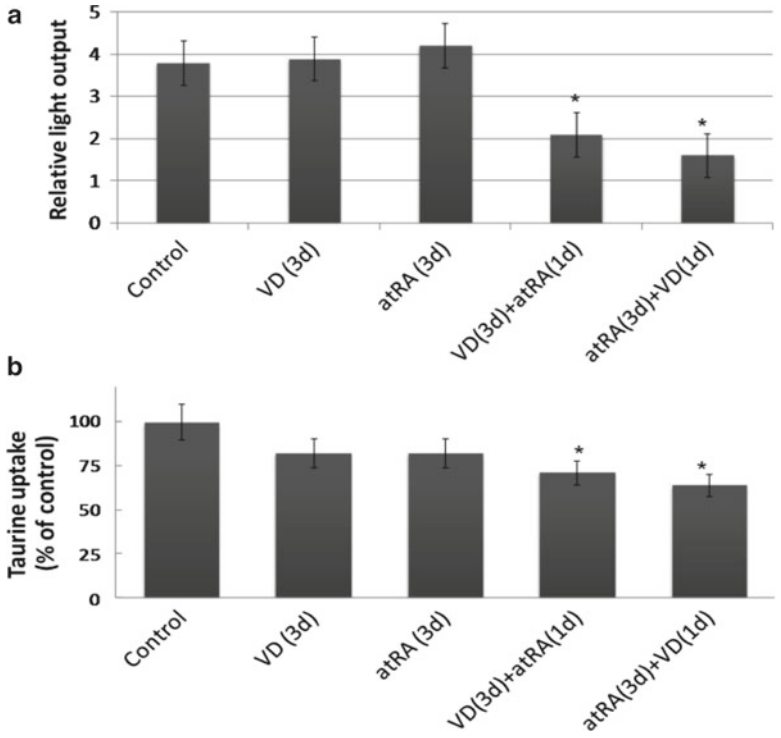

Fig. 27.3 Regulation of *TauT* by VD and RA in MCF-7 cells. MCF-7 cells were cultured in medium containing VD, RA, or VD plus RA for stated times, and then expression of TauT was determined. (**a**) Reporter gene assay. (**b**) Taurine uptake. (**c**) Western blot analysis. (**d**) Relative density of C. (**e**) Effect of 9-cis RA on taurine uptake

27.4 Discussion

The impact of $1,25(OH)_2D_3$ and all-*trans* retinoic acid on the *TauT* gene in MCF-7 cells is the opposite of that in renal cells: *TauT* is downregulated. The MCF-7 human breast carcinoma cell line has an interesting biologic feature in that it demonstrates epithelial cell polarity (van Deurs et al. 1987). In contrast to most breast carcinoma cell lines, MFC-7 cells form apical tight junctions that do not permit entry of a ricin–horseradish peroxidase conjugate, the binding site for which is found on the apical surface of the cells. These MFC-7 cells align as human mammary cells do in vivo. Moreover, these MFC-7 cells express the human milk fat globule membrane antigen, which is found on the apical surface of nonmalignant human breast cells. Hence, MFC-7 cell lines are likely to be informative about polarized taurine transport in vitro.

MCF-7 cells demonstrate estrogen-dependent growth and are responsive to antiestrogenic therapy (Demirpence et al. 1994). All-*trans* retinoic acid (RA) and $1,25(OH)_2D_3$

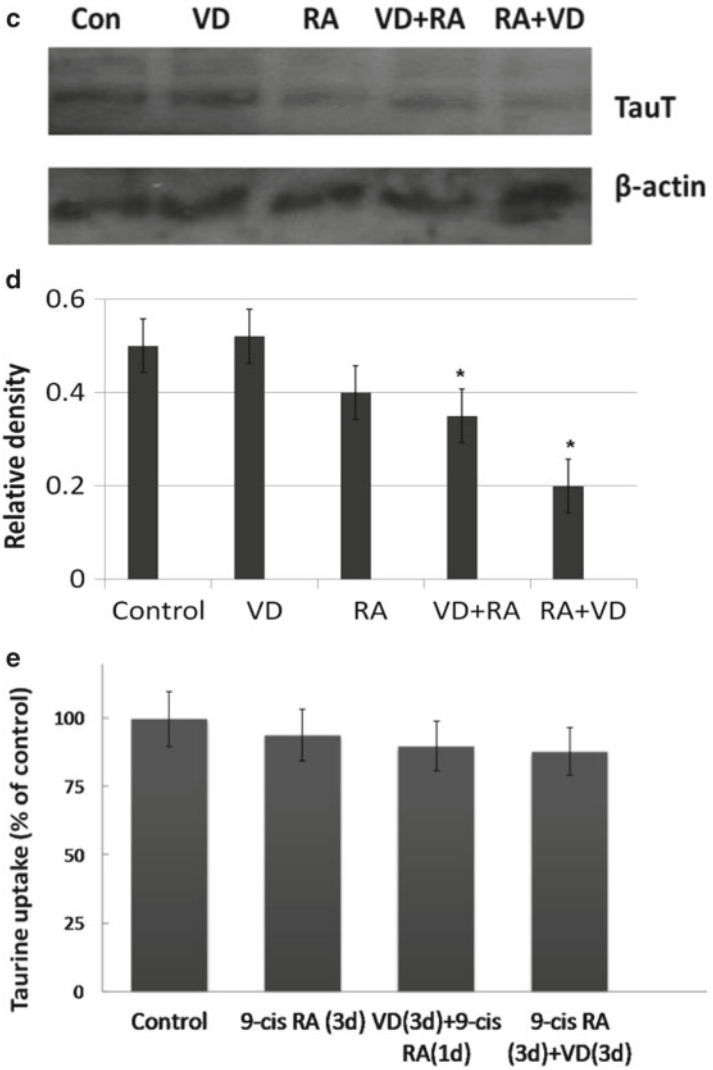

Fig. 27.3 (continued)

inhibited estrogen-stimulated growth of MCF-7 cells; this effect was potentiated by the antiestrogen drug hydroxytamoxifen. All-*trans* retinoic acid and $1,25(OH)_2D_3$ also inhibit estrogen-induced transcription. It is possible that RA and $1,25(OH)_2D_3$ either directly or indirectly inhibit the binding of estrogen to the estrogen receptor (ER) and, as a consequence, ER fails to bind to the estrogen-responsive element. We have previously shown that estrogen stimulation of the estrogen-responsive element results in upregulation of the *TauT* gene in HCF-7 cells, but not in kidney cells (Han et al. 2006).

Table 27.1 Cell line properties relative to *TauT* gene expression

	MCF-7	LLC-PK1
Tissue	Human breast carcinoma	Pig kidney proximal tubule
Estrogen	Positive	Negative
p53	↑ *TauT*	↓ *TauT*
ER	↑ *TauT*	Not active
Estrogen receptor element (ERE)	–949 to –954	Not active
E2	↑ *TauT*	No effect
VDRE/RXR, 1,25(OH)$_2$D3/atRA	↓ *TauT*	↑ *TauT*

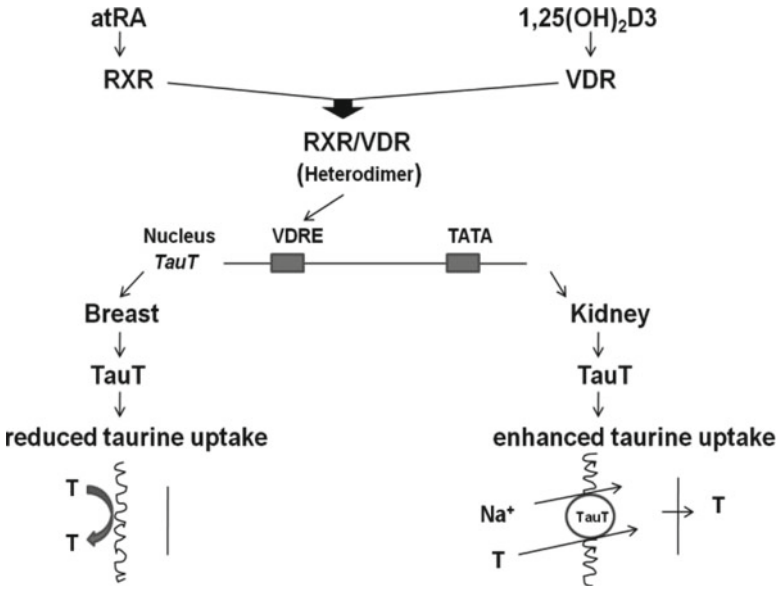

Fig. 27.4 Depiction of the regulation of *TauT* by VDR/RXR in renal cells

MCF-7 cells were derived from a malignant breast cancer metastatic lesion and as such tend to grow in a relatively uncontrolled fashion (Diesing et al. 2006). One response to 1,25(OH)$_2$D$_3$ in MCF-7 cells is to downregulate ER abundance and to suppress estrogen (E2) action in these cells (Swami et al. 2000). Because a majority of breast tumors show estrogen-dependent growth, they can be susceptible to antiestrogen therapy. Vitamins A and D have antiestrogenic action in this cell line, probably by interaction with the estrogen response element, but they appear to act by different mechanisms (Demirpence et al. 1994). Vitamin D and its metabolite, 1,25(OH)$_2$D$_3$, possess both antiproliferative and proapoptotic properties in MCF-7 cells. Estrogen upregulates VDR and induces ERK 1/2 activation in these cells (Gilad et al. 2005). Recent information indicates that the antiproliferative action of vitamin D in MCF-7

cells persists after siRNA knockdown, which suggests that these antiproliferative influences do not rely on the classical vitamin D pathway per se (Costa et al. 2009). In our present study, stimulation of MCF-7 cells with all-*trans* RA and $1,25(OH)_2D_3$ was antiproliferative and induced apoptosis in association with reduced *TauT* expression. Whether these $1,25(OH)_2D_3$-induced changes in MCF-7 cells occur, at least in part, by their influence on *TauT* is certainly a topic for greater study.

Based upon these findings, we speculate that the differences in regulation of the *TauT* gene and its transporter protein in breast tissue relate to the role of the breast in providing nutrients to the suckling infant. Taurine should be retained in breast milk to benefit the suckled mammal who requires the amino acid for central nervous system and retinal development and because of its role in the conjugation of bile acids in the infant gut (Sturman et al. 1991). In carnivores such as bears and felids that conjugate their bile acids only with taurine and not with glycine, there is a nutritional requirement for higher milk concentrations of taurine. This has been shown in the analysis of taurine in milk samples from polar bears (Hedberg et al. 2011) as well as other animals. Vitamins A and D and their metabolites could potentially influence expression of the apical taurine transporter and downregulate the *TauT* gene and its transporter protein. This, in turn, could lead to greater retention of taurine in the milk to be available for the suckling mammal.

Does vitamin D status in lactating human mothers influence the concentration of taurine in breast milk? There is no clear answer yet because in series measuring human milk taurine concentrations, vitamin D status was not assessed. Human milk taurine values depend upon the stage of lactation and mother's diet, but range from 150 to 683 μmol/l with an approximate mean of 350 μmol/l (Kim et al. 1998; Pasantes-Morales et al. 1995; Rassin et al. 1978; Shubat et al. 1989; Stapleton et al. 1997). The taurine content of breast milk of Mexican women from urban and rural areas has been compared (Pasantes-Morales et al. 1995). Taurine content in milk from urban women was 332–357 μmol/l, essentially the same as in American and Canadian women. Milk taurine content was significantly lower in the rural group (23–259 μmol/l). Vitamin D deficiency is more common in Mexican populations living in Mexico (Elizondo-Montemayor et al. 2010; Mithal et al. 2009; Romieu and Lajous 2009) and the USA (Looker et al. 2011). These results suggest that a prospective survey of milk taurine content and serum $25(OH)D_3$ values may be of interest.

27.5 Conclusion

The taurine transporter is differentially regulated by $1,25(OH)_2D_3$ and retinoic acid in a cell type- and tissue type-dependent manner. In LLC-PK1 renal proximal tubule cells, *TauT* is upregulated, whereas in MCF-7 human breast cancer cells, there is downregulation after $1,25(OH)_2D_3$ or RA exposure. This regulation of *TauT* by $1,25(OH)_2D_3$ and by retinoic acid requires the activation of both vitamin D receptors (VDR) and retinoic acid receptors (RXR) and formation of a VDR/RXR complex. It appears that regulation of *TauT* by $1,25(OH)_2D_3$ and RA occurs at the transcriptional

level via binding of the VDR/RXR complex to AP1 sites of the *TauT* promoter. Another factor that differentially regulates *TauT* in these cell lines is p53.

It is possible that these interactions between vitamins A and D and taurine include the operation of an enteral feedback loop. Taurine-conjugated bile salts increase vitamin A and D intestinal absorption, and following metabolic conversion of these vitamins, stimulation of the VDR/RXR receptor complex and binding to the promoter region of *TauT* increases or reduces synthesis of the TauT transporter protein, which, in turn, alters uptake of taurine into the cell. We speculate that in hepatic cells, taurine is more available to conjugate with bile acids necessary for fat-soluble vitamin absorption and the cycle begins anew. The negative aspects of this loop are not as yet identified, but the findings in the MCF-7 cell line demonstrate that down-regulation of *TauT* can occur.

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Chapter 28
Knockdown of *TauT* Expression Impairs Human Embryonic Kidney 293 Cell Development

Xiaobin Han and Russell W. Chesney

Abstract Studies have demonstrated that *TauT* deficiency results in small kidneys in *TauT* knockout mice. Our studies have shown that *TauT* is a direct target of several genes, including p53 and WT1, which play an important role in renal development. However, whether the *TauT* gene is directly involved in renal development is largely unknown. In the present study, we created a TauT-deficient cell model by RNAi in human embryonic kidney 293 cells, and the effect of *TauT* on renal development was investigated. Knockdown of *TauT* significantly decreased the growth rate, cell migration, and colony formation of 293 cells. Inhibition of *TauT* caused cell cycle G2 arrest. Microarray analysis showed that several genes involved in cell cycle regulation or cell division, such as CDK6 and CDC7, were significantly downregulated in TauT-deficient 293 cells as compared to control 293 cells. In conclusion, the results from this study suggest that *TauT* plays a role in the development of renal cells. Knockdown of *TauT* impairs kidney development, possibly through regulation of cell cycle-related genes.

Abbreviations

TauT	Taurine transporter gene
RNAi	RNA interference
WT1	Wilm's gene 1
p53	p53 tumor suppressor gene

X. Han, M.D., Ph.D. (✉) • R.W. Chesney
Department of Pediatrics, University of Tennessee Health Science Center, and the Children's Foundation Research Institute at Le Bonheur Children's Hospital,
Rm 307R, 50 North Dunlap, 38103 Memphis, TN, USA
e-mail: xhan@uthsc.edu

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28.1 Introduction

Studies have shown that *TauT* is regulated by a variety of stresses, including tonicity, oxidation, DNA damage, and dietary manipulation (Askwith et al. 2009; Han et al. 2009; Matsell et al. 1997; Uchida et al. 1992), suggesting that *TauT* is a stress response gene. We and others (Han and Chesney 2010; Park et al. 2003) have demonstrated that *TauT* is a target of c-Jun. c-Jun binds to two AP1 consensus sites in the *TauT* promoter and upregulates *TauT* expression in renal cells. Mutation of AP1 sites blocked binding of c-Jun to the *TauT* promoter and further abolished the effect of c-Jun on *TauT* regulation. Our previous studies have also shown that *TauT* is a target gene of p53. Activation of p53 by a chemotherapeutic agent (cisplatin) suppresses *TauT* expression both in vitro and in vivo (Han et al. 2002; Han et al. 2009). We have also shown that the JNK signaling pathway is involved in the stress regulation of *TauT* (Han and Chesney 2010). The JNK pathway is a major stress-signaling pathway in cells that plays important roles in many cellular processes, including development, apoptosis, and cell growth. In non-stressed cells, JNK targets the ubiquitination and subsequent degradation of bound proteins such as c-Jun (Han and Chesney 2010). In addition, JNK forms a complex with and degrades p53 (Fuchs et al. 1998). However, in stressed cells, JNK phosphorylates and activates associated c-Jun and p53 proteins and enhances their transcriptional regulation of stress-responsive genes (Buschmann et al. 2001; Derijard et al. 1994). Based on these results, we proposed a model for p53/c-Jun-mediated regulation of the *TauT* gene in renal cells. In stressed cells, JNK phosphorylates and activates c-Jun and p53 proteins and enhances their transcriptional regulation of *TauT*. Cisplatin-induced activation of p53 decreases *TauT* promoter activity via the p53-inhibited JNK-c-Jun pathway by competing with c-Jun for activation. Upon survival signaling, c-Jun substitutes for p53 function and enhances *TauT* expression.

Models of *TauT* deficiency, which result in taurine deficiency, have been established and studied during the past decade (Heller-Stilb et al. 2002; Ito et al. 2008). However, information about the consequences of *TauT* deficiency during cell development and its possible effects on various signaling pathways is lacking. In the present study, we used a plasmid-based shRNA, a sequence of *TauT* RNA that forms a tight hairpin loop, to silence *TauT* gene expression via RNAi in 293 renal cells. The TauT-deficient 293 cells were created and used to examine the effects of TauT deficiency on renal cell development.

28.2 Materials and Methods

28.2.1 Cell Line and Culture Conditions

The 293 human embryonic kidney cells were obtained from the American Type Culture Collection. The cells were grown in Dulbecco's modified Eagle's medium

(Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 50 units/ml penicillin, and 50 μg/ml streptomycin. The 293 cells were maintained in a humidified 37°C incubator with 5% CO_2, fed every 3 days with a complete medium, and subcultured when confluence was reached.

28.2.2 Generation of TauT-Deficient 293 Cell Line

A *TauT* knockdown cell line was created by the stable transfection of a SureSilencing shRNA plasmid DNA (5′-ggcatcaagttctatctgtat-3′ and 5′-ggaatctcattcgatgcatac-3′) into 293 cells. Cells (3×10^4) were seeded in a 6-well plate with 2 ml of growth medium and cultured for 24 h. To facilitate the transfection process, 1 μg of *TauT* shRNA plasmid was added (using the shRNA plasmid as a negative control) to separate 50 μl aliquots of Opti-MEM I Reduced-Serum Medium (Gibco). Lipofectamine2000 (1 μl) was added to the 50 μl Opti-MEM solution and incubated for 10 min at room temperature. Fifty μl Lipofectamine2000 mixture was combined with 50 μl of each shRNA mix and incubated for another 20 min at room temperature, then added to the cells, and incubated at 37°C in a CO_2 incubator for 24–96 h. For colony selection, G418 (3 μg/ml) was added to the cells. The medium was changed every 2–3 days for up to 2 weeks or until drug-resistant clones appeared in the transfected and selected plates. The concentration of G418 used for colony selection was determined using mock 293 cells. The drug-resistant clones were confirmed by an immunohistochemistry analysis of *TauT* expression and taurine uptake assay.

28.2.3 Measurement of Taurine Transport

Taurine transport studies were performed on confluent monolayers 3 days after seeding cells. The cells were rinsed with Earle's balanced salt solution (EBSS) at 37°C. Uptake was initiated by the addition of an uptake buffer (2 mM KCl, 1 mM $MgCl_2$, 96 mM NaCl, 1.8 mM $CaCl_2$, 5 mM Hepes, pH 7.6) to which 50 μM unlabelled taurine and 0.5 μCi/ml ^{14}C-taurine (Perkin Elmer, Boston, MA) were added. After a 30-min period of incubation at room temperature, uptake was terminated by the removal of uptake buffer followed by three rapid washes with cold EBSS. Cells were dissolved in 1% SDS in 0.2N NaOH and radioactivity counted in a Packard 2000-CA Liquid Scintillation Analyzer.

28.2.4 Construction of the Reporter Gene

The promoter region of *TauT* was identified in previous studies (Han et al. 2000a), and a p53-binding consensus site was found in the *TauT* promoter sequence, located

at −663 to −695. In this study, ~1.1 kb of the *TauT* promoter region DNA was used as the template for PCR (GenBank™/EBI accession number AR151716), and the PCR fragment was cloned into the promoter-less luciferase vector pGL3-Basic (Promega, Madison, WI) to generate the plasmid p963 for use in transfections and luciferase assays. The conditions used are 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 58°C, and 1 min of elongation at 72°C. The sense primer (5′-GGGGTACCTTACTGAAGGTCACACAGC-3′) designed for PCR contained a unique site for KpnI, and the antisense primer (5′-AAGATCTTGGCACGG GAGTTCA-3′) contained a unique site for BglII. PCR products were digested with KpnI and BglII and religated into KpnI and BglII sites of pGL3-Basic to generate plasmids containing segments of the *TauT* promoter sequence extending from the +48 nucleotide corresponding to the transcriptional start site. The constructs were verified by DNA sequencing. The p53-binding site deletion (del pGL-563) and p53 mutation (mt pGL-963) constructs were generated from the p963 plasmid by using sense primers 5′-GGGGTACCGAGTTGGGGAGGGA-3′ and 5′-GGGGTACCAG ATGAGGAAACCCCCACACAGAAGGTCTGGGGCTTGCCTGATGTCA-3′, respectively. The antisense primer used for these constructs was the same as described above.

28.2.5 Transient Transfection

Plasmid DNA was introduced into cultured 293 cells using cationic liposomes (Lipofectamine). Transfection was carried out for 16–18 h, and then cells were washed twice with phosphate-buffered saline and incubated in fresh medium for 24–48 h before harvesting. pGL-control, which contains a luciferase gene driven by the SV40 early region promoter/enhancer, and empty pGL-Basic vectors were used as positive and negative controls, respectively. To standardize the transfection efficiency, 0.1 μg of pRL-CMV vector (pRL *Renilla reniformis* luciferase control reporter vector; Promega) was cotransfected in all experiments. Cells were harvested 48 h after transfection and lysed in 200 μl of reporter lysis buffer (Promega). A luciferase assay was performed using a dual luciferase assay kit (Promega), and activity was measured with an Optocomp one luminometer (MGM Instruments, Inc., Hamden, CT). Promoter activity (mean ± SD of four samples in relative light units) of each construct is represented by relative light output normalized to pRL-CMV control. Graphs represent typical results of four separate experiments. The concentration of protein in the cell extracts was determined using the Bradford method (Bio-Rad, Hercules, CA).

28.2.6 Western Blot Analysis

Cells were lysed in 50 μl M-PER mammalian protein extraction reagent (Pierce, Inc., Rockford, IL) supplemented with a protease inhibitor cocktail for use with mammalian cell and tissue extracts (Sigma). The lysates were cleared by centrifugation at 14,000 × *g*

for 2 min, and the supernatants were transferred to clean tubes. Equal amounts of protein (50 μg) were separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore, Bedford, MA) using a semidry electrophoretic transfer system (Bio-Rad). Membranes were incubated in 5% nonfat dry milk in Tris base/sodium chloride (TBS) buffer with 0.2% Tween 20 (TBST) at 4°C overnight. The membranes were incubated with primary antibodies for 1 h at room temperature. Blots were washed with TBST and incubated with horseradish peroxidase-linked secondary antibody (Sigma) for another hour, and then proteins of interest were detected using a chemiluminescence detection kit (Pierce, Inc.).

28.2.7 Immunohistochemistry

Cells were cultured directly on sterile cover slips (Sigma) that were placed into a 6-well tissue culture plate for 2 days. The media was removed from cells, which were rinsed once with 1× PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2.0 mM, pH 7.4) at room temperature. The cells were fixed for 10 min at room temperature in 3.7% buffered formaldehyde and washed once in 1x PBS. Fixed cells were dehydrated by immersing in 70, 95, and 100% ethanol for 5 min each followed by air drying for 10 min each and stored at 4°C before use. For immunostaining, samples were rehydrated in decreasing ethanol series (100, 95, and 70%) for 5 min each. Then samples were immersed in 1x PBS for 5 min at room temperature. The slides were immersed in quenching solution (3% H_2O_2 in methanol) for 5 min and then washed twice in dH_2O for 10 min. Slides were blocked for 20 min with the blocking buffer. Primary antibodies (antibody against taurine transporter) were applied to slides and incubated for 1 h. Slides were washed for 10 min with PBS and treated with a secondary antibody and incubated for 1 h. After the slides were rinsed with PBS for 10 min, an ABC reagent was applied for 30 min. Finally, a Metal-Enhanced DAB Substrate Kit (Pierce) was used to detect immunostaining.

28.2.8 Cell Growth Assay

Control and TauT-deficient 293 cells (1×10^4) were cultured in medium containing G418 (1 μg/ml). The total cell number was quantified every 2 days with a hemocytometer and an Olympus inverted microscope. Cell viability was assessed by using trypan blue.

28.2.9 Cell Colony Assay

Control and TauT-deficient 293 cells (500 cells per well) were mixed with a tissue culture medium containing 0.7 agar to obtain a final agar level of 0.35%. Two ml of this cell suspension was immediately plated in 10 cm plates coated with 0.6% agar

in tissue culture medium and cultured at 37°C with 5% CO_2. Two weeks later, the top layer of cells was stained with 0.2% p-iodonitrotetrazolium violet (Sigma). Cultures were analyzed in triplicate, and colonies larger than 100 μm in diameter were counted.

28.2.10 Apoptosis Assays

Internucleosomal DNA fragmentation was detected primarily by a DNA laddering assay. An equal number of cells were suspended in 500 μl of lysis buffer (1% sodium dodecyl sulfate, 25 mM ethylenediaminetetraacetic acid, and 1 mg/ml proteinase K) and incubated overnight at 50°C. Ribonuclease A (10 mg/ml) was then added for an additional 2-h incubation at 37°C. The chromosomal DNA was extracted with phenol/chloroform, precipitated with ethanol, and analyzed by agarose gel electrophoresis. This was followed by staining the DNA with ethidium bromide to reveal the fragmentation pattern.

28.2.11 Statistics

All experiments using tissue cultures were performed in triplicate. Statistical comparisons were made using one-way ANOVA and Student's *t* test to determine significant differences in the means between experimental groups.

28.3 Results

28.3.1 Inhibition of TauT Expression in 293 Cells by RNAi

The SureSilencing™ Pre-Designed shRNA plasmids (SuperArray, Frederick, MD) were stably transfected into 293 cells, which were selected for neomycin resistance to G418 (3 μg/ml). To access the success of the *TauT* knockdown, taurine uptake was measured. The TauT-deficient 293 cell line showed a 30–35% reduction in taurine transport activity, the intensity of which reflected the relative quantity of functional *TauT* in the cellular membrane. As shown in Fig. 28.1a, taurine uptake by the TauT-deficient cells was significantly less than that of the mock group (control), which expressed a clone NC (negative control) plasmid.

The inhibition of *TauT* expression was further confirmed by the immunostaining of *TauT* by a TauT-specific antibody. As shown in Fig. 28.1b, *TauT* expression was detected in control cells, which were characterized by a darker brown in comparison to less intense staining in TauT-deficient cells. Western blot analysis of *TauT* protein

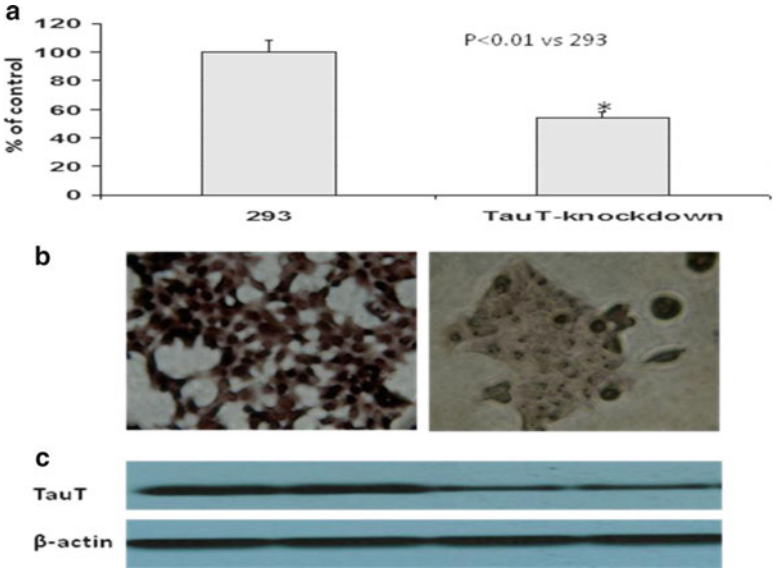

Fig. 28.1 To select the shRNA-induced TauT-deficient 293 cell line, taurine uptake was measured. The TauT-deficient cells exhibited a significant decrease in taurine uptake (**a**), which is reflected by reduced *TauT* staining (**b**). This in turn reflects expression of the *TauT* gene (**c**). Thus, these results confirm the success of shRNA-induced *TauT* knockdown in the 293 cells

confirmed such results. These results suggested that expression of *TauT* was suppressed in the 293 cells by *TauT* shRNA.

28.3.2 *Knockdown of TauT Significantly Suppresses the Growth Rate of 293 Cells*

Previous studies have shown that a TauT-deficient mouse has less body mass than a wild-type control mouse (Heller-Stilb et al. 2002; Ito et al. 2008), suggesting that *TauT* may play a role in cell proliferation. In this study, an equal number (1×10^4) of control and TauT-deficient 293 cells were cultured and counted every 3 days after seeding. The results showed that inhibition of *TauT* significantly reduced the growth rate of 293 cells in comparison to control cells over the course of 9 days (Fig. 28.2).

28.3.3 *Decreases in TauT Expression Inhibit Colony Formation and Wound Healing*

To test whether RNA-mediated reductions in *TauT* levels could influence the ability of 293 cells to form colonies in soft agar, an equal number (1×10^4) of

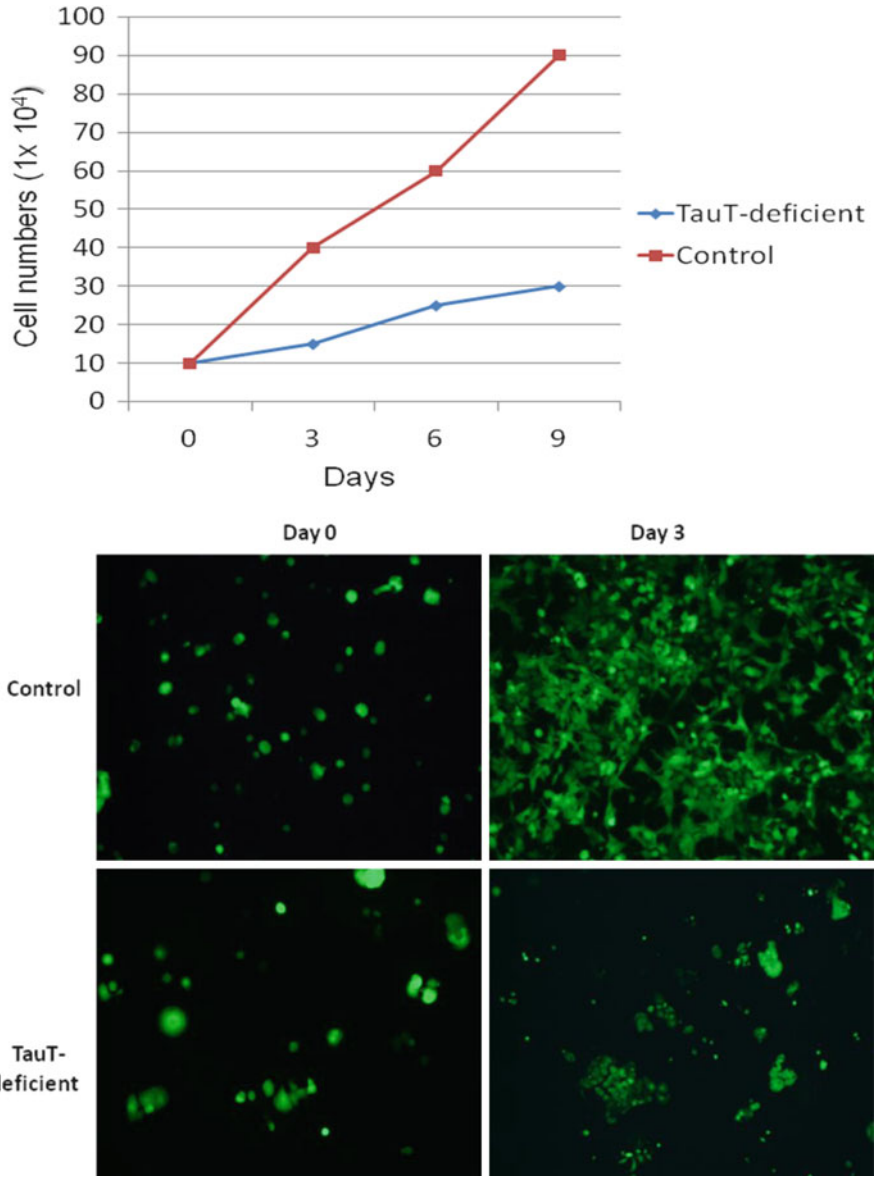

Fig. 28.2 Effect of *TauT* deficiency on cell proliferation. Cell numbers were counted using a hemocytometer for a period of 9 days (*left*). The pace of cell migration is shown on the right. These results demonstrate that *TauT* deficiency inhibits the cells' ability to proliferate

control and TauT-deficient cells were placed into a medium with soft agar, and colonies were counted after 2 weeks. RNAi directed against *TauT* resulted in a significant reduction (about 65%) in colony formation of 293 cells in comparison to control (Fig. 28.3).

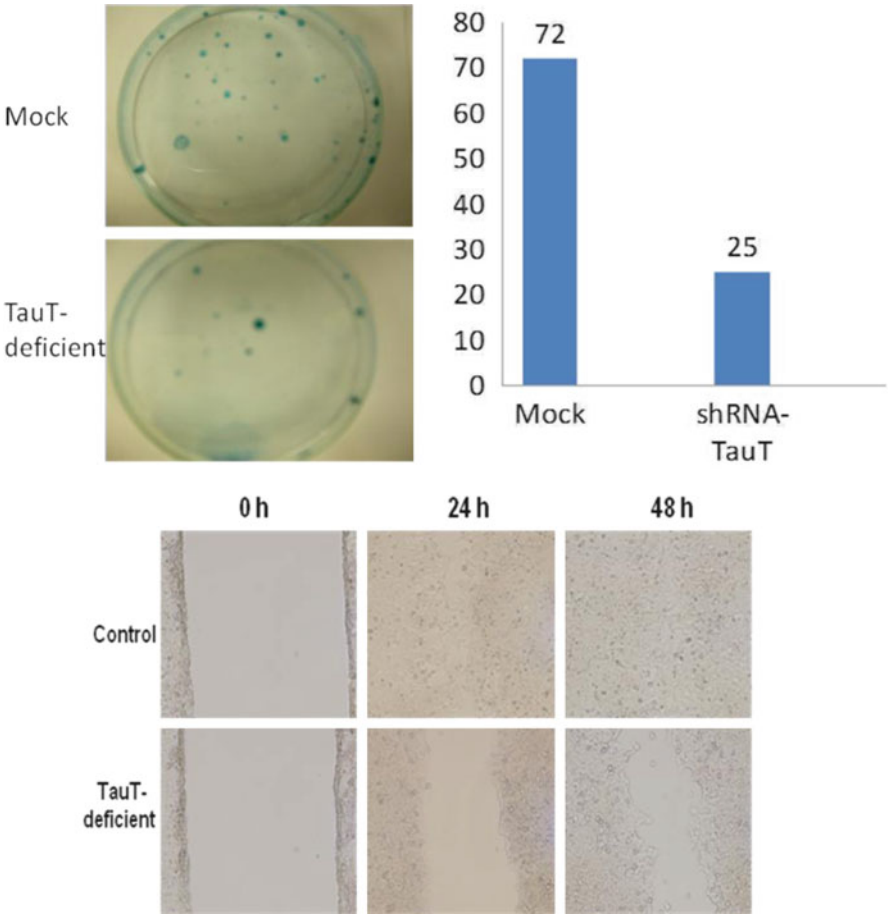

Fig. 28.3 Knockdown of *TauT* by RNAi reduces 293 cell colony formation in soft agar. Viable colonies were stained with 0.2% p-iodonitrotetrazolium violet (*Sigma*) and appear blue on the dish. After a period of 2 weeks, colonies were counted (*left*). Seventy-two colonies were counted in the mock culture, whereas only 25 were counted in the shRNA-TauT culture. Therefore, results demonstrate that *TauT* inhibition decreases colony formation and causes prolonged wound-healing time (*bottom*)

These results demonstrated that a reduction in the taurine transporter protein level decreased the ability of 293 cells to form colonies in soft agar. Also, a wound-healing test showed that control cells repaired wounds within 48 h, while the TauT-deficient cells showed a much slower wound repairing process and a prolonged wound-healing time compared to the controls.

28.3.4 Knockdown of TauT by RNAi Increases Anticancer Drug Sensitivity

The above results demonstrated that knockdown of *TauT* in 293 cells could significantly inhibit the growth of tumor cells. To determine whether inhibition of *TauT* could increase sensitivity to an anticancer drug in 293 cells, both the control and TauT-deficient cells were treated with doxorubicin. Fig. 28.4 illustrates that doxorubicin (200 ng/ml) induced cell death in both cell lines in a time-dependent manner. The number of apoptotic cells was significantly higher in TauT-deficient cells than in control cells, suggesting that knockdown of *TauT* increased the sensitivity of doxorubicin-induced apoptosis in 293 cells.

To further determine if knockdown of *TauT* increased the sensitivity of doxorubicin-induced apoptosis, both the control and shRNA-TauT 293 cell groups were treated with doxorubicin (200 ng/ml) for 24 h, and DNA fragmentation was analyzed by DNA ladder in accordance with directions provided by the manufacturer (R&D systems, MN). As shown in Fig. 28.5, the 180–200 bp DNA laddering was detected in cells treated with doxorubicin (lane 4) but not in the 200 ng/ml doxorubicin-treated control cells (lane 2). These results indicate that the apoptotic effects of doxorubicin are significantly enhanced in TauT-deficient 293 cells.

28.3.5 Knockdown of TauT Causes Cell Cycle G2 Arrest

To examine if inhibition of *TauT* alters the cell cycle, control and TauT-deficient 293 cells treated with or without cisplatin were analyzed by flow cytometry. As shown in Fig. 28.6, inhibition of *TauT* significantly increased the cell numbers in the G2 phase (10.2%) as compared to that in control cells (4.0%). *TauT* deficiency also sensitized the 293 renal cells to cisplatin-induced apoptosis.

28.3.6 Effect of Inhibition of TauT on Signal Pathways

To further explore the impact of *TauT* deficiency on the signal pathways, microarray analysis of gene expressions was performed using RNAs from control or TauT-deficient 293 cells. As expected, inhibition of *TauT* shows both positive (>1.50-fold upregulated genes; $p < 0.01$; 2,599 genes) and negative (>1.50-fold downregulated genes; $p < 0.01$; 2,345 genes) effects on genes in various signal pathways that are involved in the process of downregulation of cell proliferation and organic nutrients transporters. Surprisingly, inhibition of *TauT* dramatically decreases (87-fold) the expression of Slc2A3 (glucose transporter 3, GLUT3) in TauT-deficient 293 cells as compared to control cells. GLUT3, also known as solute carrier family 2, facilitates the transport of glucose across the plasma membranes of mammalian cells. GLUT3 is most known for its specific expression in neurons (Maher and Simpson 1994).

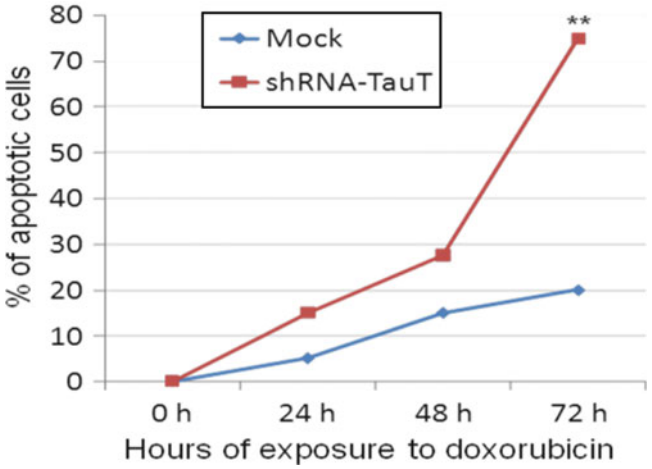

Fig. 28.4 Cell viability was counted at 24, 48, and 72 h in both cell lines. The data represent three separate experiments. The results demonstrate that a significantly greater percentage (nearly 75%) of the shRNA-TauT cell culture underwent apoptosis after a 72-h period, compared to a mere 20% in the mock cell culture. Results demonstrate that *TauT* inhibition significantly increases the sensitivity of 293 cells to doxorubicin

28.4 Discussion

It is known that the taurine transporter gene is primarily regulated by dietary taurine availability and changes in osmolarity in the kidney (Chesney et al. 1985; Handler and Kwon 1997). Molecular cloning of the promoter of the *TauT* gene has advanced the knowledge of such regulation at the molecular level and has allowed us to explore the possible biological functions of *TauT* in both physiological and clinical settings. Recent studies have shown that *TauT* is directly regulated by a variety of transcription factors, such as the p53 tumor suppressor gene, WT1, c-Jun, c-Myc, c-Myb, and estrogen, suggesting that *TauT* may play a role in cell development, drug resistance, and even tumor biology (18).

In the present study, we created a TauT-deficient cell model by RNAi in human embryonic kidney 293 cells, and the effect of *TauT* on renal development was investigated. Knockdown of *TauT* significantly decreased the growth rate, cell migration, wound healing, and colony formation of 293 cells. Inhibition of *TauT* causes cell cycle G2 arrest and sensitizes 293 renal cells to cisplatin-induced apoptosis. Microarray analysis shows that *TauT* deficiency results in the elevation of several tumor suppressor genes, including p53, FAT, and Split-2. Inhibition of *TauT* downregulates many solute carrier family genes, including the sodium/hydrogen exchanger, zinc transporters, proton/amino acid symporter, glycerol-3-phosphate transporter, neutral amino acid transporter, anion/sugar transporter, and glucose transporter 3 (GLUT3). Downregulation of solute carrier family genes may be associated with cell cycle G2 arrest in TauT-deficient cells, as these genes are mainly

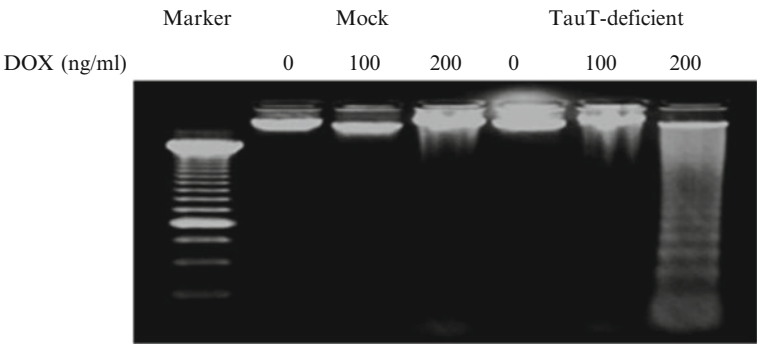

Fig. 28.5 *TauT* deficiency renders renal cell more sensitive to doxorubicin-induced apoptosis. Cells were treated with doxorubicin (0–200 ng/ml). The DNA isolated from TauT-deficient cells produced a highly distinguished ladder pattern, demonstrating that a significant percentage of TauT-deficient cells underwent apoptosis. Under the same environmental stress, the degree of cell death in the mock culture was not significant enough to produce the characteristic "ladder" appearance

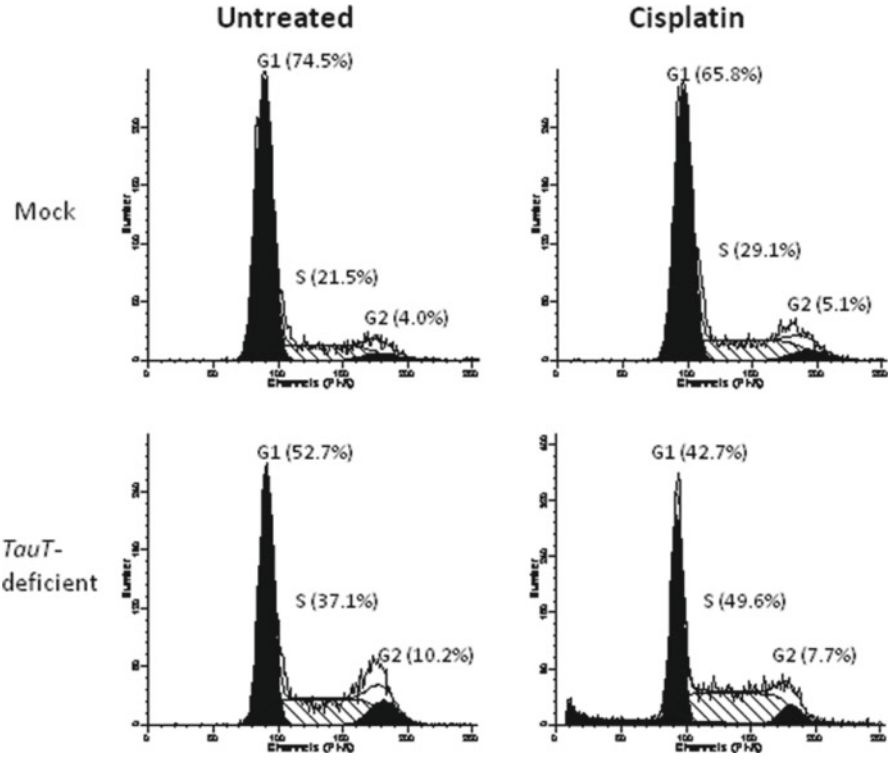

Fig. 28.6 Flow cytometry analysis of cell proliferation. Control and TauT-deficient 293 cells were treated with or without cisplatin (25 μM) for 24 h, and then cells were stained with IP for cycle analysis

involved in protein synthesis and processing in the G2 phase of the cell cycle. Interestingly, 293 cells possess the characteristics of both renal and neuronal cells. *TauT*/taurine deficiency results in brain and kidney maldevelopment (Han et al. 2000b; Heller-Stilb et al. 2002; Ito et al. 2008; Sturman 1986). Therefore, our findings here further unveil the mechanism of the physiologic function of *TauT* during development.

Theoretically, in humans, taurine deficiency mainly occurs in individuals who have a *TauT* deficiency caused by kidney disease and/or kidney injury. The human body has a limited capacity to synthesize taurine, and the kidney controls the total body pool of taurine. Therefore, models of *TauT* deficiency would be an ideal tool for studying the effects of taurine and/or *TauT* deficiency on kidney diseases and its implications. Knockdown of *TauT* strongly reduced GLUT3 expression in 293 cells, suggesting that *TauT* may affect glucose metabolism by influencing the expression of GLUT. Others have shown that high glucose levels downregulate *TauT* expression and result in intracellular taurine depletion (Askwith et al. 2009). Moreover, the bioavailability of taurine is severely reduced in diabetic patients; it is 30% lower than in matched control subjects and similar to the level found in taurine-deficient cats (Han et al. 2000b; Merheb et al. 2007). Thus, *TauT*/taurine deficiency secondary to hyperglycemia may predispose to diabetic neuropathy and diabetic nephropathy in diabetic patients.

28.5 Conclusion

In conclusion, the results from this study suggest that *TauT* plays a role in the development of renal cells. Knockdown of *TauT* impairs kidney development, possibly through regulation of cell cycle-related genes.

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Chapter 29
The Role of Taurine on Skeletal Muscle Cell Differentiation

Teruo Miyazaki, Akira Honda, Tadashi Ikegami, and Yasushi Matsuzaki

Abstract Taurine abundantly contained in the skeletal muscle has been considered as one of essential factors for the differentiation and growth of skeletal muscles. The previous studies in the taurine transporter knockout mice showed that deficiency of taurine content in the skeletal muscle caused incomplete muscular developments, morphological abnormalities, and exercise abilities. In fetal and neonatal periods, taurine must be an essential amino acid due to no biosynthesis capacity, and therefore, taurine should be endogenously supplied through placenta and maternal milk. In general cell culture condition, taurine contained in the culture medium is absent or few, and therefore, most of cultured cells are in taurine-deficient condition. In the present study, we confirmed, in cultured mouse differentiable myoblast, taurine treatment significantly enhanced the differentiation to myotube in a dose-dependent manner, while these effects were abrogated by inhibitions of taurine transport and Ca^{2+} signaling pathway.

T. Miyazaki (✉)
Joint Research Center, Tokyo Medical University Ibaraki Medical Center,
3-20-1 Chuo, Ami, Ibaraki 300-0395, Japan
e-mail: teruom@tokyo-med.ac.jp

A. Honda
Joint Research Center, Tokyo Medical University
Ibaraki Medical Center, Ami, Ibaraki, Japan

Department of Gastroenterology and Hepatology,
Tokyo Medical University Ibaraki Medical Center,
Ami, Ibaraki, Japan

T. Ikegami • Y. Matsuzaki
Department of Gastroenterology and Hepatology,
Tokyo Medical University Ibaraki Medical Center,
Ami, Ibaraki, Japan

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_29,

The present study suggested that exogenous taurine might play a key role on the mature differentiation/growth of the skeletal muscle during development period through Ca^{2+} signaling pathway, and therefore, taurine would contribute the muscle recovery after damages.

Abbreviations

TAUT	Taurine transporter
GM	Growth medium
DM	Differentiation medium
MHC	Myosin heavy chain
MCIP	Myocyte-enriched calcineurin-interacting protein

29.1 Introduction

Taurine (2-aminoethanesulfonic acid) is abundantly contained in the skeletal muscles (Jacobsen and Swimth 1968), particularly in the slow-twitch fiber rather than in the fast-twitch fiber (Airaksinen et al. 1990; Iwata et al. 1986). Although taurine is biosynthesized from sulfur-contained amino acids (methionine and cysteine) in the liver and brain via specific enzymes (cysteine sulfinate decarboxylase and cysteine dioxygenase), the biosynthesis ability is very low (Hosokawa et al. 1990; Kaisaki et al. 1995; Ramamoorthy et al. 1994). Therefore, the abundant muscular taurine content depends on the exogenous uptake through a specific transporter, taurine transporter (TAUT). Furthermore, taurine is an essential amino acid in fetus and infant due to lack of taurine biosynthesis ability in the perinatal period, and therefore, a large amount of taurine is endogenously supplied through placenta and maternal milk. It has been reported that a premature baby and incomplete postnatal tissue development and body growth were observed if taurine intake were insufficient in the pregnant and postnatal periods (Aerts and Van Assche 2002; Sturman and Messing 1991; Sturman 1993).

Taurine has been also considered as one of essential factors on the differentiation/growth of skeletal muscles because deficiency of taurine causes incomplete muscular development and exercise abilities. In the TAUT KO mice, the lower body mass and skeletal muscle growth were observed associated with significant deficiency of tissue taurine concentration compared with wild-type mice (Heller-Stilb et al. 2002; Warskulat et al. 2004), and the morphological abnormalities including muscular atrophy and disruption of myofibrillar ultrastructure and the reduction of physical capacity were also found in the TAUT KO mice (Ito et al. 2008). In the TAUT KO mice, the deficiency of tissue taurine innately induced is suggested to influence incomplete development of skeletal muscle tissue.

In general cell culture experiments, the condition should be deficient of taurine because culture medium and serum do not contain taurine at all. Therefore, most of

the cells are cultured in the taurine-deficient condition. The present study examined the effect of taurine treatment on the differentiation of mouse myoblast to myotube.

29.2 Methods

29.2.1 Culture and Differentiation of Myoblast Cells

Mouse differentiable myoblast (C2C12) was purchased from ATCC (Manassas, VA). C2C12 cells were cultured with growth medium (GM; DMEM supplemented with 10% fetal bovine serum) until confluent, and thereafter, the cells were switched to differentiation medium (DM; DMEM supplemented with 2% horse serum) with or without ~20 mM taurine for up to a week. Furthermore, the cells were exposed to ~50 mM taurine transport agonist, β-alanine; 5 μM calcineurin inhibitor, FK-506 (Cayman chemical, MI, USA); or 10 μM Ca^{2+} chelator, nifedipine (Sigma, MO, USA) in DM with or without 20 mM taurine for 5–6 days.

In C2C12 myoblast, *taut* mRNA was also silenced using siRNA (HP GenomeWide siRNA duplexes; QIAGEN, Hilden, Germany) by electroporation method (Amaxa™ Nucleofector™ Technology, Lonza, Cologne, Germany). Harvested undifferentiated C2C12 myoblast (1×10^6 cells) was transfected with the siRNA primers of *taut* or *control* and pmaxGFP® vector (Lonza), and the cells were grown up to confluent with GM. Thereafter, the cells were differentiated in DM with or without taurine.

29.2.2 Quantifications of Cell Size and Nuclei Number in Myotube

The differentiated C2C12 myotube cultured with and without taurine was fixed with methanol and 4% paraformaldehyde, and then, myosin heavy-chain (MHC) protein as a marker of differentiation in the skeletal muscle was immunohistochemically detected using monoclonal anti-skeletal MHC-fast antibody (Sigma) as the primary antibody and goat anti-mouse IgG antibody conjugated with FITC (Santa Cruz Biotechnology, CA, USA) as the secondary antibody, and the nucleus was labeled with DAPI (KPL, MD, USA). FITC and DAPI were observed using a fluorescence microscopy, and maximal short diameter and apsis length of FITC-positive in differentiated myotube and the number of DAPI-positive nucleus in the FITC-positive myotube were measured, and the number of nucleus per myotube and fusion index as ratio of the number of nucleus to the apsis length of myotube were calculated.

In the C2C12 myotube transfected with *taut* siRNA, the transfected GFP that is a marker of silenced *taut* gene was detected using fluorescence microscopy. The efficiency of silencing of *taut* gene was approximately 70% evaluated as TAUT protein expression by Western blot.

29.2.3 The mRNA Expression by Quantitative Real-Time PCR Technique

The mRNA expression level of calcineurin inhibitory protein myocyte-enriched calcineurin-interacting protein (MCIP) 1 in the differentiated myotube treated with taurine and nifedipine was quantified by real-time PCR. Total RNA was extracted from the harvested myotube using an RNeasy Plus Mini Kit (QIAGEN). Reverse transcription was performed on 500 ng of total RNA using a PrimeScript® RT reagent Kit (TAKARA Bio. Inc. Shiga, Japan). Real-time quantitative PCR was performed on cDNA aliquots with the FastStart DNA Master SYBR Green I and a LightCycler (Roche Diagnostics, Mannheim, Germany). The sequences of the oligonucleotide primer pairs used to amplify mRNA were as follows: MCIP1 forward primer, 5′-CTTCAGAACATATGACAAGGAC-3′; MCIP1 reverse primer, 5′-AGGTGTGAACTTCCTATGTGTA-3′; β-actin forward primer, 5′-CCTGTA TGCCTCTGGTCGTA-3′; and β-actin reverse primer, 5′-AGACTTCGAGCA GGAGATGG-3′. A standard curve for each run was constructed by plotting the crossover point against the log concentration. The concentration of target molecules in each sample was then calculated automatically by reference to this curve ($r=-1.00$), and results were standardized to the expression of β-actin. The specificity of each PCR product was assessed by melting curve analysis.

29.2.4 Statistical Analysis

Statistical significances were determined by unpaired Student's *t*-test or one-way ANOVA multiple comparison test. Data were expressed as the mean ± SD or the median and plots of individual value. Differences were considered statistically significant when the calculated *P* value was less than 0.05.

29.3 Results

29.3.1 The Effect of Taurine Treatment on the Differentiation of C2C12 Myoblast to Myotube

The effect of taurine treatment on the differentiation of myoblast to myotube in C2C12 cells was evaluated by the measurements of maximal length of short diameter of differentiated myotube, the number of nucleus in the myotube, and the calculation of fusion index which is the number of nucleus per the apsis length of examined myotube. The maximal short diameter in the myotube was increased by taurine treatments compared to that in the untreated control and was significantly higher in 20 mM taurine treatment than the control and 5 mM taurine treatment (Fig. 29.1a).

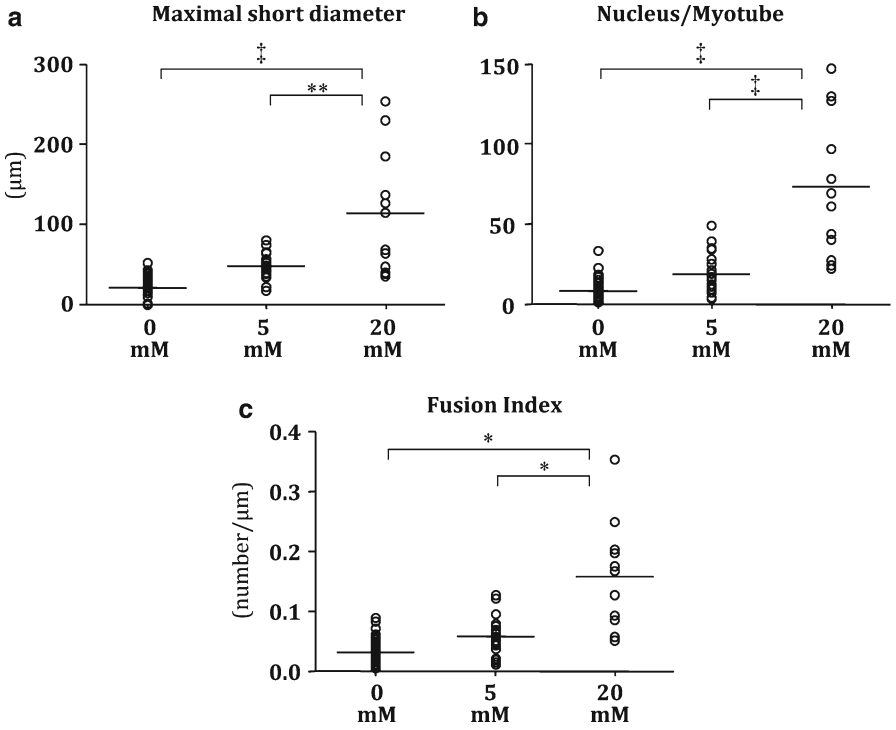

Fig. 29.1 The effect of taurine treatment on differentiation of C2C12 myoblast to myotube. C2C12 cells were cultured with 0, 5, or 20 mM taurine in the differentiation medium for 7 days. (**a**) The length of maximum short diameter in myotube, (**b**) the number of nucleus per myotube, and (**c**) fusion index was calculated by the number of nucleus per the examined myotube length. Data are shown as the median and value plots. *$p<0.05$, **$p<0.01$, ‡$p<0.0001$ by one-way ANOVA analysis

Likewise, the number of nucleus in the differentiated myotube (Fig. 29.1b) and fusion index (Fig. 29.1c) were also significantly increased by 20 mM taurine treatment compared to that in the control and 5 mM taurine treatment.

29.3.2 The Effects of Inhibitors of Taurine Transport and Ca^{+2} Signaling on the Taurine-Enhanced C2C12 Differentiation

Figure 29.2 shows the fluorescence images of C2C12 myotube treated with nifedipine or transfected with *taut* siRNA. During the differentiation period, the cells in both conditions were exposed to 20 mM taurine. The enhanced effect of taurine on the differentiation to myotube evaluated by FITC-positive MHC protein expression was cancelled by the treatment of nifedipine (Fig. 29.2a). The expression of MCIP-1 mRNA in myotube was significantly increased by nifedipine treatment compared to that in undifferentiated myoblast, and the expression level in myotube

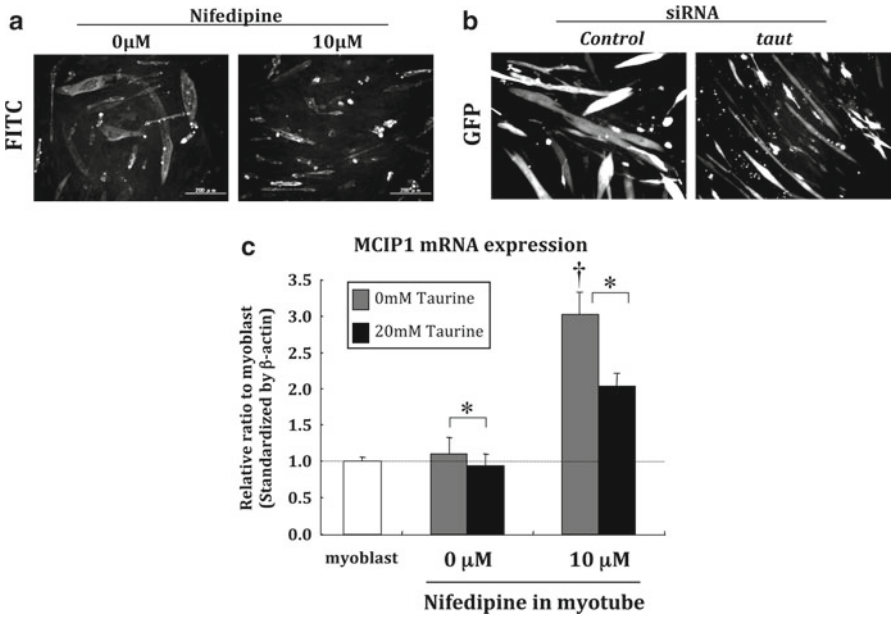

Fig. 29.2 Fluorescence image of C2C12 myotube treated with 20 mM taurine in the Ca^{2+} chelator nifedipine treatment (**a**) and *taut* siRNA transfection (**b**) and the expression level of MCIP1 mRNA in the myotube treated with taurine and nifedipine (**c**). (**a**, **c)** C2C12 cells were exposed to differentiation medium containing 20 mM taurine with or without 10 μM nifedipine for 5 days. Myosin heavy-chain protein in myotube was immunohistochemically stained with anti-MHC and FITC-conjugate IgG antibodies (**c**). C2C12 cells transfected with either *control* or *taut* siRNA and GFP vector were differentiated with 20 mM taurine (**c**). The mRNA expression level of MCIP1 was quantified by real-time PCR and shown as the relative ratio to undifferentiated myoblast. The differentiated myotube was detected by the transfected GFP. Data are shown as the mean ± SD and $*P<0.05$, $\dagger P<0.001$(vs. myoblast) by the unpaired Student's *t*-test and one-way AVOVA multiple comparison test, respectively

treated with and without nifedipine was significantly reduced by taurine treatment (Fig. 29.2b). Similarly, FK-506 treatment in the C2C12 cells markedly reduced the enhancement of differentiation to myotube by taurine treatment (data not shown).

In Fig. 29.2c, the silencing *taut* gene using siRNA technique inhibited the differentiation to myotube in the C2C12 cells treated with 20 mM taurine compared to that in the *control* siRNA-transfected cells. Furthermore, the effect of taurine on the differentiation to myotube was significantly reduced by β-alanine treatment in a dose-dependent manner (data no shown).

29.4 Discussion

In the present study, the differentiation of C2C12 fibroblast to myotube was significantly and dose-dependently enhanced by taurine treatment, and the effect of taurine was abrogated by the inhibitions of Ca^{2+}, its signaling pathway, and endogenous taurine transport. Myogenesis consists of multiple processes including ceasing of proliferation, elongation, and fusion into multinucleated myotube, and many factors including insulin growth factor 1 and myocyte-enhanced factor 2 have been reported to enhance these processes in the previous studies (Gossett et al. 1989; Kook et al. 2008; Maeda et al. 2002; Naya and Olson 1999; Semsarian et al. 1999). Taurine might be one of the important factors that regulate muscle maturation in the development period. In the processes of muscle hypertrophy including the fusion of myoblast and the formation of myotube, extracellular Ca^{2+} and Ca^{2+}-dependent calcineurin (Ca^{2+}/calmodulin-dependent phosphatase) signaling are important pathways (De Arcangelis et al. 2005; Semsarian et al. 1999). In the present study, the enhanced effects of taurine on the differentiation of C2C12 cells were cancelled by treatments of Ca^{2+} chelator nifedipine and calcineurin inhibitor FK-506, and these results supported the role of taurine on the Ca^{2+}-calcineurin pathway in the differentiation process. The enhanced mRNA expression of MCIP1 by nifedipine treatment was significantly decreased by taurine treatment. The MCIP1 expresses primarily in skeletal muscle and inhibits the activity of calcineurin (Rothermel et al. 2000). Because the gene transcription is potently stimulated by activated calcineurin as negative feedback (Yang et al. 2000), it is possible that taurine might suppress the feedback transcription of MCIP1. Furthermore, it is suggested that intracellular, but not external, taurine uptaken from culture medium might affect on the pathway because the taurine transport inhibitor β-alanine treatment and taut gene silencing significantly inhibited the cell differentiation.

29.5 Conclusion

In summary, the present study shows that exogenous taurine treatment significantly enhanced the differentiation of C2C12 myoblast to myotube, and the significant effect of taurine might be associated with the Ca^{2+} signaling pathway. This beneficial effect of taurine would possibly contribute to the muscle recovery after damages.

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Chapter 30
Taurine and Fish Development: Insights for the Aquaculture Industry

Wilson Pinto, Ivar Rønnestad, Maria Teresa Dinis, and Cláudia Aragão

Abstract Expansion of the aquaculture industry is limited by incomplete knowledge on fish larval nutritional requirements. Nevertheless, it is believed that dietary taurine deficiencies may be particularly critical for fish larvae. The reasons include the high taurine levels found during egg and yolk-sac stages of fish, suggesting that taurine may be of pivotal importance for larval development. Moreover, unlike aquaculture feeds, natural preys of fish larvae contain high taurine levels, and dietary taurine supplementation has been shown to increase larval growth in several fish species. This study aimed to further explore the physiological role of taurine during fish development. Firstly, the effect of dietary taurine supplementation was assessed on growth of gilthead sea bream (*Sparus aurata*) larvae and growth, metamorphosis success and amino acid metabolism of Senegalese sole (*Solea senegalensis*) larvae. Secondly, the expression of taurine transporter (TauT) was characterised by qPCR in sole larvae and juveniles. Results showed that dietary taurine supplementation did not increase sea bream growth. However, dietary taurine supplementation significantly increased sole larval growth, metamorphosis success and amino acid retention. Metamorphosis was also shown to be an important developmental trigger to promote taurine transport in sole tissues, while evidence for an enterohepatic recycling pathway for taurine was found in sole at least from juvenile stage. Taken together, our studies showed that the dependence of dietary taurine supplementation differs among fish species and that taurine has a vital role during the ontogenetic development of flatfish, an extremely valuable group targeted for aquaculture production.

W. Pinto (✉) • M.T. Dinis • C. Aragão
CIMAR/CCMAR, Universidade do Algarve, Campus de Gambelas,
Faro, Portugal
e-mail: wpinto@ualg.pt

I. Rønnestad
University of Bergen, Bergen, Norway

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_30,

Abbreviation

TauT Taurine transporter

30.1 Introduction

The aquaculture industry is currently the fastest-growing food-producing sector, with an increased production from less than one to 50 million tons during the last 50 years. However, one of the current setbacks of the industry is the reduced production of high-quality juveniles. This is mainly due to the poor understanding on the nutritional requirements of fish in the early developmental stages, when larvae possess a poorly differentiated digestive tract with low digestive and absorption capacities (Yúfera and Darias 2007; Rønnestad and Morais 2008). For this purpose, aquaculture nutritionists have directed their research efforts towards nutrients considered important for fish development, one of these being taurine. Similarly to several mammal species, the capacity to biosynthesise taurine varies among fish species and throughout ontogenesis (Kim et al. 2008). Moreover, although positive effects have been observed for juveniles of several fish species fed taurine-supplemented diets (Matsunari et al. 2005), any positive effects on fish larval development still require further clarification. Nevertheless, it is expected that dietary taurine supplementation may be crucial for fish larvae. This is based on the high levels of taurine found during egg and yolk-sac stages, indicating that taurine has a high physiological importance for embryo development. Additionally, in contrast to live feeds fed to fish larvae in aquaculture settings, the natural live prey of fish larvae have high taurine levels (van der Meeren et al. 2008), suggesting that fish larvae should be fed with taurine-supplemented diets.

This study aimed to further explore the physiological role of taurine during fish development. For this purpose, three experiments were conducted using two model species: gilthead sea bream (*Sparus aurata*) and the flatfish Senegalese sole (*Solea senegalensis*), a dominant and an emerging species for the aquaculture industry in southern European countries, respectively. Moreover, while Senegalese sole undergoes a visibly marked metamorphosis that is typical for flatfish (around 12–20 days after hatching), this process is morphologically less striking in gilthead sea bream (Pinto et al. 2009). The effect of dietary taurine supplementation was assessed in the two species: in sea bream larvae evaluation was based on growth performance, while in sole larvae evaluation was based on growth, metamorphosis success and amino acid metabolism. In addition, the expression of taurine transporter (TauT) was characterised by qPCR in sole larvae and juveniles.

30.2 Methods

In the first experiment, gilthead sea bream larvae were reared according to standard procedures (Moretti et al. 1999) and fed control or taurine-enriched live prey (1.5% of live prey dry weight). Growth and survival were monitored along the experimental period (until 31 days after hatching). In the second experiment, Senegalese sole larvae were also reared according to standard procedures (Dinis et al. 1999), being co-fed with live prey and control or taurine-supplemented (3% of total composition) microdiets during the pelagic phase (25 days after hatching). After settlement, larvae from both treatments were only fed live prey according to standard procedures (until 32 days after hatching). A metabolism trial was conducted in parallel with this experiment, where larvae from the control and taurine treatments were fed live prey enriched with a radiolabelled amino acid mixture [further details can be found in Pinto et al. (2010)]. The metabolic fate of radiolabelled amino acids was quantified on sole retained, catabolised and evacuated fractions, as described in Conceição et al. (2007). On the third experiment, Senegalese sole larvae were reared following standard procedures (Dinis et al. 1999) and sampled from first feeding until post-metamorphosis. Additionally, sole juveniles were also sampled and several tissues were collected, mainly focusing on organs of the digestive tract. The expression profile of TauT was subsequently analysed through qPCR on larval and juvenile tissues [for further details see Pinto et al. (2012)]. Statistical analysis was performed on data from all experiments to detect group mean differences, following procedures by Zar (1999).

30.3 Results

Results from the first experiment showed that dietary taurine supplementation did not significantly affect growth and survival of gilthead sea bream larvae.

In the second experiment, dietary taurine supplementation did not significantly affect growth, survival and metamorphosis pattern of Senegalese sole at the end of the pelagic phase (25 days after hatching). However, by the end of the trial (32 days after hatching), newly settled Senegalese sole larvae from the taurine treatment showed a significantly higher growth than larvae from the control treatment. In addition, about 20% of the larvae from the taurine treatment had completed metamorphosis, whereas none of the larvae from the control treatment had finished this process. Results from the metabolism trial showed that dietary taurine supplementation significantly increased the amino acid retention in Senegalese sole, but only when higher taurine levels were found in larvae from the Taurine treatment (9 days after hatching).

Results from the third experiment showed that the expression of TauT increased during Senegalese sole larval development, significantly increasing at the onset of

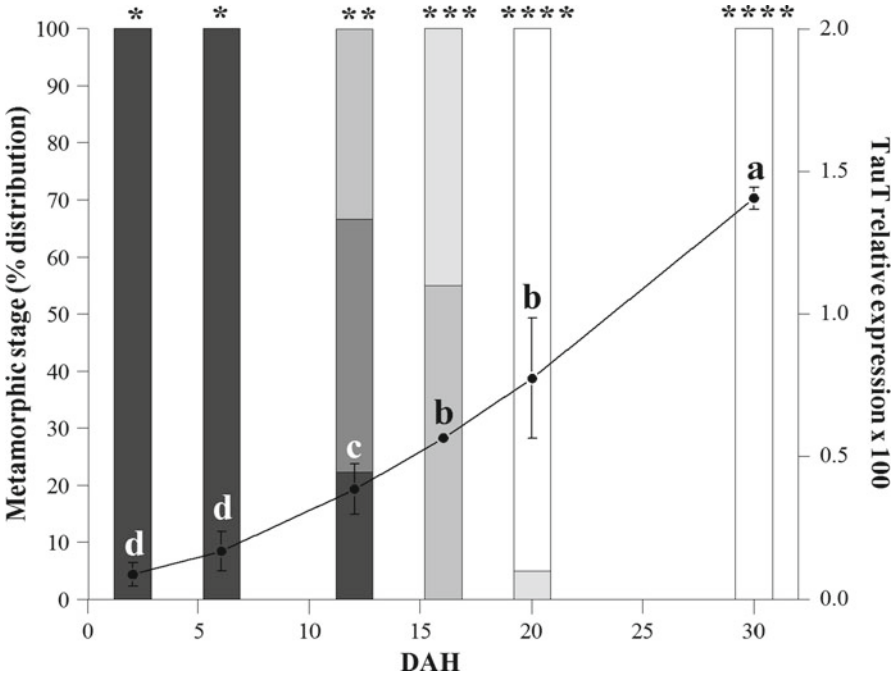

Fig. 30.1 Metamorphosis pattern and TauT expression in *Solea senegalensis* larvae. DAH—days after hatching. Results for metamorphosis pattern are expressed as percentage of each metamorphic stage (pre-metamorphic, ■; early metamorphic, ■; middle metamorphic, □; middle metamorphic, □; and late metamorphic, □) found at a certain age ($n = 20$), as described by Fernández-Díaz et al. (2001). Results for TauT expression —●— are shown as TauT copy: reference gene (eEF1a1) copy ($n = 3$ pooled samples). Results are given as means ± standard deviation. Different numbers of asterisks represent significant differences for the mean metamorphic stage at a certain age. Different letters represent significant differences for the expression of the TauT throughout larval development. Adapted with permission from Pinto et al. (2012)

metamorphosis (12 days after hatching) and achieving the highest values at post-metamorphosis (30 days after hatching; Fig. 30.1). In Senegalese sole juveniles, TauT mRNA was ubiquitously expressed in all analysed tissues, with a higher expression level in brain, heart and eye. In the digestive tract, TauT expression was higher in hindgut and stomach tissues.

30.4 Discussion

This study showed different effects of dietary taurine supplementation on gilthead sea bream and Senegalese sole larval performance. In gilthead sea bream, dietary taurine supplementation did not significantly affect larval growth or survival. These findings

indicate that dietary taurine supplementation may not be required for gilthead sea bream during the larval stage, since it does not result in apparent growth benefits.

Contrarily to what was observed for gilthead sea bream, dietary taurine supplementation significantly increased Senegalese sole larval amino acid retention, growth and metamorphosis success. The mechanisms underlying these positive effects were not further explored, although it is known that growth is essentially muscle protein deposition (Carter and Houlihan 2001) and amino acids are the building blocks for protein synthesis. Taken together, results have shown that dietary taurine supplementation may result in an enhancement of Senegalese sole larval growth. Moreover, results indicated that taurine is particularly important during sole metamorphosis. Sole larvae antioxidant defences may become saturated at this stage (Solé et al. 2004) and the antioxidant properties of taurine may be crucial during oxidative stress conditions (Métayer et al. 2008). Accordingly, TauT mRNA expression significantly increased during sole metamorphosis, indicating that metamorphosis is an important developmental trigger to promote taurine transport in larval tissues and suggesting that taurine has a high physiological importance at this stage. The benefits of dietary taurine supplementation were also shown to positively influence the settlement of Japanese flounder (Takeuchi et al. 2001), indicating that feeding taurine-supplemented diets is advantageous during the metamorphosis of flatfish species, such as Senegalese sole and Japanese flounder.

The analysis of TauT expression in Senegalese sole juvenile tissues demonstrated high mRNA levels in brain, heart and eye, organs where taurine can be found in high concentrations and has been suggested to play important biological roles (Huxtable 1992). In the digestive tract, TauT was more expressed in stomach and hindgut, suggesting that dietary taurine is readily absorbed when the digestive process begins and that taurine endogenously used for bile salt conjugation may be reabsorbed at the posterior end of the digestive tract. Therefore, these results suggest the presence of an enterohepatic recycling pathway for taurine in Senegalese sole at least from the juvenile stage, a process that may be important for maintenance of the taurine body pool in flatfish species.

30.5 Conclusion

In summary, this study showed that the effect of dietary taurine supplementation during larval stage of fish seems to be species dependent. While no apparent benefits of dietary taurine supplementation were observed for sea bream larvae, this supplementation was beneficial for flatfish larvae and suggested that taurine contributes to increase larval growth potential and metamorphosis success. Metamorphosis was also demonstrated to be an important trigger to promote taurine transport in sole tissues, while a taurine enterohepatic recycling pathway seems to be present at least from the juvenile stage onwards. This work highlights the pivotal role of taurine during the development of flatfish, an economically valuable group targeted for aquaculture production in many areas of the world.

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Chapter 31
Effect of Dietary Taurine and Arginine Supplementation on Bone Mineral Density in Growing Female Rats

Mi-Ja Choi and Kyung Ja Chang

Abstract The purpose of this study was to determine the effect of arginine or taurine alone and taurine plus arginine on bone mineral density (BMD) and markers of bone formation and bone resorption in growing female rats. Forty female SD rats (75 ± 5 g) were randomly divided into four groups (control, taurine, arginine, taurine + arginine group) and treatment lasted for 9 weeks. All rats were fed on a diet and deionized water. BMD and bone mineral content (BMC) were measured using PIXImus (GE Lunar Co, Wisconsin, USA) in spine and femur. The serum and urine concentrations of calcium and phosphorus were determined. Bone formation was measured by serum osteocalcin and alkaline phosphatase concentrations, and the bone resorption rate was measured by deoxypyridinoline cross-links. Femur BMD was significantly increased in the group with taurine supplementation and femur BMC/weight was significantly increased in the group with arginine + taurine supplementation. Rats fed an arginine or taurine supplemental diet increased femur BMD or femur BMC, but a taurine + arginine-supplemented diet does not have a better effect than arginine or taurine alone in the spine BMD. The femur BMC, expressed per body weight, was higher in arginine + taurine group than in the taurine or arginine group. The results of this study suggest that taurine + arginine supplementation may be beneficial on femur BMC in growing female rats. Additional work is needed to clarify the interactive effects between the taurine and arginine to determine whether dietary intakes of arginine and taurine affect bone quality in growing rats.

M.-J. Choi (✉)
Department of Food and Nutrition, Keimyung University,
1000 Sindangdong, Dalseogu, Daegu 704-701, South Korea
e-mail: choimj@kmu.ac.kr

K.J. Chang
Department of Food and Nutrition, Inha University,
Incheon, South Korea

A. El Idrissi and W.J. L'Amoreaux (eds.), *Taurine 8*, Advances in Experimental Medicine and Biology 776, DOI 10.1007/978-1-4614-6093-0_31,

Abbreviations

ALP	Alkaline phosphatase
DPD	Deoxypyridinoline
Arg	Arginine
Tau	Taurine
Arg + Tau	Arginine + taurine
DPD/Cr	Creatinine excretion
AI	Adequate intake
BMD	Bone mineral density
BMC	Bone mineral content
FER	Food efficiency ratio
SBMD	Spine bone mineral density
SBMC	Spine bone mineral content
FBMD	Femur bone mineral density
FBMC	Femur bone mineral content

31.1 Introduction

The increase in the life span will be associated with future increases in the prevalence of chronic disease. Osteoporosis is one of the major health problems, particularly with the gradual aging of the population. There is a growing emphasis on osteoporosis prevention. So timing of intervention will be important where the maximum benefit may be in prevention rather than therapy of osteoporosis. To ease the future burden of osteoporosis, focusing on prevention will be the key, and this should include dietary interventions to stimulate bone formation (Mundy 2006). Nutrition is important for the formation and maintenance of bone mineral density (BMD) and for the prevention of osteoporosis. A variety of dietary factors such as calcium, vitamin D, phosphate, magnesium, and protein can influence bone. It is also likely that a variety of other dietary factors such as vitamin K, caffeine, and fluoride have the potential to affect bone. Evidence suggests that some amino acids may benefit bone health. Arginine supplementation is used in several disease states. Under normal physiological conditions, arginine is a semi-essential amino acid that is derived both from endogenous and dietary sources. And arginine is well tolerated at intravenous, intra-arterial, or oral doses not exceeding 30 g/day (Luiking and Deutz 2007). The estimated daily intakes of arginine in the US diet is about 5.4 g (Visek 1986), whereas the total arginine whole-body production and consumption are 25 g/day and protein breakdown is a major endogenous source of arginine (Luiking and Deutz 2007). Arginine supplementation has been shown to have an effect on femur bone mineral content (BMC) in growing female rats (Choi 2007a) and OVX rats (Choi 2009). Taurine supplementation has been shown to

have an effect on femur BMC in OVX rats (Choi and DiMarco 2009). Although it is expected that taurine and arginine act synergistically on bone, the effect of taurine and arginine simultaneously on bone has not been studied. The objective of the present study was to investigate the effect of taurine and arginine supplementation with measures of spine and femur BMD and bone markers in growing female rats.

31.2 Methods

31.2.1 Materials

Forty-eight 6-week-old, female Sprague–Dawley rats were purchased from Bio Genomics, Oriental, Seoul, Korea. On arrival at our lab, rats were acclimated for 5 days to a standard laboratory nonpurified diet (Samyang, Seoul, Korea). After acclimation, the rats were divided into four groups through the use of a randomized complete block design, with blocks determined by initial body weight, as follows: a casein-based diet (control), a casein-based diet with an arginine (Arg), a casein-based diet with a taurine (Tau), and a casein-based diet with an arginine and taurine (Arg + Tau). Rats were individually housed in stainless steel cages in a room with controlled temperature (23°C) and humidity (55%) and were given free access to the experimental diets and water. Rats were maintained at 12-h light (07:00–19:00 h) and dark cycle. The compositions of the experimental diets are shown in Table 31.1. For 9 weeks, rats were fed experimental powdered diets. The experimental diet groups were fed similar diets which were supplemented with arginine or/and taurine. Blood samples were collected from the abdominal aorta and serums were separated at 3,000 rpm for 20 min. Serums were stored at −70°C until analysis. Serum concentrations of alkaline phosphatase (ALP) and osteocalcin were measured. Serum calcium and phosphate were also measured. Serum ALP and osteocalcin and urinary DPD cross-link value were measured as markers of bone formation and resorption. ALP activity was reported as units per liter (U/L). The concentration of urine deoxypyridinoline (DPD) was measured with an enzyme immunoassay that preferentially recognizes the free form of DPD (CLIA, Pyrilinks-D DPC, USA). DPD was corrected for creatinine excretion (DPD/Cr). The concentration of serum osteocalcin was measured with an osteocalcin kit (IRMA, OSTEO-RIACT, Cis Bio, Saclay, France), which recognizes the intact form of osteocalcin. Serum ALP activity was measured with a kit from Enzymatic assay (Prueauto S ALP) following the manufacturer's instructions.

BMD and BMC were measured using PIXImus (GE Lunar Co, Wisconsin, USA) in spine and femur on 9 weeks after feeding. The experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC) in Keimyung University and conformed to the Guide for the Care and Use of Laboratory Animals.

Table 31.1 Composition of control and experimental diets (g/kg diet)

Ingredient	Control	Arg	Tau	Arg + Tau
Casein[a]	200	200	200	200
Corn starch	529.486	523.486	509.486	503.486
Sucrose	100	100	100	100
Soybean oil	70	70	70	70
α-Cellulose[b]	50	50	50	50
Min-mix[c]	35	35	35	35
Vit-mix[d]	10	10	10	10
L-Cystine[e]	3	3	3	3
Choline[f]	2.5	2.5	2.5	2.5
TBHQ[g]	0.014	0.014	0.014	0.014
Arginine[h]		6.6		6.6
Taurine[i]			20	20

[a]Casein, Maeil Dairy industry Co. Ltd., 480 Gagok-Ri, JinwiMyun, Pyungtaek-City, Kyunggi-Do, Korea
[b]α-Cellulose, Sigma Chemical Co., St. Louis, Mo, USA
[c]Mineral-mix, AIN-93G-Mx, Teklad Test Diets, Madison, Wisconsin, USA
[d]Vitamin-mix, AIN-93-VM, Teklad Test Diets, Madison, Wisconsin, USA
[e]L-Cystine, Sigma Chemical Co., St. Louis, MO, USA
[f]Choline bitartate, Sigma Chemical Co., St. Louis, MO, USA
[g]*Tert*-butyl hydroquinone, Sigma-Aldrich Inc., St. Louis, MO, USA
[h]L-Arginine: Sigma A8094, Japan
[i]Taurine, Dong-A Pham Co. Ltd., 434–4 Moknae-dong., Ansan-City, Korea

31.2.2 Statistics Analysis

Analysis of variance (ANOVA) was performed on the means to determine whether there were significant ($p < 0.05$) differences among the groups. When ANOVA indicated statistical significance, the Duncan's multiple comparisons test was used to determine which means were significantly different. SAS package (version 9.12, Institute Inc., Cary, NC, USA) was used for all statistical analyses. Results are expressed as means ± SD. Values were reported as significant have p-values < 0.05.

31.3 Results and Discussion

31.3.1 Weight Gain, Food Intake, and Food Efficiency Ratio

Tables 31.2 and 31.3 show the weight at beginning, weight at sacrifice, weight gain, food intake, and FER of rats fed on experimental diets. Body weight gain and food intake of rats fed the experimental diets did not differ from those of rats fed the control. This finding is in agreement with what Sugiyama and coworkers (Sugiyama et al. 1989) reported that taurine supplementation had no influence on

Table 31.2 Body weight changes of experimental rats for 9 weeks

Variables	Control	Arg	Tau	Arg + Tau
Initial weight (g)	75.9 ± 2.9[1, a, 2]	75.4 ± 3.1[a]	75.1 ± 2.9[a]	75.7 ± 2.5[a]
Final weight (g)	301.9 ± 21.5[a]	296.9 ± 20.1[a]	307.8 ± 19.4[a]	303.9 ± 23.2[a]
Weight gain (g)	225.9 ± 22.5[a]	221.4 ± 18.5[a]	232.6 ± 18.8[a]	228.2 ± 22.3[a]

[1]Mean ± SD
[2]Values with different superscripts within the row are significantly different at $p < 0.05$ by Duncan's multiple range test

Table 31.3 Food intake and FER of rats fed during experimental period

Variables	Control	Arg	Tau	Arg + Tau
Food intake (g/day)	19.91 ± 1.52[1, a, 2]	19.37 ± 2.13[a]	20.15 ± 1.96[a]	19.50 ± 1.70[a]
FER	0.18 ± 0.02[a]	0.18 ± 0.02[a]	0.19 ± 0.02[a]	0.19 ± 0.02[a]

[1]Mean ± SD
[2]Values with different superscripts within the row are significantly different at $p < 0.05$ by Duncan's multiple range test

the weight gain and food intake of the animals. Table 31.2 shows that all experimental groups were fed diets containing Arg, Tau, and Arg + Tau. No difference was observed in weight of rats because of diet. Food intake was not affected by diet. FER of the rats were similar.

31.3.2 Serum Ca and P Concentrations

The concentrations of serum Ca and P were not significantly different among the experimental groups (Table 31.4). Mean serum calcium was 9.41 ± 0.39 mg/dl, 9.50 ± 0.16 mg/dl, 9.59 ± 0.30 mg/dl, and 9.44 ± 0.30 mg/dl for control, Arg, Tau, and Arg + Tau, respectively. Mean serum phosphate was 6.34 ± 0.86 mg/dl, 6.60 ± 0.42 mg/dl, 6.36 ± 0.29 mg/dl, and 6.98 ± 0.76 mg/dl for control, Arg, Tau, and Arg + Tau, respectively. The mean serum calcium and phosphate concentrations were within the normal range.

31.3.3 Urine Calcium, Phosphorus, Deoxypyridinoline, Creatinine, and Cross-Link Value

Urinary calcium and phosphate excretion were not significantly different (Table 31.5). Arginine and/or taurine supplementation did not have a measurable effect on urinary calcium and phosphate excretion. Urinary calcium excretion in the experimental diet group tended to be less in the arginine- or taurine-supplemented group than in the un-supplemented group, although urinary calcium excretion was not significantly different between the four groups. The amount of urine calcium

Table 31.4 Serum calcium and phosphorus concentrations of rats fed experimental diets

Variables	Control	Arg	Tau	Arg + Tau
Ca (mg/dl)	9.41 ± 0.39[1, a, 2]	9.50 ± 0.16[a]	9.59 ± 0.30[a]	9.44 ± 0.30[a]
P (mg/dl)	6.34 ± 0.86[a]	6.60 ± 0.42[a]	6.36 ± 0.29[a]	6.98 ± 0.76[a]

[1]Mean ± SD
[2]Values with different superscripts within the row are significantly different at $p < 0.05$ by Duncan's multiple range test

Table 31.5 Urinary calcium and phosphorus excretion of rats fed experimental diets

Variables	Control	Arg	Tau	Arg + Tau
Ca (mg/day)	0.38 ± 0.14[1, a, 2]	0.35 ± 0.06[a]	0.36 ± 0.15[a]	0.36 ± 0.11[a]
P (mg/day)	17.48 ± 4.39[a]	18.33 ± 2.28[a]	19.55 ± 3.09[a]	19.51 ± 3.31[a]

[1]Mean ± SD
[2]Values with different superscripts within the row are significantly different at $p < 0.05$ by Duncan's multiple range test

Table 31.6 Serum ALP and osteocalcin concentration of rats fed experimental diets

Variables	Control	Arg	Tau	Arg + Tau
ALP (μg/l)	50.7 ± 11.0[1, a, 2]	57.7 ± 18.4[a]	61.4 ± 18.3[a]	59.0 ± 10.7[a]
Osteocalcin (ng/ml)	30.14 ± 3.80[a]	31.29 ± 4.72[a]	32.57 ± 6.92[a]	28.25 ± 5.06[a]

[1]Mean ± SD
[2]Values with different superscripts within the row are significantly different at $p < 0.05$ by Duncan's multiple range test

was lower in the arginine-supplemented group than in the control group. No significant differences in calcium excretion were observed. Urinary excretion of phosphate was greater in the taurine- or arginine-supplemented group than in the un-supplemented group, but the difference was not significant. Fasting urinary phosphate excretion was similar in all groups, too. Urinary calcium and phosphate excretion were nearly identical in all study groups.

31.3.4 Bone Markers

Serum concentrations of ALP and osteocalcin, a marker of bone formation, were not significantly different among groups (Table 31.6). Urinary bone resorption markers, cross-link value, decreased among experimental groups, but the difference was not significant (Table 31.7). Urine DPD and cross-link value were lower in the Arg + Tau-supplemented groups than in the control group and Tau and Arg + Tau groups lower in the cross-link value than in the Arg group among supplemented groups, but the difference was not significant. Choi and Seo (2006) reported that taurine supplementation increased the femur BMD, and decreased urinary calcium excretion in male rats. They also found that taurine supplementation significantly reduced concentrations of not only DPD and cross-link value. Hip fractures are the most costly of all fracture types, resulting in significant mortality, morbidity, functional consequences, and

Table 31.7 Urine deoxypyridinoline, creatinine, and cross-link value of rats fed experimental diets

Variables	Control	Arg	Tau	Arg + Tau
Deoxypyridinoline (nM)	852.6 ± 100.0[1, a, 2]	728.4 ± 223.1[a]	605.0 ± 175.3[a]	621.3 ± 204.3[a]
Creatinine (mM)	6.0 ± 0.0[a]	6.1 ± 0.4[a]	6.0 ± 0.6[a]	5.9 ± 0.6[a]
Cross-link value (nM/mM)	142.10 ± 16.67[a]	121.40 ± 37.19[a]	100.44 ± 23.57[a]	103.24 ± 30.81[a]

[1]Mean ± SD

[2]Values with different superscripts within the row are significantly different at $p < 0.05$ by Duncan's multiple range test

socioeconomic burden (Inderjeeth et al. 2012). The health effects of arginine or taurine have been studied principally in relation to diabetes (Wells et al. 2005) or anti-inflammatory effects (Schuller-Levis and Park 2003). However, no positive effects of taurine on BMD were found in the ovariectomized rats fed a calcium-deficient diet (Choi 2009). Calcium and vitamin D are key nutrients necessary for bone health. In addition to calcium and vitamin D, vitamin K, magnesium, potassium, and vitamin C may also play a role in optimizing bone health (Nieves 2005). However, most older children and adolescents in the United States do not meet the adequate intake (AI) for calcium (Fulgoni et al. 2004). Clinical trials have shown that calcium supplementation in children can increase BMD. High protein may exert detrimental effect on bone density when calcium is low (Heaney 2007). In light of our interest in the effect of taurine and arginine on the BMD, we have taken the opportunity to examine the relation between taurine and arginine supplementation and BMD.

31.3.5 Bone Mineral Density and Bone Mineral Content

BMD and BMC were measured using PIXImus (GE Lunar Co, Wisconsin, USA) in spine and femur. The data obtained from BMD and BMC of the experimental diets are shown in Table 31.8. The spine BMC and BMD were not significantly different among the experimental groups. Rats fed taurine diet (2.0%) or arginine diet (2.0%) had no significant difference in spine and femur BMD and BMC than those fed control diet in female fed an appropriate diet. Supplementation with arginine and taurine did not significantly alter calcium excretion or markers of bone turnover in this study. Taurine (2%) was added to the food from 8 to 18 weeks of age. In one study (Choi 2007a, b), arginine supplementation markedly increased BMD in female rats. This confirms that arginine supplementation increased of bone in female rats, too. Results of this study are also consistent with the previous study (Choi 2007a, b). To our knowledge, the present study was the first attempt to supplement the amino acids of diet. This study showed that arginine and taurine supplementation is acceptable to growing female rat with enough calcium. Arginine and taurine supplementation did not affect urinary calcium excretion. This fining supports earlier studies by us (Choi 2007a, b) that taurine or arginine supplementation in diet did not increase urinary calcium excretion in rats, as long as the amount of calcium in the diet is enough. Skeletal bone mass reaches over 90% of its maximum by age 18

Table 31.8 Spine BMD and BMC of rats fed experimental diets at 9 weeks

Variables	Control	Arg	Tau	Arg + Tau
SBMD (g/cm^2)	0.156 ± 0.007[1, a, 2]	0.157 ± 0.012[a]	0.162 ± 0.007[a]	0.159 ± 0.004[a]
SBMD (g/cm^2)/Wt (kg)	0.517 ± 0.025[a]	0.524 ± 0.021[a]	0.532 ± 0.021[a]	0.521 ± 0.033[a]
SBMC (g)	0.519 ± 0.049[a]	0.550 ± 0.071[a]	0.563 ± 0.041[a]	0.539 ± 0.048[a]
SBMC (g)/Wt (kg)	1.757 ± 0.072[a]	1.831 ± 0.168[a]	1.841 ± 0.048[a]	1.733 ± 0.107[a]

[1]Mean ± SD

[2]Values with different superscripts within the row are significantly different at $p < 0.05$ by Duncan's multiple range test

(earlier in females) but does not peak until age 25–30, at some point in mid-life (Heaney et al. 2000). Body weight relationships were statistically significant between the BMC of the femoral neck (Wheatley 2005). The taurine-supplemented group had higher spine BMC than did the un-supplemented group (0.563 ± 0.041 g compared with 0.519 ± 0.049 g). We also analyzed our results after correcting for body weight, because weight can affect mechanical factors such as increased loading associated with changes to bone tissue, and increased weight can encourage mineralization and alter bone microarchitecture (Frost 2000).

In the Arg group, spine BMC increased by 6.0%, femur BMD increased by 2.6%, and femur BMC increased by 5.2% and these differences were significantly different from those in the control group ($p < 0.05$). But spine BMD of the Arg group was similar to that of the control group. After feeding of taurine supplementation for 9 weeks, serum calcium and phosphorus concentrations tended to decrease slightly. Compared with the control group, the Tau group significantly increased the FBMD at 9 weeks after taurine supplementation. Compared with the control group, the Arg + Tau group increased the FBMD ($p < 0.05$) (Table 31.9). Despite a slightly greater FBMD of the Arg and Arg + Tau group, this increase of FBMD tended to increase the amount of FBMC from the Arg + Tau group (Table 31.9). The femur BMC, expressed per body weight, was lower in the rats fed control diet than in the supplemented groups. The femur BMC when expressed as kilogram per body weight was significantly greater in the Arg + Tau group than in the Arg- or Tau-supplemented groups. The results of the current investigation confirm data reported previously indicating that bone mass is increased in rats receiving long-term Arg or Tau. Compared with control group, rats given Tau supplementation group had higher values for both femur and femur per weight BMC, as suggested previously by Choi and DiMarco (2009).

Taurine is a potent antioxidant and prevents tissue injury as a result of antioxidation (Wong et al. 2009). No serious side effects were found with taurine supplementation in study by Nakaya et al. (2000) nor in the studies by Azuma et al. (1992) and Takahashi et al. (1998). Even though a small amount of taurine is synthesized in liver in humans (Garcia and Stipanuk 1992) the main source of taurine is from ingestion of foods of animal origin (Yu et al. 1998). Also meat intake has been positively associated with incidence and mortality of chronic diseases, including heart disease (Micha et al. 2010) and osteoporosis. Excess

Table 31.9 Femur BMD and BMC of rats fed experimental diets at 9 weeks

Variables	Control	Arg	Tau	Arg + Tau
FBMD (g/cm^2)	0.196 ± 0.008[1, a, 2]	0.201 ± 0.011[ab]	0.207 ± 0.008[b]	0.200 ± 0.005[ab]
FBMD (g/cm^2)/Wt (kg)	0.653 ± 0.029[a]	0.683 ± 0.040[a]	0.679 ± 0.028[a]	0.657 ± 0.040[a]
FBMC (g)	0.425 ± 0.019[a]	0.447 ± 0.031[a]	0.448 ± 0.033[a]	0.455 ± 0.024[a]
FBMC (g)/Wt (kg)	1.413 ± 0.060[a]	1.472 ± 0.059[ab]	1.466 ± 0.060[ab]	1.485 ± 0.07[b]

[1]Mean ± SD

[2]Values with different superscripts within the row are significantly different at $p < 0.05$ by Duncan's multiple range test

protein intake is risk factors for osteoporosis. So without the increase of meat intake, supplementation of taurine is the better idea to protect bone. In a previous study, our rat model also confirmed that supplementation of a taurine diet increases femur BMC. In a previous publication by our group, it was shown that a taurine-supplemented diet could significantly increase the femur BMC in growing male rats (Choi and Seo 2006). To improve osteoporosis prevention strategies, a better understanding of nutrition-related risk is needed. Higher animal protein intakes have been reported to be associated with increased risk of diabetes (Sluijs et al. 2010). Of the many factors that affect BMD, nutrition is considered an important factor (Choi and Jo 2003; Kim and Kim 1983). In Western countries, a sizeable proportion of the population has adopted a vegetarian diet. According to previous studies in the European Union, the proportion of self-reported vegetarians in the general population is 5% (Heys and Gardner 1999). Whether vegetarian diets confer benefit or harm to bone health is a contentious issue. Ecologic studies found an inverse association between the incidence of hip fracture and vegetarian protein intake, such that countries with a high intake of vegetable protein had a lower risk of hip fracture (Newsholme and Leech 1983; Windmueller and Spaeth 1981). Whereas some data suggest that a raw vegetarian diet is associated with lower bone mass (Ho-Pham et al. 2009), other studies have found no such association (Chung 2001; Chen et al. 2003; Park et al. 2001). There was no significant effect of arginine or taurine on urinary ca excretion, osteocalcin, and spine BMD with appropriate diets. Generally, it is calcium that is the limiting nutrient in the diets of North America and Asia (Thacher et al. 2006). Taurine supplementation has been shown to have a positive effect on bone in OVX rats with appropriate calcium (Choi and DiMarco 2009). But there was no positive effects in the ovariectomized rats fed a calcium-deficient diet (Choi 2009). Thus the effects of amino acids depend on the content of calcium in the diet. Level of calcium in diet with taurine or arginine to be studied to bone health by virtue of their (taurine or arginine) beneficial effect. These data suggest that it would be worthwhile to explore further link between dietary arginine, taurine, and markers of bone health. Future work should also focus on the role of particular amino acid in regulating bone metabolism.

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Chapter 32
Taurine Enhances the Sexual Response and Mating Ability in Aged Male Rats

Jiancheng Yang, Shumei Lin, Ying Feng, Gaofeng Wu, and Jianmin Hu

Abstract It has been demonstrated that taurine is abundant in male reproductive organs, and can be biosynthesized by testis, but the taurine concentration will reduce with aging. The levels of serum LH, T, NOS, and NO were found to be obviously increased by taurine supplementation in aged rats in our previous study. In addition, aging will result in a significant decline in sexual response and function, which may be attributed to the androgen deficiency. Furthermore, NO has been proposed as a crucial mediator of penile erection. That makes us hypothesize that there is potential relationship between taurine decline and erection dysfunction in aged males. So the primary aim of the present study was to investigate the effect of taurine on male sexuality in rats. Taurine was offered in water to male aged (20 months old) rats for 110 days. The effects of taurine on the sexual response, mating ability, levels of serum reproductive hormones, and penile NOS and NO levels were investigated. The results showed that taurine can significantly reduce the EL and ML; obviously increase the ERF, MF, IF, and EJF; stimulate the secretion of GnRH, LH, and T; and elevate penis NOS and NO level in aged rats. The results indicated that taurine can enhance the sexual response and mating ability in aged male rats by increasing the level of testosterone and NO, but the exact mechanism of which needs to be further investigated.

Abbreviations

LH	Luteinizing hormone
T	Testosterone
NOS	Nitric oxide synthase

J. Yang • S. Lin • Y. Feng • G. Wu • J. Hu (✉)
College of Animal Science & Veterinary Medicine, Shenyang Agricultural University, Shenyang 110866, People's Republic of China
e-mail: hujianmin59@163.com

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NO	Nitric oxide
Con	Control group
Tau	Taurine group
β-Ala	β-Alanine group
EL	Erection latency
ERF	Erection frequency
ML	Mount latency
MF	Mount frequency
IF	Intromission frequency
EJF	Ejaculation frequency
GnRH	Gonadotropin-releasing hormone
FSH	Follicle-stimulating hormone
E2	Estradiol
LH	Luteinizing hormone
TP	Total protein

32.1 Introduction

Taurine (2-aminoethane sulfonic acid), one of the most abundant amino acids in mammalian plasma and tissues, has been demonstrated to be a potentially nutritionally important amino acid, which involved in several physiological functions, such as neurotransmitter or neuromodulator, bile formation in the liver, modulation of calcium flow, osmoregulation, stabilization of membranes, and antiarrhythmic activity in the heart (Huxtable 1992). It has been demonstrated that taurine is synthesized in the liver; however recent studies indicated that taurine might be biosynthesized in other organs such as brain (Oertel et al. 1981), retina (Pasantes-Morales et al. 1976), mammary gland (Hu et al. 2000), and testis in mammals (Li et al. 2006). In male reproductive organs, taurine immunoreactivity has been specifically localized in Leydig cells, vascular endothelial cells, other interstitial cells of testis, peritubular myoid cells, and epithelial cells of the efferent ducts (Lobo et al. 2000). Taurine may act as an antioxidant in preventing rabbit sperm lipid peroxidation (Alvarez and Storey 1983), as a capacitating agent (Meizel et al. 1980; Meizel 1985), as a membrane-stabilized factor (Mrsny and Meizel 1985), and sperm motility factor (Fraser 1986). Furthermore, our research group found that taurine supplement could stimulate the secretion of luteinizing hormone (LH) and testosterone (T), increase the activity of testicular marker enzymes, elevate testicular antioxidation (including the levels of nitric oxide synthase (NOS) and nitric oxide (NO)), and improve sperm quality in male rats of different ages, especially in aged rats (Yang et al. 2010a).

It is well known that aging results in a significant decline in sexual response and function, which may be attributed to the androgen deficiency (Tenover 1997; Swerdloff and Wang 1993), because androgens acting both centrally and peripherally are essential for normal penile erection and T-stimulating nitric oxide synthesis

in the corpora cavernosa (Mills et al. 1996; Lugg et al. 1995). As mentioned above, we found that taurine could increase the levels of testosterone and NO in aged rats. In addition, a number of studies have reported that the level of taurine is decreased in aged animals (Wallace and Dawson 1990; Dawson and Wallace 1992). However, the correlation between taurine deficiency and sexual function decline in the male aged rats is still unknown. The primary aim of the present study was to investigate the effect of taurine on sexuality and mating ability in aged male rats.

32.2 Methods

32.2.1 Experimental Animals and Treatments

Twenty-four aged male Wistar rats (20 months) were obtained from Kunming Institute of Zoology, Chinese Academic Sinica. The rats were randomly divided into three groups, eight in each. Rats in control group (Con) were given tap water, and rats in the other two groups were given water containing 1% taurine (taurine group, Tau) and 1% β-alanine (β-alanine group, β-Ala). They were maintained in controlled light (14-h light, 10-h dark) and temperature (22 ± 2°C), and were given free access to rat chow and water. The study protocol was approved by our Ethical Committee and conducted in compliance to the Helsinki Declaration.

32.2.2 Sexual Response and Mating Ability Examining

The sexual response was determined on the 60th day after taurine administration according to the reported method by means of apomorphine (Sachs et al. 1994). The erection latency (EL) and erection frequency (ERF) were recorded. Mating ability was examined on the 74th day in accordance with the previous method (Lumley and Hull 1999). Mount latency (ML), mount frequency (MF), intromission frequency (IF), and ejaculation frequency (EJF) were measured.

32.2.3 Sample Collection and Biochemical Analysis

Rats were killed after being treated for 110 days, and blood and penis were collected. Blood was used for gonadotropin-releasing hormone (GnRH), LH, follicle-stimulating hormone (FSH), and T and estradiol (E2) analysis. Penis was homogenated in cold physiological saline, and then was used for total protein (TP), NOS, and NO analysis. All the indicators were analyzed according to the introduction of reagent kits (Beijing Chemclin Biotech Co., Ltd., China).

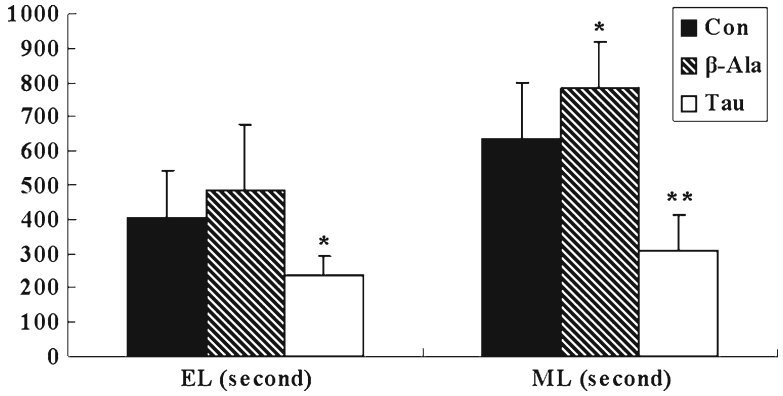

Fig. 32.1 The EL and ML of aged male rats: Results are presented as mean ± SE ($n = 8$). *: significantly different from the control group ($p < 0.05$), **: significantly different from the control group ($p < 0.01$)

32.2.4 Statistic Analysis

Data were presented as the mean ± SE and significant differences were determined by Duncan's multiple range test using SPSS 16.0 statistical analysis software. p values less than 0.05 were considered significant.

32.3 Results

32.3.1 Sexual Response and Mating Ability Analysis

The effect of taurine on sexual response and mating ability in aged male rats is present in Figs. 32.1 and 32.2. The EL was significantly decreased, but the ERF was obviously increased when treated with taurine. The EL and ERF had no changes by β-alanine administration. The ML was significantly reduced by taurine administration, but the MF, IF, and EJF all were obviously increased by taurine administration in aged male rats. The ML was significantly increased by β-alanine supplement, yet the MF, IF, and EJF showed no difference compared with the control group.

32.3.2 Blood Biochemical Indicator Analysis

As Fig. 32.3 illustrates, the level of GnRH was obviously increased by taurine administration in aged rats, but there are no statistical differences when treated by β-alanine. As shown in Figs. 32.4 and 32.5, the levels of LH and T were significantly

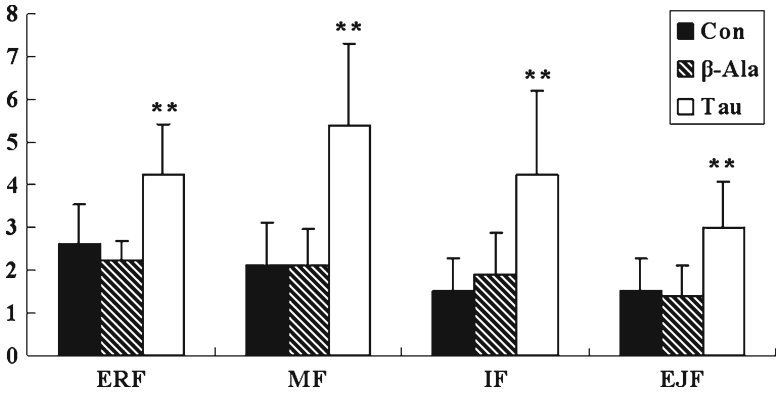

Fig. 32.2 The ERF, MF, IF, and EJF of aged male rats: Results are presented as mean ± SE ($n = 8$). **: significantly different from the control group ($p < 0.01$)

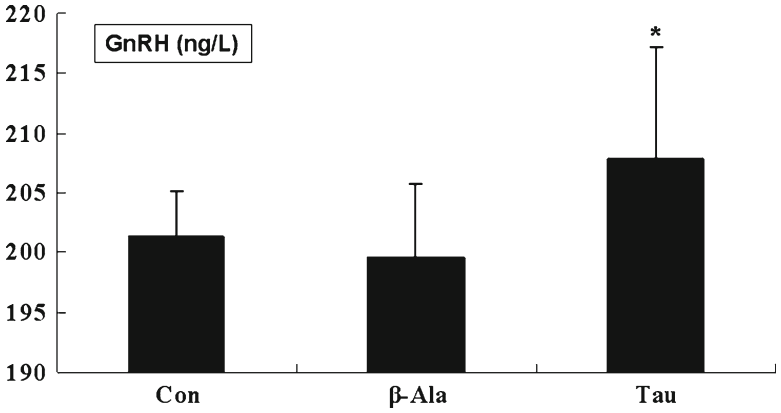

Fig. 32.3 The level of GnRH in the serum: Results are presented as mean ± SE ($n = 8$). *: significantly different from the control group ($p < 0.05$)

increased in aged male rats when treated by taurine, while it were obviously decreased by β-alanine supplement. The levels of FSH and E2 had no changes in all the experimental groups.

32.3.3 Penis NOS and NO Analysis

The effect of taurine on the levels of NOS and NO in the penis tissue of aged male rats was presented in Fig. 32.6. The levels of NOS and NO were significantly elevated by taurine administration. The NO level in the penis was obviously decreased by β-alanine treatment in aged male rats, but the ability of NOS had no difference with control group.

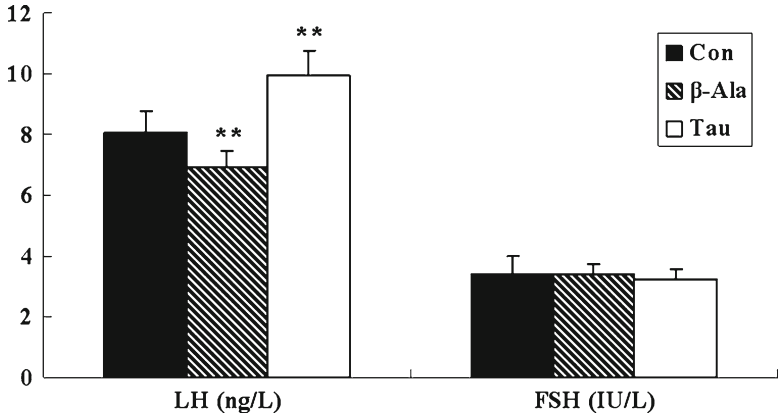

Fig. 32.4 The levels of LH and FSH in the serum: Results are presented as mean ± SE ($n = 8$). **: significantly different from the control group ($p < 0.01$)

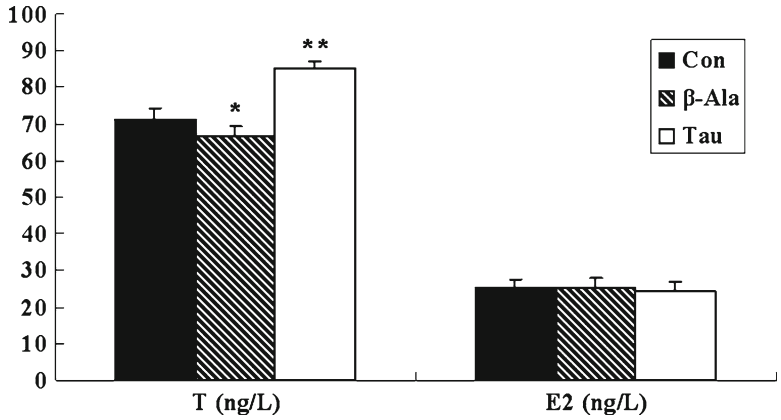

Fig. 32.5 The levels of T and E2 in the serum: Results are presented as mean ± SE ($n = 8$). *: significantly different from the control group ($p < 0.05$), **: significantly different from the control group ($p < 0.01$)

32.4 Discussion

Taurine is one of the most abundant free amino acids in male reproductive organs, and it has many effects on male reproduction including stimulating androgen secretion (Holmes et al. 1992; Yang et al. 2010b). It has been generally accepted that androgens are critical for development, growth, and maintenance of penile erectile tissue. The levels of taurine and androgen reduced with aging, and aging associated with decline in libido, erection, and sexual ability (Schiavi and Rehman 1995; Meacham and Murray 1994). So we hypothesized that there are some relations between taurine concentration reduction and age-related erectile dysfunction, and taurine supplement may increase the sexuality and mating ability in aged males.

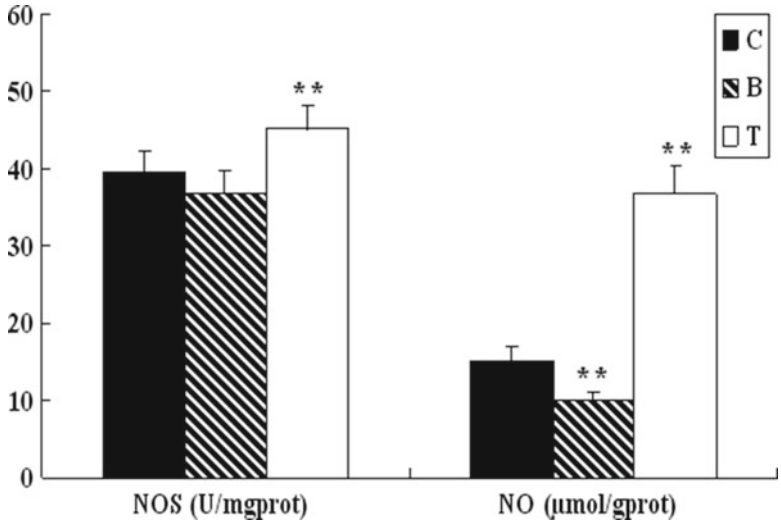

Fig. 32.6 The levels of NOS and NO in the penis tissue: Results are presented as mean ± SE ($n = 8$). **: significantly different from the control group ($p < 0.01$)

In the present study, we firstly found that taurine can significantly reduce the EL and ML and obviously increase the ERF, MF, IF, and EJF in male aged rats. The results indicated that taurine can increase the sexual response and mating ability in aged male rats.

Normal male reproductive function depends on the intermittent secretion of LH and FSH by the pituitary gland that was stimulated by hypothalamic GnRH. LH stimulates the testicular Leydig cells to secrete T. Testosterone is necessary in libido and sexual behavior, and can increase sexual interest and frequency of sexual acts (Mulliqan and Schmitt 1993). Any dysfunction of the hypothalamic–pituitary–testis axis can result in productive and sexual dysfunction. The function of hypothalamic–pituitary–testis axis will degrade with aging, which is due to the decrease in GnRH, FSH, LH, and T (Lamberts et al. 1997; Tenover 1997). Our results showed that the concentrations of GnRH, LH, and T in male aged rats were obviously increased by taurine administration, which implies that the enhancement of reproductive function may be partly attributed to more T secretion. Although taurine is known as a GnRH inhibitor in the central nervous system, we think that taurine may increase GnRH secretion by means of LH or other ways rather than direct effect on hypothalamus.

Penile erection is a highly regulated physiologic event, involving increased arterial inflow and restricted venous outflow, coordinated with corpus cavernosum smooth muscle relaxation (Andersson and Wagner 1997). NO has been demonstrated as a crucial mediator of penile erection, because NOS, the synthetic enzyme for NO, was localized to the penile innervation and NOS inhibitors selectively block erections (Burnett et al. 1992). Our laboratory had previously reported that taurine can increase the levels of serum NOS and NO in aged male rats (Yang et al. 2010b).

But there is no detailed information on the relation of taurine and penile NO. Our studies showed that the pensile NOS and NO were increased in aged rats by taurine administration, which indicated that sexual enhancement may be partly attributed to the elevated effect of taurine on penile NOS and NO levels. It has been reported that androgens can mediate the erectile response in the rat penis by stimulating the expression of NOS, thus maintaining an adequate supply of NO (Reilly et al. 1997), which is in agreement with our results.

32.5 Conclusion

In summary, the present study demonstrated that taurine supplement could increase sexual response and function; stimulate the secretion of GnRH, LH, and T; and elevate penis NOS and NO level in aged rats. The results implied that taurine can enhance the sexual response and mating ability in aged male rats by increasing the level of testosterone and NO, but the exact mechanism needs to be further investigated.

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