ʟ-Carnitine–ʟ-tartrate promotes human hair growth *in vitro*

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Abstract: The trimethylated amino acid ʟ-carnitine plays a key role in the intramitochondrial transport of fatty acids for β-oxidation and thus serves important functions in energy metabolism. Here, we have tested the hypothesis that ʟ-carnitine, a frequently employed dietary supplement, may also stimulate hair growth by increasing energy supply to the massively proliferating and energy-consuming anagen hair matrix. Hair follicles (HFs) in the anagen VI stage of the hair cycle were cultured in the presence of 0.5–50 μM of ʟ-carnitine–ʟ-tartrate (CT) for 9 days. At day 9, HFs treated with 5 μM or 0.5 μM of CT showed a moderate, but significant stimulation of hair shaft elongation compared with vehicle-treated controls ($P < 0.05$). Also, CT prolonged the duration of anagen VI, down regulated apoptosis (as measured by TUNEL assay) and up regulated proliferation (as measured by Ki67 immunohistology) of hair matrix keratinocytes ($P < 0.5$). By immunohistology, intrafollicular immunoreactivity for TGFβ2, a key catagen-promoting growth factor, in the dermal papilla and TGF-β II receptor protein in the outer root sheath and dermal papilla was down regulated. As shown by caspase activity assay, caspase 3 and 7, which are known to initiate apoptosis, are down regulated at day 2 and day 4 after treatment of HFs with CT compared with vehicle-treated control indicating that CT has an immediate protective effect on HFs to undergo programmed cell death. Our findings suggest that ʟ-carnitine stimulates human scalp hair growth by up regulation of proliferation and down regulation of apoptosis in follicular keratinocytes *in vitro*. They further encourage one to explore topical and nutraceutical administration of ʟ-carnitine as a well-tolerated, relatively safe *adjuvant* treatment in the management of androgenetic alopecia and other forms of hair loss.

Key words: anagen – apoptosis – catagen – hair follicle – ʟ-carnitine–ʟ-tartrate

Introduction

The 'conditionally essential' nutrient, ʟ-carnitine, is a trimethylated amino acid-derivative (zwitterionic alcohol), which is found in all animal species as well as in some microorganisms and higher plants. ʟ-Carnitine occurs naturally in the diet or can be synthesized from lysine and methionine within the human body (1–3). It is essential for the intramitochondrial transport of long-chain fatty acids for β-oxidation, regulation of CoA concentration and removal of the produced acyl groups, and is involved in both the metabolism of ketones for energy and the conversion of branched-chain amino acids into energy (3,4).

AcylCoA acts as a restraining factor for several enzymes participating in intermediary metabolism, and transformation of AcylCoA into acylcarnitine is an important system for removing toxic acyl groups from the body (5–7).

The transfer of long-chain fatty acids across the inner mitochondrial membrane requires enzymes and transporters that accumulate carnitine within the cell (newly identified organic cation transporter (OCTN)1 and OCTN2 carnitine transporter), conjugate it with long-chain fatty acids (carnitine palmitoyl transferase 1, CPT1), transfer the acylcarnitine across the inner plasma membrane (carnitine-acylcarnitine translocase, CACT), and conjugate the fatty acid back to coenzyme A for subsequent β-oxidation (carnitine palmitoyl transferase 2, CPT2) (4,8,9).

Primary carnitine deficiency is an autosomal recessive disorder caused by defective carnitine transport because

Abbreviations: CT, carnitine tartrate; DP, dermal papilla; HFs, hair follicles.

of mutation in the *OCTN2* gene (4,10–12). Secondary carnitine deficiency is acquired, e.g. under treatment with valproate or zidovuline, and may result from impaired carnitine biosynthesis in cirrhosis, renal failure, diabetes, chronic infectious disease, chronic heart failure and Alzheimer disease (6). Consistent experimental evidence also supports that lack of L-carnitine impairs the ability to use fat as fuel during periods of fasting or stress. This can result in acute hypoglycemia, Rye syndrome and sudden infant death or, later in life, in skeletal or cardiac myopathy (10,13,14).

On the basis of these and other observations, numerous potential uses of L-carnitine, its salts and derivatives have been explored experimentally and clinically, and a voluminous patent literature claims a wide range of beneficial uses for L-carnitine (3,15,16). For example, the less efficient inflammatory responses towards infectious agents associated with ageing may be improved by carnitine treatment (17), and acetyl-L-carnitine improves HIV-associated antiretroviral toxic neuropathy (18). Also, because of its regulatory action on the energy flow from different oxidative sources, especially under ischaemic conditions, carnitine may be of benefit in coronary heart disease, congestive heart failure, peripheral vascular disease, diabetes and chronic renal disease (16,19–21).

Mitochondrial oxidative decay, a major contributing factor to the ageing process, may be ameliorated by feeding acetyl- or L-carnitine, at least in ageing rats (22–24). Decreased serum levels of carnitine are associated with cancer cachexia (25). Recently, oral L-carnitine supplementation has been found to lower lipid peroxidation and to promote the conservation of retinol and α-tocopherol in women (26). Finally, as a variation of the 'metabolic tuneup' philosophy, prolonged consumption of high-dose L-carnitine is very popular as a putative dietary enhancer of athletic performance, even though conflicting evidence exists as to whether L-carnitine really has such ergogenic properties (15,27).

Much less is known about the functions of L-carnitine in normal skin. Here, it has been speculated to re-equilibrate the catabolic deficit of fat in elderly individuals (28). Skin ageing is associated with an increase of adipocyte diameter, and carnitine-treated mice show a significantly smaller adipocyte diameter than that of controls. In addition, propionyl-L-carnitine seems to dilate human subcutaneous arteries through an endothelium-dependent mechanism (29), possibly because of an up regulation of nitric oxide production (30,31). In ischaemia and secondary burn injuries increased energy flow requirements are major problems. In rats, L-carnitine limits the necrosis in and improves survival of burned dorsal skin flaps (32,33) as well as skin flap survival in general (34). Treatment of patients suffering from cutaneous ulcers in various vasculopathies with propionyl-L-carnitine reportedly promotes

healing (35), and acetyl-L-carnitine improves pain, nerve regeneration, and vibratory perception in patients with chronic diabetic neuropathy (36). In psoriasis, the activity of the key enzyme in fatty acid oxidation, CPT1, has been shown to be increased and treatment with the CPT-1 inhibitor, Etomoxir, blocked this activity. In a mouse model, Etomoxir had an antipsoriatic effect that was at least as good as that of betamethasone, as evidenced by reduction of epidermal thickness, keratinocyte proliferation and differentiation. Therefore, fatty acid metabolism and in particular CPT-1 may be involved in the pathogenesis of psoriasis and as a target in the management of the disease (37).

While these observations suggest beneficial effects of L-carnitine for normal skin function, states of primary or secondary L-carnitine deficiency are not recognized to induce, *sui generis*, abnormalities in the skin or its appendages. Nevertheless, we reasoned that, even under physiological conditions, the metabolically most active and most energy-consuming tissue of mammalian skin – i.e. the anagen hair follicle (HF), with its massive hair matrix proliferation, hair shaft generation and hair shaft pigmentation (38–40) – should be particularly responsive to the capacity of L-carnitine to increase energy supply, re-equilibrate catabolic deficits and/or inhibit apoptosis.

To explore this hypothesis, and to investigate the functional properties of L-carnitine in normal human skin, we have studied the effects of L-carnitine on cycling human scalp HFs *in vitro*. In microdissected, organ-cultured human scalp HFs, we studied whether L-carnitine and L-carnitine- L-tartrate (CT) affected hair shaft elongation, HF cycling, hair matrix proliferation and/or apoptosis, and the protein expression of key catagen promoters TGFβ2 and TGFβ receptor Type II (41–43) *in situ*. In addition, we performed a caspase activity essay for caspase 3/7, which are known initiate apoptosis on HFs treated for 2 or 4 days with CT. As CT has the same bioavailability as that of L-carnitine, it is absorbed faster after oral administration (44), and toxicological data for CT were available, the bulk of the experiments were performed with this carnitine salt.

Materials and methods

Materials
Williams E Medium (Gibco BRL, Rockville, MD, USA) was supplemented with L-glutamine, penicillin and streptomycin. L-carnitine and CT (44) were purchased from Lonza (Wuppertal, Germany).

Isolation and culture of human HFs
Excess anagen VI HFs from occipital human scalp skin, obtained from healthy individuals with informed consent

during routine hair transplant surgery, were isolated, microdissected and cultured within 24 h after transplantation as previously described by Philpott et al. (45), with minor modifications (46,47). The total number of organ-cultured anagen VI HFs was 180 ($n = 45$ per group), derived from five different, male individuals aged 25–55 years. Three HFs per well were cultured in Williams E medium at 37°C in 5% CO_2/95% air in 500 μl of basal medium, supplemented with insulin 10 μg/ml, hydrocortisone 10 ng/ml, streptomycin 10 μg/ml and penicillin 100 U/ml (43). In five experiments, nine HFs per group were incubated (total: $n = 45$ per group). Test groups received 500, 50, 5 or 0.5 μM CT daily, dissolved in Williams E medium. Plasma levels of total carnitine in healthy humans are 42.8 μmol in female and 43.1 μmol in males, and of free carnitine are 28.4 μmol in females and 28.6 μmol in males (48,49). According to Koeck et al. (30) 0.1 mM/l is the physiological dose of L-carnitine in human cell culture. Therefore, we chose the above concentrations. After 9 days in culture, human HFs were washed in PBS and embedded in OCT for cryosectioning.

Histomorphometry and statistical analysis

Hair shaft length was measured every second day, using a binocular dissecting microscope, and hair-cycle stages were assessed according to defined morphological criteria and photodocumented by light microscopy. Longitudinally cut HFs ($n = 27$ per group) were stained by haematoxylin & eosin (H&E), and the hair-cycle stage of each HF was classified by morphological criteria and assigned to their respective hair-cycle stages (50,51), applying quantitative hair cycle histomorphometry techniques described for murine catagen development (47,52). The data of all experiments were pooled and statistical significance was calculated by Mann–Whitney U-test for unpaired samples.

Ki-67 and TUNEL double staining

To evaluate apoptotic cells in the HF, we used an established, commercially available TUNEL kit (Apotag, Oncor Apligene, Heidelberg, Germany) as described before, while Ki-67 was used for detecting proliferating cells (47,53). For double immunofluorescence staining of TUNEL-positive cells and Ki67-IR, the protocol for the TUNEL technique was combined with the manufacturer's protocol for Ki67-immunofluorescence. Briefly, 6–7 μm frozen tissue sections were air dried for 15 min, fixed in 1% paraformaldehyde for 10 min and postfixed in ethanol–acetic acid (−20°C) for 5 min followed by incubation with digoxygenin–dUTP in the presence of TdT for 1 h at 37°C. TUNEL-positive cells were visualized by anti-digoxygenin flourescein-conjugated antibody. Subsequently, tissue sections were incubated with mouse-anti-human-Ki67 antiserum 1:20

(Dianova, Hamburg, Germany), and Ki67-IR was detected by goat-anti-mouse Rhodamin-conjugated F(ab)2 fragments (1:200; Jackson ImmunoResearch, Hamburg, Germany). Sections were counterstained with DAPI 1:5000 (Hoechst 33342). Negative controls for the TUNEL staining were made by omitting TdT enzyme; murine spleen sections served as positive control. For Ki67, positive controls were run by comparison with tissue sections from the back skin of mice in anagen VI stage of the depilation-induced hair cycle.

Sections were examined under a Zeiss Axioscope microscope (Jena, Germany), using the appropriate excitation–emission filter systems for studying the fluorescence induced by DAPI, fluorescein and rhodamine. The number of positive cells for Ki-67 and TUNEL immunoreactivity (IR) was counted per hair bulb and statistical significance was calculated by Mann–Whitney U-test for unpaired samples.

TGF-β and TGF-β type II receptor immunofluorescence

Isolated human HFs were cultured with CT or vehicle control as described above for 2 or 4 days. For positive control, HFs were treated with TGF-β 2 25 ng/ml per day for 2 or 4 days. Cryosections of cultured human HFs were air-dried for 15 min and fixed in ethanol (−20°C) for 10 min before the preincubation in 10% normal goat-serum for 30 min at room temperature. After washing in PBS, primary rabbit antibodies against TGF-β2 (1:50), TGF-β RII (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were placed on sections in a moisture chamber over night at 4°C. Immunoreactivity was detected using Alexa Flour 488 labelled goat-anti-rabbit secondary antibody (1:200, Jackson ImmunoResearch), and counterstaining was made with DAPI (Hoechst 33342) (47).

Caspase 3 and 7 activity assay

For caspase activity assay, microdissected human scalp HFs ($n = 12$ per group) were treated with 0.5 μM of CT or vehicle control for 2 or 4 days. HFs were then harvested and cut into small pieces. HFs were treated with 1 ml of collagenase for 1 h at 37°C, then vortexed and centrifuged. Collagenase was carefully thrown away and HFs were mixed with 1 ml Trypsin/EDTA. After 1 h at 37°C and centrifugation, Trypsin was thrown away and pellets were resuspended with 110 μl HF medium. Medium was then removed without taking the HF debris, remaining cells were counted and cells were again diluted until the same numbers of cells were in each tube. Subsequently, Caspase reagent (Promega cat # G8091, Maddison, WI, USA) and HF medium with cells or for negative control without cells were mixed in a 96-well plate. After 1 h of incubation at room temperature, cells were counted using a luminometer.

Results

Carnitine–tartrate promotes human hair shaft elongation *in vitro*

As previously described, human anagen VI scalp HFs can stay in culture up to about 10 days, continue to produce pigmented hair shafts and tend to enter spontaneously into a catagen-like state of HF regression (45,47,54). In the current study, we cultured human anagen VI HF from male occipital scalp skin up to 9 days and observed a hair shaft elongation of approximately 0.28 mm per day during this period, which approaches the average *in vivo* growth rate of human terminal scalp hair (54) (Figs 1a and 2a). In the first sets of experiments, we compared the influence of ʟ-carnitine and CT (50 and 500 μM) on isolated human HFs and detected a significant stimulating effect for 50 μM
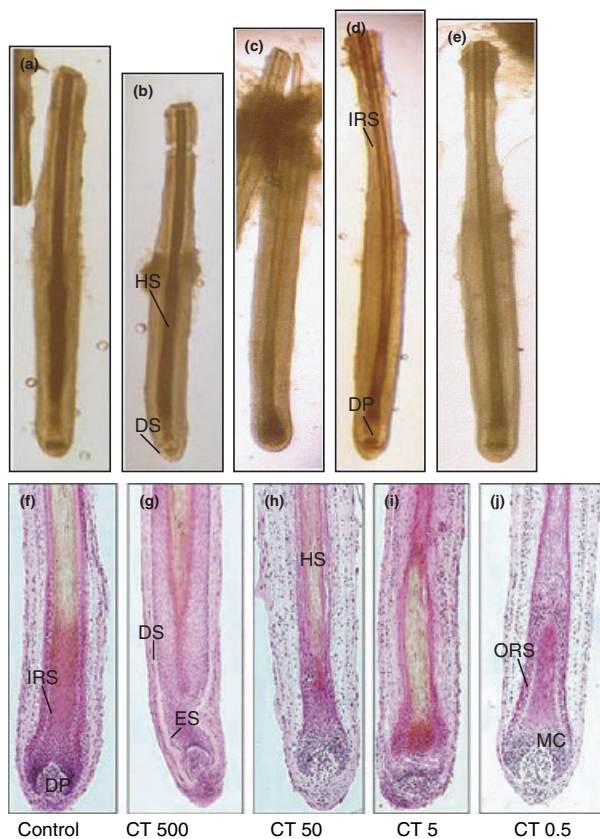

Figure 1. ʟ-Carnitine ʟ-tartrate (CT) stimulates hair shaft elongation *in vitro*. Macroscopical and histological demonstration of isolated organ-cultured human HFs treated with CT 500, 50, 5 or 0.5 μM for 9 days compared with vehicle control. (a), (f): control; (b), (g): CT: 500 μM; (c), (h): 50 μM; (d), (i):. 5 μM; (e), (j): 0.5 μM. 500 μM of CT induced early catagen induction (1b+g) characterized by cessation of pigmentation, the shape of the DP changed into a more condensed shape, the volume of the hair matrix diminishes and the pigmented lower end of the hair shaft moves upward in isolated HFs. DP, dermal papilla; DS, dermal sheath; ES, epithelial strand; IRS, inner root sheath; ORS, outer root sheath, MC, melanocytes, HS, hair shaft.

CT, while ʟ-carnitine showed similar, yet less pronounced stimulatory effects which did not quite reach the level of significance (data not shown). Therefore, we continued our studies with CT only, and tested lower concentrations.

Carnitine–tartrate treatment significantly increased hair shaft elongation *in vitro* in a dose-dependent manner up to a hair shaft elongation of 0.33 mm/day (Figs 1b–e and 2a). After 9 days of treatment, HFs treated with 5 μM or 0.5 μM of CT showed a hair shaft elongation of 37% (Fig. 1c,e) compared with 32% (in relation to the original length) in the group treated with 50 μM of CT and 25% in the vehicle-treated control group (Figs 1 and 2b) ($P < 0.05$). Although this represents only a moderate stimulation, one needs to keep in mind that these organ-cultured anagen HFs are already growing at maximal speed and that even minoxidil fails to significantly stimulate hair shaft elongation in this assay (46).Treatment with the highest dose of CT (500 μM) actually resulted in an inhibition of hair shaft elongation and in premature catagen induction (Fig. 1b,g). HF of the groups treated with lower concentrations of CT (0.05 μM) did not show any morphological changes or alterations.

CT prolongs anagen in human HFs

Quantitative histomorphometry of H&E-stained HF sections after 9 days of organ-culture revealed that CT is able to prolong anagen in human anagen follicles *in vitro* in a dose-dependent manner: As shown in Figs 1a and 2c, most of the control HFs had entered a catagen-like stage, characterized by cessation of pigmentation, condensation and retraction of the dermal papilla (DP), diminished volume of the hair matrix diminishes, and upwards movement of the pigmented lower end of the hair shaft (50–52). In comparison, the CT-treated group of HFs showed significantly fewer catagen-like follicles (Figs 1g–j and 2c). Only one HF in the control group was still in anagen, while most of the HFs had entered early or late catagen after 9 days. Most HFs treated with lower concentrations of CT (5 μM, 0.5 μM) were still in late anagen or early catagen and only one HF per group had already entered late catagen, while HFs treated with 50 μM CT had entered mostly early and late catagen, and only one HF was still in late anagen after 9 days in culture (Fig. 2c). Thus, histological evaluation suggests that the stimulating effect of CT on hair shaft elongation of human HFs results from a prolongation of the anagen phase, and is not due to passive hair shaft upwards-movement by 'apoptotic force' exerted by the involuting HF epithelium (cf. 41).

CT stimulates proliferation and inhibits apoptosis of human HF matrix keratinocytes *in vitro*

This was further confirmed by analysing keratinocyte proliferation and apoptosis in the hair matrix of organ-cul-

tured human scalp HFs (Fig. 3). Blinded counting of TUN-EL + or Ki-67 + hair matrix cells after 9 days of HF organ-culture revealed a significant down regulation of TUNEl+ (i.e. apoptotic) and a slight up regulation of Ki-67+ (i.e. proliferating) hair matrix cells in CT-treated Anagen VI HFs compared with vehicle-treated controls (Fig. 3f). Anagen VI HFs contain more proliferating and less apoptotic cells in the hair bulbs. Therefore, to have a valid comparison only Ki-67 and TUNEL positive cells in anagen VI HFs were counted in both groups. Similar results were obtained when L-carnitine was used instead of CT (data not shown). While control HF showed many scattered TUNEL+ cells throughout the hair matrix, DP and connective tissue sheath (Fig. 3e), this number was reduced in CT-treated HF (Fig. 3e).

Expression of TGF-β2 and TGF-β Type II receptor is down regulated in the HF after treatment with CT

As TGF-β1 and 2 are currently considered the most important physiological catagen inducers in human scalp HFs (41,42,55,56), we also cursorily analysed by immunohistochemistry whether TGF-β2 and TGF-β type II receptor (TGFRII) immunoreactivities were altered after 2 or 4 days of HF organ-culture on HFs either treated with CT (50 μM) or with the vehicle alone. After 4 days in culture, most of the HFs were still in anagen VI in both groups and TGF-β2 and TGF type II receptor were expressed, as expected (47), in outer root sheath and hair matrix keratinocytes, while the DP stayed negative for both antigens.

After treatment with CT (50 μM) for 2 or 4 days TGF-β2 and, TGFRII were down regulated (Fig. 4). Already after 2 days of treatment, the expression of TGF-β2 was significantly decreased (Fig. 4A,a) compared with the vehicle control. After 4 days TGF-β2 was less strongly expressed in CT-treated HFs (Fig. 4B,b, E). The same results were seen for TGF-βRII. TGF-β2 (Fig. 4C,D,E) and TGF-βRII (Fig. 4F) were both significantly down regulated after 2 days of CT treatment compared with the vehicle control and the difference became less intense after 4 days. Also expression of keratinocyte growth factor (KGF) receptor decreased after 2 and 4 days of CT treatment in the hair bulb, but the difference is not significant (data not shown). TGF-β2 is a strong catagen inducer and is up regulated during catagen, thus CT seems to have an immediate suppressive effect on TGF-β2 which is finally probably not strong enough to prevent the HFs to enter catagen, but seems to delay the process.

Caspase 3/7 activity is significantly lower after treatment of HFs with CT

Microdissected human HFs were cultured and treated with either 50 μM of CT or vehicle control. To further investi-
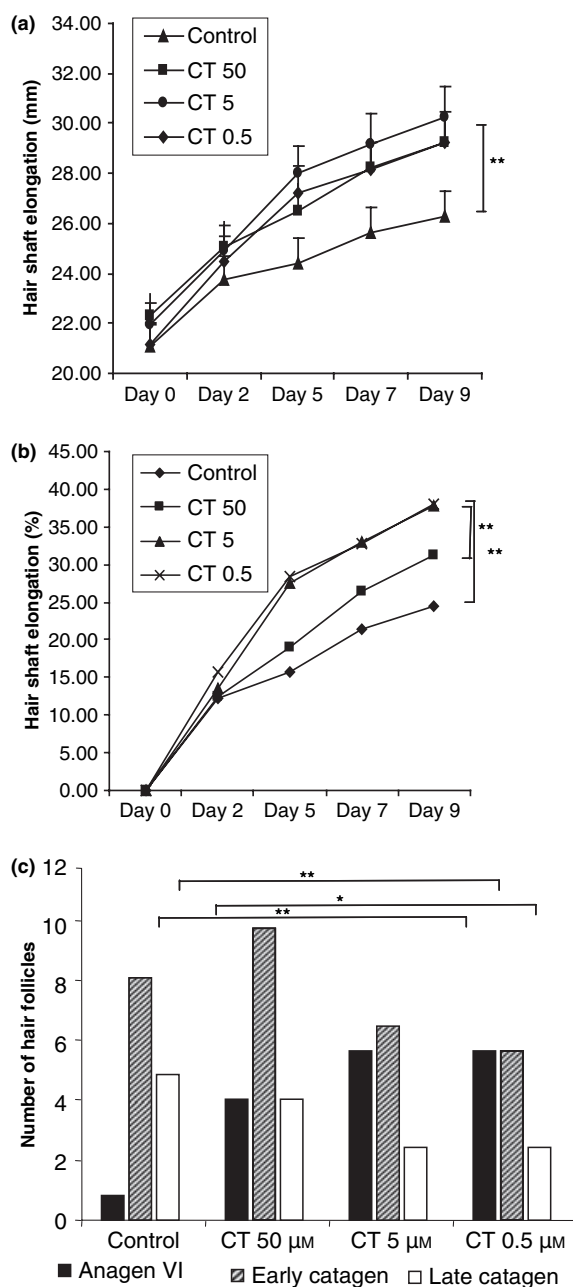

Figure 2. (a) Hair shaft elongation in millimetre of isolated human hair follicles (HFs) treated with different concentrations of L-carnitine L-tartrate (CT) after 9 days in culture. **$P = 0.05$. (b) Hair shaft elongation in percentage. HFs treated with CT showed a significant increase in hair shaft elongation compared with the control group. **$P = 0.05$. C: Assessment of hair cycle stages of human HFs treated with CT (50, 5, 0.5 μM) for 9 days. The graph shows significantly more HFs to be still in anagen VI and less in late catagen compared with the vehicle control group, in which almost all HFs have already spontaneously regressed (i.e. entered into early or late catagen). *$P = 0.5$.

gate the apoptotic activity in the cells, we performed a caspase 3/7 activity assay after 2 and 4 days of CT treatment. After 2 days of CT treatment, caspase 3/7 were significantly
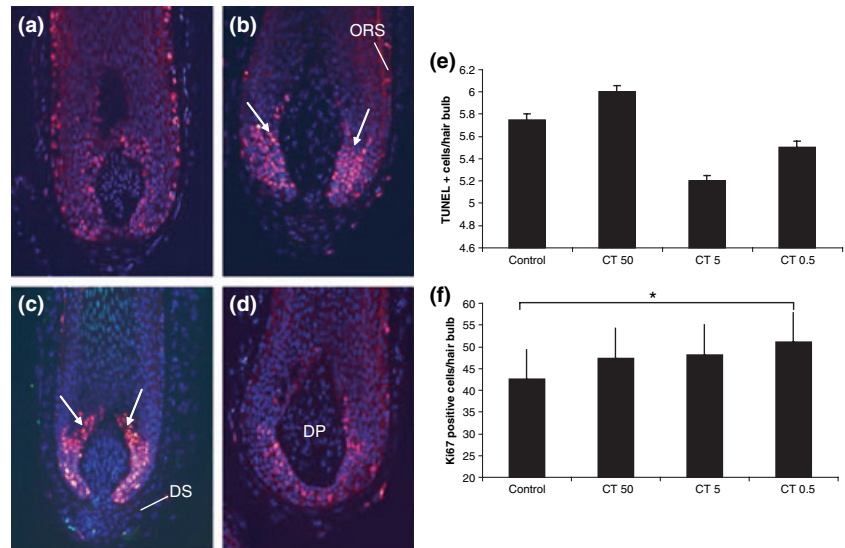
Figure 3. Ki-67/TUNEL double staining shows an increase in proliferating cells (red) and a down regulation of apoptotic nuclei (green) in the hair bulb of human hair follicles (HFs) treated with CT. (a) Control; (b) L-carnitine L-tartrate (CT), 50 μM; (c) 5 μM; (d) 0.5 μM; (e) apoptotic TUNEL-positive cells were down regulated in the hair bulbs of CT-treated HFs; while (f) Ki-67-positive cells were significantly up regulated. *$P = 0.5$.

reduced about 46.76% and after 4 days about 15.9% in CT-treated HFs compared with the vehicle control (Fig. 5). This suggests that CT may have a strong, immediate protective effect against apoptosis in follicular keratinocytes by down regulation of caspase 3/7 activity which reduces after 4 days.

Discussion

In this study, for the first time we show that CT is able to increase human hair growth *in vitro*. In isolated, organ-cultured human anagen VI HFs from occipital scalp skin, CT stimulates hair shaft elongation (directly or indirectly) by up regulation of hair matrix keratinocyte proliferation and down regulation of keratinocyte apoptosis while prolonging the duration of anagen. In addition, CT treatment suppresses expression of catagen associated factor TGF-β2 and its receptor 2 days after CT treatment in scalp HFs *in vitro*. This down regulation happens immediately 2 days after CT treament and was less prominent after 4 days. Caspase 3/7 activity is down regulated after treatment with CT indicating that these caspases are involved in the induction of apoptosis by CT.

Taken together with the HF organ culture data, however, the limited currently available results already justify claiming that

- CT stimulates human scalp hair growth *in vitro*.
- The effects seen with denervated, avascular organ-cultured HFs are unlikely to be an *in vitro*-artefact.
- It is reasonable to expect that both systemic (incl. nutraceutical) and topical modes of CT application may promote human scalp hair growth, especially when used as an adjuvant regimen to established hair growth-promoting agents such as finasteride and minoxidil.

- And that the main site of action of CT likely is the human anagen hair bulb itself, rather than epithelial HF stem cells in the bulge region or indirect effects mediated, e.g. via a HF vasculature or innervation, as none of these structures are present/functional in our HF organ-culture system.

The inhibition of hair matrix keratinocyte apoptosis by CT shown here (Fig. 3) corresponds well to the previously demonstrated anti-apoptotic activity of carnitine, e.g. heart, liver, testis, lymphocytes and fibroblasts (17,19,57–59). In the 0.5 μM CT-treated groups more apoptotic cells were observed. We do not yet have a convincing explanation for this finding. It appears, however, that 0.5 μM of CT stimulates proliferation in keratinocytes more than 5 μM of CT, but at the same time did not protect the HF against apoptosis as strongly as CT 5 μM does. Therefore, CT 0.5 μM concentration does not show a better benefit for hair growth compared with CT 0.5 μM.

Caspase 3 and caspase 7 have recently been shown to be key mediators of follicular apoptosis during catagen (41). We, therefore, investigated, whether caspase 3 and 7 activity was changed after CT treatment. Indeed we could show correlating to the above shown protection of CT against follicular apoptosis, a lower activity of caspase 3/7 immediately after 2 days of treatment which was less prominent after 4 days.

Treatment of isolated HFs with CT down regulates the protein immunoreactivy of the potent catagen inducer TGF-β2 and its receptor. In the past, we and others had already shown that the protein expression of TGF-β2 during catagen is up regulated in human HFs and that this correlates well with TGF-β2 gene transcription, as documented by quantitative PCR (41,47). The protein expression data of TGF-β2 shown in this study, therefore, likely reflect the corresponding transcriptional changes (even
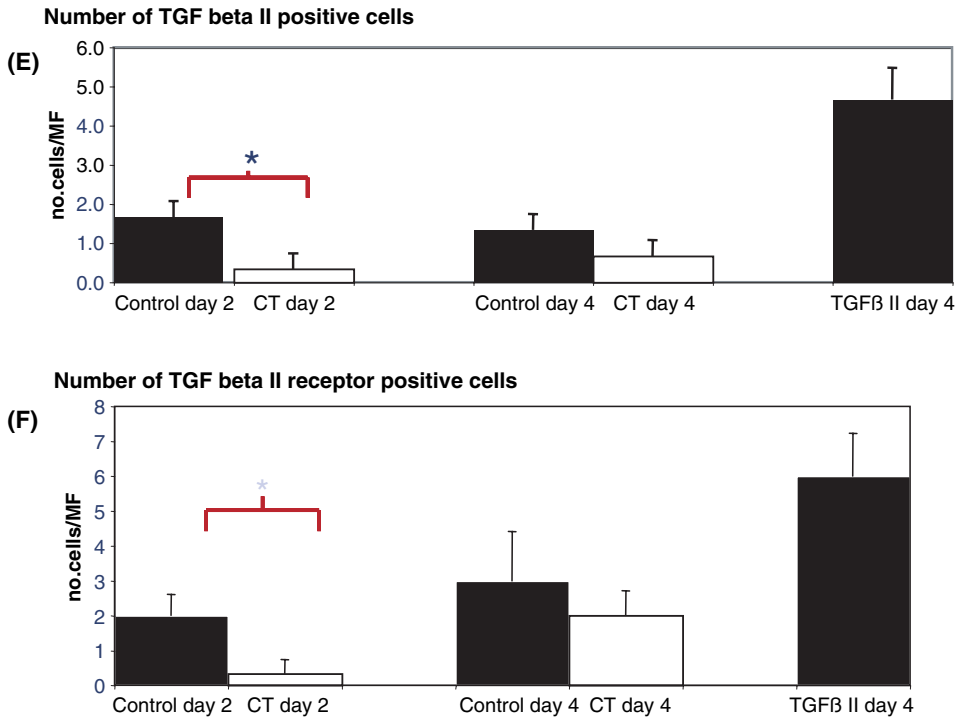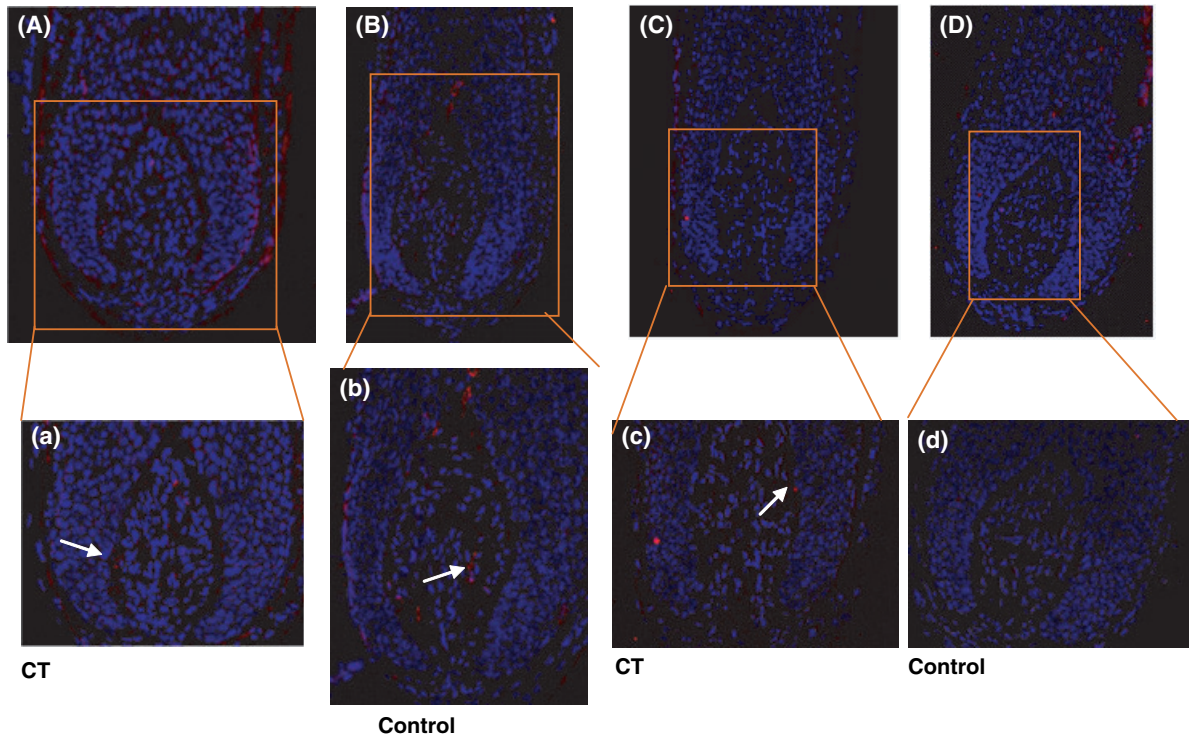
Figure 4. A, a: TGF-β2 expression in control hair follicles (HFs). A, B: 20×; a, b: 40×; B, b: TGF-β2 expression in ʟ-carnitine ʟ-tartrate (CT)-treated HFs (50 μM); C, D, c, d: TGF-βRII and KGF receptor staining; E: numbers of TGF-β-positive cells in the HF after 2 and 4 days of CT treatment compared with vehicle control were significantly down regulated after 2 days. F: numbers of TGF-β receptor type II-positive cells in the HF after 2 and 4 days of CT treatment (50 μM) compared with vehicle control were significantly down regulated after 2 days. $P = 0.5$.
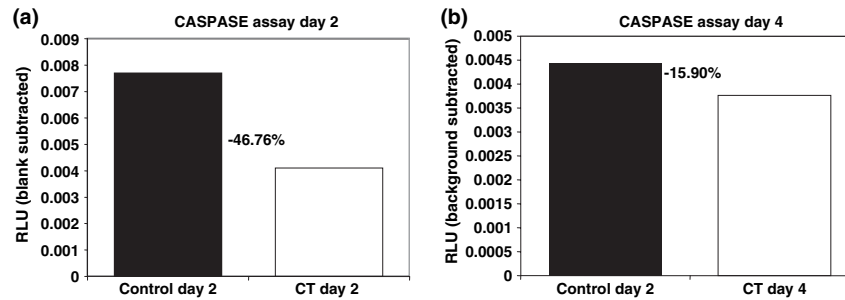
Figure 5. Caspase 3/7 activity assay after 2 and 4 days of CT treatment (50 μM). The values of caspase activity reported in the graph are calculated giving the value of 100% to the caspase present in the control sample. Caspase activity after 2 days is 48.76% down regulated and after 4 days still about 15% down regulated compared with caspase activity in control samples.

though this remains to be formally documented), and suggest that CT acts in the HF, at least in part, indirectly via the inhibition of TGF-β2 (41,47).

In rat skin, L-carnitine prevents the progressive effects of burn wounds, limits necrosis, improves survival and stimulates wound healing in burned dorsal skin flaps (19,60). Clinically, skin ulcer healing can be accelerated by systemic propionyl-L-carnitine (35), which also ameliorates hypoxia/reoxygenation-induced intestinal injury in mice (61). Also, oral propionly-L-carnitine dilates human subcutaneous arteries (29). Therefore, despite the likelihood of direct effects of CT on the anagen hair bulb (see above), it is conceivable that, upon topical application, additional indirect mechanisms of CT action (incl. vasodilation and improved oxygen supply) could come into play to explain the hair-growth promoting effects observed in our observational study.

The challenge now is to elucidate how exactly CT exerts its stimulatory effects on human HFs *in vitro*. In this context, numerous mechanistic possibilities have to be considered (see Introduction). Here, it is sufficient to mention only three examples: in view of the potential role of NO in hair growth control (62,63), the documented effects of L-carnitine on NO production (30) encourage one to explore this potential pathway. Likewise, given that low dose-glucocorticoid therapy stimulates human hair growth *in vivo* [i.e. steroid-induced hypertrichosis (64)], it is intriguing to note that L-carnitine has recently been shown to act as nutritional activators of glucocorticoid receptors (3). Finally, levocarnitine improves ischaemia and reperfusion, e.g. by preventing the accumulation of long-chain acyl-CoA, thus inhibiting the production of free radicals by damaged mitochondria, and by improving repair mechanisms for oxidative-induced damage to membrane phospholipids (20). Similar mechanisms may operate in the current HF organ culture model, where oxidative tissue damage must be expected to accumulate over time.

These are only examples for the multiple potential mechanisms of action of carnitine on human HFs, which now

deserve systematic dissection, e.g. in human HF organ culture (54). Recently, a full thickness skin organ-culture system under serum free conditions was developed with active hair growth of anagen VI HFs (65). This culture system as well as the well established isolated human HF assay can serve as a pragmatic and uniquely instructive experimental tool for further dissecting candidate mechanisms by which CT may promote human scalp hair growth.

Hair loss as a direct and sole result of carnitine deficiency, to the best of our knowledge, has not yet been documented. But this has probably never been systematically investigated so that a discrete hair phenotype-induced carnitine deficiency cannot be excluded, either. Interestingly, an association of hypocarnitinaemia with Menkes disease has been reported (66). However, it is entirely unknown whether the carnitine deficiency in this disease, which is characterized by hair shaft abnormalities associated with hair breakage, is in any way causally related to the structural hair defect in Menkes' patients (which is generally believed to be caused by a defect in copper efflux).

In conclusion, the hair growth-stimulatory, in particular the catagen-inhibitory/anagen-prolonging, properties of CT documented in the current study support the hypothesis that CT might be useful as an adjuvant treatment of general, diffuse hair loss in men and women.

Acknowledgements

Conflict of interest

This study was supported by Henkel KgaA., which has filed and obtained a patent application on the use of CT as a hair growth stimulator (patent # WO2002-EP1875). All authors declare that the reported results fully and accurately reflect the actual data that were harvested and that

no part of the study design was corrupted by preconceived notions on the study outcome.

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