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Neuroscience Letters 424 (2007) 36–40
Neuroscience Letters
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Neuroprotective effects of anthocyanins and their in vivo metabolites in SH-SY5Y cells

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Received 11 April 2007; received in revised form 6 July 2007; accepted 10 July 2007

Abstract

Recent in vivo studies have highlighted an important role for the neuroprotective actions of dietary anthocyanins. However, one consistent result of these studies is that the systemic bioavailability of anthocyanins, including cyanidin 3-*O*-glucopyranoside (Cy-3G), is very poor. Cy-3G has been demonstrated to be highly instable at physiological pH, so its in vivo metabolites, such as the aglycon cyanidin (Cy) and protocatechuic acid (PA), may be responsible for both the antioxidant activitiy and the neuroprotective effects observed in vivo. Therefore, we investigated the protective effects of Cy-3G, Cy and PA against H_2O_2-induced oxidative stress in a human neuronal cell line (SH-SY5Y). We determined their ability to counteract reactive oxygen species (ROS) formation and to inhibit apoptosis in terms of mitochondrial functioning loss and DNA fragmentation induced by H_2O_2. We demonstrated that pretreatment of SH-SY5Y cells with Cy-3G, Cy and PA inhibits H_2O_2-induced ROS formation at different cellular levels: Cy-3G at membrane level, PA at cytosolic level and Cy at both membrane and cytosolic levels. In addition, Cy showed a higher antioxidant activity at membrane and cytosolic level than Cy-3G and PA, respectively. Interestingly, both Cy and PA, but not Cy-3G, could inhibit H_2O_2-induced apoptotic events, such as mitochondrial functioning loss and DNA fragmentation. These results suggest that Cy and PA may be considered as neuroprotective molecules and may play an important role in brain health promotion. These in vitro findings should encourage further research in animal models of neurological diseases to explore the potential neuroprotective effects of compounds generated during in vivo metabolism of anthocyanins.

Keywords: Oxidative stress; Neuroprotection; Cyanidin 3-*O*-glucopyranoside; Cyanidin; Protocatechuic acid

There is growing interest in therapeutic strategies with dietary antioxidants aimed at counteracting oxidative stress-induced damage associated with neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease [17]. Oxidative stress involving reactive oxygen species (ROS) and other oxidizing agents is one of the major factors responsible for the dysfunction or death of neuronal cells that contributes to initiation and progression of these neurological diseases [3].

Recently, many studies have highlighted an important role for the neuroprotective action of dietary antioxidant components, including vitamins and phenolic compounds [22]. Anthocyanins are a group of phenolic compounds that are widely distributed among pigmented fruit and vegetables [18]. Among the various anthocyanins, cyanidin 3-*O*-glucopyranoside (Cy-3G) was indicated as having the highest antioxidant power [1] and has been shown to ameliorate age-related deficits in rat neuronal and behavioral functions [4,10,13]. Other authors reported that Cy-3G is able to cross the blood brain barrier and localize in various brain regions important for learning and memory [2]. These findings suggest that Cy-3G may deliver its antioxidant and signal modifying capabilities centrally.

Knowledge about the absorption, metabolism and tissue or cell distribution of anthocyanins is important in order to understand their neuroprotective effects. As reported in human and animal studies, anthocyanins are rapidly absorbed as glycosides that can be recovered from plasma and urine after oral administration [8,12,14]. However, one consistent result of these studies is that the systemic bioavailability of anthocyanins, including Cy-3G, is very poor and that anthocyanin profiles in tissues were quite different from those in blood plasma [12].

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0304-3940/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2007.07.017

The instability of Cy-3G at physiological pH and its rapid degradation to phenolic acids, such as protocatechuic acid (PA), is probably responsible for its relatively short half-life in biological fluids [23]. Another Cy-3G degradation pathway involves the partial hydrolysis of the glycosidic bond as the first step in anthocyanin degradation to form the aglycon cyanidin (Cy) at intestinal level [28]. Cy, which is more unstable than its glycosides, proceeds through a highly unstable alfa-diketone intermediate to easily form aldehydes and phenolic acid derivates [23]. In addition, Fleschhut et al. have also reported that the potential hydrolitic activity of gut microflora can contribute to the degradation of Cy-3G into Cy and PA [9].

To date, there are only few data on the distribution among body tissues of Cy-3G in vivo metabolites, their biological effects and mechanism of action, i.e. association with the cell membrane or intracellular activity. Recently, both Cy and PA have been detected in plasma of rats after oral ingestion of Cy-3G [26,29]. Interestingly, Mohsen et al. demostrated that metabolites of anthocyanins such as aglycons can reach different tissues including brain [7].

In the light of dietary strategies aimed at the prevention of neurodegenerative diseases, we investigated the ability of the anthocyanin Cy-3G and its in vivo metabolites Cy and PA to exert neuroprotective effects in a human neuronal cell line (SH-SY5Y) against H_2O_2-induced oxidative stress. In particular, we determined the protective effects of Cy-3G and its metabolites against ROS formation and apoptotic events, such as mitochondrial functioning loss and DNA fragmentation, induced by H_2O_2.

Human neuronal-like cells, SH-SY5Y, were routinely grown at 37 °C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

To determine ROS formation and total antioxidant activity (TAA), SH-SY5Y cells were seeded in 96-well plates at 3×10^4 cells/well and in cultures dishes at 4×10^6 cells/dish, respectively. To evaluate mitochondrial functioning and DNA fragmentation the cells were seeded in 96-well plates at 3×10^4 and 5×10^3 cells/well, respectively. All experiments were performed after 24 h of cell adhesion and a subsequent 2 h treatment of SH-SY5Y cells with various concentrations of C-3G, Cy and PA at 37 °C in 5% CO_2.

The antioxidant activity of compounds was evaluated using 2′,7′-dichloroflurescein diacetate (DCFH-DA), measuring the ROS formation evoked by exposure of SH-SY5Y cells to H_2O_2. After treatment with compounds, the cells were washed with phosphate buffered saline (PBS) and then incubated with 5 μM DCFH-DA in PBS at 37 °C for 30 min. After removal of DCFH-DA and further washing, the cells were incubated with H_2O_2 (300 μM) in PBS for 30 min. At the end of incubation, the fluorescence of the cells from each well was measured (($_{excitation}$ = 485 nm, ($_{emission}$ = 535 nm) with a spectrofluorometer (Wallac Victor® Multilabel Counter, Perkin-Elmer Inc., Boston, MA). The results were expressed as a percentage increase of ROS evoked by exposure to H_2O_2 and calculated by the formula: $((F_t - F_{nt})/F_{nt} \times 100($, where F_t is the fluorescence of treated neurons and F_{nt} is thefluorescence of untreated neurons.

To determine whether the compounds exert their antioxidant activities mainly in the membrane or cytosolic fraction, TAA was measured on both the cytosolic and membrane enriched fractions as previously reported [27]. Briefly, after incubation with compounds, SH-SY5Y cells were washed 3 times with cold PBS. Cells were subsequently collected in 1 ml of PBS and centrifuged for 10 min at 10,000 rpm at 4 °C, then supernatant was removed and the cells were washed with 1 ml of PBS. This was repeated twice more, and the pellet was finally reconstituted in 600 μl of 0.05% Triton X-100. Cells were then homogenized and allowed to stand at 4 °C for 30 min. Cytosolic and membrane enriched fractions were subsequently separated by centrifugation at 14,000 rpm for 15 min at 4 °C. TAA in cell fractions was determined by the decoloration of the radical cation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), in terms of quenching of absorbance at 740 nm. Values obtained for each sample were compared with the concentration–response curve of a standard Trolox solution, and expressed as μmol of Trolox Equivalent Antioxidant Activity per mg of protein (TEAA μmol/mg protein).

The mitochondrial metabolic function was evaluated by the conversion of the dye methylthiazoletetrazolium (MTT) to formazan [19]. Briefly, after treatment with H_2O_2 (300 μM) for 3 h, SH-SY5Y cells were washed with PBS and then incubated with DMEM 10% FBS in 5% CO_2 at 37 °C for 3 h. After medium removal, the cells were washed with PBS and then incubated with MTT (5 mg/ml) in PBS for 2 h. After further washing, the formazan crystals were dissolved with isopropanol. The amount of formazan was measured (570 nm, ref. 690 nm) with a spectrophotometer (TECAN®, Spectra model Classic, Salizburg, Austria). The mitochondrial functioning was expressed as a percentage of control cells and calculated by the formula: (absorbance of treated neurons/absorbance of untreated neurons) × 100.

DNA fragmentation following H_2O_2-induce oxidative stress was determined using Cell Death Detection ELISA^{plus} (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, after treatment with H_2O_2 (300 μM) for 3 h, SH-SY5Y cells were washed with PBS and then incubated with DMEM 10% FBS in 5% CO_2 at 37 °C for 18 h. After medium removal and further washing, 200 μl lysis buffer was added to each well for 30 min, followed by centrifugation at 2200 rpm for 10 min. Supernatant 20 μl was then incubated in a streptavidin-coated 96-well plate with a mixture of two monoclonal antibodies–anti-histone (biotin-labeled) and anti-DNA (peroxidase-conjugated). After washing the wells three times with incubation buffer, 100 μl of peroxidase substrate solution (ABTS®) was added to each well for 20 min. The amount of colored product was measured (405 nm) with a spectrophotometer (TECAN®). The values were expressed as a percentage of increase of apoptosis and calculated by the formula: (absorbance of treated sample/ absorbance of untreated sample) × 100.

Data are reported as means ± S.D. of at least three independent experiments. Statistical analysis was performed using

Table 1
Effects of Cy-3G, Cy and PA pretreatment on ROS formation induced by H_2O_2 in SH-SY5Y cells[a,b]

Concentration (μM)	Cy-3G	Cy	PA
0	102 ± 25	102 ± 25	92 ± 13
25	74 ± 24	60 ± 7[d]	88 ± 17
50	60 ± 18[c]	45 ± 13[d]	81 ± 13
100	51 ± 12[d]	25 ± 9[d]	64 ± 10[d]

[a] SH-SY5Y cells were treated with various concentrations of Cy-3G, Cy and PA for 2 h at 37 °C in 5% CO_2 before H_2O_2 treatment. ROS formation was measured after 30 min of treatment with 300 μM H_2O_2. The results were expressed as a percentage increase of ROS evoked by exposure to H_2O_2.
[b] Values are reported as means ± S.D. of three independent experiments.
[c] $p < 0.05$.
[d] $p < 0.001$ *vs.* untreated samples (ANOVA with Dunnett post hoc test).

one-way ANOVA (Dunnett post hoc test was used) and Pearson's correlation test, as appropriate. Differences were considered significant at $p < 0.05$. Analyses were performed using PRISM 3 software on a Windows platform.

Preliminary experiments showed that in the absence of H_2O_2, treatment of SH-SY5Y cells with Cy-3G, Cy and PA concentrations up to 100 μM for 2 h did not affect the oxidative damage events considered, i.e. ROS formation, mitochondrial functioning loss and DNA fragmentation (data not shown).

Treatment of SH-SY5Y cells with Cy-3G, Cy and PA (25–100 μM) showed a concentration-dependent decrease of H_2O_2-induced ROS formation (Table 1); at 100 μM concentration the order of efficacy was Cy (75%) > Cy-3G (50%) > PA (39%).

To evaluate at which subcellular level the Cy-3G, Cy and PA counteract ROS formation, we measured TEAA at membrane and cytosolic levels. As reported in Table 2, both the membrane and cytosolic fractions obtained from SH-SY5Y cells treated with Cy for 2 h showed significant increases of TEAA in comparison to untreated cells. By contrast, the treatment with Cy-3G and PA induced a significant increases of TEAA in membrane and cytosolic fraction only, respectively. Interestingly, a significant inverse correlation was found between TEAA increase at membrane levels and ROS formation for Cy-3G ($r = -0.917$, $p < 0.05$) and Cy ($r = -0.953$, $p < 0.05$), while the inverse correlation between TEAA increase at cytosolic levels and ROS formation was significant for Cy ($r = -0.955$, $p < 0.05$) and PA ($r = -0.961$, $p < 0.01$).

Table 2
Total antioxidant activity of membrane and cytosolic fraction from SH-SY5Y cells pretreated with Cy-3G, Cy and PA[a,b]

	Concentration (μM)			
	0	25	50	100
Membrane				
Cy-3G	100.0 ± 4.0	105.0 ± 5.0	128.3 ± 5.9[d]	143.0 ± 13.0[d]
Cy	100.0 ± 4.0	104.3 ± 18.8	112.0 ± 9.5	154.2 ± 22.2[d]
PA	79.8 ± 5.0	76.2 ± 5.4	81.3 ± 8.0	86.3 ± 4.2
Cytosol				
Cy-3G	100.0 ± 7.3	101.2 ± 6.5	99.6 ± 5.3	103.6 ± 4.1
Cy	100.0 ± 7.3	103.5 ± 3.2	122.4 ± 16.7[c]	133.5 ± 7.8[d]
PA	101.6 ± 5.3	98.1 ± 5.0	103.1 ± 14.1	115.9 ± 9.2[c]

[a] SH-SY5Y cells were treated with various concentration of Cy-3G, Cy and PA for 2 h at 37 °C in 5% CO_2 and cytosolic and membrane fractions were separated. The cellular fractions were submitted to the ABTS radical cation decolorization assay and the antioxidant activities of the fractions were expressed as means ± S.D. of μmol of trolox equivalent antioxidant activity per mg of protein (TEAA μmol/mg protein).
[b] Results are reported as means ± S.D. of three independent experiments.
[c] $p < 0.05$.
[d] $p < 0.001$ *vs.* untreated samples (ANOVA with Dunnett post hoc test).

We then assessed whether the observed antioxidant activity of Cy-3G, Cy and PA can really translate into protective effects against oxidative stress. As shown in Fig. 1, pretreatment of SH-SY5Y cells with 25–100 μM Cy or 100 μM PA before H_2O_2 addition, significantly reduced mitochondrial functioning loss, while Cy-3G treatment did not show any protective effect. Cell pretreatment with similar Cy and PA concentrations also showed a significant inhibitory effect on DNA fragmentation (Fig. 2), with the highest inhibition of DNA fragmentation observed with 100 μM Cy and PA (79% and 54%, respectively). Remarkably, Cy-3G did not show any protective effects against DNA fragmentation.

Our study provides the first evidence that Cy and PA, compounds generated in vivo during Cy-3G metabolism, can exert neuroprotective effects against H_2O_2-induced oxidative stress in SH-SY5Y cells. Pretreatment of SH-SY5Y cells with Cy-3G, Cy and PA inhibits H_2O_2-induced ROS formation and increases cellular antioxidant activity at different cellular levels: Cy-3G at membrane level, PA at cytosol level and Cy at both cellular levels. In particular, Cy showed a higher antioxidant activity at membrane and cytosol level than Cy-3G and PA, respectively. Interestingly, Cy and PA, but not Cy-3G, can inhibit H_2O_2-induced apoptotic events, such as mitochondrial functioning loss and DNA fragmentation. Taken together, these results suggest that the neuroprotective effects of Cy-3G, Cy and PA may depend on their cellular bioavailability, i.e. on the extent to which they associate with the membrane or are uptaken into neuronal cells.

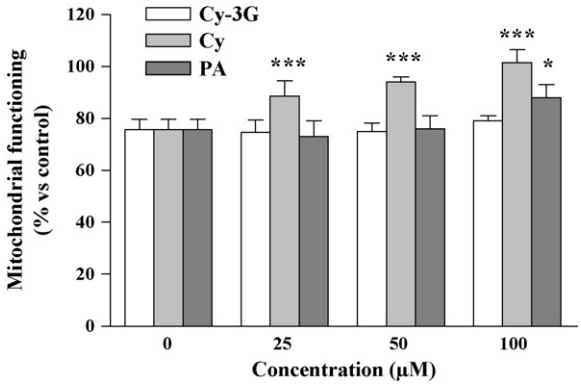

Fig. 1. Effects of Cy-3G, Cy and PA pretreatment on H_2O_2-induced mitochondrial functioning loss. SH-SY5Y cells were treated with various concentrations of Cy-3G, Cy and PA for 2 h at 37 °C in 5% CO_2 before treatment with H_2O_2 (300 μM, 3 h) and mitochondrial functioning loss was measured 3 h after H_2O_2-treatment. Mitochondrial functioning is expressed as a percentage of increases with respect to corresponding non-treated controls. Results are reported as means ± S.D. of three independent experiments (treated samples vs. untreated samples; *$p < 0.05$, ***$p < 0.001$ at ANOVA with Dunnett post hoc test).

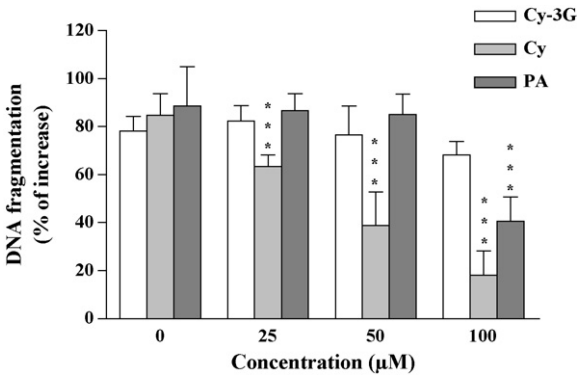

Fig. 2. Effects of Cy-3G, Cy and PA pretreatment on H_2O_2-induced DNA fragmentation. SH-SY5Y cells were treated with various concentrations of Cy-3G, Cy and PA for 2 h at 37 °C in 5% CO_2 before treatment with H_2O_2 (300 μM, 3 h). DNA fragmentation was measured 18 h after H_2O_2-treatment. DNA fragmentation is expressed as a percentage of increases with respect to corresponding non-treated controls. Results are reported as means ± SD of three independent experiments (treated samples vs. untreated samples; $^{***}p < 0.001$ at ANOVA with Dunnett post hoc test).

These findings are particularly relevant, since H_2O_2 is quantitatively the most important of the peroxides generated in brain cells [6], and its intracellular accumulation can induce oxidative stress leading to neuronal apoptosis [5]. The lack of Cy-3G neuroprotective effects against H_2O_2-induced apoptosis can be ascribed to its predominantly membrane incorporation, as also previously reported in endothelial cells [30]. We previously demonstrated that Cy-3G appeared more protective against superoxide anion ($O_2^{\bullet -}$) than H_2O_2 induced apoptosis in human keratinocytes: $O_2^{\bullet -}$ with respect to H_2O_2 does not cross the membrane and induces oxidative damage at membrane level [27].

In contrast to Cy-3G, Cy and PA can protect the neuronal cells against H_2O_2-induced apoptosis through increased antioxidant activity at cytosol level. In particular, the synergic antioxidant effects of Cy, able to increase the total antioxidant activity at both the membrane and cytosol level, could explain its higher antiapoptotic activity compared to PA. To our knowledge, these findings provide the first evidence of Cy antiapoptotic effects even though further studies are required to elucidate the cellular and molecular mechanisms of neuroprotective effects against oxidative stress. Data on PA antiapoptotic effects are consistent with recent studies where PA isolated from Alpinia Oxyphylla was shown to have neuroprotective effects on PC12 cells and aged rats, respectively [11,24]. In particular, the PA neuroprotective effects were ascribed to its direct antioxidant properties and ability to increase intracellular antioxidant enzyme activities, such as glutathione peroxidase and superoxide dismutase [24].

Recent studies in cerebral ischemia models in rats reported that animal pretreatment with anthocyanins reduced both the brain infarct volume and the apoptotic neurons evoked by transient middle cerebral artery occlusion and reperfusion [25]. Other studies looked at the effects of anthocyanin-rich diet and other antioxidant sources on brain function [21]. However, the results were not linked specifically to anthocyanins. Due to the high instability of Cy-3G at physiological pH, metabolites such as Cy and PA, or others not yet identified, may be responsible for the antioxidant activity and the neuroprotective effects observed in vivo.

Recent studies in rats demonstrated a rapid movement of anthocyanins and their metabolites from intestine to brain [7,20,29]. Cy-3G and anthocyanin metabolites such as aglycon and glucoronide were present in brain within 30 min and 2 h, respectively, after ingestion of anthocyanin extracts. These metabolites subsequently disappeared at 18 h post-ingestion. It is therefore reasonable that the transient presence of Cy-3G and Cy in brain could lead to further formation of degradation compounds, including PA. In contrast to the instability of Cy-3G and Cy, PA shows a higher stability for up to 24 h at neutral pH [16]. PA and other phenolic acids could therefore provide an important hint as to the fate of anthocyanins in brain. Unfortunately, there are no in vivo studies measuring the level of phenolic acids in brain after ingestion of anthocyanins.

Taken together, our results on the neuroprotective effects of Cy and PA in SH-SY5Y cells indicate that they may play an important role in brain health promotion, due to their ability to increase cell antioxidant capacity at the cytosol level. Although Cy-3G does not increase cytosolic (intracellular) antioxidant activity, it is a potent extracellular antioxidant which could also protect neuronal cells against oxidative stress generated by external stimuli.

These findings must also be interpreted in the light of the anthocyanin concentrations used in vitro which are several orders of magnitude higher than plasma concentrations that are achieved in vivo (<1 μM) following an anthocyanin-rich diet [21]. In contrast to neuroprotective effects in vivo, our results in vitro, in agreement with published data [11,15], showed that Cy-3G and its metabolites at low concentrations (<25 μM) did not exert any neuroprotective effects (data not shown).

It is difficult to elucidate the potential causes for the discrepancy observed between *in vitro* and *in vivo* data. As well as the concentrations of anthocyanins, their neuroprotective effects *in vivo* also depend on repeated exposure over a longer time such as chronic exposure that allows the accumulation of low concentrations of anthocyanins or their metabolites at neuronal level. Chronic exposure is not reproducible in vitro neuronal models which only allow evaluation of the neuroprotective effects after a single exposure.

Notwithstanding these technical limitations, we cannot exclude a possible therapeutic use of future cyanidin-based drugs based on the evidence from our experiments. These results should encourage further studies in animal models of neurological diseases to explore the potential neuroprotective effects of metabolites and degradation compounds generated during absorption and/or metabolism of pharmacological doses of anthocyanins.

Acknowledgments

This work was supported by MIUR-FIRB project 2003, MIUR-COFIN 2005, and Fondazione del Monte di Bologna e Ravenna (Bologna, Italy).

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