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Protective effects of ellagic and chlorogenic acids against oxidative stress in PC12 cells

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Abstract
Following exposure of differentiated neuronal PC12 cells to either *t*-BHP, hydrogen peroxide (H_2O_2) or $FeSO_4$ various kinds of reactive oxygen species (ROS) are generated leading to oxidative injury. The protective effects of two plant polyphenols, ellagic (EC) and chlorogenic acid (CGA), as well as of two metabolites, caffeic acid (CA) and ferulic acid (FA), were investigated in preincubation and coincubation experiments with respect to the following parameters: prevention of cell death, GSH depletion, lipid peroxidation and ROS formation.

The polyphenols more efficiently suppressed cytotoxicity and loss of GSH caused by peroxides than by iron, particularly in preincubation. Lipid peroxidation which increased much stronger in response to $FeSO_4$ was counteracted completely by the polyphenols. In case of iron, however, only coincubation was effective. EA and CGA and the metabolites CA and FA showed excellent elimination of ROS induced by all stressors. These findings suggest that two dietary antioxidants, EA and CGA, may have protective properties against oxidative stress induced in CNS.

Keywords: *Polyphenols, ROS, oxidative stress, antioxidants, iron chelation, PC12 cells*

Abbreviations: *CGA, chlorogenic acid; EA, ellagic acid; ROS, reactive oxygen species;* H_2O_2*, hydrogen peroxide; t*-BHP, *tert-butyl hydroperoxide*; *MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid*; *GSH, glutathione*; MDA, *malondialdehyde*; *DCF, 2′7′-dichlorofluorescein*

Introduction

It has been suggested that free radicals and other reactive oxygen species (ROS) play an important role in the pathogenesis of neurodegenerative diseases [1–3]. There are many attempts to combat oxidative stress-induced injury in the brain. Exogenous dietary antioxidants capable of scavenging free radicals are of great interest as potential neuroprotective agents [2,4–6]. As antioxidants or through other mechanisms they also contribute to anticarcinogenic or cardio-protective actions [7–9]. Plant polyphenols are multi-functional and can act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal ions chelators [5,7]. Although scientific interest has been focused mainly on flavonoids, phenolic non-flavonoid compounds (phenylpropanoids, hydroxycinamates, tannins, etc.) are often present in many edible plants at higher concentrations than the flavonoids [10,11]. Phenolic coupling is an additional advantage of these compounds [12]. Plant polyphenols with flavonoid structure are incapable of forming auto-condensates, while tannins are superior antioxidants as their eventual oxidation may lead to oligomerisation via phenolic coupling (till $n = 6$) thus enlarging the number of their reactive sites [12].

Various reports have shown protective effects of polyphenols against oxidative stress in many tissues [9,13]. Little is known about their effects on the CNS. The rat pheochromocytoma line PC12 provides a useful model system for investigating neuronal cell injury [14]. In PC12 culture, oxidative stressors, such

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as *tert*-butyl hydroperoxide induce necrosis via mechanisms that in part are mediated by peroxynitrite [15], whereas, hydrogen peroxide (H_2O_2)-triggered apoptosis in PC12 cells correlates with ROS production [16]. Iron induces lipid peroxidation and cell death in PC12 cells, probably by interaction with superoxide radicals [17].

The present study was designed to investigate whether two common nutritional antioxidants, ellagic acid (EA) and chlorogenic (CGA) acid, as well as two potential metabolites are capable of protecting neuronal PC12 cells against free radical oxidative stress. Chemical structures of these phenolic non-flavonoid compounds are shown in Figure 1. EA is a complex planar molecule containing four hydroxyl groups and two lactone groups [18]. This ellagitannin is present in a wide variety of fruits and vegetables and is a major phenolic constituent in many beverages. There are several protective effects ascribed to EA such as inhibition of procarcinogen metabolism, DNA binding, protection of DNA double helix from alkylating agent injury and scavenging of ROS [18]. Oral administration of EA decreased *N*-acetylation of carcinogens (2-aminofluorene) in rat brain tissues [20].

CGA is an ester of caffeic and quinic acid. It is abundantly present in various agricultural products such as coffee beans, tobacco leaves, potatoes and apples [21]. CGA undergoes dimerisation reactions on the occasion of oxidation [22]. Results of *in vivo* microdialysis demonstrated the stress relaxing effect of coffeine and CGA on the serotonin and dopamine levels in the rat hippocampus [23]. It has been shown that rutin and CGA, the major polyphenolic antioxidants in tobacco leaf extracts, decreased enhanced ROS production in IgE-sensitised mast cells [24]. A suppressive effect of EA and CGA on nitric oxide production has been observed in C6 astrocytes [25].

So far, protective effects of EA and CGA have not been reported for cells of neuronal origin. Only tea extracts (polyphenols from green and black tea) have been used in PC12 cells to attenuate neuronal death induced by 6-hydroxydopamine [26]. Green tea extracts were also reported to show potent antioxidative effects against iron -induced oxidative stress exerted in the nigrostriatal dopaminergic system of rat brain [27].

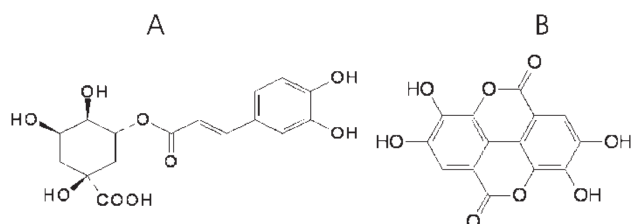

Figure 1. Chemical structures of polyphenolic acids, (A) CGA and (B) EA.

In this study, we demonstrate cytoprotective, antioxidative, antilipoperoxidant and free radical scavenging activities of the two natural polyphenols, chlorogenic and ellagic acid, against various oxidative stressors (*t*-BHP, H_2O_2, $FeSO_4$) in PC12 cells of neuronal origin. Furthermore, we show an interesting dependence of these protective effects on whether PC12 cells were preincubated with the two polyphenols or whether they are coincubated together with the stressors.

Materials and methods

Chemicals

Ellagic and chlorogenic acids, 2′7′-dichlorofluorescein diacetate, Trolox and MTT reagent were obtained from Sigma (Deisenhofen, Germany). Polyphenolic acids and 2′7′-dichlorofluorescein diacetate were dissolved in DMSO (concentration 10 and 50 mM, respectively) as stock solutions. Collagenase was provided by Roche Diagnostics (Mannheim, Germany). Dulbecco's MOD Eagle Medium and NGF were obtained from Gibco Invitrogen (Karlsruhe, Germany), while fetal calf serum and horse serum were from PAA Laboratories GmbH (Linz, Austria). $FeSO_4 \times 7\ H_2O$ (Merck, Darmstadt, Germany) was dissolved in distilled water. H_2O_2 (Fluka, Neu-Ulm, Germany) and *t*-BHP (Sigma-Aldrich, Taufkirchen, Germany) were dissolved in DMEM Medium without serum supplement and prepared as 100 mM solution from 30 and 70% stock solutions.

All other chemicals were from Roche Diagnostics (Mannheim, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Sigma (Deisenhofen, Germany).

Cell culture plates with tissue culture quality were from Techno Plastic Products AG (Trasadingen, Switzerland).

Cell culture and treatment

PC12 cells were grown routinely in Dulbecco's MOD Eagle Medium supplemented with 10% heat-inactivated horse serum, 5% inactivated fetal calf serum, 50 U/ml penicillin and 10 mg/ml streptomycin in 90% air and 10% CO_2 humidified atmosphere. The cells were subcultured twice a week by trypsin (0.05%)/EDTA (0.025%) solution. For determination of the cytotoxic effects of *t*-BHP, H_2O_2 and $FeSO_4$, the cells were plated in 96-well plates coated with collagen. For determination of GSH content collagen-coated 24-well plates (60,000 cells/well) were used. Usually, after 1 day of incubation in a CO_2 incubator at 37°C, 50 ng/ml NGF was added to the culture medium for the next 24 h, in order to differentiate the PC12 cells. Successful differentiation is shown in Figure 2 featuring the transition from a rounded to a neuronal morphology in agreement with earlier studies (e.g. [28]).

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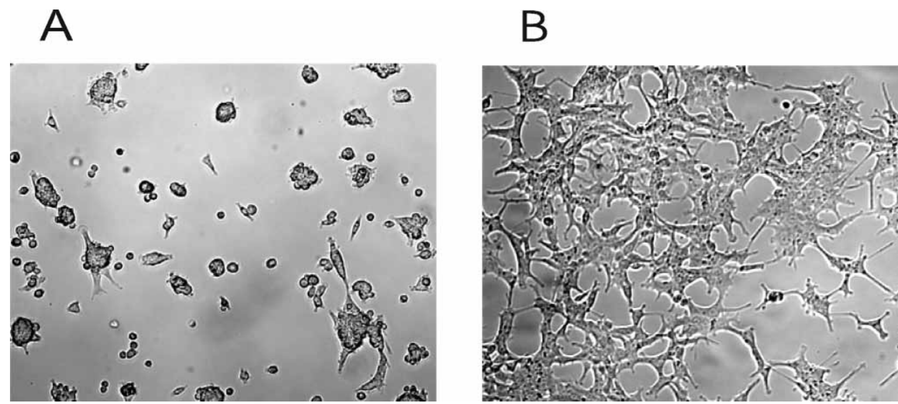

Figure 2. Differentiation of PC12 cells under the influence of NGF. (A) Naive PC12 cells. (B) PC12 cells treated with NGF (50 ng/ml) for 24 h. Pictures shown are representatives of events observed in all cultures used.

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The effect of polyphenolic acids (concentrations 6.2; 12.5 and 25 μM) was studied in two types of experiments: (a) preincubation of PC12 cells with CGA and EA or metabolites for 2 h before changing the medium and adding the peroxides or iron and (b) coincubation, in which PC12 were incubated together with polyphenols, peroxides or iron.

Viability assay

Twenty four hours after differentiation, PC12 cultures were treated with cytotoxic agents (0.1 mM and 0.15 mM *t*-BHP, 0.3 mM H_2O_2 and 0.1 mM and 1 mM $FeSO_4$) for the next 24 h. The viability of PC12 cells was measured by the MTT assay [29]. In these assays, four wells were usually examined for each sample. Values from five independent experiments were averaged and presented as means ± SD.

GSH content

After the incubation with peroxides and iron, cells were washed with 0.9% NaCl (0.5 ml) and 0.2 ml IM solution (5.36 mM KCl, 1.17 mM MgSO4, 0.79 mM Na_2HPO_4, 0.15 mM KH_2PO_4, 10 mM HEPES, 137 mM NaCl; pH 7.4). After freezing overnight, cells were scraped and sonicated for 5 s (10% of the maximum power, Sonopuls HD 2200, Bandelin electronic, Berlin, Germany). GSH content in cell homogenates was determined as described [30] except that measurements were performed by a Versamax-microplate reader (Molecular Devices).

Measurement of lipid peroxidation by malondialdehyde (MDA) assay

Cultured PC12 cells (3 million per Petri dish, ∅ 90 mm) were incubated with *t*-BHP (0.1 mM), H_2O_2 (0.3 mM) or $FeSO_4$ (0.0025, 0.1 and 1 mM) and different concentrations of the test compounds for 4 h (with peroxides) or 1 h (with iron). Alternatively, they were first pretreated with the test substances for 2 h and then treated with cytotoxic agents. After incubation, cells were washed with 0.9% NaCl and resuspended in 1 ml potassium phosphate buffer (50 mM, pH 7.4) and frozen overnight (−20°C). On the next day, cells were thawed, collected using a cell scraper and sonicated for 10 s (15% of the maximum power). MDA concentration in cell homogenates was determined by the TBA method [30]. Freezing overnight did not change the results. The protein concentration was determined following the procedure of Bradford et al. [31].

Measurement of intracellular ROS by DCF assay

PC12 cells were seeded in collagen-coated black flat bottom 96 well- plates overnight and, on the next day, were washed 3 × with Krebs-Ringer-HEPES (KRH) solution pH 7.2 [32]. In coincubation studies, plated cells were first preloaded with 0.1 mM DCFH-DA in loading Medium (working DMEM with only 1% FCS), for 30 min on 37°C, 10% CO_2, washed 3 × with KRH buffer and then treated simultaneously with either *t*-BHP (0.1 mM), H_2O_2 (0.3 mM) or $FeSO_4$ (0.1 mM) and test substances (concentrations 6, 12 and 25 μM). In preincubation studies, polyphenols were first added for 2 h, cells were washed and loaded with 0.1 mM DCFH-DA for 30 min. Then the cells were washed again with KRH and different concentrations of peroxides or iron were added. Fluorescence (485/520 nm, Spectrofluor, TECAN) was measured every 5 min for 30 min, while temperature was maintained at 37°C. Percentage increase in fluorescence per well was calculated by the formula: $Ft_{30}\text{-}Ft_0/Ft_0 \times 100$, where Ft_0 = fluorescence at time 0 min and Ft_{30} = fluorescence at time 30 min [32].

UV/Vis absorbance spectrophotometry

UV/visible spectra of the iron complex with CGA and EA were recorded by a Beckman DU 7400 spectrophotometer at room temperature. Fresh stock solutions of each polyphenol (10 mM) were prepared in DMSO, whereas $FeSO_4$ was prepared in water (10 mM stock

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solution) in the presence of 0.5% H_2SO_4, to keep iron as ferrous ion. Spectral analysis was performed at 25 and 50 μM concentrations of polyphenol compounds and iron in PBS, pH 7.4 and scans were taken after 10 s and compared with polyphenols alone [33].

Results

Suppressive effects of phenolic acids on cytoxicity of t-BHP, H_2O_2 and $FeSO_4$ towards PC12 cells

Cell viability as determined by MTT reduction was decreased after differentiated PC12 cultures were exposed to 0.1 mM *t*-BHP for 24 h to about 50% of controls. EA at all concentrations tested (6.2; 12.5 and 25 μm) provided excellent protection (Figure 3A), markedly increasing cell survival (up to 100%, as in non-treated control). Preincubation showed a somewhat better protection than coincubation ($p < 0.05$), and was even better than 1 mM Trolox, the water-soluble analogue of vitamin E, which was used as a standard antioxidant (Figure 3B). When cell viability dropped to 15% by exposing cells to 0.15 mM *t*-BHP for 24 h (Figure 3C), preincubation was even more effective than coincubation ($p < 0.01$). Again, protection against deleterious neuronal death was found to be concentration-dependent, and EA was protecting better than CGA. When two potential metabolites, caffeic acid (CA) and ferulic acid (FA), were tested similar concentration-dependent levels of protection were reached (Table I). Also in this case, preincubation was slightly but significantly more protective than coincubation at all concentrations used and surpassed the effect of 1 mM Trolox.

Exposure of differentiated PC12 cells to 0.3 mM H_2O_2 for 24 h decreased cell viability to 60%, as compared with non-treated controls. Protection by EA and CGA at all concentrations examined was pronounced and slightly better than by 1 mM Trolox (Figure 3B). The same was found for the metabolites CA and FA (Table I). With H_2O_2 as stressor, there was no significant difference in protection between preincubation and coincubation experiments (Figure 3) except for CA and FA where differences were still significant (Table I).

When PC12 cells were exposed to 0.1 mM $FeSO_4$ for 24 h (a condition comparable to the peroxides), only a moderate decrease in MTT reduction (80% viability) was observed, and the protective effects of EA and CGA were not significant (85% viability). Increasing the concentration of $FeSO_4$ to 1 mM resulted in 60% viability after 24 h. Pretreatment with EA and CGA prior to exposure to iron or co-treatment during 24 h had only a slight suppressive effect (67% viability) similar to Trolox (data not shown).

Protective effects of EA and CGA against peroxide and iron-induced depletion of GSH levels

Exogenous *t*-BHP (0.1 mM), H_2O_2 (0.3 mM) or $FeSO_4$ (1 mM) decreased GSH content in differentiated PC12 cells after 24 h of exposure to 50, 70 and 20% of controls, respectively (Figure 4). Lower concentrations of $FeSO_4$ (0.1 mM) were much less effective and, thus, could not be used to compare the influence of polyphenols. Preincubation and coincubation with polyphenols (25 μM) significantly attenuated the decrease in GSH concentrations induced by both peroxides (Figure 4). Preincubation was found to be more effective than coincubation ($p < 0.05$) against GSH loss induced by *t*-BHP similarly as in MTT assays. On the contrary, tested polyphenols did not prevent GSH decrease induced by iron in PC12 cells (Figure 4A). EA was much more effective than CGA in these experiments. When a significant drop in GSH content occurred following exposure of cells to a higher concentration of *t*-BHP (0.15 mM) for 24 h, EA was slightly more effective than Trolox, whereas CGA was almost ineffective (Figure 4B).

Protective effects of EA and CGA against lipid peroxidation

Exposing PC12 cells to 0.1 mM *t*-BHP or 0.3 mM H_2O_2 for 4 h was followed by an increase of intracellular MDA, a product of lipid peroxidation, by 102.5 and 111%, respectively (Figure 5A). Generally, hydrophilic CGA showed lower effectiveness than lipophilic EA against lipid peroxidation induced by all stressors. Peroxidation of membrane lipids was prevented in a concentration-dependent manner. Interestingly, the amount of MDA was decreased by 25 μM EA not only to values matching non-treated controls but sometimes also to even lower levels. When MDA levels were raised with peroxides, antilipoperoxidant activities of tested polyphenols were almost comparable in both, preincubation and coincubation, experiments (Figure 5A).

Exposure of PC12 cells to 1 mM $FeSO_4$ for 1 h rapidly increased MDA levels up to 1188% in comparison with the control, while 0.1 mM $FeSO_4$ led to an increase of 425% (Table II). Even a concentration as low as 2.5 μM $FeSO_4$ increased MDA levels to 290% (Figure 5B). Thus, lipid peroxidation was induced in our experimental system by trace amounts of free ferrous ions in PC12 cultures, and was much more sensitive than MTT reduction. Combined treatment with $FeSO_4$ and EA at all examined concentrations significantly decreased MDA levels, even to levels less than in controls (Figure 5B). Preincubation of PC12 cells with EA for 15–120 min prior to the addition of iron for the next 1 h did not show significant protection against lipid peroxidation except for the highest concentration (by 10–50%), as shown in Table II.

Reduction by polyphenols of intracellular ROS levels in t-BHP, H_2O_2 and $FeSO_4$ -treated PC12 cells

DCFH-DA (0.1 mM) was successfully applied as an indicator of ROS. Its fluorescence significantly increased when *t*-BHP, H_2O_2 or $FeSO_4$ were added to

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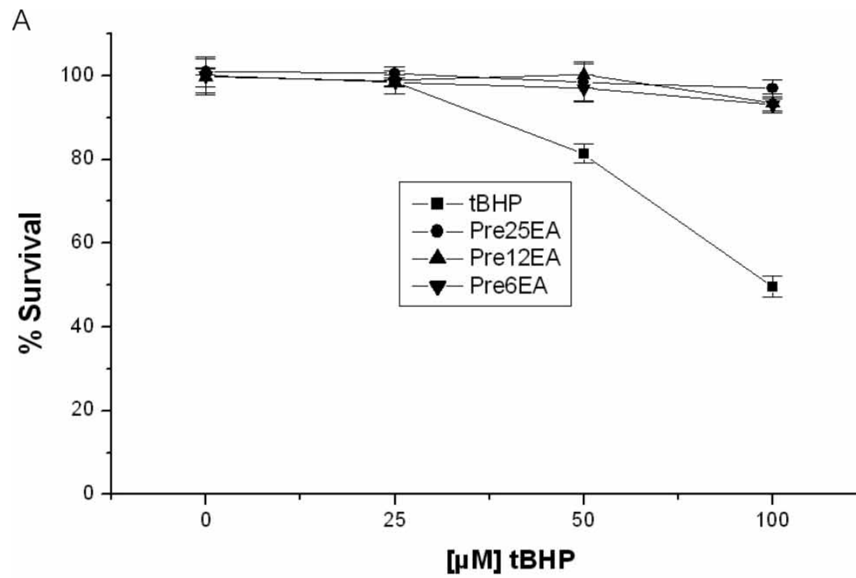

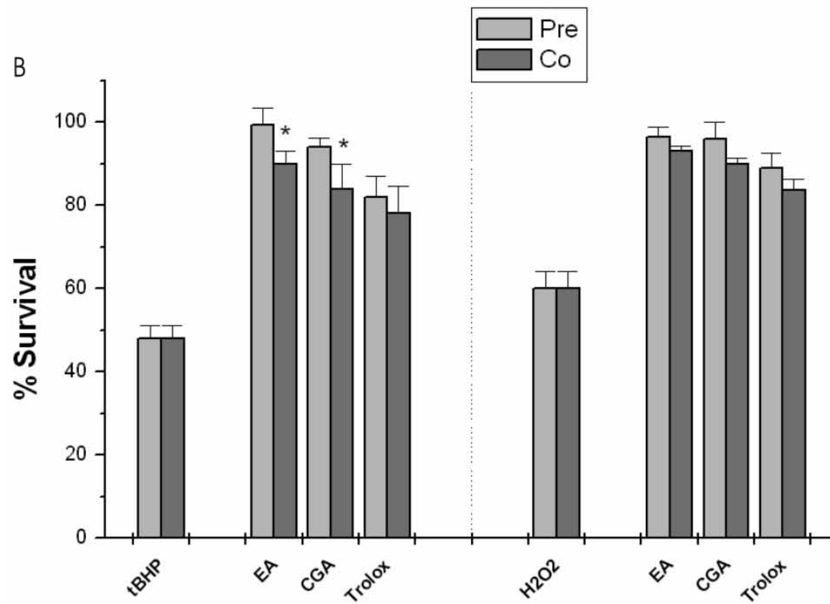

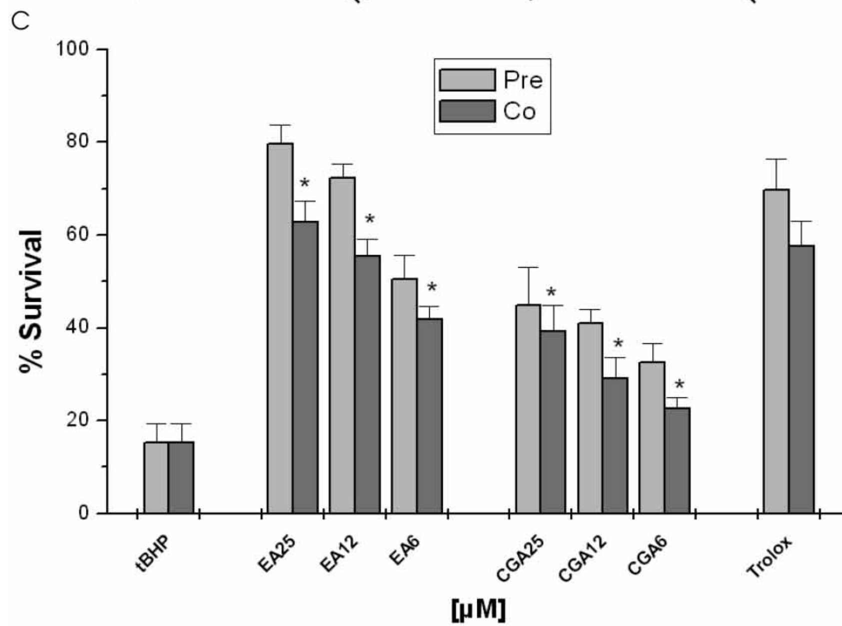

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Table I. Suppressive effects of metabolites, (CA) and (FA), on peroxide-induced cytotoxicity determined by MTT assay.

Test compound	Incubation		Viability (%)	
			0.1 mM *t*-BHP	0.3 mM H_2O_2
None			47.1 ± 6.5	64.2 ± 3.1
Caffeic acid	pre-	25.0 μM	94.6 ± 4.1*	91.7 ± 4.1†
	pre-	12.5 μM	92.1 ± 2.8*	87.6 ± 3.8†
	pre-	6.2 μM	88.2 ± 3.1*	84.3 ± 2.1†
	co-	25.0 μM	86.8 ± 3.9	88.2 ± 2.3
	co-	12.5 μM	82.2 ± 2.4	84.4 ± 3.1
	co-	6.2 μM	78.1 ± 3.5	75.6 ± 4.1
Ferulic acid	pre-	25.0 μM	96.9 ± 3.3*	96.9 ± 3.9‡
	pre-	12.5 μM	94.8 ± 4.6*	91.3 ± 1.4‡
	pre-	6.2 μM	90.6 ± 5.1*	85.4 ± 3.6‡
	co-	25.0 μM	89.8 ± 2.9	85.8 ± 3.3
	co-	12.5 μM	83.1 ± 3.5	81.7 ± 4.1
	co-	6.2 μM	76.4 ± 4.9	66.7 ± 2.1
Trolox	pre-	1.0 mM	82.4 ± 5.2	85.3 ± 4.0
	co-	1.0 mM	77.2 ± 6.9	78.8 ± 2.3

Differentiated PC12 cells were preincubated for 2 h with metabolites (6.2–25 μM) or Trolox (1 mM) and then treated with 0.1 mM tBHP or 0.3 mM H_2O_2 for the next 24 h, or were coincubated with antioxidants and peroxides for 24 h. Values represent means of SD of three independent experiments with determination in quadruplicates.
*$p < 0.001$ preincubation vs. coincubation with 0.1 mM tBHP; $^{\dagger}p < 0.05$ preincubation vs. coincubation with 0.3 mM H_2O_2; $^{\ddagger}p < 0.005$ preincubation vs. coincubation with 0.3 mM H_2O_2.

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the cultures (Figure 6). However, while the increase in ROS formation by both peroxides was in the same range as the increase in MDA production, it was much less in the case of $FeSO_4$ as stressor. Thus, $FeSO_4$ at 25 μM barely increased ROS (not shown), and at 0.1 mM the increase of ROS was 173% (Table III) compared to the 425% increase of MDA (Table II). Therefore, only 0.1 mM was used in these experiments. The decreasing effects of EA and particularly of CGA on DCFH oxidation were significantly more pronounced when they were coincubated with ROS inducers ($p < 0.05$ with *t*-BHP) compared to preincubation. Both polyphenols acted in a clear concentration-dependent manner in case of the peroxides, but less efficiently scavenged ROS formed after addition of H_2O_2 than *t*-BHP. EA and CGA provided best protection against ROS formation triggered by iron: coincubation with 0.1 mM $FeSO_4$ at all tested concentrations of plant antioxidants decreased DCF fluorescence nearly to the level of control cells which amounted to 20% (Figure 6C). Preincubation with both polyphenols, in contrast, was almost inefficient in decreasing ROS induced by iron.

The superiority of coincubation over preincubation in decreasing ROS was also markedly propounced in the case of the metabolites, CA and FA (Table III). However, with the lowest concentration of these compounds and $FeSO_4$ as stressor ROS values in preincubations even tended to increase, although this increase did not reach a sufficient level of significance.

Changes of absorption spectra of EA and CGA in the presence of $FeSO_4$

Direct interactions of polyphenols with ferrous ions at pH 7.4 were assessed by UV–Vis spectroscopy and were characterized by shifts of the absorption maximum due to formation of appropriate complexes (Figure 7). CGA showed a brought band with a maximum near 325 and a prominent shoulder near 295 (Figure 7A). In the presence of a 1:1 Fe^{2+} -to-CGA ratio, the peak from band I was shifted to the right (near 370 nm), while the shoulder was shifted left and apparently formed a peak near 295 nm (Figure 7b). No significant change was observed when this ratio was raised to 2:1. On doubling

Figure 3. Suppressive effects of EA and CGA on peroxide-induced cytotoxycity determined by MTT assay. (A) The effect of increasing concentrations of EA (6.2–25 μM) on PC12 cell death caused by 24 h of exposure to various concentrations of *t*-BHP (0.025–0.1 mM). (B) Differentiated PC12 were preincubated for 2 h with polyphenols (25 μM) or Trolox (1 mM) and then exposed to 0.1 mM *t*-BHP or 0.3 mM H_2O_2 for 24 h (light grey columns), or were coincubated together with polyphenols and peroxide for 24 h (dark grey columns) (*$p < 0.05$ preincubation v coincubation with *t*-BHP). (C) Concentration-dependent (6.2–25 μM) protection by EA and CGA in pre- or co-incubation experiments against severe cell death caused by exposing cells to 0.15 mM *t*-BHP for 24 h. Trolox was present at 1 mM (*$p < 0.01$ preincubation v coincubation with *t*-BHP). Values represent means ± SD of five independent measurements with quadruplicate determinations. Viability of cells cultured without peroxides was set as 100%.

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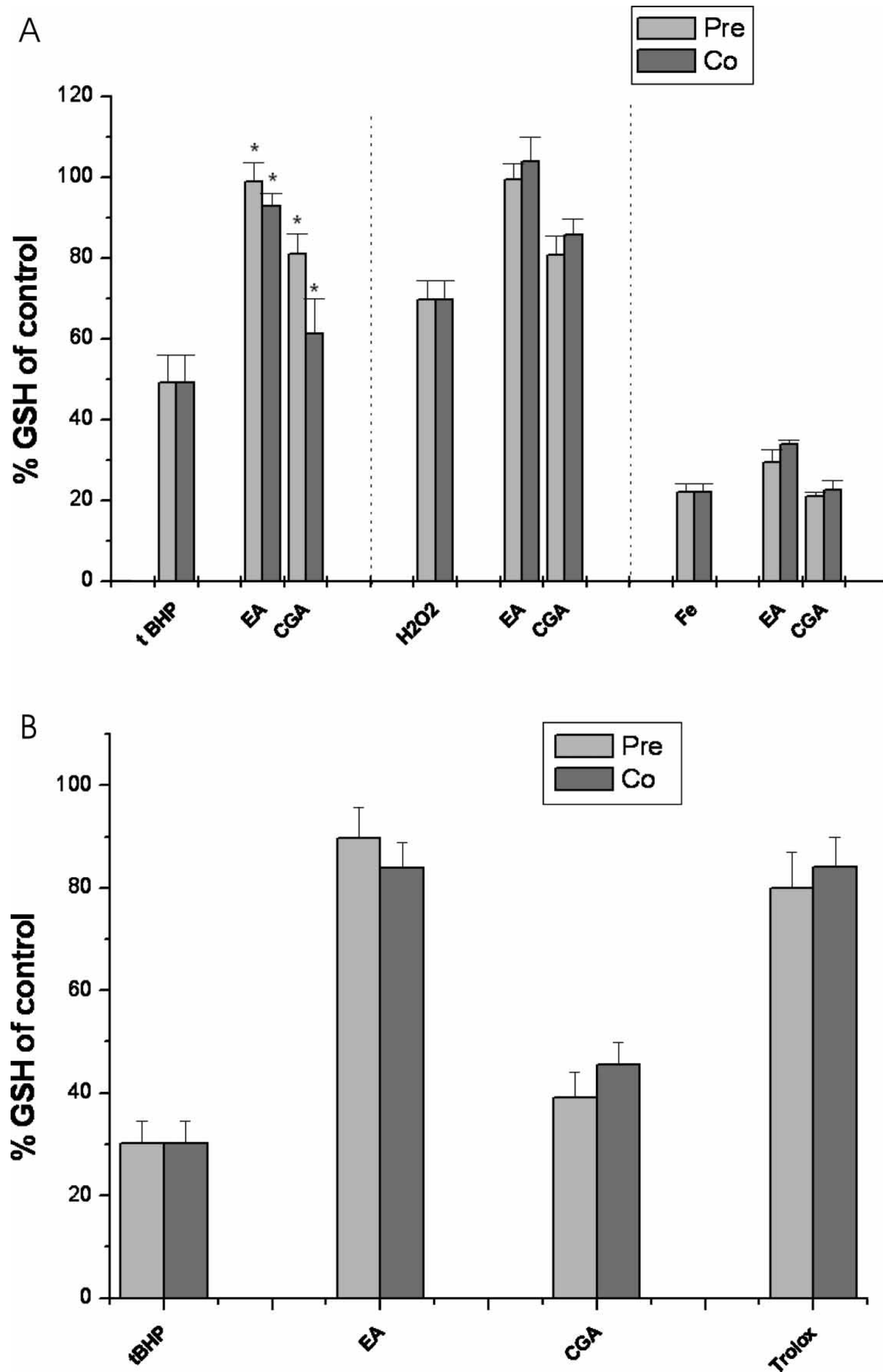

Figure 4. Protecting effect of plant polyphenols against GSH loss induced by oxidative stress. (A) Differentiated PC12 cells were preincubated for 2 h with 25 μM antioxidant and then, for the next 24 h, exposed to 0.1 mM *t*-BHP, 0.3 mM H_2O_2 or 1 mM $FeSO_4$ (light grey columns) or were for 24 h coincubated with polyphenols and stressors (dark grey columns). (B) The effect of polyphenols (25 μM) and Trolox (1 mM) on severe GSH loss induced in PC12 culture by exposure to 0.15 mM *t*-BHP for 24 h. Concentration of GSH in controls not exposed to *t*-BHP amounted to 10.97 ± 0.98 μM and was set as 100%. Values represent means ± SD of three independent experiments with evaluation in triplicate (*$p < 0.05$, preincubation v coincubation with *t*-BHP).

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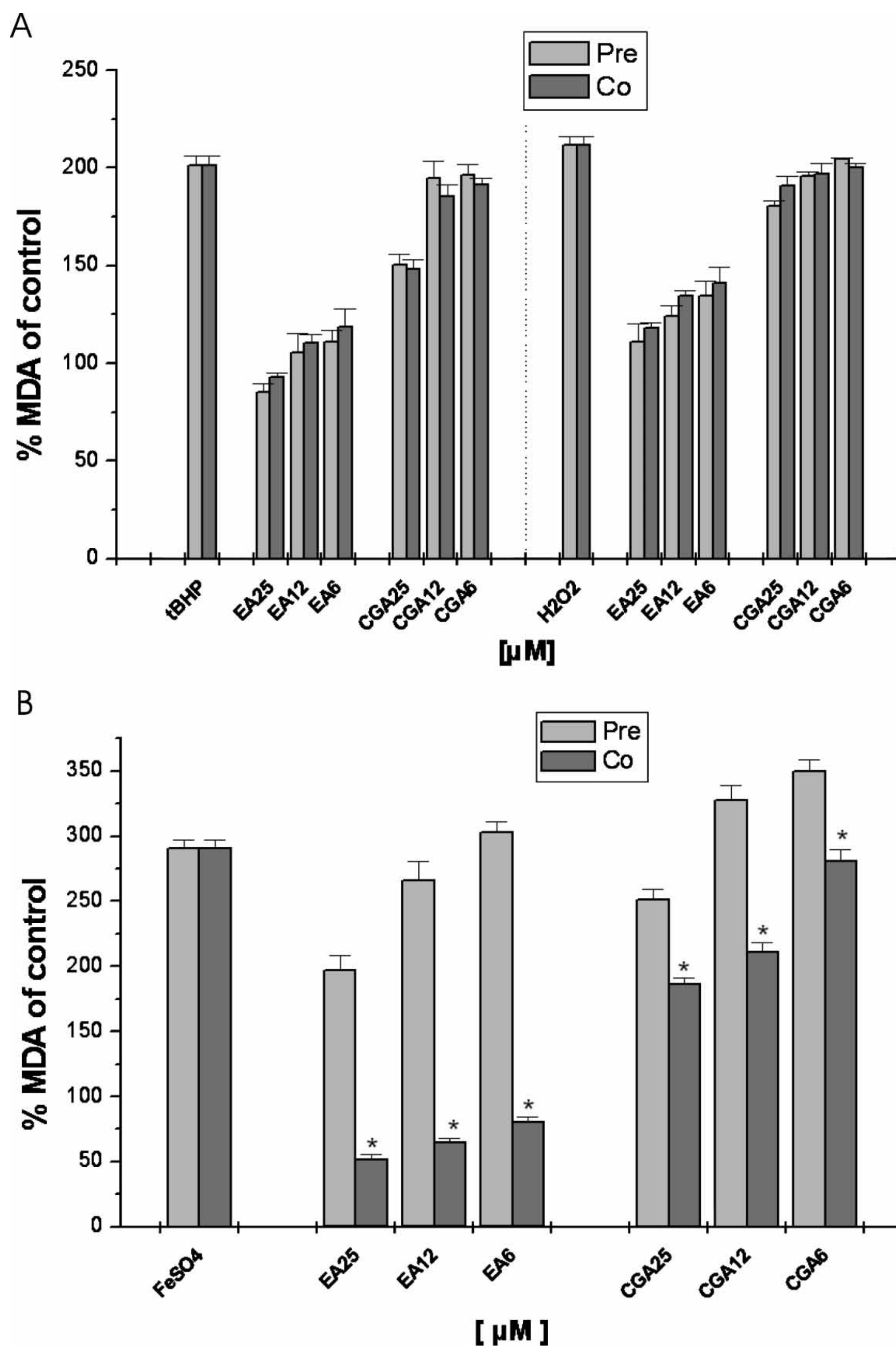

Figure 5. Comparison of the antilipoperoxidative activities of EA and CGA using MDA assay. (A) PC12 were preincubated for 2 h with the tested polyphenols (6.2–25 µM) and then, for the next 4 h, exposed to 0.1 mM *t*-BHP or 0.3 mM H_2O_2 (light grey columns) or were coincubated with polyphenols and stressors for 4 h (dark grey columns). (B) When iron was applied as stressor, PC12 were likewise preincubated with antioxidants for 2 h and then exposed to 2.5 µM $FeSO_4$ for 1 h, or were coincubated for 1 h (*$p < 0.001$, preincubation versus coincubation with $FeSO_4$). MDA production of non-treated controls was set as 100%. The error bars represent the standard error of the mean of three to four independent determinations.

the concentration of CGA (ratio 1:2), the band pattern remained and only absorbance was increased.

Spectral shifts for EA were more complex, but underwent similar changes. The first two peaks (near 255 and 275 nm) of EA (Figure 7C) combined into one band (near 285 nm) of lower intensity, when the EA to iron ratio was 1:1 or 1:2 (cf. Figure 7C and D). Doubling of the concentration of EA (50 µM) caused

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Table II. Effect of preincubation and coincubation with polyphenols on lipid peroxidation in PC12 cells induced by exposure to 0.1 and 1 mM $FeSO_4$ for 1 h, assessed by MDA assay.

		% MDA of Control*	
Test Compound	Incubation	0.1 mM Fe	1 mM Fe
None		425.4 ± 16.7	1188.7 ± 21.3
Ellagic acid	Pre-	199.3 ± 13.5†	1012.4 ± 30.4†
	Co-	92.2 ± 6.6	676.3 ± 28.4
Chlorogenic acid	Pre-	405.3 ± 10.8†	n.d.‡
	Co-	280.3 ± 14.1	n.d.‡

Polyphenol concentrations were 25 μM. Other experimental details were similar to that in Figure 5B.
* MDA values of controls amounted to 4.94 ± 2.07 nmoles/mg protein; † $p < 0.01$ preincubation v coincubation; ‡ not determined.

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a similar band pattern along with a marked increase in absorbance. Interestingly, band II (near 360 nm) of EA almost disappeared in the presence of Fe^{2+} ions. The similar increase in absorbance upon doubling the polyphenol concentration indicates that most probably two molecules of polyphenol contribute to the complex with iron.

Discussion

In our experimental set up we have successfully induced oxidative stress in neuronally differentiated PC12 cells. The three types of stressors comprise different mechanisms and, thus, show considerable differences with respect to the experimental endpoints used herein. Though *t*-BHP may act in part through formation of peroxynitrate [15] and (H_2O_2) through hydroxyl radicals [16], their effects in PC12 cells on cytotoxicity, GSH loss, MDA formation, and DCF-detectable ROS formation were relatively similar. Only the concentration of H_2O_2 necessary to induce similar alterations was about 3-times that of *t*-BHP for all endpoints. In contrast, $FeSO_4$, suspected to produce superoxide radicals [17], induced comparable changes in viability and GSH content only at a 10-fold higher concentration than *t*-BHP, but considerably enhanced lipid peroxidation as measured by MDA production even at 40-times lower concentrations than *t*-BHP. ROS production, on the other hand, was more than twice as high as at a concentration comparable to that of *t*-BHP. These different stressors, therefore, allowed to distinguish between different protective properties of EA and CGA. Furthermore, these findings revealed that ROS formation and enhanced lipid peroxidation are not the direct cause of cell death in PC12 cells, while loss of GSH much better correlates with cytotoxicity. This is also supported by comparing the different response of the various endpoints to pre- and co-incubation (see below).

The present study revealed strong free radical scavenging and protective activities of EA and CGA towards oxidative damage in PC12 cell culture of neuronal origin. Two aspects are of particular interest. Firstly, complete reversal of cytotoxicity, GSH loss and MDA production in response to the peroxides was observed with EA equally or even more effective than with CGA at concentrations as low as 25 μM. In these cases, preincubation with the antioxidants was more effective than coincubation. This is remarkable, since it shows that the polyphenols act only intracellularly whereby the more lipophilic compound EA may preferentially associate with cell membranes or at the membrane surface similar to structurally related flavonoids [4], while the more hydrophilic CGA may reside more in the cytosolic compartment. This interpretation is supported by the observation that with DCF-detectable ROS formation, CGA is more effective than EA, particularly in coincubations (with *t*-BHP), since it may colocalise with DCF in hydrophilic parts of the cell, e.g. the cytosol, where DCF seems to be localized [32,34]. These results are consistent with previously published studies, where authors have exclusively investigated the effect of antioxidants on lowering ROS during coincubations with stressors [19,35]. Moreover, our findings emphasize the remarkable antioxidative strength of both polyphenols, because the concentration added to the medium was 1/4 to 1/12 that of the stressor and it can be assumed that the amount taken up by the cells is considerably less than what is provided with the culture medium.

Secondly, in the case of $FeSO_4$ the situation is much different. With respect to ROS formation, preincubation is almost ineffective with both polyphenols, while coincubation leads to a much stronger protection than with the peroxides. This might be due to iron chelation in the medium as evidenced herein by respective changes in the absorption spectra. In fact, previous studies have demonstrated that polyphenols, beyond their antioxidative activity, can also chelate metal ions and prevent iron- and copper- catalysed formation of ROS [6,33,36]. For instance, *in vitro* studies showed that EA inhibited the generation of superoxide anions and hydroxyl radicals in both enzymic and nonenzymic systems by its metal-chelating property thus providing protection against lipid peroxidation induced by *t*-BHP

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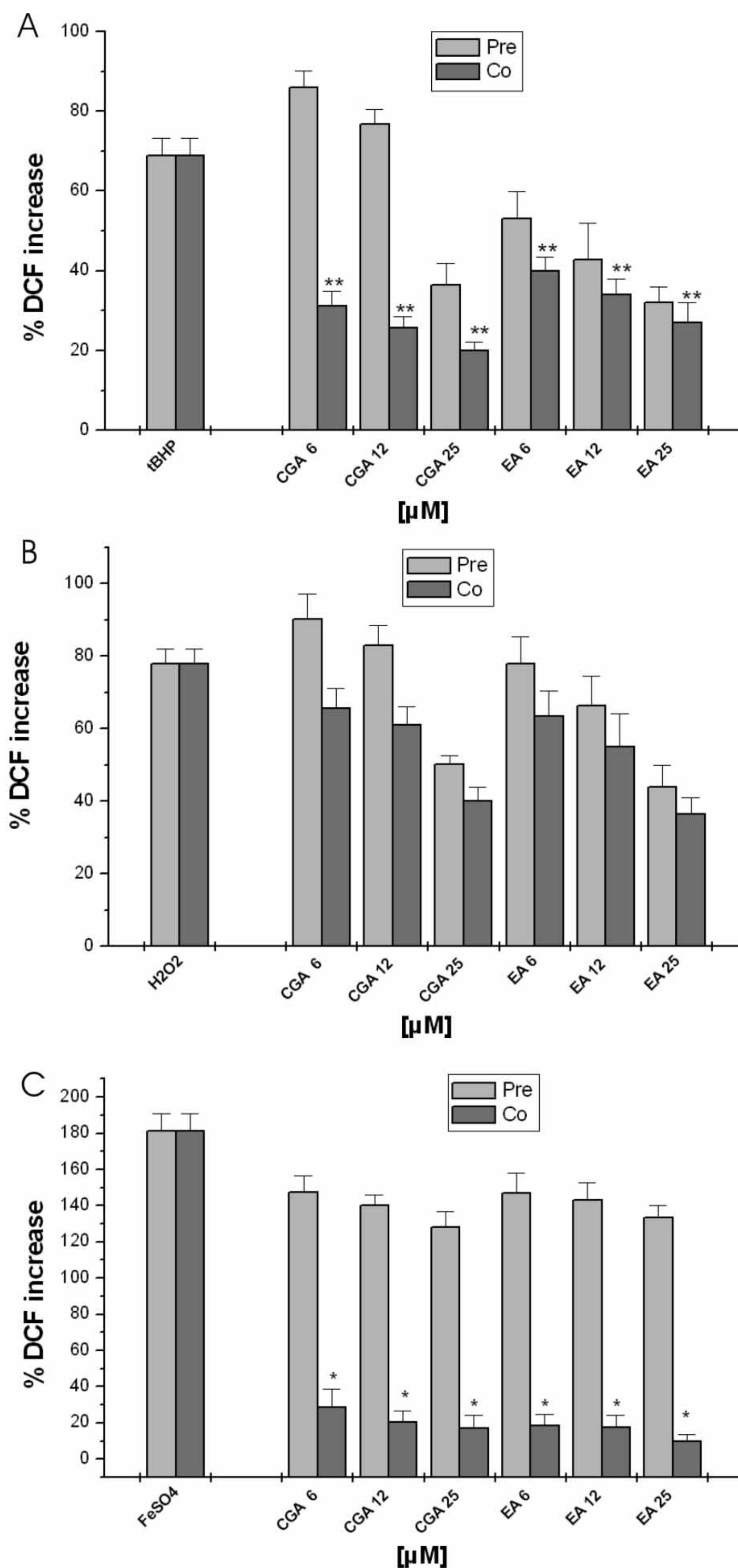

Figure 6. Comparison of free radical scavenging activities of EA and CGA (concentration range 6.2–25 μM), determined by DCF assay, on formation of ROS induced by 0.1 mM *t*-BHP (A), 0.3 mM H_2O_2 (B) and 0.1 mM $FeSO_4$ (C) in preincubation (light grey columns) and/or coincubation (dark grey columns) experiments. In control wells after 30 min of measuring fluorescence (485/520 nm), DCF increase was 20.57 ± 1.46%. Values represent means ± SD of three independent experiments with determination in triplicate. (**$p < 0.05$, preincubation v coincubation with *t*-BHP; *$p < 0.001$, preincubation v coincubation with $FeSO_4$).

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Table III. Comparation of the abilities of metabolites, CA FA, to scavenge ROS induced in PC12 cells by different stressors as determined by DCF assay.

Test compound	Incubation		DCF increase (%)		
			0.1 mM *t*-BHP	0.3 mM H_2O_2	0.1 mM $FeSO_4$
None			65.3 ± 5.5	73.2 ± 6.6	173.4 ± 11.1
Caffeic acid	pre-	25.0μM	58.9 ± 11.2	63.9 ± 8.9	122.1 ± 8.2
	pre-	12.5 μM	63.1 ± 5.1	78.8 ± 6.4	135.2 ± 7.3
	pre-	6.2 μM	67.4 ± 6.1	82.2 ± 5.1	199.8 ± 12.4
	co-	25.0μM	18.8 ± 3.6*	40.1 ± 5.5*	9.6 ± 3.7*
	co-	12.5 μM	20.2 ± 2.9*	50.4 ± 6.1*	14.1 ± 3.9*
	co-	6.2 μM	24.6 ± 3.4*	63.2 ± 5.8*	16.7 ± 5.1*
Ferulic acid	pre-	25.0μM	50.4 ± 9.1	68.9 ± 8.4	156.2 ± 9.5
	pre-	12.5 μM	54.7 ± 6.1	79.9 ± 6.0	168.1 ± 8.3
	pre-	6.2 μM	61.4 ± 4.7	90.7 ± 5.8	202.3 ± 11.6
	co-	25.0μM	24.1 ± 2.7*	45.4 ± 6.4*	10.6 ± 3.8*
	co-	12.5 μM	28.5 ± 3.3*	52.2 ± 7.1*	12.4 ± 1.8*
	co-	6.2 μM	30.1 ± 1.5*	61.3 ± 5.5*	15.1 ± 2.7*
Trolox	pre-	1.0mM	51.7 ± 9.2	56.6 ± 5.5	200.3 ± 13.4
	co-	1.0mM	25.2 ± 2.9*	30.1 ± 8.8*	21.8 ± 8.6*

PC 12 cells were preincubated with metabolites (6.2–25 μM) or Trolox (1 mM) for 2 h followed by exposure of the cells to 0.1 mM *t*-BHP/0.3 mM H_2O_2/0.1 mM $FeSO_4$ for 24 h or were co-incubated with metabolites and stressors for 24 h. DCF-fluorescence was measured 30 min after addition of the stressors to plated PC12 cells. In controls (untreated cells), DCF increase amounted to 21.96 ± 2.25. Data represent means ± SD of two to three separate experiments with determination in triplicate; * $p < 0.001$ coincubation vs. preincubation with all stressors.

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in isolated rat hepatocytes [13]. Likewise, CGA showed an inhibitory effect on the formation of 13-HPODE-derived radicals due to chelation of ferrous ions in the reaction mixture of linoleic acid hydroperoxide with iron [21]. The fact that both polyphenols are not very efficient in rescuing PC12 cells from iron-induced GSH loss and cell death may be ascribed to the fact that the concentrations of $FeSO_4$ necessary to induce such effects considerably surmount those necessary for excessive ROS formation and, thus, lead to a very unfavourable ratio between stressor and antioxidant (or chelator).

With respect to the antioxidative properties of both polyphenols our observations provide novel information. Although the chemical structures of CGA and EA are suggestive of donor antioxidants, since the hydrogen atom at the phenolic OH group is prone to donation with subsequent formation of phenoxyl radicals, *in vitro* studies demonstrated that CGA is more efficient quencher of peroxyl radicals in the aqueous phase in LDL oxidation model than EA [37]. It is difficult to extend these results to cells that are under oxidative stress, because the cell structure encompasses both hydrophilic and lipophilic compartments [38]. Results of GSH and MDA assays, in our study, clearly show the lower effectiveness of CGA as compared with EA. High lipophilicity of EA, on one hand, and the anionic nature and high water solubility of CGA, on the other hand, could be one of the reasons. In contrast, in the DCF assay CGA appeared superior to EA at least when ROS are induced by *t*-BHP. Thus, the protective effects of plant polyphenols against oxidative injury are dependent not only on their antioxidant properties, but also on their different distribution between aqueous spaces and within cell membranes.

In the study of Kono et al. the beneficial antioxidant nature of CGA has been emphasized: unlike most antioxidants its products formed by reaction with free radicals, were rapidly broken down to products unable to generate any free radicals [39]. By comparison of their absorption spectra, this group has first observed that CGA reacts with $ONOO^-$. Using pulse radiolysis, the second-order rate constants of the reactions of CGA with superoxide and hydrohyl radicals were determined [39]. Superoxide and peroxynitrite radical scavenging activity of CGA, as the most active component from distinct plant extracts, have also been demonstrated *in vitro* in DPPH decoloration assay and with different enzymatic methods [40,41]. In the present work, free radical scavenging ability of CGA has been observed in intact cells. It is important to emphasize that this hydrophilic antioxidant (located inside the cells, like DCFH) showed a somewhat higher effectiveness than lipophilic EA (probably compartmented in membranes) only in the DCF assay when *t*-BHP was the stressor. In comparison, both polyphenols similarly diminished the increase in DCF fluorescence produced by $FeSO_4$ and H_2O_2.

Using CHO cells EA was shown to exert a strong scavenging activity of ROS produced by H_2O_2 [19,35], and EA acted through more than one mechanism in the

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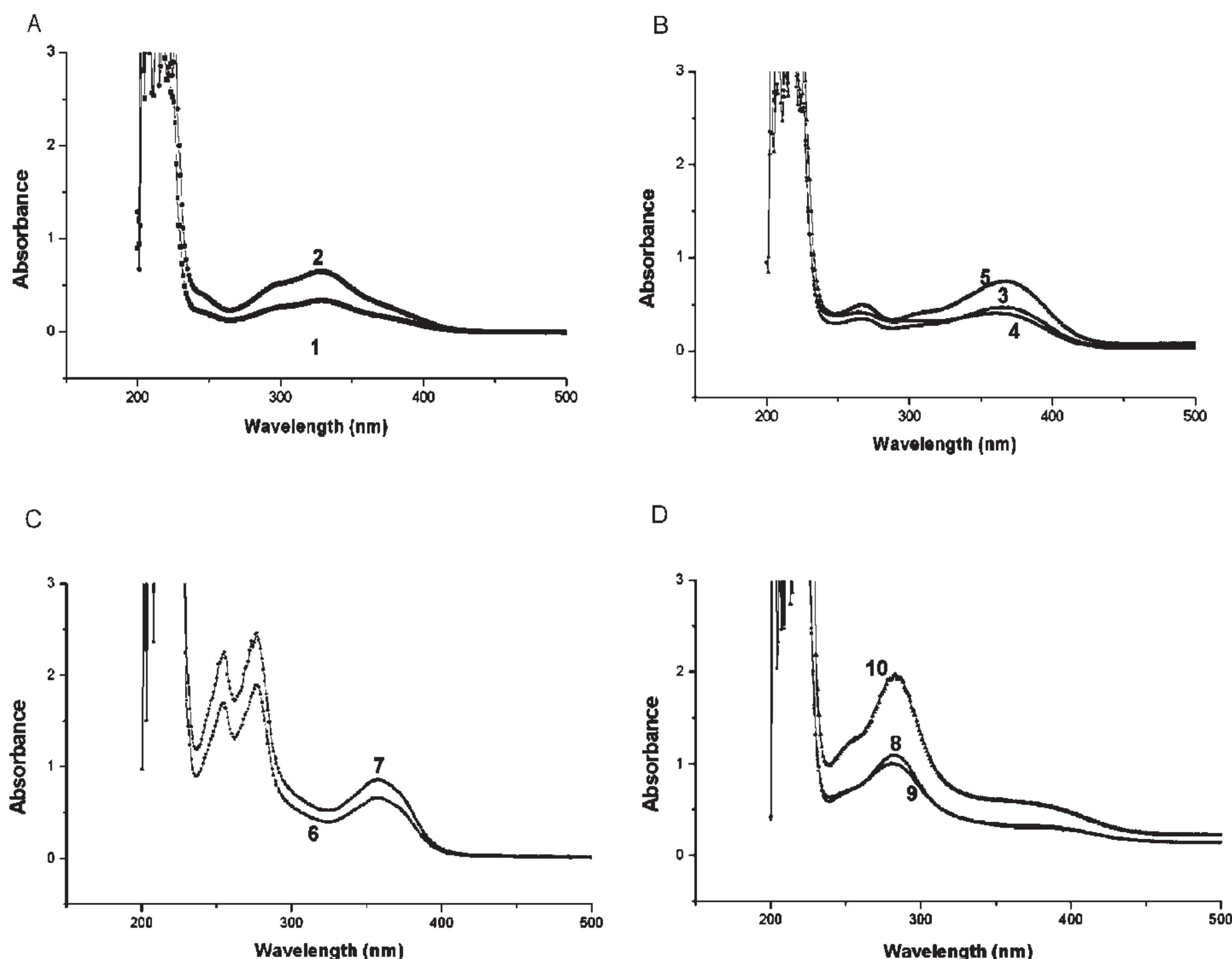

Figure 7. Absorption spectra of $FeSO_4$ complexes with CGA (A,B) and EA (C,D) in PBS. (A) Spectra of CGA at 25 μM (line 1) and 50 μM (line 2). (B) Spectra of 25 μM CGA plus 25 μM $FeSO_4$ (line 3), 25 μM CGA + 50 μM $FeSO_4$ (line 4) and 50 μM CGA plus 25 μM $FeSO_4$ (line 5). (C). Spectra of EA at 25 μM (line 6) and 50 μM (line 7). (D). Spectra of 25 μM EA plus 25 μM $FeSO_4$ (line 8), 25 μM EA + 50 μM $FeSO_4$ (line 9) and 50 μM EA plus 25 μM $FeSO_4$ (line 10).

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protection against MMC and H_2O_2 damage in these cells [19]. Doroshenko et al. [42] reported suppressive effects of EA against acute death of PC12 cells caused by arachidonic acid. Our data obtained with differentiated PC12 cells clearly show the capacity of EA to act as a scavenger of ROS produced by *t*-BHP and $FeSO_4$ treatment measured as DCFH oxidation. Since EA and CGA are rapidly metabolised [43] and can only rarely be detected in human serum [44], we also investigated two potential metabolites, CA and FA. Both compounds showed similar effects as EA and CA indicating that biotransformation does not necessarily destroy the influence of EA and CGA. This is comparable to the situation with quercetin, where we could show a partial take over of functions by some of the metabolites [45]. The potent protective action of hydroxycinnamates was also described by Schroeter et al. [46] using striatal neurons exposed to oxLDL.

As mentioned earlier, free radicals are involved in the pathology of many CNS disorders (PD, AD or stroke). Therefore, free radical scavengers are of great interest [3]. Since differentiated PC12 cells are an accepted model of neuronal cells, our results on two exogenous dietary antioxidants, ellagic and chlorogenic acid as well as on selected metabolites, may have wide implications concerning protective effects against toxicity of various oxidative stressors in the CNS. Detailed studies on the absorption capacity from diet and on the bioavailability of these polyphenic acids respectively of their metabolites, therefore, are highly indicated.

Acknowledgements

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