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Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems in vitro: structure–activity studies

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Abstract

In this study, free radical scavenging abilities of ferulic acid in relation to its structural characteristics were evaluated in solution, cultured neurons, and synaptosomal systems exposed to hydroxyl and peroxyl radicals. Cultured neuronal cells exposed to the peroxyl radical initiator AAPH die in a dose-response manner and show elevated levels of protein carbonyls. The presence of ferulic acid or similar phenolic compounds, however, greatly reduces free radical damage in neuronal cell systems without causing cell death by themselves. In addition, synaptosomal membrane systems exposed to oxidative stress by hydroxyl and peroxyl radical generators show elevated levels of oxidation as indexed by protein oxidation, lipid peroxidation, and ROS measurement. Ferulic acid greatly attenuates these changes, and its effects are far more potent than those obtained for vanillic, coumaric, and cinnamic acid treatments. Moreover, ferulic acid protects against free radical mediated changes in conformation of synaptosomal membrane proteins as monitored by EPR spin labeling techniques. The results presented in this study suggest the importance of naturally occurring antioxidants such as ferulic acid in therapeutic intervention methodology against neurodegenerative disorders such as Alzheimer's disease in which oxidative stress is implicated. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Reactive oxygen species; EPR; Ferulic acid; Synaptosomal membranes; Antioxidants; DCF fluorescence; Neuronal cultures; Oxidative stress

1. Introduction

The use of antioxidants has been recognized as an important counter measure against conditions in which oxidative stress is implicated. Specifically, oxidative stress, an imbalance between pro- and antioxidants, has been implicated in several neurodegenerative disorders such as Alzheimer's disease (AD) [1–3]. Among many classes of compounds, naturally occurring phenolics have been given attention [4,5]. Explicitly, ferulic acid (FA)—commonly found in fruits and vegetables such as tomato [6]-has been shown to possess some activity toward peroxynitrite [7] and oxidized low-density lipoprotein (oxLDL) *in vitro* [4].

In this study, the ability of ferulic acid (FA) to act as an antioxidant against peroxyl radical-induced oxidation was evaluated in neuronal cultures exposed to the peroxyl radical generator 2,2,′azobis(2-amidino-propane)dihydrochloride (AAPH). Additionally, we studied the effect of FA on oxidation of synaptosomal membranes caused by hydroxyl and peroxyl radicals by means of EPR spin labeling, protein oxidation indexing, ROS measurements, and lipid peroxidation studies. Further, the antioxidant activity of the compound was evaluated in solution by means of the DPPH assay. Finally, in structure-activity studies the antioxidant capability of FA to its structural characteristics was investigated by comparing FA to compounds with similar chemical structure vanilic acid (VA), cinnamic acid (CIA) and coumaric acid (COA).

Abbreviations used: EPR, electron paramagnetic resonance; MAL-6, 2,2,6,6-tetramethyl-4-maleimidopiperidin-1-oxyl; ROS, reactive oxygen species; DCF, dichlorofluorescein; DCFH, dichlorofluorescin; DCFH-DA, dichlorofluorescin diacetate; FA, ferulic acid; VA, vanillic acid, COA, coumaric acid; CIA, cinnamic acid; DPPH, 2,2-diphenyl-2-picrylhydrazyl; AAPH, 2,2,′azobis(2-amidino-propane)dihydrochloride.

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2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. AAPH was obtained from Wako Chemicals USA, Inc (Richmond, VA), and the oxidized protein detection kit came from Intergen (Purchase, NY). Fresh solutions of FA, VA, COA, and CIA were prepared as 1 mM stock concentration in PBS (pH = 7.4) and were diluted to appropriate concentrations for various assays. Fresh 10 mM stock solution of dichlorofluorescin diacetate (DCFH-DA) was prepared in ethanol.

2.1. DPPH assay

The detailed description of the DPPH assay can be found elsewhere [8]. Briefly, 200 μl of fresh, 2,2-diphenyl-2-picrylhydrazyl (DPPH) solution (50 μM in methanol) was added to a clear microtiter plate (Corning Inc., Acton, MA) together with 20 μl of appropriate antioxidant ranging from 0 to 400 μM in concentration. The plate was covered with aluminum foil and was left to incubate at room temp for 21 hr. The absorbance was read at 515 nM and was converted to concentration units. The antioxidant activity was expressed as a slope (μM of DPPH/μM of antioxidant). In principle, free radicals react with the antioxidant resulting in a loss of color intensity. Based on the slope values one can deduce the extent of reaction of DPPH with the potential compound [8].

2.2. Hippocampal neuronal culture

Hippocampal neuronal cultures were obtained from 18-day old Sprague-Dawley rat fetuses as previously described [9]. Various concentrations of AAPH and/or FA and its analogs were added, and the cultures were incubated with the cells for 24 hr. Neuronal toxicity was determined by the Trypan blue exclusion assay. Trypan blue exclusion was performed by counting the neurons that internalize the dye. Exclusion of the dye corresponds to cell survival. Cells were rinsed three times with 1 ml PBS (pH = 7.4), and Trypan blue (0.4%) was added along with 300 μl PBS and incubated for 10 min. Sixteen different areas were counted under the microscope for blue staining, which, if present, index dead cells. Comparison between control and subject cells was then performed [9]. In addition, the content of protein carbonyls was evaluated in a similar fashion as with synaptosomes noted below. To assess morphological changes, photomicrographs of hippocampal neuronal cultures were obtained using a camera attached to a microscope.

2.3. Preparation of synaptosomes

Synaptosomal membrane systems prepared from the fresh brain samples offer an easy and convenient method for studying the cellular processes *in vitro.* Synaptosomes, sealed synaptic vehicles, behave in a similar manner as neurons, i.e., respiration, oxygen uptake, and maintenance of proper membrane potential; therefore, important information regarding oxidation can be elucidated and related to neuronal functions [10].

All experiments were conducted in accordance with University of Kentucky Animal Care and Use Committee. Three-month old male Mongolian Gerbils were used to obtain a brain specimen. Synaptosomes were isolated using a discontinuous sucrose-gradient method [10,11]. Briefly, the animals were sacrificed by decapitation, and the brain was isolated immediately. The cortex was placed in 0.32 *M* sucrose isolation buffer containing 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5μg/ml aprotinin, 20 μg/ml trypsin inhibitor, 2 mM ethyleneglycol bistetraacetic acid (EGTA), 2 mM ethylenediamine tetraacetic acid (EDTA), and 20 mM 4-(2-hydroxyethyl)-1-piperazineethene sulfonic acid (HEPES), pH = 7.4). Samples were then homogenized by passing in the tube a motor driven pestle 10 times. The homogenate tissue was then centrifuged at 1500 *g* for 10 min. The supernatant was collected and spun at 20,000 *g* for 10 min. The pellet was removed and placed on the top of discontinuous sucrose gradient (0.85 *M,* pH = 8; 1 *M,* pH = 8; and 1.18 *M* pH = 8.5 sucrose solutions each containing 10 mM HEPES, 2mM EDTA, and 2mM EGTA) and spun in a Beckman L7–55 ultracentrifuge at 82,500 *g* for 2 hr at 4°C. The synaptosomal layer was collected at the 1/1.18 molar sucrose interface, and subsequently washed twice with PBS for 10 min at 32,000 *g* yielding synaptosomal membranes. Protein concentration was determined by the BCA method on Bio-tek Instuments PowerWaveX UV-Vis microtiter plate reader.

2.4. Measurement of lipid peroxidation

Synaptosomes (4 mg proteins/ml) were preincubated with appropriate dose of the antioxidant for 30 min at room temp. Lipid peroxidation was initiated by addition of hydroxyl radical generating mixture (30 μM $FeSO_4$/1 mM H_2O_2). After an additional 30 min at room temp, 10% w/v of ice cold TCA was added, and the samples were spun in an Eppendorf centrifuge for 5 min at 3,000 *g.* The supernatant was collected (0.5 ml) and treated with TBA reagent (20 mM TBA in 50% v/v glacial acetic acid). The samples were then heated at 100°C for 1 hr. After the cooling period, butanol was added, and the organic layer was removed and redistributed to a black microtiter plate (Corning Inc, Acton, MA). End point fluorescence was measured at λ_{ex} = 515 nm and λ_{em} = 585 nm.

2.5. Measurement of protein oxidation

Synaptosomes (4 mg protein/ml) were treated in similar fashion as for lipid peroxidation studies, only before addition of TCA small aliquots (5 μl) were withdrawn and treated with equal volume of 10% SDS. Samples were then derivatized with 20 μM 2,4-DNPH (10 μl) for 20 min. After

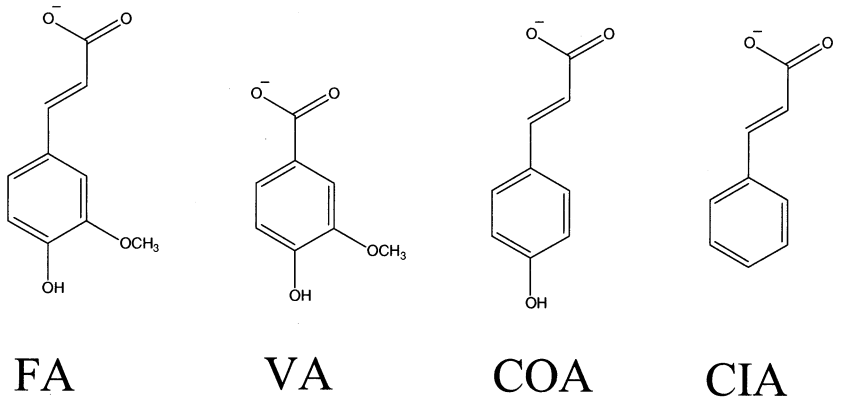

Fig. 1. Chemical structures of ferulic acid (FA), vanillic acid (VA), coumaric acid (COA), and cinnamic acid (CIA).

that period of time, reaction was stopped by addition of neutralizing reagent (7.5 μl of TRIS buffer, pH = 8.0). Levels of protein carbonyls were measured by using the slot-blot technique with 250 μg of protein loaded per slot. The resultant stain was developed by application of Sigma-Fast tablets, and the line densities were quantified by Scion-Image software package.

2.6. Measurement of ROS

Synaptosomes (1 mg protein/ml in PBS pH = 7.4, 1 ml) were treated with the appropriate dose of the antioxidant and with 10 μM of DCFH-DA. After 30 min of incubation at room temp samples were spun at 3000 *g* for 5 min to wash off the excess of dye and the antioxidant. The pellet was dissolved in 0.5 ml PBS, and 100 μl aliquots were redistributed to the black microtiter plate. DCF dependent fluorescence was triggered by addition of 10 mM AAPH followed by incubation at 37°C for 30 min. At that time end point fluorescence was measured at λ_{ex} = 485 nm and λ_{em} = 530 nm.

2.7. EPR spin labeling

Synaptosomes (2 mg protein/ml, 1 ml in PBS) were preincubated with 250 μM of FA for 30 min. Then, oxidation was initiated by addition of 10 mM of AAPH or hydroxyl radical generating solution (30 μM $FeSO_4$/1 mM H_2O_2) for 30 min. In the case of AAPH treatment, samples were incubated at 37°C; for OH radical treatment, incubation was performed at room temperature. After incubation samples were washed three times with lysing buffer (2 mM EGTA,2 mM EDTA, 10 mM HEPES, pH = 7.4) at 14,000 *g* for 4 min in Eppendorf centrifuge. This step was followed by addition of the protein specific spin label 2,2,6,6-tetramethyl-4-maleimidopiperidin-1-oxyl (MAL-6) (50 μg/ml) prepared in lysing buffer. The samples were allowed to react with MAL-6 for at least 12 hr at 4°C. Excess unreacted MAL-6 was then removed by 7 repeated washes steps with lysing buffer. Finally, the pellets were resuspended in 0.5 ml of lysing buffer, and EPR analysis was performed using a Bruker EMX EPR spectrometer with the following parameters: gain = 1×10^5, modulation amplitude = 0.3 G, microwave power = 20 mW, conversion time = 10. 28 ms, and number of scans = 10.

2.8. Statistical analysis

ANOVA was used to assess statistical significance. P values <0.05 were considered significant.

3. Results

Antioxidant effects of ferulic acid in relation to structure-activity studies were evaluated in solution, rat cultured neuronal cells, and gerbil synaptosomal membrane systems using the following ROS markers: solution DPPH assay; lipid peroxidation (TBARS); protein oxidation (protein carbonyl assay); DCF fluorescence; and MAL-6 EPR spin labeling. Additionally, the Trypan blue assay was used to determine the extent of cellular death. The hydroxyl radical generator system (30 μM Fe(II)/1 mM H_2O_2) and water-soluble peroxyl/alkoxyl generating compound AAPH were employed to stimulate an oxidative stress [11–13]. VA, COA and CIA were chosen due to their similarity in the chemical structure; importantly each of them lacks a certain structural component that is present in the structure of FA (Fig. 1).

3.1. DPPH antioxidant activity assay

Table 1 presents results obtained for FA, VA, CIA, and COA with the DPPH antioxidant activity assay after 21 hr incubation with DPPH at room temp. FA possesses the greatest antioxidant power while CIA has the smallest, practically equal to zero. The results obtained in this work correlate well with those from an other publication [8]. The only significant deviation is observed for COA, and that could be attributed to the difference in solvent used; whereas Fukumoto and Mazza dissolved COA in methanol, our solvent was a PBS buffer at pH = 7.4. Nonetheless, the

Table 1
Antioxidant activity and oxidation potential of FA, VA, COA, and CIA

Compound	E(mV)	DPPH assay	Literature DPPH assay
FA	400	−1.39 (0.13)	−1.34
VA	450	−0.92 (0.06)	−0.99
CIA	—	−0.11 (0.11)	—
COA	750	−0.67 (0.14)	−0.33

DPPH assay clearly shows FA as the most potent antioxidant tested in this work. In addition, the oxidation potentials for the relevant compounds are listed in Table 1 as well-with the one for FA being the smallest and one for COA the largest [14]. Oxidation of CIA can not be achieved within the potential range of 1000 mV [15].

3.2. Cell survival studies

Cultured hippocampal neuronal cells exposed to various concentrations of AAPH die in a dose-dependent manner after 24 hr of incubation with the prooxidant at 37°C, as seen in Fig. 2A. AAPH concentrations of 1 and 2 mM were chosen for the subsequent antioxidant studies. Figure 2B illustrates the effects of FA in preventing AAPH-induced cell toxicity. At 1 mM of AAPH, 25 and 50 μM of FA significantly attenuate free radical toxicity ($p < 0.008$), and even at the higher concentration of AAPH (2 mM), a great deal of protection by 50 μM FA was observed ($p < 0.001$). In order to demonstrate if oxidative events are responsible for cell death, immunochemical detection of protein carbonyls levels for samples exposed to 2 mM AAPH with and without 50 μM FA was observed. As seen in Figure 2C, the presence of AAPH induces significant elevation of protein carbonyls consistent with the notion of oxidative damage by ROS ($p < 0.002$). Importantly, FA significantly reduces the amount of oxidized proteins. Finally, we compared the activity of FA to those of its analogs in the cultured neuronal system (Fig. 2D). All four compounds are not toxic to the neurons, and all of them possess some antioxidant activity as demonstrated in the cell survival assay ($p < 0.0001$). Fig. 3 presents phase contrast photomicrographs of rat primary hippocampal neurons treated with 2 mM AAPH with and without 50 μM FA. AAPH-treated neurons demonstrate vaculated soma and fragmented neurites. In constrast the morphology of cells treated with AAPH in the presence of FA closely resembles that of control cells, showing that FA dramatically prevents free radical-induced alterations appearance of the treated neurons.

3.3. Measurement of ROS

Cell permeable dichlorofluorescin diacetate (DCFH-DA) crosses inside the synaptosomal vesicle where it is deesterified by cellular esterases resulting in dichlorofluorescin (DCFH). DCFH in turn is converted upon oxidation to

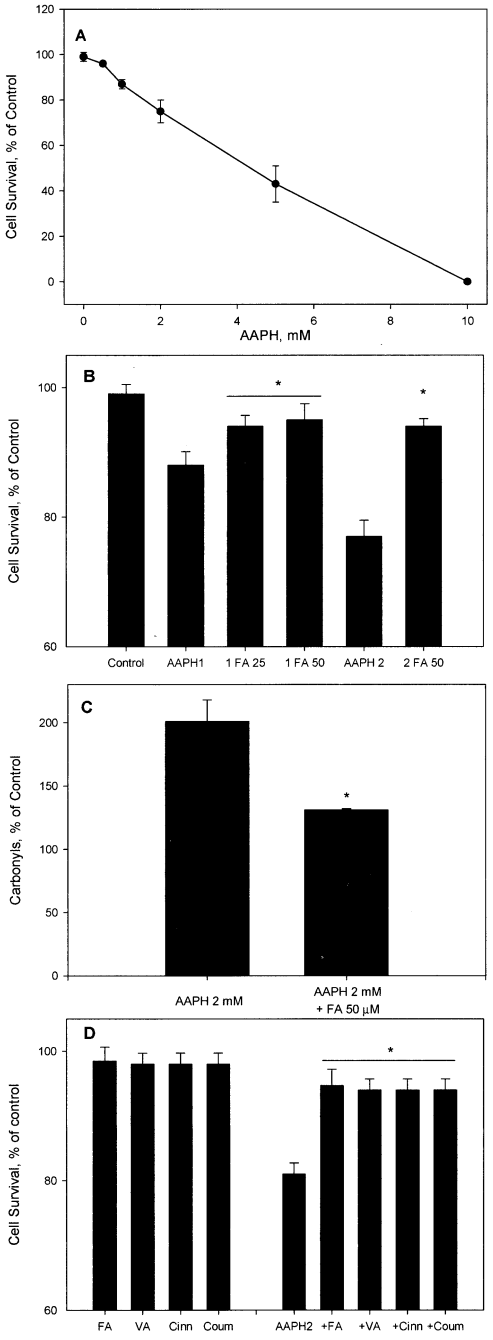

Fig. 2. Cell Survival Studies. Hippocampal neuronal cultures were obtained form 18-day old Sprague-Dawley rat fetuses. The results are presented as MEAN ± STDEV (n = 3–7). (**A**) AAPH dose response. Neuronal cell die in dose-dependent manner upon treatment with AAPH between 1 and 10 mM. (**B**) Protection of FA against AAPH induced cell death. For 25 and 50 μM FA treatment, there is a significant protection against 1 mM AAPH prooxidant (94 and 95% vs. 88% of control values, $p < 0.008$, ANOVA). Cells that had 50 μM FA also showed protection against 2 mM AAPH (94 vs 81% of control values, $p < 0.0001$, ANOVA). (**C**) FA prevention of AAPH induced protein oxidation in neuronal cells. 50 μM FA significantly attenuates AAPH (2mM) induced protein oxidation (131% vs. 201% of control values, $p < 0.002$, ANOVA). (**D**) FA and its analogs efficiently prevent cell toxicity induced by AAPH. The presence of antioxidants do not cause significant cell death (98.5–98% of control values); all compounds significantly protect cell from AAPH (2mM) induced cell death (94.6–94% vs. 81% of control values, $p < 0.0001$, ANOVA).

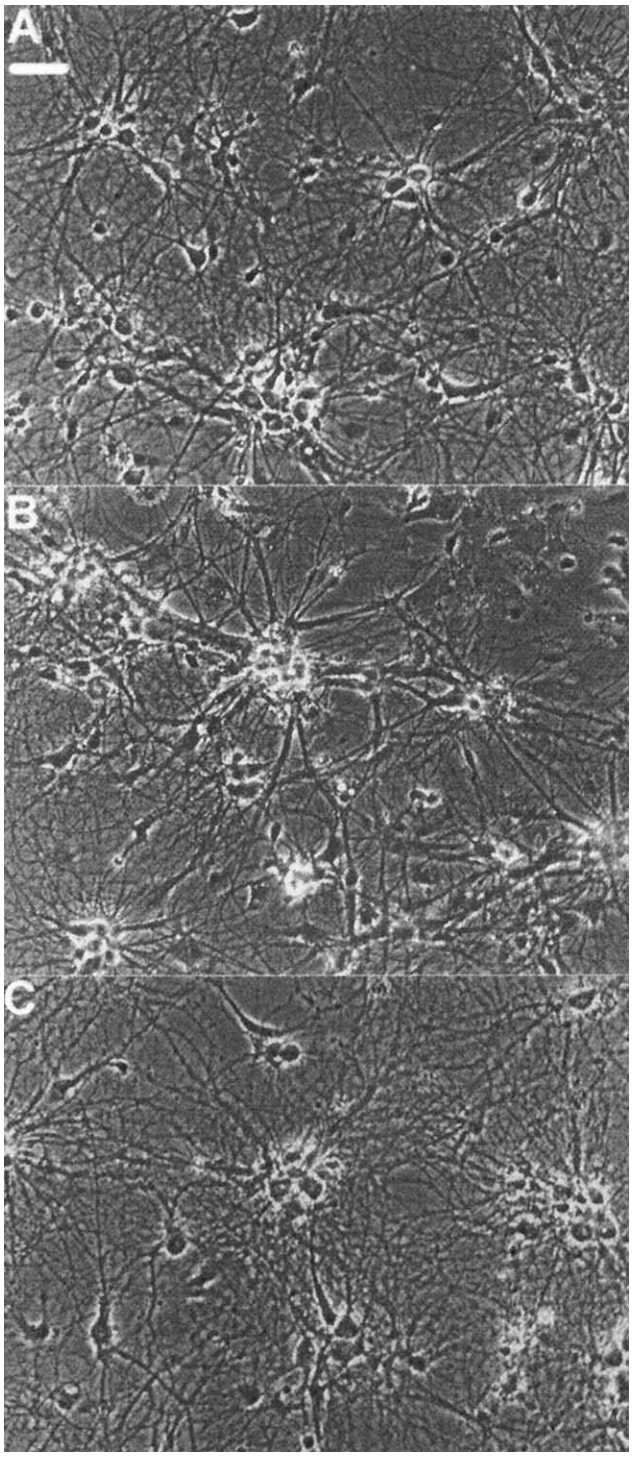

Fig. 3. Phase contrast photomicrographs of rat primary hippocampal neurons in culture. **A.** Control cells showing healthy neurons in culture with extensive network. **B.** 2 mM AAPH treatment; cells with vacuolated soma and beaded or fragmented neurites are present. **C.** 2 mM AAPH with 50 μM FA; the presence of FA not only improves survival of cells, but also improves the appearance of the treated cells. Scale bar = 100 μm.

highly fluorescent dichlorofluorescein (DCF). Thus, by measuring the fluorescence intensity, one can estimate the levels of ROS in the cells [16]. Incubation of synaptosomes with 10 mM AAPH for 30 min at room temperature causes

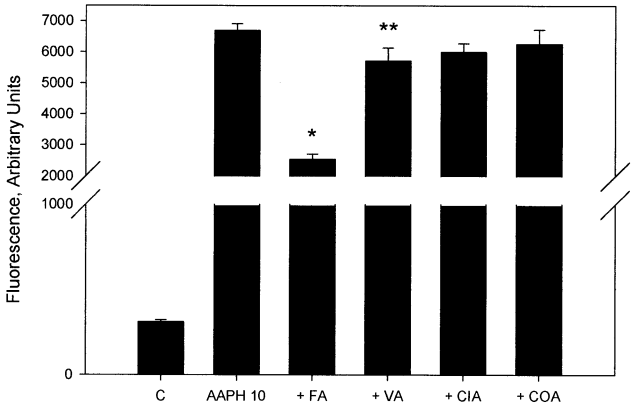

Fig. 4. ROS measurement in synaptosomes, DCF assay. Synaptosomes (1 mg protein/ml in PBS) were preincubated with 250 μM of appropriate antioxidant along with 10 μM DCFH-DA for 30 min. Samples were then spun at 3000 *g* for 5 min to wash off the excess dye and the antioxidant. The pellets were resuspended in 0.5 ml PBS, and 100 μl aliquots were redistributed to the black microtiter plate. DCF dependent fluorescence was triggered by addition of 10 mM AAPH followed by incubation at 37°C for 30 min. At that time, end point fluorescence was measured at λ_{ex} = 485 nm and λ_{em} = 530 nm. The results are shown as MEAN ± STDEV (n = 4). FA greatly reduces AAPH (10 mM) induced oxidation (2540 vs. 6700 for AAPH treatment, $p < 0.00001$, ANOVA). There is small but significant protection by VA [5712 vs. 6700, $p < 0.01$, ANOVA]. COA and CIA do not significantly protect synaptosomes from AAPH induced oxidation.

considerable amounts of fluorescence as presented in Fig. 4. This oxidation can be greatly moderated in the presence of 250 μM FA ($p < 0.00001$) and somewhat reduced in the presence of 200 μM of VA ($p < 0.01$). CIA and CO do not show any significant protection against AAPH induced ROS burst in synaptosomes.

3.4. Measurement of lipid peroxidation

Fig. 5A and 5B present the antioxidant effects of FA and its analogs on hydroxyl radical-induced oxidation of membrane lipids in synaptosomes (30 min, room temperature). Treatment with OH radicals causes significant lipid peroxidation demonstrated by an increase of TBARS, a marker of lipid peroxidation [17]. This event is due to hydroxyl radical attack on the unsaturated lipid fatty acid chain, giving rise to formation of various 2-TBA reactive substances such as malondialdahyde, HNE or other aldehydic compounds [18]. This increase in TBARS, however, is significantly moderated by FA (125, 250, and 500 μM, Figure 5A, $p < 0.02$, $p < 0.005$, $p < 0.0001$, respectively), but the antioxidant effects of three other compounds are far smaller in comparison to 250 μM FA (Figure 5B, $p < 0.05$), suggesting the inability of VA, CIA, and COA to either partition into the membrane bilayer, or to provide sufficient antioxidant activity.

3.5. Measurement of protein oxidation

Measurement of protein carbonyls levels is a key indicator of protein oxidation, and elevated levels of oxidatively

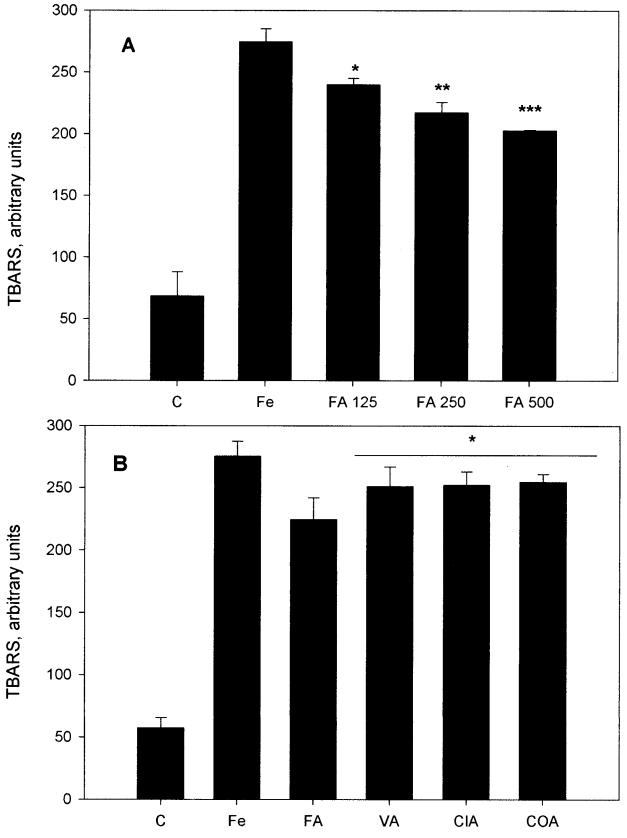

Fig. 5. Protection of FA against OH induced lipid peroxidation in synaptosomes, TBARs assay. Synaptosomes (4 mg protein/ml PBS) were preincubated with the appropriate dose of the antioxidant for 30 min followed by treatment with the OH radical inducing solution (30 μM Fe(II)/1 mM H_2O_2) for 30 min at room temp. 10% w/v of ice cold TCA was then added, and the samples were spun in an Eppendorf centrifuge for 5 min at 3,000 *g*. The supernatant was collected (0.5 ml) and treated with TBA reagent (20 mM TBA in 50% v/v glacial acetic acid). The samples were then heated at 100°C for 1 hr. After the cooling period, butanol was added, and the organic layer was removed and redistributed to the black microtiter plate (Corning Inc, Acton, MA). End point fluorescence was measured at λ_{ex} = 515 nm and λ_{em} = 585 nm. The results are shown as MEAN ± STDEV (n = 4). (**A**) FA prevents lipid peroxidation in a dose dependent manner (274, 239, 217, and 202 for no treatment, 125, 250, and 500 μM of FA, respectively, $p < 0.02$, $p < 0.005$, $p < 0.0001$ for comparison to the OH only treatment, ANOVA). (**B**) Only FA shows significant protection against lipid peroxidation in comparison to VA, COA, and CIA. ($p < 0.005$, ANOVA).

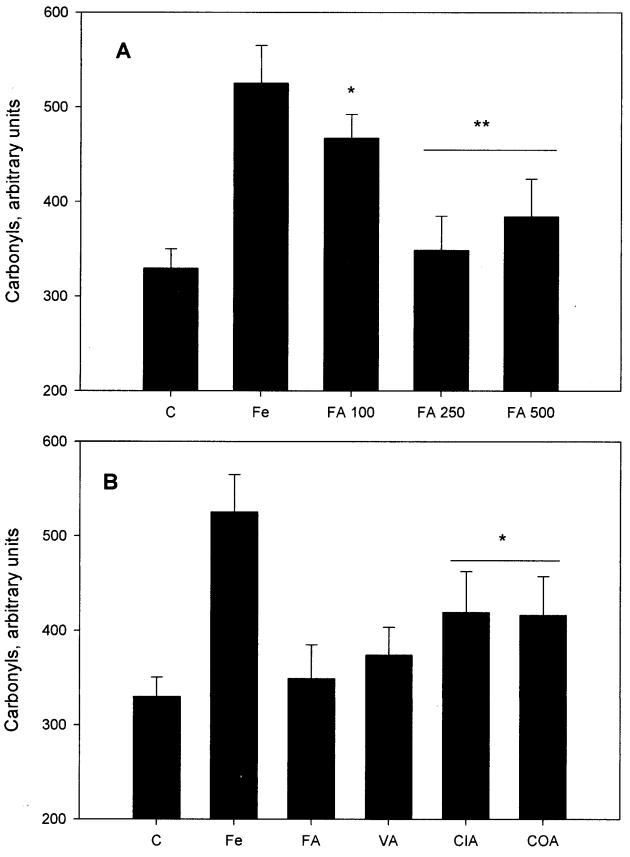

Fig. 6. FA Protection against OH induced protein oxidation in synaptosomes. Synaptosomes were treated in similar manner as for the TBARS experiment. Small aliquots were withdrawn and treated with equal amount of 12% SDS. Samples were then derivatized with 20 μM 2,4-DNPH (10 μl) for 20 min to form the hydrazone. After that period of time, the reaction was stopped by addition of neutralizing reagent (7.5 μl of TRIS buffer, pH = 8.0). Levels of protein carbonyls were measured by using the slot-blot technique with 250 μg of protein loaded per slot. The resultant stain was developed by application of Sigma-Fast tablets, and the line densities were quantified by Scion-Image software package. The results are shown as MEAN ± STDEV (n = 4).(**A**) FA protects against OH induced protein oxidation with almost complete protection at 250 μM ($p < 0.05$, between FA and OH only tretment, ANOVA). (**B**) FA prevention of synaptosomal protein oxidation is far better than that of COA and CIA ($p < 0.05$ for comparison between FA and COA/CIA, ANOVA).

modified proteins have been shown in certain regions of AD brain and are also associated with aging [19,20]. Exposure of synaptosomal system to hydroxyl radicals (30 min, room temperature) causes oxidation of proteins as presented in Fig. 6. Oxidation is almost completely prevented by FA (250 μM and 500 μM, $p < 0.001$ for both, Fig. 6A). The remaining compounds prevent the protein oxidation to some extent, however, not as profoundly as FA ($p < 0.05$ for comparison to FA, Fig. 6B). This result shows that FA can protect synaptosomal proteins directly through its scavenging mechanisms.

3.6. EPR spin labeling

The relevant EPR perameter, the W/S ratio of the protein-specific spin label MAL-6 (Fig. 7A) is decreased following protein oxidation caused by hydroxyl free radicals, ischemia reperfusion injury, amyloid β-peptide, peroxynitrite, and hyperoxia [19,21]. Synaptosomal membranes labeled with MAL-6 show a decreased W/S ratio in the EPR spin labeling experiment upon treatment with hydroxyl radical generator and AAPH (Fig. 7B). These changes can be reversed in presence of 250 μM FA ($p < 0.015$ and $p < 0.0005$ for hydroxyl and peroxyl radical treatment, respectively). These results suggest that oxidative events cause changes in structure of synaptosomal membrane pro-

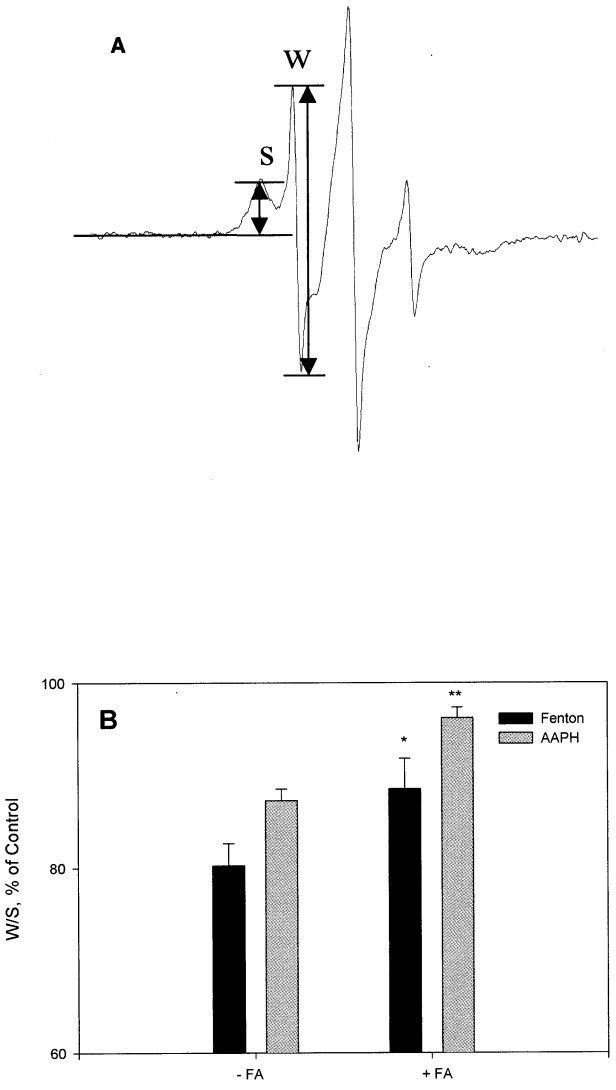

Fig. 7. MAL-6 EPR spin labeling. (**A**) A typical MAL-6 EPR spectrum with relevant W and S parameters indicated. (**B**) FA protection against peroxyl and hydroxyl radical induced alteration in conformation of synaptosomal membrane proteins. Synaptosomes [2 mg protein/ml in PBS] were treated with 250 μM of FA and with appropriate dose of peroxyl and hydroxyl radical initiator. After the incubation samples were washed off three times with lysing buffer (2 mM EGTA, 2 mM EDTA, 10 mM HEPES, pH = 7.4) at 14,000 *g* for 4 min in Eppendorf centrifuge. This step was followed by addition of MAL-6 solution (50 μg/ml) prepared in lysing buffer. The samples were then allowed to react with MAL-6 for at least 12 hr at 4°C. Excess unreacted MAL-6 was then removed by 7 washing steps with lysing buffer. Finally, the pellets were resuspended in 0.5 ml of lysing buffer, and EPR analysis was performed. The results are shown as MEAN ± STDEV (n = 3). The presence of 250 μM of FA significantly prevents the changes in protein conformation caused by hydroxyl and peroxyl radicals (88% vs. 80% of control for OH, $p < 0.015$, 96% vs. 88% for AAPH, $p < 0.0005$ with respect to control, ANOVA).

teins, possibly as the result of protein crosslinking [19]. In addition, these effects can be prevented in the presence of this antioxidant, indicating that ROS, by modifying proteins, not only changes their conformation, but also possibly compromises structural and functional properties of key brain enzymatic proteins such as creatine kinase (CK), glutamine synthetase (GS), and the glutamate transporter [20, 22,23].

4. Discussion

Increasing experimental evidence indicates the importance of oxidative stress in pathology and neurotoxicity associated with aging and many neurodegenerative diseases such as AD [1,3,36]. Free radicals can be formed by many processes [19], inducing electron leak from mitochondrial electron-chain reactions or subsequently in a metal mediated manner, i.e., Fenton chemistry. In any case, there is a cascade of events leading to production of various ROS that vary in reactivity and lifetime. ROS are capable of oxidizing proteins, lipids, and DNA, and elevated levels of corresponding oxidative markers and redox metals have been found in AD brain [20,23–27,36]. Possible preventive methods thus can involve metal ions scavenging or capturing free radicals before they attack important cellular targets. The latter can be achieved by use of antioxidants.

Although it has been recognized that the use of antioxidants is an important preventive method in minimizing pathological and toxic effects associated with oxidative stress, there are several key considerations that need to be addressed in evaluating a potential antioxidant. The foremost issue deals with the inherent toxicity of the target compound; if it exerts harmful effects on the cells, its usefulness is not justified. FA and its structural analogs, commonly found in human diet, do not cause neuronal cell death by themselves as presented in this and previous work [4]. Additionally, the antioxidant should be efficient, protecting at low concentrations, preferably capable of inhibiting lipid peroxidation processes where most of the oxidation damage occurs. As presented here, FA not only is a good antioxidant against protein oxidation, it also possesses some activity against lipid peroxidation damage in various systems as presented here and previously [28–30]. Finally, a compound that is targeted for neuronal protection should be able to cross the blood brain barrier (BBB) readily. Although we do not have any experimental evidence to what extent FA crosses the BBB, judging from the structural similarity to salicylic acid, which does cross the BBB, we speculate that FA should be able to enter the central nervous system.

In this study, the nutritionally-derived antioxidant FA has been evaluated in synaptosomal and neuronal systems and in solution in relationship to its structure. As seen in Figure 1, FA possesses three distinctive structural motifs that can possibly contribute to the free radical scavenging capability of this compound. The presence of electron donating groups on the benzene ring (3-methoxy, and more importantly 4-hydroxyl) of FA gives additional resonance structures of the resulted phenoxyl radical (Fig. 8), contributing to the stability of this intermediate or even terminating free radical chain reactions [5]. The absence 4-hydroxyl

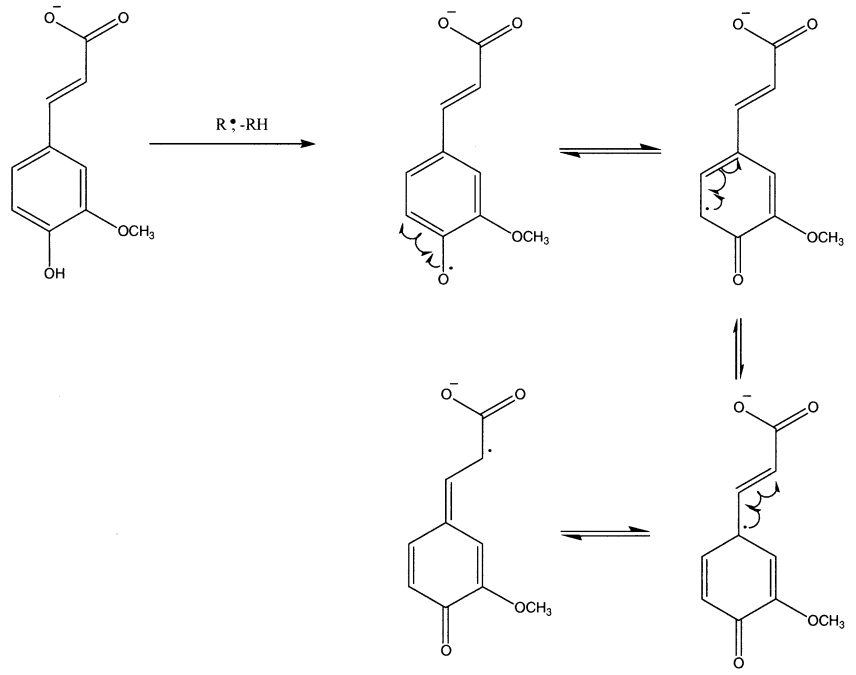

Fig. 8. Possible resonance structures of FA phenoxyl radical. Free radical initiation occurs at the 4-hydroxyl group by abstraction of hydroxyl H-atom. In the case of VA and COA resonance structure are not stabilized by the presence of additional carbon-carbon double bond or the 3-methoxy group, respectively. In the case of CIA, the absence of the 4-hydroxyl group makes free radical oxidation directly on the phenyl ring a less plausible process; possible free radical initiation at the C-C double bond of carboxylic group is conceivable.

and/or 3-methoxy groups creates a destabilized radical as in case of CIA and COA, that is not sufficiently stable to make CIA and COA efficient scavengers of ROS as seen in the DCF, TBARS, and protein carbonyl experiments presented here. The third functionality–the carboxylic acid group with the adjacent unsaturated carbon-carbon double bond–can further contribute to the stability of the radical via resonance or by providing additional attack sites for free radicals. In addition, this third functionality could facilitate an anchoring of FA into the lipid bilayer providing some protection against lipid-peroxidation. This ability is absent in VA that lacks the additional two carbon atoms adjacent to carboxylic acid, and consistent with the TBARS experiments.

From another point of view, the antioxidant activity of FA and its analogs can be related to their oxidation potential and DPPH assay activities which can be arranged in the following order FA $<$ VA $<$ COA $<$ CIA for oxidation potential and FA $>$ VA $>$ COA $>$ CIA for DPPH activity assay (Table 1) [8,14,15]. Clearly, the presence of electron-donating substituents enhances the electrochemical properties of FA in comparison with other compounds tested in this work. Consequently, as in case of CIA, the absence of aromatic substituents, makes this compound a poor antioxidant as seen in the DPPH assay, and its oxidation potential is much higher than that of the other three compounds. Nevertheless, even CIA can scavenge some ROS presumably by free radical attack on the unsaturated carbon-carbon double bond.

EPR spin labeling is an effective method for assessing the changes of structural integrity in membrane bound proteins [31]. Often these processes are a consequence of oxidative events and are well correlated with elevated levels of protein carbonyls. As seen in Fig. 7B treatment of synaptosomes with hydroxyl radical generators or peroxyl radical initiators induces changes in synaptosomal membrane conformation as monitored by a relevant EPR parameter, the W/S ratio, which arises from the weakly- and strongly- immobilized MAL-6 resonance lines (Fig. 7). During oxidation, the intensity of strongly immobilized EPR resonance line increases contributing to the overall decrease in the W/S ratio. Importantly, the presence of FA antioxidant significantly attenuates these oxidative changes in synaptosomal membranes as seen by increase of the W/S ratio of MAL-6.

Given these and other characteristics, phenolic compounds have been recognized as potent antioxidants previously. FA and other compounds of similar structures can attenuate neuronal cell death caused by the uptake of oxidized low-density lipoprotein, that are formed as a consequence of ROS [4]. The antioxidant action of FA in plasma is far greater than that of vitamin C against LDL oxidation [32]. In addition, other reports indicate increased suitability of another phenolic compound, 5-aminosalycilic acid, a therapeutic in ROS related inflammatory bowel disease [33], as an antioxidant in synaptosomal systems exposed to peroxyl/alkoxyl radical initiators *in vitro* [11], or to prevent adriamycin-related ROS burst in cardiomyocytes [34].

In summary, the antioxidant suitability of ferulic acid has been evaluated in synaptosomes and neuronal cultures ex-

posed to peroxyl, and hydroxyl radical insult via several oxidative stress indexes. From the results obtained in this work, FA, a naturally occurring nutritional compound, may be a promising candidate as an antioxidant in neurodegenerative disorders such as AD. Since it has been shown that, based on biovailability studies, ferulic acid possesses the appropriate pharmacokinetic properties [35] (i.e., excretion time, recovery), further consideration should be given for *in vivo* testing of FA, or perhaps making FA a target an initial structure for developing more potent antioxidants for the treatment of neurodegenerative disorders.

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