JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Effect of Ferulic Acid in Isolated Membranes and Intact Cells: Synergistic Interactions with α-Tocopherol, β-Carotene, and Ascorbic Acid

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Although an antioxidant mechanism has been involved in the beneficial effects of ferulic acid in human diseases, there are few reports on the antioxidant properties of this compound in isolated membranes and intact cells. Here, we evaluated the ability of ferulic acid in inhibiting lipid peroxidation in rat liver microsomal membranes and reactive oxygen species production in NIH-3T3 fibroblasts, induced by both *tert*-BOOH and AAPH. We also compared its antioxidant efficiency with that of other antioxidants, such as α-tocopherol, β-carotene, and ascorbic acid, added alone or in combination. Ferulic acid acted as a potent antioxidant in our models, being more effective in protecting from *tert*-BOOH than from AAPH. Moreover, the compound was the most effective among the antioxidants tested. Synergistic interactions were observed when the compound was used in combination with the other antioxidants, suggesting that they can cooperate in preserving physiological integrity of cells exposed to free radicals.

KEYWORDS: Ferulic acid; antioxidant; α-tocopherol; β-carotene; ascorbic acid; membranes; cells

INTRODUCTION

There is currently a great deal of interest in the health benefits of ferulic acid, one of the most ubiquitous phenolic compounds in nature, especially rich as an ester form in rice bran pitch (*1*). The compound has been largely used as a food preservative because of its ability to inhibit the autoxidation of oils (*2*, *3*). It also constitutes the active ingredient in many skin lotions and sunscreens designed for photoprotection (*4*, *5*). Moreover, epidemiological studies have provided evidence that a high consumption of whole grain products, rich in ferulic acid, may reduce the risk of chronic diseases, including cardiovascular diseases and certain types of cancer (*6*). Much attention has been paid to the possibility that ferulic acid may exert such health benefits by an antioxidant mechanism. Due to its phenolic nucleus and an extended side chain conjugation, the compound forms a resonance-stabilized phenoxy radical, which could account for its potential antioxidant and photoprotective properties (*1*, *7*). Recently, it has been reported that ferulic acid and its related compounds, such as *p*-coumaric acid, caffeic acid, and sinapic acid, inhibited the autoxidation of methyl linoleate and linoleic acid as well as the oxidation of liposomes induced by the water-soluble initiator DPPH (*8*). Moreover, natural phenolic compounds, including ferulic acid, have been shown to reinforce the antioxidant capacity of lactoferrin by inhibiting the oxidation in liposomes and oil-in-water emulsions (*9*). In addition, ferulic acid increased the antioxidant activity of plasma and the resistance of low-density lipoproteins (LDLs) to oxidation (*10*−*12*). Although numerous observations suggest that ferulic acid can act as an antioxidant in lipid solutions, little is known on systematic evaluation of the antioxidant properties of this compound in isolated membranes as well as in intact cells. In this work, in rat liver microsomal membranes and in human fibroblasts, we investigated the antioxidant activity of ferulic acid in inhibiting lipid peroxidation and/or reactive oxygen species (ROS) production induced in vitro by two different sources of free radicals: 2,2′-azobis (2-amidinopropane) (AAPH), which exogenously produces peroxyl radicals by thermal decomposition, and *tert*-butyl hydroperoxide (*tert*-BOOH), which endogenously produces alkoxyl radicals by Fenton reactions. In addition, we have compared the antioxidant efficiency of ferulic acid with that of known antioxidants, such as α-tocopherol, ascorbic acid, and β-carotene, added alone and/

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10.1021/jf0303924 CCC: $27.50
Published on Web 03/20/2004

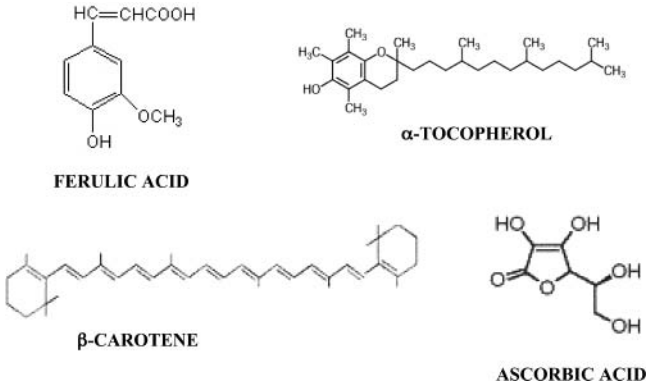

Figure 1. Chemical structures of ferulic acid, α-tocopherol, β-carotene, and ascorbic acid.

or in combination with ferulic acid. The results strongly suggest that ferulic acid can act as an excellent antioxidant in our models. Its antioxidant efficiency was more remarkable using *tert*-BOOH as a prooxidant. Moreover, the compound was the most effective among the antioxidants tested, whatever source of free radicals was used. Synergistic antioxidant interactions were observed when the compound was used in combination with the other antioxidants, suggesting the possibility of cooperative mechanisms among these molecules in biological systems.

MATERIALS AND METHODS

Chemicals. All-trans ferulic acid was obtained by Polichimica srl (Bologna, Italy), α-tocopherol and β-carotene were obtained from Fluka Chemika-Biochemika (Buchs, Switzerland). Ascorbic acid, ethylenediaminetetracetic acid (EDTA), *tert*-butyl hydroperoxide (*tert*-BOOH), butylated hydroxytoluene (BHT), 1,1,3,3-tetramethoxypropane, ammonium acetate, and 2-thiobarbituric acid (TBA) were obtained from Sigma (Sigma Chemical Co, St. Louis, MO). Trichloroacetic acid (TCA) and hydrochloric acid were obtained from Fisher Scientific (Fairlawn, NJ). 2,2′-Azobis (2-amidinopropane) (AAPH) was obtained from Polysciences, Inc (Warrington, PA). Tetrahydrofuran (THF) (99.9%) and tetrabutylammonium dihydrogen phosphate was purchased from Aldrich Chemical Co (St. Louis, MO). Hexane, methanol, acetonitrile, isobutyl alcohol, 2-propanol, and ethanol were HPLC grade and obtained from Fluka Chemika-Biochemika (Buchs, Switzerland).

Microsomal Suspensions. Liver microsomes were prepared from Wistar rats by tissue homogenization with 5 volumes of ice-cold 0.25 M sucrose containing 5 mM Hepes, 0.5 mM EDTA, pH 7.5 in a Potter-Elvehjem homogenizer (*13*). Microsomal membranes were isolated by removal of the nuclear fraction at 8000*g* for 10 min and removal of the mitochondria fraction at 18 000*g* for 10 min. The microsomal fraction was sedimented at 105 000*g* for 60 min, and the fraction was washed once in 0.15 M KCl and collected again at 105 000*g* for 30 min (*10*). The membranes, suspended in 0.1 M potassium phosphate buffer, pH 7.5, were stored at −80 °C. Microsomal proteins were determined by the Bio-Rad method (*14*).

Cell Culture. Murine transformed NIH-3T3 fibroblasts were cultured in RPMI 1640 medium (Gibco Biocult, Paisley, UK) without antibiotics, supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, UK), and 2 mM glutamine. The cells were maintained in log phase by seeding twice a week at a density of 3×10^5 cells/mL. They grew at 37 °C under 5% CO_2 and 95% air at 99% humidity.

Addition of Ferulic Acid and Other Antioxidants to Microsomes and Cells. To minimize autoxidation process, stock solutions of the antioxidants in THF were prepared immediately before each experiment. From the stock solutions, aliquots of ferulic acid, α-tocopherol, β-carotene, and ascorbic acid, whose chemical structures are shown in **Figure 1**, were rapidly added to the microsomes and to the cells to give the final antioxidant concentrations indicated. Control microsomes and cells received amounts of THF equal to those present in antioxidant-treated microsomes and cells. The amount of THF added was not greater than 0.5% (v/v) and did not affect microsomal and cell viability (data not shown). The antioxidant-enriched microsomes and cells were gently suspended by a Dounce homogenizer, and then the suspensions were incubated at 37 °C in a shaking bath under air in the dark. After 60 min of incubation, the distribution of the antioxidants between aqueous and membrane interface was evaluated. The amount of antioxidants associated with the microsomes was measured after a centrifugation of 105 000*g* for 30 min and that associated with the cells after a centrifugation of 850*g* for 10 min. In this experiment, all the antioxidants were used in a range of concentrations of 0.5−20.0 μM. α-Tocopherol and β-carotene, at the concentrations used, were both found to be associated to cell membranes (nearly 100% in the pellet fraction), while ascorbic acid was all found in the aqueous phase (nearly 100% in the supernatant). About 95% of the total amount of ferulic acid added was detected in the aqueous phase, and only 5% was found to be associated to the cell membrane fraction. The amount of ferulic acid in cell membranes was not significantly increased by a prolonged exposition of the cells (up to 6 h) with the compound.

Control and antioxidant-enriched microsomes and cells were incubated at 37 °C under air and in the dark in the absence or in the presence of *tert*-BOOH or AAPH.

Malondialdehyde Formation. Malondialdehyde (MDA) was extracted and analyzed as indicated (*15*). Briefly, aliquots of 1 mL of microsomal suspension (0.5 mg proteins) were mixed with 3 mL 0.5% TCA and 0.5 mL of TBA solution (two parts 0.4% TBA in 0.2 M HCl and one part distilled water) and 0.07 mL of 0.2% BHT in 95% ethanol. Samples were then incubated in a 90 °C bath for 45 min. After incubation, the TBA−MDA complex was extracted with 3 mL of isobutyl alcohol. The isobutanol extract was mixed with methanol (2:1) prior to injection in an HPLC system. The column was packed with Supelcosil LC-18 material, 3-μm particle size, in a 15-cm × 4.6-mm cartridge format (Supelco, Bellefonte, PA). A 2-cm cartridge precolumn containing 5-μm LC-18 Supelcosil packing was used. The mobile phase was a 1:1 (v/v) mixture of methanol and double-distilled water, with the addition of tetrabutylammonium dihydrogen phosphate (0.05%, w/v), as an ion-pairing reagent. The TBA−MDA adduct was detected by a fluorimeter set an excitation wavelength of 515 nm and an emission wavelength of 550 nm. At a flow rate of 1 mL/min, the retention time of the TBA−MDA adduct was 5 min. MDA concentration was calculated from a calibration curve generated from a peak height of the MDA standard, prepared by the hydrolysis of 1,1,3,3,-tetramethoxypropane. Ferulic acid and the other antioxidants tested did not interfere with the MDA assay, because no MDA formation was detected in the medium containing the antioxidants alone in the absence of the microsomes.

Table 1. Effects of Varying Ferulic Acid Concentrations on the Viability of NIH-3T3 Fibroblasts Exposed to a Five-Hour Treatment with AAPH[a] or *tert*-BOOH[b]

treatment	ferulic acid (μM)	cell viability (%)
none	0	98 ± 2[c]
	2.5	99 ± 1[c]
	5.0	99 ± 1[c]
	10.0	99 ± 1[c]
AAPH	0	56 ± 3[c]
	2.5	65 ± 4[c]
	5.0	83 ± 5[d]
	10.0	90 ± 5[d]
tert-BOOH	0	70 ± 4[c]
	2.5	79 ± 4[d]
	5.0	83 ± 5[d]
	10.0	93 ± 5[e]

[a] AAPH was added to the cells at the concentration of 50 mM and [b] *tert*-BOOH at the concentration of 0.1 mM. Results represented the means ± SEM of three separate experiments. [c−e] Within the same treatment, values not sharing a letter differered, $p < 0.05$ (Fisher's test).

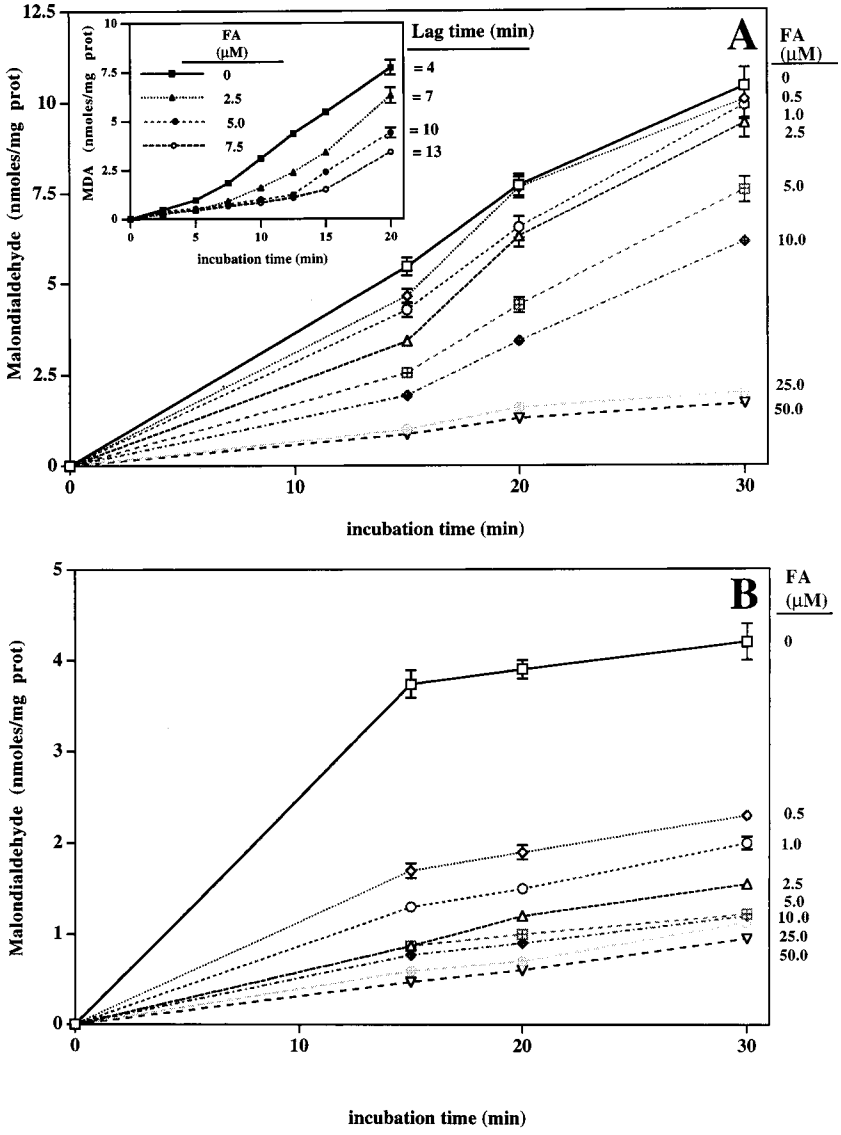

Figure 2. Effects of varying ferulic acid concentrations on MDA production induced by AAPH (**A**) and *tert*-BOOH (**B**) in rat liver microsomal membranes. The insert in **A** shows the calculated lag times. The microsomal membranes were incubated with 25 mM AAPH or 0.25 mM *tert*-BOOH at 37 °C under air in the dark. Results represented the means ± SEM of six separate experiments.

Conjugated Diene Formation. Aliquots of 1 mL of microsomal suspension (1.0 mg proteins) were extracted in chloroform/methanol (2:1, v/v) and conjugated dienes were measured by monitoring the increase of the absorbance at 233 nm. The reference cuvettes lacked the microsomes but contained the prooxidant (AAPH or *tert*-BOOH) and when indicated, ferulic acid. In addition, another set of cuvettes contained the microsomes to monitor their spontaneous autoxidation. All of the cuvettes were incubated at 37 °C, and the 233-nm readings due to oxidized products of ferulic acid and that due to autoxidation were subtracted from the sample cuvettes to obtain the 233-nm reading attributable to conjugated dienes. The difference spectrum obtained was used for the determination of conjugated dienes, using $\Sigma = 25\,200$ $M^{-1}cm^{-1}$ (*16*).

Ferulic Acid Assay. Ferulic acid was determined according to Fujiwara et al. (*17*). Briefly, to 1 mL of microsomal membranes was added 1 mL of 0.2 N HCl, and the sample was extracted with 6 mL of ethyl ether with vigorous shaking for 10 min. After centrifugation of the mixture, the organic layer was transferred into another test tube and evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 50−100 μL of methyl alcohol, and an aliquot of the solution was injected with a microsyringe into the HPLC column (*17*). Samples were eluted from a C18 column (Alltech Econosphere, 5-μM pore size, 150- × 4.6-mm) using 0.1 M acetic acid/acetonitrile (3:1, v/v), and the flow rate was 1 mL/min. The effluent from the column was monitored at 290 nm (*18*). Ferulic acid concentration in samples was calculated from a calibration curve generated from peak height of ferulic acid in calibration samples.

***β*-Carotene Assay.** The extraction of β-carotene from microsomes was performed as indicated by Palozza et al. (*19*). The sample was dissolved in methanol, and a 20-μL aliquot was analyzed by reverse-phase HPLC with spectrophotometric detection on a Perkin-Elmer LC-295 detector at 450 nm (β-carotene content) and at 350 nm (β-carotene 5,6-epoxide) (*20*). The column was packed with Alltech C18 Adsorbosphere HS material, 3-μm particle size, in a 15 × 0.46-cm cartridge format (Alltech Associates, Deerfield, IL). A 1-cm cartridge precolumn, containing 5-μm C18 Adsorbosphere packing was used. Analyses were done by gradient elution, the initial mobile phase was 85% acetonitrile/15% methanol, with the addition at 8 min of 30% 2-propanol. Ammonium acetate, HPLC grade, 0.01%, was added to the initial mobile phase.

Measurement of ROS. Cells (2×10^6) treated with varying concentrations of ferulic acid for 3 h were harvested to evaluate cellular peroxides and hydroxyl radical levels using the di(acetoxymethyl ester) analogue (C-2938) of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF) (Molecular probes, Inc., Eugene, OR) as previously described (*21*). Fluorescent units were measured in each well after 30 min of incubation with DCF (10 μm) by use of a cytofluor 2300/2350 Fluorescence Measurement System (Millipore Corp., Billerica, MA).

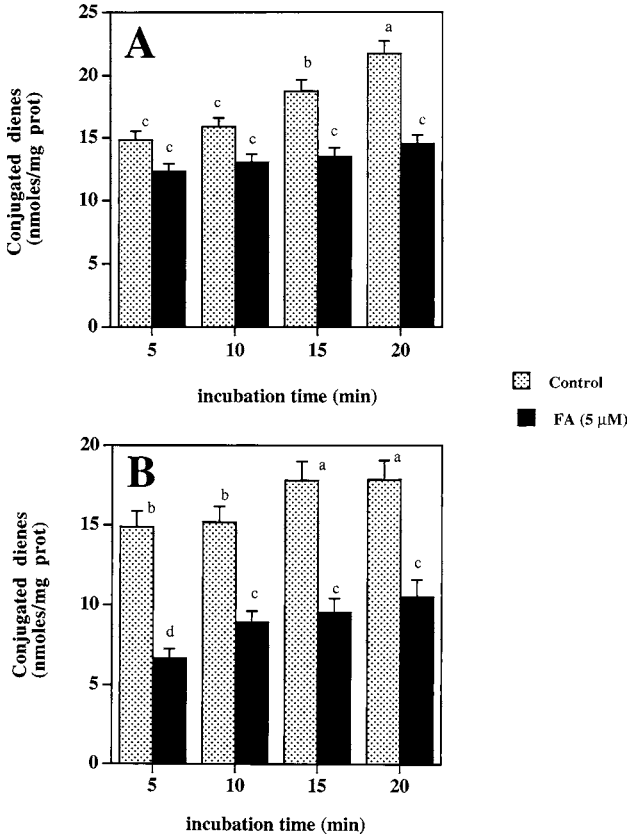

Figure 3. Effects of ferulic acid on Conjugated Diene production induced by AAPH (**A**) and *tert*-BOOH (**B**) in rat liver microsomal membranes. The microsomal membranes were incubated in the absence or in the presence of 5 μM ferulic acid with 25 mM AAPH or 0.25 mM *tert*-BOOH at 37 °C under air in the dark. Results represented the means ± SEM of four separate experiments. The treatment × time interaction was significant ($p < 0.05$). Values not sharing a letter differed, $p < 0.001$ (**A**), $p < 0.002$ (**B**) (Tukey's test).

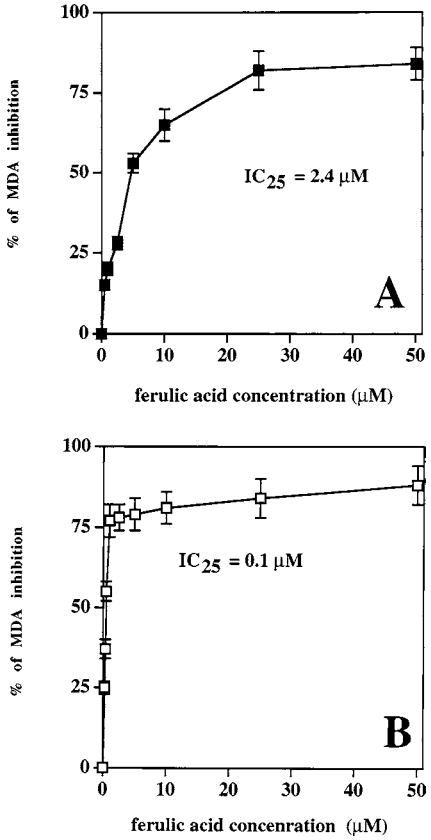

Figure 4. Inhibition of AAPH- (**A**) and *tert*-BOOH- (**B**) induced MDA production by different concentrations of ferulic acid in rat liver microsomal membranes. IC_{25} = concentration of ferulic acid responsible for an inhibition of MDA formation by 25%. The microsomal membranes were incubated with 25 mM AAPH or 0.25 mM *tert*-BOOH at 37 °C under air in the dark for 15 min. Results represented the means ± SEM of six separate experiments.

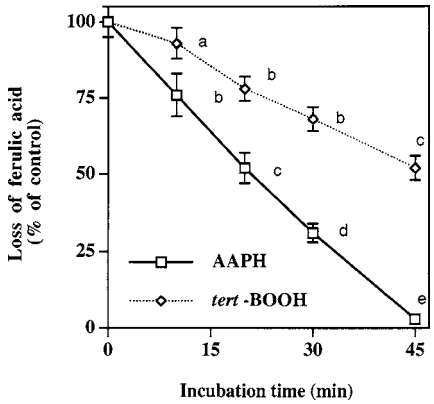

Figure 5. Loss of ferulic acid induced by AAPH and *tert*-BOOH in rat liver microsomal membranes. Ferulic acid was added to the microsomes at the concentration of 5 μM, and the microsomes were incubated with 25 mM AAPH or 0.25 mM *tert*-BOOH at 37 °C under air in the dark. Results represented the means ± SEM of six separate experiments. The treatment × time interaction was significant ($p < 0.05$). Values not sharing a letter differed, $p < 0.002$ (Tukey's test).

Fluorescence was measured without addition of prooxidants (basal ROS production) or in the presence of AAPH or *tert*-BOOH, which were added to the cells for 30 min. Ferulic acid did not alter the basal fluorescence of DCF.

Cell Viability. Cells (2×10^6), treated with or without ferulic acid for 3 h, were further exposed to a 5-h treatment with AAPH or *tert*-BOOH. Such a period of time was responsible for cytotoxic effects by both the prooxidants. The percentage of viable cells was measured by Trypan blue dye exclusion method.

Statistical Analysis. Results were expressed as means ± SEM. Multifactorial two-way ANOVA was adopted to assess any difference among treatments and times (**Figures 2**, **4**, **8**, and **9**). When the *F*-tests were significant ($P < 0.05$), post-hoc comparisons of means were made using Tukey's Honestly Significant Differences test. Data in **Table 1** were analyzed using one-way ANOVA. When significant values were found ($p < 0.05$), post hoc comparison of means were made using the Fisher's test. Differences were analyzed using Minitab Software (Minitab, State College, PA).

RESULTS AND DISCUSSION

Rat Liver Microsomal Membranes. The ability of ferulic acid to protect against lipid peroxidation induced by two different sources of free radicals, including AAPH and *tert*-butyl hydroperoxide, was examined first in rat liver microsomal membranes. It is known that the azocompound AAPH produces exogenously peroxyl radicals via an oxygen-dependent iron-independent mechanism at constant rates (*22*). On the other hand, *tert*-BOOH has been reported to act as an endogenous generator of alkoxyl-radicals (*23*). Ferulic acid was added to the system at micromolar concentrations in the range of 0.5−50 μM, dissolved in THF. In this model, the phenolic compound acted as a potent antioxidant, inhibiting the propagation phase

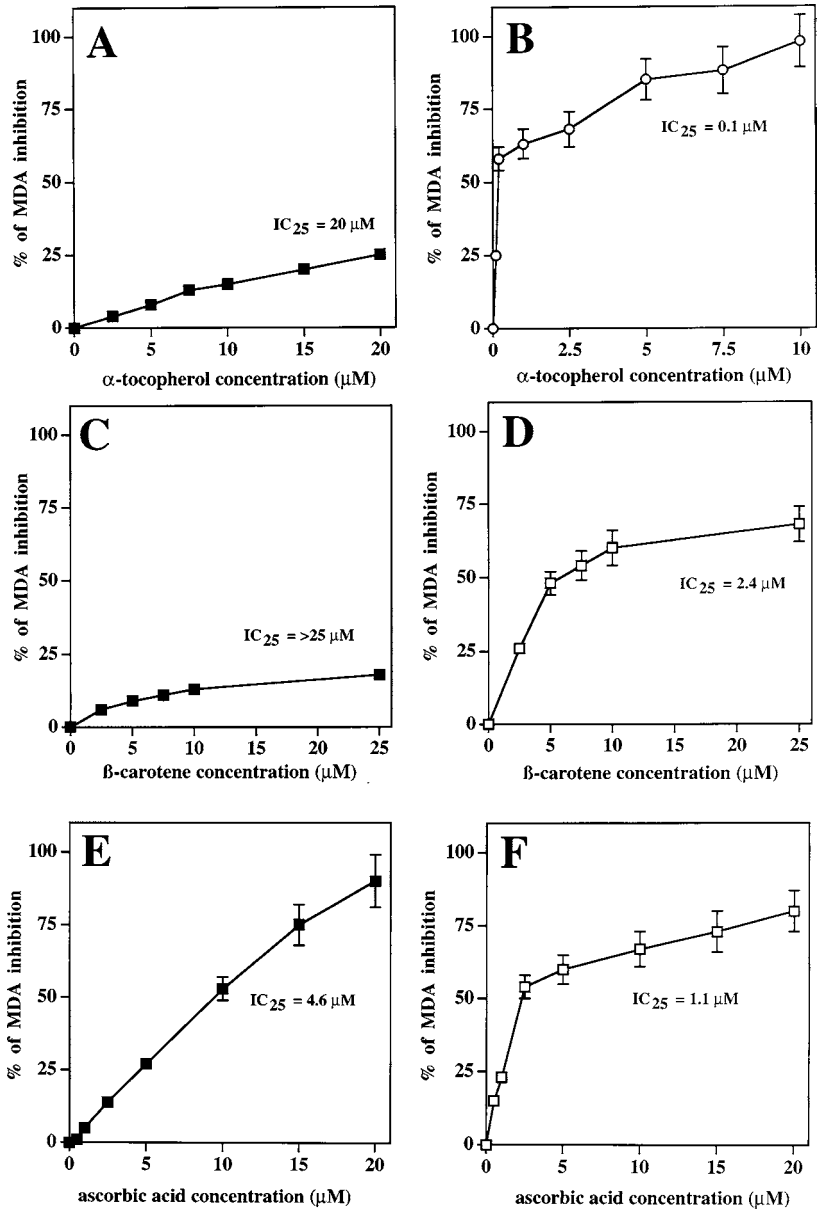

Figure 6. Inhibition of AAPH- (**A**, **C**, **E**) and *tert*-BOOH- (**B**, **D**, **F**) induced MDA production by different concentrations of α-tocopherol (**A**, **B**), β-carotene (**C**, **D**) and ascorbic acid (**E**, **F**) in rat liver microsomal membranes. IC_{25} = concentration of antioxidant responsible for an inhibition of MDA formation by 25%. The microsomal membranes were incubated with 25 mM AAPH or 0.25 mM *tert*-BOOH at 37 °C under air in the dark for 15 min. Results represented the means ± SEM of six separate experiments.

of MDA formation induced by both AAPH (25 mM) (**Figure 2A**) and *tert*-BOOH (250 μM) (**Figure 2B**) in rat liver microsomal membranes. The insert in **Figure 2A** evidences that the antioxidant was also able to inhibit the initiation phase of the lipid peroxidation induced by AAPH, as shown by the induction of lag times. Such lag times were calculated by drawing a straight line through the linear portion of the propagation phase, until it intercepts the abscissa, as described in our previous work (*24*). The effects of ferulic acid on the propagation and initiation phase of lipid peroxidation were dose- and time-dependent and evidenced at very low concentrations of the antioxidant.

It is proposed nowadays that the use of more than one marker of oxidation is required to evaluate the efficacy of an antioxidant (*25*). Therefore, we measured the ability of ferulic acid in inhibiting the production of conjugated dienes, early products in the lipid peroxidation process.

Ferulic acid not only inhibited MDA formation but also conjugated diene production induced by both AAPH (**Figure 3A**) and *tert*-BOOH (**Figure 3B**) in rat liver microsomal membranes.

The compound was a stronger antioxidant in protecting the membranes from *tert*-BOOH- than from AAPH-induced lipid peroxidation. This was clearly shown by the fact that the concentration of ferulic acid responsible for an inhibition of MDA formation by 25% (IC_{25}) was much higher using AAPH (**Figure 4A**) than it was using *tert*-BOOH (**Figure 4B**).

Figure 5 shows the loss of ferulic acid induced by the two prooxidants in rat liver microsomes. The antioxidant was linearly consumed by both AAPH and *tert*-BOOH. However, the loss of ferulic acid was much more pronounced when AAPH was used.

The antioxidant efficiency of ferulic acid was also compared with that of other antioxidants, such as α-tocopherol (**Figure**

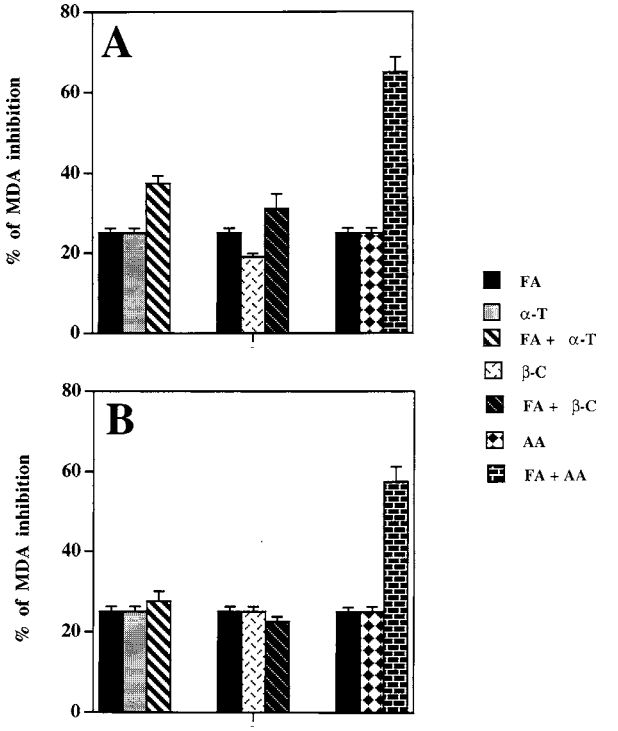

Figure 7. Percentage of inhibition of AAPH- (**A**) and *tert*-BOOH- (**B**) induced MDA formation in the presence of a combination of ferulic acid and α-tocopherol, β-carotene, or ascorbic acid in rat liver microsomal membranes. The antioxidants were added at the concentrations responsible for an inhibition of MDA formation by 25% (IC_{25}), with the only exception of β-carotene in **A**, which was added at the concentration responsible for an inhibition of MDA formation by 19% (25 μM). The microsomal membranes were incubated with 25 mM AAPH or 0.25 mM *tert*-BOOH at 37 °C under air in the dark for 15 min. Results represented the means ± SEM of six separate experiments.

6, parts **A** and **B**), β-carotene (**Figure 6**, parts **C** and **D**) and ascorbic acid (**Figure 6**, parts **E** and **F**) in the presence of AAPH (**Figures 6**, parts **A**, **C**, and **E**) or *tert*-BOOH (**Figure 6**, parts **B**, **D**, and **F**) in rat liver microsomal membranes. In such a model, using AAPH as a prooxidant, the overall order of effectiveness of these compounds as antioxidants was ascorbic acid > α-tocopherol > β-carotene, whereas using *tert*-BOOH as a prooxidant, it was α-tocopherol > ascorbic acid > β-carotene. None of these compounds interfered with MDA production: No inhibition was observed when the compounds were added to the membranes at the same time as the TBA reagent instead of at the beginning of the incubation. Interestingly, when the IC_{25} of the different antioxidants were compared with those of ferulic acid, the phenolic compound exhibited the lowest IC_{25}. The same IC_{25} was only found using α-tocopherol in the presence of *tert*-BOOH.

We have also evaluated the possibility of synergistic antioxidant interactions of ferulic acid with α-tocopherol, β-carotene, or ascorbic acid during AAPH-induced (**Figure 7A**) and *tert*-BOOH-induced (**Figure 7B**) oxidative stress in rat liver microsomal membranes. In these experiments, the antioxidants were added in combination at their respective IC_{25}, and MDA production was measured. The combined addition of ferulic acid and ascorbic acid strongly inhibited MDA production, and such an inhibition was greater than the sum of the inhibitions obtained individually by the two antioxidants. On the other hand, the combined addition of ferulic acid and α-tocopherol or ferulic acid and β-carotene-induced inhibitions of MDA formation, which were remarkably lower than the sum of the inhibitions

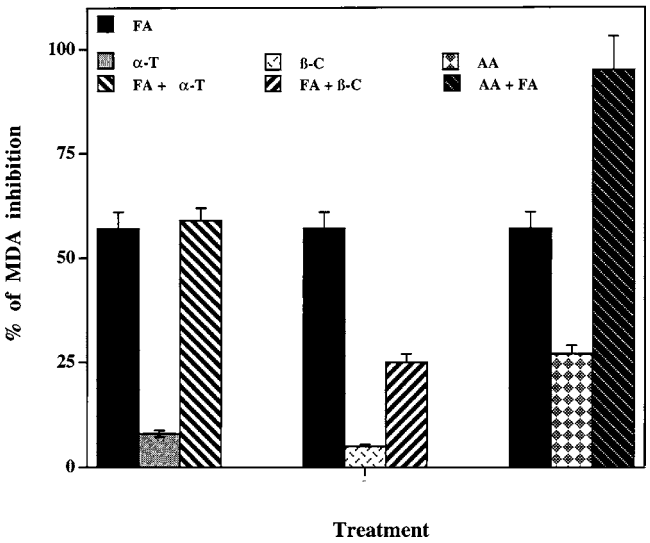

Figure 8. Percentage of inhibition of AAPH-induced MDAformation by combinations of ferulic acid with α-tocopherol, β-carotene, or ascorbic acid in rat liver microsomal membranes. The antioxidants were added at the concentration of 5 μM, and the microsomes were incubated with 25 mM AAPH under air in the dark for 15 min. Results represented the means ± SEM of six separate experiments.

found using the antioxidants individually. Similar effects were also observed during AAPH-induced lipid peroxidation when the antioxidants were added to rat liver microsomal membranes in combination at the same concentration of 5 μM (**Figure 8**). Again, ascorbic acid and ferulic acid, added in combination, showed positive synergistic effects in inhibiting MDA formation, whereas combinations of ferulic acid and α-tocopherol or β-carotene exhibited negative synergistic effects. The latter were particularly evident when a combination of ferulic acid and β-carotene was used. The carotenoid alone showed only a weak inhibition of AAPH-induced MDA formation by about 5%, whereas the phenolic compound alone inhibited lipid peroxidation by about 55%. The combination of the two compounds even reduced the antioxidant efficiency of ferulic acid, showing an inhibition of MDA formation of about 25%.

To explain the synergistic interactions among the different antioxidants, we measured AAPH-induced consumption of ferulic acid in the absence or in the presence of α-tocopherol, β-carotene, and ascorbic acid in rat liver microsomal membranes (**Figure 9**). Ferulic acid was consumed by AAPH in a time-dependent manner. While ascorbic acid protected from AAPH-induced loss of ferulic acid, both α-tocopherol and β-carotene enhanced it.

Concomitantly, we measured AAPH-induced consumption of β-carotene (**Figure 10A**) and the formation of a known oxidation product of the carotenoid, the β-carotene 5,6-epoxide, (**Figure 10B**) in the presence of ferulic acid in rat liver microsomal membranes. The phenolic compound was able to protect the carotenoid from both the spontaneous and AAPH-induced loss and to decrease the formation of β-carotene 5,6-epoxide.

Murine Fibroblasts. The fact that ferulic acid was able to protect against lipid peroxidation microsomal membranes did not prove that it had antioxidant properties in more complex, structurally ordered systems, such as intact cells. The effect of this compound on spontaneous (**Figure 11A**), AAPH-induced (**Figure 11B**) and *tert*-BOOH-induced (**Figure 11C**) ROS production in NIH-3T3 murine fibroblasts was therefore evaluated. A 3-hour addition of the antioxidant to the cells was able

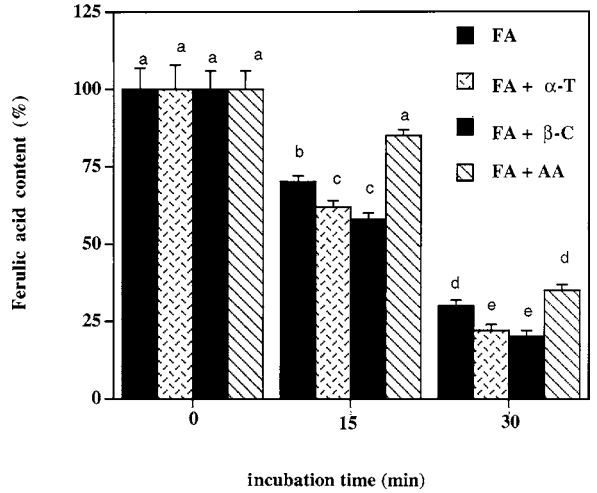

Figure 9. AAPH-induced consumption of ferulic acid in the absence or in the presence of α-tocopherol, β-carotene, and ascorbic acid in rat liver microsomal membranes. The antioxidants were added at the concentration of 5 μM, and the microsomes were incubated with 25 mM AAPH under air in the dark. Results represented the means ± SEM of six separate experiments. The treatment × time interaction was significant ($p < 0.05$). Values not sharing a letter differed, $p < 0.001$ (Tukey's test).

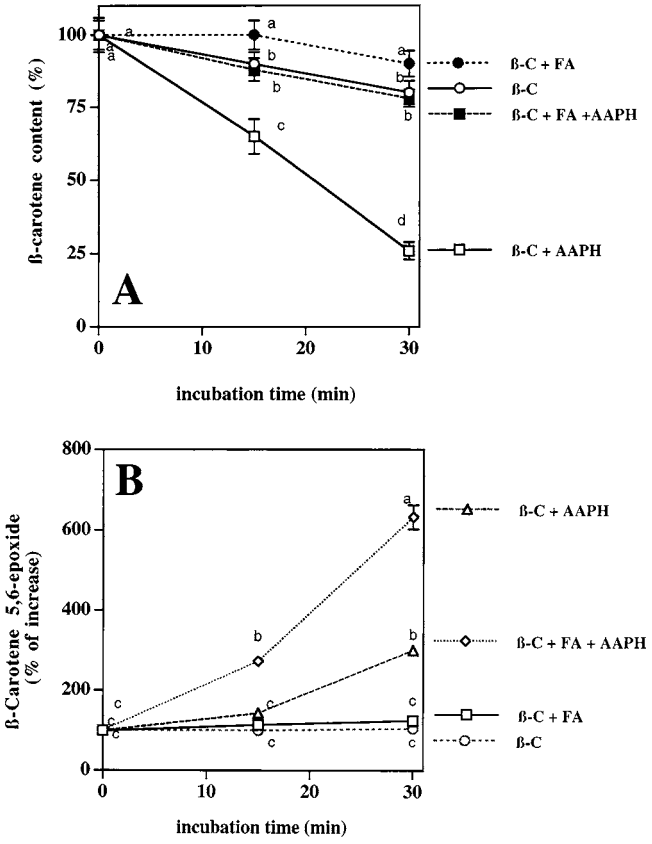

Figure 10. Spontaneous and AAPH-induced consumption of β-carotene (**A**) and formation of β-carotene 5,6-epoxide (**B**) in the absence or in the presence of ferulic acid in rat liver microsomal membranes. The antioxidants were added at the concentration of 5 μM and the microsomes were incubated with 25 mM AAPH under air in the dark. Results represented the means ± SEM of five separate experiments. The treatment × time interaction was significant ($p < 0.05$). Values not sharing a letter differed, $p < 0.004$ (**A**), $p < 0.002$ (**B**) (Tukey's test).

to strongly inhibit both spontaneous and free radical-induced ROS production in a dose-dependent manner.

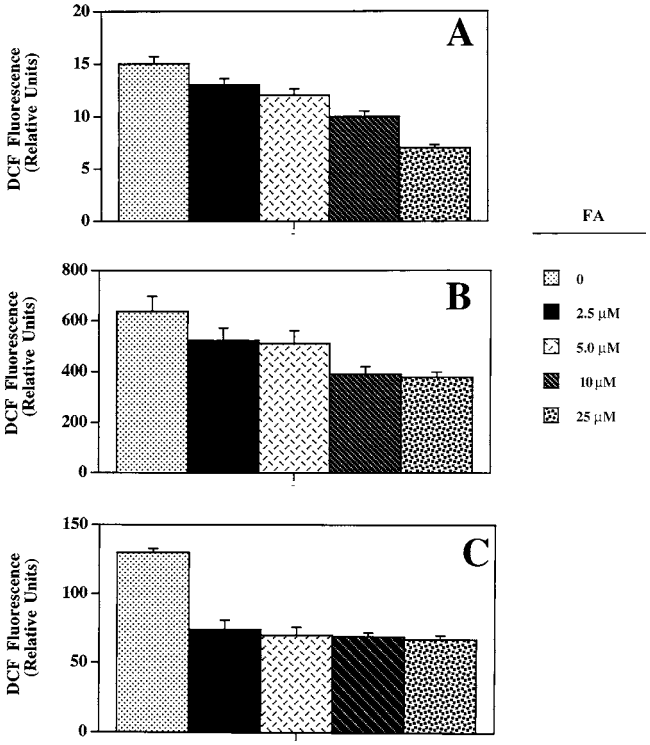

Figure 11. Effect of varying ferulic acid concentration on the production of intracellular reactive oxygen species (ROS) induced spontaneously (**A**), by AAPH (**B**) and *tert*-BOOH (**C**) in NIH-3T3 fibroblasts. The cells were cultured with ferulic acid for 3 h before the addition of 50 mM AAPH or 0.1 mM *tert*-BOOH for 30 min. Results represented the means ± SEM of five separate experiments.

Ferulic acid was also able to protect murine fibroblasts from the cytotoxic effects induced by both AAPH and *tert*-BOOH in a dose-dependent manner, as shown in **Table 1**.

Although much attention has been paid to the potential activity of phenolic compounds as natural antioxidants for practical use on account of their widespread occurrence in plants, further systematic information on their antioxidant properties in different conditions needs to be investigated. The present study demonstrates the antioxidant properties of ferulic acid in complex, structurally ordered systems, such as isolated membranes and intact cells. In microsomal membranes and in fibroblasts, the compound acted as a powerful antioxidant, as documented by its ability in inhibiting lipid peroxidation and/or free radical production. These effects occurred at micromolar concentrations of the compound and were dose- and time-dependent. Ferulic acid was able to inhibit the lipid peroxidation induced by both *tert*-BOOH, which produces alkoxyl radicals (*23*), and AAPH, which produces peroxyl radicals (*22*). Such data support the strong efficiency of ferulic acid as a scavenger of different free radicals, including anion superoxide (O_2^-), hydroxyl radical ($OH^\cdot$), NO^- (*26*), and hydroxyl and peroxyl radicals (*27*) observed in several biological models. The methoxy group at the ortho position relative to the hydroxyl group in ferulic acid was reported to improve the antioxidant effectiveness of the compound by increasing its resonance stabilization (*1*, *28*, *29*). However, in our models, ferulic acid was a more potent inhibitor of *tert*-BOOH-induced lipid peroxidation than of AAPH-induced lipid peroxidation. It is known that *tert*-BOOH produces alkoxyl radicals by Fenton reactions (*23*) and that ferulic acid exhibits ion chelating properties because of the dibasic acidity of its molecule due to its carboxyl and phenolic hydroxyl group (*1*). Therefore, it is possible that the high

efficiency of ferulic acid in protecting from *tert*-BOOH-induced lipid peroxidation is due not only to the scavenger ability of the compound itself but also to its ability in chelating iron, which may limit the formation of alkoxyl radicals by *tert*-BOOH. Interestingly, several phenolic compounds showed synergistic antioxidant properties in reinforcing the antioxidant activity of lactoferrin in lipid systems containing iron (*9*). It is also possible that the increased antioxidant efficiency of ferulic acid during *tert*-BOOH-induced lipid peroxidation may reflect a higher stability of the compound toward the hydroperoxide. Our results clearly show that ferulic acid was linearly consumed by both AAPH and *tert*-BOOH. However, *tert*-BOOH induced a lower consumption of the compound with respect to that induced by AAPH. Such an effect may be explained by the lower rate of free radical formation induced by *tert*-BOOH as well as by the different species of free radicals formed in the presence of the two different prooxidants. Using AAPH as a prooxidant, it is evidenced that ferulic acid not only inhibited the propagation phase of lipid peroxidation but also inhibited the initiation phase, inducing a clear lag time, similar to that found in the presence of α-tocopherol (data not shown) (*24*). This effect seems to be peculiar of several hydrocinnamic acids and ferulic acid esters, as evidenced during the autoxidation of methyl linoleate in bulk phase and during AAPH-induced lipid peroxidation in egg yolk PC liposomes (*7*). The antioxidant efficiency of ferulic acid was also compared with that of other antioxidants, such as α-tocopherol, *β*-carotene, and ascorbic acid in rat liver microsomal membranes. In such a model, using AAPH as a prooxidant, the overall order of effectiveness of these compounds as antioxidants was ferulic acid > ascorbic acid > α-tocopherol > *β*-carotene, whereas using *tert*-BOOH as a prooxidant, it was ferulic acid = α-tocopherol > ascorbic acid > *β*-carotene. **Figures 4** and **6** clearly reflected these differences in the antioxidant potency of the compounds in rat liver microsomes, as evidenced by their IC_{25}. The scavenging effects of α-tocopherol, ferulic acid, and its related compounds have been recently investigated in solutions as well as in liposomes (*7*). While ferulic acid was less potent than α-tocopherol in scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and in inhibiting the formation of hydroperoxides in bulk methyl linoleate, it was as potent as the tocopherol in limiting the formation of hydroperoxides in ethanol-buffered solution of linoleic acid and more potent than it in inhibiting AAPH-induced peroxidation of egg yolk liposomes. It has been suggested that the participation of α-tocopherol in the initiation or in the propagation of lipid oxidation changed with rising temperature (*7*, *28*, *29*). According to this hypothesis, in the previous studies, α-tocopherol was more effective as an antioxidant than ferulic acid at higher rather than at lower temperatures (40 vs 37 °C). Moreover, Marinova and Yanishlieva reported that α-tocopherol was more effective than ferulic acid in reducing the rate of autoxidation of lard as the temperature was increased (*30*). On the contrary, it has been reported that increasing the temperature did not influence the effect of ferulic acid on the rate of autoxidation (*30*). In addition, the different location of the two antioxidants in the phospholipid bilayer of cell membrane as well as the site and the kind of radicals may also govern their different antioxidant effectiveness in microsomal membranes. We measured the amount of ferulic acid and α-tocopherol incorporated and/or associated to microsomal membranes, and we reported that the percentage of ferulic acid associated to cell membranes was about 5%, while the remaining percentage was found in the aqueous phase. On the contrary, the incorporation percentage of α-tocopherol in cell membranes was almost 100%. The low affinity of ferulic acid for lipids was also demonstrated by the minimal extent of partitioning of the compound in phosphatidylcholine liposomes as well as in LDLs (*31*). It is recognized that peroxyl radicals are produced by AAPH in the aqueous phase, and this could explain the higher antioxidant efficiency of ferulic acid with respect to that of α-tocopherol. In microsomal membranes exposed to AAPH, ferulic acid was also more potent as an antioxidant than was ascorbic acid. These data are in agreement with a previous observation in LDLs, showing that the inhibition of LDL oxidation by ferulic acid was more remarkable than that observed in the presence of ascorbic acid (*31*). Our data also show synergistic antioxidant interactions between ferulic acid and other antioxidants, including α-tocopherol, ascorbic acid, and *β*-carotene in rat liver microsomal membranes. The combined addition of ferulic acid and ascorbic acid strongly inhibited MDA production, and such an inhibition was greater than the sum of the inhibitions obtained individually by the two antioxidants. Such an effect may be explained by the fact that ascorbic acid, which was less efficient than ferulic acid in inhibiting AAPH-induced lipid peroxidation in rat liver microsomal membranes, protected it by its oxidation, as evidenced in **Figure 9**. This hypothesis is supported by a recent finding showing that the addition of ascorbic acid provoked a delay of ferulic acid consumption induced by wheat peroxidase (POD) (*32*). This effect could occur because ascorbic acid rapidly reduced the phenoxyl radicals formed by POD back to the initial phenol, avoiding the formation of ferulate dimers until it was completely oxidized in dehydroascorbic acid.

On the other hand, in our study, the combined addition of ferulic acid and α-tocopherol or ferulic acid and *β*-carotene-induced inhibitions of MDA formation which were remarkably lower than the sum of the inhibitions found using the antioxidants individually. Such effects may be explained by the fact that both α-tocopherol and *β*-carotene enhanced the consumption of ferulic acid induced by the azocompound, as shown in **Figure 9**. In particular, ferulic acid protected *β*-carotene from AAPH-induced consumption and prevented the formation of *β*-carotene oxidation products, such as the *β*-carotene 5,6-epoxide. The finding that synergistic interactions may occur between ferulic acid and other antioxidant nutrients, such α-tocopherol, ascorbic acid, and *β*-carotene, is particularly interesting in view of the fact that many foods contain combinations of these compounds. Ferulic acid and other cinnamic acid derivatives have been found to be implicated in the antioxidant activity of tomato extracts (*33*), and ferulic acid has been reported to be absorbed from tomato consumption (*34*). In addition, lipids extracted from grasses and leaves showed strong antioxidant behavior due to their phenolic contaminants (*35*). Virgin olive oil also contains large quantities of phenolic compounds and a high degree of antioxidant potential that is destroyed during the refining process (*36*), perhaps due to the loss of synergistic antioxidant effects of other components (*37*). It has been reported that supplementation with wine phenolic compounds increases the antioxidant capacity in plasma and vitamin E in LDLs (*38*). Total tocols in combination with phenols have been significantly associated with an increase antioxidant activity (*18*). It is possible that phenolic compounds could play a role similar to that of vitamin C by regenerating the reduced form of vitamin E from its oxidized form chromanoxyl (*39*). Interestingly, it has been recently reported that processed sweet corn increased free ferulic acid content and total antioxidant activity despite the decline in vitamin C content (*40*).

Although the source of free radical production seems to deeply influence the antioxidant properties of ferulic acid, the

present study shows that this compound can act as a potent antioxidant in both isolated membranes and intact cells by inhibiting lipid peroxidation and ROS production. In membranes, its antioxidant efficiency was more pronounced than that of α-tocopherol, ascorbic acid, and β-carotene. Antioxidant interactions were observed when the compound was used in combination with these antioxidants. Antagonistic interactions were observed when ferulic acid was used in combination with α-tocopherol and/or β-carotene. On the other hand, synergistic interactions occurred between ferulic acid and ascorbic acid, suggesting that these two compounds can cooperate in preserving physiological integrity of cell membranes exposed to free radicals.

LITERATURE CITED

(1) Graf, E. Antioxidant potential of ferulic acid. *Free Rad. Biol. Med.* **1992**, *13*, 435−448.

(2) Dziedzic, S. C.; Hudson, B. J. F. Phenolic acids and related compounds as antioxidants for edible oils. *Food Chem.* **1984**, *14*, 45−51.

(3) Cho, M. Z.; Kwon, T. B.; Oh, S. K. Antioxidant effect of some phenolics on soybean oils. *Han′guk Nonghwa Hakhoechi* **1989**, *32*, 37−43.

(4) Egawa, M.; Ishida, K.; Maekawa, M.; Sato, Y. Skin preparations containing antioxidants and ellagic acids. *Jpn. Kokai* **1990**, *89*, 50118.

(5) Courtin, O. Moisturizing cosmetic comprising liquid crystals. French patent *88*, 8880; 1990.

(6) Niki, E. Free radicals, antioxidant and cancer. In *Food factors for Cancer prevention*. Ohigashi, H., Osawa, T., Terao, J., Watanabe, S., Yoshikawa, T., Eds; Springer: Tokyo, Japan, 1997; pp 55−57.

(7) Kikuzaki, H.; Hisamoto, M.; Hirose, K.; Akiyama, K.; Taniguchi, H. Antioxidant properties of ferulic acid and its related compounds. *J. Agric. Food Chem.* **2002**, *50*, 2161−2168.

(8) Son, S.; Lewis, B. A. Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: structure−activity relationship. *J. Agric. Food Chem.* **2002**, *50*, 468−472.

(9) Medina, I.; Tombo, I.; Satué-Gracia, M. T.; German, J. B.; Frankel, E. N. Effects of natural phenolic compounds on the antioxidant activity of lactoferrin in liposomes and oil-in-water emulsions. *J. Agric. Food Chem.* **2002**, *50*, 2392−2399.

(10) Ohta, T.; Nakano, T.; Egashira, Y.; Sanada, H. Antioxidant activity of ferulic acid β-glucuronide in the LDL oxidation system. *Biosci. Biotech. Biochem.* **1997**, *61*, 1942−1943.

(11) Laranjinha, J.; Vieira, O.; Almeida, L.; Madeira, V. Inhibition of metmyoglobin/H_2O_2-dependent low density lipoprotein lipid peroxidation by naturally occurring phenolic acids. *Biochem. Pharmacol.* **1996**, *51*, 395−402.

(12) Andreasen, M. F.; Landbo, A.-K.; Christensen, L. P.; Hansen, A.; Meyer, A. S. Antioxidant effects of phenolic rye (Secale cereale L.) extracts, monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low-density lipoproteins. *J. Agric. Food Chem.* **2001**, *49*, 4090−4096.

(13) Bartoli, G. M.; Giannattasio, B.; Palozza, P.; Cittadini, A. Superoxide dismutase depletion and lipid peroxidation in rat liver microsomal membranes: Correlation with liver carcinogenesis. *Biochim. Biophys. Acta* **1988**, *966*, 214−221.

(14) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(15) Tatum, V. L.; Changoit, C.; Chow, C. K. Measurement of Malondialdehyde by high performance liquid chromatography with fluorescence detection. *Lipids* **1990**, *25*, 226−229.

(16) Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. *Methods Enzymol.* **1978**, *52*, 302−310.

(17) Fujiwara, S.; Hamada, T.; Sugimoto, I.; Awata, N. High-performance liquid chromatographic determination of ferulic acid in plasma. *Chem. Pharm. Bull.* **1983**, *31*, 1079−1081.

(18) Emmons, C. L.; Peterson, D. M.; Paul, G. L. Antioxidant capacity of oat (Avena sativa L.) extracts. 2. In vitro antioxidant activity and contents of phenolic and tocol antioxidants. *J. Agric. Food Chem.* **1999**, *47*, 4894−4898.

(19) Palozza, P.; Calviello, G.; Maggiano, N.; Lanza, P.; Ranelletti, F. O.; Bartoli, G. M. Beta-carotene antagonizes the effects of eicosapentaenoic acid on cell growth and lipid peroxidation in WiDr adenocarcinoma cells. *Free Rad. Biol. Med.* **2000**, *28*, 228−234.

(20) Handelman, G. J.; van Kuijk, F. J. G. M.; Chatterjee, A.; Krinsky, N. I. Characterization of products formed during the autoxidation of β-carotene. *Free Rad. Biol. Med.* **1991**, *10*, 427−437.

(21) Kane, D. J.; Sarafian, T. A.; Anton, R.; Hahn, H.; Gralla, E. B.; Valentie, J. S.; Ord, T.; Bredesen, D. E. Bcl-2 inhibition of neuronal death: decreased generation of reactive oxygen species. *Science* **1993**, *262*, 1274−1277.

(22) Niki, E. Free radical initiators as source water- or lipid-soluble peroxyl radicals. *Methods Enzymol.* **1990**, *186*, 100−108.

(23) Palozza, P.; Luberto, C.; Ricci, P.; Sgarlata, E.; Calviello, G.; Bartoli, G. M. Effect of β-carotene and canthaxanthin on *tert*-butyl hydroperoxide-induced lipid peroxidation in murine normal and tumor thymocytes. *Arch. Biochem. Biophys.* **1996**, *325*, 145−151.

(24) Palozza, P.; Krinsky, N. I. The inhibition of radical-initiated peroxidation of microsomal lipids by both α-tocopherol and β-carotene. *Free Rad. Biol. Med.* **1991**, *11*, 407−414.

(25) Frankel, E. N. Interfacial lipid oxidation and antioxidation. *J. Oleo Sci.* **2001**, *50*, 387−391.

(26) Ogiwaka, T.; Satoh, K.; Kadoma, Y.; Murakami, Y.; Unten, S.; Atsumi, T.; Sakagami, H.; Fujisawa, S. Radical scavenging activity and cytotoxicity of ferulic acid. *Anticancer Res.* **2002**, *22*, 2711−2717.

(27) Kanski, J.; Aksenova, M.; Stoyanova, A.; Butterfield, D. A. Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems in vitro: structure−activity studies. *J. Nutr. Biochem.* **2002**, *13*, 273−281.

(28) Chen, J. H.; Ho, C.-T. Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. *J. Agric. Food Chem.* **1997**, *45*, 2374−2378.

(29) Pekkarinen, S. S.; Stockmann, H.; Schwarz, K.; Heinonen, M.; Hopia, A. I. Antioxidant activity and partitioning of phenolic acids in bulk and emulsified methyl linoleate. *J. Agric. Food Chem.* **1999**, *47*, 3036−3043.

(30) Marinova, E. M.; Yanishlieva, N. V. Effect of temperature on the antioxidative action of inhibitors in lipid autoxidation. *J. Sci. Food Agric.* **1992**, *60*, 313−318.

(31) Castelluccio, C.; Bolwell, G. P.; Gerrish, C.; Rice-Evans, C. Differential distribution of ferulic acid to the major plasma constituents in relation to its potential a san antioxidant. *Biochem. J.* **1996**, *316*, 691−694.

(32) Garcia, R.; Rakotozafy, L.; Telef, N.; Potus, J.; Nicolas, J. Oxidation of ferulic acid or arabinose-esterified ferulic acid by wheat germ peroxidase. *J. Agric. Food. Chem.* **2002**, *50*, 3290−3298.

(33) Qureshi, M. J.; Blain, J. A. Antioxidant activity in tomato extracts. *Nucleus* (*Karachi*) **1976**, *13*, 29−33.

(34) Bourne, L. C.; Rice-Evans, C. Bioavailability of ferulic acid. *Biochem. Biophys. Res. Commun.* **1998**, *253*, 222−227.

(35) Hudson, B. J. F.; Mahgoub, S. E. O. Naturally occurring antioxidants in leaf lipids. *J. Sci. Food Agric.* **1980**, *31*, 646−650.

(36) Nergiz, C.; Unal, K. Determination of phenolic acids in virgin olive oil. *Food Chem.* **1991**, *39*, 237−240.

(37) Yi, O. S.; Han, D.; Shin, H. K. Synergistic antioxidative effects of tocopherol and ascorbic acid in fish oil/lecithin/water system. *J. Am. Oil Chem. Soc.* **1991**, *68*, 881−883.

(38) Carbonneau, M.-A.; Leger, C. L.; Monnier, L.; Bonnet, C.; Michel, F.; Fouret, G.; Dedieu, F.; Descomps, B. Supplementation with wine phenolic compounds increases the antioxidant capacity of plasma and vitamin E of low-density lipoprotein without changing the lipoprotein Cu^{2+}-oxidizability: possible explanation by phenolic location. *Eur. J. Clin. Nutr.* **1997**, *51*, 682−690.

(39) Belyakov, V. A.; Roginsky, V. A.; Bors, W. Rate constants for the raction of peroxyl free radical with flavonoids and related compounds as determined by kinetic chemiluminescence method. *J. Chem. Soc., Perkin Trans.* **1995**, *2*, 2319−2326.

(40) Dewanto, V.; Wu, X.; Liu, R. H. Processed sweet corn has higher antioxidant activity. *J. Agric. Food Chem.* **2002**, *50*, 4959−4964.

Received for review May 22, 2003. Revised manuscript received February 3, 2004. Accepted February 3, 2004.

JF0303924