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Ethyl Ferulate, a Lipophilic Polyphenol, Induces HO-1 and Protects Rat Neurons Against Oxidative Stress

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ABSTRACT

In the CNS, the heme oxygenase (HO) system has been reported to be active and to operate as a fundamental defensive mechanism for neurons exposed to an oxidant challenge. We have recently shown that both curcumin and caffeic acid phenethyl ester, two phenolic natural compounds, potently induce HO-1 expression and activity in rat astrocytes. We have extended our previous findings examining the effects of two other plant-derived phenolic compounds, with analogous chemical structures, in rat astrocytes and neurons. Ethyl ferulate (ethyl 4-hydroxy-3-methoxycinnamate) (EFE), the naturally occurring ester of ferulic acid, was able to induce HO-1 protein expression. Maximal expression of HO-1 mRNA and protein and a significant increase in HO activity were detected after 6 h of incubation with 15 μ*M* EFE in astrocytes and 5 μ*M* EFE in neurons. Higher concentrations of EFE (50 μ*M*) caused a substantial cytotoxic effect with no change in HO-1 protein expression and activity. Exposure of astrocytes to resveratrol, a phytoalexin derived from grapes, resulted in an increase of HO-1 mRNA, but it was not able to induce HO-1 protein expression and activity. Interestingly, preincubation (12 h) of neurons with EFE resulted in an enhanced cellular resistence to glucose oxidase-mediated oxidative damage; this cytoprotective effect was considerably attenuated by zinc protoporphyrin IX, an inhibitor of HO activity. This study identifies a novel natural compound that could be used for therapeutic purposes as a potent inducer of HO-1 for the protection of brain cells against oxidative and neurodegenerative conditions. *Antioxid. Redox Signal.* 6, 811–818.

INTRODUCTION

MAMMALIAN CELLS have developed highly protective inducible systems against a variety of stressful stimuli, including oxidative challenges. When appropriately activated, each one of these systems has the potential to restore cellular homeostasis and rebalance redox equilibrium (5, 8, 22). Activation of antioxidant pathways is particularly important for tissue with relatively weak endogenous antioxidant defenses, such as the brain (6). Increasing evidence, in fact, supports the notion that reduction of cellular expression and activity of antioxidant proteins and consequent augmentation of oxidative stress are fundamental causes for aging processes and neurodegenerative diseases (6, 8, 13).

Among the various genes encoding proteins that possess antioxidant characteristics, there has been a growing interest over the last years in the heme oxygenase (HO) system, the family of enzymes that control the initial and rate-limiting steps in heme catabolism (24, 31). Although the biological role of HO proteins remains to be completely elucidated, their

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relevance for cellular stress responses has been widely demonstrated in a variety of tissues, including brain (7, 33, 43). To date, three isoforms of HO have been identified: the inducible HO-1 (38), the constitutive HO-2 (25), and the less active HO-3 (26), cloned only in rat and probably representing the result of a species-specific gene retrotransposition (35). HO-1, also named heat shock protein 32, is finely upregulated by stress conditions. The HO-1 gene contains a heat shock element in its promoter region and is rapidly induced upon exposure to heme, metal ions, sulfhydryl compounds, ultraviolet light, and various prooxidants (17, 21, 28). The efficacy of HO-1 in promoting cytoprotection resides primarily in the intrinsic ability of its metabolic products, carbon monoxide and bilirubin, to exert potent antioxidant and antiinflammatory activities (11, 39). In the brain, astrocytes strongly express HO-1 in response to injury, and the HO pathway has been shown to act as a fundamental defensive mechanism for neurons exposed to an oxidant challenge (10, 20). Moreover, deregulation of the HO system has been 4 associated with the pathogenesis of several neurodegenerative disorders, including Alzheimer's disease and multiple sclerosis (37, 41, 42). Consequently, modulation of HO-1, by increasing carbon monoxide and/or biliverd in availability, can represent an interesting pharmacological target and can be of clinical relevance (8, 24).

A number of experimental and epidemiological studies have recently supported the beneficial effects of some commonly used natural products in preventing various pathologic conditions ranging from cardiovascular diseases to cancer. Spices and herbs often contain phenolic substances with potent antioxidative and chemopreventive properties (3, 16, 30). We have recently shown that both curcumin and caffeic acid phenethyl ester (CAPE), two phenolic natural compounds well known for their antioxidative, antiinflammatory, and anticancer properties, potently induce HO-1 expression and activity in rat astrocytes (36). Here we have extended our previous findings, examining in astrocytesand neuronal cell lines the effects of other plant-derived phenolic compounds, with analogous chemical structures but endowed with better bioavailability. Ferulic acid, which is the precursor of lignin biosynthesis, has long been recognized for its antioxidative and antiinflammatory activities (18, 44). Cinnamic acid derivatives, including ferulic acid, are abundant in plants, playing important roles in the cross-linking of the cell walls of various grasses. Although ferulic acid has been demonstrated to be effective in *in vitro* experiments, the low lipophilicity impairs its *in vivo* efficiency, bioavailability, and stability. Ethyl ferulate (EFE), the naturally occurring ester derivative of ferulic acid, ranges widely within various systems of many plants as a trace constituent (12, 19). In comparison with the corresponding acid form, EFE is more lipophilic and has been shown to present better scavenging properties toward both hydroxyl radicals and superoxide anions. In a recent study of the inhibitory effects of antioxidants on lipid oxidation, EFE was shown to prevent more effectively autooxidation of model substrates by extending the induction time of this process (18).

In this study, we analyzed the potency of EFE as an inducer of HO-1 expression, at both mRNA and protein levels, and HO activity in cultured astrocytes and hippocampal neurons, and we explored whether a similar effect could be obtained with *trans*-resveratrol (RSV), another well-known phenolic antioxidant derived from plants, recently suggested for prevention of several diseases (32). It has been, in fact, reported that RSV, a phytoalexin present in red wine, can protect the heart from ischemia and also can protect excitotoxic brain damage (32). Finally, we have examined whether the protective effect of EFE on oxidative damage in neuronal cells might be mediated by the induction of HO-1 expression.

MATERIALS AND METHODS

Chemicals and reagents

EFE (ethyl 4-hydroxy-3-methoxycinnamate) was purchased from Aldrich (St. Louis, MO, U.S.A.). RSV (*trans*-3,2,5-trihydrostilbene) was obtained from Sigma Chemical (St. Louis, MO, U.S.A.). The chemical structures of these phenolic compounds are shown in Fig. 1. Stock solutions of polyphenolic compounds were prepared as described previously (36). Glucose oxidase (GOX), which generates hydrogen peroxide in the culture medium, and all other reagents were from Sigma unless otherwise specified. Zinc protoporphyrin IX (ZnPP IX), a specific inhibitor of HO activity, was from Porphyrin Product (Logan, UT, U.S.A.). Rabbit polyclonal antibodies directed against HO-1 and HO-2 were obtained from Stressgen (Victoria, BC, Canada).

Cell culture

Rat type 1 astrocytes (DI TNC1) (34) and rat hippocampal neurons (H 19–7) (13) were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.) and cultured according to the manufacturer's instructions. Cells were grown in 75-cm^2 flasks and maintained at 37°C in a humidified atmosphere of air and 5% CO_2. Confluent cells were exposed to various concentrations of EFE and RSV. After each

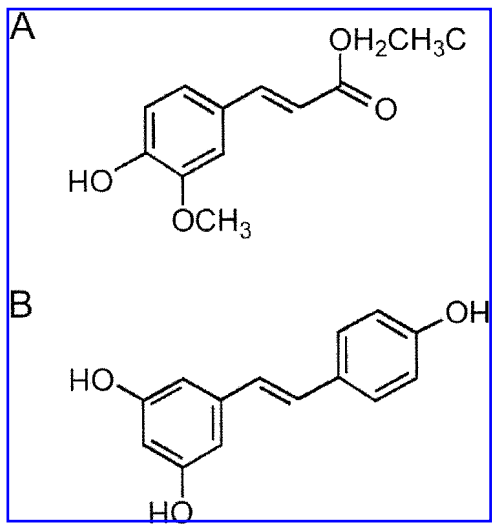

FIG. 1. The chemical structures of ethyl ferulate (A) and *trans*-resveratrol (B).

treatment (6 or 24 h), cells were harvested for the determination of HO activity, HO-1 and HO-2 mRNA levels, and protein expression. Neurons growing in 24 wells were also exposed to different concentrations of EFE, and cell viability was determined at 24 h. Other neurons were pretreated for 12 h with 5 μ*M* EFE or 5 μ*M* EFE plus 10 μ*M* ZnPP IX and then exposed for 2 h to 50 mU/mL GOX, before cell viability was determined.

HO activity assay

HO activity was determined at the end of each treatment as described previously (36). In brief, microsomes from harvested cells were added to a reaction mixture containing NADPH, glucose-6-phosphate dehydrogenase, rat liver cytosol as a source of biliverdin reductase, and the substrate hemin. The reaction mixture was incubated in the dark at 37°C for 1 h and was terminated by the addition of 1 ml of chloroform. After vigorous vortex mixing and centrifugation, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm ($\epsilon = 40\ mM^{-1}\ cm^{-1}$).

Western blot analysis

After treatment with polyphenols, samples of astrocytes and neurons were also analyzed for HO-1 and HO-2 protein expression using a western immunoblot technique as described previously (7). In brief, an equal amount of proteins (30 μg) for each sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred overnight to nitrocellulose membranes, and the nonspecific binding of antibodies was blocked with 3% nonfat dried milk in phosphate-buffered saline. Membranes were then probed with polyclonal rabbit anti-HO-1 and anti-HO-2 antibodies (Stressgen) (1:1,000 dilution in Tris-buffered saline, pH 7.4) for 2 h at room temperature. After three washes with phosphate-buffered saline, blots were visualized using an amplified alkaline phosphatase kit from Sigma (Extra-3A), and the relative density of bands was analyzed by the use of an imaging densitometer (model GS-700; Bio-Rad, Herts, U.K.). Blots shown are representative of three independent experiments.

Real-time quantitative RT-PCR

Total RNA from cell cultures was extracted using Trizol (Sigma) and treated with RNase-free DNase to remove any residual genomic DNA. Single-stranded cDNAs were synthesized by incubating total RNA (1 μg) with SuperScript II RNase H reverse transcriptase (200 U), oligo-$(dT)_{12–18}$ primer (100 n*M*), dNTPs (1 m*M*), and RNase inhibitor (40 U) at 42°C for 1 h in a final volume of 20 μl. Reaction was terminated by incubating at 70°C for 10 min.

Forward and reverse primers used to amplify HO isoforms are listed in Table 1. The expected amplification products for HO-1 and HO-2 are 123 and 331 bp, respectively. Aliquots of cDNA (0.1 and 0.2 μg) and known amounts of external standard (purified PCR product, 10^2 to 10^8 copies) were amplified in parallel reactions using the forward and reverse primers. Each PCR reaction (final volume, 20 μl) contained 0.5 μ*M* of primers, 2.5 m*M* Mg^{2+}, and 1 × Light Cycler DNA Master SYBR Green (Roche Diagnostics, Indianapolis, IN, U.S.A.). PCR amplifications were performed with a Light Cycler (Roche Molecular Biochemicals) using the following four cycle programs: (i) denaturation of cDNA (one cycle: 95°C for 10 min); (ii) amplification (40 cycles: 95°C for 0 s, 58°C for 5 s, 72°C for 10 s); (iii) melting curve analysis (one cycle: 95°C for 0 s, 70°C for 10 s, 95°C for 0 s); (iv) cooling (one cycle: 40°C for 3 min). The temperature transition rate was 20°C/sec except for the third segment of the melting curve analysis, where it was 0.2°C/s. Fluorimeter gain value was 6. Real-time detection of fluorimetric intensity of SYBR Green I, indicating the amount of PCR product formed, was measured at the end of each elongation phase. Quantification was performed by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of the external standards. For this analysis, fluorescence values measured in the log-linear phase of amplification were considered using the second derivative maximum method of the Light Cycler Data Analysis software (Roche Molecular Biochemicals). Specificity of PCR products obtained was characterized by melting curve analysis followed by gel electrophoresis, visualized by ethidium bromide staining, and DNA sequencing.

Cell viability assay

Neurons were exposed to EFE for the indicated times, and cell viability was assessed with the use of an Alamar Blue assay according to the manufacturer's instructions (Serotec, Oxford, U.K.) as reported previously (36). At the end of each treatment, cells were washed twice and incubated for an additional 5 h in complete medium containing 10% Alamar Blue solution. Optical density in each sample was measured using a plate reader (Molecular Devices, Crawley, U.K.). The intensity of the color developed in the medium is proportional to the viability of cells, which is calculated as the difference in absorbance between 570 and 600 nm and expressed as percentage of control.

TABLE 1. SEQUENCES OF OLIGONUCLEOTIDE PRIMERS FOR HO ISOFORM mRNAS

Name	*GenBank accession no.*	*Sequence*	*Expected PCR product*
HO-1-F	NM_012580.1	TGCTCGCATGAACACTCTG	123 bp
HO-1-R	NM_012580.1	TCCTCTGTCAGCAGTGCCT	
HO-2-F	J05405.1	CACCACTGCACTTTACTTCA	331 bp
HO-2-R	J05405.1	AGTGCTGGGGAGTTTTAGTG	

F, forward primer; R, reverse primer.

Statistical analysis

Differences in the data among the groups were analyzed by using one-way analysis of variance combined with the Bonferroni test. Values were expressed as the means ± SEM, and differences between groups were considered to be significant at $p < 0.05$.

RESULTS

EFE up-regulates HO activity, HO-1 mRNA, and protein expression in astrocytes and hippocampal neurons

The chemical structure of EFE is reported in Fig. 1. The exposure of astrocytes and neurons for 6 h to different concentrations of EFE (1, 5, 15 and 25 μ*M*) resulted in a gradual and significant ($p < 0.05$) increase in HO activity, with a maximal value at 15 μ*M* in astrocytes and 5 μ*M* in neurons (Fig. 2). This enzymatic activation was strongly associated with a marked up-regulation of HO-1 mRNA and protein, as confirmed by quantitative RT-PCR (Fig. 3) and western blot analysis (Fig. 4). Although to a lesser extent, the increase of HO activity was also found in cells 24 h after EFE treatment (Fig. 2). In contrast, EFE failed to increase HO-1 expression and activity when higher concentrations (50 μ*M*) of this drug were used. The reduced ability of EFE to increase HO activity at high concentrations (50 μ*M*) correlated with a cytotoxic effect exerted by this compound (see below).

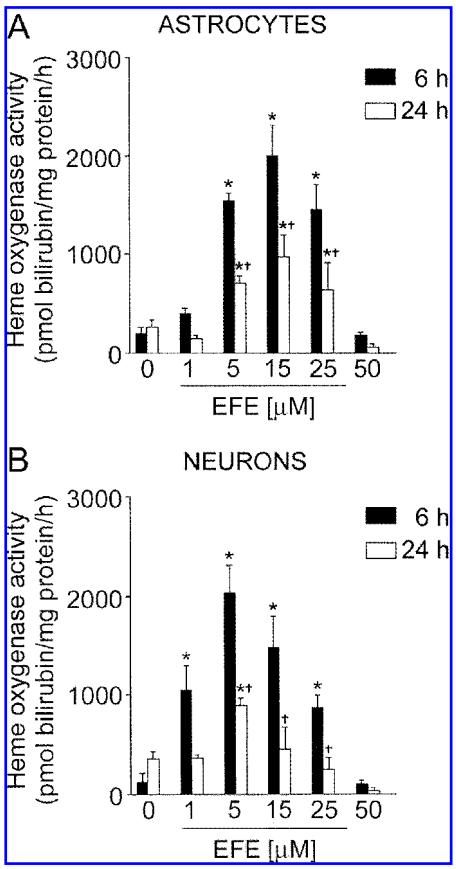

FIG. 2. Effect of EFE on HO activity in astrocytes and neurons. HO activity was measured in astrocytes (**A**) and neurons (**B**) after short (6 h) or prolonged (24 h) exposure to various concentrations of EFE (0–50 μ*M*). Each column represents the mean ± SEM of five independent experiments. *$p < 0.05$ versus 0 μ*M* EFE; †$p < 0.05$ versus 6 h.

Effect of EFE on cell viability

To determine a potential toxic effect of phenolic compounds on astrocytes, cells grown to confluence in 24 wells were incubated with increasing concentrations of EFE for 24 h. When the concentration of these drugs did not exceed 25 μ*M*, cell viability (determined using the Alamar Blue assay) as well as cell morphology observed under the microscope were fully preserved throughout the incubation period (Fig. 5). The toxic effect of EFE was more pronounced in neurons because treatment with this drug at 25 μ*M* resulted in 32% reductions in the number of viable cells. In contrast, treatment of astrocytes and neurons with 50 μ*M* EFE was strongly cytotoxic, causing 63 and 82% reduction in cell viability, respectively (Fig. 5).

RSV induces HO-1 mRNA, does not up-regulate HO-1 protein, and down-regulates HO activity in astrocytes

We have investigated mRNA expression of HO isoforms in astrocytes exposed to different concentrations of RSV (1, 5, 15 and 25 μ*M*). HO-2, as expected, did not show significant differences in its mRNA expression (data not shown). On the contrary, HO-1 mRNA expression showed a gradual and significant ($p < 0.05$) increase related to RSV concentration (Fig. 6). Interestingly, the induction of HO-1 messenger did not result in a significant expression of HO-1 protein (data not shown). Moreover, exposure of astrocytes to RSV resulted in a significant reduction of HO activity (Fig. 6).

HO expression by EFE protects neuronal cells from GOX-induced cell death

We examined the effects of EFE pretreatment on GOX-induced cell death in hippocampal neurons. The neuronal cells were pretreated for 12 h with EFE at a concentration of 5 μ*M* and then exposed to 50 mU/ml GOX for 2 h. Exposure of the cells for 2 h to 50 mU/ml GOX caused a 74% decrease in cell viability ($p < 0.05$; Fig. 7). However, pretreatment of the cells with EFE recovered the viability of the cells to 76% of control ($p < 0.05$; Fig. 7). The involvement of HO-1 in the cytoprotective effect of EFE was confirmed using an inhibitor of HO activity, ZnPP IX, which at the concentration of 10 μ*M* significantly blocked EFE-mediated suppression of GOX-induced cell death (Fig. 7). These data show that the cytoprotective effect of EFE might be due, in part, to the induction of HO-1.

DISCUSSION

In recent years, there has been an unprecedented interest in identifying new pharmacological strategies to increase defense mechanisms by activating multiple antioxidant defense

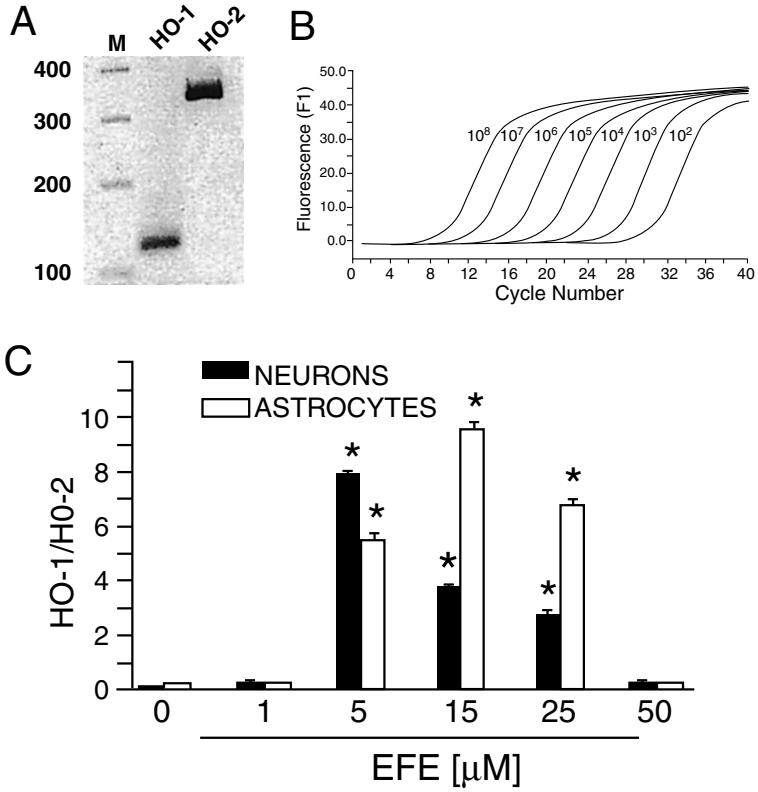

FIG. 3. Real-time quantification of HO-1 and HO-2 mRNA levels by RT-PCR in astrocytes and neurons treated with EFE. Specific primers for HO-1 and HO-2 were used to amplify rat RNA (**A**). Total RNA from different samples and known amounts of external standards (purified PCR product, 10^2 to 10^8 copies) were amplified in parallel reaction. Fluorimetric intensity of SYBR Green I, indicating the amount of PCR product formed, was measured at the end of each elongation phase. Quantification was performed by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of the external standards (**B**). Cellular expression of HO-1 transcript relative to the expression of HO-2 (mean ± SEM) for the different EFE concentrations after 6 h of treatment is shown (**C**). *$p <$ 0.05 versus 0 μ*M* EFE.

genes, a process that has been referred to as programmed cell life. Previous studies have shown that induction of HO-1 can represent an efficient antioxidant system and a potential pharmacological target in a variety of oxidant- and inflammatory-mediated diseases, including brain aging and neurodegenerative disorders (8, 24).

Considering, in fact, that brain has a limited ability to withstand oxidative stress because of its high content of easily oxidizable substrates, such as polyunsatured fatty acids and catecholamines, and because brain has relatively low levels of antioxidants such as glutathione and vitamin E (4, 14), the activation of early inducible genes, endowed with antioxidant and repair properties, such as HO-1, is crucial to survive

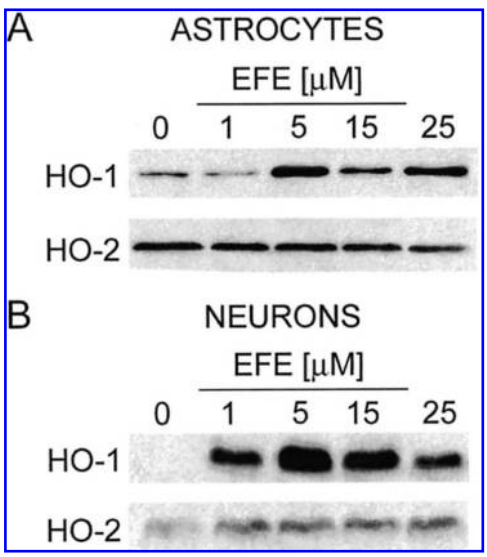

FIG. 4. Effect of EFE on HO-1 and HO-2 protein expression in astrocytes and neurons. Western blots showing HO-1 and HO-2 protein levels in astrocytes (**A**) and neurons (**B**) after treatment with EFE (0–25 μ*M*) for 6 h are presented. These panels are representative of four different experiments with similar results. Western immunoblot technique was performed as described under Materials and Methods.

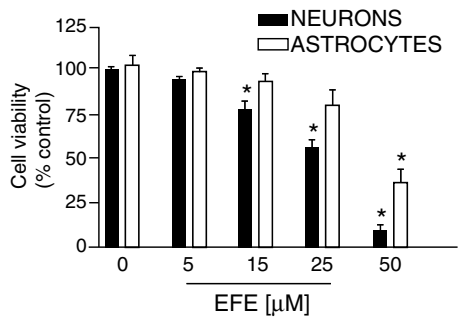

FIG. 5. Effect of EFE on cell viability. Astrocytes and neurons were exposed for 24 h to various concentrations (0–50 μ*M*) of EFE in complete medium. Cell viability was measured spectrophotometrically using an Alamar Blue assay as described under Materials and Methods. Data are expressed as the means ± SEM of six independent experiments. *$p < 0.05$ versus 0 μ*M* EFE.

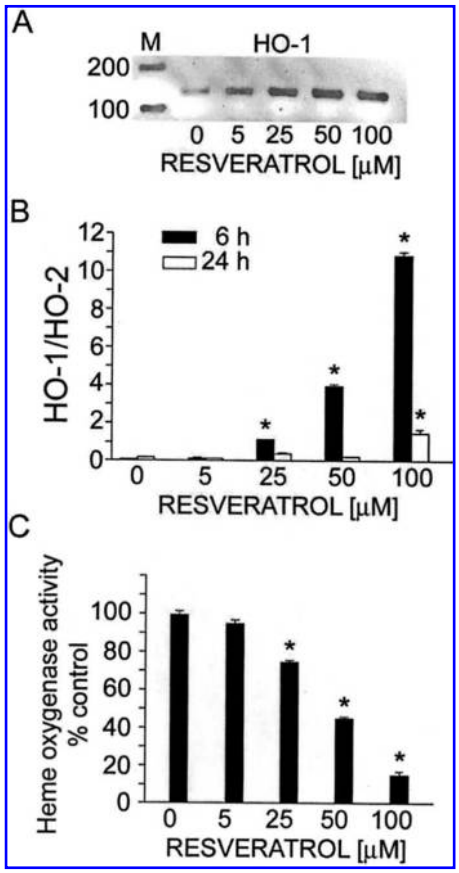

FIG. 6. Effect of RSV on HO-1 and HO-2 mRNA levels and HO activity in rat astrocytes. Total RNA from astrocytes treated with different concentrations of RSV (0–100 μ*M*) was reverse-transcribedand PCR amplified with different primer pairs specific for HO-1 and HO-2. A 100-bp DNA ladder is shown at the left of the gel (M), with bands labeled in bp units (**A**). Cellular expressionof HO-1 transcript relative to the expression of HO-2 (mean ± SEM) for different RSV concentrations after 6 h and 24 h of treatment is shown (**B**). Astrocytes were incubated for 6 h with various concentrations of RSV, and HO activity was determined as described under Materials and Methods (**C**). **p* < 0.05 versus 0 μ*M* EFE.

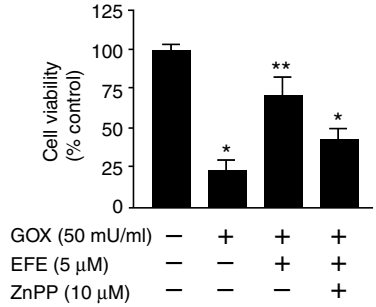

FIG. 7. Neuroprotective effect of EFE against GOX-induced cell death. Rat hippocampal neurons were treated with 5 μ*M* of EFE for 12 h in the presence or absence of 10 μ*M* ZnPP IX. After this pretreatment, cells were incubated for 2 h with 50 mU/ml GOX. After these treatments, cells were washed and viability was assessed by Alamar Blue assay. **p* < 0.05 versus control; ***p* < 0.05 versus GOX alone.

different types of injuries. It has been suggested that polyphenols can scavenge free radicals, but the underlying cellular mechanism is still unclear (3, 30). Our group and others have recently demonstrated that low concentrations of curcumin and CAPE significantly increase HO-1 expression and activity in different cell types (2, 29, 36), by the activation of the transcriptional factor Nrf2 (1, 2). These observations identified a novel mechanism by which some of a plant's compounds exert their antioxidant activity against stressful conditions (9, 27). Although a recent *in vivo* study has shown a protective role of curcumin against oxidative damage and amyloid pathology in an Alzheimer transgenic mouse (23), the possibility of using curcumin as a therapeutic drug is uncertain because of its poor bioavailability. In this respect, the search for novel, nontoxic, and more potent inducers of this pathway, possibly endowed with a better bioavailability, will facilitate the development of pharmacological strategies to increase the intrinsic capacity of cells to maximize HO-1 expression and, ultimately, cytoprotection. Accordingly, other studies (D.A. Butterfield *et al.*, unpublished observations) show that EFE exerts strong neuroprotection against the oxidative stress and neurotoxicity induced by amyloid β1–42.

Our present data clearly indicated that EFE, a lipophilic polyphenol, is able, at low concentrations, to induce HO-1 protein expression and activity in cell lines of rat astrocytes and hippocampal neurons. In contrast, exposure of astrocytes to RSV, an antioxidant polyphenol, resulted in both a concentration- and time-dependent increase in HO-1 mRNA, but interestingly, it was not able to induce protein expression and HO activity. As a recent study has shown that RSV is able to induce HO-1 in mouse cortical neurons (45), our findings support the notion of different activities of RSV depending on the cell type and the animal species. It is unclear if this activity on HO-1 mRNA expression can be relevant in terms of RSV antioxidant effects, improving the amount of HO-1 messenger to be translated via posttranscriptional mechanisms.

In this study, we also demonstrated the cytoprotective effects of EFE against oxidative damage in neuronal cells. Treatment of the cells with low concentrations of EFE resulted in high resistance to GOX/hydrogen peroxide-induced cell death. ZnPP IX, a specific inhibitor of HO activity, abrogated the protective effect of EFE, demonstrating a direct involvement of HO-1 induction, in the antioxidant mechanisms of this polyphenol. At this point, we have not yet identified the specific cascade that triggers HO-1 up-regulation, but it is probably an involvement of the Nrf2 pathway, similarly to that already seen for curcumin. Interestingly, in both astrocytes and neurons, EFE (1–25 μ*M*), without causing a significant cytotoxicity, increased HO activity with a bell-shaped dose-response curve, and this finding is paralleled by the bell-shaped increase in HO-1 mRNA and protein (Figs. 2–4). The reduction in HO-1 mRNA and protein at the higher EFE doses could be partially explained by possible HO-1 gene repression (40), which is particularly relevant in brain cells. Experiments designed to study the effect of EFE on HO-1 gene transcriptional repressors (*e.g.,* Bach-1/2) and activators (*e.g.,* Nrf2), in both neurons and astrocytes, are now warranted. Ad-

ditional *in vitro* and *in vivo* studies are necessary to determine whether EFE can be used as a preventive agent against acute neurodegenerative conditions, or to reduce the progression of chronic and age-associated neurodegenerative disorders, such as Alzheimer's disease.

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ABBREVIATIONS

CAPE, caffeic acid phenethyl ester; EFE, ethyl ferulate; GOX, glucose oxidase; HO, heme oxygenase; RSV, resveratrol; ZnPP IX, zinc protoporphyrin IX.

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