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Caffeic Acid Phenethyl Ester and Curcumin: A Novel Class of Heme Oxygenase-1 Inducers

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ABSTRACT

Heme oxygenase-1 (HO-1) is a redox-sensitive inducible protein that provides efficient cytoprotection against oxidative stress. Curcumin, a polyphenolic natural compound that possesses anti-tumor and anti-inflammatory properties, has been reported recently to induce potently HO-1 expression in vascular endothelial cells (*Free Rad Biol Med* **28:**1303–1312, 2000). Here, we extend our previous findings by showing that caffeic acid phenethyl ester (CAPE), another plant-derived phenolic agent, markedly increases heme oxygenase activity and HO-1 protein in astrocytes. The effect seems to be related to the peculiar chemical structures of curcumin and CAPE, because analogous antioxidants containing only portions of these two molecules were totally ineffective. At a final concentration of 30 μM, both curcumin and CAPE maximally up-regulated heme oxygenase activity while promoting marked cytotoxicity at higher concentrations (50–100 μM). Similar results were obtained with Curcumin-95, a mixture of curcuminoids commonly used as a dietary supplement. Incubation of astrocytes with curcumin or CAPE at concentrations that promoted maximal heme oxygenase activity resulted in an early increase in reduced glutathione followed by a significant elevation in oxidized glutathione contents. A curcumin-mediated increase in heme oxygenase activity was not affected by the glutathione precursor and thiol donor *N*-acetyl-L-cysteine. These data suggest that regulation of HO-1 expression by polyphenolic compounds is evoked by a distinctive mechanism which is not necessarily linked to changes in glutathione but might depend on redox signals sustained by specific and targeted sulfydryl groups. This study identifies a novel class of natural substances that could be used for therapeutic purposes as potent inducers of HO-1 in the protection of tissues against inflammatory and neurodegenerative conditions.

Heme oxygenase-1 (HO-1) is a ubiquitous and redox-sensitive inducible stress protein (Motterlini et al., 2002). In mammals, the crucial participation of HO-1 gene expression in alleviating organ dysfunction and counteracting metabolic disorders is supported by consistent reports showing a protective role for the products of the enzymatic activity of HO-1. Heme serves as a substrate for HO-1 in the formation of carbon monoxide, free ferrous iron, and biliverdin; the latter is rapidly converted to bilirubin by biliverdin reductase (Choi and Alam, 1996; Foresti and Motterlini, 1999). A substantial body of evidence demonstrates that increased carbon monoxide and bilirubin effectively contribute to modulate important physiological processes within the cardiovascular, immune, and nervous systems. These include the regulation of vessel tone (Motterlini et al., 1998), inhibition of platelet aggregation (Durante and Schafer, 1998), and prevention of cell death and tissue injury (Clark et al., 2000b). The overall concept emerging from these and other studies is that the induction of HO-1 is an essential step in the cellular adaptation to stress inflicted by pathological events.

Apart from the substrate heme which functions as a native inducer of the HO-1 gene, increased HO-1 expression occurs under a wide range of unrelated conditions that are characterized by alteration of the cellular redox state (Tyrrell, 1999). This is typified by in vitro and in vivo evidence showing that in several, if not all, stress-related circumstances, stimulation of HO-1 is directly associated with a change in intracellular glutathione levels. An imbalance in the redox status of thiols after a challenge with oxidants (Tyrrell, 1999), ultraviolet A radiation (Lautier et al., 1992), and hypoxia (Motterlini et al., 2000a), as well as with nitric oxide (NO) (Foresti et al., 1997) or NO-related species (Foresti et al., 1999), is known to promote activation of the HO-1 system in different cell types. These studies reveal that both oxidative and nitrosative reactions, which are implicated in a

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ABBREVIATIONS: HO-1, heme oxygenase-1; Curcumin, 1,7-bis[4-Hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione; CAPE, caffeic acid phenethyl ester; NAC, *N*-acetyl-L-cysteine; GSH, reduced glutathione; GSSG, oxidized glutathione; PBS, phosphate-buffered saline.

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variety of pathophysiological conditions, seem to play a crucial role in the mechanism(s) leading to HO-1 induction (Foresti et al., 1997; Motterlini et al., 2000a); recent reports hypothesized an important biological function for heme oxygenase in counteracting these two types of stress (Foresti and Motterlini, 1999; Motterlini et al., 2002). The idea that HO-1 expression can be primarily regulated by redox signaling events is also corroborated by data showing that transcription of this gene is suppressed by thiols and certain antioxidants (Camhi et al., 1998). Although the key factors participating in signal transduction mechanisms and the specific chemical modifications required for transcriptional activation of HO-1 remain to be fully identified, this enzyme can be regarded as a potential therapeutic target in a variety of oxidant- and inflammatory-mediated diseases. In this respect, the search for novel and more potent inducers of this pathway will facilitate the development of pharmacological strategies to increase the intrinsic capacity of cells to maximize HO-1 expression and, ultimately, cytoprotection.

Recent and unprecedented data from our group revealed that low concentrations of curcumin, a naturally occurring antioxidant, potently induces HO-1 expression in endothelial cells, leading to increased resistance to oxidative stress-mediated damage (Motterlini et al., 2000b). 1,7-bis[4-Hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione (Curcumin) is a yellow spice extracted from the rhizome of *Curcuma longa* L. (turmeric) and is commonly used as a flavoring and coloring agent in food (Ammon and Wahl, 1991). It contains two electrophilic α,β-unsaturated carbonyl groups, which can react with nucleophiles, such as glutathione. Its anti-inflammatory properties and cancer-preventive activities have been consistently reported using in vitro and in vivo models of tumor initiation and promotion (Huang et al., 1988; Huang et al., 1997). By virtue of Michael reaction acceptor functionalities and its electrophilic characteristics, curcumin and several other structurally related polyphenolic compounds have been recently shown to induce the activities of phase II detoxification enzymes, which seem to be crucial in protecting against carcinogenesis (Dinkova-Kostova and Talalay, 1999). Thus, activation of "classic" detoxifying enzymes and induction of HO-1 by phenolic natural substances might be directly correlated and are likely to involve common transcription mechanisms that are sensitive to the distinctive chemistry originating from these compounds.

In this study, we analyzed the potency of curcumin as an inducer of HO-1 expression and heme oxygenase activity in astrocytes, and we explored whether a similar effect could be obtained with caffeic acid phenethyl ester (CAPE), another structurally related phenolic originating from plants. CAPE is, in fact, an active component of propolis derived from the bark of conifer trees and carried by honeybees to their hives. The similarity to curcumin is striking because CAPE is also a Michael reaction acceptor that has a broad spectrum of biological activities, including anti-inflammatory (Natarajan et al., 1996; Michaluart et al., 1999), antioxidant (Chen et al., 2001), and anti-cancer effects (Frenkel et al., 1993; Huang et al., 1996). We report our finding that CAPE is a potent inducer of HO-1. The extent of heme oxygenase activation after treatment of astrocytes with commercially available curcumin (Curcumin-95) and other well-known natural antioxidants was also tested. Because astrocytes are strongly involved in the regulation of neuronal redox homeostasis, by investigating the effect of curcumin and CAPE on intracellular glutathione levels, we finally examined how the change in redox state influences heme oxygenase activity and cell survival.

Experimental Procedures

Chemicals and Reagents. Curcumin, CAPE, *ortho*-coumaric acid (2-hydroxycinnamic acid), and *para*-coumaric acid (4-hydroxycinnamic acid) were purchased from Sigma Chemical (St. Louis, MO). Resveratrol (*trans*-3,2,5-trihydrostilbene) and rosmarinic acid [(*R*)-α-[[3-(3,4-dihydroxyphenyl)-1-oxo-2*E*-propenyl]oxy]-3,4,-dihydroxy-benzenepropanoic acid] were obtained from Alexis Corporation (Läufelfingen, Switzerland). The chemical structures of these phenolic compounds are shown in Fig. 1. Curcumin-95, a commercially available mixture of curcuminoids (68% curcumin, 17% dimethoxy curcumin, 3% bis-dimethoxy curcumin, and 12% other curcumins), was purchased from Advanced Orthomolecular Research (Smith Falls, ON, Canada). Stock solutions of curcumin and other polyphenolic compounds were prepared as described previously (Motterlini et al., 2000b). *N*-Acetyl-L-cysteine (NAC), reduced (GSH) and oxidized (GSSG) glutathione, and all other reagents were from Sigma unless otherwise specified. Rabbit polyclonal antibodies directed against HO-1 were obtained from Stressgen (Victoria, Canada).

Cell Culture. Type 1 astrocytes (DI TNC1) were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin and supplemented with 10% fetal bovine serum. 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 curcumin, CAPE, Curcumin-95, or other phenolic compounds. After each treatment (6 or 24 h), cells were harvested for the determination of heme oxygenase activity, HO-1 protein expression, and intracellular glutathione. Astrocytes growing in 24 wells were also exposed to polyphenolic compounds, and cell viability was determined at 24 h.

Heme Oxygenase Activity Assay. Heme oxygenase activity was determined at the end of each treatment as described previously by our group (Foresti et al., 1997; Motterlini et al., 2000a). Briefly, 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 and centrifugation, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm (ϵ = 40 mM^{-1}cm^{-1}).

Western Blot Analysis. After treatment with curcumin or CAPE, samples of astrocytes were also analyzed for HO-1 protein expression using a Western immunoblot technique as described previously (Foresti et al., 1997; Motterlini et al., 2000a). Briefly, an equal amount of proteins (30 μg) for each sample was separated by SDS-polyacrylamide gel electrophoresis and transferred overnight to nitrocellulose membranes, and the nonspecific binding of antibodies was blocked with 3% nonfat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen) (1:1000 dilution in Tris-buffered saline, pH 7.4) for 2 h at room temperature. After three washes with PBS, 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, UK). Blots shown are representative of three independent experiments.

Determination of Intracellular Glutathione. GSH and GSSG levels were measured after 6- and 24-h exposure of astrocytes to curcumin and CAPE using a method described by our group previ-

ously (Calabrese et al., 1998; Motterlini et al., 2000a). Briefly, cells harvested in cold PBS were freeze-thawed three times, and an aliquot of this suspension was added to a buffer solution containing 12 mM EDTA and 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid). Total glutathione was measured spectrophotometrically (optical density = 412 nm) using the glutathione reductase-recycling assay. To determine the amount of GSSG, an aliquot of the cell suspension was added to an equal volume of buffer containing EDTA and *N*-ethylmaleimide (10 mM). The sample was mixed and centrifuged, and the supernatant was passed through a C_{18} Sep-Pak cartridge (Waters, Milford, MA) to remove the excess *N*-ethylmaleimide. The sample was added to a cuvette containing 5,5′-dithiobis-(2-nitrobenzoic acid) and glutathione reductase, and the assay was performed as for the measurement of total glutathione. Intracellular glutathione was determined by comparison with a standard curve obtained with GSH and GSSG solutions and was expressed as nmoles/mg of protein.

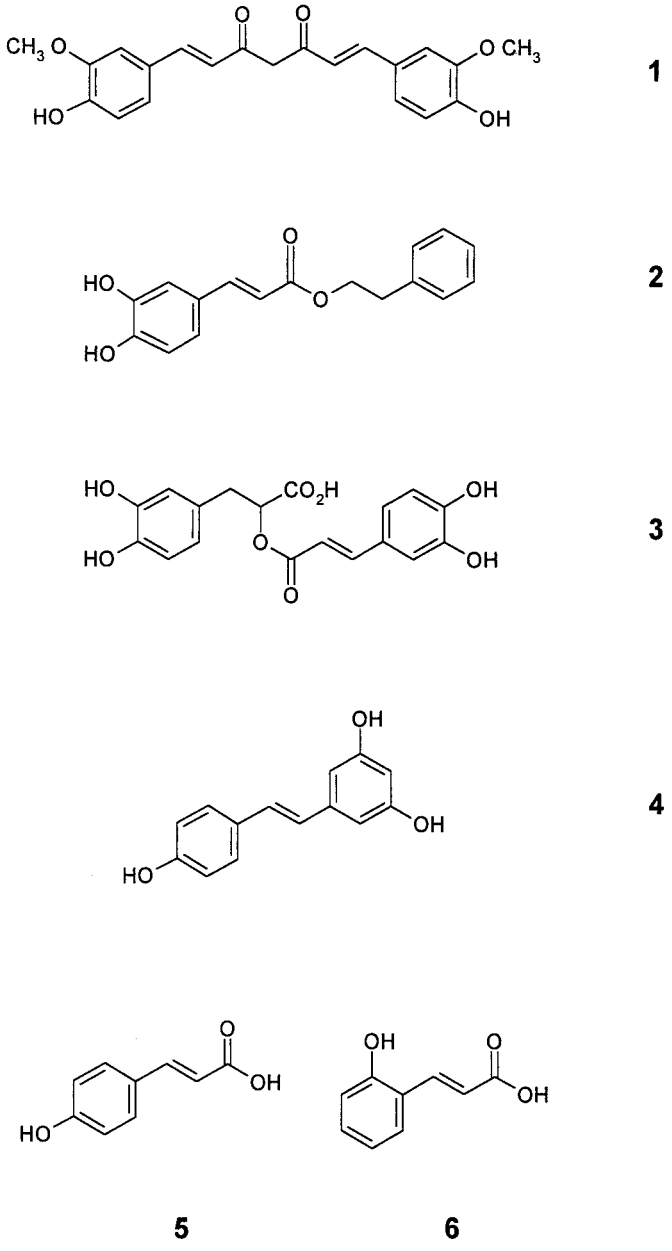

Fig. 1. Chemical structure of naturally occurring polyphenolic compounds that possess antioxidant and/or anti-inflammatory properties. Curcumin (1), CAPE (2), rosmarinic acid (3), and resveratrol (4) represent natural substances derived either from plants or fruits. Two additional phenolic compounds that possess chemical structure and properties similar to those of 1, 2, 3, and 4 are also reported: *para*-coumaric acid (5) and *ortho*-coumaric acid (6).

Cell Viability Assay. Astrocytes were exposed to curcumin or CAPE for the indicated times, and cell viability was assessed with the use of an Alamar Blue assay according to manufacturer's instructions (Serotec, Oxford, UK) as reported previously (Motterlini et al., 2000b). At the end of each treatment, cells were washed twice and incubated for an additional 5 h in complete medium containing 1% Alamar Blue solution. Optical density in each sample was measured using a plate reader (Molecular Devices, Crawley, UK). 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.

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 mean ± S.E.M., and differences between groups were considered to be significant at $p < 0.05$.

Results

CAPE and Curcumin Up-Regulate Heme Oxygenase Activity and HO-1 Expression in Astrocytes. The chemical structures of curcumin, CAPE, and other phenolic compounds are reported in Fig. 1. The exposure of astrocytes for 6 h to 15 and 30 μM curcumin resulted in a gradual and significant ($p < 0.05$) increase in heme oxygenase activity (7.4- and 9.1-fold, respectively); this enzymatic activation was strongly associated with a marked up-regulation of HO-1 protein, as confirmed by Western blot analysis (Fig. 2, A and B). Although to a lesser extent, over-expression of HO-1 was also found in astrocytes 24 h after curcumin treatment (Fig. 2C). In contrast, curcumin failed to increase HO-1 expression when higher concentrations (50–100 μM) of this drug were used; consequently, the elevation in heme oxygenase activity was much less pronounced (1.9-fold). Similar to the effect evoked by curcumin, exposure of cells to low concentrations of CAPE (15–50 μM) resulted in a substantial increase in heme oxygenase activity and HO-1 protein levels (Fig. 3, A-C). Maximal enzyme activation and protein expression were found at 30 μM CAPE, whereas 100 μM was significantly less effective. The reduced ability of curcumin and CAPE to increase heme oxygenase activity at high concentrations (50–100 μM) correlated with a cytotoxic effect exerted by these two drugs (see below). We then tested other phenolic compounds that possess antioxidant properties but contain only portions of the chemical structure typical of curcumin or CAPE. Specifically, we selected rosmarinic acid, resveratrol, *o*-coumaric, and *p*-coumaric acids (5–100 μM), all of which contain a phenolic group and/or a Michael reaction functionality. We observed that none of these agents was able to activate heme oxygenase (Table 1). On the contrary, some of these phenolics caused a significant decrease in enzymatic activity compared with control. It is interesting that the exposure of astrocytes for 6 h to low concentrations of Curcumin-95 (15–30 μM), a mixture of curcuminoids that is commercially available as a dietary supplement, also resulted in a significant elevation of heme oxygenase activity compared with controls (Fig. 4); however, this effect was less pronounced compared with pure curcumin. Similar to the effect caused by pure curcumin, high concentrations of Curcumin-95 (50 μM) did not cause any significant increase in heme oxygenase activity.

Effect of *N*-Acetyl-L-Cysteine on Curcumin-Mediated Activation of Heme Oxygenase. To determine the role of thiols in the modulation of heme oxygenase activity by phenolic compounds, cells were exposed to various concentrations of curcumin for 6 h in the presence of 1 mM *N*-acetyl-L-cysteine, a precursor of glutathione synthesis that possesses antioxidant properties. As shown in Fig. 5, the substantial increase in heme oxygenase activity observed with both 15 and 30 μM curcumin was not significantly affected by the presence of NAC. At 30 μM, for instance, curcumin increased heme oxygenase activity from 247 ± 5 (control) to 2461 ± 194 pmol of bilirubin/mg of protein/h ($p <$ 0.05), and the addition of NAC to the culture medium did not change the potency of activation by this phenolic agent (2392 ± 22 pmol of bilirubin/mg of protein/h). Similar results were obtained when astrocytes were incubated with CAPE in the presence of NAC (data not shown). At higher concentrations of curcumin (50 μM), the increase in heme oxygenase activity was less pronounced at 492 ± 30 and 752 ± 78 pmol of bilirubin/mg of protein/h in the absence or presence of NAC, respectively.

Effect of Curcumin, CAPE, and Curcumin-95 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 curcumin, CAPE, or Curcumin-95 for 24 h. When the concentration of these drugs did not exceed 30 μM, cell viability (determined using the Alamar Blue assay) as well as cell morphology observed under the microscope were fully preserved throughout the incubation period (Figs. 6 and 7). In contrast, treatment of astrocytes with 50 and 100 μM curcumin was cytotoxic, causing 20 and 63% reductions in cell viability, respectively (Fig. 6A). A similar pattern was observed after exposure of astrocytes to 50 and 100 μM Curcumin-95, which promoted 21 and 69% losses in viability, respectively (Fig. 7). The toxic effect of CAPE was more pronounced because treatment with this drug at 50 and 100 μM resulted, respectively, in 61 and 78% reductions in the

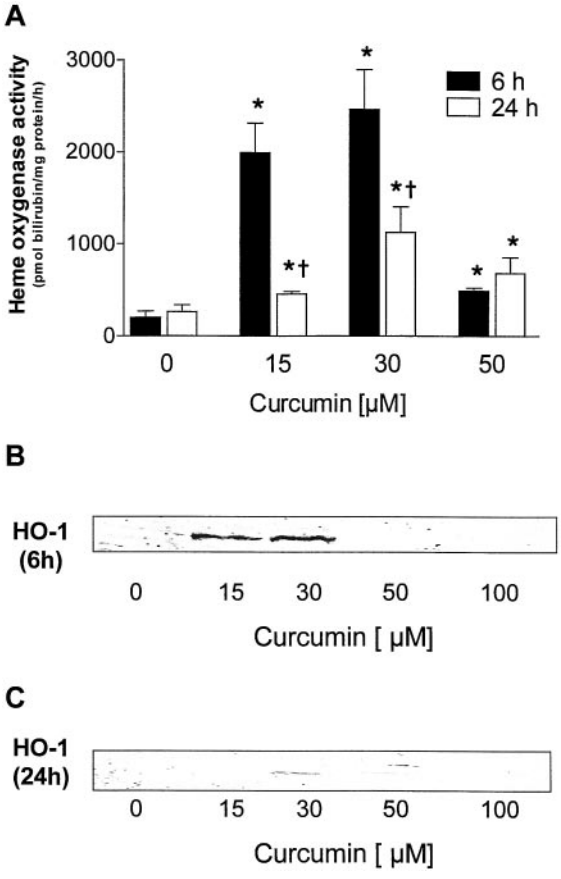

Fig. 2. Effect of curcumin on heme oxygenase activity and HO-1 protein expression in astrocytes. A, heme oxygenase activity was measured in astrocytes after short (6 h) or prolonged (24 h) exposure to various concentrations of curcumin (15, 30, and 50 μM). Control groups are represented by cells incubated with complete medium alone (0 μM). Each bar represents the mean ± S.E.M. of five independent experiments. *, $p <$ 0.05 versus 0 μM curcumin; †, $p < 0.05$ versus 6 h. B and C, Western blots showing HO-1 protein expression in astrocytes after treatment with curcumin (0–100 μM) for 6 and 24 h, respectively. Western immunoblot technique was performed as described under *Experimental Procedures*.

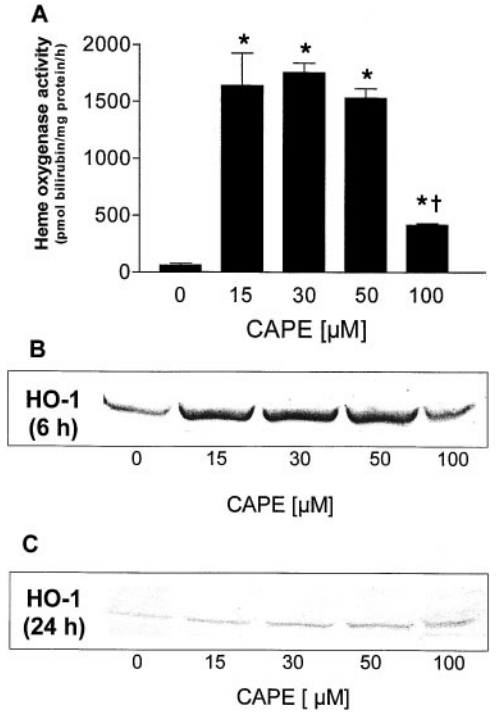

Fig. 3. Effect of CAPE on heme oxygenase activity and HO-1 protein expression in astrocytes. A, heme oxygenase activity was measured in astrocytes after short (6 h) or prolonged (24 h) exposure to various concentrations of CAPE (15, 30, and 50 μM). Control groups are represented by cells incubated with medium alone (0 μM). Each bar represents the mean ± S.E.M. of five independent experiments. *, $p < 0.05$ versus 0 μM CAPE; †, $P < 0.05$ versus 15, 30, and 50 μM CAPE. B and C, Western blots showing HO-1 protein expression in astrocytes after treatment with CAPE (0–100 μM) for 6 and 24 h, respectively. Western immunoblot technique was performed as described under *Experimental Procedures*.

TABLE 1
Effect of phenolic compounds on heme oxygenase activity in astrocytes
Astrocytes were incubated for 6 h with various polyphenolics at the concentrations indicated, and heme oxygenase activity was determined as described under *Experimental Procedures*.

	Heme Oxygenase Activity			
	5 μM	15 μM	30 μM	50 μM
	% control			
Rosmarinic acid	97.6 ± 8.9	93.7 ± 1.6	83.0 ± 4.2*	82.3 ± 2.6*
Resveratrol	99.4 ± 1.9	83.2 ± 3.5*	67.2 ± 7.4*	19.0 ± 2.5*
o-Coumaric acid	97.9 ± 0.5	99.5 ± 5.4	86.3 ± 4.1*	74.0 ± 9.9*
p-Coumaric acid	97.1 ± 0.3	63.3 ± 0.83*	51.6 ± 1.7*	43.5 ± 2.0*

* $p < 0.05$ vs. control.

number of viable cells (Fig. 6B). The presence of 1 mM NAC in the culture medium significantly attenuated the cytotoxic action mediated by both curcumin (100 μM) and CAPE (50 and 100 μM).

Effect of CAPE and Curcumin on Intracellular Glutathione Levels. To determine the effect of polyphenolic compounds on the redox status of the cell, GSH and GSSG levels were determined at 6 and 24 h after treatment of astrocytes with different concentrations of curcumin and CAPE. Exposure to 15 and 30 μM curcumin for 6 h resulted in a significant increase in both intracellular GSH and GSSG, whereas 50 μM caused oxidation without affecting the GSH content (Fig. 8). A prolonged exposure (24 h) to curcumin (15, 30, and 50 μM) caused a concentration-dependent decrease in GSH that was paralleled by a gradual and substantial increase in GSSG levels. CAPE (15 and 30 μM) evoked a similar effect on intracellular glutathione leading to the elevation of GSH in the early stage of the treatment followed by a marked reduction at 24 h (Fig. 9). Once again, exposure of cells to 50 μM CAPE did not affect GSH at 6 h, whereas prolonged incubation (24 h) caused a significant depletion of GSH and concomitant elevation in GSSG ($p <$ 0.05 versus control).

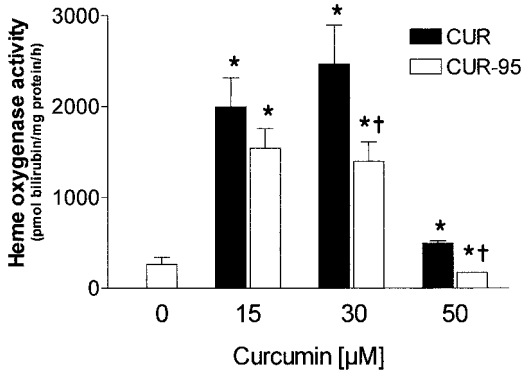

Fig. 4. Comparison between the potency of curcumin (CUR) and Curcumin-95 (CUR-95) as inducers of heme oxygenase. Confluent astrocytes were incubated for 6 or 24 h in the presence of various concentrations (15, 30, and 50 μM) of pure curcumin or Curcumin-95. Curcumin-95 consists of a mixture of curcuminoids (see *Experimental Procedures*) and is commercially available as a dietary supplement. Heme oxygenase activity was measured at the end of incubation as described under *Experimental Procedures*. Each bar represents the mean ± S.E.M. of five independent experiments. *, $p < 0.05$ versus 0 μM curcumin; † $p < 0.05$ versus CUR.

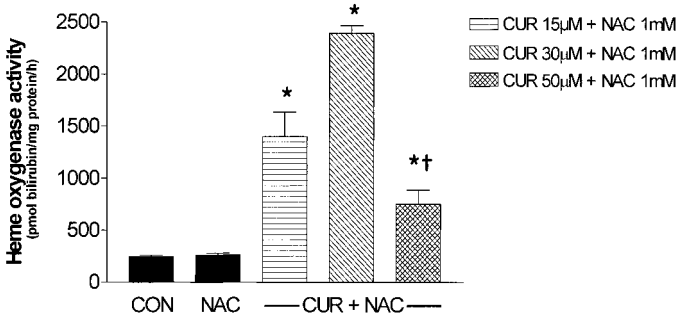

Fig. 5. Effect of *N*-acetyl-L-cysteine on curcumin-mediated heme oxygenase activation. Astrocytes were exposed to 15, 30, or 50 μM curcumin (CUR) for 6 h in the presence of NAC (1 mM), and heme oxygenase activity was determined as reported under *Experimental Procedures*. Cells were also incubated with complete medium (control) or 1 mM NAC alone. Each bar represents the mean ± S.E.M. of five experiments performed independently. *, $p < 0.01$ versus control (CON); †, $p < 0.01$ versus 30 μM CUR plus NAC.

Discussion

Persistent oxidant damage caused by the increased production of free radical species along with recurrent inflammation triggered by cytokines characterizes the development of numerous pathologies, including neurodegenerative diseases, vascular dysfunction, and carcinogenesis. Irrespective of the source and mechanisms that lead to the generation of intracellular toxic mediators, mammalian cells have developed

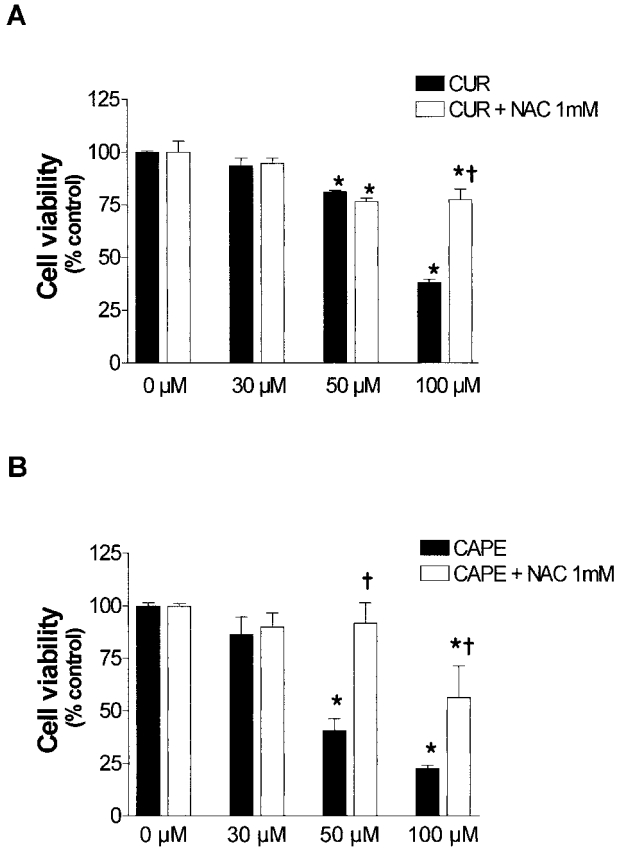

Fig. 6. Effect of curcumin and CAPE on cell viability. Astrocytes were exposed for 24 h to various concentrations (0–100 μM) of curcumin (A) or CAPE (B) in complete medium with or without 1 mM NAC. Cell viability was measured spectrophotometrically using an Alamar Blue assay as described under *Experimental Procedures*. Data are expressed as the mean ± S.E.M. of six independent experiments. *, $p < 0.05$ versus 0 μM; †, $p < 0.05$ versus curcumin or CAPE alone.

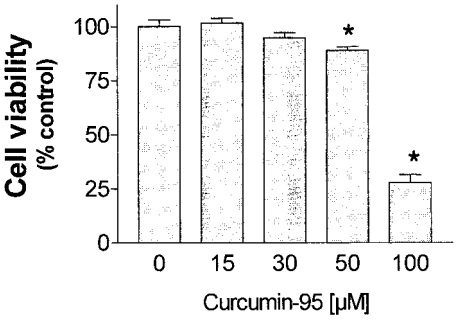

Fig. 7. Effect of Curcumin-95 on cell viability. Astrocytes were exposed for 24 h to various concentrations (0–100 μM) of Curcumin-95 in complete medium. Cell viability was measured spectrophotometrically using an Alamar Blue assay as described under *Experimental Procedures*. Data are expressed as the mean ± S.E.M. of six independent experiments. *, $p < 0.05$ versus 0 μM.

highly refined inducible systems against a variety of stressful conditions; upon stimulation, each one of these systems can be engaged concertedly to alleviate and hinder the manifestation of a distinctive metabolic disorder. Increasing scientific evidence supports a pivotal role for HO-1 (the inducible isoform of heme oxygenase) in the resolution of acute inflammatory states (Willis et al., 1996), suppression of acute hypertension (Motterlini et al., 1998), and prevention of cardiac graft rejection (Soares et al., 1998), as well as protection against oxidative (Poss and Tonegawa, 1997; Clark et al., 2000a) and nitrosative stress (Foresti et al., 1999; Foresti and Motterlini, 1999; Motterlini et al., 2000a). In the brain, astrocytes strongly express HO-1 in response to injury (Koistinaho et al., 1996), and stimulation of the HO-1 pathway seems to render neurons more resistant to oxidant challenge (Chen et al., 2000). These and other studies (Yachie et al., 1999) strongly suggest that HO-1 gene induction is essential for restoring cellular homeostasis and that the beneficial effects of increased heme oxygenase activity may represent a promising therapeutic expedient to preclude tissue injury and, consequently, impede the progression of several diseases. The efficacy of HO-1 in promoting cytoprotection resides primarily in the intrinsic ability of its metabolic products (e.g., carbon monoxide and bilirubin) to exert potent antioxidant and anti-inflammatory activities (Foresti et al., 1999; Clark et al., 2000b; Otterbein et al., 2000; Fujita et al., 2001).

This study reports that low concentrations of CAPE and curcumin, two naturally occurring phenolic agents that are well known for retaining remarkable antioxidant and anti-inflammatory properties, significantly increase HO-1 expression and heme oxygenase activity in astrocytes. A mixture of curcuminoids (Curcumin-95) that is commercially available as dietary supplement also markedly induced heme oxygenase activation, although it was slightly less effective than pure curcumin. These findings, together with our recent report showing a direct contribution of curcumin-mediated HO-1 expression in protecting endothelial cells against oxidative stress (Motterlini et al., 2000b), extend our view on the concept that certain active components of medicinal plants are potent inducers of the HO-1 gene. Notably, both CAPE and curcumin are known to be specific inhibitors of nuclear transcription factor NF-kB (Singh and Aggarwal, 1995; Natarajan et al., 1996) and cyclooxygenase activity (Michaluart et al., 1999; Plummer et al., 1999). These two phytochemicals can also efficiently inhibit lipid peroxidation and cellular growth (Singh and Aggarwal, 1995; Natarajan et al., 1996), as well as exert an antitumorigenic action in many different cancers (Huang et al., 1988; Frenkel et al., 1993; Huang et al., 1996, 1997). The majority of in vitro and in vivo studies conducted so far have attributed the protective effect of bioactive polyphenols to their chemical reactivity toward free radicals and their capacity to prevent the oxidation of important intracellular components. However, our previous (Motterlini et al., 2000b) and present observations reveal a potential novel aspect in the mode of action of CAPE and curcumin; that is, the ultimate stimulation of the HO-1 pathway is likely to account for the established and powerful antioxi-

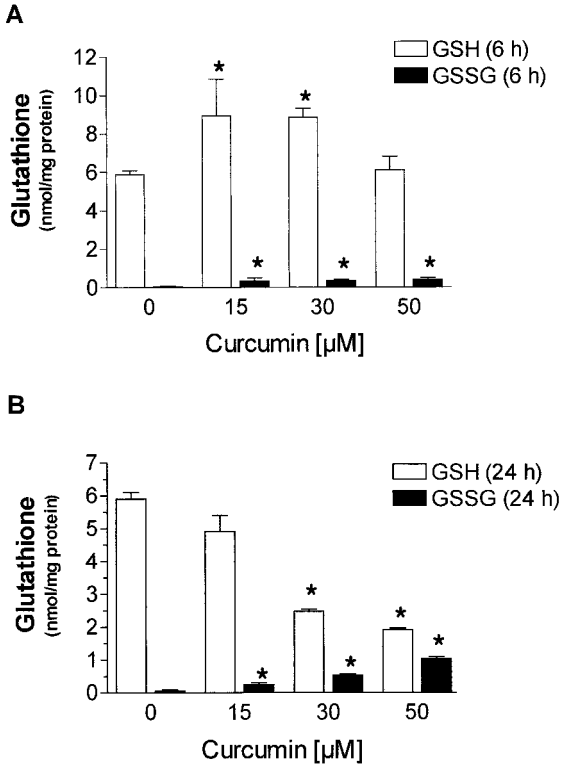

Fig. 8. Effect of curcumin on intracellular glutathione levels. GSH and GSSG levels were measured after 6- (A) or 24-h (B) exposure of astrocytes to curcumin (0–100 μM). The change in GSH and GSSG levels represents an index of the cellular redox status. Each bar represents the mean ± S.E.M. of four to five experiments performed independently. *, $p < 0.05$ versus 0 μM.

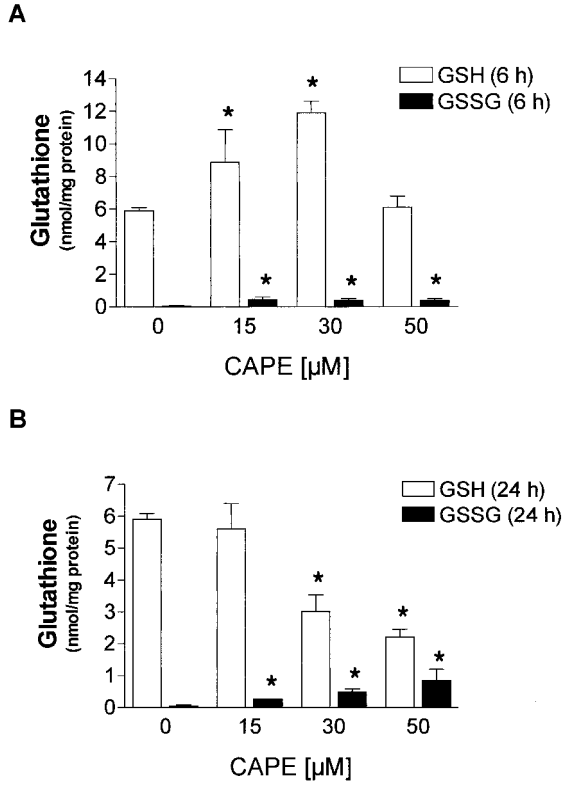

Fig. 9. Effect of CAPE on intracellular glutathione levels. GSH and GSSG levels were measured after 6- (A) or 24-h (B) exposure of astrocytes to CAPE (0–100 μM). The change in GSH and GSSG levels represents an index of the cellular redox status. Each bar represents the mean ± S.E.M. of four to five experiments performed independently. *, $p < 0.05$ versus 0 μM.

dant/anti-inflammatory properties of these two plant-derived compounds. Two recent studies have confirmed the ability of curcumin to induce HO-1 mRNA expression in both in vitro and in vivo models (Jones et al., 2000; Hill-Kapturczak et al., 2001).

The potency of CAPE and curcumin in increasing HO-1 expression and consequently heme oxygenase activity once added to astrocytes seems to be strictly associated with a rapid change in the intracellular redox status. Curcumin and other structurally related compounds are known to covalently modify sulfydryl groups by oxidation and alkylation reactions (Awasthi et al., 2000; Dinkova-Kostova et al., 2001). Under our experimental conditions, it was found that, despite an initial oxidation of glutathione (GSSG) after exposure of cells to low doses of curcumin and CAPE, this treatment did not significantly affect cell viability. Moreover, at concentrations that caused a gradual increase in heme oxygenase activity (15 and 30 μM), both CAPE and curcumin promoted an early increase in GSH levels, and this effect was reflected in the maintenance of cell viability even after prolonged incubations with the two agents. These data are in agreement with studies demonstrating that low concentrations of polyphenolics, such as curcumin, can increase the activity of γ-glutamyl-cysteinyl synthetase and other GSH-linked detoxifying enzymes (Singhal et al., 1999). Of significant interest also are the findings showing that, in the early stages of the treatment with high concentrations (50 and 100 μM) of curcumin and CAPE, a significant loss in cell viability was associated with a failure to increase the GSH content and was accompanied by a late and more dramatic reduction in the GSH/GSSG ratio (see Figs. 8 and 9). Notably, at the high concentrations used, both curcumin and CAPE were unable to stimulate an increase in heme oxygenase activity. These results are consistent with the notion that transient and moderate changes in the redox status of the cell are prerequisites for the induction of cytoprotective genes (such as HO-1) and that a more severe oxidation inflicted to GSH results in suppression of the cellular stress response, ultimately leading to cell death (Motterlini et al., 2002). The fact that *N*-acetyl-L-cysteine, a precursor of glutathione synthesis with potent antioxidant properties, significantly attenuated the loss of cell viability but failed to prevent HO-1 expression mediated by CAPE and curcumin indicate that HO-1 induction, in these circumstances, may not be directly related to redox changes involving glutathione. From recent findings demonstrating a high and specific reactivity of polyphenol compounds with sulfydryl groups (Dinkova-Kostova et al., 2001), it is possible that, because of their peculiar chemical structures, both curcumin and CAPE have a preferential affinity toward selective cysteine residues of targeted proteins that finely control the transcription of inducible genes (Dinkova-Kostova et al., 2001). Because activation of the HO-1 gene by classic inducers (such as ultraviolet A radiation, arsenite, hypoxia, and NO) can be effectively abolished in the presence of thiol compounds (Lautier et al., 1992; Choi and Alam, 1996; Foresti et al., 1997; Motterlini et al., 2000a), the data presented here indicate a distinct but still unidentified mechanism of HO-1 induction by this class of phenolic antioxidants.

From a mechanistic standpoint, the analogy existing between the ability of CAPE and curcumin to highly increase HO-1 expression and the potency of certain electrophiles to induce phase II detoxifying enzymes is rather intriguing. The chemical classes of compounds known to activate phase II enzymes are disparate and structurally different (Dinkova-Kostova et al., 2001), but it can generally be stated that mono- or polyphenols retaining Michael reaction acceptor functionalities are the most effective ones. Although the efficacy of CAPE to induce detoxifying systems in cells or tissues has not been tested yet, curcumin and curcuminoids seem to be potent inducers of phase II enzymes (Dinkova-Kostova and Talalay, 1999; Dinkova-Kostova et al., 2001). In our attempt to delineate a common chemical feature of HO-1 inducers with potency and biological functions similar to those elicited by curcumin and CAPE, we selected a series of natural phenolics that (1) exhibit antioxidant/anti-inflammatory activities (rosmarinic acid and resveratrol) (Kimura et al., 1987; Subbaramaiah et al., 1998); (2) function as Michael reaction acceptors (rosmarinic acid, *o*-coumaric acid, and *p*-coumaric acid); and (3) are portions of the curcumin (*p*-coumaric acid) or CAPE (rosmarinic acid) molecules. We found that none of these chemicals activated heme oxygenase in astrocytes. Therefore, it seems that CAPE and curcumin belong to a particular class of natural compounds that possess the strong ability to elevate heme oxygenase activity. Whether other plant-derived electrophiles, particularly the ones that have been recently shown to augment the activity of detoxifying enzymes (Dinkova-Kostova et al., 2001), can act as potent stimulants for HO-1 expression remains to be investigated. It is important to note that the genes encoding for HO-1 protein and detoxifying enzymes contain 5′-upstream antioxidant/electrophile responsive elements (Prestera et al., 1993; Choi and Alam, 1996), which can be selectively recognized by the ubiquitous transcriptional factor Nrf2. In view of the evidence demonstrating a crucial role for Nrf2 in both phase II enzymes and HO-1 expression (Alam et al., 1999; Ramos-Gomez et al., 2001), it is plausible to speculate on a common mechanism of induction for these cytoprotective proteins by a selected class of natural phenolics. Experiments designed to verify whether Nrf2 activation is required for curcumin/CAPE-mediated HO-1 gene expression are now warranted.

In conclusion, from their well-known antioxidant and anti-inflammatory properties, we have identified CAPE and curcumin as novel and potent HO-1 inducers that can be used to markedly increase heme oxygenase activity in astrocytes and other cell types (Motterlini et al., 2000b). It needs to be emphasized that CAPE, curcumin, and other plant constituents eventually become part of the human diet and can be consumed daily as herbal supplements, such as in the case of Curcumin-95. Because the HO-1 gene can be stimulated at transcriptional levels by a plethora of noxious stimuli, the use of plant-derived natural substances to trigger HO-1 expression and other intracellular defensive systems would clearly offer a greater advantage for therapeutic purposes. Further in vitro and in vivo studies using curcumin/CAPE-like molecules will give us important information on the feasibility of developing new pharmacological strategies for maximizing heme oxygenase activity in targeted tissues as an alternative to or in combination with HO-1 gene therapy.

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Correction to "Caffeic Acid Phenethyl Ester and Curcumin: A Novel Class of Heme Oxygenase-1 Inducers"

In the above article [Scapagnini G, Foresti R, Calabrese V, Giuffrida Stella AM, Green CJ, and Motterlini R (2002) *Mol Pharmacol* **61:**554–561], there is an error in the authors' affiliation. It should read, "Department of Chemistry, Section of Biochemistry and Molecular Biology (G.S., V.C., A.M.G.S.)."

We regret this error and apologize for any inconvenience it may have caused.