ANTIOXIDANTS & REDOX SIGNALING
Volume 8, Numbers 3 & 4, 2006

Forum Original Research Communication

Curcumin Activates Defensive Genes and Protects Neurons Against Oxidative Stress

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ABSTRACT

Spices and herbs often contain active phenolic substances endowed with potent antioxidative properties. We had previously shown that curcumin, the yellow pigment in curry, strongly induced HO-1 expression and activity in rat astrocytes. In the CNS, HO-1 has been reported to operate as a fundamental defensive mechanism for neurons exposed to an oxidant challenge. Treatment of astrocytes with curcumin upregulated expression of HO-1 protein at both cytoplasmic and nuclear levels, as shown by immunofluorescence analysis under laser-scanning confocal microscopy. A significant expression of quinone reductase and glutathione *S*-transferase, two members of phase II detoxification enzymes, was found in astrocytes exposed to 5–15 μ*M* curcumin. Moreover, the effects of curcumin on HO-1 activity were explored in cultured hippocampal neurons. Elevated expression of HO-1 mRNA and protein were detected after 6 h incubation with 5–25 μ*M* curcumin. Higher concentrations of curcumin (50–100 μ*M*) caused a substantial cytotoxic effect with no change in HO-1 protein expression. Interestingly, pre-incubation (18 h) with curcumin resulted in an enhanced cellular resistance to glucose oxidase-mediated oxidative damage; this cytoprotective effect was considerably attenuated by zinc protoporphyrin IX, an inhibitor of heme oxygenase activity. This study gives additional support to the possible use of curcumin as a dietary preventive agent against oxidative stress-related diseases. *Antioxid. Redox Signal.* 8, 395–403.

INTRODUCTION

In recent years there has been a growing interest, supported by a large number of experimental and epidemiological studies, about the beneficial effects of some commonly used food-derived products in preventing various pathologic conditions, ranging from cancer to neurodegenerative diseases. Spices and herbs, in fact, often contain active phenolic substances endowed with potent antioxidative and chemopreventive properties (35). Curcumin (CUR) is a phytochemical compound extracted from the rhizome of *Curcuma longa*, and it is the pigment responsible for curry's characteristic yellow color (2). It has been used for centuries as a food preservative and herbal medicine in India. Curcumin is a representative member of plant-derived polyphenol family, which also includes resveratrol, caffeic acid phenethyl ester, carnosol, ethyl ferulate, epigallocatechin gallate, and other green tea phenols (50, 38). These bioactive phytochemicals exhibit Michael acceptor function and possess antioxidant properties, which may reduce the production

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of free radicals and improve cell viability under conditions of enhanced oxidative stress (13). Moreover, all of these compounds appear to have a number of different molecular targets, impinging on several signaling pathways, and showing pleiotropic activity on cells and tissues. Curcumin has anti-inflammatory properties which include the capacity to inhibit 5- and 8-lipoxygenases, cyclooxygenases, and metalloproteinases (21, 6), and exhibits promising anticancer activity (20, 23), related to its ability to induce glutathione *S*-transferase enzymes, to inhibit prostaglandin production, and to suppress oxidative DNA adduct (M(1)G) formation (41). Several reports have shown the ability of curcumin to induce a general xenobiotic response in the target cells, activating multiple defense genes such as phase II enzymes and heme oxygenase-1 (HO-1) (14, 33, 40). The involvement of curcumin in restoring cellular homeostasis and rebalancing redox equilibrium, suggests that it might be a useful adjunct also in the treatment of neurodegenerative illnesses characterized by inflammation, such as Alzheimer's disease (5, 47, 48). It has been recently demonstrated that low nontoxic doses of curcumin suppress inflammation and oxidative damage in the brains of APPSw mice, a transgenic mouse model of Alzheimer's disease, inhibit formation of amyloid beta oligomers and fibrils, and reduce levels of insoluble and soluble amyloid and plaque burden in many brain regions usually affected by the disease (28, 49). The molecular pathways and mechanisms involved are not yet understood, but possibly relate to curcumin's capacity to induce cellular stress responses with activation of antioxidant and defensive genes (7).

Accordingly with this concept, we have previously shown that curcumin potently induces HO-1 expression and activity in rat astrocytes (40). HO proteins play a significant physiological role in cellular stress responses, as widely demonstrated in a variety of tissues including brain (1, 25, 29). To date, three isoforms of heme oxygenase have been identified: the inducible HO-1 (42), the constitutive HO-2 (30) and the less active HO-3 (32), cloned only in rat and probably representing the result of a *species*-specific gene retrotransposition (39). Many reports have demonstrated the potent antioxidant activity of the heme-derived metabolites (biliverdin and bilirubin) (45, 12) generated by HO catalysis and the cytoprotective actions of carbon monoxide on vascular endothelium and nerve cells (29). Therefore, it is now widely accepted that induction of HO-1 expression represents an adaptive response that increases cell resistance to oxidative injury (8, 36). In the brain, astrocytes strongly express HO-1 in response to injury (24), and the heme oxygenase pathway has been shown to act as a fundamental defensive mechanism for neurons exposed to an oxidant challenge (11). Moreover, deregulation of the HO system has been associated with the pathogenesis of several neurodegenerative disorders, including Alzheimer's disease and multiple sclerosis (29, 46). The HO-1 isozyme is transcriptionally regulated by a large variety of stimuli. These include its substrate, heme; oxidative and nitrosative stress; signaling proteins like nerve growth factor and tumor necrosis factor-α (9); phenolic compounds such as curcumin and caffeic acid phenetheyl ester (40), ethyl ferulate (38), carnosol (31), and rosolic acid (16). Numerous putative regulatory sites have been found in the promoter of HO-1 gene (19).

Recently, a new class of AP-1-related sites, shown to mediate the expression of genes involved in the cellular stress response, included HO-1 (26). These regions are termed Stress Response Elements or Antioxidant Response Elements (AREs) (27) and are tightly regulated by the redox-sensitive transcription factor Nrf2 (NF-E2-related factor-2) (3). Nrf2, a member of the Cap'n'Collar family of transcription factors, is sequestered in the cytoplasm by binding to protein Keap1 in nonstimulated conditions. However, several stimuli, including oxidative stress, lead to the disruption of this complex, freeing Nrf2 for translocation to the nucleus and dimerization with basic leucine zipper transcription factors such as Maf and Jun family members (3). It has been shown that some polyphenols, including curcumin, upregulate HO-1 through Nrf2 signaling pathway in porcine renal epithelial cells (4) and PC-12 cells (31).

In the present study, we analyzed the effect of curcumin on the antioxidant enzyme HO-1 gene expression in cultured astrocytes and neurons. Our results indicate that curcumin activates HO-1 and phase II enzymes expression in astrocytes, probably by activation of transcription factor Nrf2, and this activation is able to effort a significant cytoprotection in cultured neurons exposed to oxidative stress.

MATERIALS AND METHODS

Chemicals and reagents

Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) was obtained from Sigma Chemical (St. Louis, MO, USA). Glucose oxidase (GOX), which generates hydrogen peroxide in the culture medium, and all other reagents were from Sigma unless otherwise specified. Zinc protoporphirin IX (Zn-PP-IX), a specific inhibitor of HO activity, was from Porphyrin Product (Logan, UT, USA). Rabbit polyclonal antibodies directed against HO-1 and HO-2 were obtained from Stressgen (Victoria, British Columbia, Canada). Anti-Nrf2 and anti-Sp1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Rat type 1 astrocytes (DI TNC1) (37) and rat hippocampal neurons (H 19–7) (15) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured according to the manufacturer's instructions. Cells were grown in 75-cm^2 flasks and maintained at 37°C in a humid atmosphere of air and 5% CO_2. Confluent cells were exposed to various concentrations of curcumin. After each treatment (6 or 24 h), cells were harvested for the determination of heme oxygenase activity, and HO-1 and HO-2 mRNA and protein levels. Neurons, growing in 24 wells, were also exposed to different concentrations of curcumin, and cell viability was determined at 24 h. A group of cultured astrocytes were pretreated for 12 h with CUR 25 μ*M* or *S*-nitroso-*N*-acetylpenicillamine (SNAP) 0.5 m*M* before immunocytochemistry was performed. Other neurons were pretreated for 12 h with CUR 5 μ*M* or CUR 5 μ*M* + Zn-PP-IX 10 μ*M* and then exposed for 2 h to 50 mU/ml GOX, before cell viability was determined.

Immunocytochemistry

The nontreated and treated astrocytes were fixed with 4% formaldehyde in PBS (pH 7.4) at room temperature for 5 min. After the cells were washed three times with PBS 1X, they were permeabilized with 1% Triton-X100 in PBS (pH 7.4), and the nonspecific binding sites were blocked with a "blocking solution" containing BSA 1%, goat serum 1%, and fetal calf serum 0.1% in PBS 1X. Cells were then stained with rabbit anti-HO-1/HO-2 antibodies overnight at 4°C in a humid atmosphere. The dilution of these antibodies was 1:500. After the cells were washed three times with PBS 1X, a secondary anti-rabbit IgG produced in goat and conjugated with fluorescein (Vector Laboratories, Burlingame, CA, USA) was added to the cells and incubated at room temperature for 2 h in the dark. The cells were then washed three times with PBS 1X, sealed with VECTASHIELD (Vector Laboratories) and observed under a confocal microscope.

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 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 (FP) and reverse (RP) primers used to amplify HO isoforms and Phase II enzymes, and expected amplification products, are listed in Table 1. 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 FP and RP. 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, USA). PCR amplifications were performed with a Light-Cycler (Roche Diagnostics) using the following four cycle programs: (a) denaturation of cDNA (1 cycle: 95°C for 10 min); (b) amplification (40 cycles: 95°C for 0 sec, 58°C for 5 sec, 72°C for 10 sec); (c) melting curve analysis (1 cycle: 95°C for 0 sec, 70°C for 10 sec, 95°C for 0 sec); (d) cooling (1 cycle: 40°C for 3 min). Temperature transition rate was 20°C/sec except for the third segment of the melting curve analysis where it was 0.2°C/sec. 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. Specificity of PCR products obtained was characterized by melting curve analysis, followed by electrophoretic-gel visualized by ethidium bromide staining and by DNA sequencing.

Western blot analysis for HO-1 and HO-2

After treatment with curcumin, samples of neurons were analyzed for HO-1 and HO-2 protein expression using a Western immunoblot technique as described previously (38). Briefly, an equal amount of proteins (30 µg) for each sample was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes; the nonspecific binding of antibodies was blocked with 3% nonfat dried milk in Tris-buffered saline (TBS-T). Membranes were then probed with a polyclonal rabbit anti-HO-1 and anti-HO-2 antibodies (Stressgen) (1:1000 dilution in TBS-T, pH 7.4) for 2 h at room temperature. After three washes with TBS-T, 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.

Heme oxygenase activity assay

Heme oxygenase activity was determined at the end of each treatment as described previously by our group (40). 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 mixture was incubated in the dark at 37°C for 1 h and the reaction was stopped by the addition of 1 ml of chloroform. After vigorous vortex and

TABLE 1. SEQUENCES OF OLIGONUCLEOTIDE PRIMERS FOR HO ISOFORMS AND PHASE II DETOXIFICATION ENZYMES MRNAS

Name	*GenBank Acc. N.*	*Sequence*	*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	
QUIN-R-F	J02640.1	CTTTCTGTGGGCCATCATTT	224-bp
QUIN-R-R	J02640.1	GAGGCCCCTAATCTGACCTC	
UDPG-F	J02589.1	TCACCATGACCAGCCTATGA	167-bp
UDPG-R	J02589.1	TGACTGCAAAACAGGTGAGC	
GLUST-F	J03752	GTCCTCCTGGGATTCAGTCA	248-bp
GLUST-R	J03752	TCGTCAGTCCGAAGGAACTT	
EPHX-F	NM_022936	TCCCAGTGGGTACCACTCAT	199-bp
EPHX-R	NM_022936	GCCAGTTGTTGGTGACAATG	

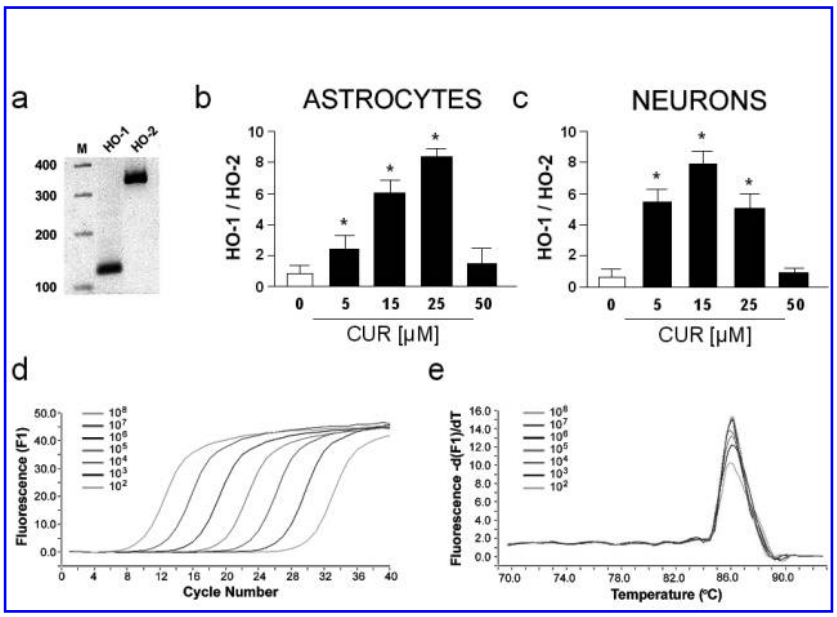

FIG. 1. Real-time quantification of HO-1 and HO-2 mRNA levels by RT-PCR in astrocytes (b) and neurons (c) treated with curcumin. Specific primers for HO-1 and HO-2 were used to amplify rat RNA (**a**). Total RNA from different samples and known amounts of external standards (purified PCR product, 102 to 108 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 (**d**). The specificity of the products amplified was evaluated by melting curve analysis (**e**). Astrocytes (**b**) and neuron (**c**) expression of HO-1 transcript relative to the expression of HO-2 (mean ± SEM) for the different curcumin concentration after 6 h treatment. *, $p < 0.05$ versus 0 μ*M* CUR.

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}).

Cell viability assay

Neurons were exposed to curcumin 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 (40). 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.

Preparation of nuclear extract and Western blot for Nrf2

Astrocytes were washed twice with PBS 1X. Cells were then harvested in 1 ml PBS 1X and centrifuged at 3000 rpm for 3 min at 4°C. The cell pellet was carefully resuspended in 200 μl of cold buffer containing 10 m*M* HEPES (pH 7.9), 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 μ*M* DTT, and complete protease inhibitor cocktail (Roche, Mannheim, Germany). The pellet was then incubated on ice for 15 min to allow cells to swell. After this time, 15 μl of 10% NP-40 was added and the tube was vortexed for 10 s. The homogenate was then centrifuged at 3000 rpm for 3 min at 4ºC and the nuclear pellet was resuspended in 30 μl of cold buffer consisting of 20 m*M* HEPES (pH 7.9), 0.4 *M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 μ*M* DTT, and protease inhibitors. The pellet was then incubated on ice for 15 min and vortexed for 10–15 s every 2 min. The nuclear extract was finally centrifuged at 13,000 rpm for 5 min at 4ºC. The supernatant containing the nuclear proteins was resolved by SDS-polyacrylamide gel and submitted to immunoblot analysis using anti-Nrf2 (1:500 dilution) and anti-Sp1 (1:500 dilution) antibodies.

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 ± SEM, and differences between groups were considered to be significant at $p < 0.05$.

RESULTS

Curcumin upregulates heme oxygenase activity, HO-1 mRNA and protein expression in astrocytes and hippocampal neurons.

The exposure of astrocytes and neurons for 6 h to different concentration of curcumin (5, 15, 25, and 50 μ*M*) resulted in a gradual and significant ($p < 0.05$) increase in HO-1 mRNA, as shown by quantitative RT-PCR (Fig. 1), with a maximal value at 25 μ*M* in astrocytes and 15 μ*M* in neurons. This gene activation was strongly associated with a marked upregulation of HO-1 protein. Treatment of astrocytes with curcumin 25 μ*M* upregulated expression of HO-1 protein at both cytoplasmatic and nuclear level, as shown by immunofluorescence analysis under a laser-scanning confocal microscope, while treatment with 0.5 m*M* *S*-nitroso-*N*-acetylpenicillamine (SNAP), a potent inducer of HO-1, led to HO-1 protein upregulation only in the cytoplasm (Fig. 2). Moreover we extended our previous findings by showing that curcumin markedly increases heme oxygenase activity and HO-1 protein in neurons (Fig. 3). Heme oxygenase activity and protein expression was maximally upregulated after 6 h treatment with 15 μ*M* curcumin. Although to a lesser extent, the in-

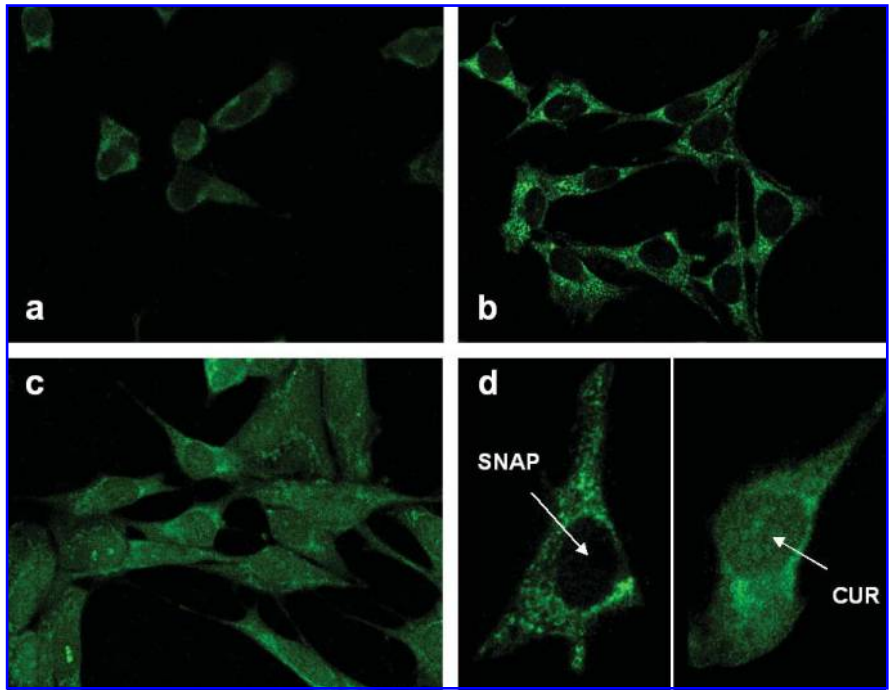

FIG. 2. Immunocytochemistry staining performed in astrocytes. The cells were stained with rabbit anti-HO-1 antibody before (**a**) and after 6 h curcumin (CUR) 25 μ*M* (**b**) or *S*-nitroso-*N*-acetylpenicillamine (SNAP) 0.5 m*M* (**c**) stimulation. The immunoreactive signals were revealed by the addition of the fluorescent secondary antibodies. Panel **d** shows the cells of panels **b** and **d** at higher magnification.

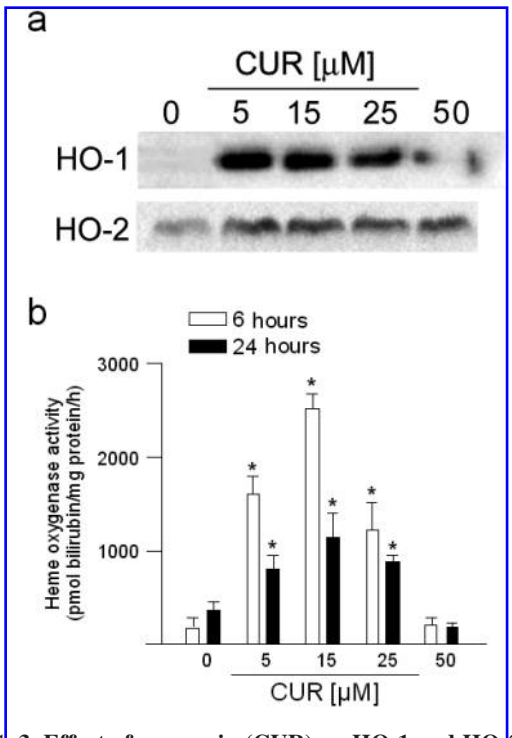

FIG. 3. Effect of curcumin (CUR) on HO-1 and HO-2 protein expression and heme oxygenase activity in neurons. Western blots showing HO-1 and HO-2 protein levels in neurons after treatment with curcumin (0–50 μ*M*) for 6 h (**a**). These panels are representatives of three different experiments with similar results. Heme oxygenase activity was measured in neurons (**b**) after short (6 h) or prolonged (24 h) exposure to various concentrations of CUR (0–50 μ*M*). Each bar represents the mean ± SEM of five independent experiments. *, $p < 0.05$ versus 0 μ*M* CUR.

crease of HO activity was also found in cells after 24 h CUR treatment (Fig. 3b).

In contrast, CUR failed to increase HO-1 expression and activity when higher concentrations (50 μ*M*) of this drug were used. The reduced ability of CUR to increase heme oxygenase activity at high concentrations (50 μ*M*) correlated with a cytotoxic effect exerted by this compound (see below).

Effect of curcumin on cell viability

To determine a potential toxic effect of phenolic compounds on neurons, cells grown to confluence in 24 wells were incubated with increasing concentrations of CUR for 24 h. When the concentration of these drugs did not exceed 15 μ*M*, cell viability (determined using the Alamar Blue assay)

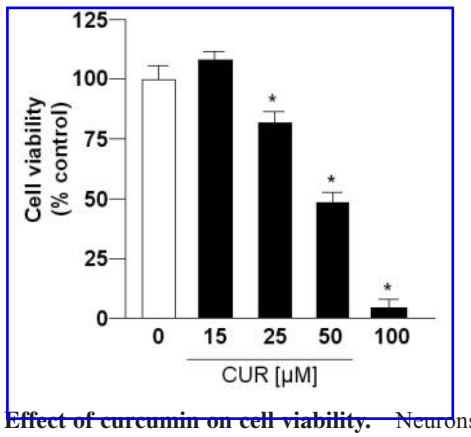

FIG. 4. Effect of curcumin on cell viability. Neurons were exposed for 24 h to various concentrations (0–100 μ*M*) of curcumin (CUR) in complete medium. Cell viability was measured spectrophotometrically using an Alamar Blue assay. Data are expressed as the mean ± SEM of six independent experiments. *, $p < 0.05$ versus 0 μ*M* CUR.

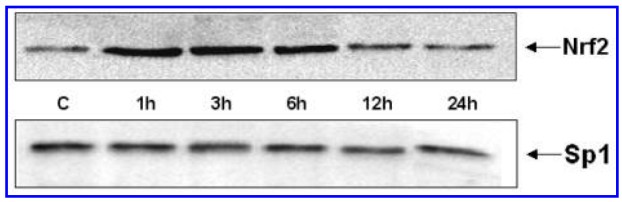

FIG. 5. Curcumin increases the levels of the Nrf2 transcription factor in astrocytes nuclear extracts. Western blots showing increases in Nrf2 protein levels in nuclear extracts of cells treated with 25 μ*M* curcumin whereas the levels of the housekeeping transcription factor Sp1 were stable.

as well as cell morphology observed under the microscope were fully preserved throughout the incubation period (Fig. 4). Treatment of neurons with 50 μ*M* CUR was highly cytotoxic, causing 52% reductions in cell viability.

Curcumin activates Nrf2 expression in astrocytes

Astrocytes were exposed to curcumin at the final concentration of 25 μ*M* to evaluate the expression of Nrf2 protein over time. As shown in Figure 5, treatment with curcumin caused a significant time-dependent increase in Nrf2 protein expression in the nuclear extracts. Quantification of three independent Western blots showed that after 1 h exposure to 25 μ*M* curcumin, Nrf2 expression significantly increased and remained upregulated until 12 h, whereas the levels of the housekeeping transcription factor Sp1 were stable.

Curcumin upregulates phase II detoxification enzymes mRNA in astrocytes

The exposure of astrocytes for 6 h to different concentrations of curcumin (5, 15, 25, and 50 μ*M*) resulted in a significant ($p < 0.05$) increase of quinone reductase and glutathione *S*-transferase, two members of Phase II detoxification enzymes, with a maximal value at 5 μ*M* and 15 μ*M* (Fig. 6).

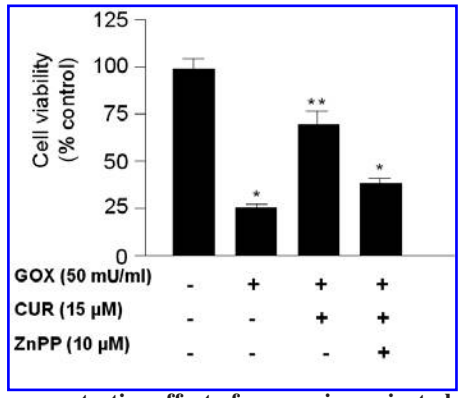

FIG. 7. Neuroprotective effect of curcumin against glucose oxidase induced cell death. Rat hippocampal neurons were treated with 15 μ*M* of curcumin (CUR) for 12 h in the presence or absence of 10 μ*M* of zinc protoporphyrin IX (Zn-PP-IX). After this pretreatment, cells were incubated for 2 h with glucose-oxidase (GOX) 50 mU/ml. After these treatments, cells were washed and viability was assessed by Alamar Blue assay. *, $p < 0.05$ versus GOX; **, $p < 0.05$ versus GOX plus CUR.

Nevertheless curcumin was not effective in inducing cytosolic epoxide hydrolase and UDP glucuronosyltransferase, two other members of phase II enzymes family.

HO expression by curcumin protects neuronal cells from glucose oxidase-induced cell death

We examined the effects of CUR pretreatment on GOX-induced cell death in H 19–7 rat hippocampal neurons. The neuronal cells were pretreated 12 h with CUR at concentration of 15 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 74% decrease in cell viability ($p < 0.05$; Fig. 7). However, pretreatment of the cells with CUR recovered the viability of the cells to 72% of control ($p < 0.05$; Fig.7). The involvement of HO-1 in the cytoprotective effect of CUR was confirmed using an inhibitor of HO activity, Zn-PP-IX, which at the con-

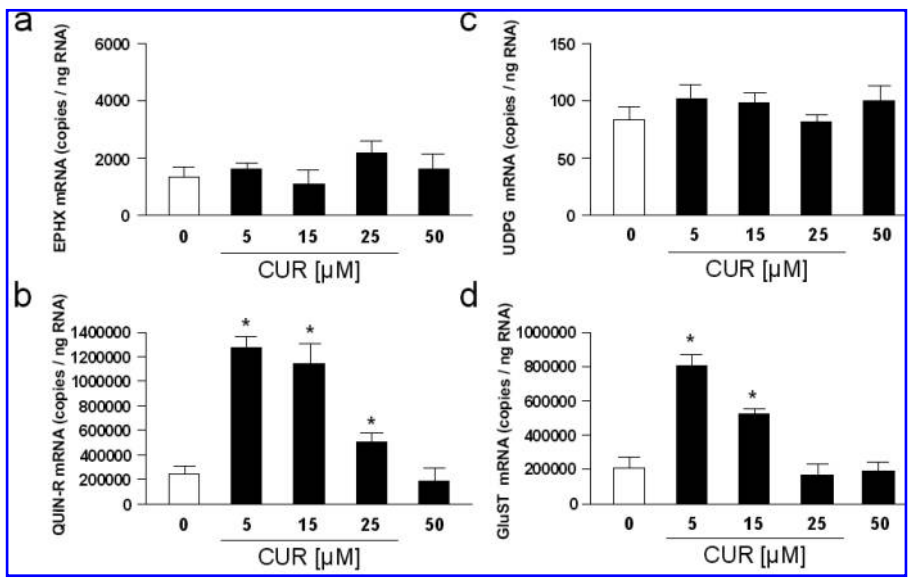

FIG. 6. Real-time quantification of Phase II detoxification enzymes mRNA levels by RT-PCR in astrocytes treated with curcumin. Transcript levels for cytosolic epoxide hydrolase (EPHX) (**a**), quinone reductase (QUIN-R) (**b**), UDP glucuronosyltransferase (UDPG) (**c**), and glutathione *S*-transferase (GLUST) (**d**) in astrocytes treated with different curcumin concentrations, assessed by real-time quantitative RT-PCR.

centration of 10 μ*M* significantly blocked CUR-mediated suppression of GOX-induced cell death (Fig. 7). These data show that the cytoprotective effect of CUR might be due, in part, to the induction of HO-1.

DISCUSSION

Epidemiological studies repeatedly show association between food intake and the incidence and severity of chronic diseases, giving rise to the concept that dietary chemicals, by modulating expression of target genes, can influence the balance between healthy and disease states, and play a role in the onset, incidence, progression, and severity of chronic pathologies (22). Diseases preventive strategy based on dietary agents is particularly attractive because of our long-standing exposure to them, their relative lack of toxicity, and encouraging indications from epidemiology.

Curcumin, the yellow curry spice derived from turmeric, possesses both anti-inflammatory and antioxidant properties (44, 47), and has recently received much attention as a promising agent for reducing the risk of oxidative stress-related diseases, ranging from cardiovascular pathologies to cancer (20, 41).

In a recently completed Phase I clinical trial, oral curcumin at a daily dose of 3.6 g was, in general, well-tolerated and decreased inducible PGE2 by approximately 60%, as observed in blood samples taken 1 h after curcumin assumption on days 1 and 29 of treatment (41).

In view of its efficacy and apparent low toxicity, curcumin has been also proposed for the prevention of neurodegenerative pathologies, particularly for AD (43, 7). In fact, both oxidative damage and inflammation are elevated in AD patients, suggesting a potential utility of a long-term therapy with a compound endowed with anti-inflammatory/antioxidant properties (18). This idea has been reinforced by epidemiological studies showing that, in India where this spice is widely used in daily diet, there is a reduced age-adjusted prevalence of AD (in patients between 70 and 79 years of age is 4.4-fold less than that of the United States) (10), as well as a lower prevalence of Parkinson's disease (34). Consistent with its possible use in neurodegenerative diseases, curcumin has been reported to decrease oxidative damage and amyloid deposition in a transgenic mouse model of Alzheimer's disease (49), and to reverse Aβ-induced cognitive deficits and neuropathology in rats (17). A possible mechanism of curcumin's activity relates to its ability to overexpress highly protective inducible genes, involved in the cellular stress response (8).

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 (9). Considering the ability of curcumin to induce HO-1 expression and activity in different cell types, we have explored in cultured astrocytes and neurons the effect of this spice on this enzyme. By performing the quantitative Real time PCR analysis of HO-1 transcript, Western blot and HO activity assay, we have found in astrocytes and neurons that curcumin increased heme oxygenase activity, HO-1 mRNA and protein in a concentration-dependent way with a maximal induction at 25 μ*M* for astrocytes and 15 μ*M* for neurons, respectively. Treatment of astrocytes with curcumin 25 μ*M* upregulated expression of HO-1 protein at both cytoplasmic and nuclear levels, as shown by immunofluorescence analysis under laser-scanning confocal microscopy, while treatment with 0.5 m*M* SNAP, a nitric oxide donor that strongly activates HO-1 expression, induced HO-1 protein expression only in the cytoplasm. This different subcellular distribution suggests possible different mechanisms underlying to HO-1 induction. In particular we have seen that curcumin specifically increased the levels of the Nrf2 transcription factor in the astrocyte nucleus. The crucial role of Nrf2/ARE-mediated HO-1 gene expression in response to phenolic compounds, has already been referred in different cell types. In line with this hypothesis we have also found in astrocytes exposed to 5–15 μ*M* curcumin a significant increase in the expression of quinone reductase and glutathione *S*-transferase, two members of phase II detoxification enzymes typically induced by the activation of Nrf2/ARE pathway (3, 14).

Therefore, in addition to its intrinsic antioxidant nature, a low concentration of curcumin activates in astrocytes Nrf2 and upregulates the expression of antioxidant HO-1 and phase II enzymes, improving cellular defensive mechanisms. Interestingly, both in astrocytes and neurons, curcumin (1–25 μ*M*), without causing a significant cytotoxicity, increased HO activity with a bell-shaped dose-response curve and this finding is accompanied by a parallel bell-shaped increase in HO-1 mRNA and protein (Figs. 2–4). This evidence is consistent with better efficacy of curcumin at low concentration, compared to higher dosage, in *in vivo* models of AD (28, 49).

In this study, we also demonstrated the cytoprotective effects of curcumin against oxidative damage in neuronal cells. Treatment of the cells with low concentrations of CUR resulted in high resistance to GOX/hydrogen peroxide induced cell death. Zn-PP-IX, a specific inhibitor of HO activity, abrogated the protective effect of CUR, demonstrating a direct involvement of HO-1 induction in the antioxidant mechanisms promoted by this polyphenol. In conclusion, a corollary of our study is that pharmacological activation of HO-1 by this food-related compound (probably due to Nrf2 nuclear translocation) efficiently protects neurons from oxidative stress and should be evaluated as a new therapeutic approach in degenerative processes such as Alzheimer's and Parkinson diseases that correlate with oxidative damage. However, we favor the nonexclusive hypothesis that curcumin also upregulates other intracellular antioxidant systems that do not belong to the Nrf2/HO-1 module and that also contribute to the long-term antioxidant properties of this compound.

Further *in vitro* and *in vivo* studies using curcumin-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.

ACKNOWLEDGMENTS

This work was supported, in part, by grants from the Équipe Enervit Foundation (GS), and by grants from Wellcome Trust and FIRB RBNE01ZK8F (VC).

ABBREVIATIONS

AD, Alzheimer's disease; CUR, curcumin; EPHX cytosolic epoxide hydrolase; GLUST, glutathione *S*-transferase; GOX, glucose oxidase; HO, heme oxygenase; QUIN-R, quinone reductase; UDPG, UDP glucuronosyltransferase; Zn-PP-IX, zinc protoporphirin IX.

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Received after revision September 12, 2005; accepted September 14, 2005.

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