Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element

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The transcription factor Nrf2, which normally exists in an inactive state as a consequence of binding to a cytoskeleton-associated protein Keap1, can be activated by redox-dependent stimuli. Alteration of the Nrf2–Keap1 interaction enables Nrf2 to translocate to the nucleus, bind to the antioxidant-responsive element (ARE) and initiate the transcription of genes coding for detoxifying enzymes and cytoprotective proteins. This response is also triggered by a class of electrophilic compounds including polyphenols and plant-derived constituents. Recently, the natural antioxidants curcumin and caffeic acid phenethyl ester (CAPE) have been identified as potent inducers of haem oxygenase-1 (HO-1), a redox-sensitive inducible protein that provides protection against various forms of stress. Here, we show that in renal epithelial cells both curcumin and CAPE stimulate the expression of Nrf2 in a concentration- and time-dependent manner. This effect was associated with a significant increase in HO-1 protein expression and haem oxygenase activity. From several lines of investigation we also report that curcumin (and, by inference, CAPE) stimulates *ho-1* gene activity by promoting inactivation of the Nrf2–Keap1 complex, leading to increased Nrf2 binding to the resident *ho-1* AREs. Moreover, using antibodies and specific inhibitors of the mitogen-activated protein kinase (MAPK) pathways, we provide data implicating p38 MAPK in curcumin-mediated *ho-1* induction. Taken together, these results demonstrate that induction of HO-1 by curcumin and CAPE requires the activation of the Nrf2/ARE pathway.

Key words: caffeic acid phenethyl ester, cytoprotection, haem oxygenase-1 regulation, plant-derived constituents.

INTRODUCTION

Haem oxygenase-1 (HO-1) is a ubiquitous and redox-sensitive inducible stress protein that degrades haem to CO, iron and biliverdin [1–3]. The importance of this protein in physiological and pathological states is underlined by the versatility of HO-1 inducers and the protective effects attributed to haem oxygenase products in conditions that are associated with moderate or severe cellular stress.

We have recently shown that curcumin and caffeic acid phenethyl ester (CAPE), two plant-derived polyphenolic compounds, are potent inducers of HO-1 in vascular endothelial and neuronal cells [4,5] and we hypothesized that part of the pleiotropic and beneficial actions attributed to these and other chemically related natural substances could be explained by their intrinsic ability to strongly activate the haem oxygenase pathway [5]. In view of the increasing evidence corroborating the importance of CO and bilirubin to counteract cellular dysfunction [6–10], the activation of HO-1 by natural compounds offers a great advantage for therapeutic purposes, as curcumin and CAPE could become part of the human diet and be consumed daily as herbal supplements. Both curcumin and CAPE exert a diversity of beneficial effects including inhibition of mutagenesis and chemically induced carcinogenesis [11,12], as well as prevention of vascular and neurodegenerative diseases [13,14]. A recent study revealed that under severe hypoxic conditions the potency of curcumin to increase endothelial HO-1 expression and consequently protect cells against oxidative stress is highly amplified [4].

Unlike most ‘classical’ HO-1 inducers, which are strictly dependent on their oxidant potential to transcriptionally activate *ho-1* gene expression, curcumin and CAPE are known to possess also antioxidant as well as anti-tumour and anti-inflammatory properties [11,12,15,16]. By virtue of Michael reaction acceptor functionalities and its electrophilic characteristics, curcumin and several other structurally related polyphenolic compounds induce the activities of phase II detoxification enzymes, which appear to be crucial in protection against carcinogenesis and oxidative stress [17]. Among these defensive systems are γ-glutamylcysteine synthetase, glutathione S-transferases and NADP(H):quinone oxidoreductase. The co-ordinated induction of these cytoprotective genes is mediated through *cis*-regulatory DNA sequences located in the promoter or enhancer region, which are known as antioxidant-responsive elements (AREs) or stress-responsive elements [the active sequences in the mouse *ho-1* gene, referred to as the stress-responsive element (StRE), are structurally and functionally similar to the ARE, a more commonly used term: for simplicity, and to avoid confusion, the latter terminology will be used throughout the remainder of the article]. The consensus ARE resembles the Maf-recognition element and can be specifically bound by a combination of the basic-leucine zipper (bZIP) transcriptional factors including Jun, Fos, Maf and Nrf2. Among them, Nrf2 plays a central role in the transcriptional regulation of antioxidant and detoxifying genes.

Abbreviations used: CAPE, caffeic acid phenethyl ester; HO-1, haem oxygenase-1; ARE, antioxidant-responsive element; MAPK, mitogen-activated protein kinase; bZIP, basic-leucine zipper; EMSA, electrophoretic mobility shift assay; ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinase; AP-1, activator protein 1.

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Studies using Nrf2-deficient mice have confirmed their inability to express cytoprotective genes upon stimulation with carcinogens [18], and cells lacking the Nrf2 gene display a higher susceptibility to oxidant-mediated cell injury and death [19]. Nrf2 is a potent positive regulator of the *ho-1* gene and other detoxifying enzymes [20]; however, a direct link between curcumin-mediated *ho-1* induction and activation of Nrf2 expression via stimulation of ARE-binding activity remains to be examined. Here, we analysed the involvement of Nrf2 and ARE activation in *ho-1* induction by curcumin and CAPE in renal epithelial cells.

MATERIALS AND METHODS

Chemicals and reagents

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], CAPE and all the reagents for luciferase assays were obtained from Sigma (St. Louis, MO, U.S.A.). Stock solutions of curcumin and CAPE (5 mM) were prepared in ethanol. Tissue-culture media were from Life Technologies (Rockville, MD, U.S.A.) and fetal bovine serum was obtained from Mediatech (Herndon, VA, U.S.A.). Oligonucleotides were synthesized by IDT (Coralville, IA, U.S.A.). Radiolabelled nucleotides were obtained from NEN Life Science Products (Boston, MA, U.S.A.). Polyclonal antibodies for HO-1 were from Stressgen (Victoria, Canada). Anti-Nrf2 and all the other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All other chemicals were of reagent grade and obtained from Sigma unless otherwise specified.

Cell culture and experimental protocols

Porcine renal epithelial proximal tubule cells (LLC-PK_1) and rat kidney epithelial cells (NRK-52E) were purchased from the American Tissue Culture Collection (Manassas, VA, U.S.A.). Cells were cultured using Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cells were grown in 75 cm^2 flasks and kept at 37 °C in a humidified atmosphere of air and 5 % CO_2. Confluent LLC-PK_1 cells were exposed to various concentrations of curcumin, CAPE or other phenolic compounds for different times. After each treatment, cells were harvested for the measurements of haem oxygenase activity, HO-1 and Nrf2 protein expression and electrophoretic mobility shift assays (EMSAs; see below).

Haem oxygenase activity assay and Western blot for HO-1

Haem oxygenase activity was determined at the end of each treatment using a modification of a method described previously by our group [21]. Briefly, harvested cells were subjected to three cycles of freeze–thawing and the suspension was added to a reaction mixture (1 ml final volume, pH 7.4) containing $MgCl_2$ (2 mM), NADPH (0.8 mM), glucose 6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 unit), 3 mg of rat liver cytosol and the substrate haemin (20 μM). The reaction was conducted at 37 °C in the dark for 1 h, terminated by the addition of 1 ml of chloroform, and the extracted bilirubin was measured by the difference in absorbance between 464 and 530 nm ($\varepsilon = 40\ mM^{-1} \cdot cm^{-1}$). The total protein content was determined using a Bio-Rad DC protein assay (Bio-Rad, Herts., U.K.) and haem oxygenase activity expressed as pmol of bilirubin/mg of protein per h.

Samples of cells were also analysed by the Western immunoblotting technique as described previously [22]. Briefly, 30 μg of protein was separated by SDS/PAGE, transferred overnight on to nitrocellulose membranes, and the non-specific binding of antibodies was blocked with 3 % non-fat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (1 : 1000 dilution in Tris-buffered saline, pH 7.4) for 2 h at room temperature. After three washes with PBS containing 0.05 % (v/v) Tween-20, blots were visualized using an amplified alkaline phosphatase kit from Sigma (Extra-3A) and the relative density of bands was analysed using an imaging densitometer (model GS-700; Bio-Rad).

Preparation of nuclear extract and Western blot for Nrf2

LLC-PK_1 cells were washed twice with 1 × PBS. Cells were then harvested in 1 ml of PBS and centrifuged at 800 ***g*** for 3 min at 4 °C. The cell pellet was carefully resuspended in 200 μl of cold buffer A, consisting of 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 μM dithiothreitol 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 % Nonidet P-40 was added and the tube was vortex-mixed for 10 s. The homogenate was then centrifuged at 800 ***g*** for 3 min at 4 °C. The resulting nuclear pellet was resuspended in 30 μl of cold buffer B, consisting of 20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 μM dithiothreitol and protease inhibitors. The pellet was then incubated on ice for 15 min and vortex-mixed for 10–15 s every 2 min. The nuclear extract was finally centrifuged at 15 000 ***g*** for 5 min at 4 °C. The supernatant containing the nuclear proteins was loaded on an SDS/polyacrylamide gel, and Western blot analysis using Nrf2 antibodies (1 : 500 dilution) was performed as described above.

Plasmids

Construction of plasmids pHO15luc, pHO15lucΔE1, pHO-15lucΔE2, pHO15lucΔ(E1 + E2), pE1luc and its corresponding mutant pE1lucM739, pE2luc and its corresponding mutant pE2lucM45, and p3XStREluc and its corresponding mutant p3X-StREM2luc has been described previously [23–26]. Plasmid pCMVβ-gal encodes *Escherichia coli* β-galactosidase and was used to normalize for variations in transfection efficiency. Expression plasmids for Nrf2 and Keap1 were kindly provided by Dr Stuart Orkin (Harvard Medical School and Howard Hughes Medical Institute, Boston, MA, U.S.A.) and Dr Masayuki Yamamoto (The Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan). The plasmid encoding Nrf2(29–597) was generated by deletion of 5′ sequences up to the unique *Bgl*II site in the mouse Nrf2 cDNA.

Cell transfection and enzyme assays

NRK-52E cells were seeded in 12-well plates (1 × 10^5 cells/well), cultured for 20 h and transfected with a DNA mixture consisting of (per well) 100 ng of the appropriate luciferase construct, 50 ng of pCMVβ-gal and 100 ng of the appropriate empty vector or effector plasmid using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) according to the manufacturer's recommendations. Then, 24 h later, the transfection medium was removed and replaced with medium containing vehicle (ethanol) or 10 μM curcumin.

Where indicated, vehicle or mitogen-activated protein kinase (MAPK) inhibitors were added 30 min prior to the addition of curcumin. After a 5 h incubation period, cells were harvested for preparation of cellular extract and measurement of luciferase and β-galactosidase activities [27]. β-Galactosidase-normalized luciferase activities are presented.

EMSAs

These assays were carried out on both NRK-52E and LLC-PK_1 cells. Confluent LLC-PK_1 cells were treated in complete Dulbecco's modified Eagle's medium with 15 μM curcumin or CAPE for 3 or 6 h. NRK-52E cells were plated (4×10^6 cells/100 mm plastic dish) and cultured in complete medium for 40–48 h, and then treated with vehicle or 10 μM curcumin in serum-free medium for 3 or 6 h. Whole-cell extracts were prepared and EMSA reactions carried out as described previously [25]. A ^{32}P-labelled double-stranded oligonucleotide containing the sequence 5′-GATCTTTTATGCTGAGTCATGGTTT-3′ (core ARE underlined) was used as the probe in EMSA reactions. In antibody supershift assays, 1 μl (2 μg) of pre-immune IgG or specific rabbit polyclonal antibodies was added to the reaction mixture and incubated for 20 min at room temperature prior to electrophoresis. In the case of MafG, 1 μl of whole serum (pre-immune and anti-MafG) was used.

Western blot for MAPKs

NRK-52E cells were plated (5×10^5 cells/60 mm plate) and cultured for 48 h. The culture media was replaced with serum-free medium and curcumin (10 μM) was added to individual plates at staggered time points so that all cells were exposed to serum-free medium for the same period. Cells were washed with cold PBS and lysed directly in 100 μl of 1 × electrophoresis sample buffer containing 2 mM EGTA and 50 mM NaF. Protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (Sigma). Samples of 20 μg were size-fractionated on 10 % denaturing polyacrylamide gels and protein-blot analysis was carried out using the ECL Western Blotting System (Amersham Biosciences) according to the manufacturer's recommendations. Antibodies to non-phosphorylated and phosphorylated MAPKs were obtained from New England Biolabs and used at dilutions and under conditions recommended by the manufacturer.

Statistical analysis

Differences in the data among the groups were analysed by using one-way ANOVA combined with the Bonferroni test. Values were expressed as means ± S.E.M. and differences between groups were considered to be significant at $P < 0.05$.

RESULTS

Effect of curcumin and CAPE on haem oxygenase activity and HO-1 expression in renal epithelial cells

Curcumin caused a significant increase in haem oxygenase activity, with a maximal value being observed at 15 μM (see Figure 1A). HO-1 protein expression was also significantly elevated by curcumin (Figure 1B); quantification of three independent Western blots showed that in the presence of 5, 10, 15, 20, 30 and 50 μM curcumin, HO-1 protein increased 1.4 ± 0.3, 1.8 ± 0.3, 5.6 ± 1.4, 12.4 ± 2.2, 12.5 ± 1.9 and 4.7 ± 1.0-fold, respectively. Thus at concentrations higher than 30 μM, curcumin appears to be less effective in stimulating haem oxygenase activity and HO-1 protein expression. In a similar fashion, CAPE also caused a significant increase in haem oxygenase activity (see Figure 2A) and HO-1 protein expression (Figure 2B). Quantification of three independent Western blots showed that

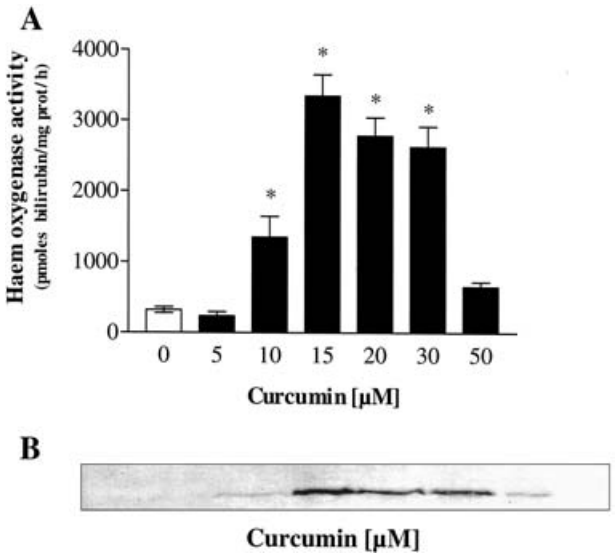

Figure 1 Effect of curcumin on haem oxygenase activity and HO-1 expression in renal epithelial cells

LLC-PK_1 cells were exposed to various concentrations of curcumin for 6 h. Haem oxygenase activity (**A**) and HO-1 protein expression (**B**) were measured as described in the Materials and methods section. The control group is represented by cells incubated with medium alone (0 μM). Each bar represents the mean ± S.E.M. from five independent experiments. *$P < 0.05$ versus 0 μM curcumin.

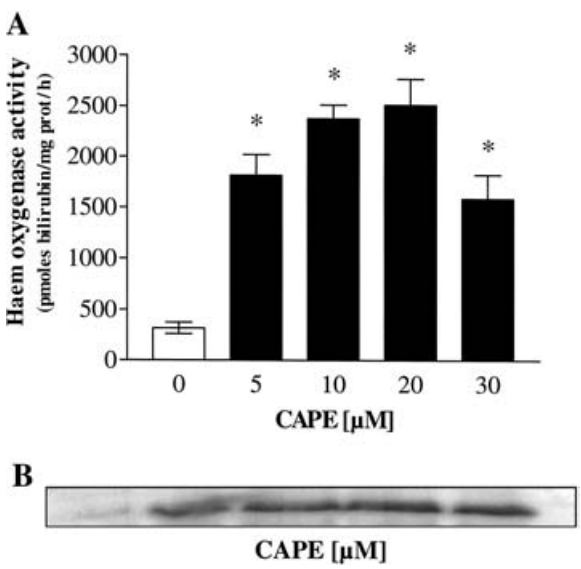

Figure 2 Effect of CAPE on haem oxygenase activity and HO-1 expression in renal epithelial cells

LLC-PK_1 cells were exposed to various concentrations of CAPE for 6 h. Haem oxygenase activity (**A**) and HO-1 protein expression (**B**) were measured as described in the Materials and methods section. The control group is represented by cells incubated with medium alone (0 μM). Each bar represents the mean ± S.E.M. from five independent experiments. *$P < 0.05$ versus 0 μM CAPE.

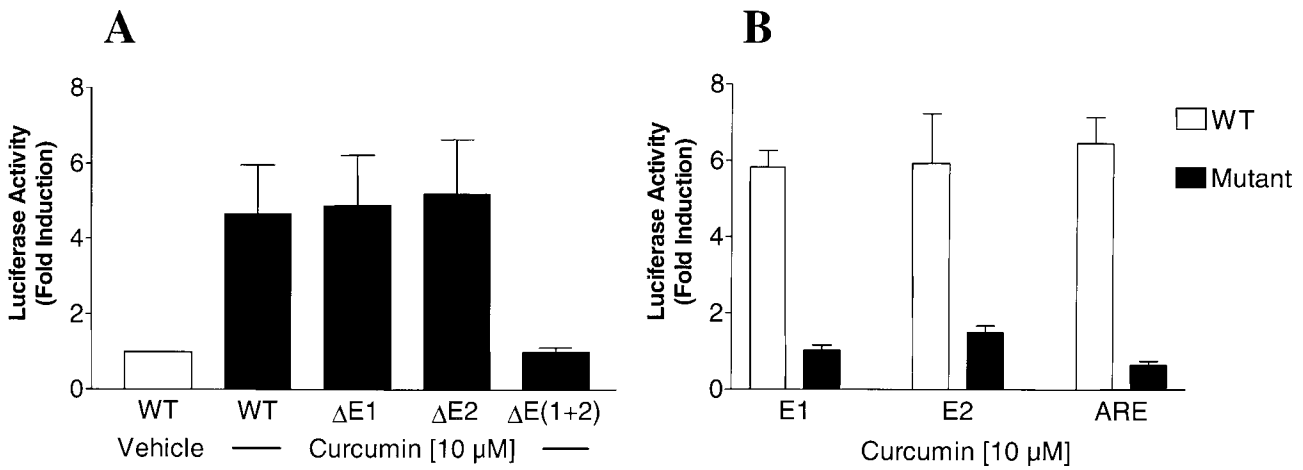

Figure 3 Activation of the mouse *ho-1* gene promoter by curcumin is mediated by the AREs

Duplicate wells of renal epithelial cells were transfected with various mouse *ho-1* gene promoter/luciferase fusion constructs and treated for 5 h with either vehicle or 10 μM curcumin (see the Materials and methods section for details). (**A**) Cells were transfected with the wild-type (WT) 15 kb *ho-1* promoter construct or mutants with deletion of either one or both of the previously identified E1 (268 bp) and E2 (161 bp) enhancers [ΔE(1+2)]. (**B**) Experiments were repeated in which cells were transfected with constructs containing E1, E2 or the ARE fused to a minimal *ho-1* promoter sequence. Normalized activity for the WT construct in the presence of vehicle was arbitrarily assigned a value of 1. Each bar represents the mean $\pm$ S.D. from three to five independent experiments.

in the presence of 5, 10, 20 and 30 μM CAPE, HO-1 protein increased 4.8 ± 1.7, 6.1 ± 2.3, 7.5 ± 2.6 and 8.6 ± 2.3-fold, respectively.

Activation of the mouse *ho-1* gene promoter by curcumin is mediated by the AREs

Induction of HO-1 by most agents is regulated primarily at the level of gene transcription [1]. To characterize the mechanism of HO-1 induction by curcumin, renal epithelial cells were transfected with various mouse *ho-1* gene promoter/luciferase fusion constructs and treated for 5 h with either vehicle or 10 μM curcumin (see the Materials and methods section). Curcumin stimulated the activity of the wild-type 15 kbp *ho-1* promoter by greater than 4-fold (Figure 3A). Deletion of both of the previously identified E1 (268 bp) and E2 (161 bp) enhancers [ΔE(1 + 2)] completely abolished induction, demonstrating that these regions are essential for this response. Interestingly, mutants containing only one enhancer were as responsive as the wild-type promoter, suggesting that, at least with respect to induction by curcumin, the enhancers are redundant. Consistent with this idea, in isolation both E1 and E2 can confer curcumin-responsiveness to the luciferase reporter gene (Figure 3B). Mutation of the ARE within the E1 or E2 enhancers abrogates this response. Furthermore, the ARE sequence by itself, but not its mutant counterpart, is capable of conferring curcumin-responsiveness. Together these results indicate that the ARE is both necessary and sufficient for induction of the *ho-1* gene by curcumin.

Curcumin stimulates ARE-binding activity in NRK cells

EMSA reactions using an ARE probe and whole-cell extracts from NRK cells were carried out to identify DNA-binding proteins potentially responsible for curcumin-mediated *ho-1* gene induction (see Figure 4). Several specific ARE–protein complexes of varying intensities were detected when using extracts from vehicle-treated cells (Figure 4, lane 1). Treatment of NRK cells with curcumin generated an apparently novel and slower-migrating complex (Figure 4, lane 7; marked by an arrow). The consensus core ARE sequence, YGCTGAGTCA, resembles

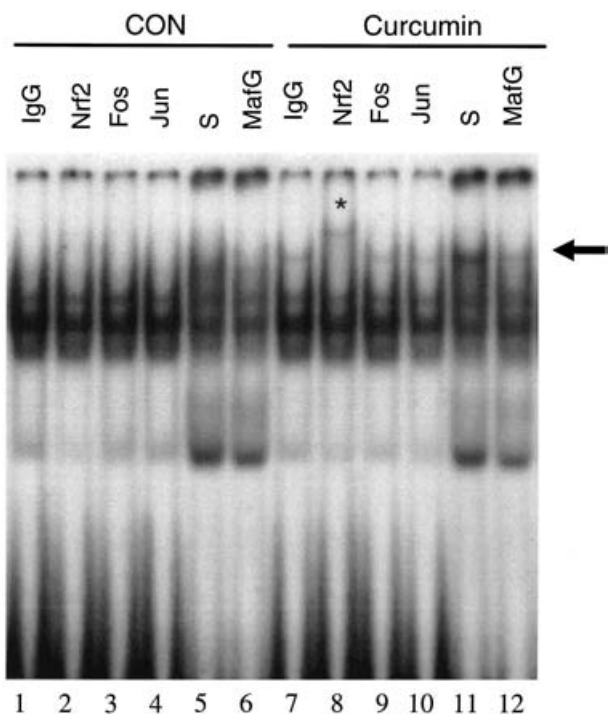

Figure 4 Curcumin stimulates ARE-binding activity in NRK cells

EMSA reactions using a ARE probe and whole-cell extracts from ethanol- (CON) or curcumin- (10 μM) treated NRK cells were carried out as described in the Materials and methods section. Pre-immune IgG (IgG, lanes 1 and 7) was used as a negative control for Nrf2 (lanes 2 and 8), Fos (lanes 3 and 9) and Jun (lanes 4 and 10) antibodies in supershift assays; pre-immune serum (S) was used as a negative control for MafG antibodies. The curcumin-induced complex is marked with an arrow and the supershifted complex is indicated with an asterisk. The use of whole serum (lanes 5, 6, 11 and 12) generates non-specific DNA–protein complexes.

the consensus binding sites for the activator protein 1 (AP-1; Jun–Jun or Jun–Fos dimers), Maf and cap'n'collar-bZIP families of transcription factors, which function as obligate dimers. Supershift EMSA reactions using antibodies directed

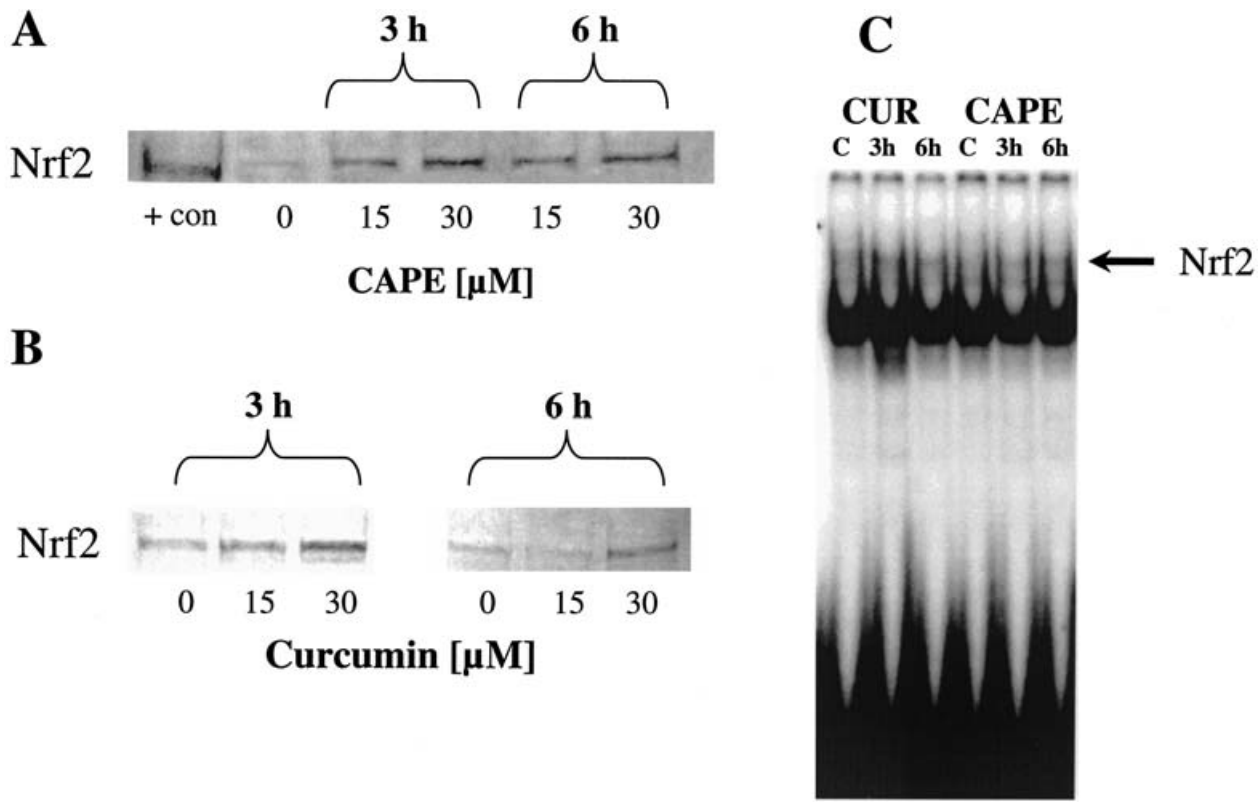

Figure 5 Curcumin and CAPE activate Nrf2 expression and stimulate ARE-binding activity in renal epithelial LLC-PK$_1$ cells

LLC-PK$_1$ cells were exposed to curcumin or CAPE at the final concentration of 15 or 30 μM. Gel electrophoresis was performed on nuclear extracts from cells treated for 3 and 6 h with CAPE (**A**) or curcumin (**B**), and Western-blot analysis using Nrf2 antibodies was carried out as described in the Materials and methods section. This blot is representative of three independent experiments. +con, positive control. (**C**) EMSA showing that treatment of LLC-PK$_1$ cells with curcumin (CUR) or CAPE (15 μM) increases the amount of the Nrf2/ARE complex (see arrow). C, control.

against one or more members of these protein families were carried out to identify the protein constituent(s) within the curcumin-induced DNA–protein complex. The migration of this complex was quantitatively retarded using anti-Nrf2 IgG, but not with pan-Jun or pan-Fos antibodies that recognize all members within their respective families. Nrf2 does not form homodimers, but heterodimerizes with small Maf proteins. Although we have previously provided evidence for a role for Nrf2–MafG heterodimers in cobalt-mediated *ho-1* gene induction [25], MafG was not detected in the curcumin-induced complex. Taken together, these results support a role for Nrf2, but not for AP-1 proteins, in *ho-1* activation by curcumin. The identity of the Nrf2 dimerization partner in this response is not presently known, but does not appear to be MafG.

Curcumin and CAPE activate Nrf2 expression and stimulate ARE-binding activity in renal epithelial cells (LLC-PK$_1$)

LLC-PK$_1$ cells were exposed to curcumin or CAPE at the final concentration of 15 or 30 μM to evaluate the expression of Nrf2 protein over time. As shown in Figures 5(A) and 5(B), treatment with CAPE or curcumin caused a significant dose- and time-dependent increase in Nrf2 protein expression in the nuclear extracts. Quantification of three independent Western blots showed that after 3 h exposure to 15 and 30 μM curcumin, Nrf2 expression increased 2.1 ± 0.7 and 4.4 ± 1.1-fold, respectively; in the presence of 15 and 30 μM CAPE, Nrf2 expression increased 3.1 ± 1.0 and 6.2 ± 1.5-fold, respectively. In addition, EMSA revealed that treatment of LLC-PK$_1$ cells with curcumin or CAPE (15 μM) increases the amount of the Nrf2/ARE complex (see Figure 5C). Nrf2 in this complex was confirmed by antibody supershift analysis (results not shown).

Curcumin relieves Keap1 inhibition of Nrf2

One model for the regulation of Nrf2 function stipulates that, under normal conditions, Nrf2 exists in an inactive, cytoplasm-localized state, in part or fully as a consequence of binding to the cytoskeleton-associated protein Keap1 [28,29]. Upon cellular stimulation by xenobiotics, electrophiles or oxidative-stress-generating agents, the cytoplasmic-retention mechanism is inactivated and Nrf2 is transported to the nucleus, where it activates target-gene transcription. To examine whether curcumin modulates Nrf2–Keap1 interaction, we carried out the experiment depicted in Figure 6. In these transfections, curcumin elicited a nearly 5-fold stimulation of the E1-regulated luciferase reporter activity (compare Figure 6, lanes a and b). Consistent with the proposed function of Keap1, co-expression of Keap1 nearly abolished both basal and curcumin-dependent luciferase activity (Figure 6, lanes c and d). Conversely, ectopic expression of Nrf2 resulted in very high levels of luciferase activity (Figure 6, lane e), which was not further enhanced by curcumin (Figure 6, lane f), indicating maximal activation of E1 under these conditions and also supporting the idea that curcumin induces *ho-1* transcription via Nrf2. Importantly, Keap1 almost completely inhibited Nrf2 *trans*-activation of E1 (Figure 6, lane g), and this response was partially mitigated by curcumin

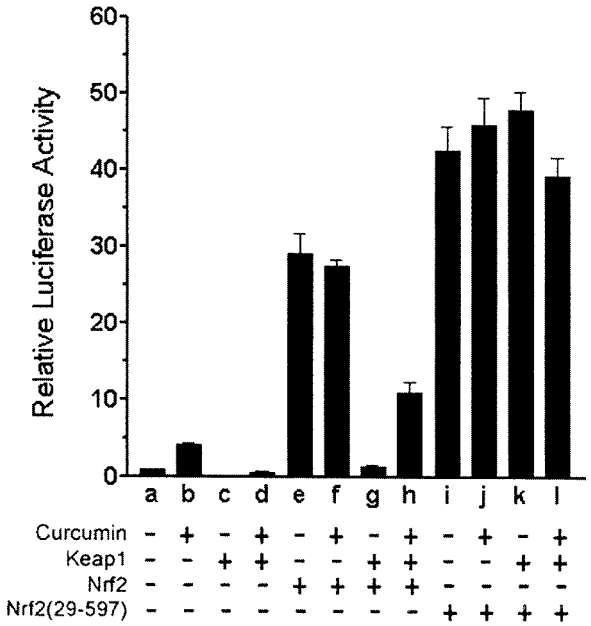

Figure 6 Curcumin relieves Keap1-mediated inhibition of Nrf2 *trans*-activation

NRK-52E cells were transfected with pE1-luc and the indicated expression plasmids as described in the Materials and methods section and treated for 5 h with either vehicle or 10 μM curcumin. Each bar, normalized to the luciferase activity observed in the absence of curcumin and any effector plasmid, represents the mean $\pm$ S.E.M. from four independent experiments.

(Figure 6, lane h). As a control we tested a mutant of Nrf2 lacking the N-terminus, the region of the protein required for interaction with Keap1 [28]. Nrf2 (29–597) promoted an even higher level of *trans*-activation than wild-type Nrf2 (compare Figure 6, lanes i and e), possibly reflecting inhibition of the latter by endogenous Keap1. Curcumin did not stimulate and Keap1 did not inhibit the activity of Nrf2(29–597) (Figure 6, lanes j–l). It is necessary to point out that the Keap1 interaction sequences of Nrf2 have been mapped not within the first 29 amino acids but to residues directly downstream of this segment [28]. The lack of inhibition of the *trans*-activation potential of Nrf2(29–597) by Keap1 suggests that residues 1–29 are required either for proper Nrf2–Keap1 association or for functional manifestation of such an interaction.

Curcumin activates p38 kinase

To determine the role, if any, of MAPKs in curcumin-mediated *ho-1* gene activation, we first examined the effect of curcumin on MAPK activities. MAPKs are activated by dual phosphorylation of threonine and tyrosine residues located in the 'activation lip' of the conserved core kinase sequence, and the activated species can be detected by antibodies directed against phosphorylated peptides encompassing these residues. NRK-52E cells were treated with vehicle (ethanol) or 10 μM curcumin for up to 2 h, and cell extracts were analysed for phosphorylated and total MAPKs by Western blotting. Curcumin stimulated the activation of extracellular-signal-regulated kinase (ERK) 1 (p44), ERK2 (p42) and p38 in a time-dependent manner (Figure 7). Whereas appreciable stimulation of the activated ERK species was not observed until the last time point tested (2 h), increased phosphorylation of p38 (approx. 5-fold) was detected within 30 min after treatment with curcumin, and the amount of activated p38 remained above the basal level for up to 2 h. In contrast

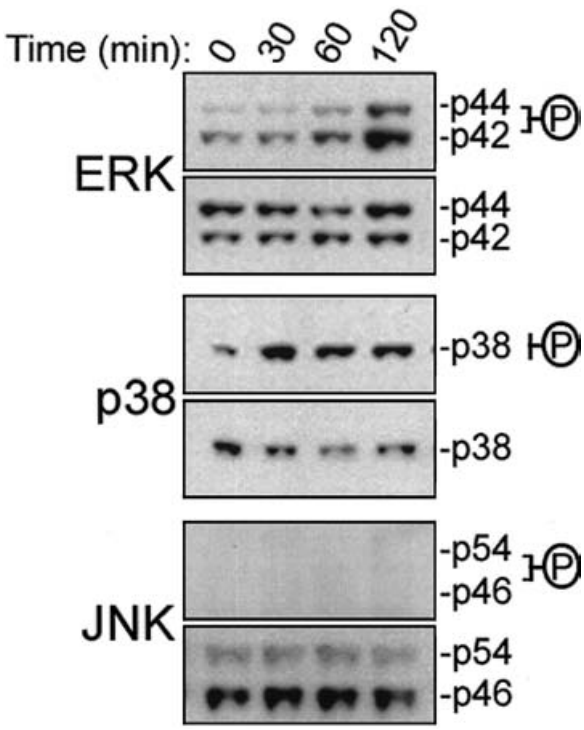

Figure 7 Activation of MAPKs by curcumin in NRK-52E cells

NRK-52E cells were cultured, treated with 10 μM curcumin for the indicated time period, and processed for immunoblot analysis as described in the Materials and methods section. Filters were initially used to detect phosphorylated (P) MAPKs, then stripped and probed with antibodies that detect total MAPK proteins. Individual MAPKs are identified by their sizes (kDa) on the right. Similar results were obtained in a second independent experiment.

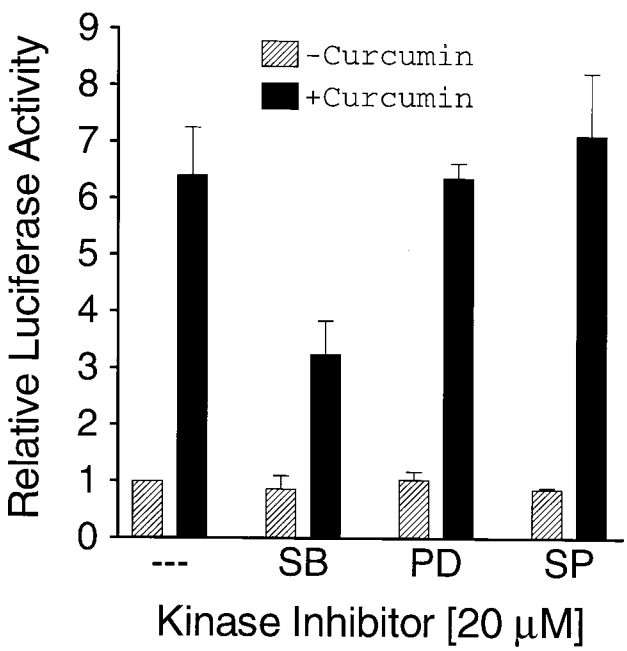

Figure 8 Effect of MAPK inhibitors on pE1-luc activity

NRK-52E cells were transfected with pE1-luc and treated as described in the Materials and methods section. Each bar, normalized to the luciferase activity observed in the absence of curcumin and kinase inhibitors, represents the mean $\pm$ S.E.M. from four independent experiments. SB, SB203580; PD, PD098059; SP, SP600125.

with the ERK and p38 enzymes, phosphorylated c-Jun N-terminal kinase (JNK) was not detected in untreated or stimulated cells. The increase in the levels of phosphorylated ERK and p38 kinase was not due to a concomitant elevation in the amount of the respective enzymes.

SB203580, a p38 inhibitor, attenuates curcumin-stimulated E1 activity

To address the role of individual MAPK pathways in *ho-1* gene regulation by curcumin, we examined the effects of SB203580, PD098059 and SP600125, inhibitors of the p38, ERK and JNK pathways, respectively, on pE1-luc expression. Treatment of NRK-52E cells with PD098059 or SP600125 had no effect on basal or curcumin-stimulated pE1-luc expression (Figure 8). SB203580 also did not alter basal E1 activity, but reduced curcumin-dependent induction by 50 %. Taken together with the MAPK-phosphorylation data, these results implicate the p38 pathway, but not the ERK or JNK pathways, in curcumin-mediated *ho-1* gene induction.

DISCUSSION

The transcription factor Nrf2 is a member of the cap'n'collar family of basic leucine transcription factors and plays an essential role in the ARE-mediated expression of phase II detoxifying enzymes and stress-inducible genes [30]. The activity of Nrf2 is normally suppressed in the cytosol by specific binding to the chaperone Keap1 [28]. However, upon stimulation by electrophilic agents or compounds that possess the ability to modify thiol groups [17], Keap1 repression of Nrf2 activity is lost, allowing Nrf2 protein to translocate into the nucleus and potentiate the ARE response [28]. This mechanism of gene activation leads to the synthesis of highly specialized proteins that efficiently protect mammalian cells from various forms of stress and, consequently, reduce the propensity of tissues and organisms to develop disease or malignancy [31]. Inducible proteins that require transcription via Nrf2 activation include γ-glutamylcysteine synthetase [32,33], glutathione S-transferases [34], NADP(H):quinone oxidoreductase [35] and HO-1 [20], which generates the antioxidant biliverdin and the signalling molecule CO. The concerted defensive action of these and other ubiquitous enzymes against specific diseases is exemplified by very recent studies using Nrf2-deficient mice, which lack the inherent ability to respond to classical inducers of phase II enzymes and are highly susceptible to carcinogenesis [18]. Moreover, Nrf2-knockout mice develop lupus-like autoimmune nephritis [36] and may die of liver failure due to a decreased capability of hepatocytes in drug detoxification [37]. Thus a direct involvement of this redox-sensitive transcription factor in mediating the adaptive response of tissues against progression of cell dysfunction is currently emerging.

In the present study, we show that Nrf2 protein is markedly induced in renal epithelial cells exposed to low concentrations of curcumin and CAPE, two plant-derived polyphenolic compounds that possess the chemical features required to trigger the induction of antioxidant and defensive genes [5]. In addition, transfection studies confirm that curcumin exerts a significant stimulation of Nrf2 activity and that this effect is completely blocked by the native repressor of Nrf2, Keap1. The translocation of Nrf2 protein into the nucleus following curcumin or CAPE treatment was associated with stimulation of the ARE-binding activity as well as a marked increase in HO-1 expression and haem oxygenase activity levels. In view of the strong evidence demonstrating an essential physiological role for HO-1 and its products in the mitigation of vascular dysfunction and inflammatory states [8–10], these and our previous findings point to the potential pharmacological use of naturally occurring compounds in modulating haem oxygenase activity and function [4,5]. These data are also consistent with the recognized biological activity of a number of phytochemicals or extracts of edible plants which are being identified as potent inducers of phase II enzymes and are able to transduce the signal for transcription to AREs through a chemical interaction with Nrf2 proteins [17,31].

Our results emphasize the crucial role of Nrf2-mediated gene expression in response to natural substances that possess a peculiar chemical structure. The data presented here also support the critical role of Keap1 in this response, as curcumin can significantly reverse the Keap1-mediated inhibition of Nrf2 activity. Thus it appears that curcumin promotes *ho-1* gene expression by altering the Nrf2–Keap1 interaction. Both curcumin and CAPE contain electrophilic α,β-unsaturated carbonyl groups which can react selectively with nucleophiles such as thiols, leading to formation of Michael adducts. In the case of curcumin, interaction with GSH has been reported [10] and kinetic studies *in vivo* have shown that the majority of orally administered curcumin is rapidly transformed into glutathione conjugates which can inhibit glutathione S-transferase activity [38]. Moreover, we have shown in cultured astrocytes that HO-1-mediated induction by curcumin and CAPE is associated with transient and marked changes in the intracellular GSH/GSSG ratio. Notably, Keap1 contains several highly reactive cysteine residues; although the chemical modification that signals dissociation of the Keap1–Nrf2 complex is currently unknown, a specific modulation of thiols by electrophiles in Keap1 might promote a conformational change that releases Nrf2 protein, thereby favouring its translocation to the nucleus [17]. Of major interest, and in line with this hypothesis, is that the potency of plant curcuminoids and other Michael-reaction acceptors as inducers of detoxifying enzymes depends on their specific reactivity with thiol groups [17]. The fact that the thiol donor *N*-acetyl-L-cysteine is unable to prevent the increase in haem oxygenase activity promoted by treatments with curcumin or CAPE [5] may be indicative of a unique chemical feature of these polyphenolic compounds in reacting with selective cysteines located in transcriptional factors or other proteins involved in signal-transduction pathways.

Our data demonstrate the obligatory role of the ARE in HO-1 transcription by curcumin. Previous studies have identified two enhancer regions, E1 and E2, located approx. 4 and 10 kb upstream of the transcription-initiation site of the *ho-1* gene [26]. Each enhancer contains at least three distinct AREs, which are essential for *ho-1* gene activation in response to a variety of agents, including haemin and heavy metal ions. We demonstrate here that deletion of both enhancers completely suppressed HO-1 induction by curcumin in renal epithelial cells and that each enhancer has the ability to respond to the effect elicited by the yellow pigment. The direct implication of Nrf2 in the regulation of the *ho-1* gene via the ARE has been recently reported using Nrf2 dominant-negative mutants (Nrf2M). It was found that, in fibroblasts over-expressing Nrf2M, the accumulation of HO-1 mRNA was almost completely inhibited in response to 'classic' HO-1 inducers such as haemin, cadmium and arsenite [20]. From that study it also appears that Nrf2 plays a more prominent role than AP-1 in modulating *ho-1* gene expression [20]. This is in line with the data presented here showing that, in renal cells exposed to curcumin, the migration of the DNA–protein complex in the EMSA reaction

is retarded only in the presence of Nrf2 antibodies, whereas it remains unaffected with members of the AP-1 family (Jun and Fos). In our system, we could not confirm that the nuclear factor-κB pathway contributes to transcriptional activation of HO-1 by curcumin, as partially shown in human renal proximal cells [39]. In addition, although *ho-1* gene induction by cobalt in Chinese hamster ovary cells involves the formation of Nrf2–MafG heterodimers [25], we were unable to detect MafG protein in the curcumin-induced complex. The biological significance of Nrf2 dimerizing with small Maf proteins or different bZIP proteins has received little attention and remains to be fully investigated.

The experiments designed to determine a possible role of MAPK pathways in curcumin-mediated *ho-1* gene induction showed that curcumin activates the ERK and p38 subfamilies, but not the JNK pathway. Additionally, the use of specific inhibitors for the MAPK subfamilies confirmed the involvement of p38, but not of the ERK or JNK pathways, in *ho-1* induction mediated by curcumin. Taken together with the promoter-analysis data, these results are in agreement with a previous report showing that cadmium, a classical HO-1 inducer, promotes *ho-1* gene expression in MCF-7 cells via sequential stimulation of the p38 kinase pathway and Nrf2 [24].

Collectively, the data presented in this paper are consistent with the specific requirement of AREs in the activation of protective genes by electrophilic substances [35] and highlight the fundamental role of the Nrf2/ARE complex in the regulation of HO-1 protein expression in conditions characterized by changes in the intracellular redox state [3]. Notably, a recent study reported that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), an α,β-unsaturated ketone containing two electrophilic carbons in the cyclopentenone ring, potently activates *ho-1* gene in mouse hepatoma cells and that the mechanism of induction is mediated by AREs and the transcription factor Nrf2 [40]. As in the case of curcumin and CAPE, which exert a variety of beneficial actions, 15d-PGJ_2 is known to regulate cellular growth, inflammation and homoeostasis. These emerging findings emphasize the notion that the pharmacological activity attributed to several well-known or newly discovered drugs could rely on their innate ability to strongly activate the haem oxygenase and other intracellular defensive systems [10,41].

In conclusion, we report that the induction of HO-1 in renal epithelial cells by curcumin and CAPE is dependent on the regulation of the ARE–Nrf2 complex, and propose Keap1 and the p38 kinase pathway as important modulators of this response. Since curcumin has been shown to ameliorate oxidative stress-induced kidney injury in mice [42] and a previous study confirms the high inducibility of HO-1 by low doses of curcumin in human renal cells [39], plant-derived compounds could be seriously considered as promising pharmacological agents in the development of therapeutic approaches for the prevention or treatment of renal diseases.

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