Curcumin induces heme oxygenase 1 through generation of reactive oxygen species, p38 activation and phosphatase inhibition

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Abstract. Curcumin is a naturally occurring compound which is known to induce heme oxygenase 1 (HO-1), although the underlying mechanism has not been fully elucidated. This study investigates in detail the mechanism of HO-1 induction by curcumin in human hepatoma cells. There was increasing toxicity of curcumin at concentrations higher than 10 μM. Curcumin was found to induce HO-1 at doses of 10 to 25 μM. At both non-toxic and toxic doses, HO-1 induction was found to correlate with production of reactive oxygen species (ROS), suggesting a causative relationship. This was reinforced by the finding that pretreatment with the antioxidants N-acetylcysteine, vitamin E and catalase prevented HO-1 induction by curcumin. ROS production appeared to be mitochondrial in origin, and curcumin treatment resulted in depolarisation of the mitochondrial membrane potential. Nrf2 was induced by curcumin treatment, which was also partly ROS dependent. Using siRNA, Nrf2 was demonstrated to contribute to HO-1 induction. A panel of kinase inhibitors was used to examine the contribution of MAP kinases to the induction of HO-1 by curcumin. PKC and p38 MAPK activity are required for full induction of HO-1. Furthermore, curcumin also inhibited protein phosphatase activity. In conclusion, curcumin treatment results in ROS generation, activation of Nrf2 and MAP kinases and the inhibition of phosphatase activity in hepatocytes, and when curcumin is not administered in toxic doses, these multiple pathways converge to induce HO-1.

Introduction

Curcumin is a natural extract of turmeric, the spice produced from Curcuma longa L (Zingiberaceae), part of the ginger family (1). Curcumin has been shown in recent years to have cytoprotective properties by inducing the protective protein heme oxygenase 1 (HO-1) (2,3). HO-1 is a stress responsive enzyme and part of the cell’s natural defence mechanisms. It catalyses the degradation of heme, generating the end-products biliverdin, carbon monoxide and iron. Increased HO-1 expression prolongs cardiac xenograft survival (4), allows prolonged cold preservation of liver transplants (5), and limits the severity of ischaemia reperfusion injury and development of chronic allograft nephropathy in kidney transplants (6). For this reason there has been great interest in understanding the cellular mechanisms through which curcumin acts, both to improve the understanding of the molecular regulation of HO-1 and to facilitate the design of safe clinical agents which can harness the HO-1 inducing properties of curcumin.

HO-1 induction is a complex multi-regulated process involving a variety of transcription factors (7). It is readily upregulated in response to oxidative stress, although it can also be induced by many other stimuli. Most ‘classical’ inducers depend on their prooxidant activity to induce HO-1. However, curcumin has both prooxidant and antioxidant properties (8,9). Indeed, curcumin has been found to have potent chemopreventive properties, as a result of its ability to induce apoptosis of tumour cells through generation of reactive oxygen species (8,10). Hence it is unclear whether the HO-1 inducing effect of curcumin represents a general response to cell injury or activation of a more specific signal transduction pathway.

Many of the classical HO-1 inducers such as cadmium have been shown to activate the transcription factor Nrf2 (11). Curcumin has been shown to induce HO-1 through Nrf2 binding to the antioxidant response element in the HO-1 promoter (12). However, the signal transduction mechanism through which curcumin triggers Nrf2 activation has not been examined. Furthermore there are significant interspecies differences in the HO-1 promoter (7), the result of which is that certain stimuli such as heat and hypoxia, which induce HO-1 in rodent models, do not induce HO-1 in human cells (13,14).

MAP kinases also have a role in the regulation of HO-1 induction (15), and curcumin differentially activates certain MAP kinases including p38, JNK and ERK, depending on cell line and treatment conditions (12,16,17). Curcumin has many potential mechanisms through which it may induce HO-1, including ROS generation, Nrf2 activation and kinase activation. Therefore in this paper we examined in
detail the potential pathways through which curcumin may induce HO-1 in a human hepatocyte cell line.

Materials and methods

Cell culture and reagents. All reagents were purchased from Sigma-Aldrich Ltd. (Poole, Dorset, UK) unless otherwise stated. Curcumin was diluted in dimethyl sulfoxide (DMSO) to a stock concentration of 100 mM. HUH7 human hepatoma cells were obtained from the European Tissue Culture Collection (Porton Down, UK), and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 μg/ml penicillin G and 50 μg/ml streptomycin. Whole cell extracts were obtained using RIPA buffer with protease inhibitors.

Western blotting. Western blotting was performed using a standard protocol with a Bio-Rad Miniprotein II system. Protein concentrations of samples were determined using a Bio-Rad DC protein assay kit. Antibody to HO-1 was purchased from Stressgen Biotechnologies (Victoria, BC, Canada). Secondary HRP-labelled antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Upstate (Waltham, MA, USA). Equality of protein loading was tested by ponceau staining and by probing membranes with an antibody to β-actin (Santa Cruz). Chemiluminescence was performed using the Amersham ECL system (Amersham Biosciences Ltd., Buckinghamshire, UK).

Cytotoxicity assay. Cell viability assays were performed on HUH7 cells using an MTT (methythetrazolium) dye technique. Cells were cultured in 96-well plates at 3x10^4 cells per well in the presence or absence of curcumin or vehicle for the indicated times. The medium was changed and 10 μl of MTT solution (5 mg/ml in PBS) was added. After 4-h incubation at 37°C, 150 μl extraction buffer (10% SDS, pH 3.0) was added and incubated overnight, following which the optical densities were measured at 570 nm using a plate reader (Dynex MRX II, Dynatech). All treatments were performed in sextuplicate on each occasion. Results are expressed as a percentage of medium only controls.

Measurement of caspase 3/7 activity. Caspase activity was measured using a proprietary kit, Apo-ONE homogenous caspase 3/7 assay (Promega), as per the manufacturer’s instructions.

Measurement of the mitochondrial membrane potential. Mitochondrial membrane potential was measured by two methods; 3,3'-dihexyloxacarbocyanine (DiOC6) and Mito-Tracker green and red. For DiOC6, cells were treated with curcumin or vehicle for 1 h, trypsinised and then suspended in PBS at 10^6 cells/ml. They were incubated in DiOC6 (50 nM final concentration) for 15 min at room temperature. Propidium iodide (5 μg/ml final concentration) was added and the cells were read immediately on an Epics XL-MCL flow cytometer (Coulter). However, this method does not allow for variations in total mitochondrial mass. For this reason, a normalised mitochondrial membrane potential was measured using MitoTracker green and red. Cells were treated as above, and then suspended in PBS. The 2 dyes were added at a final concentration of 100 nM, and the cells were incubated at 37°C in the dark for 30 min. The cells were then placed on ice and read immediately on the flow cytometer. The ratio of red:green fluorescence represents the normalised mitochondrial membrane potential.

Measurement of reactive oxygen species. ROS generation was measured using two methods; dichlorodihydrofluorescein diacetate (H₂DCFDA) and dihydroethidium. For the H₂DCFDA, cells were plated in 96-well plates at a density of 2x10^4 cells per well. The next day the cells were washed three times with HBSS and then treated as required, in the presence of H₂DCFDA at a final concentration of 10 μM. The plate was placed in a preheated fluorometer (at 37°C) and continuous measurement performed for 1 h, at excitation 480 nm and emission 530 nm. Following this, the supernatant was removed and a protein assay was performed on the cells. The plate was snap frozen, and the protein retrieved by applying hot (95°C) Laemmli buffer. The protein concentration was measured using a proprietary kit (Bio-Rad). The protein concentration was then suspended in PBS. The 2 dyes were added at a final concentration of 100 nM, and the cells were incubated at 37°C in the dark for 30 min. The cells were then placed on ice and read immediately on the flow cytometer. The ratio of red:green fluorescence represents the normalised mitochondrial membrane potential.

RNA interference. Transfections with siRNA encoding specific sequences for Nrf2 and HIF-1α were performed using the manufacturer’s instructions (Santa Cruz). Control siRNA was transfected to exclude non-specific effects.

Transfections. Transient transfections were performed using Fugene reagent (Roche) as per the manufacturer’s instructions, at a ratio of 6 μl Fugene/μg plasmid DNA. HUH7 cells were plated at 4x10^5 cells per 35-mm dish, and transfected the day after plating. The pHOGL3/11.6 reporter construct containing 11.6 kb of the human HO-1 promoter was a kind gift from Dr Agarwal (University of Alabama, USA). The HIF-1α reporter construct was a kind gift from Professor Esumi (National Cancer Research Institute East, Japan). PSV-β-galactosidase vector (0.5 μg; Promega) was co-transfected with the reporter constructs, to control for transfection efficiency. The cells were then treated 24 h later and the β-galactosidase or luciferase activity assayed using a proprietary kit as per the manufacturer’s instructions (Promega). Results were calculated as units of luciferase activity per units of β-galactosidase activity.

Phosphatase activity. Cells were treated with curcumin for the required time and then phosphatase activity was assayed using a non-radioactive molybdate dye based proprietary kit as per the manufacturer’s instructions (Promega).

Data analysis. All values are expressed as means ± standard error (SEM). SPSS software was used for statistical analysis (SPSS, IL, USA) Analysis of variance and Student’s t-test
were used for statistical analysis, and the differences between groups were considered to be significant at P<0.05.

Results

Curcumin induces HO-1 in a dose- and time-dependent manner. Treatment with curcumin induced HO-1 in hepatocytes at doses of 10-25 μM. This induction was seen from 2 to 12 h, but was maximal at 6 to 8 h after administration (Fig. 1).

Curcumin toxicity is associated with caspase activation. Curcumin treatment reduces cell viability in a dose-dependent manner. This effect is seen after treatment with doses ≥25 μM. Exposure to 25 μM curcumin for 24 h reduces survival by 10% (p<0.01, Fig. 2A). The same induction of caspase activity was seen after 6 h of curcumin treatment (data not shown).

Curcumin induces ROS production. Reactive oxygen species production was measured as a possible mechanism for curcumin toxicity. ROS production was significantly increased with curcumin treatment (p<0.05, Fig. 3). Using H₂DCFDA the ROS generation was measured continuously for 2 h.
were generated at a high rate within a minute of commencing curcumin treatment, and continued to be produced for the period studied. There was a clear dose dependency between the concentration of curcumin and ROS production when measured with dihydroethidium. Curcumin induced ROS production in doses from 10-50 μM. Each bar represents the mean ± SEM of six repeats from one experiment. Each experiment was performed 3 times with similar results. †p<0.05 vs nil control.

Curcumin treatment results in partial mitochondrial depolarisation. In view of a possible mitochondrial origin for the ROS production, attention was then focused on curcumin induced changes in mitochondria. Curcumin treatment with 10-50 μM curcumin resulted in loss of the mitochondrial membrane potential as measured by MitoTracker red and green dye (p<0.01, Fig. 4A). Use of DiOC6 similarly found depolarisation of the mitochondria at 25-50 μM, although 10 μM had no effect (Fig. 4B).

Induction of HO-1 by curcumin is mediated through Nrf2. Nrf2 is an oxidant-responsive transcription factor that has previously been shown to be involved in curcumin-mediated induction of HO-1. Nrf2 was induced following curcumin treatment, as a result of de novo synthesis which could be prevented by inhibiting transcription or translation with actinomycin D and cyclohexamide respectively (Fig. 5A). The induction of Nrf2 in response to curcumin appears partially dependent on ROS production (Fig. 5B). To demonstrate a definite effect of Nrf2 in mediating HO-1 induction by curcumin, siRNA to Nrf2 was used. This prevented the upregulation of Nrf2 by curcumin treatment and partially abrogated the induction of HO-1 by curcumin. Control siRNA and siRNA for HIF-1α or a control sequence had no effect (Fig. 5C).

Antioxidants inhibit HO-1 induction by curcumin. To confirm the role of oxidative stress in induction of HO-1 by curcumin,
antioxidants were used. Cells pretreated with the antioxidants N-acetylcysteine (Fig. 6A) or vitamin E (Fig. 6B) demonstrated a reduction in HO-1 levels following curcumin treatment. Treatment with catalase also reduced the induction of HO-1 in a dose-responsive manner, suggesting hydrogen peroxide as an important contributing ROS (Fig. 6C). Co-treatment of cells with 10,000 units of catalase with 10 μM curcumin led to a reduction in HO-1 reporter activity of 411% of normal controls to 287% (p<0.05).

PKC and p38 contribute to the induction of HO-1 by curcumin. A panel of kinase inhibitors was used to examine the contribution of MAP kinase activity to the induction of HO-1 by curcumin. Inhibition of PKC and p38 significantly reduced HO-1 induction (from 280% to 168% and 184%, p<0.01 and p<0.05 respectively, Fig. 7A). Inhibition of PI3 kinase also reduced the HO-1 induction, although this did not reach significance (280% to 231%, p=0.181), and inhibition of ERK had no effect. The reporter results were confirmed by Western blotting (Fig. 7B). Activation of p38 by curcumin was also confirmed by Western blotting (Fig. 7C).

**Curcumin inhibits phosphatase activity.** Phosphatase activity was examined after curcumin administration. PP2A activity was significantly inhibited within 5 min of curcumin treatment and remained reduced for 1 h (80% of basal activity, p<0.05; Fig. 8). PP1 activity was unchanged by curcumin treatment. Tyrosine phosphatase activity was reduced only after 1 h of curcumin treatment (77% of basal activity, p<0.05).

**Discussion**

This study examines in detail for the first time the mechanisms through which curcumin acts to induce HO-1. We have previously shown that curcumin induces HO-1 in hepatocytes and that this is cytoprotective (3). In this paper we have demonstrated that curcumin is toxic in high doses. This is reflected by caspase activation, which suggests that curcumin induces apoptotic cell death. Whilst curcumin has predominantly been described as inducing apoptosis through a classical mitochondrial pathway, associated with caspase activation (18,19), atypical variants have been described where there is no change in caspase activity and no mitochondrial depolarisation (20,21). It appears that in hepatocytes the classical caspase-mediated pathway is activated by high-dose curcumin treatment.

Curcumin induced ROS production. These ROS may originate from the mitochondria, as they could be inhibited by the uncoupler CCCP, and curcumin treatment also led to mitochondrial depolarisation, which can occur as the result of mitochondrial ROS production. Curcumin has been found to generate superoxide in cell lines that are sensitive to its proapoptotic properties (22) and, in isolated rat mitochondria, curcumin induces mitochondrial depolarisation in an ROS-dependent manner (23).

The two methods used for ROS measurement produced slightly different results. This probably reflects differences in sensitivity to the different ROS species. H2DCFDA is more sensitive for hydrogen peroxide and hydroxyl radicals, whilst dihydroethidium is more sensitive for superoxide although neither is species-specific (24,25).

The ROS generating properties of curcumin reflect its chemical structure, and certain other flavonoids can also generate ROS (26). Ligeret examined the effects of curcumin derivatives on rat liver mitochondria, and found that the phenolic group was essential to effect depolarisation of the mitochondria (23). This allows curcumin to reduce Fe3+ to Fe2+, which subsequently allows generation of hydroxyl radicals by the Fenton reaction. Ligeret proposed that it is the reductive ‘antioxidant’ properties of curcumin which results in increased ROS production. That the Fenton reaction is involved with HO-1 induction by curcumin is in keeping with our finding that this was prevented by catalase, suggesting that hydrogen peroxide contributes to the rise in HO-1.

In this study we have found that the transcription factor Nrf2 is induced by curcumin treatment. Nrf2 is rapidly induced by oxidative stress, and binds to consensus sequences in the HO-1 promoter to mediate its induction. Nrf2 has previously been shown to be involved in the HO-1 induction by curcumin...
There are significant interspecies differences in the HO-1 promoter (7), and we have confirmed this finding for the first time in human cells, and for the first time using siRNA and HO-1 protein. We also examined the transcription factors HIF-1 and HSF-1, which were found not to contribute to the HO-1 induction (unpublished data).

We have demonstrated for the first time that HO-1 induction by curcumin can be inhibited by the use of the antioxidants N-acetylcysteine, vitamin E and catalase. We propose that curcumin generates mitochondrial ROS and that this is the trigger for Nrf2 induction and subsequent upregulation of HO-1 (Fig. 9). Curcumin treatment results in dose-dependent mitochondrial generation of ROS. If a high dose of curcumin is used, the levels of ROS generated are overwhelmingly toxic, leading to the mitochondrial permeability transition pore, and subsequent caspase-mediated apoptosis. If lower doses of curcumin are administered, the mitochondria generates levels of ROS which are survivable. This results in diverse effects, activating Nrf2 and the protein kinases p38 and PKC, and inhibiting protein phosphatases, all of which contribute to the induction of HO-1 and the protection of the cell from injury.
kinase C (28) is involved in activation of Nrf2, by phosphoryl-
ating it to allow dissociation from the cytoplasmic inhibitor
Keap1. This allows nuclear migration of Nrf2 where it binds
to the requisite promoters (28). Whilst this would explain the
contribution of PKC activity to HO-1 induction by curcumin,
a direct effect on other transcription factors cannot be excluded.
p38 has previously been found to contribute to HO-1 induction
by cadmium and curcumin, although the mechanism is unclear
(11,12). In rat hepatocytes, only the p38\(\beta\) isoform promotes
HO-1 induction, whilst p38\(\alpha\), \(\beta\) and \(\delta\) isoforms inhibit HO-1
(15). The contribution of different p38 isoforms to the induction
of HO-1 by curcumin has not been assessed in this study.

Control of cellular processes is regulated through a delicate
balance of protein kinase and protein phosphatase activity.
After having demonstrated the involvement of kinase activation
in curcumin’s induction of HO-1, we examined the cellular
phosphatase activity. We found that PP2A was rapidly inhibited
by curcumin and that tyrosine phosphatase activity was
inhibited after one hour of treatment. It is likely that this plays
a role in the HO-1 induction seen with curcumin, as the PP2A
inhibitor okadaic acid is a potent HO-1 inducer (29). PP2A
may be inhibited by ROS (Kim 2003), and although not directly
tested, this would be consistent with the rapid decrease in its
activity seen in our experiments, as ROS were generated
constantly at high levels from the beginning of curcumin
treatment. Furthermore, PP2A acts directly on both p38 and
caspase 3 resulting in their inactivation (30). This may represent
part of the link between curcumin treatment and p38 and
caspase 3 activation.

In conclusion, we report that curcumin treatment results in
ROS generation, activation of Nrf2 and MAP kinases and the
inhibition of phosphatase activity in hepatocytes, and when
curcumin is not administered in toxic doses, these multiple
pathways converge to induce HO-1. Thus curcumin can be
used at low doses to pharmacologically induce HO-1,
although its induction is through generation of non-lethal
levels of reactive oxygen species.

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