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Curcumin attenuates dimethylnitrosamine-induced liver injury in rats through Nrf2-mediated induction of heme oxygenase-1

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Abstract

Curcumin (diferuloymethane), a yellow colouring agent present in the rhizome of *Curcuma longa* Linn (Zingiberaceae), has been reported to possess anti-inflammatory, antioxidant, antimutagenic and anticarcinogenic activities. Curcumin exerts its chemoprotective and chemopreventive effects via multiple mechanisms. It has been reported to induce expression of the antioxidant enzymes in various cell lines. Heme oxygenase-1 (HO-1) is an important antioxidant enzyme that plays a pivotal role in cytoprotection against noxious stimuli of both endogenous and exogenous origin. In the present study, we found that oral administration of curcumin at 200 mg/kg dose for four consecutive days not only protected against dimethylnitrosamine (DMN)-induced hepatic injury, but also resulted in more than three-fold induction of HO-1 protein expression as well as activity in rat liver. Inhibition of HO-1 activity by zinc protoporphyrin-IX abrogated the hepatoprotective effect of curcumin against DMN toxicity. NF-E2-related factor 2 (Nrf2) plays a role in the cellular protection against oxidative stress through antioxidant response element (ARE)-directed induction of several phase-2 detoxifying and antioxidant enzymes including HO-1. Curcumin administration resulted in enhanced nuclear translocation and ARE-binding of Nrf2. Taken together, these findings suggest that curcumin protects against DMN-induced hepatotoxicity, at least in part, through ARE-driven induction of HO-1 expression.
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Keywords: Curcumin; Tetrahydrocurcumin; Heme oxygenase-1; Nrf2; Hepatoprotection; ARE

1. Introduction

Curcumin (deferuloymethane), a yellow colouring ingredient of the spice turmeric obtained from the rhizome of *Curcuma longa* Linn (Zingiberacea), a perennial herb distributed mainly throughout tropical and subtropical regions of the world. It has been used in indigenous herbal medicine for the treatment of inflammatory and liver disorders (Joe et al., 2004). Curcumin has potent antioxidant, anti-inflammatory, antimutagenic and anticarcinogenic properties (Araujo and Leon, 2001; Chainani-Wu, 2003; Surh et al., 2001). The protective effects of curcumin against chemically-induced hepatotoxicity are well documented, and have been attributed to its intrinsic antioxidant properties (Nanji et al., 2003; Rukkumani et al., 2004). Thus, curcumin was shown to retard lipid peroxidation and ameliorate chemically-induced oxidative stress (Okada et al., 2001; Rukkumani et al., 2004). In addition, curcumin has been shown to increase expression of the xenobiotic detoxifying enzymes, such as glutathione *S*-transferase, glutathione reductase and NAD(P)H: quinone oxidoreductase in both liver and kidney of mice (Iqbal et al., 2003).

Heme oxygenase-1 (HO-1) is a stress-responsive enzyme widely distributed in many mammalian tissues and is

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responsible for the breakdown of heme to biliverdin, free iron and carbon monoxide (Maines and Gibbs, 2005). HO-1 expression is induced by a wide variety of stimuli including heme, heavy metals, cytokines and chemical carcinogens (McNally et al., 2006; Prawan et al., 2005). Moreover, HO-1 has been considered to be a potential therapeutic target for a number of chemopreventive agents (Prawan et al., 2005). Curcumin has been shown to up-regulate HO-1 in endothelia cells, renal epithelia cells and astrocytes (Hill-Kapturczak et al., 2001; Motterlini et al., 2000; Scapagnini et al., 2002) as well as in rat kidney *in vivo* (Jones et al., 2000). Upregulation of many phase-2 detoxifying and antioxidant enzymes, including HO-1, is mediated by antioxidant response element (ARE) (Li et al., 2004). The transcription factor Nrf2 plays a pivotal role in the activation of ARE-driven antioxidant gene expression. Nrf2 is normally sequestered in the cytosol as an inactive complex with its suppressor Keap-1. Upon cell stimulation, Nrf2 dissociates from Keap-1 and translocated into the nucleus where it binds to the ARE located in the promoters of target genes (Jaiswal, 2004). Induction of HO-1 by curcumin has been shown to occur via Nrf2 in porcine renal epithelial cells (Balogun et al., 2003), human monocytes (Rushworth et al., 2006) and in wild-type mouse embryo fibroblasts (Andreadi et al., 2006).

Dimethylnitrosamine (DMN) is a potent hepatotoxin, carcinogen and mutagen (George et al., 2001). DMN exerts carcinogenic effects and induces hepatic necrosis through metabolic activation by CYP2E1 (Guengerich et al., 1991) in experimental animals. Activation of DEN by CYP2E1 in mouse liver has been shown to stimulate Kupffer cells leading to generation of superoxide and other reactive oxygen species (ROS) capable of damaging liver cells (Teufelhofer et al., 2005). It has been shown that curcumin protects against a number of hepatotoxic agents including nitrosamines (Chuang et al., 2000; Sreepriya and Bali, 2006). However, the mechanisms by which curcumin elicits hepatoprotective and other chemoprotective/chemopreventive effects in association with HO-1 induction remains poorly understood. Here, we report that curcumin attenuates DMN-induced liver injury in rats via induction of Nrf2-mediated up-regulation of HO-1.

2. Materials and methods

DMN and curcumin were purchased from Sigma Chemical Co. (St Louis, MO, USA). The antibody against Nrf-2 was the product of Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Zinc-protoporphyrin (ZnPP) was obtained from Alexis (Carlsbad, CA, USA). Antibody against HO-1 was purchased from Stessgen Biotechnology Co. (Victoria, BC, Canada). ARE oligonucleotide was provided from Bionics, Korea. All other reagents used were in the purest form available commercially.

2.1. Animal treatment

Male Albino rats Wistar strain were purchased from the Charles River (Tokyo, Japan). They were housed in a temperature-controlled (25 °C) room with alternating 12 h- light/12 h-dark cycles. All animals were acclimatized for 7 days before experiment, fed standard pellet chow and given fresh water *ad libitum*. The experimental protocols were approved by the Animal Care and Use Committee of the Seoul National University. For determining the dose-dependent effect of curcumin, rats were administered curcumin dissolved in corn oil at 100 mg/kg or 200 mg/kg for four consecutive days by gavage. The control rats received corn oil in lieu of curcumin. Another set of rats were subsequently treated with curcumin (200 mg/kg) once a day for 1, 2, 3 and 4 days. For the hepatotoxicity studies, 24 male rats were divided into four groups. Control rats (group 1) received the vehicle only. Rats in groups 3 and 4 were administered 200 mg/kg curcumin for 4 consecutive days. Rats in groups 2, 3 and 4 received a single intraperitoneal dose of DMN (20 mg/kg) 48 h before sacrifice. In addition, rats in group 4 were treated with 0.1 mg/kg of ZnPP (Gaedeke et al., 2005) as a single intraperitoneal injection for 4 days. At the end of each experiment, rats were sacrificed by cervical dislocation. Blood was collected by heart puncture for serum isolation. Liver was excised, rinsed in physiological saline and stored at −80 °C until use for western blot analysis and nuclear extract preparation.

2.2. Western blot analysis

Livers were homogenized in ice-cold lysis buffer [150 mM NaCl, 0.5% Triton-X 100, 50 mM Tris-HCl (pH 7.4), 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4 and protease inhibitor cocktail tablet]. Lysates were centrifuged at 14,800*g* for 30 min, and aliquots of supernatant containing 30 μg protein were boiled in sodium dodecylsulphate (SDS) sample buffer for 5 min before electrophoresis on 12% SDS–polyacrylamide gel. After electrophoresis, proteins were transferred to PVDF membrane, and the blots were blocked with 5% fat-free dry milk-PBST buffer (phosphate-buffered saline containing 0.1% Tween 20) for 1 h at room temperature and then washed in PBST buffer. The membranes were incubated for 2 h at room temperature with 1:1000 dilution of primary antibodies for HO-1, cytochrome P4502E1 and anti-human Nrf2 (1:5000). Blots were washed three times with PBST at 10 min intervals followed by incubation with 1:5000 dilution of horsederadish peroxidase-conjugated secondary antibodies (rabbit) for 1 h and again washed in PBST thee times. The transferred proteins were visualized with an ECL chemiluminescence detection kit (Amersham Pharmacia Biotech, Inc) and short exposure of the membrane to X-ray films.

2.3. Histological studies and immunohistochemical staining

Liver specimen was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. After deparaffinization and dehydration, the paraffin blocks were stained with hematoxylin and eosin for microscopic examination. For immunohistochemical analysis, the enzymatic activity of endogenous peroxidases in the liver section was first blocked with 3% hydrogen peroxide, followed by incubation with rabbit polyclonal anti-rat HO-1 (StressGen Biotechnologies) at 37 °C for 3 h. The peroxidase binding sites were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark). Finally counterstaining was performed using Mayer's hematoxylin.

2.4. Determination of HO activity

Livers were homogenized in 3 vol. of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose and a protease inhibitor (Complete Boehringer Mannheim GmbH) and centrifuged at 10,000*g* for 30 min at 4 °C, followed by centrifugation at 100,000*g* of the supernatant for 60 min at 4 °C, to obtain the microsomal fraction as a pellet. The pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 10% glycerol. HO activity was measured spectrophotometrically as described previously (Naughton et al., 2002). Briefly, the obtained microsomal pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.4) containing 2 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 2 μg/ml aprotinin and 1 mM PMSF. This microsomal fraction (200 μl) was added to the reaction mixture (200 μl) containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 unit of glucose-6-

phosphate dehydrogenase, 20 μM hemin, 100 mM potassium phosphate buffer (pH 7.4), and 2 mg of rat liver cytosol as a source of biliverdin reductase. The mixture was incubated at 37 °C for 1 h in the dark, and the samples were left in an ice bath for at least 2 min to terminate the reaction. Bilirubin formed was determined by calculation of the difference in absorbances between 464 nm and 530 nm. The HO activity was expressed as picomoles of bilirubin per milligram of protein per 60 min.

2.5. Measurement of serum enzyme activities

Serum was prepared from the whole blood by centrifugation at 3000*g* for 10 min at 4 °C. Serum aspartate amino transferase (AST), alanine amino transferase (ALT) and γ-glutamyl transferase (γ-GT) activities were determined using commercially available kits.

2.6. Determination of lipid peroxidation

Lipid peroxidation was assessed in terms of malondialdehyde (MDA) formation in the rat liver 10,000*g* supernatant fraction. The measurement of thiobarbituric acid reacting substances (TBARS) was performed as described previously (Aeschbach et al., 1994). MDA was quantitated by using $\Sigma = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (Buege and Aust, 1978).

2.7. Determination of reduced glutathione (GSH)

GSH levels were measured using the commercially available colorimetric assay kit BIOXYTECH GSH-400 (OXIS Research, Portland, OR, USA). Briefly 50 μl of R1 solution (solution of chromogenic reagent in HCl) was added to the 700 μl of the 10,000*g* liver supernatant fraction followed by gentle vortex-mixing. Following the addition of 50 μl of R2 solution (30% NaOH), the mixtures were incubated at 25 ± 3 °C for 10 min. After centrifugation, the absorbance of the clear supernatant was read at 400 nm.

2.8. Preparation of nuclear extracts from rat liver

The nuclear extract from rat liver was prepared as described previously (Chun et al., 2003). Briefly, liver samples were homogenized in 1 ml of hypotonic buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl-fluoride (PMSF)]. To the homogenates was added 80 μl of 10% Nonidet P-40 (NP-40) solution, and the mixture was then centrifuged for 2 min at 14,000g. The supernatant was collected as a cytosolic fraction. The precipitated nuclei were washed once with 500 μl of buffer A plus 40 μl of 10% NP-40, centrifuged, resuspended in 200 μl of buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 20% glycerol] and centrifuged for 5 min at 14800*g*. The supernatant containing nuclear proteins was collected and stored at −70 °C after determination of protein concentrations.

2.9. Electrophoretic mobility shift assay (EMSA)

EMSA for determining the rat ARE-binding activity was performed using a DNA–protein binding detection kit (Gibco BRL, Grand Island, NY), according to the manufacturer's protocol. Briefly, the ARE oligonucleotide probe was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech, Buckinghamshire, UK). The binding reaction was carried out in a total volume of 25 μl of 10 mM Tris–HCl (pH 7.5) containing 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol, 0.1 mg/ml sonicated salmon sperm DNA, 10 μg of nuclear extracts and 100,000 cpm of [γ-^{32}P]ATP-end labeled oligonucleotide. An 100-fold excess of unlabeled oligonucleotide (competitor) was added where necessary. After 50-min incubation at room temperature, 2 μl of 0.1% bromophenol blue was added, and samples were electrophoresed through 6% non-denaturating polyacrylamide gel at 150 V for 2 h. Finally, the gel was dried and exposed to an X-ray film.

2.10. Statistics

The data were analyzed using one-way ANOVA followed by Dunnett's test. A *p* value less than 0.05 was considered statistically significant. All the statistics were carried out in SAS (The SAS System for windows, v8; SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Curcumin induces HO-1 expression and HO activity in rat liver

The effect of various doses of curcumin on rat liver HO-1 protein expression and HO activity is depicted in Fig. 1. A maximal induction of HO-1 expression was achieved with oral administration of curcumin at 200 mg/kg (Fig. 1A). The increased HO-1 protein expression directly correlated with the HO activity (Fig. 1B). As shown in Fig. 2, treatment of rats with curcumin (200 mg/kg) resulted in gradual increases in both HO activity and HO-1 protein expression up to 4 days.

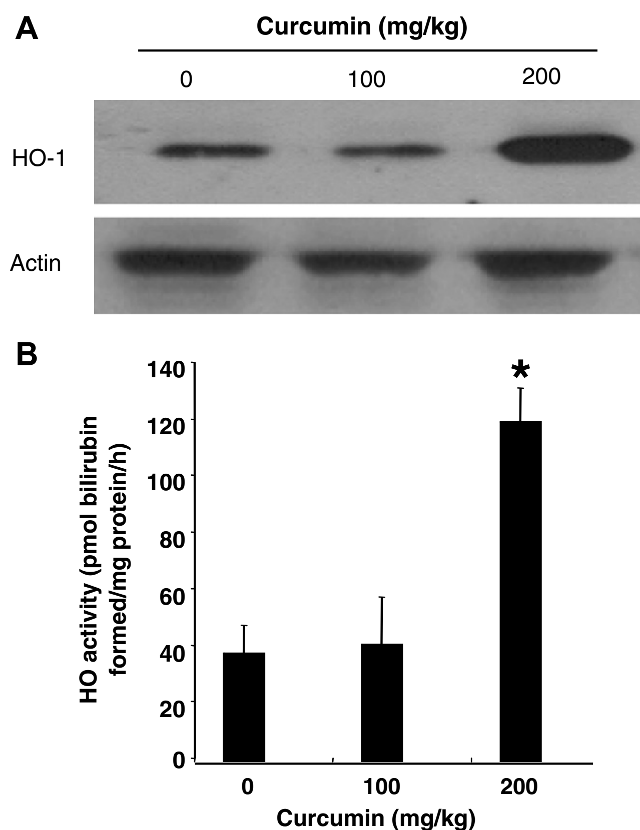

Fig. 1. Effects of curcumin treatment on HO-1 expression (A) and activity (B) in rat liver. (A) HO-1 protein levels were measured by western blot analysis of liver samples from rats treated with indicated doses of curcumin given intraperitoneally. Three independent experiments showed similar results, and a representative blot is shown. (B) HO activity was measured as described in materials and methods. Values are means ± SD ($n = 6$). *significantly different from the vehicle control ($p < 0.001$).

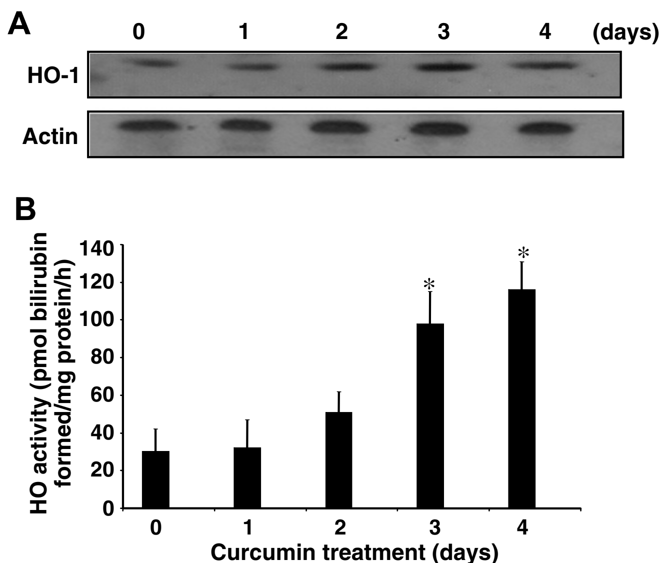

Fig. 2. Time-related induction of hepatic HO-1 expression (A) and HO activity (B) in rats. Three independent experiments showed similar profiles, and a representative blot is illustrated. HO activity as well as HO-1 expression was measured in liver samples of rats treated with curcumin (200 mg/kg) by gavage. Values are means ± SD of six rats. $*p < 0.001$ vs control.

3.2. Curcumin increases the Nrf2 ARE binding activity in rat liver

Several investigators have defined Nrf2 as a major transcription factor regulating ARE-driven phase 2 gene expression. Therefore we were attempted to determine whether curcumin could activate Nrf2 in association with its HO-1 up-regulation in rat liver *in vivo*. Activation of Nrf2 was determined by western analyses of nuclear extracts from liver homogenates of rats treated with curcumin at different doses. As illustrated in Fig. 3A, curcumin treatment caused increased Nrf2 accumulation in the nuclear fraction. To elucidate the role of Nrf2 in transcriptional activation of ARE, EMSA was performed using the oligonucleotide harboring the HO-1 specific ARE sequence. Incubation of the nuclear extract from rat liver after curcumin administration (200 mg/kg) with [γ-^{32}P]-labeled HO-1 ARE oligonucleotide resulted in the enhanced HO-1 ARE-binding activity of Nrf2 (Fig. 3B). Since DMN is known to be bioactivated by CYP 2E1, we thought it necessary to examine whether curcumin can modulate the metabolism of DMN by interfering with this enzyme. Curcumin did not change the expression of CYP 2E1 compared with control (data not shown).

3.3. Inhibition of HO activity abrogates the protective effect of curcumin on DMN-induced hepatotoxicity and oxidative damage

Using ZnPP that is a pharmacologic inhibitor of HO-1, we determined whether the protective effect of curcumin against DMN-induced hepatotoxicity was mediated via

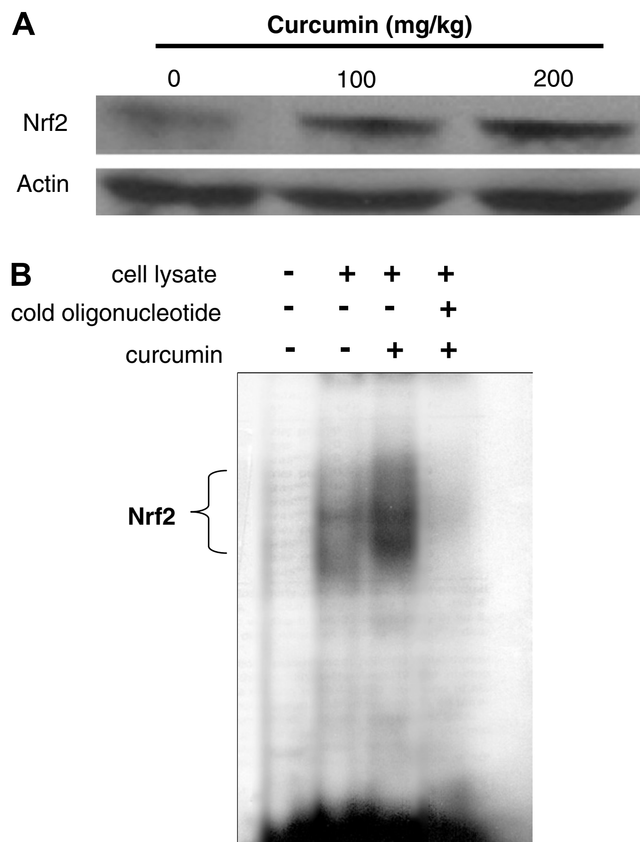

Fig. 3. Activation of Nrf2 by curcumin in rat liver. (A) Effects of curcumin on the nuclear levels of Nrf2. Nuclear extracts from rat liver were prepared after treatment by gavage with indicated doses of curcumin. Immunoblots of nuclear lysates from rat liver were probed with Nrf2 specific antibody. (B) Effect of curcumin (200 mg/kg) on the ARE-binding activity of Nrf2 in rat liver.

HO-1 upregulation. As shown in Fig. 4, curcumin administered at a dose of 200 mg/kg significantly lowered the activities of AST, ALT and γ-GT that were released into serum as a consequence of DMN-induced hepatic damage. However the hepatoprotective effect of curcumin was abolished by co-administration with ZnPP (Fig. 4). Histological examination of liver samples also corroborated the above findings. Thus, liver specimens from rats treated with DMN revealed massive and severe hepatocyte necrosis at the centrilobular zone (Fig. 5B) compared with the control liver (Fig. 5A). In curcumin-pretreated rats, the integrity of the hepatocytes were relatively well preserved (Fig. 5C). Pharmacologic inhibition of HO-1 activity with ZnPP negated the curcumin-mediated protection against DMN-induced hepatic injury (Fig. 5D). Enhancement of oxidative stress has been reported to be implicated in DMN-induced fibrosis and possibly hepatocarcinogenesis in rats (Vendemiale et al., 2001). In agreement with this notion, DMN caused a decrease in intracellular GSH levels (Fig. 6A) with concomitant increases in MDA production (Fig. 6B) both of which were abrogated by curcumin. Again the protective effect of curcumin was abolished in rats treated with ZnPP (Fig. 6).

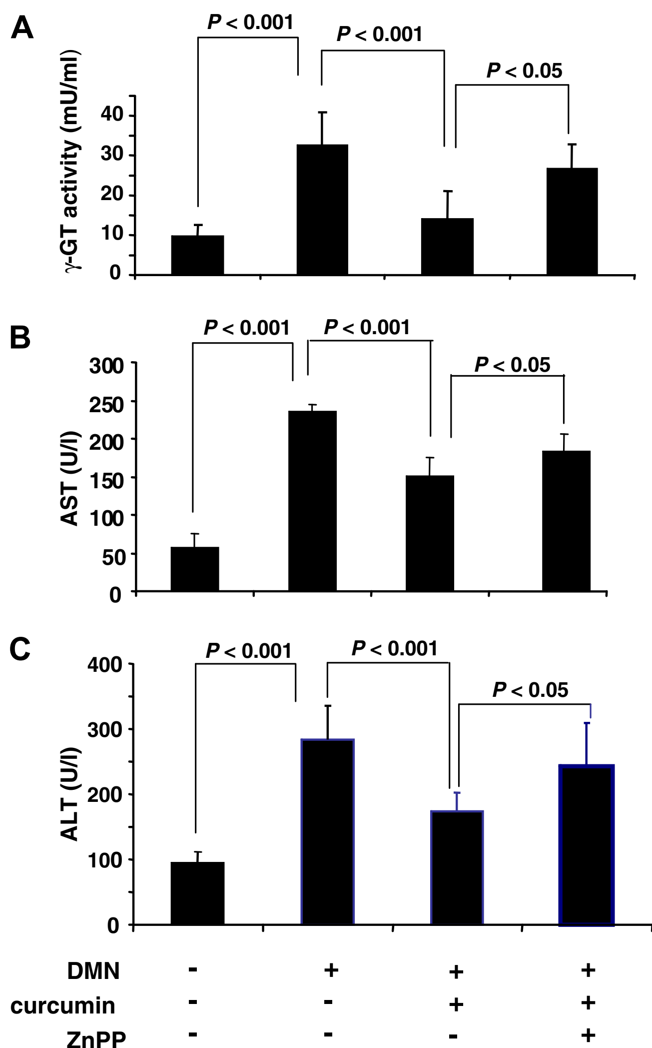

Fig. 4. Effect of ZnPP on curcumin protection against DMN-induced increases in the activities of γ-glutamyl transferase (A), serum apartate amino transferase (B) and serum alanine amino transferase (C). Values are means ± SD of six rats.

3.4. Tetrahydrocurcumin failed to induce HO-1 expression and Nrf2 ARE binding

Since curcumin possesses an electrophilic carbon centre in the α,β-unsaturated carbonyl moiety, it can act as Michael reaction acceptor capable of activating Nrf2 through covalent modification of its repressor Keap1 (Dinkova-Kostova et al., 2001). When each double bond conjugated to the carbonyl moiety of curcumin was saturated by catalytic hydrogenation, the resulting tetrahydrocurcumin cannot act as a Michael reaction acceptor. Unlike curcumin that retains the electrophilic carbon center, tetrahydrocurcumin failed to induce ARE binding of Nrf2 (Fig. 7A) and HO-1 expression (Fig. 7B and C), lending support that the α,β-unsaturated carbonyl moiety of curcumin is critical in its activation of Nrf2 and subsequent ARE-driven induction of HO-1 in rat liver.

4. Discussion

HO-1-mediated cytoprotection has been shown to be critical for tissues that are vulnerable to oxidative stress (Perrella and Yet, 2003). Therefore, HO-1 is recognized as an important target of a number of chemopreventive and cytoprotective agents (Prawan et al., 2005). Curcumin has been reported to attenuate liver injury induced by diverse hepatotoxicants (Kaur et al., 2006; Park et al., 2000; Reys-Gordillo et al., 2007; Shapiro et al., 2006) via multiple mechanisms. In the present study, we attempted to explore the possible role of HO-1 in hepatoprotection afforded by curcumin in rats. Previous studies by other investigators revealed that curcumin administration gave rise to an increase in several cytoprotective enzymes, especially in the liver (Iqbal et al., 2003). In the present study, curcumin given intraperitoneally up-regulated HO-1 protein expression as well as activity. The induction of HO-1 expression by curcumin affords the protection against hepatic damage associated with oxidative stress in rats challenged the hepatocarcinogen DMN. Cytochrome P4502E1 mediates the biotransformation of DMN to reactive species (Frei et al., 2001; Guengerich et al., 1991). Therefore, one cannot rule out the possibility that curcumin can attenuate DMN-induced hepatic injury by inhibiting this enzyme. However, we found that curcumin did not influence the constitutive expression of cytochrome P4502E1, compared with the control (data not shown). Based on these findings, it is likely that protection of DMN-induced hepatotoxicity by curcumin is associated with its induction of antioxidant enzyme, such as HO-1 rather than suppression of the metabolic activation of DMN.

Nrf2 plays a key role in the transcriptional regulation of the HO-1 gene expression through interaction with ARE (Alam and Cook, 2003). Under normal physiologic conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex with its repressor Keap 1 (Itoh et al., 1999). Upon stimulation by inducers, however, Nrf2 dissociates from Keap 1 and translocates into the nucleus where it dimerizes with some cofactors like small Maf protein and binds to ARE. This will lead to activation of a battery of highly specialized proteins, including HO-1 and other antioxidant enzymes, such as glutamate cysteine ligase, glutathione *S*-transferase, and quinone reductase that efficiently protect mammalian cells from various forms of toxicants and consequently reduce the propensity of tissues and organs to develop malignancies and other ailments (Talalay and Fahey, 2001). In this study, we found that curcumin administration induced nuclear translocation of Nrf2 and its ARE binding in rat liver. Although several *in vitro* cell culture studies have shown that induction of HO-1 by curcumin is mediated by Nrf2 (Andreadi et al., 2006; Balogun et al., 2003), our present study demonstrates for the first time that curcumin induces the expression of HO-1 in rat liver *in vivo*, at least in part, via activation of Nrf2 signaling. Some chemopreventive and chemoprotective agents,

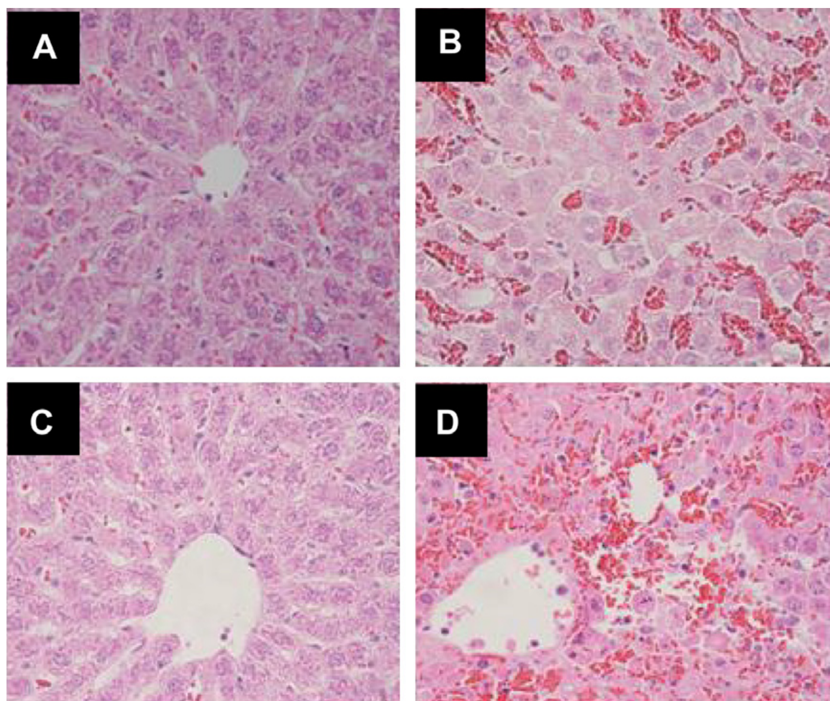

Fig. 5. Effects of curcumin and ZnPP on DMN-induced histological changes in the livers of rats (A) liver from the vehicle treated rats (B) DMN-treated rat liver (C) liver from the rat treated with curcumin plus DMN (D) liver from the rat treated with ZnPP, curcumin and DMN. Liver sections were stained with hematoxylin and eosin. 400×.

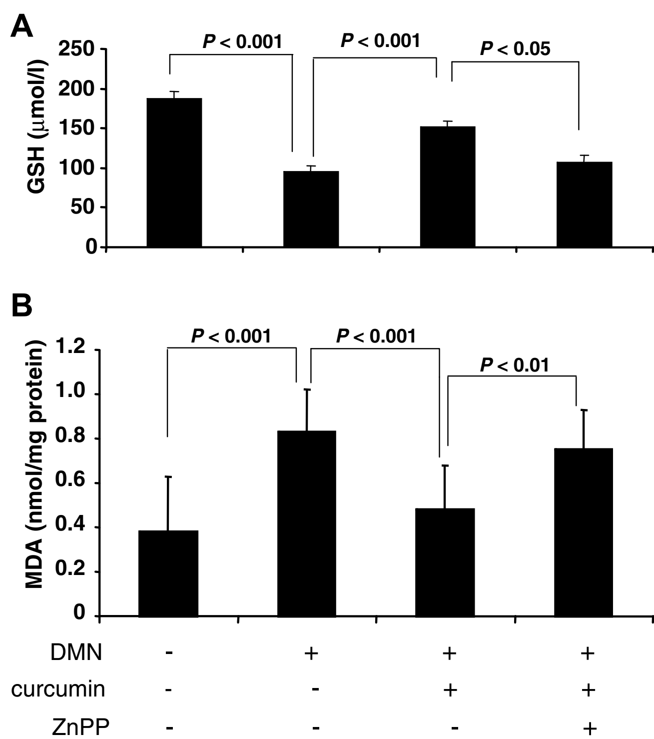

Fig. 6. Effect of curcumin and ZnPP on DMN-induced oxidative stress in rat liver. Oxidative stress was assessed in terms of depletion of cellular GSH (A) and malondialdeyde (MDA) formation (B) and as described in materials and methods. Values are means ± SD of six rats.

such as 3H-1,2-dithiole-3-thione (Kwak et al., 2001) and a related compound oltipraz (Ramos-Gomez et al., 2003), have been reported to induce phase-2 detoxifying enzymes through stimulation of Nrf2 transactivation.

Compounds bearing an electrophilic α,β-unsaturated carbonyl group can undergo Michael addition reactions with nucleophiles. As curcumin has two α,β-unsaturated carbonyl moieties, it can act as a Michael reaction acceptor. We hypothesize that the curcumin, due to the presence of such electrophilic carbonyl moieties, can directly interact with a critical cysteine thiol of Keap1. Such covalent modification of Keap1 would lower its affinity for Nrf2, releasing Nrf2 for nuclear translocation. In order to test the possibility of covalent modification of Keap1 by curcumin, we eliminated its electrophilic α,β-unsaturated carbonyl moieties by reducing the double bonds to form tetrahydrocurcumin. In support of our supposition, tetrahydrocurcumin that cannot function as a Michael reaction acceptor caused neither Nrf2 ARE binding nor HO-1 induction to any appreciable extent. This finding is also in good agreement with a previous study by Gong et al. (2002) which demonstrates that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, a cyclopentenone prostaglandin possessing α,β-unsaturated carbonyl groups, is a powerful inducer of HO-1 gene expression in mouse hepatoma cells via stimulation of Nrf2 ARE-binding activity (Gong et al., 2002). In contrast to our observation, tetrahydrocurcumin was reported to exert antioxidative (Murugan and Pari, 2006; Osawa and Kato, 2005) and hepatoprotective (Pari and Amali, 2005) effects. While tetrahydrocurcumin is a poor inducer of antioxidant enzyme gene expression, it may have an effective ROS scavenging prop-

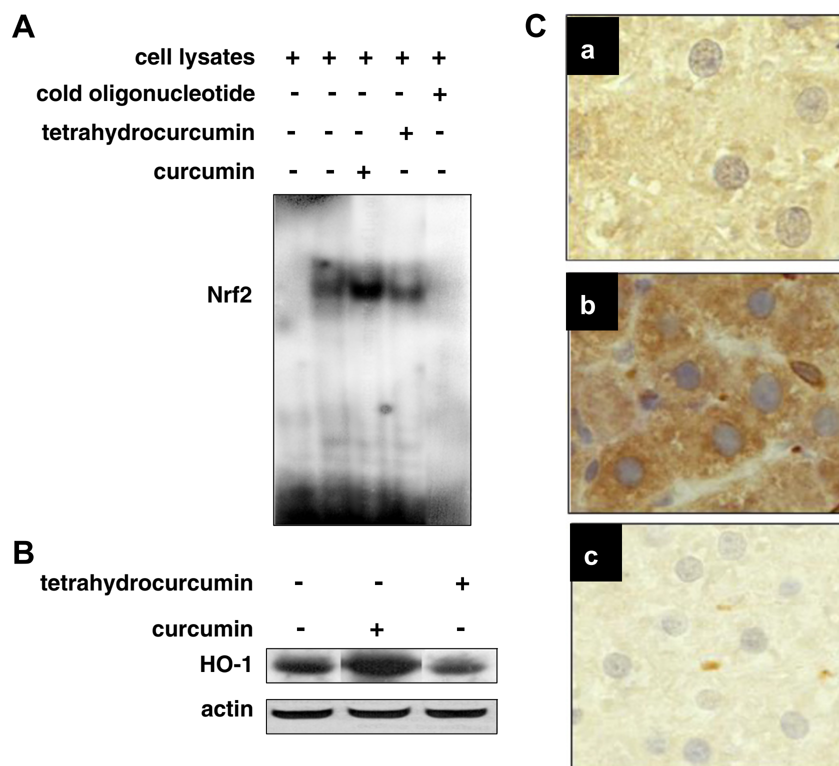

Fig. 7. Comparison of the effects of curcumin and tetrahydrocurcumin on Nrf2 activation and HO-1 induction. Curcumin and tetrahydrocurcumin were given orally at 200 mg/kg each to rats. Nrf2-ARE binding (A) and HO-1 protein expression in rat liver (B). HO-1 protein was analyzed by western blot technique as described in Materials and Method. Three independent experiments showed similar results and a typical example is shown. Immunohistochemistry of HO-1 in the liver of curcumin and tetrahydrocurcumin-treated rats (C). Treated rat livers were used for immunohistochemical analysis of HO-1, using rabbit polyclonal anti-rat HO-1 antibody as a primary antibody. Immunohistochemical staining was carried out as described in materials and methods. Positively stained HO-1 staining yielded a brown-colored product. (a) the vehicle treated control rat liver; (b) curcumin treated rat liver; (c) teterahydrocurcumin-treated rat liver.

erty. Besides direct interaction with Keap1, curcumin may indirectly stimulate the phosphorylation of Nrf2 at serine and/or threonine residues which may facilitate the nuclear translocation of this transcription factor and its subsequent interaction with the coactivator, CBP/p300. Induction of transient, moderate oxidative stress has been proposed to be an important mechanism by which some chemopreventive and chemoprotective phytochemicals induce antioxidant gene expression. Thus, the ability of flavonoids to activate an ARE [or more correctly, electrophile response element (EpRE)]-mediated response has been reported to correlate with their prooxidant potential (Lee-Hilz et al., 2006). According to this study, flavonoids with a higher intrinsic potential to generate oxidative stress and redox cycling are more potent inducers of EpRE-mediated antioxidant gene expression. ROS derived from curcumin can activate Nrf2-ARE/EpRE signaling by stimulating the upstream kinases through phosphorylation or oxidizing the cysteine thiol of Keap1. Further studies will be necessary to clarify the molecular mechanisms underlying curcumin-induced activation of Nrf2 and subsequent induction of HO-1 in rat liver *in vivo*.

Acknowledgments

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