Original Article — *J. Clin. Biochem. Nutr.*, **44**, 79–84, January 2009

Dimethoxycurcumin, a Synthetic Curcumin Analogue, Induces Heme Oxygenase-1 Expression through Nrf2 Activation in RAW264.7 Macrophages

Sun-Oh Jeong[1], Gi-Su Oh[1], Hun-Yong Ha[1], Bon Soon Koo[2], Hak Sung Kim[3], Youn-Chul Kim[3], Eun-Cheol Kim[4], Kang-Min Lee[5], Hun-Taeg Chung[1], and Hyun-Ock Pae[1,*]

[1]*Department of Microbiology and Immunology, Wonkwang University School of Medicine, Iksan, Republic of Korea*
[2]*Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan, Republic of Korea*
[3]*College of Pharmacy, Wonkwang University, Iksan, Republic of Korea*
[4]*Department of Oral and Maxillofacial Pathology, College of Dentistry, Wonkwang University, Iksan, Republic of Korea*
[5]*Division of Biological Sciences, College of Natural Science, Chonbuk National University, Jeonju, Republic of Korea*

Received 24 July, 2008; Accepted 10 September, 2008

Summary **Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] induces heme oxygenase-1 (HO-1) expression via activation of the nuclear factor-erythroid-2-related factor 2 (Nrf2), whereas tetrahydrocurcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-3,5-heptanedione], one of curcumin *in vivo* metabolites, has no effect on HO-1 expression and Nrf2 activation. The aim of this study was to investigate whether dimethoxycurcumin [1,7-bis(4,3-dimethoxyphenyl)-1,6-heptadiene-3,5-dione], a synthetic curcumin analogue with higher metabolic stability over curcumin, could induce HO-1 expression to the same extent as curcumin in RAW264.7 macrophages. Dimethoxycurcumin and curcumin, but not tetrahydrocurcumin, induced HO-1 expression and Nrf2 nuclear translocation, suggesting that the unsaturated nature of the diarylheptanoid chain of the compounds are crucial for HO-1 expression and Nrf2 activation. Blockage of Nrf2 synthesis by small interfering RNA abolished HO-1 expression by dimethoxycurcumin, indicating that dimethoxycurcumin may induce HO-1 expression via Nrf2 activation. In comparison, dimethoxycurcumin and curcumin had about the same effect on HO-1 expression, suggesting that dimethoxycurcumin retains the HO-1-inducing activity of its parent compound curcumin in RAW264.7 macrophages.**

***Key Words*: dimethoxycurcumin, curcumin, tetrahydrocurcumin, heme oxygenase-1, nuclear factor-erythroid-2-related factor 2**

Introduction

Heme oxygenase-1 (HO-1) that catalyzes the first and rate-limiting step in the oxidative degradation of free heme is now recognized as a fundamental endogenous cytoprotective system [*1*]. This enzyme can be expressed primarily by its substrate, free heme, and also by a wide variety of endogenous and exogenous stimuli, suggesting that the molecular mechanisms that regulate HO-1 expression are complex. The nuclear factor-erythroid-2-related factor 2 (Nrf2) has been shown to mediate HO-1 expression by certain phytochemicals [*2*].

The dietary phytochemical curcumin (chemical structure shown in Fig. 1) has a long history of medicinal use in

*To whom correspondence should be addressed.
Tel: +82-63-851-5066 Fax: +82-63-851-5066
E-mail: hopae@wku.ac.kr

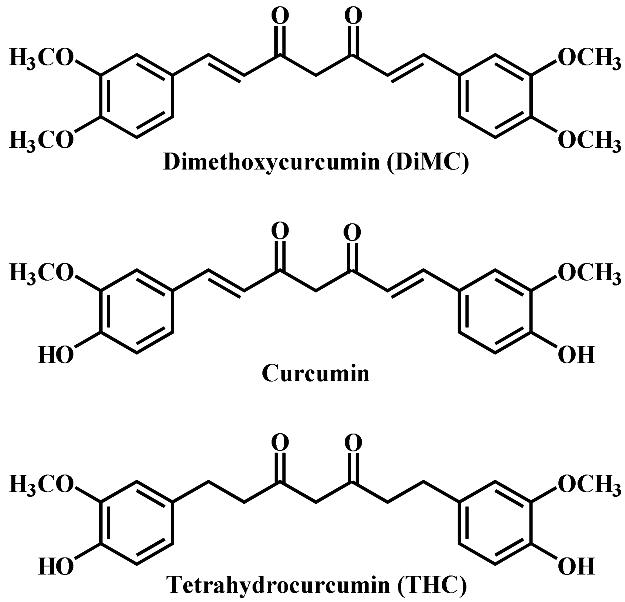

Fig. 1. Chemical structures of dimethoxycurcumin, curcumin and tetrahydrocurcumin.

India and Southeast Asia for a wide variety of medical conditions [*3*]. Extensive investigations on the pharmacological activities of curcumin have demonstrated that curcumin can induce HO-1 expression via Nrf2 activation [*4*], thus being considered a non-cytotoxic HO-1 inducer. Unfortunately, curcumin is rapidly metabolized *in vivo* into tetrahydrocurcumin (THC; chemical structure shown in Fig. 1) and other reduced forms [*5*]. Moreover, the anti-inflammatory property of curcumin is lost when curcumin is reduced to THC or others [*6*]. Thus, there is a need to develop curcumin analogues with higher metabolic stability than the original curcumin. Dimethoxycurcumin (DiMC; chemical structure shown in Fig. 1), one of several synthetic curcumin analogues, has been reported to exert an anticancer activity comparable to curcumin and to have increased metabolic stability in comparison with curcumin [*7*]. However, whether DiMC could exert other biological effects similar to those of curcumin remains to be investigated. Moreover, whether DiMC would induce HO-1 expression in RAW264.7 macrophages is currently unknown, and was thus investigated in this study.

Materials and Methods

Chemicals and reagents

Curcumin was isolated from the rhizomes of turmeric, as described earlier [*8*]. THC was prepared from curcumin by hydrogenating the two double bonds conjugated to the β-diketone, as described previously [*9*]. DiMC was synthetically prepared, as described previously [*10*], at the College of Pharmacology, Wonkwang University (Iksan, Republic of Korea). The purity of each compound, detected by HPLC, was >90%. All solvents used in this study were LC-MS grade and purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), hemin, NADPH, glucose-6-phosphate dehydrogenase and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Antibodies against HO-1, Nrf2 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Nrf2 small interfering RNA (siRNA) and its transfection kit were also from Santa Cruz Biotechnology.

Cell culture and treatment

The mouse monocytic-macrophage cell line RAW264.7 (American Type Culture Collection, VA) was cultured in DMEM containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and antibiotics (100 U/ml penicillin-G and 100 μg/ml streptomycin). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. RAW264.7 macrophages were cultured in either 6-well or 12-well flat-bottom plates at the concentration of 5×10^5 cells/ml. After 12 h of preconditioning, the cells were incubated with curcumin analogues (1–10 μM).

Cell viability assay

Cell viability was determined by a modified MTT reduction assay. MTT is a pale yellow substance that is reduced by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even fresh dead cells do not reduce significant amounts of MTT. RAW264.7 macrophages were cultured in a 96-well flat-bottom plate at concentration of 5×10^5 cells/ml. After 12 h of preconditioning, the cells were treated with various concentrations of DiMC or other agents for 18 h. Thereafter, culture medium was aspirated and 100 μl of MTT dye (1 mg/ml in phosphate-buffered saline) was added; the cultures were incubated for 4 h at 37°C. The formazan crystals produced through dye reduction by viable cells were dissolved using acidified isopropanol (0.1 N HCl). Index of cell viability was calculated by measuring the optical density of color produced by MTT dye reduction at 570 nm.

Heme oxygenase activity assay

Heme oxygenase activity was determined at the end of each treatment as described previously [*11*]. Briefly, harvested cells were subjected to three cycles of freeze-thawing before addition to a reaction mixture consisting of phosphate buffer (1 ml final volume, pH 7.4) containing magnesium chloride (2 mM), NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 Units), rat liver cytosol as a source of biliverdin reductase, and the substrate hemin (20 μM). The reaction mixture was incubated in the dark at 37°C for 1 h and was terminated by the addition of 1 ml of chloroform. After being vigorously vortexed and centrifuged, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm (ε = 40 $mM^{-1} \cdot cm^{-1}$).

Western blot analysis

RAW264.7 macrophages were incubated with or without reagents. They were harvested, washed ice-cold phosphate-buffered saline (PBS) and kept on ice for 1 min. The suspension was mixed with buffer A (10 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin, and 10 μg/ml leupeptin) and lysed by three freeze-thaw cycles. Cytosolic fraction was obtained by centrifugation at 12,000 × g for 20 min at 4°C. The pellets were re-suspended in buffer C (20 mM HEPES, pH 7.5, 0.4 M NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin, and 10 μg/ml leupeptin) on ice for 40 min and centrifuged at 14,000 × g for 20 min at 4°C. The resulting supernatant was used as soluble nuclear fraction. Protein content was determined with BCA protein assay reagent (Pierce, Rockford, IL). Total cellular or nuclear fractions were separated on 10% SDS-polyacrylamide gels, and transferred to the nitrocellulose membranes (Amersham Biosciences, Inc., Piscataway, NJ). The membrane was then blocked in blocking buffer containing 20 mM sodium phosphate buffer, pH 7.6, 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk for 1 h at room temperature. Thereafter, the membrane was incubated with antibodies against HO-1 (1:1000 dilution), Nrf2 (1: 500 dilution) or β-actin (1:1000 dilution) at 4°C overnight. The membrane was then washed four times with PBS-Tween 20 buffer and further incubated with secondary antibody for 1 h at room temperature. Specific bands were detected using enhanced chemiluminescence detection system (Amersham Biosciences), and the membrane was exposed to X-ray film.

Nrf2 siRNA transfection

RAW264.7 macrophages were grown in 6- or 12-well plates and transiently transfected with Nrf2 siRNA (Santa Cruz Biotechnology) mixed with siRNA transfection reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions. After incubation at 37°C and 5% CO_2 for 30 h, cells were treated with DiMC and curcumin. The samples were then prepared for Western blot analysis.

Statistical analysis

Data were analyzed using Student's *t* test, one-way analysis of variance or Newman-Keuls multiple comparison test. Differences were considered significant when $p<0.05$.

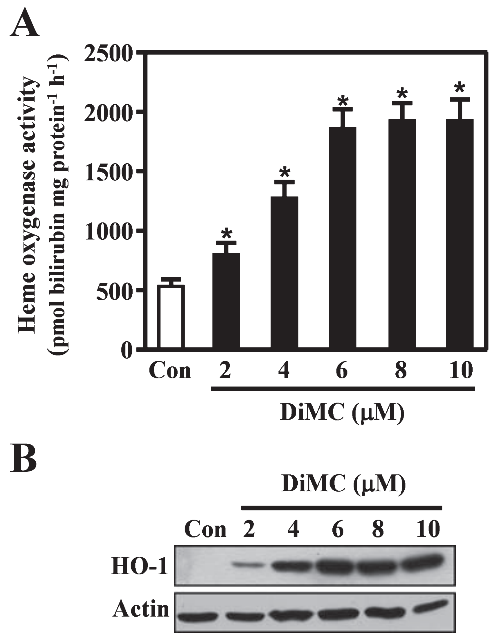

Fig. 2. Effects of DiMC on heme oxygenase activity and HO-1 expression in RAW264.7 macrophages. Cells were treated with indicated concentrations of DiMC. (A) Heme oxygenase activity was determined 6 h after exposure to DiMC, as described in Materials and Methods. Bars represent the means ± SD of 5–6 independent experiments. *$p<0.05$ with respect to control (Con; open bar). (B) HO-1 expression was detected 6 h after exposure to DiMC, as described in Materials and Methods. Blots shown are representative of 3 independent experiments.

Results

The chemical structures of curcumin analogues tested in this study are shown in Fig. 1. While the original form of curcumin contains two methoxy groups at two aromatic rings, DiMC contains four. In comparison with curcumin, THC contains two methoxy groups but lacks conjugated double bonds in the central seven-carbon chain. Curcumin and THC were used to explore possible mechanism(s) of action of DiMC. In RAW264.7 macrophages, DiMC and curcumin, but not THC, exhibited cytotoxicity at more than 20 μM (data not shown).

Treatment of RAW264.7 macrophages with different concentrations of DiMC (2–10 μM) for 6 h resulted in a significant increase in heme oxygenase activity (Fig. 2A); this enzymatic activation was strongly associated with a marked increase in HO-1 expression, as confirmed by Western blot analysis (Fig. 2B). Similar to the effects evoked by DiMC, treatment with curcumin resulted in a substantial increase in heme oxygenase activity (Fig. 3A) and HO-1 protein levels (Fig. 3B). In contrast, THC failed to increase heme oxygenase activity and HO-1 expression

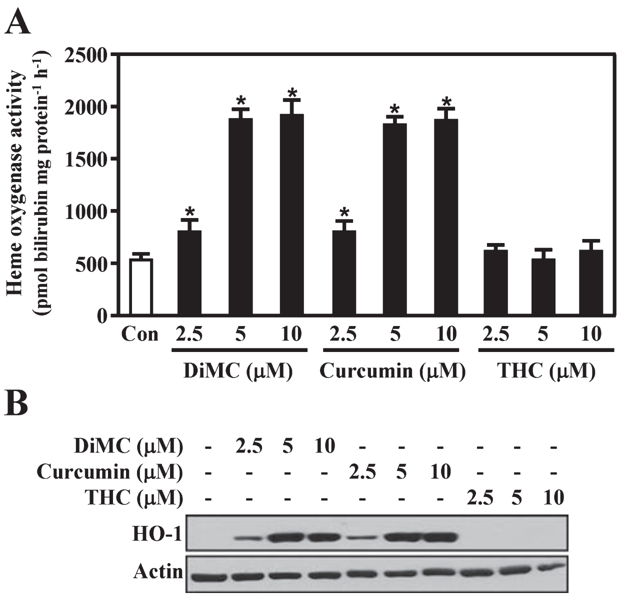

Fig. 3. Comparative effects of DiMC, curcumin and THC on heme oxygenase activity and HO-1 expression in RAW264.7 macrophages. Cells were treated with indicated concentrations of DiMC, curcumin and THC. (A) Heme oxygenase activity was determined 6 h after exposure to different compounds, as described in Materials and Methods. Bars represent the means ± SD of 5–6 independent experiments. *$p<0.05$ with respect to control (Con; open bar). (B) HO-1 expression was detected 6 h after exposure to DiMC, as described in Materials and Methods. Blots shown are representative of 3 independent experiments.

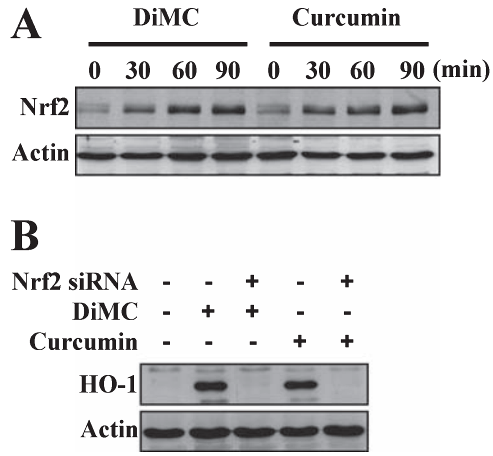

Fig. 4. Effects of DiMC and curcumin on Nrf2 activation and role of Nrf2 in HO-1 expression in RAW264.7 macrophages. (A) Cells were treated for indicated times with 5 μM of DiMC or curcumin. At each time point, nuclear accumulation of Nrf2 protein was determined by Western blot analysis, as described in Materials and Methods. (B) Cells were transiently transfected with Nrf2 siRNA, and then exposed to 5 μM of DiMC or curcumin. HO-1 expression was detected 6 h after exposure to the different compounds, as described in Materials and Methods. Blots shown are representative of 3 independent experiments.

(Fig. 3).

In other experimental sets, we examined the effects of DiMC and curcumin on Nrf2 activation in RAW264.7 macrophages, and found that both DiMC and curcumin induced the nuclear accumulation of the transcription factor Nrf2 (Fig. 4A). However, THC had no significant effect on Nrf2 nuclear accumulation (not shown). The role of Nrf2 in HO-1 expression by DiMC and curcumin was studied using siRNA against Nrf2. As shown in Fig. 4B, transient transfection with Nrf2 siRNA completely abolished HO-1 expression by DiMC as well as curcumin.

Discussion

As HO-1 is widely recognized as an effective cellular strategy to counteract a variety of stressful events [*12*], the induction of HO-1 by pharmacological modulators may represent a novel target for therapeutic intervention. The naturally occurring curcumin has been reported to induce HO-1 expression [*13–16*], but whether DiMC, a modified curcumin, could also induce HO-1 expression to the same extent as the original form curcumin has not been investigated so far. The present study demonstrates, for the first time, that DiMC can induce HO-1 expression and Nrf2 activation in RAW264.7 macrophages.

The present study determined which structural features of the DiMC molecule could contribute to its ability to serve as an inducer of HO-1. For this purpose, we compared the potency of DiMC and curcumin for HO-1 expression, because DiMC is structurally related to curcumin. DiMC affected the inducer potency only very slightly as compared with that of curcumin (Fig. 3), suggesting that the substituted methoxy groups in the DiMC are not essential for HO-1-inducing activity. On the contrary, the α,β-unsaturated carbonyl group may be an important structure of DiMC, because THC, lacking this functional group, was virtually inactive in inducing HO-1 expression. Compounds carrying this reactive group have been reported to induce HO-1 expression through activation of Nrf2 nuclear translocation [*17*, *18*]. The cyclopentenone 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, possessing the α,β-unsaturated carbonyl group, has been shown to induce HO-1 expression through Nrf2-dependent pathway [*19*]. Moreover, curcumin which possesses two α,β-unsaturated carbonyl groups has been already reported to induce HO-1 expression by activating Nrf2 nuclear translocation [*15*]. By structural analogy, DiMC can be recognized to have a chemical property resembling that of curcumin; this structural similarity prompted us to examine whether DiMC could also activate Nrf2 nuclear translocation. Indeed, DiMC induced the activation of Nrf2 nuclear translocation, and this activation was obviously associated with DiMC-induced HO-1 expression (Fig. 4B). We, therefore, speculate that DiMC is effective in inducing HO-1 expression, at least in part, because it bears the α,β-unsaturated carbonyl group.

DiMC and curcumin were found to have about the same effect at least partially on HO-1 expression in RAW264.7 macrophages; this may be because they have the same functional group playing a crucial role in HO-1 expression. Thus, our results confirm that DiMC, a synthetic curcumin analogue with higher metabolic stability over curcumin [*7*], retains the HO-1-inducing activity of its parent compound curcumin.

Conclusion

The results of the present study demonstrate that: (i) the synthetic DiMC induces HO-1 expression through Nrf2-dependent pathway in RAW264.7 macrophages; (ii) the α,β-unsaturated carbonyl group of DiMC are crucial for Nrf2-dependent HO-1 expression; and (iii) the HO-1-inducing activities of DiMC and curcumin are almost identical.

Acknowledgment

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006-005-JO3202).

Abbreviations

DiMC, Dimethoxycurcumin; DMEM, Dulbecco's modified Eagle's medium; HO-1, Heme oxygenase-1; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; Nrf2, Nuclear factor-erythroid-2-related Factor; siRNA, Small interfering RNA; THC, Tetrahydrocurcumin.

References

[1] Chung, H.T., Pae, H.O., and Cha, Y.N.: Role of heme oxygenase-1 in vascular disease. *Curr. Pharm. Des.*, **14**, 422–428, 2008.

[2] Pae, H.O., Kim, E.C., and Chung, H.T.: Integrative survival response evoked by heme oxygenase-1 and heme metabolites. *J. Clin. Biochem. Nutr.*, **42**, 197–203, 2008.

[3] Goel, A., Kunnumakkara, A.B., and Aggarwal, B.B.: Curcumin as "Curecumin": from kitchen to clinic. *Biochem. Pharmacol.*, **75**, 787–809, 2008.

[4] Pae, H.O., Jeong, G.S., Jeong, S.O., Kim, H.S., Kim, S.A., Kim, Y.C., Yoo, S.J., Kim, H.D., and Chung, H.T.: Roles of heme oxygenase-1 in curcumin-induced growth inhibition in rat smooth muscle cells. *Exp. Mol. Med.*, **39**, 267–277, 2007.

[5] Pan, M.H., Huang, T.M., and Lin, J.K.: Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug Metab. Dispos.*, **27**, 486–489, 1999.

[6] Ireson, C., Orr, S., Jones, D.J., Verschoyle, R., Lim, C.K., Luo, J.L., Howells, L., Plummer, S., Jukes, R., Williams, M., Steward, W.P., and Gescher, A.: Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat *in vivo*, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. *Cancer Res.*, **61**, 1058–1064, 2001.

[7] Tamvakopoulos, C., Dimas, K., Sofianos, Z.D., Hatziantoniou, S., Han, Z., Liu, Z.L., Wyche, J.H., and Pantazis, P.: Metabolism and anticancer activity of the curcumin analogue, dimethoxycurcumin. *Clin. Cancer Res.*, **13**, 1269–1277, 2007.

[8] Jeong, G.S., Oh, G.S., Pae, H.O., Jeong, S.O., Kim, Y.C., Shin, M.K., Seo, B.Y., Han, S.Y., Lee, H.S., Jeong, J.G., Koh, J.S., and Chung, H.T.: Comparative effects of curcuminoids on endothelial heme oxygenase-1 expression: ortho-methoxy groups are essential to enhance heme oxygenase activity and protection. *Exp. Mol. Med.*, **38**, 393–400, 2006.

[9] Pae, H.O., Jeong, S.O., Jeong, G.S., Kim, K.M., Kim, H.S., Kim, S.A., Kim, Y.C., Kang, S.D., Kim, B.N., and Chung, H.T.: Curcumin induces pro-apoptotic endoplasmic reticulum stress in human leukemia HL-60 cells. *Biochem. Biophys. Res. Commun.*, **353**, 1040–1045, 2007.

[10] Chen, W.F., Deng, S.L., Zhou, B., Yang, L., and Liu, Z.L.: Curcumin and its analogues as potent inhibitors of low density lipoprotein oxidation: H-atom abstraction from the phenolic groups and possible involvement of the 4-hydroxy-3-methoxyphenyl groups. *Free Radic. Biol. Med.*, **40**, 526–535, 2006.

[11] Sawle, P., Foresti, R., Mann, B.E., Johnson, T.R., Green, C.J., and Motterlini, R.: Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. *Br. J. Pharmacol.*, **145**, 800–810, 2005.

[12] Abraham, N.G. and Kappas, A.: Pharmacological and clinical aspects of heme oxygenase. *Pharmacol. Rev.*, **60**, 79–127, 2008.

[13] Hsu, H.Y., Chu, L.C., Hua, K.F., and Chao, L.K.: Heme oxygenase-1 mediates the anti-inflammatory effect of Curcumin within LPS-stimulated human monocytes. *J. Cell Physiol.*, **215**, 603–612, 2008.

[14] Farombi, E.O., Shrotriya, S., Na, H.K., Kim, S.H., and Surh, Y.J.: Curcumin attenuates dimethylnitrosamine-induced liver injury in rats through Nrf2-mediated induction of heme oxygenase-1. *Food Chem. Toxicol.*, **46**, 1279–1287, 2008.

[15] Balogun, E., Hoque, M., Gong, P., Killeen, E., Green, C.J., Foresti, R., Alam, J., and Motterlini, R.: Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem. J.*, **371**, 887–895, 2003.

[16] Motterlini, R., Foresti, R., Bassi, R., and Green, C.J.: Curcumin, an antioxidant and anti-inflammatory agent,

induces heme oxygenase-1 and protects endothelial cells against oxidative stress. *Free Radic. Biol. Med.*, **28**, 1303–1312, 2000.

[17] Pae, H.O., Jeong, G.S., Kim, H.S., Woo, W.H., Rhew, H.Y., Kim, H.S., Sohn, D.H., Kim, Y.C., and Chung, H.T.: Costunolide inhibits production of tumor necrosis factor-alpha and interleukin-6 by inducing heme oxygenase-1 in RAW264.7 macrophages. *Inflamm. Res.*, **56**, 520–526, 2007.

[18] Jeong, G.S., Pae, H.O., Jeong, S.O., Kim, Y.C., Kwon, T.O., Lee, H.S., Kim, N.S., Park, S.D., and Chung, H.T.: The alpha-methylene-gamma-butyrolactone moiety in dehydrocostus lactone is responsible for cytoprotective heme oxygenase-1 expression through activation of the nuclear factor E2-related factor 2 in HepG2 cells. *Eur. J. Pharmacol.*, **565**, 37–44, 2007.

[19] Lim, H.J., Lee, K.S., Lee, S., Park, J.H., Choi, H.E., Go, S.H., Kwak, H.J., and Park, H.Y.: 15d-PGJ2 stimulates HO-1 expression through p38 MAP kinase and Nrf-2 pathway in rat vascular smooth muscle cells. *Toxicol. Appl. Pharmacol.*, **223**, 20–27, 2007.