Heme oxygenase-1-mediated reactive oxygen species reduction is involved in the inhibitory effect of curcumin on lipopolysaccharide-induced monocyte chemoattractant protein-1 production in RAW264.7 macrophages

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Abstract. Monocyte chemoattractant protein 1 (MCP-1) is a CC chemokine which stimulates mononuclear leukocytes and is significant in the pathogenesis of inflammatory disease. Curcumin, extracted from *Curcumae longae*, has been shown to possess anti-inflammatory activity, in inflammation associated with the induction of MCP-1 expression. However, the underlying mechanisms involved in the effect of curcumin on MCP-1 expression remain unclear. In the current study, we investigated the effect of curcumin on the production of MCP-1 induced by lipopolysaccharide (LPS) in macrophages and the possible mechanisms involved. The results revealed that curcumin decreased MCP-1 production in a concentration-dependent manner and reduced the generation of reactive oxygen species (ROS) induced by LPS in RAW264.7 macrophages. Additionally, zinc protoporphyrin, a heme oxygenase-1 (HO-1) inhibitor, blocked the inhibitory effect of curcumin on the LPS-induced MCP-1 expression. The exposure of cells to curcumin was found to enhance HO-1 expression. Furthermore, additional experiments indicated that the inhibitory effect of curcumin on LPS-induced MCP-1 expression was significantly attenuated in the presence of N-acetylcysteine (an effective ROS scavenger). The results presented in our study suggest that curcumin enhances the expression of HO-1 to reduce the LPS-induced production of ROS, which leads to the inhibition of MCP-1 expression in RAW264.7 macrophages.

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Introduction

A number of studies have suggested that inflammation of the blood vessel wall plays a pivotal role in the initiation and maintenance of vascular diseases, including atherosclerosis, transplant arteriopathy and restenosis following mechanical injury (1). Monocyte chemoattractant protein 1 (MCP-1) is an important chemokine that mediates monocyte and macrophage infiltration and is largely responsible for the recruitment of monocytes and macrophages to the vessel wall during the early stages of atherogenesis. In addition, Boring *et al* (2) found that the MCP-1 receptor, CC chemokine receptor (CCR2), markedly decreased lesion formation in apolipoprotein E (ApoE)-deficient mice by inhibiting macrophage infiltration. Furthermore, MCP-1 induces adhesion molecule, pro-inflammatory cytokine, chemokine, matrix metalloproteinase and tissue factor expression. Taken together, these findings indicate that MCP-1 contributes to the initiation and development of inflammation.

Curcumin is the active ingredient extracted from turmeric, a curry spice, which is not only used in preparing Asian curry dishes but is also a component of certain ancient herbal remedies for various diseases (3). Previous pharmacological studies have shown that curcumin has a variety of health-beneficial effects, including anti-inflammatory, antioxidant, anticarcinogenic, antithrombotic and cardiovascular protective effects (4). Among these biological activities, the anti-inflammatory effects of curcumin have been assessed in various *in vitro* systems and in experimental animal systems (5,6). However, the cellular and molecular mechanisms of the anti-inflammatory effects of curcumin have not yet been well characterized.

In the present study, we monitored the inhibitory effect of curcumin on lipopolysaccharide (LPS)-induced MCP-1 expression in macrophages and explored the cellular and molecular mechanisms involved. Our results revealed that curcumin significantly inhibited the LPS-induced increase in MCP-1 expression and enhanced heme oxygenase-1 (HO-1) expression in a concentration-dependent manner. Additionally, HO-1 is a rate-limiting enzyme that degrades heme to biliverdin, ferrous iron and carbon monoxide (7). HO-1 and its end products play

a key role in protecting cells against inflammatory responses and oxidative stress (8). Therefore, the present study was designed to investigate whether the anti-inflammatory effects of curcumin are partially dependent on HO-1-mediated reactive oxygen species (ROS) reduction, subsequently suppresing LPS-induced MCP-1 production in macrophages.

Materials and methods

Cell culture and treatment. RAW264.7 cells (American Type Culture Collection) were cultured in DMEM (Gibco-BRL, Carlsbad, CA, USA) containing 10% FBS (Gibco-BRL), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C. The medium was changed to serum-free medium after the cells had been grown to confluence, and the cells were incubated overnight prior to the experiments. Before LPS was added to the medium, the cells were pre-treated with curcumin (Sigma, St. Louis, MO, USA). The macrophages were pre-treated with zinc protoporphyrin (ZnPP; Sigma), N-acetylcysteine (NAC) (J&K Scientific, Ltd., Beijing, China), apocynin (Sigma), PD98059 (Beyotime Biotech, Haimen, China), SB203580 (Beyotime Biotech) or SP600125 (Beyotime Biotech) at 1 h before curcumin treatment.

Cell viability assay. The cells were plated with a variety of concentrations of curcumin (0-80 μM) in 96-well microtiter plates and were then cultured for 24 h at 37˚C in a 5% CO_2 incubator. Cell viability was determined using the conventional methylthiazolyl tetrazolium (MTT) reduction assay. Following the treatment of the cells with curcumin, MTT solution was added (final concentration 5 mg/ml) and incubation was continued for 4 h at 37˚C. The dark blue formazan crystals formed in the intact cells were solubilized with DMSO and then the absorbance of the blue color was measured at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified by UV absorption at 260 and 280 nm. RT-PCR was performed according to the manufacturer's instructions. According to GenBank, the RT-PCR primers were designed as follows: HO-1 sense, 5'-GGGTGACAGAAGAGGCTAAGACC-3' and antisense, 5'-AGATTCTCCCCTGCAGAGAGAAG-3'. The PCR conditions were as follows: 30 cycles of 94˚C for 30 sec; 55˚C for 30 sec; and 72˚C for 45 sec. The amplified products were visualized by 1.5% agarose gel electrophoresis, stained with ethidium bromide and images were then captured under ultraviolet light. Densitometric analysis of three different observations was performed using Quantity One Software (Bio-Rad). The quantity of each transcript was normalized to that of GAPDH.

Western blot analysis. The cells were harvested and washed twice with PBS. The harvested cells were then lysed and 50 μg total protein was separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto nitrocellulose membranes. After blocking for 1 h with 8% skimmed milk in TBS buffer (10 mM Tris, 150 mM NaCl), the membranes were incubated with primary antibodies at 4˚C overnight. The membranes were then washed four times for 15 min each with TBST buffer (10 mM Tris, 150 mM NaCl and 0.1% Tween-20) and incubated with the appropriate HRP-conjugated secondary antibody at 37˚C for 30 min. The protein bands were detected using an enhanced chemiluminescene western blotting detection kit (Amersham, Buckinghamshire, UK). The antibodies were all purchased from Beyotime Biotech.

Measurement of ROS. Prior to the chemical treatment, the cells were incubated in culture medium containing 30 μM 2',7-dichlorofluorescein (DCF; a fluorescent dye, Beyotime Biotech) for 30 min to establish a stable intracellular level of the probe. Subsequently, the cells were washed with PBS, removed from the Petri dishes by scraping and evaluated for DCF fluorescence intensity, which was used as an index of the intracellular levels of ROS. The fluorescent DCF was detected using a laser scanning confocal microscope (Leica TCS-NT, Heidelberg, Germany) with excitation and emission wavelengths of 488 and 520 nm, respectively (9). The cell number in each sample was counted and utilized to normalize the fluorescence intensity of DCF.

Determination of MCP-1 levels by enzyme-linked immunosorbent assay (ELISA). The MCP-1 concentrations of the cell supernatants were determined using a commercially available rat MCP-1 ELISA development kit (Beyotime Biotech). The assays were performed using the instructions provided by the manufacturer. All samples were assayed in triplicate.

Statistical analysis. Data are expressed as the means ± SD of three assays. The statistical analysis was conducted by one-way ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Assessment of cell toxicity of curcumin. In order to exclude the possibility that reductions in the levels of inflammatory cytokines in the cells were due to the direct toxic effects of curcumin on the cells, we evaluated the cytotoxicity of curcumin at various concentrations (0-80 μM) using MTT assay. The results showed that the curcumin-induced cytotoxicity was negligible at concentrations of 0-80 μM in RAW264.7 macrophages (Fig. 1A).

Curcumin inhibits LPS-induced MCP-1 production in RAW264.7 macrophages. To investigate the inhibitory effect of curcumin on MCP-1 expression *in vitro*, ELISA was performed. As expected, treatment with 500 ng/ml LPS caused a time-dependent increase in MCP-1 production in the RAW264.7 cells (Fig. 1B). In addition, when the RAW264.7 cells were pre-incubated with curcumin and then stimulated with LPS, the curcumin pre-treatment significantly decreased the levels of MCP-1 released by the LPS-stimulated RAW264.7 cells (Fig. 1C).

Curcumin upregulates HO-1 expression in a concentration-dependent manner. To examine whether curcumin induces HO-1 expression, RT-PCR and western blot analyses were performed to determined the levels of HO-1 mRNA and

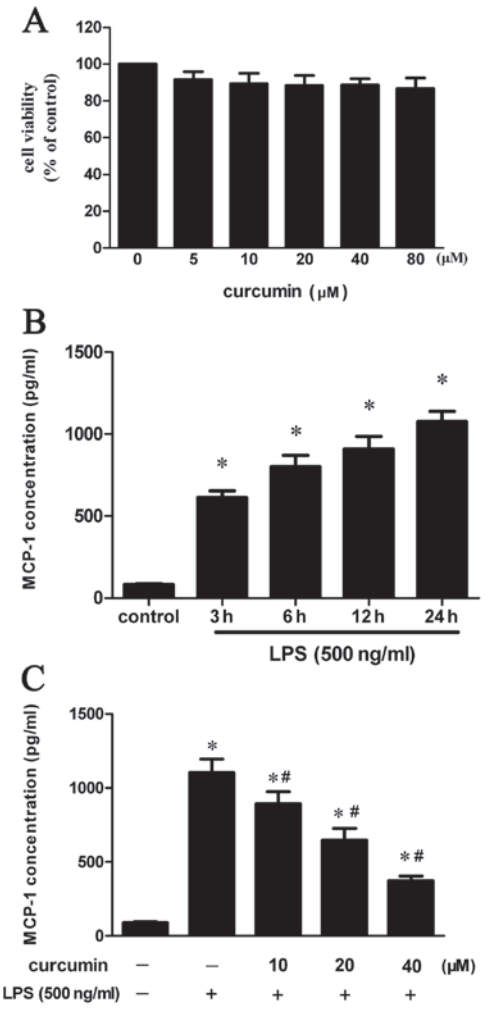

Figure 1. Curcumin suppresses LPS-induced MCP-1 production in RAW264.7 cells. (A) Cytotoxic assessment of curcumin in macrophages. The cytotoxic effect of curcumin on the cells was determined by MTT assay. (B) MCP-1 production was markedly increased following stimulation with 500 ng/ml LPS in a time-dependent manner. Cells were stimulated with LPS (500 ng/ml) for 3, 6, 12 and 24 h and MCP-1 production was assayed by ELISA. (C) Curcumin decreases LPS-induced MCP-1 production. Cells were pre-treated with curcumin at 10, 20 and 40 μM for 30 min prior to treatment with LPS (500 ng/ml) for 24 h. Data are expressed as the means ± SD of three independent experiments. *P<0.05 compared with the control group. $^{\#}$P<0.05 compared with the the LPS-treated group. LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MTT, methylthiazolyl tetrazolium; ELISA, enzyme-linked immunosorbent assay.

protein. We found that curcumin upregulated HO-1 expression in a concentration-dependent manner (Fig. 2).

HO-1 inhibitor, ZnPP, attenuates the inhibitory effect of curcumin on LPS-induced MCP-1 expression. To further investigate whether HO-1 contributes to the regulatory effect of curcumin on LPS-induced MCP-1 production, the cells were pre-treated with ZnPP (a HO-1 inhibitor) and curcumin prior to the addition of LPS. The results indicated that the inhibitory effect of curcumin on LPS-induced MCP-1 production was blocked by ZnPP (Fig. 3A).

NAC (ROS scavenger) inhibits LPS-induced MCP-1 production in RAW264.7 macrophages. Similarly, RAW264.7 cells

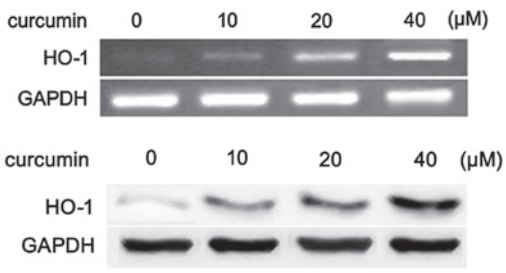

Figure 2. Curcumin significantly upregulates heme oxygenase-1 (HO-1) mRNA and protein expression in a concentration-dependent manner. Cells were incubated with various concentrations of curcumin (10, 20 and 40 μM) for 12 h and HO-1 expression was determined by western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR). Results are representative of three independent experiments.

were pre-incubated with NAC and then stimulated with LPS. We found that NAC markedly reduced the LPS-induced MCP-1 expression in RAW264.7 macrophages (Fig. 3A).

HO-1 inhibitor (ZnPP) attenuates the inhibitory effect of curcumin on LPS-induced ROS production. Increased ROS generation was observed in RAW264.7 cells stimulated with LPS, whereas the inhibitory effect was significantly blocked by the ROS scavenger, NAC, and the specific NADPH oxidase inhibitor, apocynin (Fig. 3B). In addition, LPS-mediated ROS production was significantly inhibited by curcumin. However, when the cells were pre-treated with ZnPP (a HO-1 inhibitor), the inhibitory effect was effectively eliminated (Fig. 3C).

Discussion

Macrophages play important roles in inflammatory diseases by producing multiple pro-inflammatory cytokines and enzymes in response to various stimuli, including LPS, a bacterial endotoxin (10). Activated macrophages release multiple cytokines and play critical roles in inflammatory processes. Among the numerous pro-inflammatory mediators implicated in inflammatory processes, MCP-1 appears to be one of the most significant chemokines that stimulates mononuclear leukocytes and triggers their adhesiveness and transmigration through the endothelial layer (11). It is widely expressed in atherosclerotic lesions, including vascular endothelial cells, smooth muscle cells and monocytes/macrophages. Therefore, methods of inhibiting MCP-1 production during exposure to chronic or long-term stimulation have been the subject of therapeutic strategies for treatment of these diseases. In the present study, we investigated the effect of curcumin on the LPS-stimulated production of MCP-1. Subsequently, we demonstrated that curcumin inhibited the LPS-induced MCP-1 production in RAW264.7 macrophages and explored the possible mechanisms involved. To our knowledge, we show for the first time that HO-1-mediated ROS reduction is involved in the inhibitory effect of curcumin on LPS-induced MCP-1 expression in RAW264.7 macrophages. The present data extend our knowledge of the effects of curcumin on macrophages.

HO-1, an inducible antioxidant enzyme, mediates the degradation of heme into ferrous iron, carbon monoxide and

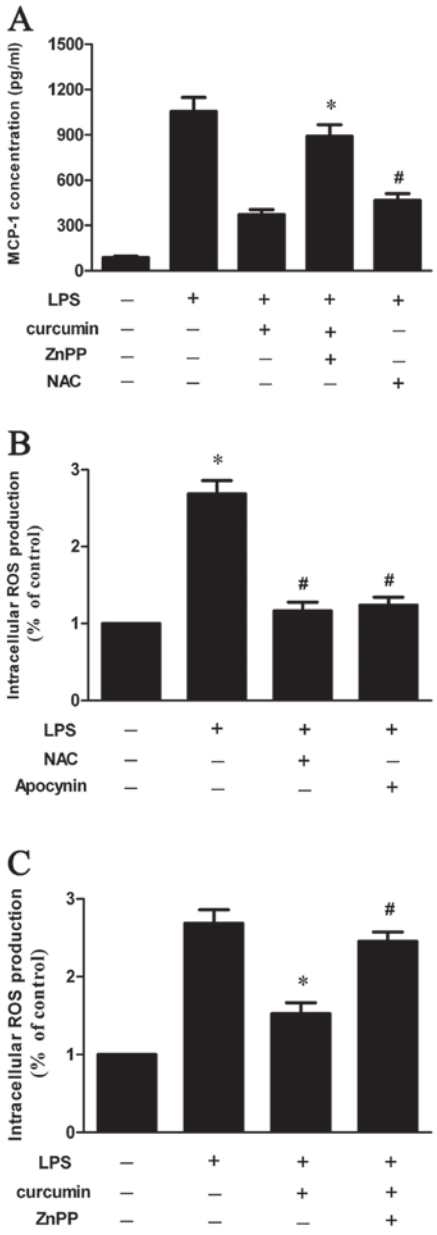

Figure 3. HO-1 inhibitor (ZnPP) blocked the inhibitory effect of curcumin on LPS-induced MCP-1 expression and ROS production. Data are the means ± SD of three independent experiments. (A) Cells were pre-treated with curcumin (40 μM), ZnPP (10 μM) or NAC (10 mM) for 30 min and cultured in the presence of LPS (500 ng/ml) for 24 h. MCP-1 production was assayed by ELISA. $^{\#}$P<0.05 vs. the LPS-treated group, *P<0.05 vs. the LPS- and 40 μM curcumin-treated group. (B) LPS-induced ROS production in RAW264.7 cells is dependent on NADPH oxidase. Cells were pre-treated with NAC (10 mM) or apocynin (1 mM) and cultured with LPS (500 ng/ml) for 2 h. *P<0.05 compared with the control group. $^{\#}$P<0.05 compared with the the LPS-treated group. (C) ZnPP attenuates the inhibitory effect of curcumin on LPS-induced ROS generation. Cells were pre-treated with curcumin and ZnPP (10 μM) for 30 min and cultured in the presence of LPS (500 ng/ml) for 2 h. Intracellular ROS levels were determined by DCF oxidation. *P<0.05 compared with the LPS-treated group, $^{\#}$P<0.05 compared with the LPS- and 40 μM curcumin-treated group. Heme oxygenase-1, HO-1; ZnPP, zinc protoporphyrin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; ELISA, enzyme-linked immunosorbent assay; ROS, reactive oxygen species; NAC, N-acetylcysteine.

biliverdin. The products resulting from HO-1 activity have antioxidant and anti-inflammatory effects (12). HO-1 is a cytoprotective protein whose expression is consistently associated with therapeutic benefits in a number of pathological conditions, including atherosclerotic vascular disease and inflammation (13).

In the present study, we found that curcumin significantly upregulates HO-1 mRNA and protein expression, which is consistent with several previous studies (14-16). Data from previous studies suggest that the induction of HO-1 enhances antioxidant and anti-inflammatory effects. It has been reported that celastrol-induced HO-1 expression is responsible for the suppression of IFN-γ-induced ICAM-1 expression and subsequent monocyte adhesion in keratinocytes (17). Furthermore, celastrol attenuates the hypertension-induced expression of inflammatory cytokines in vascular smooth muscle cells via HO-1 induction (18). Chen *et al* also demonstrated that HO-1 is involved the LPS-induced expression of cyclooxygenase-2 and MCP-1 (19). In the present study, our finding that curcumin inhibited LPS-induced MCP-1 production in RAW264.7 macrophages is in agreement with the findings of Panicker and Kartha (20), who revealed that curcumin attenuates glucose-induced MCP-1 synthesis in aortic endothelial cells through NF-κB. In addition, Lim and Kwon (21) identified that a possible anti-inflammatory mechanism of curcumin may be the inhibition of the secretion of the inflammatory MCP-1 chemokine. To investigate whether the inhibitory effect of curcumin on LPS-induced MCP-1 expression is mediated via HO-1, we pre-treated cells with ZnPP (a HO-1 inhibitor) and found that the inhibitory effect of curcumin was partially, but not completely reversed in the presence of ZnPP. Thus, our results demonstrate that the curcumin-induced HO-1 expression contributes, to a certain extent, to the MCP-1 reduction in RAW264.7 macrophages stimulated with LPS.

Under physiological conditions, there is a balance between the generation of ROS and detoxification by antioxidant systems. In general, oxidative stress occurs when this balance is disrupted, either directly by infectious agents or by cytokines released from inflamed cells that may lead to increased ROS generation and/or decreased antioxidant defense. Normally, ROS are involved in signal transduction pathways which mediate certain essential cellular functions, including host cell defense, mitochondrial respiration, cytokine generation and cell proliferation/apoptosis (22). LPS and various inflammatory cytokines, including TNF-α, interleukin (IL)-1 and IL-10, activate NADPH oxidase to generate significant, occasionally toxic, amounts of ROS which propagate their signals that activate transcription factors. In addition, accumulating evidence indicates that ROS act as signaling molecules to trigger pro-inflammatory cytokine production (23,24).

In our study, we found that ZnPP blocked the inhibitory effect of curcumin on LPS-induced ROS production, which demonstrated that curcumin-induced HO-1 expression is involved in the reduction of ROS in RAW264.7 macrophages stimulated with LPS. In addition, LPS-induced ROS production was suppressed by NAC and apocynin, which suggests that LPS induces ROS production in macrophages through NADPH oxidases. Moreover, the LPS-induced MCP-1 expression was also blocked by NAC (a ROS scavenger), which indicates that ROS generation is involved in LPS-induced MCP-1 expression. Furthermore, we demonstrate that curcumin-induced HO-1 expression may contribute to a reduction in MCP-1 expression levels in RAW264.7 macrophages stimulated with LPS. Taken

together, our results imply that the HO-1-mediated reduction of ROS is involved in the inhibitory effect of curcumin on LPS-induced MCP-1 production.

Several intracellular signaling pathways are involved in the regulation of the inflammatory reaction in stimulated macrophages, including the MAPK pathway linked to the activation of transcription factors. MAPKs may be activated by various extracellular molecules and may induce the downstream phosphorylation of a number of key signaling molecules related to cell proliferation, inflammation and apoptosis (25,26). In addition, numerous studies have implicated ROS as participants in a variety of intracellular signaling pathways that include MAPK members (27-29) and other signaling systems (30). It has been reported that curcumin inhibits phorbol myristate acetate (PMA)-induced MCP-1 expression by inhibiting ERK and NF-κB transcriptional activity (21). Takaya *et al* (31) found that albumin overload-induced MCP-1 expression was regulated by the ERK pathway in mouse proximal tubular cells. However, Hong *et al* (32) reported that the upregulation of MCP-1 by PMA was inhibited by the blockade of the p38 MAPK, but not by the blockade of the ERK and JNK pathways in human endothelial ECV304 cells. These inconsistent observations may be due to diverse experimental conditions and cell types.

Moreover, although the present study was conducted in cell culture, these findings should provide some useful molecular mechanisms to explain the beneficial anti-inflammatory effects of curcumin. In the future, we will attempt to confirm these observations with *in vivo* experiments.

In conclusion, this study provides evidence to suggest that curcumin enhanced the expression of HO-1 to reduce the LPS-induced production of ROS, which inhibited the expression of MCP-1 in RAW264.7 macrophages.

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