Induction of Heme Oxygenase-1 Inhibits Monocyte Chemoattractant Protein-1 mRNA Expression in U937 Cells

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Abstract. Heme oxygenase-1 (HO-1) is a stress-inducible isoform of HO with potential cytoprotective effects. Monocyte activation/migration mediated by monocyte chemoattractant protein-1 (MCP-1) is one of the earliest and important events in the pathogenesis of atherosclerosis. We examined the effect of HO-1 on the production of lysophosphatidylcholine (Lyso-PC)-induced MCP-1 in the human promonocytic cell line U937. Increased HO-1 induction by hemin resulted in a significant decrease in the Lyso-PC-mediated induction of MCP-1 mRNA expression. SnPP (IX), the specific inhibitor of HO-1 enzymatic activity, prevented the hemin-mediated attenuation of MCP-1 mRNA expression. These results suggest that HO-1 may work as an anti-atherogenic agent through the attenuation of MCP-1 production.

Keywords: heme oxygenase-1, oxidized low-density lipoprotein, monocyte chemoattractant protein-1

Monocytes/macrophages play important roles in the development of atherosclerosis. The selective recruitment of monocytes/macrophages into the wall of arteries is one of the earliest and critical events in the pathogenesis of atherosclerosis (1, 2). Monocyte chemoattractant protein-1 (MCP-1) is a potent chemotactic factor for monocytes and is produced by vascular smooth muscle cells, monocytes/macrophages, and vascular endothelial cells (3). In the process of atherogenesis, MCP-1 is largely responsible for the recruitment of monocytes/macrophages into the vessel wall. Previous studies have shown that oxidized low-density lipoprotein (ox-LDL) plays a key role in atherogenesis (4). Lysophosphatidylcholine (Lyso-PC) is a major phospholipid component of ox-LDL and can upregulate MCP-1 expression in vascular wall cells (5, 6).

Heme oxygenase (HO) is the rate-limiting enzyme that catalyzes the degradation of heme to carbon monoxide (CO), free iron, and biliverdin, which is then rapidly converted to bilirubin by biliverdin reductase (7, 8). Both bilirubin and biliverdin can act as antioxidants. CO activates the cyclic guanosine 3′,5′-monophosphate (cGMP) pathway and modulates the expression of platelet-derived growth factor-B that inhibits the proliferation of vascular smooth muscle cells (9). The oxidant, free iron, stimulates ferritin synthesis that in turn exerts additional antioxidant effects (7). HO-1 is a stress inducible isoform activated by stimuli associated with oxidative stress, and has a potent cytoprotective effect in various in vitro and in vivo models of atherosclerosis (10, 11).

We hypothesized that induction of HO-1 inhibited the MCP-1 mRNA expression of monocytes/macrophages in human vessel walls. Thus, the purpose of the present study is to determine whether the induction of HO-1 can inhibit MCP-1 mRNA expression in the human promonocytic cell line U937.

Lyso-PC (palmitoyl, C16:0), phorbol 12-myristate 13-acetate (PMA), hemin, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tin-protoporphyrin IX (SnPP IX) was obtained from Frontier Scientific Porphyrin Products, Inc. (Logan, UT, USA).
U937 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka) and cultured in RPMI 1640 medium (Nikken Seibutsu Igaku, Tokyo) supplemented with 100 µg/ml streptomycin, 100 U/ml of penicillin, 25 mM N-(2-hydroxyethyl)piperazine-N'- (2-ethanesulfonic acid) (HEPES), and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA) in 10-cm dishes (Becton Dickinson, Franklin Lakes, NJ, USA) and maintained at 37°C in a humidified atmosphere of 95% air/5% CO₂. U937 cells, at a concentration of 1.0 × 10⁶ in M199 medium (Sigma) containing 2% (vol/vol) FCS, were seeded into six-well dishes (2 ml/each well) (Becton Dickinson). Lyso-PC was dissolved in ethanol, and the same amount of ethanol was added to wells as a vehicle to the control. After incubation of the cells with or without Lyso-PC for 20 h, 10 µM hemin or DMSO was added as a vehicle to the medium and incubated for 4 h. Prior to the addition of hemin, various concentrations of SnPP (IX) or DMSO were added as a vehicle to the medium. U937 cells were incubated for 24 h in total. The cells were then harvested and total RNA was extracted.

Total RNA from U937 cells was extracted using the RNeasy Mini Kit from Qiagen (Hilden, Germany). cDNA synthesis was performed using an Omniscript RT kit (Qiagen), ribonuclease inhibitor (Promega, Madison, WI, USA), and random primers (Invitrogen, Carlsbad, CA, USA). Total RNA from samples was quantified by measuring the optical density (OD) at 260 nm. The sense and antisense primers were as follows: MCP-1, forward primer: 5'-CATAGCAGCCACCTTCCATCC, reverse primer: 5'-TCTGACTGAGATCTTCTTGTG; 18S ribosomal RNA, forward primer: TGCATGGCCGTCTTAGTTG, reverse primer: AGTACGATGCCAGGTCTCGTT; HO-1, forward primer: 5'-GAGGCCAAGACTGCGTTCC, reverse primer: 5'-GGGTGT CATGGGTGCAGCAGC.

To evaluate the expression levels of the target genes, real-time RT-PCR was performed with real-time TaqMan technology with the sequence detection system model 7700 (Perkin-Elmer/Applied Biosystems, Forester City, CA, USA). The PCR reaction mixture contained TaqMan cytokine gene expression reagents with MCP-1 specific target primers and probe (FAM dye layer), and endogenous reference primers and probe (18S ribosomal RNA, VIC dye layer) in MicroAmp Optical reaction tubes with optical caps (Perkin-Elmer/Applied Biosystems). The PCR was conducted using the following parameters: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s, and 60°C for 1 min. The threshold cycle (Ct), that is, the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was determined as previously described.

**Fig. 1.** Lyso-PC induces MCP-1 mRNA in U937 cells. A: Dose-response relationship of Lyso-PC-mediated induction of MCP-1 mRNA expression in U937 cells. Cells were incubated with 0–50 µM Lyso-PC for 8 h. MCP-1 mRNA expression was determined by real-time RT-PCR and normalized by 18S ribosomal RNA. The number of experiments is three. Data are the mean ± S.E.M. (n = 4). **P<0.01. B: Time course of Lyso-PC (50 µM)-induced MCP-1 mRNA expression after 0, 4, 8, 12, and 24 h. MCP-1 mRNA and 18S ribosomal RNA were quantified by real-time RT-PCR. The number of experiments is three. Values are the mean ± S.E.M. (n = 4). **P<0.01.
The relative quantification for MCP-1 and 18S ribosomal RNA gene expression assays were calculated from the accurate Ct. Fold induction of MCP-1 mRNA was determined from the Ct values normalized to the values of 18S ribosomal RNA. Data were analyzed with a Sequence Detector V1.7 program (Perkin-Elmer/Applied Biosystems).

Data were expressed as the mean ± S.E.M. ANOVA was used for statistical analysis of the results. Values of P<0.05 were considered statistically significant.

We determined MCP-1 mRNA expression levels in U937 cells by real-time RT-PCR. Figure 1A shows the induction of MCP-1 mRNA levels after an 8-h treatment with various concentrations of Lyso-PC. MCP-1 mRNA expression was the highest with 50 µM Lyso-PC. At a concentration of 50 µM, Lyso-PC induced MCP-1 mRNA about 2.8-fold compared to the control. The addition of higher doses of Lyso-PC (100 µM) reduced MCP-1 mRNA levels to the control level (data not shown).

Figure 1B shows the time course of MCP-1 mRNA induction by 50 µM Lyso-PC. The induction of MCP-1 mRNA by Lyso-PC was increased at 8, 12, and 24 h after incubation. In contrast, the induction of MCP-1 mRNA by ethanol as a vehicle was not increased significantly at any point during the incubation period.

To examine the effect of HO-1, U937 cells were treated with 10 µM hemin for 4 h before harvest. Treatment with hemin significantly attenuated Lyso-PC-induced MCP-1 mRNA expression compared to the control (P<0.05, Fig. 2A). To determine whether the hemin-mediated attenuation of MCP-1 mRNA levels was via the induction of HO-1, the cells were pre-treated for 2 h before the addition of hemin with SnPP (IX). The inhibitory effect of hemin on Lyso-PC-induced MCP-1 expression was attenuated in a dose-dependent manner (Fig. 2A). We have examined the effect of SnPP (IX) alone on MCP-1 mRNA expression in U937 cells. SnPP (IX) alone did not induce the expression of MCP-1, rather decreased it (Fig. 2B).

To confirm that HO-1 was induced by hemin in U937 cells, HO-1 mRNA was evaluated by real-time RT-PCR. Treatment with Lyso-PC alone did not upregulate HO-1 mRNA expression in U937 cells (Fig. 3A). In contrast, treatment of U937 cells with hemin induced higher levels of HO-1 mRNA expression than that of the

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**Fig. 2.** The effect of Lyso-PC (50 µM), hemin (10 µM), and SnPP (IX) on MCP-1 mRNA expression. A: MCP-1 mRNA and 18S ribosomal RNA were quantified by real time RT-PCR. Values represent the mean ± S.E.M. (n = 4). *P<0.05. B: The effect of SnPP (IX) alone on MCP-1 mRNA expression. MCP-1 mRNA and 18S ribosomal RNA were quantified by real time RT-PCR. Values represent the mean ± S.E.M. (n = 5). **P<0.01.
SnPP (IX) is known to block the enzymatic activity of HO-1 in U937 cells. Interestingly, SnPP (IX) augmented the hemin-mediated induction of HO-1 mRNA levels in a dose-dependent manner (Fig. 3A). SnPP (IX) alone did not upregulate the expression of HO-1 mRNA at all (Fig. 3B). The second bar is a positive control to show that the PCR has been performed in a proper way. These results have clearly shown that the upregulation of MCP-1 by SnPP (IX) (Fig. 2A) may take place in the presence of HO-1 induced by hemin.

The present study showed that hemin-mediated induction of HO-1 attenuated Lyso-PC-induced MCP-1 mRNA expression in the human promonocytic cell line U937 and that the inhibition of HO-1 activity by SnPP (IX) increased MCP-1. Thus, HO-1 may contribute to the downregulation of MCP-1 mRNA expression in U937 cells. As MCP-1 is considered to be a key molecule in atherogenesis (13), HO-1 may play an important role in inhibiting the progression of atherosclerosis.

Here, we show that Lyso-PC increased MCP-1 mRNA expression in U937 cells. Lyso-PC is generated from phosphatidylcholine via the action of phospholipase A$_2$ (14). However, the underlying mechanism for MCP-1 production by Lyso-PC in U937 cells is not clear and requires further investigation.

MCP-1 is recognized as an immediate early gene. Our data showed that in U937 cells, the induction of MCP-1 by Lyso-PC was not rapid and took 24 h. Although the molecular mechanism for this delayed induction of MCP-1 in monocytes/macrophages is unknown, the sustained production of MCP-1 by monocytes/macrophages might promote the continuous recruitment of the same cell types in vivo.

Our data indicate that modulators of HO-1 production and activity can affect the Lyso-PC-mediated increase in MCP-1 mRNA expression levels. HO-1 mediates the catabolism of heme, yielding the sequential cytoprotective products bilirubin, CO, and ferritin (7, 8). In the current study, we have not specified which products attenuate Lyso-PC-induced MCP-1 mRNA expression in U937 cells. Ishikawa et al. reported that augmentation of HO-1 inhibited the progression of atherosclerosis both in vitro and in vivo (10, 12). It is possible that the anti-atherosclerotic effect of HO-1 may be through the suppression of MCP-1.

SnPP (IX) is a specific competitive inhibitor of HO-1. When cells were treated with concomitant administration of hemin and SnPP (IX), HO-1 mRNA was more...
highly expressed compared to the control treated with hemin alone. This increase in HO-1 mRNA expression with SnPP (IX) may be a compensatory effect, and it may be induced by the suppression of HO-1 enzymatic activity.

We conclude that HO-1 attenuated MCP-1 mRNA expression in the human monocytic cell line U937. These results suggest that the induction of HO-1 in monocytes/macrophages may provide a promising strategy for the prevention of atherosclerosis in humans.

References