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Induction of Heme Oxygenase-1 in Monocytes Suppresses Angiotensin II-Elicited Chemotactic Activity Through Inhibition of CCR2: Role of Bilirubin and Carbon Monoxide Generated by the Enzyme

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ABSTRACT

Monocyte chemoattractant protein 1 (MCP-1) and the receptor for MCP-1, CCR2, play a pivotal role in the recruitment of monocytes to the subendothelium, which is the initial event in atherosclerosis. Heme oxygenase (HO) is a microsomal enzyme that catalyzes the degradation of heme into biliverdin, which is subsequently reduced to bilirubin, free iron, and carbon monoxide, and induction of HO-1 is potentially associated with cellular protection, especially against oxidative insults. The present study was designed to examine the role of HO-1 in monocytes in angiotensin II (Ang II)-induced chemotactic response. Ang II significantly stimulated superoxide formation in monocytes, as measured by nitro blue tetrazolium reduction assay, as well as the chemotactic response to MCP-1 with the increased expression of CCR2 determined by RT-PCR and western blotting analysis. Hemin-treated monocytes displayed an enhanced HO activity with the increased accumulation of bilirubin determined by immunostaining, when compared with control monocytes. The induction of HO-1 in monocytes suppresses not only Ang II-stimulated superoxide formation, but also Ang II-enhanced chemotactic activity. Exogenously applied bilirubin and carbon monoxide mimicked the inhibitory effect of HO-1 on the chemotactic response. These findings suggest that monocytic HO-1 might be a new therapeutic target for atherosclerosis. *Antioxid. Redox Signal.* 5, 439–447.

INTRODUCTION

HEME OXYGENASE (HO) is a microsomal enzyme that catalyzes the degradation of heme into biliverdin, which is subsequently reduced to bilirubin, free iron, and carbon monoxide (CO) (35). There has been a recent explosion of interest in heme oxygenase-1 (HO-1), an isozyme of HO inducible by a variety of stimulants, as its breakdown products play many vital physiological and pathological roles in diverse biological processes. Biliverdin and bilirubin are regarded as endogenous antioxidants that scavenge a variety of active oxygen species, including superoxide anions (30). CO is known to function both as a vasodilating mediator and as an inhibitor of cytochrome P450, which promotes the oxidation of fatty acids (5, 18, 28). HO-1 gene knockout mice were found to be more sensitive to a wide variety of oxidative stimuli (21), suggesting that enhanced HO-1 activity contributes to increase resistance to oxidative insults.

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Angiotensin II (Ang II), a vasoconstrictor produced by the renin–angiotensin system, is implicated in atherosclerosis (17). Increased oxidative stress, through an elevated formation of reactive oxygen species, is a putative risk factor involved in the proatherosclerotic effects of Ang II. Those reactive oxygen species include superoxide anions formed through the activation of NAD(P)H oxidase in every cell type of the vasculature, including monocytes (9). Animals rendered hypertensive by chronic infusion of Ang II augmented superoxide formation, resulting in enhanced leukocyte adhesion to the endothelium (22).

Invasion of monocytes into the vessel wall is one of the earliest steps in the development of atherosclerosis. Substantial evidence supports a role for monocyte chemoattractant protein 1 (MCP-1) and CCR2, the receptor for MCP-1, in the recruitment of monocytes to the subendothelial space of arteries. MCP-1 is a member of the CC family of chemokines and is characterized by its ability to evoke chemotaxis mainly in monocytes (24). It is expressed and secreted by the cells of the vessel wall, such as vascular endothelial cells, vascular smooth muscle cells (VSMCs), and monocytes/macrophages, in response to a variety of proinflammatory stimuli, including Ang II (7, 25). The effect of MCP-1 is mediated by the seven-transmembrane-spanning G protein-coupled receptor, CCR2, which was shown to exist in two highly homologous isoforms (6). CCR2B appears to be the predominant form found on human monocytes, and the exact cellular localization and function of CCR2A remain uncertain (33). A homozygous deletion of the mouse CCR2 gene exhibited diminished monocyte chemotaxis and adhesion to the vasculature (3), and selective disruption of the CCR2 gene in the apolipoprotein E null mice showed markedly less macrophage accumulation in the aorta and decreased lesion formation (8). Based on these properties, CCR2 has been suggested to play an important role in accelerating the process of atherosclerosis.

The present study was designed to examine the roles of HO-1 in the Ang II-induced chemotactic response to MCP-1 through CCR2. We demonstrated that induction of HO-1 in monocytes not only prevented the elevation of intracellular superoxide formation induced by Ang II, but also suppressed Ang II-stimulated CCR2 expression as well as the Ang II-enhanced chemotactic response to MCP-1.

MATERIALS AND METHODS

Cell culture and incubation conditions

Human peripheral blood monocytes (PBM) from healthy volunteers were obtained by the Ficoll–Hypaque method and cultured as described elsewhere (32). The purity of monocytes was >90% as estimated by flow cytometry using anti-CD14 antibody (Pharmingen, San Diego, CA, U.S.A.). A human monocytic cell line (THP-1 cells) was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and cultured as described elsewhere (11, 32). The cells were incubated with various concentrations of Ang II; thereafter, they were washed three times with phosphate-buffered saline and used for the analyses as described below. Cell viability, determined by trypan blue exclusion, was >95%. The endotoxin level in the medium was routinely monitored using an endotoxin kit and never exceeded 0.05 ng/ml (29).

Exposure to CO

THP-1 monocytes were exposed to CO as previously described (4). In brief, gases from stock gas tanks containing 1% CO and 5% CO_2 in air (Tomoe Inc., Tokyo, Japan) were mixed in a cylinder before delivery into an exposure chamber. Flow into the humidified chamber was 1 L/min, and the temperature was maintained at 37°C. CO levels were measured continuously by electrochemical detection using a CO analyzer placed in the chamber.

Isolation of RNA and analysis of CCR2 and HO-1 transcripts

Total cellular RNA was isolated from PBM or THP-1 cells using TRIzol reagent (Invitrogen). Transcripts from 0.5 μg (PBM) or 2 μg (THP-1 cells) of total RNA were reverse-transcribed by Superscript II (Life Technologies Inc.), and CCR2B and HO-1 expression was estimated by PCR, as described elsewhere (2, 10). The amplified DNA was analyzed by agarose gel electrophoresis; the intensity of the band stained with ethidium bromide was estimated by densitometric scanning (LKB Ultrascan XL laser densitometer) and compared with that of the internal standard. As the internal standard, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as described elsewhere (10).

Analysis of CCR2 protein by western blotting analysis

The amount of CCR2 protein in monocytes was determined by western blotting analysis as described elsewhere (32). In brief, cellular protein (30 μg) from monocytes was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [12% (wt/vol) gel]. Proteins transferred to a nitrocellulose membrane were identified by polyclonal anti-CCR2B antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), which recognized the 38-kDa human CCR2. Horseradish peroxidase-conjugated secondary antibody was used to develop the membrane. Blots were developed by the enhanced chemiluminescence method (Amersham), and relative protein levels were quantified by scanning densitometry (LKB Ultrascan XL laser densitometer).

HO activity in THP-1 monocytes and PBM

HO activity in THP-1 monocytes was determined by measuring the formation of bilirubin as described previously (14). In brief, the cells were harvested and washed twice with phosphate-buffered saline at pH 7.0. The cells were lysed in 1% Nonidet P-40 solution containing 150 m*M* NaCl, phenylmethylsulfonyl fluoride (1 m*M*), and 50 m*M* Tris-HCl (pH 8.0) for 1 h on ice. The supernatants were collected by centrifugation at 10,000 *g* for 10 min. The reaction mixture contained the following in a final volume of 300 μl: glucose

6-phosphate (1 m*M*; Roche Molecular Biochemicals), glucose-6-phosphate dehydrogenase (0.167 units/ml; Roche Molecular Biochemicals), NADP (0.8 m*M*), hemin (30 μ*M*), $MgCl_2$ (2 m*M*), NADPH-cytochrome P450 reductase (0.1 mg/ml; Gentest, Woburn, MA, U.S.A.), rat liver cytosol (6.7 mg of protein/ml), potassium phosphate buffer (18.3 m*M*, pH 7.4), and the supernatant from the cells. Incubation was carried out at 37°C for 30 min. An equal volume of chloroform was added to the reaction mixture, and the bilirubin generated was extracted into the chloroform fraction. After centrifugation at 10,000 *g* for 10 min, the amount of bilirubin in the chloroform extract was examined.

Immunohistochemistry

Immunostaining was performed using the Vecstain ABC kit (Vector Laboratories) according to the protocol provided by the manufacturer. Accumulation of bilirubin IXα and HO-1 protein in THP-1 monocytes was examined by immunohistochemistry with the use of monoclonal antibody against bilirubin IXα, 24G7 (34), and human HO-1 (StressGen), respectively, as previously described (15, 31). In brief, cells were fixed with 4% paraformaldehyde, treated with saponin for membrane permeabilization, and incubated with the primary antibody in 2% bovine serum albumin overnight at 4°C. Cells were then washed and dyed with a Fuchsin kit (Dako) as indicated by the manufacturer.

Chemotaxis assay

Monocyte chemotaxis was measured in a 48-well microchemotaxis chamber (12). THP-1 monocytes were incubated for 24 h in the presence or absence of Ang II at various concentrations. After incubation, cells were resuspended at 2×10^6 cells/ml and loaded in the upper chamber of the microchemotaxis chamber. MCP-1 (0.1 μg/ml) was added to the lower chamber, and cells were allowed to transmigrate for 2 h. After transmigration, the upper side of the membrane was scraped off and washed three times in accordance with the instructions provided by the manufacturer. THP-1 monocytes attached to the underside of the membrane were fixed and then stained with hematoxylin. The number of cells that migrated through the membrane was determined by counting the cells in four high-power 400× fields per well under a microscope. Each experiment was done at least in triplicate.

Determination of cellular superoxide anion content

The content of superoxide anion was determined by the nitro blue tetrazolium (NBT) reduction assay (23). After incubation for 24 h in the absence or presence of Ang II, cells were washed three times with Hanks' balanced salt solution and further incubated for 2 h in Krebs–Henseleit buffer containing 1 mg/ml NBT. Then the cells were washed three times with Hanks' balanced salt solution and lysed with 5% (wt/vol) sodium dodecyl sulfate/80 m*M* phosphate buffer (pH 7.8) followed by centrifugation for 5 min at 13,000 *g*, and the absorbance of the supernatant at 540 and 450 nm was determined as described previously (23).

Reagents

All reagents used in the present study were purchased from Sigma unless otherwise mentioned.

Statistical analyses

Data in the present study are expressed as the means ± SE of at least five independent experiments. Analysis of multiple paired data within the same experiments was performed with the nonparametric Friedman's test and the Wilcoxon's test. $p < 0.05$ was considered significant.

RESULTS

Effects of Ang II on the expression of CCR2 mRNA

THP-1 monocytes were incubated with Ang II (10^{-7} *M*) for 6, 12, 24, and 48 h. After incubation, CCR2 mRNA was determined by RT-PCR. As shown in Fig. 1A, THP-1 cells slightly expressed basal levels of CCR2 mRNA before treatment with Ang II. However, Ang II elicited a significant and sustained increase of CCR2 mRNA from 12 h to 48 h of incubation (Fig. 1A). Parallel experiments using PBM revealed that Ang II-treated PBM exhibited the enhanced CCR2 expression in a manner similar to that observed in THP-1 cells. We then treated THP-1 cells with various concentrations of Ang II for 24 h. This stimulatory effect of Ang II on CCR2 mRNA expression occurred in a concen-

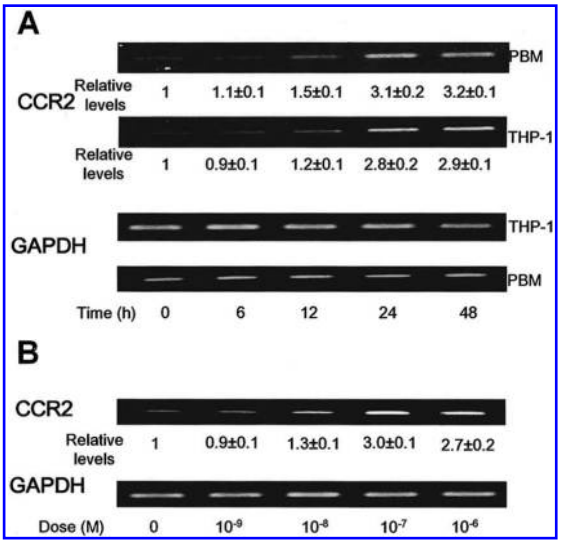

FIG. 1. Effects of Ang II on CCR2 mRNA levels in monocytes. (**A**) THP-1 monocytes or PBM were incubated in the absence or presence of 10^{-7} *M* Ang II for various periods of time. (**B**) THP-1 cells were incubated with various concentrations for 24 h. After incubation, RT-PCR was performed to determine CCR2 mRNA expression. Values indicate the relative expression of CCR2 mRNA normalized for GAPDH levels. Ang II-untreated cells were used as the control. Relative amounts of mRNA are presented as the means ± SE of five separate experiments.

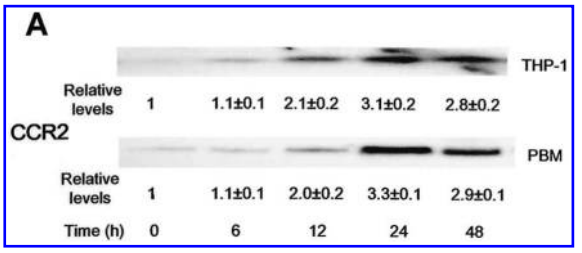

FIG. 2. Effects of Ang II on CCR2 protein levels in monocytes. (**A**) THP-1 monocytes or PBM were incubated in the absence or presence of 10^{-7} *M* Ang II for various periods of time. (**B**) THP-1 cells were incubated with various concentrations for 24 h. After incubation, the amount of CCR2 protein was determined by western blotting analysis. Ang II-untreated cells were used as the control. The relative protein levels are presented as the means ± SE of five separate experiments.

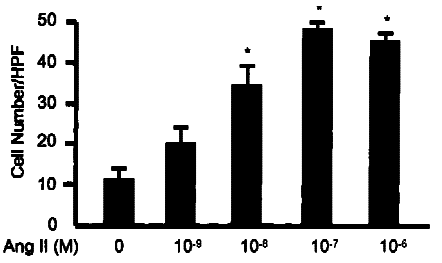

FIG. 3. Chemotactic activity of THP-1 cells. Cells were incubated in the absence or presence of 10^{-9}–10^{-6} *M* Ang II for 24 h. The MCP-1-induced chemotactic activity was determined using a microchemotaxis chamber. The results are expressed as numbers of migrated monocytes per high-power field (HPF) under light microscopy and are presented as the means ± SE (error bar) of five separate experiments. *$p < 0.05$ compared with control.

tration-dependent manner (10^{-9}–10^{-6} *M*), and its maximum effect was attained at concentrations of 10^{-7} *M* and 10^{-6} *M* (Fig. 1B).

Effects of Ang II on the expression of CCR2 protein

The amounts of CCR2 protein in THP-1 monocytes and PBM were determined by western blotting analysis. In accordance with the expression of CCR2 mRNA, Ang II significantly increased the amount of CCR2 protein in both THP-1 monocytes and PBM (Fig. 2A), and the largest amount of CCR2 protein was observed at concentrations of 10^{-7} *M* and 10^{-6} *M* through 24 h to 48 h of incubation (Fig. 2B). Parallel experiments using PBM revealed that the levels of CCR2 expression were increased in Ang II-treated PBM in a manner similar to those observed in THP-1 monocytes. We, therefore, used THP-1 monocytes in the rest of the experiments.

Effects of Ang II on the chemotactic response to MCP-1

The functional relevance of the Ang II-increased expression of CCR2 in THP-1 monocytes was examined by means of a chemotaxis assay. As shown in Fig. 3, Ang II significantly stimulated the migration of monocytes in a concentration-dependent manner.

Effects of Ang II on cellular formation of superoxide anion

The effect of Ang II on the formation of superoxide anion in THP-1 monocytes was measured by the NBT reduction assay. As shown in Fig. 4 (left panel), the intracellular level of superoxide anions was significantly elevated after 12 h of incubation of THP-1 monocytes with Ang II and reached a maximum at 24 h and 48 h of incubation. After cells were incubated for 24 h with various concentrations of Ang II, the levels of superoxide were elevated in a concentration-dependent manner and reached a maximum at 10^{-7} *M* and 10^{-6} *M* of Ang II (Fig. 4, right panel).

Induction of HO-1 in THP-1 monocytes supressed Ang II-stimulated levels of CCR2 mRNA and protein

To investigate the role of HO in THP-1 monocytes in CCR-2 expression, we added hemin to the culture media and

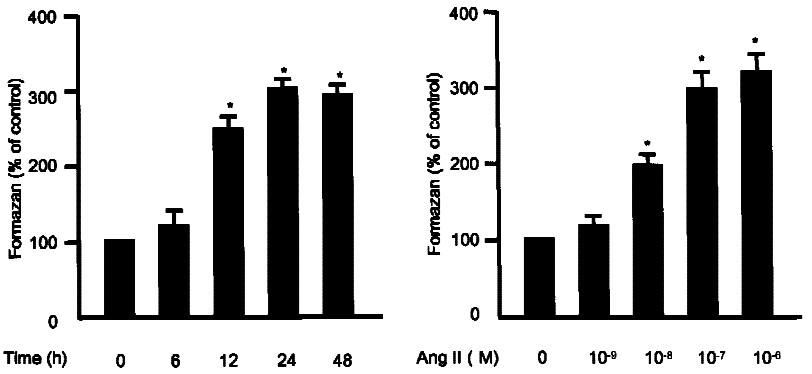

FIG. 4. Effects of Ang II on superoxide formation in THP-1 monocytes. (Left panel) Cells were incubated with Ang II (10^{-7} *M*) for various periods of time. (**Right panel**) Cells were incubated in the absence or presence of 10^{-9}–10^{-6} *M* Ang II for 24 h. In both experiments, the formation of cellular superoxide was determined by the NBT reduction assay and expressed as the percentage of formazan formed in the control cells. Results are presented as the means ± SE (error bars) of five separate experiments. *$p < 0.05$ compared with control.

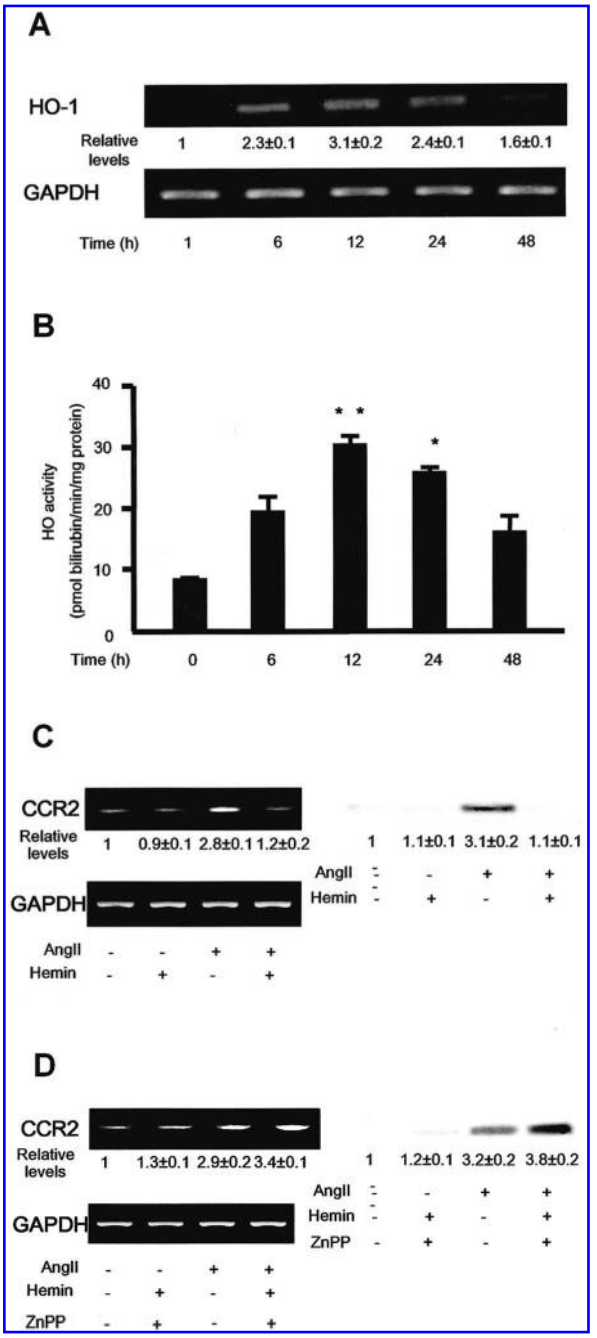

FIG. 5. Effects of hemin on the expression of HO-1 mRNA (A), HO activity (B), and CCR2 expression (C and D). THP-1 monocytes were incubated with hemin (10^{-8} *M*) for various periods of time (**A** and **B**). After incubation, RT-PCR was performed to examine the expression of HO-1 mRNA (A) and HO activity was determined based on the formation of bilirubin (**B**). Values indicate the relative expression of HO-1 mRNA normalized for GAPDH levels (**A**) and means ± SE (error bars) of six separate experiments. $*p < 0.05$, $**p < 0.01$ compared with the control (**B**), respectively. (**C** and **D**) Cells were incubated for 24 h with Ang II (10^{-7} *M*) in the absence or presence of hemin (10^{-8} *M*) and ZnPP (0.5 μ*M*) for the last 12 h. After incubation, RT-PCR (**left panel**) and western blotting (**right panel**) analyses were performed as described in the legends for Figs. 1 and 2, respectively.

determined HO-1 expression in THP-1 monocytes. As shown in Fig. 5A, HO-1 expression was induced 6 h after treatment and reached the maximum value at 12 h, which coincided with the increase in HO activity (Fig. 5B). We then examined the levels of CCR2 induced by Ang II in the HO-enhanced THP-1 monocytes. We found that hemin did not cause a significant reduction in basal CCR2 expression and that Ang II failed to stimulate CCR2 expression in the HO-enhanced THP-1 monocytes (Fig. 5C). Treatment with the HO inhibitor, zinc protoporphyrin IX (ZnPP), not only cancelled the inhibitory effect of hemin on CCR2 expression, but also augmented CCR2 expression under basal and Ang II-stimulated conditions (Fig. 5D).

Accumulation of bilirubin IXα in hemin-treated THP-1 monocytes

To demonstrate the actual enhanced degradation of heme through the HO reaction in hemin-treated THP-1 monocytes, we analyzed the accumulation of bilirubin IXα using the 24G7 monoclonal antibody. As shown in the upper left panel of Fig. 6, weak staining was detected in the cytoplasm of control THP-1 cells, which did not exhibit immunoreactivity to HO-1 (Figure 6, lower left panel). However, hemin-treated THP-1 monocytes displayed notable immunoreactivity to bilirubin IXα compared with control THP-1, and intense staining was detected mainly in perinuclear regions (Fig. 6, upper right panel), which was consistent with the expression pattern of HO-1 (Fig. 6, lower right panel).

Exogenously applied bilirubin and CO mimicked the inhibitory effect of HO-1 on the expression of Ang II-stimulated CCR2 expression

To demonstrate which enzymatic product, that is, bilirubin or CO, contributes to suppress Ang II-induced CCR2 expression, cells were incubated with bilirubin in the absence or presence of CO for the last 12 h of a 24-h treatment with Ang II. As shown in Fig. 7, bilirubin reduced the Ang II-induced increase in CCR2 in a concentration-dependent manner, and combined application of CO completely abolished these expressions to a level similar to that observed in hemin-treated cells.

The induction of HO activity not only prevented the elevation of superoxide formation induced by Ang II, but also suppressed Ang II-enhanced chemotactic response to MCP-1

The effect of induced HO-1 on Ang II-stimulated formation of superoxide anion in THP-1 monocytes was estimated by the NBT reduction assay, because bilirubin is the most abundant endogenous antioxidant in mammalian tissues. As shown in Fig. 8A, hemin abolished Ang II-stimulated superoxide formation and the inhibitory effect of hemin was cancelled by treatment with ZnPP, indicating that the enhanced HO-1 reduced Ang II-stimulated superoxide formation. Bilirubin exogenously applied to the culture medium inhibited Ang II-induced superoxide formation in a concentration-dependent manner, and combined application of CO with

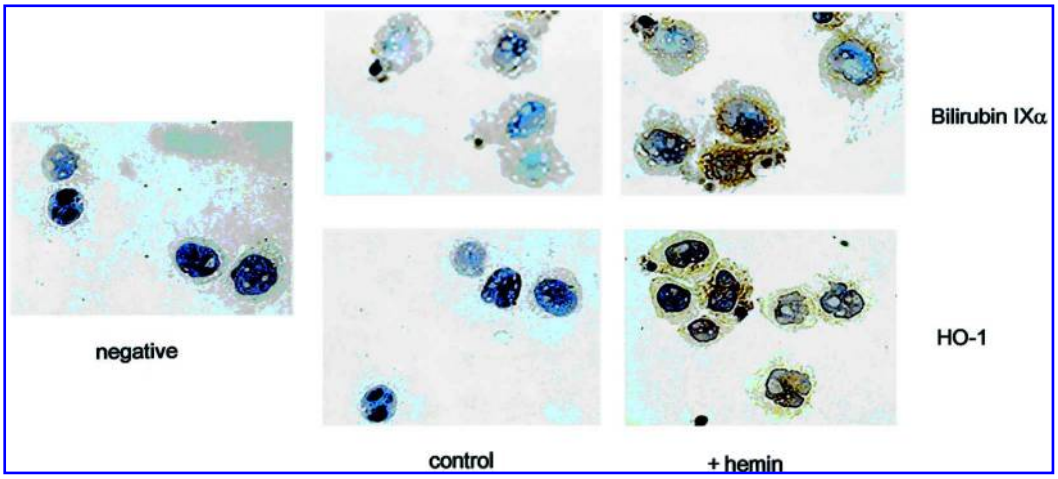

FIG. 6. Immunostaining for HO-1 and bilirubin IXα in THP-1 monocytes. THP-1 monocytes were incubated in the absence or presence of hemin (10^{-8} *M*) for 12 h. After incubation, immunohistochemistry using a monoclonal antibody against bilirubin IXα (**upper panels**) or HO-1 (**lower panels**) was performed (scale ×400). Immunostaining without primary antibody is shown as the negative.

bilirubin did not show any further inhibitory effect on Ang II-induced superoxide formation. In accordance with the reduced formation of superoxide, chemotactic responses were altered by HO activity, that is, hemin-treated THP-1 monocytes displayed a reduced chemotactic activity as compared with control monocytes and exogenously applied bilirubin blocked it in a concentration-dependent manner (Fig. 8B). Interestingly, combined application of CO wiith bilirubin further blocked Ang II-enhanced chemotactic activity, which was accompanied by the further reduction of CCR2 expression (see Fig. 7) to levels similar to those observed in hemin-treated cells.

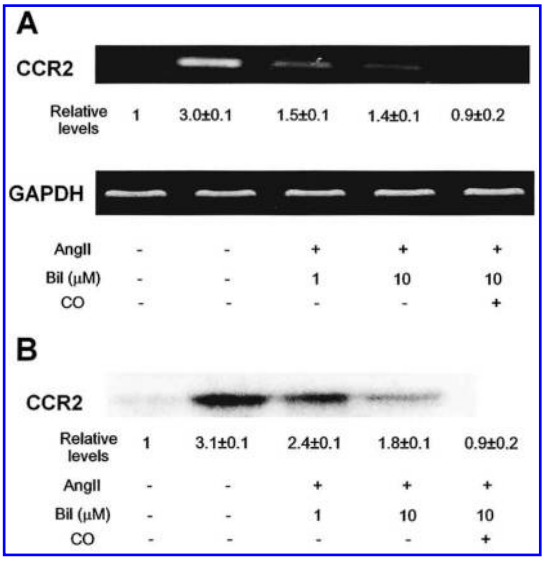

FIG. 7. Effects of exogenously applied bilirubin and CO on CCR2 expression. THP-1 monocytes were incubated for 24 h with Ang II (10^{-7} *M*) in the absence or presence of bilirubin (Bil) for the last 12 h. In some experiments, CO was applied to the culture with 10 μ*M* bilirubin. After incubation, RT-PCR (**A**) and western blotting analysis (**B**) were performed as described above.

DISCUSSION

The present study clearly demonstrates that Ang II stimulates production of superoxide anions as well as CCR2 expression in monocytes and enhances the chemotactic response to MCP-1. HO-1 not only prevented the increase of superoxide formation induced by Ang II, but also suppressed the Ang II-enhanced chemotactic response and CCR2 expression. Combined application of bilirubin and CO mimicked the inhibitory effect of HO-1, although bilirubin alone failed to abolish CCR2 expression and chemotactic activity completely.

MCP-1 and CCR-2 are key mediators of the recruitment of monocytes to the subendothelium (3, 8, 24). Ang II stimulates MCP-1 mRNA expression in VSMCs (7), and the induced MCP-1 gene expression was suppressed by inhibitors of NAD(P)H oxidase and catalase (7), suggesting that Ang II-induced MCP-1 expression depends on redox-sensitive signaling events involving activation of NAD(P)H oxidase and generation of hydrogen peroxide (H_2O_2). In the present study, treatment with Ang II significantly increased superoxide formation in monocytes, as well as the expression of CCR2 gene and protein. We, therefore, speculated that Ang II-induced oxidative stress was involved in the up-regulation of CCR2 expression in a way similar to that of MCP-1 induced by Ang II in VSMCs. Monocyte CCR2 expression is reported to be regulated by a cellular redox state. Oxidative insults, including H_2O_2, buthionine sulfoximine, and homocysteine, stimulate CCR2 expression in human monocytes (26, 32); in contrast, treatment with antioxidants, such as pyrrolidine dithiocarbamate and superoxide dismutase, decreases the expression of CCR2 (26, 32). In this study, we found that Ang II increased intracellular superoxide production in monocytes.

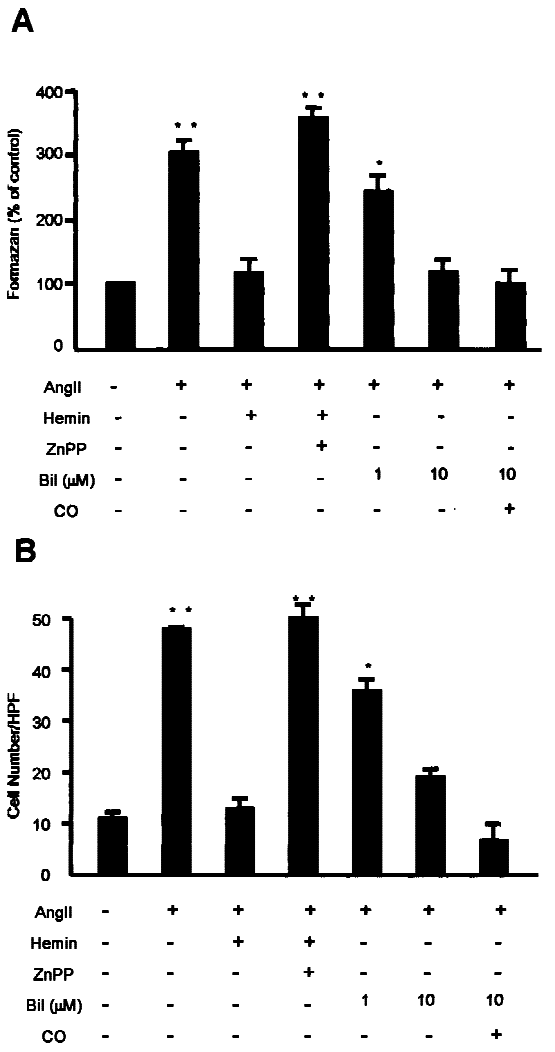

FIG. 8. Effects of HO-1 on intracellular superoxide formation (A) and chemotactic response to MCP-1 (B). THP-1 monocytes were incubated for 24 h with Ang II (10^{-7} *M*) in the absence or presence of hemin (10^{-8} *M*) for the last 12 h. In some experiments, ZnPP (0.5 μ*M*) or bilirubin (Bil; 1 or 10 μ*M*) was added to the cultures in the absence or presence of CO. (**A**) After incubation, the formation of superoxide was determined by the NBT reduction assay. The results are expressed as percentages of the control and are shown as the means ± SE (error bars) of six separate experiments. $*p < 0.05$, $**p < 0.01$ compared with the control. (**B**) The MCP-1-induced chemotactic activity was determined using a microchemotaxis chamber. The results are expressed as the number of migrated monocytes per high-power field (HPF) under light microscopy and are shown as the means ± SE (error bars) of six separate experiments. $*p < 0.05$, $**p < 0.01$ compared with control.

NAD(P)H oxidase in monocytes is involved in the production of superoxide anions (9). Treatment with statins inhibits NAD(P)H oxidase activity through the inhibition of the translocation of p21 rac 2 and p67, resulting in a reduced production of superoxide anions (9). Taken together, Ang II stimulates superoxide production through NAD(P)H oxidase, resulting in an increase in CCR2 expression in monocytes with enhanced chemotactic activity.

There are several ways through which HO activity alleviates cellular oxidative stress. First, the HO reaction is attributable to a decrease in the substrate protoheme IX, which stimulates oxidative damage (1). Second, the HO reaction can reduce oxidative stress through biological actions of its enzymatic products, biliverdin IXα and CO. Biliverdin IXα and its reduced product bilirubin IXα serve as endogenous antioxidants (30), and have been shown to inhibit the adhesion of neutrophils elicited by ischemia/reperfusion or exposure to H_2O_2 (13). On the other hand, CO is known to function both as a vasodilating mediator and as an inhibitor of cytochrome P450, which can promote oxidation of fatty acids (5, 18, 28). Immunohistochemistry revealed that bilirubin IXα accumulated intensely in perinuclear regions of hemin-treated THP-1 monocytes, which was consistent with the distribution of the immunoreactivity to HO-1, in a pattern very similar to that observed in foam cells and mast cells (19, 31). As 24G7 specifically recognizes bilirubin IXα and its oxidative metabolites (34), immunoreactivity to 24G7 in THP-1 monocytes indicates that the α-methene bridge of heme is actually cleaved by HO to generate CO, free iron, and biliverdin IXα. We demonstrated that the induction of HO-1 by hemin reduced the elevation of superoxide formation, and that exogenously applied bilirubin blocked superoxide formation, suggesting that bilirubin accumulated mainly by the reaction of HO-1 reduces Ang II-induced oxidative stress. Hayashi *et al.* reported that induction of HO-1 suppressed leukocyte adhesion elicited by oxidative stress through the action of bilirubin (13). Takamiya *et al.* revealed that bilirubin generated through HO-1 desensitized mast cells and ameliorated leukocyte recruitment (31). Taken together, bilirubin endogenously generated through the action of HO-1 is a key player against oxidative stress-induced monocyte/leukocyte recruitment to endothelium. Weak immunoreactivity to bilirubin IXα in the cytoplasm may represent the accumulation of bilirubin IXα produced by the reaction of HO-2, a constitutively expressed isozyme of HO (27). Experiments using ZnPP, an inhibitor of HO, revealed that treatment with ZnPP slightly augmented CCR2 expression under both basal and Ang II-stimulated conditions with the increase in superoxide formation, suggesting that bilirubin endogenously formed by HO-2, as well as HO-1, may contribute to reduce superoxide formation in THP-1 monocytes. On the other hand, 10 μ*M* bilirubin did not fully suppress the Ang II-stimulated CCR2 expression nor the Ang II-enhanced chemotactic response, although the concentration of bilirubin used in the current study is considered to be comparable to that of endogenously formed bilirubin in hemin-treated THP-1 cells (31). Combined application of CO with bilirubin further suppressed the Ang II-enhanced chemotactic activity, as well as Ang II-stimulated CCR2 expression, although CO did not show any additional inhibitory effect on superoxide formation. Moreover, we found that CO in the absence of bilirubin suppresses CCR2 expression in THP-1 monocytes (our unpublished data). Oxidized low-density lipoprotein has been shown to stimulate chemotaxis of THP-1 cells via the activation of p38 mitogen-activated protein kinase (p38 MAPK) (16), and CO works as an antiinflammatory substance through p38 MAPK (20). Taken to-

gether, these findings suggest that mechanisms other than increased oxidative stress are involved in Ang II-enhanced chemotactic activity. Further research will be needed to clarify fully the mechanisms by which CO reduces Ang II-enhanced CCR2 expression and chemotactic activity in monocytes.

In the present study, we demonstrated that the induction of HO-1 suppressed the elevation of superoxide formation induced by Ang II, as well as of Ang II-stimulated expression of CCR2, leading to the inhibition of chemotactic activity. These findings suggest that an increase of HO activity in monocytes ameliorate monocytes recruitment to the subendothelium, which is an important step for the progression of atherosclerosis. Our study shed light on the importance of HO-1 in monocytes, and HO activity in monocytes might be a new therapeutic target regarding atherosclerosis.

ACKNOWLEDGMENTS

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ABBREVIATIONS

Ang II, angiotensin II; CO, carbon monoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO, heme oxygenase; H_2O_2, hydrogen peroxide; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein 1; NBT, nitro blue tetrazolium; PBM, peripheral blood monocyte(s); VSMC, vascular smooth muscle cell; ZnPP, zinc protoporphyrin IX.

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