Heme: a novel inducer of MCP-1 through HO-dependent and HO-independent mechanisms

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Kanakiriya, Sharan K. R., Anthony J. Croatt, Jill J. Haggard, Julie R. Ingelfinger, Shiow-Shih Tang, Jawed Alam, and Karl A. Nath. Heme: a novel inducer of MCP-1 through HO-dependent and HO-independent mechanisms. Am J Physiol Renal Physiol 284: F546–F554, 2003; 10.1152/ajprenal.00298.2002.—This study examined the effect of heme on the expression of heme oxygenase-1 (HO-1) and monocyte chemotactic protein-1 (MCP-1) in immortalized rat proximal tubular epithelial cells (IRPTCs). Hemin elicited a dose- and time-dependent induction of HO-1 and MCP-1 mRNA. HO activity contributed to MCP-1 mRNA expression at early time points (4–6 h) because inhibition of HO activity by zinc protoporphyrin (ZnPP) prevented hemin-induced expression of MCP-1 mRNA. Catalytically active intracellular iron was markedly increased in hemin-treated IRPTCs and contributed to the induction of HO-1 and MCP-1 mRNA because an iron chelator blocked hemin-induced upregulation of both genes, whereas a cell-permeant form of iron directly induced these genes. N-acetylcysteine completely blocked hemin-induced expression of HO-1 and MCP-1 mRNA, thereby providing added evidence for redox regulation of expression of these genes. The redox-sensitive transcription factor NF-κB was recruited in hemin-induced upregulation of MCP-1 because two different compounds that abrogate the activation of NF-κB (TPCK and BAY 11-7082) completely blocked hemin-induced upregulation of MCP-1 mRNA. In contrast to this HO-mediated induction of MCP-1 through redox-sensitive, iron-dependent, and NF-κB-involved pathways observed after 4–6 h, hemin also elicited a delayed induction of MCP-1 at 18 h through HO-independent pathways. We conclude that hemin is a potent inducer of MCP-1 in IRPTCs: HO-dependent, heme-degrading pathways lead to an early, robust, and self-remitting induction of MCP-1, whereas HO-independent mechanisms lead to a delayed expression of MCP-1.

HEMOXYGENASE (HO) is the rate-limiting enzyme in the degradation of heme (1, 4, 26, 34, 45), and induction of its isozyme, HO-1, is recognized as a protective response against heme protein-mediated and other injurious agents. HO-1 is induced by heme degradation products and is involved in the resolution of inflammatory processes and cell injury (2, 25, 35, 43, 52, 63). While inducible by numerous stimuli (1, 4, 12, 22, 56), HO-1 is readily induced by heme proteins and their heme moiety (2, 5, 35). In pathological states, increased cellular content of heme may originate from two main sources (2, 9, 25, 29, 35, 56, 65). Heme may be released from intracellular hemoglobin that are widespread in cells and include such members as hemoglobin, myoglobin, mitochondrial cytochromes, microsomal cytochromes, peroxidescavenging enzymes, nitric oxide synthase, guanylate cyclase, and cyclooxygenases (9, 29, 56); such heme-containing proteins may be destabilized in the course of cell injury, such that heme is freed from its linkage with its respective protein moiety (9, 29, 56). Cellular content of heme in certain organs may also accrue from heme proteins originating from other tissues; for example, myoglobin released into the systemic circulation from injured skeletal muscle cells (as occurs in rhabdomyolysis), or hemoglobin released from lysed red blood cells (as occurs in hemolysis) can be readily incorporated by renal epithelial cells (2, 21, 25, 35, 65).

Augmentation in cellular content of heme can damage cells and their organelles through mechanisms that are, at least in part, prooxidant in nature (6, 8, 20, 36, 38, 40). Such prooxidant actions of heme are indicated, for example, by the capacity of heme to provoke cellular generation of hydrogen peroxide and peroxidation of membrane lipid; these effects may be ameliorated by antioxidants (6, 8, 20, 36, 38, 40). The induction of HO-1 in tissues exposed to heme provides a protective response, in part, by facilitating the degradation of heme and procuring antioxidant and other cytoprotective mechanisms (1, 4, 34, 45). The cellular mechanisms underlying the inductive effects of heme on HO-1 in tissues in general are poorly understood; notably, with regard to the kidney, there are no studies to date that explore the mechanisms underlying the inductive effect of heme on HO-1 in kidney-derived cells. The present study aimed to elucidate these mecha-
HEMЕ INDUCES RЕNAL EXPRESSION OF MCP-1

Dose-dependent and time-dependent effects of hemin on HO-1 and MCP-1 mRNA expression. The dose-dependent effect of hemin was examined by exposing IRPTCs to increasing concentrations of hemin (5, 10, and 20 μM) for 1 h in the medium described above supplemented with 0.1% FBS. The medium was then replaced by hemin-free medium, and, after 4 h of incubation, RNA was extracted for the assessment of HO-1 and MCP-1 mRNA expression. In protocols that examined the time-dependent effect of hemin on gene expression, IRPTCs were exposed to hemin (10 μM) for 1 h, after which the medium was replaced by hemin-free DMEM medium containing 0.1% FBS. After 2, 4, and 6 h of incubation, RNA was extracted for Northern analyses.

Studies examining mechanisms underlying hemin-induced gene expression in IRPTCs. In these protocols, IRPTCs were exposed to hemin (10 μM)-containing medium for 1 h, followed by incubation in hemin-free medium for an additional 4 h. Depending on the specific protocol, the hemin-containing medium also contained 10 μM ZnPP, 1 mM DFO, 1 mM NAC, 25 μM TPCK, 10 μM BAY 11-7082, or where relevant, the vehicle for these reagents. These reagents were added one-half hour before the addition of hemin and were maintained during the 1-h exposure to hemin. After this exposure to hemin and relevant reagent, the hemin-containing medium was replaced by hemin-free medium containing the respective reagents, as appropriate. After incubation for 4 h, RNA was extracted for the assessment of HO-1 and MCP-1 mRNA expression.

The effect of ZnPP on hemin-induced MCP-1 expression was examined at time points later than 4 h. In these studies, IRPTCs were exposed to hemin (10 μM) in the presence or absence of ZnPP (10 μM) for 1 h; the hemin-containing medium was replaced by hemin-free medium (containing ZnPP), and after 6, 10, 14, and 18 h of incubation, extraction of RNA was performed.

Additional protocols examined the effect of the cell-permeant form of iron, ferrous ammonium sulfate/8-hydroxyquinoline (7, 55), on gene expression. IRPTCs were exposed to ferrous ammonium sulfate (10 μM)/8-hydroxyquinoline (10 μM) in the presence or absence of DFO (1 mM). After 1 h of incubation, the ferrous ammonium sulfate/8-hydroxyquinoline-containing medium in the presence or absence of DFO was replaced by a ferrous ammonium sulfate/8-hydroxyquinoline-free medium, also containing, as appropriate, DFO (1 mM). After 4 h of incubation, RNA was extracted for the assessment of expression of HO-1 and MCP-1 mRNA.

RNA extraction and Northern analysis for HO-1 and MCP-1. To examine expression of HO-1 and MCP-1 mRNA, IRPTCs were washed with PBS, and RNA was extracted using the TRizol method (Invitrogen, Carlsbad, CA). Ten micrograms of total RNA from each sample were separated on an agarose gel and transferred to a nylon membrane. Membranes were hybridized overnight with a 32P-labeled mouse HO-1 or rat MCP-1 cDNA probe. Autoradiograms were evaluated for loading and transfer by assessing the density of the 18S rRNA on an ethidium bromide-stained membrane, as previously described (13, 27).

Determination of HO activity. HO activity was measured by bilirubin generation in microsomes isolated from IRPTCs, as described previously (27). Cells were washed, scraped with a rubber policeman, and centrifuged at 1,000 g for 10 min at 4°C. The cell pellet was suspended in potassium phosphate buffer (100 mM, pH 7.4) and sonicated on ice before centrifugation at 12,000 g for 10 min at 4°C. The supernatant was centrifuged at 105,000 g for 60 min at 4°C. The pellet was suspended in potassium phosphate buffer (pH 7.4) containing

Materials and Methods

Reagents employed. Hemin (iron protoporphyrin chloride), zinc protoporphyrin (ZnP), deferoxamine mesylate (DFO), N-acetylcycteine (NAC), TPCK, ferrous ammonium sulfate, and 8-hydroxyquinoline were obtained from Sigma (St. Louis, MO), as were all other chemicals employed unless otherwise stated; BAY 11-7082 was obtained from Calbiochem (San Diego, CA). Stock solutions of hemin and ZnP were prepared in 0.05 M NaOH; TPCK was dissolved in DMSO; DFO was dissolved in cell culture media, whereas deionized water was used to prepare stock solutions of NAC, ferrous ammonium sulfate/8-hydroxyquinoline, and BAY 11-7082.

Cell culture. IRPTCs (immortalized rat proximal tubular cells, 93-p-2-1, developed and characterized as previously described) (58) were grown at 37°C in 95% air-5% CO2 in DMEM (Invitrogen, Grand Island, NY) containing low glucose (1 g/l), 20 mM HEPES, and 0.1 mM nonessential amino acids; the medium was supplemented with 5% FBS, 40 U/ml penicillin, and 40 μg/ml streptomycin. IRPTCs were studied as a confluent monolayer in all experiments. In all experiments, IRPTCs were incubated in the same medium as the one in which IRPTCs were grown, except that the medium was supplemented with 0.1% FBS instead of 5% FBS.

Animals, focusing on the involvement of oxidant-related pathways.

Our laboratory (37, 41) has recently reported that in certain in vivo models of renal injury, upregulation of HO-1 is accompanied by induction of the chemokine monocyte chemoattractant protein-1 (MCP-1). MCP-1 is widely incriminated as a stimulus for mononuclear cellular infiltrate in diverse inflammatory conditions affecting the kidney and other organs (15, 50, 59, 62); additionally, special emphasis is assigned to MCP-1 in the evolution of atherosclerosis and in the pathogenesis of assorted vascular diseases (15, 23, 51). The basis for this upregulation of MCP-1 we have described in these in vivo models of renal injury has not been explored: such upregulation of MCP-1 may reflect an immediate early gene response to tissue injury (47); renal ischemia, which occurs invariably in such models (46); the stimulatory effect of cytokines and other humoral factors elaborated in the course of such injury (65); and possibly, oxidative stress, which occurs in such states (3, 65). With regard to the last consideration, exposure to heme proteins occurs in these in vivo models, thereby raising the question of whether heme, possibly through oxidant pathways, induces renal expression of MCP-1.

The present study examined whether direct exposure of renal epithelial cells to heme elicits upregulation of MCP-1 in conjunction with HO-1, specifically determining whether such expression of MCP-1 is influenced by HO-1 induced in these cells. The latter possibility, that a dialogue exists between cellular expression of MCP-1 and HO-1 in heme-exposed cells, was considered because mcp-1 is an oxidant-inducible gene (42, 49), and ho-1 represents not only an oxidant-inducible gene but one that modulates cellular redox through its antioxidant [for example, bile pigments (19) and ferritin (5)] and prooxidant products [for example, iron (5)].
2 mM MgCl₂ and designated as the microsomal fraction. An aliquot of the microsomal fraction was added to the reaction mixture (400 μl) containing rat liver cytosol (2 mg of cytosolic protein), 20 μM hemin, 2 mM glucose-6-phosphate, 0.2 units glucose-6-phosphate dehydrogenase, and 0.8 mM NADPH and incubated for 1 h at 37°C in the dark. The formed bilirubin was extracted with chloroform, and ΔOD 464–530 nm was measured (extinction coefficient, 40 mM/cm for bilirubin), where OD is optical density. HO activity was expressed as picomoles of bilirubin formed per hour per milligram protein.

**Determination of catalytically active iron in IRPTCs.** Catalytically active iron was measured in cellular lysates from IRPTCs using the bleomycin assay (17). Experimental media for incubations, wash buffer, and lysis buffer were prepared with Chelex-treated water in new plastic containers and subsequently treated with Chelex, except for the bleomycin, magnesium chloride, and iron standard.

In these studies, IRPTCs were exposed to hemin (10 μM) in the absence or presence of ZnPP (10 μM) for 1 h; the hemin-containing medium was then replaced by hemin-free medium in the absence or presence of ZnPP (10 μM). After 6 h, IRPTCs were washed with HBSS, lifted with a scraper into 5 ml of HBSS, and gently pelleted by centrifugation. The pelleted cells were resuspended in 0.5 ml of 25 mM HEPES buffer (pH 7.3), lysed in a sonicating water bath for 10 min, and centrifuged at 10,000 g for 10 min. The supernatants were assayed for iron in an incubation mixture consisting of 0.1 ml calf thymus DNA (1 mg/ml), 20 μl bleomycin sulfate (1 U/ml), 20 μl MgCl₂ (100 mM), 20 μl HEPES buffer (25 mM, pH 7.3), 20 μl sample, and 20 μl ascorbic acid (8 mM). This mixture was incubated for 2 h at 37°C with shaking, and the reaction was subsequently terminated with the addition of 0.1 ml of 0.2 M EDTA. Blank reactions for each sample were simultaneously incubated without bleomycin along with a calibration curve constructed using FeCl₃. After the addition of 0.2 ml of thiobarbituric acid (1% in 0.5 N NaOH) and HCl (25% wt/vol), the samples were heated at 100°C for 15 min and cooled to room temperature. Chromagen formed was measured spectrophotometrically at 532 nm, standardized against the calibration curve, and expressed as nanomoles iron per milligram protein, the latter measured using the Lowry method.

**Statistical analysis.** Data are expressed as means ± SE. For comparisons involving two groups, Student's t-test was applied, whereas for comparisons involving more than two groups, ANOVA and the Student-Newman-Keuls test were applied. All results are considered significant at P < 0.05.

**RESULTS**

The exposure of IRPTCs to hemin for 4 h led to intense upregulation of HO-1 and MCP-1 mRNA in a dose-dependent manner (Fig. 1). This upregulation of MCP-1 and HO-1 mRNA was discernible as early as 2 h after the exposure to hemin for 1 h at a concentration of 10 μM (Fig. 2).

To determine whether HO-1 is involved in the upregulation of MCP-1 mRNA, we studied the effect of ZnPP, the competitive inhibitor of HO activity. As shown in Fig. 3, ZnPP completely blocked the upregulation of MCP-1 mRNA induced by hemin without affecting hemin-induced upregulation of HO-1 mRNA. Along with these findings, we demonstrate that the induction of HO-1 mRNA by hemin is accompanied by a marked increase in HO activity and that ZnPP completely ablates cellular HO activity in either the absence or presence of hemin (Fig. 4). Thus the induction of MCP-1 mRNA by hemin is critically dependent on intact HO activity because inhibition of HO activity by ZnPP prevents such expression of MCP-1 mRNA.

To examine mechanisms that may underlie such induction of HO-1 and MCP-1 mRNA, we considered the possibility that iron may be involved in such regulation: iron is a potent catalyst for oxidative stress (56), and both genes are inducible by oxidative stress (42, 49, 56); moreover, iron is released as heme is catabolized by HO activity (1, 4, 25). Indeed, we demonstrate large increments in cellular iron levels in hemin-treated cells and the marked attenuation in hemin-induced rise in cellular iron after concomitant treatment with ZnPP (Fig. 5); cellular levels of iron in hemin-exposed cells concomitantly treated with ZnPP were still significantly higher than levels in cells treated with ZnPP alone and cells studied under control conditions (Fig. 5).

We thus studied the effect of the iron chelator DFO on hemin-induced expression of MCP-1 and HO-1

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**Fig. 1. Northern analysis demonstrating dose-dependent hemin-induced upregulation of heme oxygenase-1 (HO-1) and monocyte chemotactrant protein-1 (MCP-1) mRNA in immortalized rat proximal tubular epithelial cells (IRPTCs) assessed by the expression of 18S rRNA.**

**Fig. 2. Northern analysis demonstrating induction of HO-1 and MCP-1 mRNA in IRPTCs at 2, 4, and 6 h after exposure to 10 μM hemin for 1 h. C, studies undertaken in IRPTCs exposed to hemin-free medium; H, studies in IRPTCs exposed to hemin-containing medium.**
mRNA. As shown in Fig. 6, the induction of MCP-1 and HO-1 mRNA by hemin were both reduced by DFO. To examine the capacity of iron per se to induce MCP-1 and HO-1 mRNA, we examined the effect of a cell-permeant form of iron. As demonstrated in Fig. 7, iron induced both MCP-1 and HO-1 mRNA, and this inductive effect of iron was blocked by the iron chelator DFO. Thus the upregulation of MCP-1 and HO-1 in response to hemin is dependent, at least in part, on increments in intracellular iron.

To determine whether alterations in cellular redox contribute to the upregulation of these genes, we studied the effect of the sulphydryl-containing antioxidant N-acetylcysteine. As demonstrated in Fig. 8, N-acetylcysteine completely prevented the upregulation of MCP-1 and HO-1 mRNA.

Because activation of the redox-sensitive transcription factor NF-κB (24) regulates expression of MCP-1 (15, 57, 61), we examined whether hemin-induced upregulation of MCP-1 can be interrupted by inhibiting activation of NF-κB. Evidence in support of this pathway was provided by two approaches. TPCK, a protease inhibitor that blocks activation of NF-κB (18, 64), completely prevented hemin-induced upregulation of MCP-1 (Fig. 9). Additional studies were undertaken with BAY 11-7082, a more specific inhibitor of NF-κB activation than TPCK; this compound inhibits the nuclear translocation of NF-κB by inhibiting the phosphorylation of IκB (44). BAY 11-7082 completely prevented the upregulation of MCP-1 mRNA by hemin (Fig. 10). Thus inhibition of NF-κB-dependent pathways by two different approaches completely prevented hemin-induced upregulation of MCP-1. While TPCK blocked hemin-induced upregulation of HO-1 mRNA (Fig. 9), BAY 11-7082 only partially inhibited hemin-induced HO-1 mRNA accumulation and, by itself, BAY 11-7082 stimulated HO-1 expression in IRPTCs (Fig. 10).

In our study of the inductive effect of hemin on MCP-1 expression, we also examined the time course of expression of MCP-1 mRNA in response to hemin in the absence and presence of ZnPP, the competitive inhibitor of HO activity. As shown in Fig. 11, the expression of MCP-1 mRNA after exposure to hemin in the absence of ZnPP peaked at 6 h and returned to basal levels by 18 h. In contrast, while there was no discernible expression of MCP-1 mRNA at 6 h elicited by hemin when ZnPP was concomitantly present, the expression of MCP-1 mRNA under such conditions thereafter increased. Standardized densitometric assessment confirmed these findings: expressed as a percentage of standardized densitometric expression of
MCP-1 mRNA in the presence of hemin alone, standardized densitometric expression of MCP-1 mRNA in the presence of hemin and ZnPP increased from 10% at 6 h to 272% at 18 h.

DISCUSSION

We demonstrate that hemin is a vigorous inducer of HO-1 and MCP-1 via iron-mediated, redox-dependent mechanisms. First, at a time point at which hemin induced these genes (4–6 h), IRPTCs exhibited increased amounts of catalytically active iron, the latter representing a potent facilitator of oxidant stress. Second, the iron chelator DFO attenuated the induction of these genes by hemin, findings that indicate the fundamental involvement of catalytically active iron in the induction of these genes; moreover, direct evidence that increased cellular levels of catalytically active iron induced these genes was provided by studies in which marked upregulation of HO-1 and MCP-1 occurred in cells exposed to a cell-permeant form of iron, effects also abrogated by DFO. Third, the antioxidant sulfhydryl agent N-acetylcysteine prevented the induction of these genes by hemin. In the aggregate, these findings support an important role for iron-dependent, redox-involved pathways in hemin-induced expression of HO-1 and MCP-1.

We wish to point out that ZnPP inhibits HO activity emanating not only from HO-1, but also from HO-2, the constitutive isof orm; the third isof orm of HO, HO-3, possesses trivial HO activity. Thus the results of studies which employ ZnPP as a competitive inhibitor of HO activity represent the combined inhibitory effect of ZnPP on HO enzyme activities originating from HO-1 (as this isoform is induced) as well as from HO-2 (the basally expressed, constitutive isoform). Because the catalytic activity of HO (from either HO-1 or HO-2) on hemin acutely elevates cellular iron content, we suggest that iron, released as hemin is degraded by HO, contributes to the induction of MCP-1. In support of this interpretation, we provide evidence that ZnPP totally blocked HO activity in cells (either in the control setting or after treatment with hemin) and that ZnPP attenuated the rise in catalytically active iron in hemin-treated cells.

Fig. 7. Northern analysis demonstrating the effect of 10 μM ferrous ammonium sulfate/8-hydroxyquinoline (Fe), 1 mM DFO, and Fe + DFO on expression of HO-1 and MCP-1 mRNA in IRPTCs assessed 4 h after exposure to hemin for 1 h.

Fig. 8. Northern analysis demonstrating the effect of 10 μM hemin, 1 mM N-acetylcysteine (NAC), and hemin + NAC on expression of HO-1 and MCP-1 mRNA in IRPTCs assessed 4 h after exposure to hemin for 1 h.

Fig. 9. Northern analysis demonstrating the effect of 10 μM hemin, 25 μM TPCK, and hemin + TPCK on expression of HO-1 and MCP-1 mRNA in IRPTCs assessed 4 h after exposure to hemin for 1 h.

Fig. 10. Northern analysis demonstrating the effect of 10 μM hemin, 10 μM BAY 11-7082, and hemin + BAY 11-7082 on expression of HO-1 and MCP-1 mRNA in IRPTCs assessed 4 h after exposure to hemin for 1 h.
ZnPP completely blocked HO activity, but ZnPP by itself stimulated HO-1 mRNA expression, albeit to a lesser extent than did hemin (Fig. 3). Induction of ho-1 gene/protein expression by analogs of heme that inhibit HO activity is well established (28, 48, 54). At least two mechanisms may account for this phenomenon: 1) heme may function as a structural cofactor for one or more proteins that participate in the HO-1 induction pathway, and ZnPP may be able to functionally substitute for the heme molecule; and 2) alternatively, the ho-1 gene may be negatively regulated as a consequence of product feedback inhibition. The inhibition of HO activity by ZnPP markedly reduces the availability of products of HO activity, thereby decreasing product feedback inhibition and, in turn, leading to increased ho-1 gene accumulation. It should also be pointed out that induction of HO-1 mRNA expression per se by ZnPP would not elicit upregulation of MCP-1 mRNA because the treatment of cells with ZnPP effectively blocks HO activity, and it is through HO activity that HO-1 exerts its cellular effects.

Because cleavage of heme and release of iron are not necessary for hemin-elicited induction of HO-1 mRNA, it is likely that hemin and iron can independently modulate HO-1 expression. Alternatively, or in conjunction, sources of iron other than heme-iron may be responsible; for example, hemin, through its direct oxidative effects may mobilize iron from the “low-molecular-weight cellular iron pool” and other sources of iron (56), and such catalytically active iron may induce HO-1 mRNA. Indeed, in studies in which catalytically active iron was measured in IRPTCs exposed to hemin, levels of catalytically active iron in cells treated with hemin were reduced when ZnPP was concomitantly present; however, concentrations of catalytically active iron in hemin-treated cells in the presence of ZnPP were still significantly greater than levels in cells studied under control conditions, or in cells exposed to ZnPP alone.

To examine further the basis for hemin-induced upregulation of MCP-1, we considered the possibility that the oxidant-responsive transcription factor NF-κB (24) was involved. NF-κB binding sites are present in the promoter of the mcp-1 gene, and activation of NF-κB is regarded as an intermediary step in transcriptional control of expression of MCP-1 (15, 57, 61). To probe the involvement of NF-κB-dependent mechanisms, studies were undertaken with TPCK; TPCK is a protease inhibitor that blocks the degradation of IκB and thereby prevents the nuclear translocation of NF-κB (18, 64). In hemin-exposed cells, TPCK completely blocked the induction of the MCP-1 gene. To complement this finding, we employed an additional approach that utilized the compound BAY 11-7082; this compound prevents the translocation of NF-κB to the nucleus by inhibiting the phosphorylation of IκB (44). In our studies, BAY 11-7082 also completely blocked upregulation of MCP-1 in IRPTCs exposed to hemin. On the basis of these studies, we suggest that NF-κB-dependent pathways are involved in hemin-induced upregulation of MCP-1. Interestingly, BAY 11-7082 (a more specific inhibitor of activation of NF-κB than TPCK) only partially inhibited hemin-induced HO-1 mRNA accumulation and BAY 11-7082 by itself stimulated HO-1 expression in IRPTCs, thereby pointing to an additional mechanistic difference between the induction of the ho-1 and mcp-1 genes by hemin.

This marked expression of MCP-1 in hemin-treated cells observed at 2–6 h after exposure to hemin subsided by 10 h and completely abated by 14 and 18 h (Fig. 11). This temporal profile of expression of MCP-1 in hemin-treated cells was markedly altered when HO activity was inhibited by ZnPP. As shown in Fig. 11, while ZnPP prevented the expression of MCP-1 by hemin at 6 h, examination at later time points demonstrate an increasing level of expression of MCP-1 mRNA despite the continued presence of this inhibitor of HO activity; indeed, at 18 h, the level of expression of MCP-1 was increased almost threefold in the presence of ZnPP. Thus inhibition of HO altered the pattern of MCP-1 expression in IRPTCs exposed to hemin: while preventing the induction of MCP-1 observed at the early time point (4–6 h), such inhibition was associated with increased expression of MCP-1 at the later time point (18 h). From these findings, we suggest that induction of MCP-1 in IRPTCs in response to hemin occurs through HO-dependent and HO-independent pathways: HO-dependent heme-degrading pathways...
lead to an early, prominent, and self-remitting induction of MCP-1, whereas HO-independent mechanisms lead to a delayed expression of MCP-1.

As is well established, the induction of HO-1 by heme is coupled to secondary events that ultimately restore catalytically active iron to their basal levels (1, 5, 10, 14). For example, the induction of HO-1 entrains the synthesis of ferritin, the iron-binding protein that is the major intracellular repository for iron (5); induction of HO-1 is also linked to increased expression of iron-exporting proteins that facilitate the cellular egress of iron (10, 14). Thus to the extent that iron directly elicits the induction of MCP-1 in hemin-treated cells, the temporal regression in expression of this gene in hemin-treated cells (when HO activity is intact) likely reflects the reduction in cellular levels of iron due to increased availability of iron-binding and iron-exporting proteins.

The mechanisms that may underlie the delayed induction of MCP-1 in HO-inhibited cells merit comment. Heme can be degraded via HO-independent processes that involve nonenzymatic autooxidative reactions (1, 30–32, 56). For example, in pathophysiologically relevant concentrations, the interaction of hydrogen peroxide with ferrylheme leads to the degradation of heme, the liberation of iron, and the production of superoxide anion (30–32); superoxide anion can undergo dismutation to hydrogen peroxide, thereby providing a positive-feedback loop in the degradation of heme. Heme strongly stimulates cellular generation of hydrogen peroxide, as we have shown previously (36). Thus hemin itself may initiate a chain of oxidative events that culminate in the degradation of hemin through HO-independent, nonenzymatic processes. These nonenzymatic, autooxidative, heme-degrading processes lack the rapid, efficient, controlled, and coordinated features exhibited by the HO system. We speculate that such nonenzymatic autooxidative reactions, by promoting oxidative stress and/or increased availability of redox iron, may contribute to the delayed induction of MCP-1 we observed in hemin-treated cells. While the functional significance of these findings is beyond the scope of the present studies, it is intriguing that this early expression of MCP-1 may be relevant to a number of inflammatory or inimical to cellular vitality. In this regard, an analogy to TGF-β1 may be relevant: whereas the sustained upregulation of TGF-β1 provides a dominant pathway for chronic inflammation and fibrosis, TGF-β1, acutely and transiently upregulated, exerts anti-inflammatory and cytoprotective actions (11).

We conclude by suggesting that the induction of MCP-1 by heme may be relevant to a number of inflammatory states in the kidney characterized by repetitive or unremitting exposure to heme proteins (21, 33, 37, 39, 41). This inductive effect of heme on MCP-1 may also be germane to the proinflammatory effects, including thrombophlebitis, that attend the clinical use of heme-based compounds (16, 53). Finally, we raise the possibility that our findings may be relevant to atherosclerosis. Atherosclerosis is more likely to involve the vasculature at sites of turbulence, wherein red blood cells undergo mechanical trauma with the attendant insinuation of hemoglobin and heme in the walls of blood vessels (60). Because the sustained upregulation of MCP-1 is considered a critical chemokine in atherogenesis (15, 23, 51), we speculate that increased amounts of heme in the vasculature originating from these and other mechanisms may drive the expression of the proatherogenic chemokine MCP-1.

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