Heme oxygenase-1 modulates the expression of the anti-angiogenic chemokine CXCL-10 in renal tubular epithelial cells

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The development of tubulointerstitial fibrosis is characteristic of chronic renal disease, and inhibition of its progression has been proposed to be of major importance in the preservation of renal function (42, 44). In experimental animal models and also in humans, it has been shown that there is a significant loss of peritubular capillaries, as well as defective capillary repair, in association with the development of interstitial fibrosis (42, 44–46, 49, 55). It is proposed that the loss of the intrarenal vasculature results in impaired delivery of oxygen and nutrients to the tubules, which, in turn, results in chronic ischemia and cell death (42, 49). Several regions of the medulla normally exist in some degree of hypoxia; therefore, any defect in the vasculature will disrupt the high metabolic demands of the tubular epithelial cell and may promote cell death (3, 22, 28, 33, 42). Thus the peritubular capillaries are essential to maintain the normal structure and function of renal tubules.

The integrity of peritubular capillaries seems to be regulated by several factors, including cytokines, chemokines, and angiogenic growth factors (12, 27, 34). Indeed, the process of angiogenesis may play a critical role in vascular repair (7, 21, 56). Following injury, an early ineffective angiogenesis may result in chronic disease, whereas effective angiogenesis/capillary repair attenuates the injury process (24, 25). Thus a balance between angiogenic vs. anti-angiogenic factors may be essential to maintain the physiological function of blood vessels, including the renal microvasculature (31, 32, 56). Angiogenesis occurring during reparative or pathological processes is driven by various factors. For example, reactive oxygen species and nitric oxide (NO) are important modulators of the synthesis and activity of vascular endothelial growth factor (VEGF), a major angiogenic molecule for renal vasculature (20, 26). Moreover, heme oxygenase (HO)-1, a stress-inducible enzyme that is induced by reactive oxygen species and NO, has recently been shown to be involved in regulating angiogenesis (2, 19, 48).

HO-1 is a rate-limiting enzyme in heme degradation, and it converts heme to biliverdin, carbon monoxide (CO), and iron (1, 43, 47, 52, 57). In various tissue injury models, including an inflammatory renal injury model, induced HO-1 has been shown to confer protection (1, 2, 43, 57). Renal tissues express a significant amount of HO-1 (1, 4, 35), and we have recently shown that human renal proximal tubular epithelial cells (RPTEC) overexpress HO-1 during CD40-induced inflammation (35). The products of the HO-1 enzymatic activity (CO, iron, etc.) can regulate angiogenesis, in both positive and negative ways (9, 19). For example, CO may augment angiogenesis through VEGF and its receptor expression, whereas iron may attenuate VEGF-dependent angiogenesis (8, 9, 19). In support of its pro-angiogenic properties, it has been reported that HO-1 can also regulate the expression of some angiogenic chemokines. Kanakiriya et al. (30) demonstrated that, in renal epithelial cells, heme can induce HO-1-mediated robust induction of CCL-2 (monocyte chemoattractant protein-1), a known angiogenic chemokine belonging to the CC subfamily. Pae et al. (48) found that NO induces HO-1 expression, and that the overexpressed HO-1 stimulates VEGF secretion, which in turn results in the production of CXCL-8 (IL-8), another angiogenic chemokine belonging to the CXC subfamily. Overall, these
data suggest that HO-1 may play an important role in regulating angiogenesis, through either direct or indirect pathways. However, the role of HO-1 in regulating chemokine expression in the context of angiogenesis is still underappreciated.

Members of the CXC chemokine subfamily can behave either as angiogenic or as antiangiostatic (anti-angiogenic) agents (51, 54). The family has four highly conserved cysteine amino acid residues, with the first two cysteines separated by a nonconserved amino acid residue (54). The NH₂-terminus of CXC chemokines dictates their specificity for binding to their cognate receptors. For example, CXC chemokines that contain a glutamic acid-leucin-arginine motif (ELR motif; ELR⁺) in the NH₂-terminus of the molecule that immediately precedes the first cysteine amino acid residue are highly angiogenic (51, 54). In contrast, CXC chemokines that lack the conserved ELR motif (ELR⁻) are anti-angiogenic (51, 54). The ELR⁺ chemokine CXCL-8 and the ELR⁻ chemokine CXCL-10 (interferon-γ-inducible protein-10) represent the prototype chemokines for this model. In the present study, we show that the induction of HO-1 in RPTEC results in the upregulation of the anti-angiogenic chemokine CXCL-10, along with angiogenic chemokines. There is a robust expression of CXCL-10 at early times following HO-1 induction, which gradually decreases at later times. We also find that HO-1-mediated CXCL-10 expression is regulated at the transcriptional level and involves the protein kinase C (PKC) signaling pathway. Moreover, HO-1-mediated CXCL-10 expression can inhibit endothelial cell (EC) proliferation. This is the first report to our knowledge demonstrating that HO-1 can regulate an anti-angiogenic chemokine, together with its ability to induce angiogenic chemokines. We suggest that, during tubular injury, HO-1 can promote a balance between angiogenic factors vs. anti-angiogenic factors to maintain the normal physiological functions of the renal microvasculature.

**MATERIALS AND METHODS**

**Reagents.** PKC inhibitor (Calphostin C), Raf kinase inhibitor (5-iodo-3-[3,5-dibromo-4-hydroxyphenyl]methylene]-2-indolinone), phosphatidylinositol 3-kinase inhibitor (LY 294002), p38 MAP kinase (MAPK) inhibitor (SB203580), and MAPK kinase inhibitor (PD98059) were purchased from Calbiochem (La Jolla, CA). CXCL-10 neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). HO activity inhibitor [tin protoporphyrin IX (SnPPIX)] was purchased from Frontier Scientific (Logan, UT). All other reagents for measuring HO activity were purchased from Sigma Chemical (St. Louis, MO).

**Cell cultures.** Human RPTEC were purchased from Clonetics (Walkersville, MD) and were cultured in complete epithelial medium (REGM BulletKit; Clonetics), according to recommended instructions. Human ECs were isolated from umbilical cords and cultured as described (5). Briefly, the cells were cultured in medium 199 (Cambrex, Walkersville, MD), supplemented with 20% fetal bovine serum, 1% EC growth factor, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were subcultured and used at passages 3–6.

**Plasmids.** The CXCL-10 promoter luciferase construct containing the 5'-flanking region of the human CXCL-10 gene from −875 to +97 (relative to the transcription start site) was obtained as a generous gift from Richard M. Ransohoff (Cleveland Clinic, Cleveland, OH) (38). The rat HO-1 cDNA overexpression plasmid (pcDNA3-HO-1) was obtained as a generous gift from Miguel P. Soares (Beth Israel Deaconess Medical Center, Boston, MA) (6).

**rnase protection assay.** Total RNA was prepared using the RNeasy isolation kit (Qiagen, Valencia, CA). Equal amounts of RNA (3–8 μg) were analyzed by RNase protection assay using the Riboquant multiprobe template set (BD Biosciences, San Diego, CA), according to the manufacturer’s instructions. Chemokine transcripts were analyzed by autoradiography, and the expressions were quantified by densitometry (Alpha Innotech, San Leandro, CA). The signals were standardized to the expression of the housekeeping gene GAPDH.

**Transfection and luciferase assay.** For all transfection assays, the RPTEC were plated at 2.5 × 10⁶ cells/well in six-well plates. The cells were transfected with either the CXCL-10 promoter luciferase construct or the HO-1 overexpression plasmid using the Effectene transfection reagent (Qiagen). The control cells were always transfected with an empty expression vector. A recommended 1:25 ratio of DNA to Effectene was used for all of the experiments. Utilizing a green fluorescent protein expressing plasmid, we determined that ~70% RPTEC could be efficiently transfected by Effectene. For luciferase assays, cells were harvested 24–48 h after transfection, and luciferase activity was measured using a standard assay kit (Promega, Madison, WI). Transfection efficiency was determined by cotransfection of the β-galactosidase gene under control of cytomegalovirus immediate early promoter and by measurement of β-galactosidase activity.

**Real-time PCR.** Total RNA was prepared as described before, and cDNA was synthesized using cloned AMV first-strand synthesis kit (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed using the 7300 real-time PCR system and the Assays-on-Demand Gene Expression Product (TaqMan MGB Probes), according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Primers for human CXCL-10 and GAPDH were also obtained from Applied Biosystems.

**Western blot analysis.** Protein samples were run on 10% SDS-polyacrylamide gel following a standard protocol (35). Size-separated proteins were transferred to a polyvinylidenefluoride membrane (NEF Life Sciences Product, Boston, MA) at 60 V for 1 h. The membranes were blocked with 5% milk and coated with either anti-HO-1 or anti-HO-2 (Calbiochem, San Diego, CA). Following washes, the membranes were incubated with peroxidase-linked secondary antibody, and the reactive bands were detected by chemiluminescence (Pierce, Rockford, IL).

**FACS analysis.** To measure intracellular chemokine production, GolgiStop (BD Biosciences) was added to the cell culture medium 12 h before analysis. The cells were fixed/permeabilized in Cytosix (BD Biosciences) for 20 min at 4°C and washed with Perm/Wash solution (BD Biosciences). The fixed cells were then incubated with phycoerythrin-conjugated antibody to CXCL-10 (BD Biosciences) or phycoerythrin-labeled matched IgG isotype (BD Biosciences) as control for 30 min at 4°C. The stained cells were analyzed in a FACS Calibur (Becton Dickinson, San Jose, CA).

**Assay for HO activity.** The HO enzyme activity in the cells overexpressing HO-1 was measured following a standard protocol (23). Briefly, HO-1 plasmid-transfected cells were harvested 24 h after transfection and centrifuged at 2,000 g for 10 min. The pellet was resuspended in 100 mM phosphate buffer and 2 mM MgCl₂. It was frozen and thawed three times and then sonicated briefly on ice. The cells were centrifuged for 10 min at 4°C at 18,000 g, and the supernatant was saved. The samples were then treated with 0.8 mM NADPH, 2 mM glucose 6-phosphate, 0.2 units glucose 6-phosphate dehydrogenase, 2 mg protein of rat liver cytosol prepared from 105,000 g supernatant fraction as a source of biliverdin reductase, and 20 μM hemin in a final volume of 400 μl. Samples were incubated at 37°C for 60 min in the dark, and the reaction was terminated by addition of 1 ml chloroform. The extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm (extinction coefficient, 40 mM⁻¹·cm⁻¹ for bilirubin).
ELISA. The concentrations of CXCL-10 in tissue culture supernatants were determined by using Quantikine human CXCL-10 immunoassay kit (R&D Systems).

Cell proliferation assay. Human ECs (5 × 10^4) were seeded and grown in 96-well plates and treated with the culture supernatants of RPTEC, transfected with either the empty expression vector or the HO-1 overexpression plasmid. [3H]thymidine (0.5 μCi/well) was added for the final 15 h before cell harvesting. [3H]thymidine incorporation was measured using a microplate scintillation and luminescence counter (Perkin Elmer/Wallac, Boston, MA).

Statistical analysis. Statistical evaluation for data analysis was determined by Student’s t-test. Differences with P < 0.05 were considered statistically significant.

RESULTS

Induction of HO-1 promotes the expression of anti-angiogenic CXCL-10 in RPTEC. We first evaluated the role of HO-1 in the regulation of chemokine expression in RPTEC. To this end, RPTEC were transfected either with a HO-1 overexpression plasmid (1.0 and 2.0 μg) or with the empty expression vector as a control, and the mRNA expression of a panel of chemokines was determined by RNase protection assay. As observed in Fig. 1A, HO-1 significantly induced the expression of the anti-angiogenic chemokine CXCL-10 compared with the empty vector-transfected control. However, the induction of CXCL-10 was less at a higher concentration (2.0 μg) of HO-1, compared with a lower concentration (1.0 μg). HO-1 also markedly increased the expression of the angiogenic chemokines CXCL-8 and CCL-2 in these cells, and the expression of CXCL-8 was induced in a concentration-dependent manner following HO-1 induction (Fig. 1A). There were no changes in the expression of other chemokines. By Western blot analysis, we confirmed that the expression of HO-1 was markedly increased in HO-1 overexpression plasmid-transfected cells; however, there was no change in the expression of HO-2, the constitutive isoform of HO in these cells (Fig. 1B). Our findings were consistent with other reports that HO-1 can regulate the angiogenic chemokines CXCL-8 and CCL-2 (30, 48). However, for the first time, these observations suggest that HO-1 can also regulate the expression of the anti-angiogenic chemokine CXCL-10.

We next quantified HO-1-induced mRNA expression of CXCL-10 by real-time PCR. RPTEC were transfected either with the HO-1 overexpression plasmid (0.5 and 1.0 μg) or with the empty expression vector. We found that HO-1 significantly
induced the expression of CXCL-10 compared with the empty vector-transfected control (Fig. 1C).

We next wished to determine whether the induction of HO-1 promotes CXCL-10 protein expression in RPTEC. To this end, cells were transfected either with the HO-1 overexpression plasmid or with the empty expression vector, and intracellular protein expression of CXCL-10 was measured by FACS analysis. As shown in Fig. 1D, HO-1 significantly increased the expression of CXCL-10 compared with the empty vector-transfected control. Together, these results demonstrate that HO-1 can induce the expression of the anti-angiogenic chemokine CXCL-10 at both the mRNA and protein levels.

**Induction of HO enzyme activity is critical for CXCL-10 expression in RPTEC.** Our earlier experiments did not allow us to determine whether HO-1-mediated CXCL-10 expression in RPTEC is dependent on HO enzyme activity. To dissect this, we first examined whether HO enzyme activity is increased in cells overexpressing HO-1. RPTEC were transfected either with the HO-1 overexpression plasmid (1.0 and 2.0 μg) or with the empty expression vector as a control; HO activity in these cells was determined following the method as described earlier. As shown in Fig. 2A, we found a significant increase in HO activity following HO-1 overexpression compared with the empty vector-transfected control.

We next analyzed whether the increased HO activity is responsible for CXCL-10 expression in these cells. To this end, we made use of SnPPIX, a potent inhibitor of HO activity. RPTEC were transfected with the HO-1 overexpression plasmid or with the empty expression vector in the absence or presence of two different concentrations (10 and 20 μM) of SnPPIX, and CXCL-10 mRNA expression in these cells was quantified by real-time PCR. As shown in Fig. 2B, HO-1-mediated expression of CXCL-10 was significantly inhibited by SnPPIX at both of the concentrations. These results suggest that HO-1-mediated CXCL-10 expression in RPTEC is dependent on HO activity.

**HO-1 regulates CXCL-10 transcriptional activation.** We next used a full-length CXCL-10 promoter-luciferase construct to determine whether HO-1-mediated overexpression of CXCL-10 in RPTEC involves transcriptional regulatory mechanism(s). First, we evaluated the effect of different concentrations of HO-1 on CXCL-10 transcriptional activation. The RPTEC were cotransfected with the CXCL-10 promoter luciferase construct and increasing concentrations (1.0–2.0 μg) of the HO-1 overexpression plasmid. Control cells were cotransfected with the HO-1 promoter construct and an empty expression vector. After 24 h, the effect of HO-1 on CXCL-10 promoter activation was assessed by the measurement of luciferase activity in cell lysates. As shown in Fig. 3A, HO-1 at a concentration of 1.0 μg significantly induced CXCL-10 transcriptional activation compared with the empty vector-transfected control. However, HO-1-induced CXCL-10 transcription markedly decreased at higher concentrations of HO-1 expression. Next, we evaluated the effect of different time intervals of HO-1 induction on CXCL-10 transcriptional activation. The RPTEC were cotransfected with the CXCL-10 promoter luciferase construct and either the HO-1 overexpression plasmid (1.0 μg) or the empty expression vector; following transfection, the cells were incubated for 24, 48, and 72 h. As shown in Fig. 3B, the induction of HO-1 significantly increased CXCL-10 promoter activity at 24 h compared with
the empty vector-transfected control. However, the HO-1-induced CXCL-10 transcription was decreased at 48 and 72 h. Overall, our data indicate that the induction of HO-1 in RPTEC promotes an early and robust induction of CXCL-10 promoter activity, which gradually decreases in both a dose- and a time-dependent manner.

We next analyzed whether the induction of HO-1 in RPTEC could also promote the release of CXCL-10 protein. The cells were transfected with either the HO-1 overexpression plasmid (1.0 µg) or the empty expression vector for different time intervals (24–72 h), and the release of CXCL-10 in the culture supernatant was measured by ELISA. As shown in Fig. 3C, HO-1 induction significantly increased the release of CXCL-10 in 24 h compared with the empty-vector-transfected control. However, the HO-1-induced CXCL-10 release gradually decreased at later time points. These observations suggest that HO-1 can induce an early release of CXCL-10 from RPTEC.

**HO-1-induced CXCL-10 expression is mediated through the PKC pathway.** We have recently reported that CXCL-10 promoter activity may be regulated by kinases (5). To study the roles of the intermediary signaling molecules in HO-1-induced CXCL-10 transcriptional activation, we used a series of pharmacological inhibitors of known kinases. RPTEC were cotransfected with the CXCL-10 promoter luciferase construct and either the HO-1 overexpression plasmid (1.0 µg) or the empty expression vector. Following transfection, the cells were treated with inhibitors of PKC (Calphostin C, CalC), p38 MAPK (SB203580), MAPK kinase (PD98059), phosphatidylinositol 3-kinase (LY294002), or Raf kinase (Raf kinase inhibitor-I). As shown in Fig. 4A, induction of HO-1 increased CXCL-10 transcription compared with the empty vector-transfected control, and CalC significantly inhibited HO-1-induced CXCL-10 transcriptional activation, whereas the other inhibitors had no significant effects. Using the increasing doses of CalC, we found that it inhibited HO-1-induced CXCL-10...
transcriptional activation in a dose-dependent manner (Fig. 4B). CalC also dose-dependently inhibited HO-1-induced CXCL-10 release from RPTEC, as observed by ELISA (Fig. 4C). Together, these results suggest that PKC may be an important signaling molecule in HO-1-induced CXCL-10 expression.

**HO-1-induced CXCL-10 inhibits EC proliferation.** Here, we sought to determine the functional significance of HO-1-induced CXCL-10 release. As CXCL-10 is known to be a potent anti-angiogenic factor (37, 51), we evaluated whether HO-1-induced CXCL-10 in renal epithelial cells could inhibit the proliferation of human ECs. To this end, we transfected RPTEC with either the HO-1 overexpression plasmid (1.0 and 2.0 μg concentrations) or the empty expression vector and collected the cell culture supernatants following 24 h of transfection (as HO-1-induced expression of CXCL-10 was found to peak at this time point). Next, we cultured human vascular ECs with these culture supernatants of RPTEC in the absence or presence of a CXCL-10 neutralizing antibody, and we performed a cell proliferation assay. As shown in Fig. 5, we found that the supernatant from the lower concentration (1.0 μg) of HO-1 plasmid-transfected cells markedly inhibited EC proliferation compared with the supernatant of the empty vector-transfected control cells. Moreover, addition of the CXCL-10 neutralizing antibody into the culture supernatant significantly blocked this inhibitory effect. In contrast, supernatants collected from RPTEC transfected with the higher concentration (2.0 μg) of HO-1 plasmid had much lower anti-proliferative effect on EC, and there was no significant change in this effect in the presence of CXCL-10 neutralizing antibody (Fig. 5). Together, these experiments suggest that a

![Graph A](image1)

**A.** RPTEC were pretreated with calphostin C (1 μM), SB203580 (1 μM), PD98059 (1 μM), LY294002 (1 μM), or Raf-1 kinase inhibitor (100 nM). Control cells were pretreated with the vehicle alone. The cells were cotransfected with the full-length CXCL-10 promoter-luciferase construct (1.0 μg) and either the HO-1 overexpression plasmid (1.0 μg; solid bars) or the empty expression vector (open bar). After 24 h of transfection in presence and absence of inhibitors, the cells were harvested, and fold increase in luciferase activity was calculated, as previously mentioned. Graph is representative of at least three independent experiments. Values are triplicate readings of two different samples ± SD. *P < 0.01 compared with the empty vector-transfected cells; **P < 0.01 compared with the HO-1 overexpression plasmid-transfected and vehicle-treated cells.

![Graph B](image2)

**B.** RPTEC were pretreated with either the different doses (0.1 or 1 μM) of calphostin C or the vehicle alone. The pretreated cells were cotransfected with the full-length CXCL-10 promoter-luciferase construct (1.0 μg) and either the HO-1 overexpression plasmid (1.0 μg) or the empty expression vector. After 24 h of transfection in presence and absence of the inhibitor, the cells were harvested, and fold increase in luciferase activity was calculated as previously mentioned. Graph is representative of at least three independent experiments. Values are triplicate readings of two different samples ± SD. *P < 0.01 compared with the empty vector-transfected cells; **P < 0.01 compared with the HO-1 overexpression plasmid-transfected and vehicle-treated cells.

![Graph C](image3)

**C.** RPTEC were pretreated with either the different doses (0.1 or 1 μM) of calphostin C or the vehicle alone. Following treatment, the cells were transfected with either the HO-1 overexpression plasmid (1.0 μg) or the empty expression vector. After 24 h, the CXCL-10 protein release in the culture supernatant was measured by ELISA. The fold increases in HO-1-induced CXCL-10 release were compared with that of cells transfected with the empty expression vector alone. Graph is representative of at least three independent experiments, resulting from duplicate readings of two different samples. Values are average of CXCL-10 release ± SD. *P < 0.01 compared with the empty vector-transfected cells; **P < 0.01 compared with the HO-1 overexpression plasmid-transfected and vehicle-treated cells.
HO-1 is induced in various cell types, including renal tubular epithelial cells following tissue injury (1, 2, 11, 43). It classically functions as a potent anti-inflammatory molecule, and it may facilitate the repair of injured tissues through inhibition of the infiltration of inflammatory cells (8, 43, 47). However, it has been reported that the products of HO enzymatic activity can also regulate both angiogenic and anti-angiogenic processes (9, 19). Recent studies have demonstrated that the process of angiogenesis is critical in regulating inflammatory as well as the tissue repair processes (7, 41, 50). In its normal guise, angiogenesis is thought to facilitate the repair of injured tissues and to restore oxygenation. In renal diseases such as glomerulonephritis, ischemic nephropathy, and tubulointerstitial fibrosis, accelerated loss of microvasculature as a result of inefficient delivery of angiogenic factors results in hypoxia, leading to further tissue destruction (29, 33, 39, 49). In contrast, it is important to note that some kidney diseases, such as diabetic nephropathy, have been associated with high levels of the angiogenic cytokine VEGF, and treatment of animals with anti-VEGF antibodies has been shown to improve early renal dysfunction in a model of experimental diabetes (15). Moreover, it has been shown that, following injury, an early ineffective angiogenesis/capillary repair may aggravate the disease process, whereas effective repair attenuates the injury (24, 25). Thus it appears that the balance between angiogenic vs. anti-angiogenic factors is essential for effective tissue repair (32, 56). As discussed earlier, the role of HO-1 in angiogenesis is complex and intriguing. It has been demonstrated that gene transfer of HO-1 into corneal ECs promotes angiogenesis (16). Moreover, recent studies have reported that VEGF can regulate HO-1 expression and activity and that inhibition of HO-1 abrogates VEGF-induced endothelial activation and subsequent angiogenesis (8, 9, 53). In addition, it has also been shown that inhibition of HO-1 can induce marked leukocytic infiltration, resulting in enhanced VEGF-induced inflammatory angiogenesis (8, 9). Thus VEGF-induced HO-1 may play a bifunctional role in regulating both angiogenesis and leukocytic infiltration. Dukel et al. (18) observed that HO-1 activity may also modulate the expression of VEGF. Cisowski et al. (13) found that HO-1 may play an important role in hydrogen peroxide-induced VEGF expression and angiogenesis. Deshane et al. (17) recently demonstrated that stromal cell-derived factor-1 promotes angiogenesis via a HO-1-dependent but VEGF-independent mechanism. However, the role of HO-1 in regulating angiogenesis for effective repair mechanism has not been well studied.

Recent reports indicate that HO-1 may regulate chemokine expression. It has been demonstrated that the induction of HO-1 in renal epithelial cells and also in other cell types can promote the expression of CCL-2 and CXCL-8, known to have potent angiogenic activities (30, 48). In this study, we have observed that the induction of HO-1 in RPTEC can also promote the expression of CXCL-10. HO-1 can also induce the expression of both CCL-2 and CXCL-8 in these cells, as observed by others (30, 48). Furthermore, we have found that HO-1-mediated expression of CXCL-10 is regulated at the transcriptional level and is dependent on induced HO enzyme activity. However, we did not evaluate the exact role(s) of the products (biliverdin, CO, and iron) of HO enzyme activity in CXCL-10 expression. We have observed that SnPPIX significantly inhibits HO-1-induced CXCL-10 expression. SnPPIX

**Fig. 5.** HO-1-induced CXCL-10 in RPTEC inhibits the proliferation of endothelial cells (ECs). RPTEC were transiently transfected with either different concentrations (1.0 and 2.0 μg) of the HO-1 overexpression plasmid or the empty expression vector. Following 24 h of transfection, cell culture supernatants were collected. ECs were treated with the culture supernatants of RPTEC for 72 h in presence of either the CXCL-10 neutralizing antibody (10 μg/ml) or the control IgG. The open bar represents EC treated with supernatant of RPTEC transfected with empty vector; solid bars represent EC treated with supernatants of RPTEC transfected with 1.0 μg concentration of the HO-1 overexpression plasmid; shaded bars represent EC treated with supernatants of RPTEC transfected with 2.0 μg concentration of the HO-1 overexpression plasmid. The cell proliferation was assessed by measuring [H]thymidine incorporation within EC, as described in METHODS. Graph is representative of three independent experiments. Values are average of triplicate readings [counts per minute (CPM)] of the sample ± SD. *P < 0.05 compared with EC treated with the supernatant of RPTEC transfected with the empty expression vector; **P < 0.05 compared with EC treated with the supernatant of RPTEC transfected with the HO-1 overexpression plasmid and incubated in absence of CXCL-10 neutralizing antibody.

DISCUSSION

In the present study, we show that the induction of HO-1 in human RPTEC promotes the upregulation of both angiogenic as well as anti-angiogenic chemokines. Following low-level HO-1 induction, there is an early and robust expression of the anti-angiogenic chemokine CXCL-10. However, the expression of CXCL-10 gradually decreases following increase in either the concentration or the time interval of HO-1 induction. It has been observed that HO-1-induced early expression of CXCL-10 is mediated through the PKC signaling pathway. Finally, it has been shown that HO-1-induced CXCL-10 may limit early angiogenesis by inhibiting the proliferation of vascular ECs.
is a potent inhibitor of HO activity, and it has been shown that it strongly inhibits CO production (40). This suggests that CO may act as one of the important regulators of HO-1-mediated CXCL-10 expression in renal cells. On the other hand, we found that an iron chelator (deferoxamine) had no significant effect on HO-1-mediated CXCL-10 expression (data not shown), indicating that iron may not be an important factor in this process.

CXCL-10 binds with high affinity to CXCR-3 (54), a receptor known to be expressed on several cell types, including activated and memory CD4+ and CD8+ T cells, natural killer cells, some subsets of dendritic cells, some epithelial cells, and ECs (10, 14, 51). Classically, CXCL-10 is known to play an important role in the recruitment of lymphocytes (36, 51). However, as discussed earlier, CXCL-10 is also a potent anti-angiogenic factor with antifibrotic properties (24, 25, 37, 51). It has been suggested that CXCL-10 may prevent premature angiogenesis and fibrous tissue deposition, until a provisional matrix necessary for the growth of granulation tissue is formed (24, 25). In this way, it may promote an effective repair mechanism during the early phase of tissue injury. In support of this observation, we have found that CXCL-10 expressed in tubular cells by lower concentrations of HO-1 and at an early time point of HO-1 induction can inhibit the proliferation of human vascular ECs by acting in a paracrine manner. We have also observed that HO-1 at a higher concentration can markedly upregulate the angiogenic chemokine CXCL-8, while reducing the expression of the anti-angiogenic CXCL-10. Thus the timely onset of angiogenesis and a critical balance between angiogenic and anti-angiogenic factors in the tubular microenvironment may be considered to be an important factor for the effective repair mechanisms after chronic renal injury. We suggest that HO-1-induced and CXCL-10-mediated early inhibition of EC proliferation may prevent premature angiogenesis. However, future studies are needed to determine the in vivo significance of HO-1-induced anti-angiogenic CXCL-10 expression and angiogenic CXCL-8 expression to promote effective tissue repair.

In summary, these findings suggest that HO-1 can regulate an anti-angiogenic chemokine CXCL-10, as well as some angiogenic chemokines in renal epithelial cells. Thus, during tubular injury, early and low-level induction of HO-1, while cytoprotective to tubular cells, may also be anti-angiogenic. However, at later times following injury and at higher concentrations, HO-1 may be part of the angiogenic switch that facilitates tissue repair. Collectively, our findings define HO-1 as a mediator of a critical balance between angiogenic vs. anti-angiogenic factors that are important to maintain the renal microvasculature and prevent chronic renal injury.

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