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Carbon Monoxide Signaling in Promoting Angiogenesis in Human Microvessel Endothelial Cells

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ABSTRACT

Heme oxygenase isoforms (HO-1/HO-2) catalyze the conversion of heme to carbon monoxide (CO) and bilirubin. In this study, HO-1-deficient endothelial cells were transduced with HO-1 in the antisense orientation to determine whether supplementation with CO or bilirubin would regulate cell proliferation and angiogenesis. Western blotting, enzyme activity, CO and prostaglandin E_2 (PGE_2) production, and cell-cycle analysis were used to assess transgenic expression and functionality of the recombinant protein. A Matrigel matrix was used for assessment of *in vitro* capillary formation. Transduction with HO-1 antisense resulted in decreased capillary formation, cell proliferation, and cell-cycle progression, and increased PGE_2 production compared with control. HO-1 deficiency was also associated with increased expression of p21 and p27, but had no significant effect on p16 and p53. We also compared two different CO donors for their ability to rescue angiogenesis. Compared with control, HO-1-deficient endothelial cells showed increased angiogenesis following tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$) (CORM-1) starting at 50 μ*M*, whereas tricarbonylchloro(glycinato)ruthenium(II) (CORM-3), starting at 25 μ*M*, was a potent enhancer of angiogenesis. The addition of bilirubin did not restore angiogenesis. These data suggest that HO-mediated angiogenesis and cell proliferation were dependent on HO-1- and not HO-2-derived CO. *Antioxid. Redox Signal.* 7, 704–710.

INTRODUCTION

Angiogenesis is a fundamental process by which new blood vessels are formed. Angiogenesis is increased during embryogenesis and in pathological events, such as hypoxia, ischemia, inflammation, tumor growth, and wound healing, in response to angiogenic factors. These factors increase vascular permeability, endothelial cell activation, migration and proliferation, and capillary formation. These circumstances lead simultaneously to the production of a number of soluble mediators, including cytokines and acute-phase proteins such as heme oxygenase (HO). Current evidence strongly suggests that HO plays an important physiological and pathophysiological role in angiogenesis, cellular proliferation, cell-cycle progression, and somatic body growth (10, 31, 48, 52). HO isoforms catalyze the conversion of heme to carbon monoxide (CO) and bilirubin with a concurrent release of iron, which can drive the synthesis of ferritin for iron sequestration. To date, two HO isoforms, HO-1 and HO-2, have been shown to be catalytically active in heme degradation, and each is encoded by a different gene (36, 50).

HO-1 is expressed, under basal conditions, at low levels in endothelial cells (2, 7, 10, 54, 56), as well as in the kidney (9, 40), liver, and spleen, and can be induced in these cells and in other tissues by oxidative stress-causing agents, including hyperthermia (16), oxidized lipoproteins (22), inflammatory

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cytokines (21), hypoxia (37), nitric oxide (NO) (18), and heavy metals (1, 3, 17). HO-2 is constitutively expressed in blood vessels, endothelium, testis, and most other tissues, and its levels are relatively unaffected by factors inducing HO-1 (3). Evidence of the cell-survival effect of enhanced HO-1 *in vivo* is reflected in the attenuation of inflammatory responses associated with a variety of experimental and clinical circumstances (6, 20, 32, 37). CO, generated by HO-1, has been shown to prevent endothelial cell apoptosis (8), an effect reversed by inhibitors of the enzyme, such as tin mesoporphyrin (SnMP) (8, 41).

We have shown previously that up-regulation of HO-1 in endothelial cells enhances cell proliferation and angiogenesis (10, 52). More recently, we demonstrated that transduction of human HO-1 into spontaneously hypertensive rats promotes growth and may play a significant role in cell-cycle progression (30, 31, 38, 57). Tulis *et al.* (53) have also shown that HO-1 gene transfer attenuated a remodeling response to vascular injury by stimulating medial wall smooth muscle cell (SMC) apoptosis and inhibiting medial wall DNA replication (53). Furthermore, Duckers *et al.* demonstrated that HO-1 adenovirus-mediated overexpression inhibited the growth of SMCs *in vitro* and *in vivo* and up-regulation of $p21^{Cip1}$ (11), suggesting the importance of HO-1 in vascular wall remodeling. In recent studies, we also demonstrated that HO-1 regulates proliferation in a cell-specific and cell cycle-specific manner; in fact, pharmacological HO-1 induction increased endothelial cell proliferation, but inhibited SMC proliferation (34). Many of these effects seem to be mediated by HO-derived CO, a cellular messenger with signaling functions resembling that of NO. CO exerts antiinflammatory effects by inhibition of tumor necrosis factor-α, interleukin-1β, or macrophage inflammatory protein-1, and by up-regulation of interleukin-10 (29, 33). Like NO, CO regulates soluble guanylyl cyclase, which has also been shown to have a role during endothelial cell proliferation. Jozkowicz *et al.* showed that inhibition of HO activity can significantly reduce both the synthesis of vascular endothelial growth factor (VEGF) and the capability of endothelial cells to respond to exogenous stimulation and that these effects are mediated, at least in part, by changes in CO production (25). The possibility that HO-1 and/or HO-2 may have different roles in neovascularization and angiogenesis is of interest and remains to be studied.

To address the role of HO-1 and HO-2 in angiogenesis, we used HO-1 retroviral constructs in the antisense orientation and analyzed how supplementation of bilirubin or CO impacts on the angiogenic process in HO-1-deficient endothelial cells. Our results clearly demonstrate that endothelial cells deficient in HO-1 exhibit a significant reduction in angiogenesis and that bilirubin failed to restore angiogenesis. By contrast, the addition of CO-releasing drugs showed a dose-dependent increase in angiogenesis, thus suggesting the importance of HO-1-derived CO in this process.

MATERIALS AND METHODS

Cell culture conditions

The amphotropic retroviral packaging cell line PA317 (ATCC, Manassas, VA, U.S.A.) or PT67 (Clontech, Palo Alto, CA, U.S.A.) was used for generation of the replication-deficient recombinant retroviruses, as previously described (45). The CO donor, tricarbonyldichlororuthenium (II) dimer ($[Ru(CO)_3Cl_2]_2$, called CORM-1), was purchased from Sigma, and tricarbonylchloro(glycinato)ruthenium(II) (CORM-3) was prepared by Dr. J.R. Falck.

Western blot, HO activity, and endogenous CO production

Cells were harvested using cell lysis buffer as described previously (34). The lysate was used for western, CO, and HO activity (45).

Prostaglandin E_2 (PGE_2) and VEGF measurements

PGE_2 levels and VEGF protein were determined in the media of endothelial cell cultures using an enzyme-linked immunoassay (Quantikine Immunoassay Kit, R&D Systems, Minneapolis, MN, U.S.A.).

Cell proliferation

Cell proliferation was determined using a cell counting kit (Dojindo Molecular Technologies, Inc., Gaithersburg, MD, U.S.A.). In brief, 5×10^3 cells were inoculated in a 96-well microtiter plate and incubated in a humidified atmosphere (37°C, 5% CO_2) for 12, 24, 36, and 48 h, and then 10 µl of the kit solution was added to each well of the microtiter plate. Absorbance was measured at 450 nm using a microplate reader.

In vitro *angiogenesis and capillary formation*

A growth factor-induced basement membrane Matrigel matrix (BD Bioscience, Bedford, MA, U.S.A.) was used for assessment of *in vitro* capillary formation, as previously described (10).

Statistical analysis

Data are expressed as means ± SE. Significance was assessed by one-way analysis of variance and Student's *t* test; $p < 0.05$ was considered to be statistically significant.

RESULTS

Characterization of endothelial cells diminished by HO-1

HO-1 and HO-2 protein expression in control endothelial cells and cells transduced with HO-1 antisense was evaluated by western blot analysis. As shown in Fig. 1A, HO-1 protein was significantly decreased in cells transduced with HO-1 antisense compared with control cells. The level of HO-2 protein was similar in the two types of endothelial cells. These findings indicate that HO-1 gene transfer, in both orientations, strongly down-regulates HO-1 expression without affecting HO-2 protein levels. Furthermore, transduction of endothelial cells with HO-1 antisense resulted in a significant up-regulation of p21 and p27 protein expression when com-

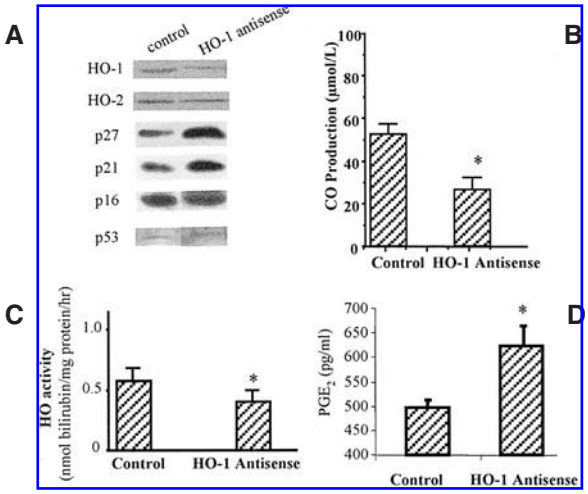

FIG. 1. **(A)** Western blot analysis of HO-1, HO-2, p27, p21, p16, and p53. Cell lysates from control cells and cells transduced with human HO-1 gene in antisense orientations were visualized by immunoblotting with antibodies against the respective antibody proteins as described in Materials and Methods. **(B)** CO production was determined as described in Materials and Methods. CO production is expressed as pmol/mg of protein and is the mean ± SD of three experiments (*$p < 0.05$ compared with control). **(C)** Cell lysate HO activity was assayed by measuring bilirubin using the difference in absorbency from λ 460 to λ 530 nm with an absorption coefficient of 40 mM cm^{-1}. HO activity (nmol of bilirubin/mg/h) is expressed as the mean ± SE of three experiments (*$p < 0.05$). **(D)** PGE_2 in control and cells underexpressing HO-1. The levels of PGE_2 in the culture medium were measured by enzyme-linked immunoassay as described in Materials and Methods. The results are expressed in pg of prostaglandins in 1 ml of culture medium and are the mean ± SE of three experiments (*$p < 0.05$) compared with the corresponding control.

pared with control cells (Fig. 1A). As shown in Fig. 1B, HO activity, measured as the rate of conversion of heme to bilirubin, was 40% lower ($p < 0.001$) in cells transduced with HO-1 antisense compared with control cells.

The levels of CO in control cells and cells transduced with HO-1 antisense directly corresponded to the levels of HO activity. As shown in Fig. 1C, CO levels in the control cells amounted to 52.7 ± 8.0 pmol/mg of protein, whereas these levels were 50% lower in cells transduced with HO-1 antisense. These results indicate that CO production was decreased by the suppression of HO-1 expression. As overexpression of human HO-1 decreases cellular heme content (45), the basal levels of cyclooxygenase activity-derived PGE_2 levels were determined and were shown to be significantly increased in cells transduced with HO-1 antisense compared with control cells (Fig. 1D).

Analyses of cell proliferation and DNA distribution in endothelial cells

As shown in Fig. 2A, cell proliferation significantly decreased in cells transduced with HO-1 antisense compared with control cells. To investigate the significance of selective modulation of HO-1 on the cell cycle, we examined the effect

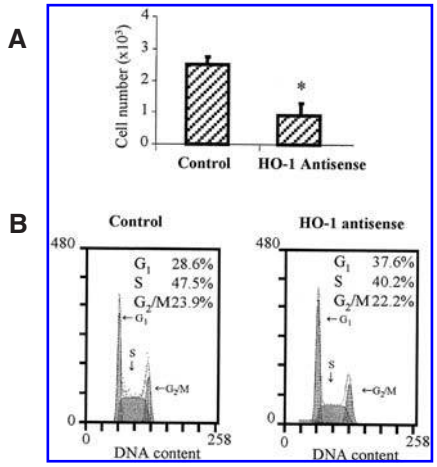

FIG. 2. **(A)** Cell proliferation was measured after 7 days, during which cells were harvested after 5 days and seeded in 96-well plates for an additional 48 h in the presence of glucose. Cell proliferation was determined as described in Materials and Methods ($n = 4$; *$p < 0.05$, glucose versus untreated cells). **(B)** DNA distributions of control or cells underexpressing HO-1. Data are representative of three independent experiments. Representative DNA distributions are shown.

of HO-1 antisense transduction. This resulted in an increase in the G_1 phase and a decrease in the S and G_2/M phases compared with control cells (Fig. 2B) ($p < 0.05$).

VEGF and in vitro *angiogenesis and capillary endothelial cell formation*

Down-regulation of HO-1 significantly decreased the basal levels of VEGF when compared with control (Fig. 3A). These results are consistent with previous reports (25) showing that inhibition of HO activity results in a reduction of

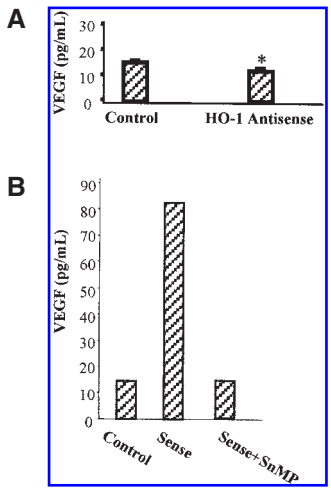

FIG. 3. **(A)** Levels of VEGF in culture medium expressed as the mean ± SE ($n = 3$; *$p < 0.05$ compared with the corresponding control). **(B)** Effect of HO inhibitors on VEGF production.

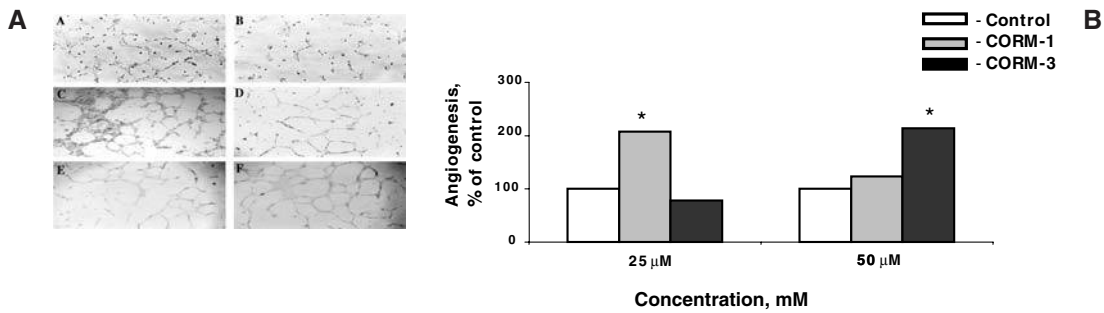

FIG. 4. Effect of CO-releasing molecules on control and HO-1 AS on angiogenesis *in vitro*. **(A)** Representative pictures: Panel A, control; panel B, AS; panel C, CORM-3; panel D, AS and CORM-3; panel E, CORM-1; panel F, AS and CORM-1 **(B)** Quantitative analysis of the capillary formation. Results are expressed as mean ± SE, $n = 3$; $^*p < 0.05$ vs. corresponding control.

VEGF synthesis. To elucidate better the role of HO-1-mediated VEGF synthesis, we transduced endothelial cells with a retrovirus containing the HO-1 gene in the sense orientation. Retroviral-mediated up-regulation of HO-1 resulted in a significant increase of VEGF production, which was abolished by the addition of SnMP, a potent inhibitor of HO activity (Fig. 3B).

As shown in Fig. 4A, the HO-1 antisense-transduced cells were assayed for capillary formation. Cells with decreased HO-1 demonstrated significantly decreased tubular growth and angiogenesis. Figure 4 panel B illustrates the quantitative measurement of the increase in capillary formation over control. The decrease in angiogenesis in HO-1 AS cell can be prevented by supplementation of CO (Fig. 4A, panels D, F). These findings indicate that a decrease in HO-1 gene expression by delivery of HO-1 in antisense orientation suppresses angiogenesis.

To evaluate the restoration of angiogenic activity, we performed angiogenesis assay on endothelial cells transduced with HO-1 antisense in the presence of bilirubin or of two different CO-releasing molecules. In the presence of bilirubin, the HO-1 antisense-transduced cells did not show significant augmentation of capillary-like formation compared with untreated cells and nontransduced cells (data not shown). In contrast, in the presence of CO-releasing molecules, the HO-1 antisense-transduced cells significantly increased tubular growth and angiogenesis in a dose-dependent manner (Fig. 4B). Interestingly, the two compounds were demonstrated to have different angiogenesis-stimulating abilities, which is probably due to their different abilities to release CO into the medium.

DISCUSSION

In the present study, we demonstrate, for the first time, that when HO-1 is impaired, CO may act as a signaling mechanism to enhance angiogenesis. This suggests that HO-1- and not HO-2-derived CO plays a key role during the angiogenic process. Several key findings substantiate this conclusion. The first is that transducing endothelial cells with the HO-1 gene in the antisense orientation led to a substantial decline in HO-1 protein expression and HO activity, reflected in the decreased rate of CO production, without affecting HO-2 protein expression. As a result of the HO-1 deficiency, cell-cycle progression, cell proliferation, and angiogenesis were inhibited. Again, these effects appear to be independent of HO-2. Others have also shown that HO-1, but not HO-2, increases angiogenesis (10) in endothelial cells and enhances cell proliferation (35) and up-regulation of HO-1 increases VEGF (5, 12, 13). In addition, HO-1 deficiency was also associated with increased expression of p21 and p27, two well-known inhibitors of cell-cycle progression, without affecting p53 and p16 expression. We also demonstrated, for the first time, that specific down-regulation of HO-1 significantly decreased the basal levels of VEGF and that this effect was dependent on HO-1-derived CO. Taken together, these results suggest that the HO-1 antisense-mediated reduction in VEGF and up-regulation of p21 and p27 were sufficient to decrease angiogenesis significantly even though PGE_2, a well-known inducer of cell proliferation, was increased (Fig. 5). The second key finding is that the addition of bilirubin to HO-1-deficient cells did not restore angiogenesis, thus suggesting the possible role of HO-1-derived CO during this process. The third key finding is that the addition of CO-releasing molecules to HO-1-deficient endothelial cells rescued the ineffectiveness of HO-2 to sustain endothelial cell angiogenic activity. Interestingly, CORM-3, starting at a concentration of 25 μ*M*, increased angiogenesis, whereas the tricarbonyldichlororuthenium(II) dimer (CORM-1) showed a significant increase starting at a concentration of 50 μ*M*, suggesting a different CO-releasing kinetic for the two drugs. CORM-3 at 50 μ*M* resulted in a significant decrease in endothelial cell proliferation compared with 25 μ*M*, suggesting that optimal elevated CO levels may be toxic for the cell. Consistent with these results is the fact that the concentrations of CO found in urban environments have been correlated with hospital admissions, as well as with morbidity and mortality from cardiovascular and pulmonary disease (55). However, rodents and humans deficient in HO-1 associated with a decrease in CO formation demonstrate a disturbed growth pattern (43, 44), which results in high morbidity (56). Therefore, an optimum concentration of CO is required for an ideal environment for cell growth. Under physiological conditions, the basal level of CO in the human body is ~20 μmol h^{-1} (24). The basal level of

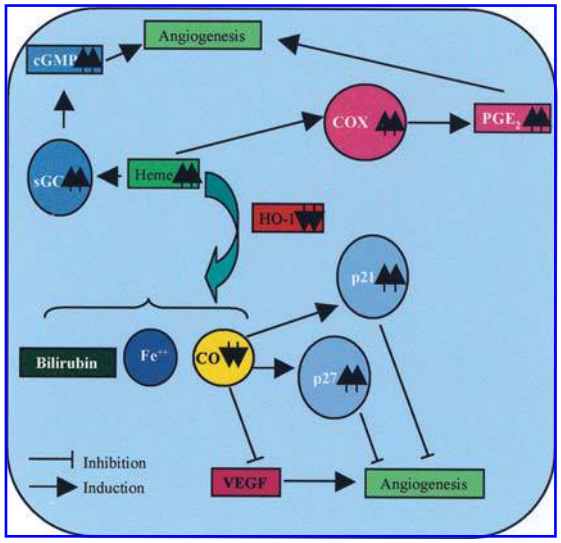

FIG. 5. Mechanism of HO-1 modulates cell proliferation. CO-derived HO-1 may directly enhance cyclic GMP (cGMP) and angiogenesis or a decrease in CO, as a result of suppression of HO-1, may diminish VEGF formation and its angiogenetic response. Alternatively, a decrease in CO enhances p21 and p27 and subsequently decreases cell proliferation and angiogenesis. COX, cyclooxygenase; sGC, soluble guanylyl cyclase.

CO is generated from HO-2 (28, 51) as a result of heme degradation and the physiological turnover of heme (3, 1). The basal level of CO from HO-2 is responsible for numerous biological functions, including activation of cyclic GMP when NO synthase is inhibited (26–28). However, under stress conditions, CO is derived from HO-1 up-regulation (4, 5, 23, 39). The increase in CO during pathological situations is considered an adaptive response to stress conditions and increased HO-1 activity (42). The decrease in HO-1 activity and CO levels in diabetes enhances cell death (46, 47), and a CO donor may substitute for abnormalities in cell function (4).

Recently, several reports have demonstrated that HO-1 is up-regulated in various types of rapid-growth cells, such as in renal adenocarcinoma (19). The mechanism by which HO-1 mediates angiogenesis is not fully defined, but it may be related to the HO product, CO. CO, generated by HO-1, has been shown to enhance endothelial cell proliferation and decrease apoptosis (8). These observations suggest that the HO system, via the production of CO, subserves mechanisms that enhance cell-cycle progression and cell proliferation. These results are in agreement with an earlier study showing that inhibition of HO activity, such as by SnMP**,** can significantly reduce both the synthesis of VEGF and the capability of endothelial cells to respond to exogenous stimulation, suggesting that these effects are mediated, at least in part, by changes in CO production (25). However, in this previous study, only a nonspecific HO inhibitor was used, which also produces HO-independent effects, which can make the interpretation of these experiments very uncertain. Therefore, in the present work, we selectively inhibited HO-1 by using a retroviral vector that conta3ined the HO-1 gene in the antisense orientation.

There are several important clinical findings in the present report. The delivery of CO will directly enhance angiogenesis in ischemic organs, such as kidney ischemia and heart ischemia. Up-regulation of HO-1, or supplementation of CO, has an important stimulating effect on endothelial cells, but has an inhibitory effect on vascular SMCs (14, 15, 34). Therefore, CO and bilirubin can be very beneficial in situations where HO-1 is actively diminished, such as in hypertension (4, 46, 49). The use of retroviral HO-1 transfer allowed us to confirm the important role of this isoform in endothelial cells, which is also suggested by the disturbed growth in HO-1 knockout mice (43, 44) and by the fatal consequences of HO-1 deficiency in humans (56). In both cases, the lack of HO-1 activity results in extreme vulnerability of vessels to common stressful stimuli, leading to severe vascular endothelial damage and detachment, thus pointing to the key role of HO-1 in the endothelium.

Collectively, our findings suggest that HO-1-derived CO, but not bilirubin, another HO-1-derived metabolite, mediates angiogenesis. Additional studies to elucidate the detailed regulatory pathway of CO and signaling in angiogenesis are necessary to develop strategies for the potential targeted pharmacological modulation of HO-1.

ACKNOWLEDGMENTS

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ABBREVIATIONS

CO, carbon monoxide; CORM-1, tricarbonyldichlororuthenium(II) dimer; CORM-3, tricarbonylchloro(glycinato)ruthenium(II); HO, heme oxygenase; NO, nitric oxide; PGE_2, prostaglandin E_2; SMC, smooth muscle cell; SnMP, tin mesoporphyrin; VEGF, vascular endothelial growth factor.

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