Carbon Monoxide Generated by Heme Oxygenase 1 Suppresses Endothelial Cell Apoptosis

By Sophie Brouard,* Leo E. Otterbein,‡ Josef Anrather,§ Edda Tobiasch,* Fritz H. Bach,* Augustine M.K. Choi,‡ and Miguel P. Soares*

From the *Immunobiology Research Center, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215; the ‡Department of Internal Medicine, Pulmonary and Critical Care Section, Yale University, School of Medicine, New Haven, Connecticut 06520; and the §Department of Neurobiology, Weill Medical College of Cornell University, New York, New York 10021

Abstract

Heme oxygenase 1 (HO-1) inhibits apoptosis by regulating cellular prooxidant iron. We now show that there is an additional mechanism by which HO-1 inhibits apoptosis, namely by generating the gaseous molecule carbon monoxide (CO). Overexpression of HO-1, or induction of HO-1 expression by heme, protects endothelial cells (ECs) from apoptosis. When HO-1 enzymatic activity is blocked by tin protoporphyrin (SnPPIX) or the action of CO is inhibited by hemoglobin (Hb), HO-1 no longer prevents EC apoptosis while these reagents do not affect the antiapoptotic action of bcl-2. Exposure of ECs to exogenous CO, under inhibition of HO-1 activity by SnPPIX, substitutes HO-1 in preventing EC apoptosis. The mechanism of action of HO-1/CO is dependent on the activation of the p38 mitogen-activated protein kinase (MAPK) signaling transduction pathway. Expression of HO-1 or exposure of ECs to exogenous CO enhanced p38 MAPK activation by TNF-α. Specific inhibition of p38 MAPK activation by the pyridinyl imidazol SB203580 or through overexpression of a p38 MAPK dominant negative mutant abrogated the antiapoptotic effect of HO-1. Taken together, these data demonstrate that the antiapoptotic effect of HO-1 in ECs is mediated by CO and more specifically via the activation of p38 MAPK by CO.

Key words: apoptosis • endothelial cells • heme oxygenase 1 • carbon monoxide • p38 mitogen-activated protein kinase

Introduction

In their normally quiescent state, endothelial cells (ECs) maintain blood flow, allowing the continuous traffic of plasma and cellular constituents between blood and parenchymal tissues. To accomplish this function, ECs must promote a certain level of vasorelaxation and inhibit leukocyte adhesion as well as coagulation and thrombosis (for a review, see reference 1). However, when ECs are exposed to proinflammatory stimuli, they become “activated” and promote vasoconstriction, leukocyte adhesion, and activation, as well as coagulation and thrombosis (for reviews, see references 1–3). These functional changes are due to the expression by activated ECs of a series of proinflammatory genes encoding adhesion molecules, cytokines/chemokines, and costimulatory and procoagulant molecules (for reviews, see references 1–4). To prevent unfettered EC activation that could lead to EC injury and apoptosis, the expression of these proinflammatory genes must be tightly regulated (5). One of the mechanisms by which this occurs relies on the expression by activated ECs of “protective genes” (6–10). One such gene is the stress responsive gene heme oxygenase 1 (HO-1).

Heme oxygenases are the rate-limiting enzymes in the catabolism of heme into biliverdin, free iron, and carbon monoxide (CO), with biliverdin being subsequently catabolized to bilirubin. HO-1 is induced constitutively under resting conditions in many tissues and can also be induced by a variety of proinflammatory stimuli in vitro and in vivo (11). The protective role of HO-1 is well documented in a number of systems (12–15), and HO-1-null mice are susceptible to a variety of proinflammatory stimuli (16–20). Thus, HO-1 is an important proinflammatory signal transduction pathway (21–23).
lyzed into bilirubin (for reviews, see references 11–13). Several proinflammatory stimuli that lead to EC activation also upregulate the expression of HO-1 (for reviews, see references 12 and 13). That HO-1 acts as a cytoprotective gene is suggested by the observation that expression of HO-1 in vitro prevents EC injury mediated by activated polymorphonuclear cells (14), hydrogen peroxide (14, 15), or heme (15–17). In addition, expression of HO-1 in vivo suppresses a variety of inflammatory responses, including endotoxic shock (17–19), hyperoxia (20), acute pleurisy (21), ischemia reperfusion injury (22), and graft rejection (23, 24), further supporting the cytoprotective role of this gene.

We have shown previously that expression of HO-1 prevents ECs from undergoing apoptosis (23). This may be an important mechanism by which HO-1 exerts its cytoprotective function, since EC apoptosis, such as it occurs during acute (25) and chronic inflammation (26), is highly deleterious. We now demonstrate that the antiapoptotic effect of HO-1 is mediated through the ability of HO-1 to generate the gaseous molecule CO. In addition, we demonstrate that CO suppresses EC apoptosis through a mechanism that is dependent on the activation of the p38 mitogen-activated protein kinase (MAPK) signal transduction pathway.

Materials and Methods

Cell Culture. The murine 2F-2B EC line (American Type Culture Collection) and primary bovine aortic ECs (BAECs) were cultured as described previously (23, 27).

Expression Plasmids. The β-galactosidase was cloned into the pcDNA3 vector (Invitrogen) as described previously (28). Two vectors encoding rat HO-1 cDNA were used. The original vector encoding the full-length rat HO-1 cDNA under the control of a the β-actin enhancer/promoter (β-actin/HO-1) has been described elsewhere (29). A 1.0-kbp XhoI-HindIII fragment encoding the full-length rat HO-1 cDNA was cut from the prHO-1 vector (30) and subcloned into the pcDNA3 vector to achieve expression of the HO-1 cDNA under the control of the CMV enhancer/promoter (pcDNA3/HO-1). The mouse Bcl-2 cDNA was cloned in the pac vector, as described elsewhere (10). p38/CSBP1 MAPK was amplified from HeLa cDNA by PCR and cloned into the pcDNA3/HA vector derived from pcDNA3 (Invitrogen) by inserting a DNA fragment coding for an epitope derived from the hemagglutinin protein of the human influenza virus hemagglutinin (HA: MYPYDVPDYASL). A dominant negative mutant of p38/CSBP1, harboring a T180A and a Y182F substitution, was generated by overlap extension mutagenesis. Green fluorescent protein (GFP) cDNA (CLONTECH Laboratories, Inc.) was cloned into the pcDNA3-expressing vector.

Transient Transfections. BAECs and 2F-2B ECs were transiently transfected as described elsewhere (23, 28). All experiments were carried out 24–48 h after transfection. β-Galactosidase–transfected cells were detected as described elsewhere (23, 28). The percentage of viable cells was detected by evaluating the number of β-galactosidase–expressing cells that retained normal morphology, as described elsewhere (23, 28). The number of random fields counted was determined to have a minimum of 200 viable transfected cells per control well. The percentage of viable cells was normalized for each DNA preparation to the number of blank samples.

Figure 1. HO-1 suppresses EC apoptosis. (A) 2F-2B ECs were transfected with a GFP-expressing plasmid and monitored for GFP expression by flow cytometry. The percentage of transfected ECs was assessed by measuring fluorescence intensity in ECs transfected with control (pcDNA3; filled histogram) versus GFP (open histogram) expression plasmids. (B) ECs were cotransfected with β-galactosidase plus control (pcDNA3) or HO-1 (β-actin/HO-1) expression vectors. EC apoptosis was induced by TNF-α plus Act.D and apoptosis of β-galactosidase–transfected ECs was quantified. Gray bars represent ECs treated with Act.D and black bars represent ECs treated with TNF-α plus Act.D. Results shown are the mean ± SD from duplicate wells taken from 1 representative experiment out of 10. (C) HO-1 expression was detected in BAECs by Western blot. No Tr, nontransfected. NT, nontreated. (D) 2F-2B ECs were cotransfected with β-galactosidase plus control (pcDNA3) or HO-1 (β-actin/HO-1) expression vectors. EC apoptosis was induced by etoposide or subjected to serum deprivation (0.1% FCS for 24 h). Results shown are the mean ± SD from duplicate wells taken from 1 representative experiment out of 3 independent experiments. Similar results were obtained using BAECs.
transfected cells counted in the absence of the apoptosis-inducing agent (100% viability). All experiments were performed at least three times in duplicate.

Adenovirus. The recombinant HO-1 adenovirus has been described previously (31). The recombinant β-galactosidase adenovirus was a gift of Dr. Robert Gerard (University of Texas Southwestern Medical Center, Dallas, Texas). Adenoviruses were produced, extracted, purified, and titrated, as described previously (28). Confluent BAECs were infected with a multiplicity of infection of 200 PFU/cell, as described elsewhere (28).

Cell Extracts and Western Blot Analysis. Cell extracts were prepared, electrophoresed under denaturing conditions (10–12.5% polyacrylamide gels), and transferred into polyvinylidifluoridene membranes (Immobilon P; Millipore), as described elsewhere (28). HO-1 was detected using a rabbit anti-human HO-1 polyclonal antibody (StressGen Biotechnologies). Total and activated/phosphorylated forms of extracellular signal-regulated kinases (ERK-1 and -2), c-Jun N-terminal kinases (JNK-1, -2, and -3), and p38 MAPK were detected using rabbit polyclonal antibodies directed against the total or phosphorylated forms of these MAPKs, according to the manufacturer’s suggestions (New England Biolabs, Inc.). β-Tubulin was detected using an anti-human β-tubulin monoclonal antibody (Boehringer). Primary antibodies were detected using horseradish peroxidase–conjugated donkey anti–rabbit or goat anti–mouse IgG secondary antibodies (Pierce Chemical Co.). Peroxidase was visualized using the enhanced chemiluminescence assay (Amer sham Pharmacia Biotech) according to the manufacturer’s instructions, and stored in the form of photoradiographs (Biomax® MS; Eastman Kodak Co.). Digital images were obtained using an image scanner (Arcus II; Agfa) equipped with FotoLook and Photoshop® software. The amount of phosphorylated ERK, JNK, and p38 MAPK was quantified using ImageQuant® software (Molecular Dynamics). When indicated, membranes were stripped (62.5 mM Tris–HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol, 30 min, 50°C). Phosphorylated ERK, JNK, and p38 MAPK were normalized to the total amount of total ERK, JNK, and p38 MAPK detected in the same membrane.

Flow Cytometry. 2F-2B ECs were transfected with the GFP expression plasmid and harvested 24 h after transfection by trypsin digestion (0.05% in PBS). ECs were washed in PBS, pH 7.2, 5% FCS, and fluorescent labeling was evaluated using a FACSort™ equipped with CELLQuest™ software (Becton Dickinson).

Cell Treatment and Reagents. Actinomycin D (Act.D; Sigma- Aldrich) was dissolved in PBS and added to the culture medium 24 h after transfection. The Act.D concentration corresponded to the optimal concentration necessary to sensitize ECs to TNF-α–mediated apoptosis, e.g., 10 μg/ml for 2F-2B ECs and 0.1 μg/ml for BAECs. When indicated, EC apoptosis was induced by etoposide (200 μM, 8 h; Calbiochem–Novo biochem) or by serum deprivation (0.1% FCS for 24 h). The iron chelator deferoxamine mesylate (DFO; Sigma-Aldrich) was dissolved ex tempore in water and added to culture medium (1–100 μM) 1 h before the induction of apoptosis. Hemoglobin (Hb; Sigma- Aldrich) was dissolved (1 mM) extemporarily in PBS, 10 mM Na2S3O4, dialyzed against PBS (2 h, 4°C, 1:800 dilution), and added to the culture medium (1–100 μM) 6 h after transfection. The guanylylase inhibitor 1H(1,2,4)oxadiazolo(4,3-a)quin oxalin-1 (ODQ; Calbiochem–Novo biochem) was dissolved in DMSO (Sigma–Aldrich) and added to the culture medium (10–100 μM) 6 h after transfection. Iron protoporphyrin ([FePPP]/

Figure 2. The antiapoptotic effect of HO-1 is dose dependent. (A) 2F-2B ECs were cotransfected with increasing doses of HO-1 expression vector (β-actin/HO-1). EC apoptosis was induced by TNF-α plus Act.D. Results shown are the mean ± SD from duplicate wells taken from one representative experiment out of three. Similar results were obtained using BAECs. (B) The expression of HO-1 was detected in BAECs by Western blot. Values indicate the amount of HO-1 vector (pcDNA3/HO-1) used in each transfection (ng of DNA per 3 x 105 cells). No Tr., nontransfected.

Figure 3. The antiapoptotic effect of HO-1 is dependent on HO enzymatic activity. (A) 2F-2B ECs were cotransfected with β-galactosidase plus pcDNA3, HO-1 (β-actin/HO-1), or bcl-2 expression vectors. Cells were either left untreated (Control) or treated with the inhibitor of HO enzymatic activity SnPPIX. CoPPIX, a protoporphyrin that does not inhibit HO enzymatic activity, was used as a control treatment. Gray bars represent ECs treated with Act.D and black bars represent ECs treated with TNF-α plus Act.D. Results shown are the mean ± SD from duplicate wells taken from one representative experiment out of three. (B) 2F-2B ECs were transfected with β-galactosidase plus pcDNA3 expression vectors. ECs were either left untreated (Control) or were treated with SnPPIX and CoPPIX as in A. The results shown are the mean ± SD from duplicate wells taken from one representative experiment out of three.
Figure 4. Scavenging of CO by Hb suppresses the antiapoptotic effect of HO-1. 2F-2B ECs were cotransfected with β-galactosidase plus control (pcDNA3), HO-1 (β-actin/HO-1), or bcl-2 expression vectors. ECs were either left untreated (0) or were treated with increasing concentrations of Hb. Gray bars represent ECs treated with Act.D and black bars represent ECs treated with TNF-α plus Act.D. The results shown are the mean ± SD from duplicate wells taken from one representative experiment out of four.

Figure 5. Exogenous CO suppresses EC apoptosis in the absence of HO-1. (A) 2F-2B ECs were transfected with a β-galactosidase expression vector and exposed to exogenous CO. Gray bars represent ECs treated with Act.D alone and black bars represent ECs treated with TNF-α plus Act.D. (B) 2F-2B ECs were transfected with a β-galactosidase expression vector and exposed to exogenous CO (10,000 ppm) with or without Hb. Gray bars represent ECs treated with Act.D and black bars represent ECs treated with TNF-α plus Act.D. (C) 2F-2B ECs were cotransfected with β-galactosidase and HO-1 (β-actin/HO-1) expression vectors. Where indicated (+), HO-1 enzymatic activity was inhibited by SnPPiX and/or exposed to exogenous CO. Gray bars represent ECs treated with Act.D and black bars represent ECs treated with TNF-α plus Act.D. Results shown (A, B, and C) are the mean ± SD from duplicate wells taken from one representative experiment out of three.
expression vector per 3 × 10⁶ cells (Fig. 2). All subsequent experiments were carried out using these experimental conditions.

The Antiapoptotic Function of HO-1 Requires Its Enzymatic Activity. To test whether the antiapoptotic action of HO-1 was dependent on its enzymatic action, HO-1 activity was blocked using SnPPIX. When HO-1 activity was blocked by SnPPIX, HO-1 was no longer able to prevent EC apoptosis (Fig. 3), and the antiapoptotic effect of bcl-2 was not impaired by SnPPIX (Fig. 3). CoPPIX, which has a similar structure to SnPPIX but does not inhibit HO activity, did not suppress the antiapoptotic effect of HO-1 or that of Bcl-2 (Fig. 3). These protoporphyrins had no detectable effect per se on EC viability (Fig. 3).

Endogenous CO Mediates the Antiapoptotic Effect of HO-1. Since HO-1 enzymatic activity is needed for its antiapoptotic effect, this suggests that this antiapoptotic effect is mediated through one or more end products of heme catabolism by HO-1, i.e., bilirubin, iron, and/or CO. We tested if CO would account for the antiapoptotic effect of HO-1. ECs were transiently transfected with HO-1 and treated with Hb to scavenge CO. Under these conditions, the antiapoptotic effect of HO-1 was suppressed (Fig. 4). The ability of Hb to block the antiapoptotic effect of HO-1 was dose dependent, in that increasing concentrations of Hb (3–50 μM) decreased the ability of HO-1 to prevent EC apoptosis (Fig. 4). Hb did not impair the antiapoptotic effect of Bcl-2 (Fig. 4), nor did it sensitize control ECs (pcDNA3) to apoptosis (Fig. 4).

Exogenous CO Can Substitute for HO-1 in Preventing EC Apoptosis. If CO mediates the antiapoptotic action of HO-1, then exogenous CO should prevent EC apoptosis. The data illustrated in Fig. 5 show that this is the case. When control ECs (transfected with pcDNA3) were exposed to exogenous CO (10,000 ppm), TNF-α-mediated apoptosis was suppressed (Fig. 5). Exogenous CO also suppressed EC apoptosis when HO-1 activity was inhibited by SnPPIX, suggesting that CO can prevent EC apoptosis in the absence of other biological functions of HO-1 (Fig. 5). We then tested whether the level of exogenous CO used (10,000 ppm) was comparable to that produced when HO-1 is expressed in ECs. Given that Hb (50 μM) blocks the protective effect of endogenously produced CO (Fig. 4), we reasoned that if this was the case for exogenous CO then the effects of exogenous CO may mimic those of endogenous CO. The antiapoptotic effect of exogenous CO was suppressed by Hb (50 μM), suggesting that the concentration of exogenous CO (10,000 ppm) used in these experiments is not supraphysiologic (Fig. 5).

ECs That Express HO-1 Can Suppress Apoptosis of ECs That Do Not Express HO-1. Given that CO can act as an intercellular signaling molecule, we hypothesized that ECs that express HO-1 may generate sufficient levels of CO to protect neighboring ECs that do not express HO-1 from undergoing apoptosis. To test this hypothesis, ECs were transfected with control (pcDNA3) or HO-1 expression vectors and cocultured with β-galactosidase-transfected ECs. When cocultured with control ECs (pcDNA3), TNF-α plus Act.D induced apoptosis of β-galactosidase–transfected ECs (that do not express HO-1). However, when cocultured with ECs expressing HO-1, β-galactosidase–transfected ECs were protected from TNF-α plus Act.D–mediated apoptosis (Fig. 6).

Expression of Endogenous HO-1 Inhibits EC Apoptosis via CO. We questioned whether upregulation of endogenous HO-1 by heme would suppress EC apoptosis. The data illustrated in Fig. 7 suggest that this is the case. Explo-
Carbon Monoxide Suppresses Endothelial Cell Apoptosis

Heme becomes cytotoxic at concentrations higher than 10 \( \mu M \) (35; Fig. 7). The antiapoptotic effect of heme was dependent on the generation of CO, since heme was no longer able to suppress EC apoptosis when CO was scavenged by Hb (Fig. 7).

Iron Chelation Protects ECs from Apoptosis. The observation that CO can prevent EC apoptosis (Figs. 4 and 5) contrasts with the notion that the antiapoptotic effect of HO-1 relies exclusively on its ability to prevent intracellular iron accumulation (34). Given that overexpression of HO-1 in ECs resulted in significant upregulation of ferritin expression (data not shown), we questioned whether elimination of reactive intracellular iron such as it occurs when ferritin is expressed would contribute to prevent TNF-\( \alpha \)–mediated apoptosis of ECs. To mimic the iron chelator effect of ferritin, we used the iron chelator DFO and tested whether DFO would suppress EC apoptosis. The data illustrated in Fig. 8 suggest that this is the case. Induction of EC apoptosis by TNF-\( \alpha \) plus Act.D was suppressed by DFO (Fig. 8). When HO-1 activity was inhibited by SnPPIX or the action of CO was suppressed by Hb, DFO was still able to prevent EC apoptosis (Fig. 8).

CO and Iron Chelation Have Additive Effects in Protecting ECs from Apoptosis. Given the ability of both CO and iron chelation to suppress EC apoptosis, we asked whether these two biological functions, engendered by HO-1, would act together to suppress EC apoptosis. Under inhibition of HO activity by SnPPIX, exposure to low levels of CO (250 ppm) did not suppress EC apoptosis significantly. When used alone, DFO (100 \( \mu M \)) suppressed EC apoptosis, but to a lesser extent than HO-1 (Fig. 9). However, when ECs were exposed to both CO (250 ppm) and DFO (100 \( \mu M \)), inhibition of EC apoptosis was comparable to that achieved with the expression of HO-1.
The Antiapoptotic Effect of HO-1 Is Not Mediated by Guanylyl cyclase Activation or cGMP Generation. Most biological functions attributed to CO have been linked to its ability to bind guanylyl cyclase and increase the generation of cGMP (15, 36, 37). Since cGMP can regulate apoptosis (38, 39), we tested whether or not the antiapoptotic effect of HO-1 acted via the activation of guanylyl cyclase and/or the generation of cGMP. The data illustrated in Fig. 10 suggest that this is not the case. Expression of HO-1 in ECs did not result in a detectable increase of cGMP-related functions as illustrated by the absence of VASP phosphorylation, a protein phosphorylated by cyclic nucleotide–dependent protein kinases (protein kinase G-α/β; Fig. 10). This finding is in keeping with that reported by others (40). Inhibition of guanylyl cyclase activity by ODQ did not suppress the antiapoptotic effects of HO-1, and the cGMP analogue 8-Br-cGMP failed to suppress EC apoptosis (Fig. 10). That 8-Br-cGMP acted as a cGMP analogue was shown by its ability to induce VASP phosphorylation (Fig. 10). That ODQ was efficient in suppressing guanylyl cyclase activity in ECs was shown by its ability to prevent constitutive VASP phosphorylation in 2F-2B ECs (data not shown).

HO-1 Increases TNF-α–mediated Activation of p38 MAPK in ECs. Given that HO-1 and/or CO can modulate p38 MAPK activation in monocyte/macrophages (Mφ [33]), we tested whether HO-1 and/or CO would have similar effects in ECs. Stimulation of ECs with TNF-α resulted in transient activation of JNK and p38 MAPK (Fig. 11), a finding consistent with those of others (41). ERKs (42 and 44 kD) were constitutively active in resting ECs, and no significant upregulation was detectable after TNF-α stimulation (Fig. 11). Recombinant adeno virus–mediated overexpression of HO-1 potentiated the ability of TNF-α to activate p38 MAPK (Fig. 12), but not to activate JNK (data not shown). Overexpression of β-galactosidase had no detectable effect on the activation of either p38 MAPK or JNK by TNF-α. Exposure of ECs to exogenous CO activated p38 MAPK even in the absence of TNF-α (Fig. 12).

The Mechanism by Which CO Prevents EC Apoptosis Acts via the Activation of p38 MAPK. Since p38 MAPK can regulate apoptosis (42), we investigated whether the ability of HO-1 to modulate p38 MAPK activation was linked to its ability to prevent EC apoptosis. We found that this is the case. The antiapoptotic action of HO-1 was suppressed when p38 MAPK activation was blocked by the pyridinyl imidazol SB203580 (10–20 μM), a specific inhibitor of p38 MAPK (32). This effect was dose dependent in that increasing concentrations of SB203580 were increasingly efficient in suppressing the antiapoptotic action of HO-1 (Fig. 13). Inhibition of p38 MAPK activation per se did not sensitize ECs to TNF-α–mediated apoptosis (data not shown). As expected, activation of p38 MAPK by TNF-α was significantly inhibited (85–95%) in ECs exposed to SB203580 (5–20 μM), compared with control ECs stimulated by TNF-α in the absence of SB203580 (Fig. 13). Similarly to SB203580, overexpression of a dominant negative mutant of p38/CSBP1 (43) also suppressed TNF-α–mediated p38 MAPK activation, as tested by a kinase assay using the activating transcription factor (ATF)-2 as a substrate (data not shown). Overexpression of this dominant negative mutant suppressed the ability of HO-1 to prevent EC apoptosis (Fig. 13). This inhibitory effect was dose dependent in that increasing amounts of the p38/CSBP1 dominant negative mutant were more efficient in suppressing the antiapoptotic action of HO-1 (Fig. 13).
Carbon Monoxide Suppresses Endothelial Cell Apoptosis

The mechanism by which apoptotic ECs promote vascular thrombosis is thought to involve the expression of procoagulant phospholipids by apoptotic bodies (49) and presumably the exposure of the procoagulant subendothelial matrix that is associated with EC apoptosis. In addition, apoptotic cells can activate the complement cascade directly through the binding of C1q to apoptotic bodies (50), and promote platelet adhesion (49) that will sustain inflammation and thrombosis as well.

The observation that HO-1 prevents apoptosis induced by different proapoptotic stimuli (23, 24; Fig. 1) suggests that HO-1 suppresses one or several signaling pathways that are common to a broad spectrum of proapoptotic stimuli. The antiapoptotic effect of HO-1 has recently been associated with increased cellular iron efflux through the upregulation of an iron pump that remains to be fully characterized (34). According to this study (34), HO-1 inhibits apoptosis by limiting the availability of prooxidant-free iron to participate in the generation of reactive oxygen species through the Fenton reaction, a well-established component in the signaling cascades leading to apoptosis (51). We hypothesized that HO-1 may have additional effects that could contribute to suppress EC apoptosis, such as by generating CO. Data from L.E. Otterbein, A.M.K. Choi, and colleagues have suggested that this may be the case in fibroblasts (52). However, data from other laboratories have suggested that in 293 cells CO is not antiapoptotic (34). Moreover, CO has been suggested to be proapoptotic in ECs (53). This data suggests that HO-1 can suppress EC apoptosis and that the antiapoptotic effect of HO-1 is mediated through the generation of CO (Figs. 4, 5, and 7).

The observation that the elimination of endogenous CO by Hb abrogates the cytoprotective effect of HO-1 (Figs. 4 and 5) supports the notion that, in the absence of CO, other biological functions engendered by HO-1, i.e., upregulation of ferritin expression and subsequent iron chelation, are not sufficient per se to prevent EC apoptosis (Figs. 4 and 7). Given the above, it is difficult to understand why iron chelation by DFO can protect ECs from apoptosis, even under conditions in which the action of CO is prevented (i.e., inhibition of HO-1 activity by SnPPIX or elimination of CO by Hb; Fig. 8). At least two possible interpretations may explain these observations: (a) DFO may sustain inflammation and promoting vascular thrombosis (25, 45). The mechanism by which apoptotic ECs promote vascular thrombosis is thought to involve the expression of procoagulant phospholipids by apoptotic bodies (49) and presumably the exposure of the procoagulant subendothelial matrix that is associated with EC apoptosis. In addition, apoptotic cells can activate the complement cascade directly through the binding of C1q to apoptotic bodies (50), and promote platelet adhesion (49) that will sustain inflammation and thrombosis as well.

The observation that HO-1 prevents apoptosis induced by different proapoptotic stimuli (23, 24; Fig. 1) suggests that HO-1 suppresses one or several signaling pathways that are common to a broad spectrum of proapoptotic stimuli. The antiapoptotic effect of HO-1 has recently been associated with increased cellular iron efflux through the upregulation of an iron pump that remains to be fully characterized (34). According to this study (34), HO-1 inhibits apoptosis by limiting the availability of prooxidant-free iron to participate in the generation of reactive oxygen species through the Fenton reaction, a well-established component in the signaling cascades leading to apoptosis (51). We hypothesized that HO-1 may have additional effects that could contribute to suppress EC apoptosis, such as by generating CO. Data from L.E. Otterbein, A.M.K. Choi, and colleagues have suggested that this may be the case in fibroblasts (52). However, data from other laboratories have suggested that in 293 cells CO is not antiapoptotic (34). Moreover, CO has been suggested to be proapoptotic in ECs (53). This data suggests that HO-1 can suppress EC apoptosis and that the antiapoptotic effect of HO-1 is mediated through the generation of CO (Figs. 4, 5, and 7).

The observation that the elimination of endogenous CO by Hb abrogates the cytoprotective effect of HO-1 (Figs. 4 and 5) supports the notion that, in the absence of CO, other biological functions engendered by HO-1, i.e., upregulation of ferritin expression and subsequent iron chelation, are not sufficient per se to prevent EC apoptosis (Figs. 4 and 7). Given the above, it is difficult to understand why iron chelation by DFO can protect ECs from apoptosis, even under conditions in which the action of CO is prevented (i.e., inhibition of HO-1 activity by SnPPIX or elimination of CO by Hb; Fig. 8). At least two possible interpretations may explain these observations: (a) DFO may...
MAPK phosphorylation was monitored by Western blot (0, 5, 15, 30, 60, and 120 min after TNF-α stimulation) using antibodies directed against the phosphorylated forms of each MAPK. (C) 2F-2B ECs were cotransfected with β-galactosidase, control, HO-1 (β-actin/HO-1), and where indicated with a phosphorylation-deficient p38/CSBP1 dominant negative mutant (DNM) expression vector. The values indicate the amount of vector used, in nanograms of DNA per 300 × 10^6 cells. Apoptosis was induced as in A. Gray bars represent ECs treated with Act.D and black bars represent ECs treated with TNF-α plus Act.D. The results shown are the mean ± SD from duplicate wells taken from one representative experiment out of three. (D) BAECs were transfected with a control (pDNA3) vector and stimulated with TNF-α in the presence (○) or absence (○) of the p38 kinase inhibitor SB203580 (20 μM).

have a higher ability to “eliminate” free iron compared with ferritin, and/or (b) DFO may have additional effects that contribute to prevent EC apoptosis, independently of its ability to eliminate free iron. In any case, these observations suggest that DFO does not, at least fully, mimic the effect of HO-1-mediated ferritin expression in preventing EC apoptosis.

Our data also suggest that CO, generated by cells that express HO-1, acts as an intercellular signaling molecule to prevent apoptosis of cells that do not express HO-1 (Fig. 6). If a similar effect of CO would occur in vivo, these data would suggest that vascular ECs at sites of inflammation might protect neighboring cells, such as infiltrating leukocytes that migrate into sites of inflammation or smooth muscle cells in blood vessels from undergoing apoptosis.

The antiapoptotic effect of HO-1 in ECs is not mediated by guanylylcleacylase and/or by the generation of cGMP (Fig. 10). This is in contrast to data showing that inhibition of guanylylcleacylase suppresses the antiapoptotic effect of HO-1 and/or CO in fibroblasts (51). Our interpretation is that the mechanism by which HO-1 and/or CO prevents apoptosis is cell type specific.

Based on the finding by Otterbein et al. that HO-1/CO activates p38 MAPK in Mφ (33), we tested whether HO-1 and/or CO would have similar effects in ECs. We found that this is the case (Fig. 12). However, contrary to Mφ, exogenous CO per se induced the activation of p38 MAPK in ECs, whereas HO-1 did not (Fig. 12). Possible explanations for the differences between HO-1 and CO in regulating p38 MAPK activation include the following. Whereas CO generated by HO-1 activates p38 MAPK, other end products of HO-1 activity may inhibit p38 activation. Alternatively, the level of CO, generated by HO-1, is significantly lower than exposure to exogenous CO as we used it. Whatever the explanation, these data show that CO can specifically modulate the activation of p38 MAPK.

We also found that the antiapoptotic effect of HO-1/CO acts via the activation of a transduction pathway involving the activation of p38 MAPK (Fig. 13). This is consistent with findings by others showing that activation of p38 MAPK is key in regulating apoptosis in a variety of cell types including the kidney epithelial cell line HeLa (54), cardiac muscle cells (55), and lymphoid Jurkat T cells (54, 56). The mechanism by which the activation of p38 MAPK modulates the induction of apoptosis is not well understood.

In conclusion, our findings suggest that CO, generated through heme catabolism by HO-1, acts as an antiapoptotic molecule that can suppress EC apoptosis. We show that the mechanism of action of CO involves the activation of p38 MAPK. These findings further support the notion that HO-1 acts as a protective gene and thereby contributes to prevent a series of inflammatory reactions that are associated with EC apoptosis.

The authors thank Dr. Neil R. Smith for critical reading of the manuscript.

This work was supported by a grant (Roche Organ Transplantation Research Foundation 998521355) awarded to M.P. Soares and a National Institutes of Health grant (HL58688) awarded to F.H. Bach. S. Brouard was supported by a grant from Association pour la Recherche sur le Cancer (ARC) and by a grant from Institut National de la Santé et de la Recherche Médicale (Inserm), France. F.H. Bach is the Lewis Thomas Professor at Harvard Medical School and is a paid consultant for Novartis Pharma. This work was supported in part by Novartis Pharma, Basel, Switzerland. This is paper no. 800 from our laboratories.
References


30. Shibahara, S., M. Yoshizawa, H. Suzuki, K. Takeda, K. Me-


