Carbon Monoxide Generated by Heme Oxygenase 1 Suppresses Endothelial Cell Apoptosis

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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)[1]
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 catabo-

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[1]*Abbreviations used in this paper:* Act.D, actinomycin D; BAEC, bovine
aortic endothelial cell; CoPPIX, cobalt protoporphyrin; DFO, deferoxamine mesylate; EC, endothelial cell; ERK, extracellular signal–regulated
kinase; FePP, iron protoporphyrin; GFP, green fluorescent protein; Hb,
hemoglobin; HO-1, heme oxygenase 1; JNK, c-Jun NH$_2$-terminal kinase; MAPK, mitogen-activated protein kinase; Mφ, monocyte/macrophage(s); ppm, parts per million; SnPPIX, tin protoporphyrin; VASP,
vasodilatator-stimulated phosphoprotein.

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 assessed 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
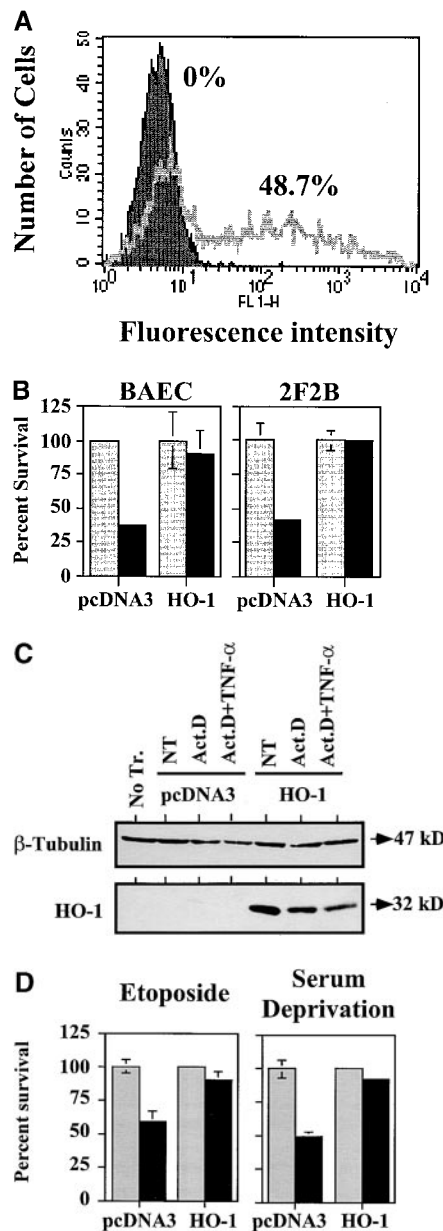

Figure 1. HO-1 suppresses EC apoptosis. (A) 2F-2B ECs were transfected with a GFP-expressing plasmid and monitored for GFP expression by flow cytometry. The percentage of transfected ECs was assessed by measuring fluorescence intensity in ECs transfected with control (pcDNA3; filled histogram) versus 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. Gray bars represent untreated ECs and black bars represent ECs treated with etoposide (200 μM, 8 h) or subjected to serum deprivation (0.1% FCS for 24 h). Results shown are the mean ± SD from duplicate wells taken from one representative experiment out of three independent experiments. Similar results were obtained using BAECs.
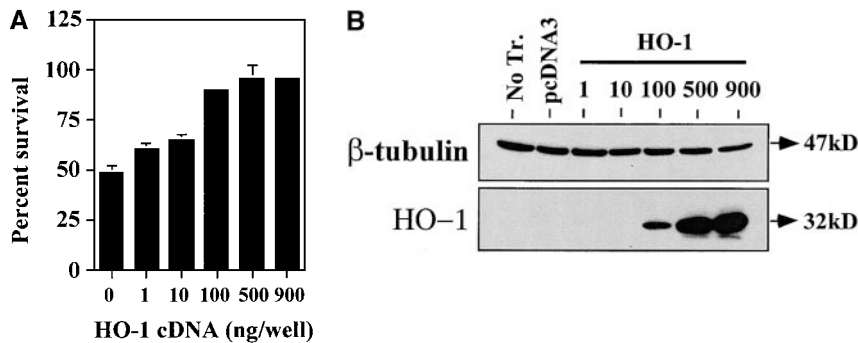
Figure 2. The antiapoptotic effect of HO-1 is dose dependent. (A) 2F-2B ECs were cotransfected with increasing doses of HO-1 expression vector (β-actin/HO-1). EC apoptosis was induced by TNF-α plus Act.D. Results shown are the mean ± SD from duplicate wells taken from one representative experiment out 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 3×10^5 cells). No Tr., nontransfected.

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 polyvinyldifluoridine membranes (Immobilon P; Millipore), as described elsewhere (28). HO-1 was detected using a rabbit anti–human HO-1 polyclonal antibody (StressGen Biotechnologies). Vasodilatator-stimulated phosphoprotein (VASP) was detected using a rabbit anti–human VASP polyclonal antibody (Calbiochem-Novabiochem). Total and activated/phosphorylated forms of extracellular signal–regulated kinases (ERK-1 and -2), c-Jun NH₂-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 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 (Amersham 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 used 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-Novabiochem) or by serum deprivation (0.1% FCS for 24 h). The iron chelator deferoxamine mesylate (DFO; Sigma-Aldrich) was dissolved extemporarily 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 Na₂S₂O₄, dialyzed against PBS (2 h, 4°C, 1:800 dilution), and added to the culture medium (1–100 μM) 6 h after transfection. The guanylcyclase inhibitor 1H(1,2,4)oxadiazolo(4,3-α)quinoxalin-1 (ODQ; Calbiochem–Novabiochem) was dissolved in DMSO (Sigma-Aldrich) and added to the culture medium (10–100 μM) 6 h after transfection. Iron protoporphyrin ([FePP]/
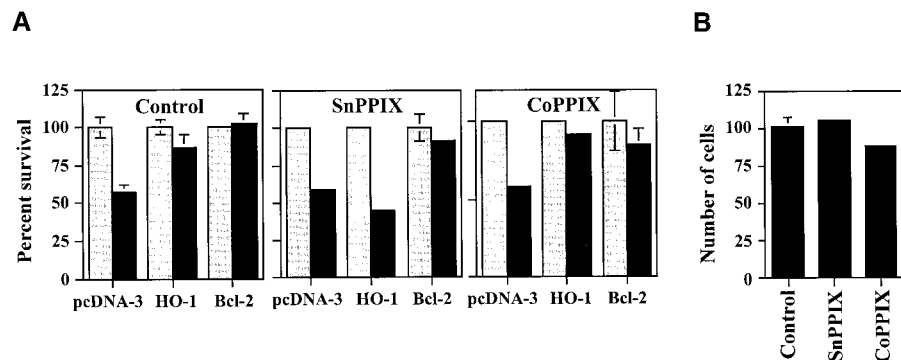

Figure 3. The antiapoptotic effect of HO-1 is dependent on HO enzymatic activity. (A) 2F-2B ECs were cotransfected with β-galactosidase plus pcDNA3, HO-1 (β-actin/HO-1), or bcl-2 expression vectors. Cells were either left untreated (Control) or treated with the inhibitor of HO enzymatic activity SnPPIX. 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.
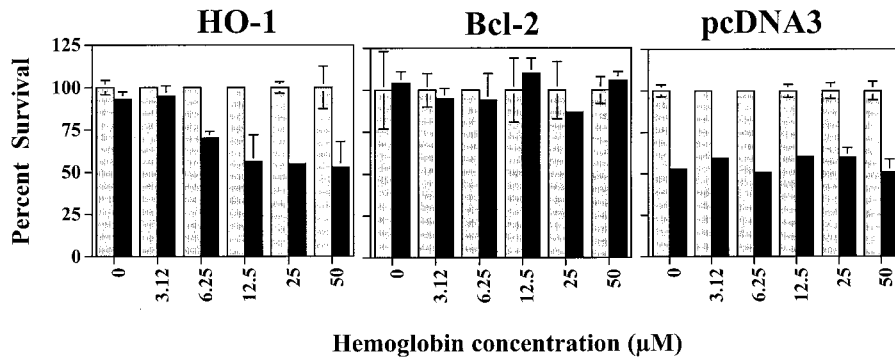
Figure 4. Scavenging of CO by Hb suppresses the antiapoptotic effect of HO-1. 2F-2B ECs were cotransfected with β-galactosidase plus control (pcDNA3), HO-1 (β-actin/HO-1), or bcl-2 expression vectors. ECs were either left untreated (0) or were treated with increasing concentrations of Hb. Gray bars represent 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.

heme), cobalt protoporphyrin (CoPPIX), and tin protoporphyrin (SnPPIX; all from Porphyrin Products, Inc.) were dissolved (10 mM) in 100 mM NaOH and conserved at −20°C until use. Metalloporphyrins were added to the culture medium (50 μM) 6 h after transfection. The cGMP analogue 8-bromo-cGMP sodium salt (8-Br-cGMP; Sigma-Aldrich) was dissolved in water and added to the culture medium (10–100 μM) 30 min before the induction of apoptosis. Human recombinant TNF-α (R&D Systems) was dissolved in PBS and 1% BSA, and added to the culture medium (10–100 ng/ml) 24 h after transfection. The p38 MAPK inhibitor pyridinyl imidazol SB203580 (32) was dissolved in DMSO and added to the culture medium (5–20 μM) 6 h after transfection.

CO Exposure. Cells were exposed to compressed air or varying concentration of CO (250 and 10,000 parts per million [ppm]), as described elsewhere (33).

Results

HO-1 Protects ECs from Apoptosis. TNF-α induces apoptosis of cultured ECs when transcriptional activity is inhibited by Act.D (28). We have used this experimental system to ask whether transient overexpression of HO-1 could prevent ECs from undergoing apoptosis. We first evaluated the ability of ECs to be transiently transfected. To do so, ECs were transfected with a GFP-expressing plasmid and the percentage of GFP-expressing ECs was evaluated by flow cytometry. As illustrated in Fig. 1, 45–50% of ECs expressed the GFP protein 24 h after transfection. We then tested whether transfection of HO-1 would prevent ECs from undergoing TNF-α–mediated apoptosis. To do so, ECs were cotransfected with HO-1 and β-galactosidase and apoptosis was evaluated by counting the number of viable β-galactosidase–expressing ECs. TNF-α plus Act.D induced apoptosis of control ECs transfected with the pcDNA3 (60–70% apoptotic ECs). Overexpression of HO-1 prevented EC apoptosis (5–10% apoptotic ECs; Fig. 1). The expression of HO-1 was confirmed by Western blot (Fig. 1). Overexpression of HO-1 also prevented EC apoptosis induced by other proapoptotic stimuli such as etoposide or serum deprivation, which is in keeping with similar observations in 293 cells (34). The antiapoptotic effect of HO-1 was dose dependent in that increasing levels of HO-1 expression resulted in increased protection from TNF-α plus Act.D–mediated apoptosis (Fig. 2). The maximal antiapoptotic effect of HO-1 (90–100% protection) was reached using 500–1,000 ng of the β-actin/HO-1 ex-
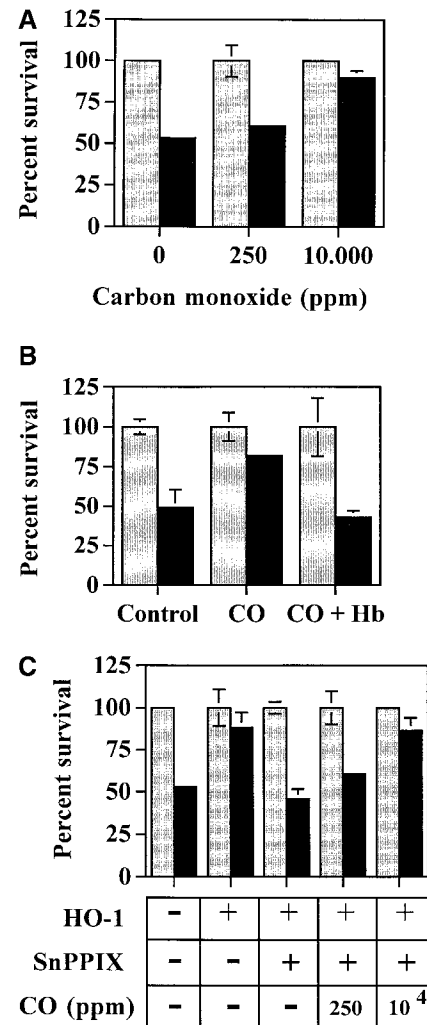

Figure 5. Exogenous CO suppresses EC apoptosis in the absence of HO-1. (A) 2F-2B ECs were transfected with a β-galactosidase expression vector and exposed to exogenous CO. Gray bars represent ECs treated with Act.D alone and black bars represent ECs treated with TNF-α plus Act.D. (B) 2F-2B ECs were transfected 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.
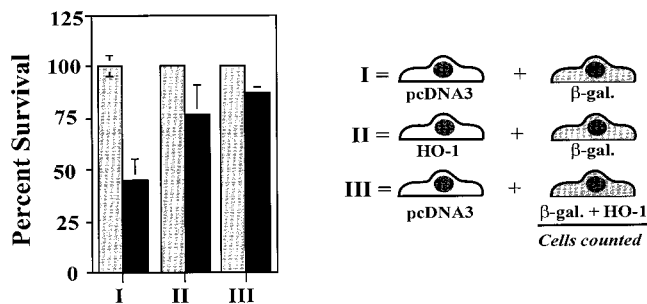
Figure 6. ECs that express HO-1 suppress apoptosis of ECs that do not express HO-1. 2F-2B ECs were transfected with control (pcDNA3; I and II) or HO-1 (III) expression vectors. 16 h after transfection, ECs were harvested, washed, and cocultured at a ratio of 1:1 with ECs transfected with β–galactosidase (I and II) or with β–galactosidase plus HO-1 (III). Cocultures were maintained for an additional 24 h before induction of apoptosis by TNF-α plus Act.D. The percentage of survival was evaluated by counting the number of β–galactosidase–positive cells that retained normal morphology. 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 duplicates taken from one representative experiment out of three.

pression vector per 3×10^5 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. Expo-
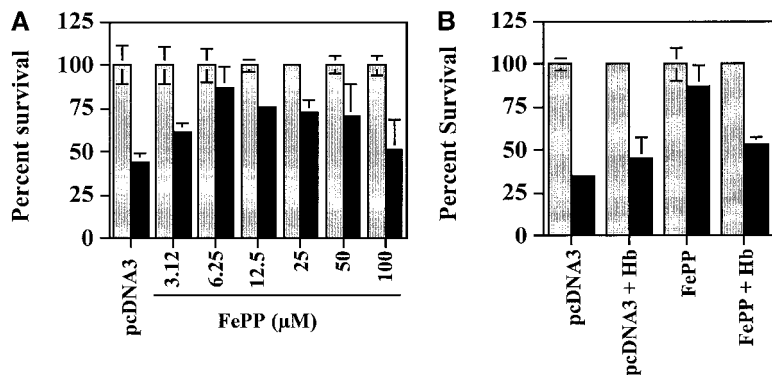
Figure 7. Upregulation of endogenous HO-1 expression inhibits EC apoptosis via CO. (A) 2F-2B ECs were cotransfected with a β–galactosidase expression vector and exposed to FePP. Apoptosis was induced by TNF-α and Act.D. Gray bars represent ECs treated with Act.D and black bars represent ECs treated with TNF-α plus Act.D. The results shown are the mean ± SD from duplicate wells taken from one representative experiment out of three. (B) 2F-2B ECs were cotransfected with a β–galactosidase expression vector and exposed to FePP (6.25 μM). Where indicated, ECs were treated with Hb (50 μM). The results shown are the mean ± SD from duplicate wells taken from one representative experiment out of three.

A

B

HO-1	−	+	+	+	+
SnPPIX	−	−	+	+	+
DFO (µM)	−	−	−	10	100

C

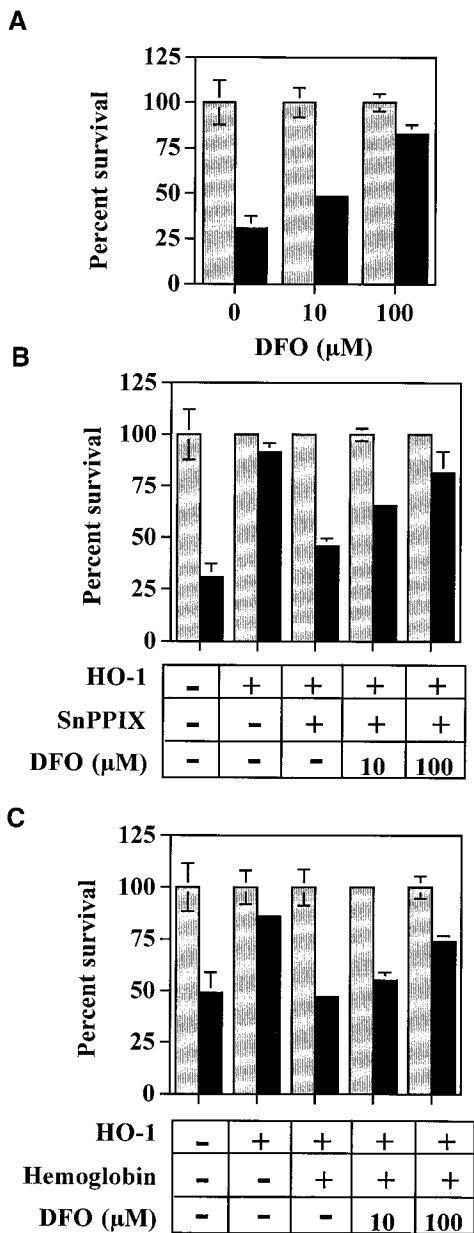| HO-1	−	+	+	+	+
Hemoglobin	−	−	+	+	+
DFO (µM)	−	−	−	10	100

Figure 8. Iron chelation by DFO suppresses EC apoptosis. (A) 2F-2B ECs were transfected with a β-galactosidase expression vector and exposed to DFO. 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 cotransfected as described above in A. Where indicated (+), HO-1 enzymatic activity was inhibited by SnPPIX and iron was chelated by DFO, as described above in A. 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 as described above in A. Where indicated (+), CO was removed from the culture medium by Hb and/or iron was chelated by DFO as described above in A and B. 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.

sure to heme protected ECs from TNF-α plus Act.D–mediated apoptosis (Fig. 7). This protective effect was observed only at heme concentrations ranging from 5 to 7 µM and was lost at higher concentrations, suggesting that
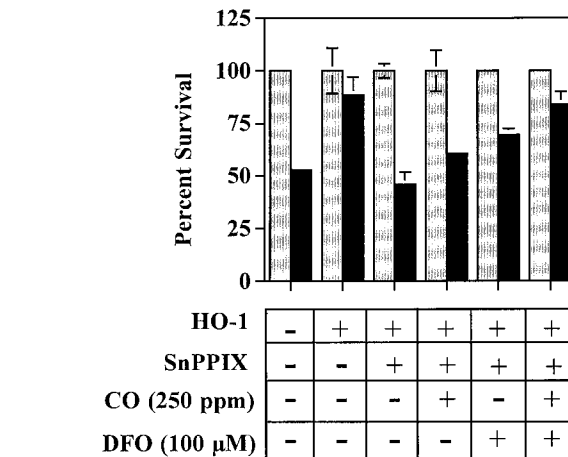

HO-1	−	+	+	+	+	+
SnPPIX	−	−	+	+	+	+
CO (250 ppm)	−	−	−	+	−	+
DFO (100 µM)	−	−	−	−	+	+

Figure 9. Iron chelation and CO have additive effects in suppressing EC apoptosis. 2F-2B ECs were cotransfected with β-galactosidase and HO-1 (β-actin/HO-1) expression vectors. Where indicated (+), cells were treated with the inhibitor of HO enzymatic activity SnPPIX. ECs were exposed to CO (250 ppm) and to the iron chelator DFO. 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.

heme becomes cytotoxic at concentrations higher than 10 µM (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-α–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-α 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 µM) 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 µM), inhibition of EC apoptosis was comparable to that achieved with the expression of HO-1.
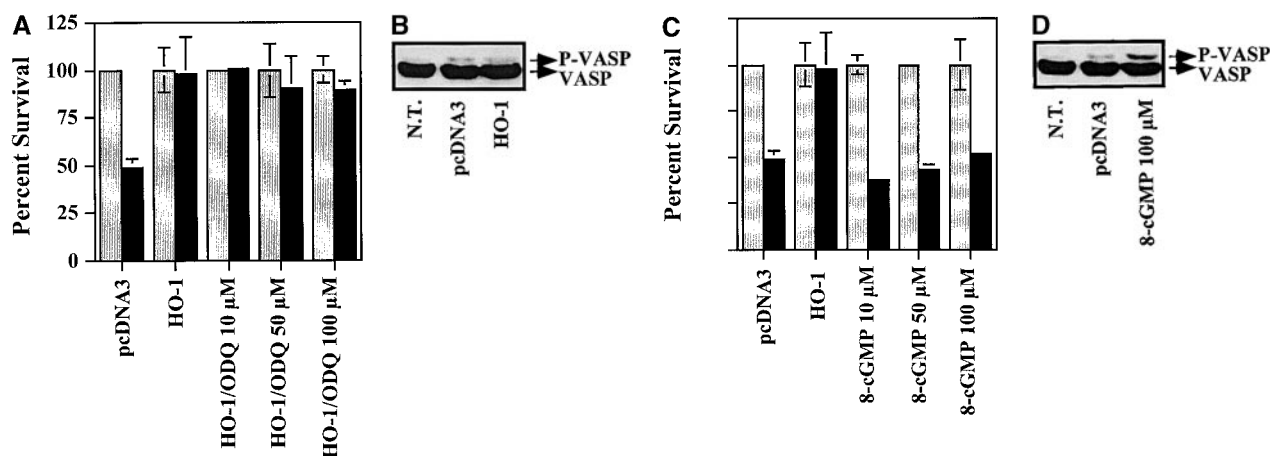
Figure 10. The antiapoptotic effect of HO-1 does not act via a cGMP–dependent pathway. (A) 2F-2B ECs were cotransfected with β-galactosidase and HO-1 (β-actin/HO-1) expression vectors. HO-1–transfected cells were exposed to increasing doses of the guanylcyclase inhibitor ODQ. Gray bars represent ECs treated with Act.D alone 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. (B) Activation of guanylcyclase was monitored in BAECs by analyzing the phosphorylation of VASP. P-VASP (50 kD) and VASP (46 kD) are the phosphorylated and nonphosphorylated forms of VASP, respectively. (C) 2F-2B ECs were with β-galactosidase or with β-galactosidase plus HO-1 (β-actin/HO-1) expression vectors as described above in A. Where indicated, ECs were exposed to the cGMP analogue 8-Br-cGMP, as described in Materials and Methods. 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) Phosphorylation of VASP was analyzed by Western blot as described above in B. N.T., nontreated.

The Antiapoptotic Effect of HO-1 Is Not Mediated by Guanylcyclase Activation or cGMP Generation. Most biological functions attributed to CO have been linked to its ability to bind guanylcyclase 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 guanylcyclase 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 guanylcyclase 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 guanylcyclase 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 adenovirus–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).
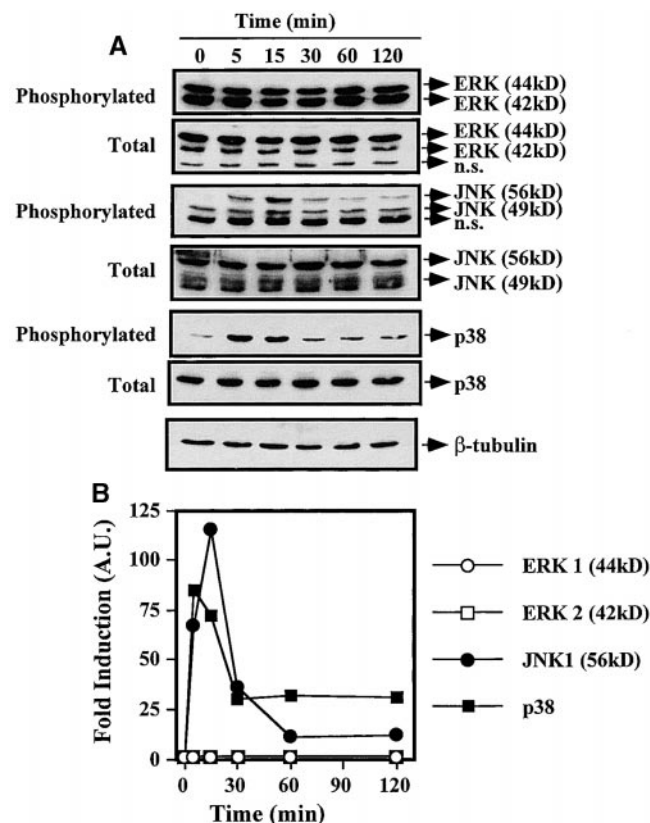
Figure 11. Activation of MAPK by TNF-α. (A) BAECs were stimulated with TNF-α (10 ng/ml; time 0) and MAPK phosphorylation was monitored by Western blot (0, 5, 15, 30, 60, and 120 min after TNF-α stimulation) using antibodies directed against the phosphorylated forms of each MAPK. One single membrane was used for all the stainings shown. Experiments were repeated three times with virtually identical results. n.s., nonspecific band. (B) Phosphorylation of different MAPKs was quantified. The results were presented as fold induction in arbitrary units (A.U.), compared with time 0, before TNF-α stimulation. The results in B correspond to the membranes shown in A.

Discussion

EC apoptosis is a prominent feature associated with acute and/or chronic inflammation such as it occurs during hyperoxia (44), endotoxic shock (25), arteriosclerosis (26), ischemia reperfusion injury (45, 46), and acute or chronic graft rejection (23, 47, 48). Presumably, EC apoptosis contributes to the development of these inflammatory reactions by

sustaining 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
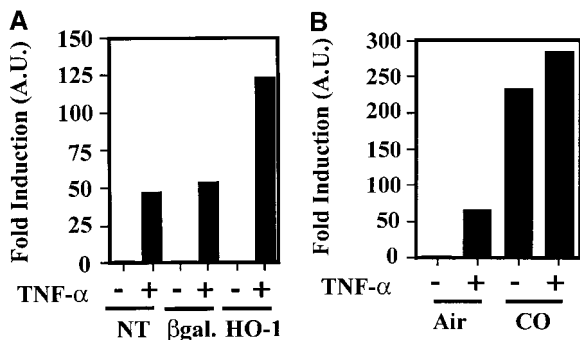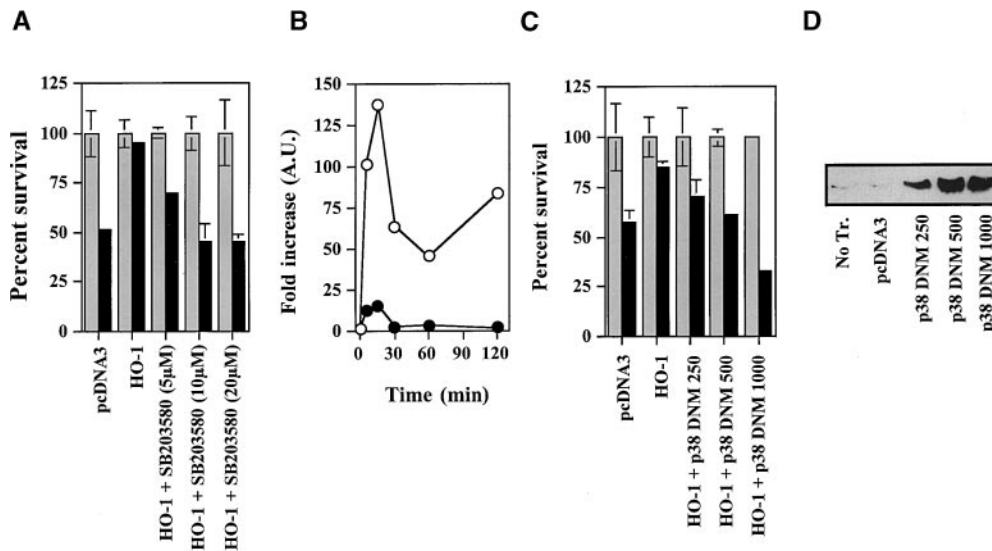
Figure 12. HO-1 and CO modulate p38 MAPK activation in ECs. (A) BAECs were either nontransduced (NT), transduced with a β-galactosidase (βgal.), or HO-1 recombinant adenovirus, and were left untreated (−) or treated (+) with TNF-α (10 ng/ml for 15 min). p38 MAPK phosphorylation was monitored by Western blot using antibodies directed against the phosphorylated forms of each MAPK. Results are presented as fold induction of MAPK activation by TNF-α in arbitrary units (A.U.), compared with time 0, before TNF-α stimulation. (B) BAECs were stimulated (+) or not (−) by TNF-α (10 ng/ml, 30 min) in the presence or absence of CO (10,000 ppm). Phosphorylation of p38 MAPK was quantified as in A. The results are presented as fold induction of MAPK activation by TNF-α in arbitrary units (A.U.).

Figure 13. The antiapoptotic effect of HO-1 acts via the activation of p38 MAPK. (A) 2F-2B ECs were cotransfected with β-galactosidase, control (pc-DNA3), or HO-1 (β-actin/HO-1) expression vectors. Where indicated, ECs were treated with the p38 kinase inhibitor SB203580. Gray bars represent ECs treated with Act.D alone 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. (B) BAECs were transfected with a control (pcDNA3) vector and stimulated with TNF-α in the presence (●) or absence (○) of the p38 kinase inhibitor SB203580 (20 μM). 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 × 10^3 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) The expression of the p38/CSBP1 dominant negative mutant was confirmed by Western blot using an anti-p38 specific antibody. No Tr., nontransfected ECs.

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 immigrate 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 guanylylcyclase and/or by the generation of cGMP (Fig. 10). This is in contrast to data showing that inhibition of guanylcyclase 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 difference 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 signifi-

cantly 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.

References

1. Cines, D.B., E.S. Pollak, C.A. Buck, J. Loscalzo, G.A. Zimmerman, R.P. McEver, J.S. Pober, T.M. Wick, B.A. Konkle, B.S. Schwartz, et al. 1998. Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood.* 91:3527–3561.

2. Hughes, C.C., C.O. Savage, and J.S. Pober. 1990. The endothelial cell as a regulator of T-cell function. *Immunol. Rev.* 117:85–102.

3. Mantovani, A., F. Bussolino, and M. Introna. 1997. Cytokine regulation of endothelial cell function: from molecular level to the bedside. *Immunol. Today.* 18:231–240.

4. Springer, T.A. 1990. Adhesion receptors of the immune system. *Nature.* 346:425–434.

5. Soares, M.P., C. Ferran, K. Sato, K. Takigami, J. Anrather, Y. Lin, and F.H. Bach. 2000. Protective responses of endothelial cells. *In* Genes and Resistance to Disease. World Health Organization and Foundation IPSEN. V. Boulyjenkov, K. Berg, and Y. Christen, editors. Springer-Verlag, Heidelberg. 91–104.

6. Bach, F.H., W.W. Hancock, and C. Ferran. 1997. Protective genes expressed in endothelial cells: a regulatory response to injury. *Immunol. Today.* 18:483–486.

7. Stroka, D.M., A.Z. Badrichani, F.H. Bach, and C. Ferran. 1999. Overexpression of A1, an NF-κB-inducible anti-apoptotic bcl gene, inhibits endothelial cell activation. *Blood.* 93:3803–3810.

8. Ferran, C., D.M. Stroka, A.Z. Badrichani, J.T. Cooper, C.J. Wrighton, M. Soares, S.T. Grey, and F.H. Bach. 1998. A20 inhibits NF-κB activation in endothelial cells without sensitizing to tumor necrosis factor-mediated apoptosis. *Blood.* 91:2249–2258.

9. Cooper, J.T., D.M. Stroka, C. Brostjan, A. Palmetshofer, F.H. Bach, and C. Ferran. 1996. A20 blocks endothelial cell activation through a NF-κB-dependent mechanism. *J. Biol. Chem.* 271:18068–18073.

10. Badrichani, A.Z., D.M. Stroka, G. Bilbao, D.T. Curiel, F.H. Bach, and C. Ferran. 1999. Bcl-2 and Bcl-XL serve an anti-inflammatory function in endothelial cells through inhibition of NF-κB. *J. Clin. Invest.* 103:543–553.

11. Maines, M.D. 1997. The heme oxygenase system: a regulator of second messenger gases. *Annu. Rev. Pharmacol. Toxicol.* 37:517–554.

12. Choi, A.M., and J. Alam. 1996. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am. J. Respir. Cell Mol. Biol.* 15:9–19.

13. Willis, D. 1999. Overview of HO-1 in inflammatory pathologies. *In* Inducible Enzymes in the Inflammatory Response. D.A. Willoughby and A. Tomlinson, editors. Birkhauser, Basel. 55–96.

14. Balla, G., H.S. Jacob, J. Balla, M. Rosenberg, K. Nath, F. Apple, J.W. Eaton, and G.M. Vercellotti. 1992. Ferritin: a cytoprotective antioxidant strategem of endothelium. *J. Biol. Chem.* 267:18148–18153.

15. Yang, L., S. Quan, and N.G. Abraham. 1999. Retrovirus-mediated HO gene transfer into endothelial cells protects against oxidant-induced injury. *Am. J. Physiol.* 277:L127–L133.

16. Abraham, N.G., Y. Lavrovsky, M.L. Schwartzman, R.A. Stoltz, R.D. Levere, M.E. Gerritsen, S. Shibahara, and A. Kappas. 1995. Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: protective effect against heme and hemoglobin toxicity. *Proc. Natl. Acad. Sci. USA.* 92:6798–6802.

17. Poss, K.D., and S. Tonegawa. 1997. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc. Natl. Acad. Sci. USA.* 94:10925–10930.

18. Otterbein, L., S.L. Sylvester, and A.M. Choi. 1995. Hemoglobin provides protection against lethal endotoxemia in rats: the role of heme oxygenase-1. *Am. J. Respir. Cell Mol. Biol.* 13:595–601.

19. Otterbein, L., B.Y. Chin, S.L. Otterbein, V.C. Lowe, H.E. Fessler, and A.M. Choi. 1997. Mechanism of hemoglobin-induced protection against endotoxemia in rats: a ferritin-independent pathway. *Am. J. Physiol.* 272:L268–L275.

20. Otterbein, L.E., J.K. Kolls, L.L. Mantell, J.L. Cook, J. Alam, and A.M. Choi. 1999. Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. *J. Clin. Invest.* 103:1047–1054.

21. Willis, D., A.R. Moore, R. Frederick, and D.A. Willoughby. 1996. Heme oxygenase: a novel target for the modulation of the inflammatory response. *Nat. Med.* 2:87–90.

22. Amersi, F., R. Buelow, H. Kato, B. Ke, A. Coito, X. Shen, D. Zhao, J. Zaky, J. Melinek, C. Lassman, et al. 1999. Up-regulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. *J. Clin. Invest.* 104:1631–1639.

23. Soares, M.P., Y. Lin, J. Anrather, E. Csizmadia, K. Takigami, K. Sato, S.T. Grey, R.B. Colvin, A.M. Choi, K.D. Poss, and F.H. Bach. 1998. Expression of heme oxygenase-1 (HO-1) can determine cardiac xenograft survival. *Nat. Med.* 4:1073–1077.

24. Hancock, W.W., R. Buelow, M.H. Sayegh, and L.A. Turka. 1998. Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. *Nat. Med.* 4:1392–1396.

25. Haimovitz, F.A., C. Cordon-Cardo, S. Bayoumy, M. Garzotto, M. McLoughlin, M. Gallily, C. Edwards III, E.H. Schuchman, Z. Fuks, and R. Kolesnick. 1997. Lipopolysaccharide induces disseminated endothelial cell apoptosis requiring ceramide. *J. Exp. Med.* 186:1831–1841.

26. Dimmeler, S., C. Hermann, and A.M. Zeiher. 1998. Apoptosis of endothelial cells. Contribution to the pathophysiology of atherosclerosis? *Eur. Cytokine Netw.* 9:697–698.

27. Anrather, J., V. Csizmadia, C. Brostjan, M.P. Soares, F.H. Bach, and H. Winkler. 1997. Inhibition of bovine endothelial cell activation in vitro by regulated expression of a transdominant inhibitor of NF-κB. *J. Clin. Invest.* 99:763–772.

28. Soares, M.P., A. Muniappan, E. Kaczmarek, K. Koziak, C.J. Wrighton, F. Steinhauslin, C. Ferran, H. Winkler, F.H. Bach, and J. Anrather. 1998. Adenovirus mediated expression of a dominant negative mutant of p65/RelA inhibits proinflammatory gene expression in endothelial cells without sensitizing to apoptosis. *J. Immunol.* 161:4572–4582.

29. Lee, P.J., J. Alam, G.W. Wiegand, and A.M. Choi. 1996. Overexpression of heme oxygenase-1 in human pulmonary epithelial cells results in cell growth arrest and increased resistance to hyperoxia. *Proc. Natl. Acad. Sci. USA.* 93:10393–10398.

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

guro, and K. Endo. 1993. Functional analysis of cDNAs for two types of human heme oxygenase and evidence for their separate regulation. *J. Biol. Chem.* 113:214–218.

31. Otterbein, L.E., P.J. Lee, B.Y. Chin, I. Petrache, S.L. Camhi, J. Alam, and A.M. Choi. 1999. Protective effects of heme oxygenase-1 in acute lung injury. *Chest.* 116:61S–63S.

32. Lee, J.C., J.T. Laydon, P.C. McDonnell, T.F. Gallagher, S. Kumar, D. Green, D. McNulty, M.J. Blumenthal, J.R. Heys, S.W. Landvatter, et al. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature.* 372:739–746.

33. Otterbein, L.E., F.H. Bach, J. Alam, M.P. Soares, H.L. Tao, M. Wysk, R. Davis, R. Flavell, and A.M.K. Choi. 2000. Carbon monoxide mediates anti-inflammatory effects via the mitogen activated protein kinase pathway. *Nat. Med.* 6:422–428.

34. Ferris, C., S. Jaffrey, A. Sawa, M. Takahashi, S. Brady, R. Barrow, S. Tysoc, H. Wolosker, D. Baranano, S. Dore, et al. 1999. Haem oxygenase-1 prevents cell death by regulating cellular iron. *Nat. Cell Biol.* 1:152–157.

35. Balla, J., H.S. Jacob, G. Balla, K. Nath, J.W. Eaton, and G.M. Vercellotti. 1993. Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. *Proc. Natl. Acad. Sci. USA.* 90:9285–9289.

36. Morita, T., M.A. Perrella, M.E. Lee, and S. Kourembanas. 1995. Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. *Proc. Natl. Acad. Sci. USA.* 92:1475–1479.

37. Verma, A., D.J. Hirsch, C.E. Glatt, G.V. Ronnett, and S.H. Snyder. 1993. Carbon monoxide: a putative neural messenger. *Science.* 259:381–384.

38. Kim, Y.M., R.V. Talanian, and T.R. Billiar. 1997. Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* 272:31138–31148.

39. Chiche, J.D., S.M. Schlutsmeyer, D.B. Bloch, S.M. de la Monte, J.D. Roberts, Jr., G. Filippov, S.P. Janssens, A. Rosenzweig, and K.D. Bloch. 1998. Adenovirus-mediated gene transfer of cGMP-dependent protein kinase increases the sensitivity of cultured vascular smooth muscle cells to the antiproliferative and pro-apoptotic effects of nitric oxide/cGMP. *J. Biol. Chem.* 273:34263–34271.

40. Suttner, D.M., and P.A. Dennery. 1999. Reversal of HO-1 related cytoprotection with increased expression is due to reactive iron. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 13:1800–1809.

41. Roulston, A., C. Reinhard, P. Amiri, and L.T. Williams. 1998. Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor alpha. *J. Biol. Chem.* 273:10232–10239.

42. Kyriakis, J.M., and J. Avruch. 1996. Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.* 271:24313–24316.

43. Wang, Y., S. Huang, V.P. Sah, J. Ross, Jr., J.H. Brown, J. Han, and K.R. Chien. 1998. Cardiac muscle cell hypertro-

phy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J. Biol. Chem.* 273:2161–2168.

44. Otterbein, L.E., L.L. Mantell, and A.M. Choi. 1999. Carbon monoxide provides protection against hyperoxic lung injury. *Am. J. Physiol.* 276:L688–L694.

45. Cursio, R., J. Gugenheim, J.E. Ricci, D. Crenesse, P. Rostagno, L. Maulon, M.C. Saint-Paul, B. Ferrua, and A.P. Auberger. 1999. A caspase inhibitor fully protects rats against lethal normothermic liver ischemia by inhibition of liver apoptosis. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 13:253–261.

46. Yaoita, H., K. Ogawa, K. Maehara, and Y. Maruyama. 1998. Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. *Circulation.* 97:276–281.

47. Bach, F.H., C. Ferran, M. Soares, C.J. Wrighton, J. Anrather, H. Winkler, S.C. Robson, and W.W. Hancock. 1997. Modification of vascular responses in xenotransplantation: inflammation and apoptosis. *Nat. Med.* 3:944–948.

48. Brouard, S., M.C. Cuturi, P. Pignon, R. Buelow, P. Loth, A. Moreau, and J.P. Soulillou. 1999. Prolongation of heart xenograft survival in a hamster-to-rat model after therapy with a rationally designed immunosuppressive peptide. *Transplantation.* 67:1614–1618.

49. Bombeli, T., B.R. Schwartz, and J.M. Harlan. 1999. Endothelial cells undergoing apoptosis become proadhesive for nonactivated platelets. *Blood.* 93:3831–3838.

50. Korb, L.C., and J.M. Ahearn. 1997. C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. *J. Immunol.* 158:4525–4528.

51. Kroemer, G., P. Petit, N. Zamzami, J.L. Vayssiere, and B. Mignotte. 1995. The biochemistry of programmed cell death. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 9:1277–1287.

52. Petrache, I., L.E. Otterbein, J. Alam, G.W. Wiegand, and A.M. Choi. 2000. Heme oxygenase-1 inhibits TNF-α-induced apoptosis in cultured fibroblast. *Am. J. Physiol. Lung Cell Mol. Physiol.* 278:312–319.

53. Thom, S.R., D. Fisher, Y.A. Xu, K. Notarfrancesco, and H. Ishiropoulos. 2000. Adaptive responses and apoptosis in endothelial cells exposed to carbon monoxide. *Proc. Natl. Acad. Sci. USA.* 97:1305–1310.

54. Nemoto, S., J. Xiang, S. Huang, and A. Lin. 1998. Induction of apoptosis by SB202190 through inhibition of p38beta mitogen-activated protein kinase. *J. Biol. Chem.* 273:16415–16420.

55. Wang, Y., B. Su, V.P. Sah, J.H. Brown, J. Han, and K.R. Chien. 1998. Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific activator for c-Jun NH$_2$-terminal kinase in ventricular muscle cells. *J. Biol. Chem.* 273:5423–5426.

56. Huang, S., Y. Jiang, Z. Li, E. Nishida, P. Mathias, S. Lin, R.J. Ulevitch, G.R. Nemerow, and J. Han. 1997. Apoptosis signaling pathway in T cells is composed of ICE/Ced-3 family proteases and MAP kinase kinase 6b. *Immunity.* 6:739–749.