Adenovirus-Mediated Heme Oxygenase-1 Gene Expression Stimulates Apoptosis in Vascular Smooth Muscle Cells
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Background—Heme oxygenase-1 (HO-1) catalyzes the degradation of heme into biliverdin, iron, and carbon monoxide (CO). Although HO-1 is induced in vascular smooth muscle cells (SMCs), the biological role of HO-1 in these cells has not been completely characterized.

Methods and Results—In the present study, we overexpressed HO-1 in rat aortic SMCs by generating a recombinant defective adenovirus containing the rat HO-1 gene (AdHO-1) and examined the effect on SMC proliferation. Infection of SMCs with AdHO-1 resulted in a dose-dependent increase in the expression of HO-1 mRNA, protein, and activity. Infection of SMCs with AdHO-1 inhibited serum-stimulated SMC proliferation in a dose-dependent manner. In contrast, the control adenovirus expressing the green fluorescent protein failed to induce HO-1 expression and had minimal effects on SMC growth. Infection with AdHO-1 stimulated SMC apoptosis in a dose-dependent fashion, as demonstrated by DNA fragmentation, positive annexin V labeling, and caspase-3 activation. HO-1–mediated apoptosis was associated with a marked increase in the expression of the proapoptotic protein p53. Finally, the exogenous administration of biliverdin and bilirubin stimulated SMC apoptosis. In contrast, the administration of CO or iron failed to induce cell death.

Conclusions—These results demonstrate that overexpression of HO-1 or the exogenous administration of biliverdin or bilirubin stimulates SMC apoptosis. Adenovirus-mediated transfer of the HO-1 gene may provide a novel therapeutic approach in treating occlusive vascular disease. (Circulation. 2002;105:79-84.)

Key Words: oxygenase ■ apoptosis ■ muscle, smooth ■ biliverdin ■ bilirubin

Heme oxygenase (HO) is the rate-limiting enzyme that catalyzes the degradation of heme into equimolar amounts of biliverdin, iron, and carbon monoxide (CO).1 To date, 3 different isoforms of HO (HO-1, HO-2, and HO-3) have been identified.2-3 Both HO-2 and HO-3 are constitutively expressed, whereas HO-1 is inducible.1-3 Recent studies indicate that HO-1 may play a significant role in the circulation. Administration of inducers of HO-1 causes a marked decrease in blood pressure in hypertensive rats, whereas HO-1 inhibitors increase blood pressure and peripheral resistance, suggesting that HO-1 serves a tonic vasodepressor function.4,5 HO-1 also modulates platelet–vessel wall interactions. Using a platelet–vascular smooth muscle cell (SMC) coculture system, we found that the induction of HO-1 in vascular SMCs inhibits the aggregation of platelets, indicating a potentially important antithrombotic role for this enzyme.6 These HO-1–mediated effects on vascular tone and platelet function appear to be mediated via the release of CO and the subsequent activation of soluble guanylate cyclase.4-7 HO-1 also modulates the oxidative state of the vessel wall by converting the prooxidant heme to biliverdin that is subsequently metabolized to the potent antioxidant bilirubin by biliverdin reductase.8

Although recent studies suggest a crucial role for HO-1 in the circulation, definitive conclusions cannot be drawn, because the inducers used to stimulate HO-1 gene expression have numerous other actions. Furthermore, the metalloprotoporphyrins, which are used to block HO-1 activity, are not specific for HO.9-11 These HO inhibitors are also photosensitive and can undergo nonenzymatic degradation to release CO.12 Moreover, a recent study demonstrated that metalloporphyrins can actually induce the expression of HO-1,13 further complicating the interpretation of results when these compounds are used. To further determine the role of HO-1 in the vessel wall, we infected vascular SMCs with an adenoviral vector containing the gene for HO-1. We now report that overexpression of HO-1 stimulates vascular SMC apoptosis and that this is associated with a marked increase in the cellular level of the proapoptotic protein p53. In addition, we demonstrate that exogenous administration of the HO-1 products biliverdin and bilirubin increases the rate of apoptosis in vascular SMCs.
Methods

Materials

FCS, minimum essential media, elastase, collagenase, SDS, β-mercaptoethanol, acrylamide, trypsin, EDTA, sucrose, urea, bilirubin, biliverdin, benzene, Tween 20, and barium chloride were purchased from Sigma Chemical Co; the adenoviral vectors pAdTrack-CMV and pADEasy-1 were kindly provided by Dr Bert Vogelstein (Johns Hopkins University); GAPDH cDNA was from Ambion Inc; a monoclonal p53 antibody was from Oncogene; caspase-3 activity kit (Invitrogen), and the termination codons. The PCR product was ligated into pPCRII/H11032 to amplify HO-1 cDNA from reverse-transcribed rat aortic SMC mRNA. Oligonucleotide primers (sense, 5′-CAGTGCCTCCAGAGTTCC-3′; antisense, 5′-GAGAGCCAGGCAAGATTCCTC-3′) were designed spanning the translation initiation and termination codons. The PCR product was ligated into pCPPRII (Invitrogen), and the HindIII/XbaI restriction fragment of HO-1 was ligated into the HindIII/XbaI sites of the shuttle vector pAdTrack-CMV. The resultant plasmid was linearized with PmeI and cotransformed with the adenoviral backbone vector pAdEasy-1 into Escherichia coli BJ5183 cells by electroporation. Homologous recombinants containing HO-1 cDNA were detected by restriction endonuclease digestion and agarose gel electrophoresis. Recombinant HO-1 adenovirus (AdHO-1) was then transformed into mammalian 293 cells, and the recombinants containing HO-1 cDNA were detected by restriction endonuclease digestion and agarose gel electrophoresis. Recombinant HO-1 adenovirus (AdHO-1) was then transformed into E coli DH10B cells for large-scale amplification. The PacI-digested pAdHO-1 was then transfected into mammalian 293 cells, and the AdHO-1 adenovirus was expanded, purified, and titrated. The recombinant adenovirus encoding green fluorescent protein (AdGFP) was used as a control. This adenovirus was similar to AdHO-1 but lacked the HO-1 cDNA.

Cell Culture

Vascular SMCs were isolated by elastase and collagenase digestion of rat thoracic aorta, and SMCs were serially cultured in minimum essential medium. Bovine aortic endothelial cells (BAECs) were purchased from VEC Technologies Inc and grown in Dulbecco’s modified Eagle’s medium.

Construction of a Recombinant HO-1 Adenovirus and Gene Transfer

A polymerase chain reaction (PCR) was performed to clone and amplify HO-1 cDNA from reverse-transcribed rat aortic SMC mRNA. Recombinant adenovirus (AdHO-1) adenovirus was expanded, purified, and titrated. The recombinant adenovirus encoding green fluorescent protein (AdGFP) was used as a control. This adenovirus was similar to AdHO-1 but lacked the HO-1 cDNA.

SMC Proliferation

SMCs were seeded at a density of 5×10⁴ cells per well in 12-well plates. After infection with adenovirus, SMCs were incubated in culture media containing 0%, 2%, or 5% serum for 2 days. SMCs were then harvested with trypsin (0.025%/EDTA (1 mmol/L) and counted in a Coulter Counter (model Z onset, Coulter Electronics).

Apoptosis

Apoptosis was monitored by measuring DNA fragmentation on 2% agarose gels, the movement of phosphatidylserine to the extracellular surface by annexin V binding, and by caspase-3 activation, as previously described.

HO Activity

HO activity was quantified spectrophotometrically by measuring the release of bilirubin into the culture media. Culture media were mixed in tubes containing barium chloride and benzene. The benzene phase containing the extracted bilirubin was separated from the aqueous phase by centrifugation at 13 000g for 30 minutes. Bilirubin was determined spectrophotometrically as a difference in absorbance between 450 and 600 nm with an extinction coefficient of 27.3 (mmol/L)/cm.

Protein Analysis

SMCs were lysed in electrophoresis buffer (125 mmol/L Tris-HCl [pH 6.8], 12.5% glycerol, and 2% SDS), boiled, and sonicated. Proteins (20 μg) were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and blocked for 1 hour in PBS containing Tween 20 (0.1%) and nonfat milk (5%). Blots were incubated with the HO-1 (1:500 dilution) or p53 (10 μg/mL) antibody for 1 hour. Membranes were then incubated for 1 hour with horseradish peroxidase–conjugated goat anti-rabbit (1:7500 dilution) or goat anti-mouse (1:5000) antibody. Blots were developed by the ECL method (Amersham), and relative protein levels were quantified by scanning densitometry (LKB Ultrascan XL laser densitometer).

mRNA Analysis

HO-1 mRNA levels were determined by ribonuclease protection analysis (Ambion Inc). Total RNA (10 μg) was hybridized with ~1×10⁶ cpm of [32 P]UTP-labeled antisense HO-1 and GAPDH (316-bp) riboprobes. The HO-1 (284-bp) antisense RNA probe was prepared as described earlier. Protected RNA was analyzed by electrophoresis using 6% acrylamide/8 mmol/L urea gels.

CO Exposure

SMCs were exposed to CO via a previously described exposure chamber. Stock gas tanks containing 1% CO and 5% CO₂ in air (Matheson Tri-Gas Inc) were mixed in a stainless steel mixing cylinder before delivery into a 13.5-L exposure chamber. Flow into the humidified chamber was at 1 L/min, and the temperature was maintained at 37°C. A CO analyzer (Interscan) was used to continuously measure CO levels in the chamber by electrochemical detection.

Statistics

Results are expressed as the mean±SEM. Statistical analysis was performed with Student’s 2-tailed t test and ANOVA when >2 treatment regimens were compared. Probability values of P<0.05 were considered statistically significant.

Results

Treatment of vascular SMCs with AdHO-1 stimulated a dose-dependent increase in HO-1 mRNA expression, whereas the control adenovirus (AdGFP) failed to induce the expression of HO-1 (Figure 1A). The HO-1 adenovirus also stimulated a marked increase in the number of SMCs (Figure 1B). Increased HO-1 expression after exposure to AdHO-1 was accompanied by a significant increase in HO-1 activity, as reflected by the HO-1 activity (Figure 2). In contrast, infection of SMCs with AdGFP did not stimulate HO-1 protein expression or the synthesis of bilirubin (data not shown).

Incubation of vascular SMCs with serum (2% or 5%) resulted in a marked increase in the number of SMCs (Figure 3). Infection of SMCs with AdHO-1, however, inhibited the proliferative response to serum in a concentration-dependent manner (Figure 3). Interestingly, the high dose (50 MOI) of AdGFP resulted in a small but significant decrease in cell number (Figure 3). The loss in cell number, however, was significantly less than observed with 50 MOI of AdHO-1 (Figure 3).

Infection of vascular SMCs with AdHO-1 stimulated DNA fragmentation in a dose-dependent manner (Figure 4A). Faint DNA laddering was also detected with a high dose (50 MOI)
of AdGFP; DNA fragmentation was absent, however, at lower doses of AdGFP and in control cells (Figure 4A). Consistent with an earlier study, a cocktail of cytokines (interleukin-1 \( \beta \) 10 ng/mL, tumor necrosis factor- \( \alpha \) 40 ng/mL, and interferon- \( \gamma \) 400 U/mL) induced substantial DNA laddering (Figure 4A). Infection of SMCs with AdHO-1 also resulted in a dose-dependent increase in annexin V binding (Figure 4B). Fluorescence microscopy (Olympus 1X70, Leeds Instrument Inc) using the Annexin V-Cy3.18 fluorescent labeling kit (Sigma) revealed that annexin V binding was observed only in SMCs overexpressing HO-1 (Figure 4C).

Infection of SMCs with AdHO-1 also resulted in a significant increase in caspase-3 activity (Figure 5A). In contrast, infection of BAECs with AdHO-1 failed to stimulate caspase-3 activity (data not shown). Moreover, AdHO-1 (20 MOI) inhibited BAEC caspase-3 activity after serum deprivation (Figure 5B). AdGFP (20 MOI) had no effect on BAEC caspase-3 activity (Figure 5B).

The induction of SMC apoptosis by AdHO-1 was not blocked by treatment of SMCs with the CO scavenger hemoglobin (50 \( \mu \)mol/L) or with the iron chelator desferrioxamine (100 \( \mu \)mol/L) (Figure 6A). In addition, exposure of vascular SMCs to CO (100 ppm) or ferrous iron (50 \( \mu \)mol/L) failed to stimulate DNA fragmentation (Figure 6B) or annexin V binding (data not shown). In contrast, the administration of biliverdin induced DNA fragmentation (Figure 7A) and annexin V labeling (Figure 7B) in a concentration-dependent fashion. Similarly, treatment of SMCs with bilirubin stimulated a concentration-dependent increase in DNA laddering (Figure 7A).

Finally, infection of SMCs with pAdHO-1 (50 MOI) for 48 hours significantly stimulated the expression of p53 protein by 3-fold (Figure 8). In contrast, AdGFP (50 MOI) failed to elevate the levels of p53 (Figure 8).

**Discussion**

The present study demonstrates that overexpression of HO-1 stimulates apoptosis in vascular SMCs. HO-1–mediated apoptosis is associated with an increase in the expression of the proapoptotic protein p53. In addition, the exogenous administration of biliverdin or bilirubin stimulates apoptosis, whereas the other HO-1 products, CO and iron, fail to induce apoptosis.

Using adenovirus-mediated gene transfer, we were able to selectively express high levels of functional HO-1 in vascular SMCs. Infection of vascular SMCs with the recombinant HO-1 adenovirus resulted in a dose-dependent increase in HO-1 mRNA, protein, and activity. In contrast, the control adenovirus, which lacked the HO-1 gene, failed to stimulate HO-1 expression and activity.

The overexpression of HO-1 in vascular SMCs significantly attenuates their proliferative response to serum. The decrease in SMC proliferation by HO-1–overexpressing cells...
is associated with a significant increase in the rate of SMC apoptosis, as demonstrated by DNA laddering, positive annexin V staining, and caspase-3 activation. These results demonstrate that HO-1 blocks the proliferation of vascular SMCs by stimulating programmed cell death. Interestingly, we found that overexpression of HO-1 in endothelial cells blocks the activation of caspase-3 after serum deprivation, indicating that HO-1 protects endothelial cells against apoptosis. This latter finding is consistent with recent reports demonstrating that HO-1 inhibits apoptosis in fibroblasts, renal tubular cells, and endothelial cells. Thus, HO-1 may regulate apoptosis in a cell-specific manner.

Interestingly, infection of vascular SMCs with the control empty adenovirus containing GFP (AdGFP) modestly inhibited SMC growth. This is consistent with earlier studies demonstrating that adenoviral proteins and GFP can induce apoptosis. The decrease in cell number observed with the control adenovirus, however, is much less than that observed with the HO-1 adenovirus (AdHO-1), indicating the contribution of the HO-1 gene to the apoptotic response.

The mechanism by which HO-1 stimulates apoptosis in vascular SMCs is not entirely clear. It does not involve the HO-1–mediated release of iron, because the iron chelator desferrioxamine fails to abrogate the apoptotic effect. Moreover, the addition of iron does not induce apoptosis in these cells. Although CO has recently been reported to induce apoptosis in endothelial cells, it does not appear that CO mediates the HO-1 effect, because the CO scavenger hemoglobin does not protect SMCs against apoptosis. In addition, the exogenous administration of CO (100 ppm) at a concentration that stimulates apoptosis in endothelium fails to induce apoptosis in SMCs. We found, however, that the exogenous administration of biliverdin or bilirubin stimulates SMC apoptosis. We further observed that the induction of apoptosis by HO-1 is associated with a significant increase in

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**Figure 4.** Effect of AdHO-1 infection on vascular SMC apoptosis. A, DNA laddering in SMCs infected with AdHO-1 (5 to 50 MOI), AdGFP (5 to 50 MOI), or a cytokine mixture (CM) (interleukin-1β 10 ng/mL, tumor necrosis factor-α 40 ng/mL, and interferon-γ 400 U/mL) and incubated with 2% serum for 48 hours. Data are representative of 3 separate experiments. B, Annexin V binding in SMCs infected with AdHO-1 (5 to 50 MOI) and incubated with 2% serum for 48 hours. Results are mean±SEM of 4 experiments. *Statistically significant effect of AdHO-1. C, Fluorescence microscopy demonstrating that only HO-1–overexpressing SMCs (stained green) undergo apoptosis (red membrane staining).

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**Figure 5.** Effect of AdHO-1 infection on vascular SMCs or endothelial cell caspase-3 activity. A, SMCs were infected with AdHO-1 (5 to 50 MOI) and then incubated with 2% serum for 48 hours. B, BAECs were infected with AdHO-1 (20 MOI) and then incubated with serum-free media (SF) for 48 hours. Results are mean±SEM of 4 experiments. *Statistically significant effect of AdHO-1.

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**Figure 6.** Effect of iron and CO on vascular SMC apoptosis. A, DNA laddering in SMCs infected with AdHO-1 (50 MOI) or AdGFP (50 MOI) in presence or absence of desferrioxamine (Des; 100 μmol/L) or hemoglobin (Hb; 50 μmol/L) and incubated with 2% serum for 48 hours. Data are representative of 3 separate experiments. B, DNA laddering in SMCs treated with CO (100 ppm) or iron (50 μmol/L) and incubated with 2% serum for 48 hours.

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**Figure 7.** Effect of biliverdin and bilirubin on SMC apoptosis. A, DNA laddering in SMCs treated with biliverdin (5 to 200 μmol/L) or bilirubin (5 to 50 μmol/L) in 2% serum for 48 hours. Data are representative of 3 separate experiments. B, Annexin V binding in SMCs treated with biliverdin (5 to 200 μmol/L) in 2% serum for 48 hours. Results are mean±SEM of 4 experiments. *Statistically significant effect of biliverdin.
mediated apoptosis is associated with a molecular mechanism by which antioxidants stimulate SMC apoptosis. HO-1 activity by metalloprotoporphyrins potentiates intimal neointima after balloon injury.30

Induction of HO-1 by antioxidants stimulates apoptosis via a p53-dependent pathway. The notion that reactive oxygen species may play a critical role in the concentration of bilirubin in the culture medium, suggesting that the HO-1–catalyzed formation of biliverdin and/or its subsequent metabolism to bilirubin may mediate the apoptotic response. Although the concentration of bilirubin in the media is less than that required to induce SMC apoptosis, the intracellular levels of bilirubin may be significantly higher. Alternatively, a lower intracellular concentration of bilirubin may be sufficient to induce apoptosis. Interestingly, apoptosis was restricted to the HO-1–infected cells. The lack of a bystander effect may be explained by the insufficient release of biliverdin or bilirubin from the HO-1 infected cells.

Our finding that the antioxidant protein HO-1 induces apoptosis complements recent studies demonstrating that antioxidants stimulate programmed cell death in vascular SMCs. Treatment of vascular SMCs with the antioxidants N-acetyl-L-cysteine or pyrrolidinedithiocarbamate enhances apoptosis in a dose-dependent manner.25 Moreover, suppression of endogenous intracellular hydrogen peroxide formation through overexpression of catalase stimulates apoptosis in vascular SMCs.26 Thus, our study further supports the notion that reactive oxygen species may play a critical role in promoting the survival of vascular SMCs.27 Although the molecular mechanism by which antioxidants stimulate SMC apoptosis is not known, HO-1–mediated apoptosis is associated with a marked increase in the cellular levels of the transcription factor p53, which is a well-established inducer of apoptosis.28 Interestingly, the induction of apoptosis by N-acetyl-L-cysteine has also been linked to an increase in p53 expression,29 suggesting that antioxidants stimulate apoptosis via a p53-dependent pathway.

Recent studies suggest that HO-1 is an important regulator of the vascular response to injury. Induction of HO-1 by hemin administration or gene transfer inhibits formation of neointima after balloon injury.30–32 In contrast, inhibition of HO-1 activity by metalloprotoporphyrins potentiates intimal thickening after vascular injury.30 Although the mechanism by which HO-1 prevents intimal thickening is not known, our findings suggest that HO-1–mediated SMC apoptosis may be involved. HO-1, however, may also exert other actions to limit the extent of stenosis after vascular trauma. In this respect, recent studies found that overexpression of HO-1 induces cell cycle arrest in SMCs32 and stimulates the proliferation of cultured endothelial cells.33 This raises the possibility that HO-1 may also promote tissue repair by stimulating the reendothelialization of blood vessels at sites of vascular injury. In addition, HO-1 may serve to preserve blood flow at sites of vascular damage by inhibiting vascular tone and platelet aggregation via the release of CO.5–8 Thus, adenovirus-mediated overexpression of HO-1 may represent a promising novel therapeutic approach in treating fibroproliferative disorders of the vessel wall.

In conclusion, these studies demonstrate that adenovirus-mediated transfer of HO-1 stimulates apoptosis in vascular SMCs. The ability of HO-1 to selectively stimulate SMC apoptosis and promote blood flow suggests that genetic approaches targeting HO-1 to the vessel wall may provide an effective strategy in alleviating occlusive vascular disease.

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