Protection of Human Vascular Smooth Muscle Cells From H₂O₂-Induced Apoptosis Through Functional Codependence Between HO-1 and AKT

Keith R. Brunt, Keith K. Fenrich, Gholam Kiani, M. Yat Tse, Stephen C. Pang, Christopher A. Ward, Luis G. Melo

Objective—Oxidative stress (OS) induces smooth muscle cell apoptosis in the atherosclerotic plaque, leading to plaque instability and rupture. Heme oxygenase-1 (HO-1) exerts cytoprotective effects in the vessel wall. Recent evidence suggests that PKB/Akt may modulate HO-1 activity. This study examined the role of Akt in mediating the cytoprotective effects of HO-1 in OS-induced apoptosis of human aortic smooth muscle cells (HASMCs).

Methods and Results—HASMCs were transduced with retroviral vectors expressing HO-1, Akt, or GFP and exposed to H₂O₂. Cell viability was assessed by MTT assay. OS was determined by CM-H2DCFDA fluorescence, and apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), caspase-3 activity, and Bel-2/Bad levels. Mitochondrial membrane potential (ΔΨₘ) was assessed by fluorescence-activated cell sorter (FACS) using JC-1. HO-1 reduced H₂O₂-induced OS and apoptosis. Akt knockdown removed the protective effect of HO-1 on ΔΨₘ during exposure to H₂O₂. Conversely, HO-1 knockdown removed the protective effect of Akt on ΔΨₘ. Inhibition of PI3K-Akt reduced induction of HO-1 protein expression by H₂O₂ and blocked its anti-apoptotic effects. The Akt-mediated upregulation of HO-1 was dependent on activation of HO-1 promoter by Nrf2.

Conclusion—HO-1 and Akt exert codependent cytoprotective effects against OS-induced apoptosis in HASMCs. These findings may have implications for the design of novel therapeutic strategies for plaque stabilization. (Arterioscler Thromb Vasc Biol. 2006;26:2027-2034.)

Key Words: apoptosis | flow cytometry | mitochondrial membrane potential | oxidative stress | vascular smooth muscle cells

Oxidative stress (OS) has been implicated in vascular injury leading to atherosclerosis, hypertension, restenosis, and vasospasm.¹ Pro-oxidant species such as hydrogen peroxide (H₂O₂) exert dose-dependent effects on vascular cells, including cell proliferation, activation, and apoptosis.² In the atherosclerotic plaque, excessive production of pro-oxidant species induces apoptosis of vascular smooth muscle cells in the fibrous cap, resulting in plaque instability.³-⁶ Because vascular smooth muscle cell (VSMC) loss precipitates plaque rupture and thrombosis,⁷-¹⁰ the protection of VSMC from apoptosis in the plaque has become an important therapeutic target for plaque stabilization.¹¹,¹² Heme oxygenase-1 (HO-1) is the rate-limiting enzyme involved in the conversion of heme into biliverdin, carbon monoxide, and free iron.¹³ The byproducts of heme breakdown have pleiotropic cytoprotective effects on the vessel wall.¹³,¹⁴ Bilirubin is a powerful antioxidant¹⁵ and carbon monoxide exerts vasodilatory, anti-inflammatory, anti-mitogenic, and anti-apoptotic effects in VSMCs and endothelial cells.¹⁶-¹⁹ The protective effects of HO-1 on VSMCs may be particularly important for maintenance of atherosclerotic plaque stability. Because of its anti-inflammatory and anti-apoptotic effects, HO-1 may reduce loss of VSMCs in the fibrous cap, and prevent plaque erosion and rupture. Indeed, several studies support the notion that HO-1 exerts an essential protective role in the vessel wall during atherogenesis.²⁰ For example, HO-1 is upregulated in atherosclerotic plaques,²¹ suggesting that the increase in HO-1 gene expression may be a cytoprotective response to the oxidative and inflammatory microenvironment in the plaque. This is further supported by Yet et al.,²² who reported that the absence of HO-1 exacerbates atherosclerotic lesion formation in apoE⁻⁻/⁻ mice. Others have shown that HO-1 over-expression markedly reduces atherosclerotic lesion formation and thrombosis.²³-²⁷

The mechanism underlying the protection of VSMC by HO-1 from oxidative stress-induced apoptosis is not known. Carbon monoxide has been reported to mediate the anti-apo-
apototic effects of HO-1 in response to inflammatory cytokine stimulation in VSMCs, but its role in protecting VSMCs from pro-oxidant–induced apoptosis has not been established. Paradoxically, one study reported increased apoptosis in rat VSMCs after exogenous overexpression of HO-1, suggesting that HO-1 may exert different dose-dependent effects on cell survival. More recently, several studies suggested that PI3K through the survival gene Akt may play a role in the induction of HO-1 gene expression and its anti-apoptotic effects in the presence of cellular stress. In addition, Akt also phosphorylates HO-1, suggesting a role of Akt in post-translational regulation of HO-1 activity. More significantly, simvastatin inhibits VSMC activation and proliferation by inducing HO-1 expression in an Akt-dependent manner. However, despite these findings, a functional dependence between Akt and HO-1 in protection of VSMCs from OS-induced apoptosis has not been established. Such a mechanism could have potential therapeutic implications, given the role of HO-1 and Akt in vascular homeostasis.

Thus, in this study we examined the role of Akt activation in mediating the cytoprotective effects of HO-1 in pro-oxidant induced apoptosis in HASMCs.

Methods

For more details, please see online Methods supplement at http://atvb.ahajournals.org.

Statistical Analysis

All results are presented as means±SE unless stated otherwise. Two-way analysis of variance (ANOVA) was used to compare combined and separate effects of time and treatment on HO-1 protein expression. One-way ANOVA coupled to Bonferroni multiple comparison post-hoc test was used to compare the effects of different treatments on CM-H2DCFDA fluorescence, cell viability, and apoptosis. Unpaired 2-tailed t test was used to compare differences in caspase-3 activity between HO-1 and GFP-transduced cells. P<0.05 was considered to indicate statistically significant difference.

Results

HO-1 Overexpression Protects HASMCs Against Oxidative Stress, Maintains Cellular Viability, and Reduces Apoptosis

The effect of HO-1 overexpression in H2O2-induced OS and apoptosis in HASMC is shown in Figure 1. Transduction efficiency of HASMC by MSCV retrovirus was >90% after 2 rounds of transduction with 5 multiplicities of infection, and
resulted in ~2.5-fold increase in HO-1 protein levels compared with MSCV-GFP–transduced cells (supplemental Figure I, available at http://atvb.ahajournals.org). H2O2 increased OS, the number of TUNEL-positive cells, and reduced cellular viability in a dose-dependent manner 24 hours after exposure (supplemental Figure II). H2O2 increased OS significantly in GFP-transduced cells as measured by CM-H2DCFDA (Figure 1A). H2O2-induced OS was significantly reduced in HO-1 overexpressing cells compared with the GFP cells (Figure 1B). The increase in OS in GFP cells was accompanied by a ~60% increase in TUNEL positive cells (Figure 1C, 1E, 1G). Concomitant with the decrease in OS, there was a significant reduction in the number of TUNEL-positive nuclei (Figure 1D, 1F, 1G) and caspase-3 activity (Figure 1H) and a parallel increase in overall cell viability (Figure II) in HO-1–transduced cells. Even at supra-physiological doses (>300 μmol/L) capable of causing >90% cell death in the control cells, HO-1 reduced apoptosis by >80% (Figure 1G). The cytoprotective effect of HO-1 was accompanied by a time-dependent increase in the level of the anti-apoptotic protein Bcl-2 and a decrease in the level of the pro-apoptotic protein Bad (Figure 1J).

The Cytoprotective Effect of Exogenous HO-1 Over-expression Against Oxidative Stress-Induced Apoptosis Is Dependent on Akt Activity

We postulated that the anti-apoptotic effect of HO-1 against OS may be mediated, at least in part, by a positive feedback interaction with the PI3K-Akt survival pathway. Figure 2 shows the effect of Akt on HO-1 protein expression and apoptotic cell death after exposure to H2O2 in GFP and HO-1–transduced cells. HO-1 protein expression increased time dependently up to 12 hours after exposure to 300 μmol/L H2O2 in both the GFP and HO-1–transduced cells (Figure 2A, 2B; see also supplemental Figure III). As expected, the amount of HO-1 protein at any one time point was higher in HO-1–transduced cells than in the GFP-transduced cells. Inhibition of PI3K with LY294002 reduced HO-1 protein expression significantly in both the GFP and HO-1–transduced cells (Figure 2A, 2B) and further increased H2O2-induced apoptosis in GFP transduced cells (Figure 2C). LY294002 did not increase apoptosis in the HO-1 transduced cells in response to 300 μmol/L H2O2 (Figure 2D). However, at 600 μmol/L H2O2, the anti-apoptotic effect of HO-1 overexpression was completely removed by LY294002. To further define the potential interaction between HO-1 and Akt in cellular protection against OS-induced apoptosis, we used small interfering RNA oligonucleotides (siRNA) for human HO-1 and Akt 1/2. Transfection of HASMCs with fluorescein-conjugated scrambled sequences showed high levels of siRNA transfection efficiency as confirmed by intense green fluorescence in the cytosol, leading to marked decrease in protein expression (supplemental Figure IV). Interestingly, pretreatment with Akt 1/2 siRNA reduced the cytoprotective effects of HO-1 even at 300 μmol/L of H2O2 (Figure 2E), suggesting that a PI3K-independent mechanism(s) may contribute to the modulatory effects of Akt in HO-1-mediated cytoprotection in HASMC.

HO-1 and Akt Exert Reciprocal Effects in Preservation of Mitochondrial Membrane Potential

To determine whether there were reciprocal effects of HO-1 and Akt in the cytoprotective response to H2O2, we used FACS analysis to assess changes in fluorescence of JC-1, a potentiometer dye that detects changes in mitochondrial membrane potential ($\Delta$Ψm). Hyperpolarized intact mitochondria concentrate JC-1 in the intermembrane space resulting in JC-1 aggregation and fluorescence in the red spectrum (FL2). Depolarization caused by pore formation results in JC-1...
aggregate release and dissociation to its monomeric form, which fluoresces in the green spectrum (FL1). Cells that are healthy are most intense for the red aggregate and localize to the third log (Figure 3A, 3J), whereas cells with perforated mitochondria shift to the lower logs (10^1 to 10^0), and dead cells to the lattermost log (10^0). In comparison to control cells (Figure 3A) we observed a large decrease in \( \Psi_{m} \) in HASMCs exposed to H2O2 (Figure 3B, 3J). Transfection with a scrambled siRNA had no detrimental effect on \( \Psi_{m} \) (Figure 3C, 3J). HO-1 overexpression attenuated mitochondrial depolarization (Figure 3D, 3J) as indicated by a higher percentage of cells in the third decade and fewer cells in the lower decades. However, inhibition of Akt with siRNA removed the protective effect of HO-1 over-expression on \( \Psi_{m} \) (Figure 3E, 3J). This effect was recapitulated when HO-1 siRNA was used in HO-1–transduced cells (Figure 3F, 3J). \( \Psi_{m} \) was preserved in Akt-overexpressing cells exposed to H2O2 (Figure 3G, 3J). The cytoprotective effect of Akt on \( \Psi_{m} \) was attenuated by targeting HO-1 with siRNA (Figure 3H, 3J). The deleterious effect of H2O2 on \( \Psi_{m} \) was recapitulated by siRNA targeting Akt in Akt-overexpressing cells (Figure 3I, 3J).

**Figure 3.** FACS analysis of mitochondrial membrane potential (\( \Delta \Psi_{m} \)) in HASMC cells after exposure to H2O2 for 24 hours. A, GFP-transduced cells. B, GFP-transduced cells exposed to 300 \( \mu \text{mol/L} \) H2O2. C, Control siRNA cells. D, HO-1–transduced cells exposed to 300 \( \mu \text{mol/L} \) H2O2. E, HO-1–transduced cells pre-treated with Akt siRNA and exposed to 300 \( \mu \text{mol/L} \) H2O2. F, HO-1–transduced cells pre-treated with HO-1 siRNA and exposed to 300 \( \mu \text{mol/L} \) H2O2. G, Akt-transduced cells exposed to 300 \( \mu \text{mol/L} \) H2O2. H, Akt-transduced cells pretreated with HO-1 siRNA and exposed to 300 \( \mu \text{mol/L} \) H2O2. I, Akt-transduced cells pretreated with Akt siRNA and exposed to 300 \( \mu \text{mol/L} \) H2O2. J, Percentage of cells within each decade for these groups in FL-2 channel. FL2 channel represents red fluorescence of JC-1 aggregates in hyperpolarized mitochondria. Depolarization results in a downward shift in scatter plot and leftward shift in histogram plot.

**HO-1 and Akt Exert Reciprocal Effects on Other Protein Levels**

To understand the potential interaction between HO-1 and Akt on cellular protection against OS-induced apoptosis, we used pharmacological inhibitors of PI3K and siRNA for human HO-1 and Akt 1/2 (for transfection and gene knockdown efficiency (supplemental Figure IV) to determine the reciprocal effects of HO-1 and Akt on each other’s protein levels. HO-1 overexpression increased Akt phosphorylation by 70% to 80% relative to GFP control cells in response to H2O2 without affecting the total Akt protein levels (Figure 4A). Inhibition of PI3K-Akt by LY294002 markedly reduced HO-1 protein levels in both HO-1 and GFP-transduced cells (Figure 4B). This was further confirmed using gene knockdown with siRNA (Figure 4C). HO-1 siRNA reduced H2O2 induced Akt phosphorylation by \( \approx \)30% relative to control cells transfected with a scrambled sequence. Reciprocally, Akt knockdown with siRNA nearly suppressed HO-1 protein expression in response to H2O2 (Figure 4C), thus indicating that Akt and HO-1 reciprocally stimulate each other’s
activity in a codependent manner. Akt siRNA markedly reduced steady state HO-1 mRNA levels after exposure to 300 μmol/L H₂O₂, suggesting that Akt regulates HO-1 expression by a transcriptional mechanism (Figure 4D).

 Akt Increases HO-1 Levels Via Cap’n Collar Transcription Factor Nrf2

We investigated the mechanism underlying the stimulation of HO-1 by Akt. Exposure to 300 μmol/L H₂O₂ increased HO-1 promoter activity in a time-dependent fashion, peaking at 3 hours (Figure 5A). The increase in HO-1 promoter activity was preceded by an increase in Akt phosphorylation (Figure 5B) and coincided with increased Nrf2 protein levels (Figure 5B). The transcription factor appeared diffuse and exclusively localized to the cytosol in unstimulated conditions (Figure 5C to 5F). On exposure to H₂O₂, Nrf2 concentrated in the perinuclear region and translocated to the nucleus (Figure 5G to 5N), in parallel with the increased promoter activity (Figure 5A). The translocation of Nrf2 peaked at 3 to 6 hours after H₂O₂ and preceded the induction of HO-1 (Figure 5B, 5G to 5J), which declined steadily thereafter (Figure 5B, 5K to 5N).

To determine the role of Nrf2 in mediating the effect of Akt in H₂O₂-induced HO-1 expression, we treated cells with Akt or Nrf2 siRNA. Akt knockdown was associated with reduced Nrf2 and HO-1 expression, compared with cells treated with scrambled siRNA (Figure 6A). This was accompanied by reduced HO-1 promoter activity (Figure 6B). Similarly, Nrf2 knockdown decreased HO-1 protein expression (Figure 6A) and promoter activity (Figure 6B). Immunohistochemical

**Figure 4.** Reciprocal effect of HO-1 and Akt inhibition on each other protein expression in cells exposed to H₂O₂. A, Effect of HO-1 overexpression on Akt phosphorylation. B, Effect of pharmacological inhibition of PI3K-Akt on HO-1 protein expression. C, Effect of Akt and HO-1 gene knockdown with siRNA on each others protein expression. D, Effect of Akt gene knockdown on HO-1 transcription. Membranes were re-probed for total Akt and β-actin.

**Figure 5.** Transcriptional activation of HO-1 by Akt and Nrf2. A, Time-dependent HO-1 promoter activity after exposure to 300 μmol/L H₂O₂ (n=12 at each time point). B, Time course of Akt, Nrf2 and HO-1 protein expression after exposure to 300 μmol/L H₂O₂. C to N, Time course of Nrf2 distribution after treatment with 300 μmol/L H₂O₂ showing time-dependent perinuclear accumulation and translocation to the nucleus (400×; *P<0.05, vs 0 hours).
analysis of Nrf2 localization showed that both Akt, and Nrf2, markedly reduced H$_2$O$_2$-induced perinuclear localization and nuclear translocation of Nrf2 (Figure 6G to 6J) compared with untreated (Figure 6C, 6D) or scrambled siRNA-treated cells (Figure 6E, 6F).

**Discussion**

Plaque rupture rather than luminal stenosis is the most severe clinical manifestation of advanced atherosclerosis, leading to thrombosis and potentially fatal acute coronary events. Vascular smooth muscle apoptosis occurs throughout atherogenesis, but is accentuated in advanced lesions because of the heightened inflammatory and pro-oxidant micro-environment of the plaque. This poses a problem for plaque stability because of the heightened inflammatory and pro-oxidant micro-environment of the plaque.4,5 These effects may act to limit cell replication and excessive luminal occlusion in the developing lesion and prevent excessive apoptosis in the advanced lesion. In this context, our current findings suggest that exogenous HO-1 supplementation may be a useful therapeutic strategy for protection of VSMCs in the pro-inflammatory and pro-oxidant milieu of the advanced atherosclerotic lesion.

The mechanism by which HO-1 inhibits apoptosis in VSMC is not fully understood. Our current results indicate that the protective effect of HO-1 against pro-oxidant-induced apoptosis in HASMCs is critically dependent on Akt activity. Furthermore, the cytoprotective effect of Akt appears to be, at least partially, dependent on HO-1 activity, suggesting that these 2 enzymes function in a codependent and cooperative fashion to confer protection from OS in HASMCs. This premise is supported by our results showing that inhibition of Akt activity markedly reduces the ability of HO-1 to inhibit apoptosis and preserve mitochondrial membrane potential. Indeed, pretreatment of cells with Akt siRNA

Figure 6. Akt mediated induction of HO-1 through Nrf2. A, Effect of Akt siRNA and Nrf2 siRNA on Nrf2 and HO-1 protein expression. B, Effect of Akt siRNA and Nrf2 siRNA on human HO-1 promoter activity 3 hours after exposure to 300 μmol/L H$_2$O$_2$. C to J, Nrf2 distribution 3 hours after exposure to 300 μmol/L H$_2$O$_2$ in untreated HASMCs (C, D), HASMCs treated with scrambled siRNA sequence (E, F), HASMCs treated with Akt siRNA (G, H), HASMCs treated with Nrf2 siRNA (I, J). (400x; *P<0.05, Akt, Nrf2, vs scrambled siRNA and GFP controls).
led to almost complete knockdown of HO-1 promoter activity, mRNA, and protein expression, indicating that the role of Akt in HO-1 mediated cytoprotection may be caused by its ability to promote HO-1 transcription.

The mechanism linking exogenous H$_2$O$_2$ to Akt activity and induction of HO-1 is not known. HO-1 levels are primarily regulated at the transcriptional level by a number of redox sensitive transcription factors. H$_2$O$_2$ diffuses freely across the cell membrane and activates intracellular signaling molecules that may converge to induce HO-1 gene transcription via stimulation of redox-sensitive transcription factors such as NF-κB, AP-1, and Nrf-2. Our data indicate that the effect of Akt on HO-1 levels occurs primarily at the level of transcription, because Akt inhibition markedly reduces HO-1 promoter activity and steady state mRNA levels. This is in agreement with the data reported by Salinas et al on PC12 cells. However, our data show that Akt siRNA decreases Nrf2 perinuclear localization and nuclear translocation in response to H$_2$O$_2$. Furthermore, HO-1 promoter activity is comparably inhibited by Akt and Nrf2 siRNA, suggesting that the effect of Akt on H$_2$O$_2$-induced HO-1 transcriptional activation is, at least in part, mediated via Nrf2. In this regard, we note that Nrf2 has been reported to play an essential role in induction of HO-1 in response to heme and the antioxidant carnosol by a mechanism that is dependent on upstream activation by PI3K/Akt. Furthermore, PI3K/Akt regulates the nuclear translocation of Nrf2 in response to oxidative stress. In addition, Akt and HO-1 may also interact at the post-translational level. Akt phosphorylates HO-1 at serine 188 both in vitro and in vivo, resulting in a modest increase in HO activity. Interestingly, our data show that HO-1 overexpression results in increased levels of phosphorylated Akt without affecting total Akt. It is not clear from our results whether HO-1 directly phosphorylates Akt or whether HO-1 inhibits Akt dephosphorylation by reducing OS. This suggests that Akt and HO-1 may operate in a positive feedback mechanism, whereby the level of HO-1 expression reciprocally augments Akt activation, which in turn increases HO-1 expression.

The current findings may have therapeutic implications for atherosclerosis. A recent study reported that simvastatin markedly induced HO-1 and inhibited proliferation and inflammation-mediated activation in vascular smooth muscle cells in vitro and in the medial layer of blood vessels. Interestingly, these pleiotropic effects of simvastatin were found to be dependent on p38 and PI3K-Akt. Our current results show that HO-1-mediated protection of HASMCs from oxidative stress induced apoptosis is dependent on Akt activity. A plausible working model for the interaction between Akt and HO-1 in cytoprotection from oxidative stress may involve activation of Akt by H$_2$O$_2$ either directly or proximally at the level of PI3K (supplemental Figure V). Activated Akt may act as a relay to phosphorylate Nrf2, promoting its dissociation from the cytosolic repressor Keap1 and its translocation into the nucleus, where it induces HO-1 gene transcription by binding to the antioxidant response element (ARE) in the HO-1 promoter. A positive feedback loop between Akt and HO-1 driven by reactive oxygen species may operate at the post-translational level by reciprocal phosphorylation events between these 2 enzymes. Akt activity may be further enhanced via BVR-mediated phosphorylation. Termination of the positive feedback loop between Akt and HO-1 is likely mediated by bilirubin, which may buffer cytosolic ROS accumulation. However, confirmation of this potential mechanism of cytoprotection remains to be established.

In conclusion, our results reveal for the first time to our knowledge a functional codependence between HO-1 and Akt in mediating cytoprotection against oxidative stress induced cell death. Given the prevalence of oxidative stress and apoptosis in advanced atherosclerotic disease, this novel interaction between 2 key cytoprotective systems may provide the rationale for the development of therapeutic strategies for plaque stabilization and prevention of plaque rupture and thrombosis.

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Disclosures

None.

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METHODS (ON-LINE SUPPLEMENT)

Reagents. All chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) with the exception of MTT reagent and TUNEL (Roche, Laval, Quebec, Canada), Hoescht 33342, CM-H2DCFDA, and JC-1 (Invitrogen, Burlington, Ontario, Canada) and LY294002 (Calbiochem, San Diego, CA, USA). HO-1 stealth siRNA was purchased from Invitrogen, Akt siRNA was purchased from Cell Signalling (Beverly, MA, USA) and Nrf2 siRNA was purchased from (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Hydrogen peroxide was diluted to a range of 10-1000 µM from a stabilized 30% w/v solution in HEPES buffer saline solution (HBSS) immediately before use. LY294002, CM-H2DCFDA, and JC-1 were initially dissolved in DMSO and diluted in PBS with a final DMSO concentration of ≤ 0.01%.

Cell Culture. Primary human aortic vascular smooth muscle cells derived from a young donor were purchased from Clonetics (San Diego, CA, USA) and seeded at constant density (10,000/cm²) and grown to 80% confluence according to the manufacturer’s recommendations in full media containing 5% fetal bovine serum supplemented with insulin, human fibroblast growth factor, human endothelial growth factor, and gentamycin. Cells were washed three times with HBSS and rendered quiescent in serum free DMEM for 24 hours prior to experiments. All experiments were conducted with cells at the 5th or 6th passage from derivation.

Retroviral Production and Transduction. Retroviral vectors were constructed by ligating the full length human heme oxygenase-1 (HO-1, 987 bp, gift from Dr. M. Perrella, Harvard Medical School, MA) into the Hind III cloning site of pMSCV vector (BD Biosciences Clontech, Palo Alto, CA), downstream from the 5’ LTR promoter. cDNA of AKT was PCR-amplified (forward
5'-GCAAGATCTGATACCATGAACGACGTAGCC-3'; reverse 5'-CGGTCACCGTGTCGGACTCCTAGGATC-3') and cloned in pMSCV using BgII and BamHI. The green fluorescent protein (GFP) gene together with the internal ribosomal entry site (IRES) were excised from pEGFP vector (BD-Clonetch) by Xho I/BamHI digestion and cloned at the corresponding sites into the pMSCV vector. Generation and titering of VSV-G pseudotyped retroviral particles was carried out by the Harvard Gene Therapy Initiative. The titers were approximately $0.5-1 \times 10^9$ IU/ml. HASMC were plated in full media and after 24 hours cells were transduced with 5 multiplicities of infection (5 MOI) of MSCV expressing either HO-1, AKT, or GFP in full media supplemented with 8µg/ml of Polybrene™ (Aldrich Chemical, Milwaukee, WJ, USA)) for 12 hours. At 48 hours a second round of transduction was performed in full media supplemented with 4µg/ml Polybrene™ and 5 MOI of virus for 24 hours. Cells were allowed a 24 hours recovery in normal growth serum prior to being rendered quiescent. Transduction efficiency was 80-90% based on GFP fluorescence.

**siRNA Transfection.** Double stranded Stealth™ RNAi (sense GGU GGC GAC AGU UGC UGU AGG GCUU; antisense AAG CCC UAC AGC AAC UGU CGC CACC) was reconstituted to 20 µM in 10 mM Tris-HCl, pH 8.0, 20 nM NaCl, 1mM EDTA. Transfection was performed for 24 hours in quiescent cells using 3:1:3 RNAi:PLUS-Reagent:Lipofectamine in DMEM. AKT siRNA (Cell Signaling, #6210) and Nrf2 siRNA (Santa Cruz Biotechnology, sc37030) were delivered according to manufacturer’s instructions. siRNA transfection efficiency was observed by uptake of fluorescein conjugated scrambled siRNA sequence. RNAi protein knockdown was confirmed by western blot analysis of total protein 24 hours after transfection with siRNA and 24 hours after exposure to H$_2$O$_2$. 
**Oxidative Stress.** OS was determined using CM-H$_2$DCFDA. The dye (5 µM) was preloaded to cells in 8-well culture slides for 30 minutes and kept at 37°C in 5% CO$_2$-21% O$_2$. Wells were washed once with HBSS and incubated in serum free media with H$_2$O$_2$, at 37°C, 5% CO$_2$, for 1 hour and then incubated with Hoescht 33342 (10 µg/ml) for 5 min. Fluorescence was viewed with a Leica DRB inverted microscope using a 10X objective calibrated for degree fluorescence detection with excitation and emission wavelengths of 480 nm and 520 nm, respectively.

**Cell viability assay.** Cell viability was determined by the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) to formazan utilizing NADH and NADPH pyridine nucleotide cofactors. MTT was added to a final concentration of 0.5mg/ml, incubated for 4 hours and solubilized for 24 hours at 37°C. Absorbance was read using a SPECTROMAT® plate reader at 550-690 nm.

**TUNEL assay.** DNA cleavage was detected by terminal dUTP nick end labelling using a kit from Roche. Briefly, cells were seeded onto 8-well culture slides. After exposure to H$_2$O$_2$ the cells were fixed in 2% paraformaldehyde-PBS for 10 minutes.Slides were centrifuged at 500 g for 5 min to pellet floating cells. The cells were then washed with PBS, permeabilized with 0.1% Triton X-100, 0.1% sodium citrate in PBS and incubated for 1 hour at 37°C, in a dark humid chamber. Nuclei were counterstained using 10 µg/ml Hoechst 33342 in PBS for 5 min at 24°C, in the dark. Five random fields were counted in each well using a 40x objective. The TUNEL-positive cells were counted using SigmaScan® (SystStat Software, Point Richmond, CA, USA) and expressed as the percentage of total number of cells.
**Caspase-3 activity.** Caspase-3 activity was determined using the BD ApoAlert fluorescent Caspase-3 Assay Kit (BD Bioscience-Clontech, Mountain View, CA, USA) according to the manufacturer’s instructions. Briefly, cells were lysed and incubated with fluorescent DEVD-AFC (λ 400nm) caspase-3 substrate for 1 hour at 37°C. Caspase cleavage releases AFC (λ 505nm) which was measured using a FLUOstar OPTIMA plate reader. Enzyme activity was extrapolated from a standard curve.

**Flow Cytometry.** The fluorescent indicator of mitochondrial membrane potential (ΔΨₘ) and integrity JC-1 was used for flow cytometric analysis of mitochondrial function. Cells were exposed to H₂O₂, trypsinized and incubated in PBS, with 1µM JC-1 for 15 min at 37°C. Cells were then washed twice with PBS and FACS analysis was performed to determine the amount of JC-1 aggregate measured in the FL2 channel. JC-1 monomers, fluoresce in the green spectrum and concentrate in the intermembrane space of hyperpolarized mitochondrial membranes where JC-1 aggregates fluoresce in the red spectrum. Mitochondrial pore formation during apoptosis depolarizes the mitochondria allowing JC-1 aggregates to escape and revert to monomeric form.

**Western Immunoblot Analysis.** Cell lysates were prepared using TPER protein extraction reagent (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktails (Sigma). Protein concentration was determined using the Bradford method. Protein samples (25 µg) were denatured in Laemmli buffer, resolved in a 10% SDS-PAGE and transferred to PVDF membrane (Immobilon-P, 0.45 µM, Millipore, Billerica, MA, USA). Equal loading was verified by Ponceau-S, Coomassie stain and reprobing for β-Actin (1:1000, Sigma). Membranes
were probed for human HO-1 (SPA-896, StressGen, Victoria, BC, Canada, 1:5000), total AKT and phosphorylated AKT-S473 (Cell Signaling # 9272 and #9271, respectively; 1:1000), Bcl-2, Bad, Nrf2 (Santa Cruz Biotechnology, sc783, sc7869, sc13032, Santa Cruz, CA, USA, 1:500). The blots were then incubated in secondary HRP conjugated anti-rabbit (Cell Signaling) or HRP conjugated anti-mouse (Amersham, Oakville, Ontario, Canada) according to manufacturer recommendations. Immunoreactivity was detected using Chemiglow reagent and Alpha Innotech™ 8900 gel documentation system.

**RNA extraction and RT-PCR.** RNA was isolated using Trizol reagent (Invitrogen) according to the instructions provided by the supplier. For reverse transcriptase-polymerase chain reaction (RT-PCR) the One-Step Platinum Taq RT-PCR kit (Invitrogen) was used to detect human HO-1 and GAPDH transcripts, 100 ng of total RNA, verified for integrity using an Agilent 2150 Bioanalyzer, was used for first-strand cDNA synthesis and PCR amplification. A HO-1 (185 bp) and GAPDH (325bp) fragment was amplified for 25 cycles with the following HO-1 and GAPDH human specific primers: HO-1 Forward: GCTCTTTGAGGAGTTGCAGG, Reverse: GTGTAAGGACCCATCGGAGA; GAPDH Forward: CAGCCTCAAGATCATCAGCA, Reverse: TTCTAGACGGCAGGTCAGGT.

**HO-1 Promoter Construct and Plasmid Transfection.** The human hemoxygenase-1 promoter was taken from the 5'UTR sequence HO-1 gene (Accession # X14782). Sequence verification was conducted by PCR amplification restriction digest. An independent sequencing facility (Robarts, London, ON, Canada) confirmed sequence identity. The sequence (541bp) was cloned in the *KpnI* and *HindIII* sites in pCR2.1 and pGL3-basic. Plasmid was transfected to DH5α.
ultracompetant cells by electroporation; 0.45kV, 500µFD, 800Ω, and 0.01msec. Bacterial cultures were grown in Ampicillin containing TE-Broth and plasmid DNA was extracted and purified by maxi-prep (Qiagen, Mississauga, ON, CAN). Transfection of plasmid-DNA was carried out in serum and anti-biotic free media by pre-complexing Lipofectamine2000™ (Invitrogen) and DNA ((3µl:1.5µg)/50,000cells) for six hours followed by addition of serum containing media. Transfection efficiency was 2-3% and some cell death <20% resulted from toxicity of the DNA-lysosomal complexes. Luciferase activity was measured using Bright-Glo™ Luciferase System (Promega, Madison, MI, USA) with a Berthold Lumat LB 9507 luminometer.

**Nrf2 Immunocytochemistry.** Cells were washed three times with PBS and fixed using 0.45µm filtered 100%, -20°C methanol, for ten minutes on ice. Cells were washed three times with PBS and incubated in 10% Goat Serum (Sigma) for 90 minutes. Nrf2 primary antibody (rabbit polyclonal, sc-13032, 1:40 in 1.5% Goat Serum/PBS, Santa Cruz) were incubated for 60 minutes, washed three times with PBS and incubated in AlexaFlouro-488™ goat anti-rabbit secondary antibody (A11008, 1:1000 in 1.5% Goat Serum/PBS, Invitrogen). Cells were washed three times in PBS and mounted with Molecular Probes, ProLong Gold® anti-fade reagent with DAPI (P36931, Invitrogen). Images were acquired at constant exposure with a 40x oil immersion objective using OpenLab™ software on a Leica DMRB inverted microscope.
Supplemental Figure 1 (Online only)

A. Transmission electron microscopy (TEM) image showing cellular structures.
B. Fluorescence microscopy image highlighting green fluorescence, possibly indicating cellular markers.
C. Western blot analysis showing protein bands at 32Kd.
D. Bar chart showing integrated density values for different conditions: Non-Transduced, MSCV-GFP, and MSCV-HO-1. Significant differences are indicated by asterisks (*) and a hashtag (#).
Supplemental Figure 2 (Online only)

A. CM-H2DCFDA-Vehicle
B. CM-H2DCFDA-300μM
C. CM-H2DCFDA-600μM
D. CM-H2DCFDA-900μM
E. TUNEL-Vehicle
F. TUNEL-300μM
G. TUNEL-600μM
H. TUNEL-900μM
I. Hoescht-Vehicle
J. Hoescht-300μM
K. Hoescht-600μM
L. Hoescht-900μM

M. CM-H2DCFDA Oxidative Stress

N. Fluorescent TUNEL

O. MTT Cell Viability Assay

Concentration of Hydrogen Peroxide

Concentration of Hydrogen Peroxide

Concentration of Hydrogen Peroxide
Supplemental Figure 3 (Online only)

A

Integrated Density Values

0H 1H 3H 6H 12H 24H

Time

GFP HO-1

B

HO-1 Peptide

MSCV-GFP

C

MSCV-HO-1
Supplemental Figure 5 (Online only)
Supplemental Figure 1. Murine stem cell virus transduction in human aortic smooth muscle cells. A. Brightfield image of VSMC (200x); B. View of same field under green fluorescence; C. HO-1 protein expression in HASMC transduced with pseudotyped MSCV-HO-1 or MSCV-GFP; D. Average integrated density values of basal HO-1 protein expression in GFP and HO-1 transduced cell (*, P < 0.05, n = 6).

Supplemental Figure 2. Dose dependent effect of H$_2$O$_2$ on oxidative stress, apoptosis and cell viability. A-D. Oxidative stress by CM-H$_2$DCFDA fluorescence (200x) after exposure to increasing levels of H$_2$O$_2$; E-H. Apoptosis by TUNEL positive nuclei (400x) 24 hours after exposure to increasing levels of H$_2$O$_2$; I-L. Hoescht 33342 staining of nuclei of same fields as in E-H. Arrows indicate TUNEL-positive nuclei; M. Quantification of CM-H$_2$DCFDA fluorescence (n = 6 fields for each condition, repeated twice) ; N. Quantification of TUNEL positive nuclei in response to increasing concentrations of H$_2$O$_2$ (n = 8 for each condition); O. Dose-dependent effect of H$_2$O$_2$ on cell viability as assessed by MTT assay ( n = 6 for each doe, done in triplicate) . *, P<0.05 vs. vehicle.

Supplemental Figure 3. Time-dependent changes in HO-1 protein expression in response to H$_2$O$_2$. A. Time- dependent change in HO-1 protein expression in response to H$_2$O$_2$ (300 µM) in HASMC transduced with MSCV-GFP or MSCV-HO-1; B, C. Representative blots of HO-1 expression in MSCV-GFP (B) and MSCV-HO-1 (C) cells (*, P < 0.05, n = 4-6).
Supplemental Figure 4. Transfection efficiency and protein expression knockdown using siRNA. A. Brightfield view (200x) of HASMC; B. Same view using fluorescein conjugated scrambled oligonucleotide; C. Protein knockdown determined by Western blot of RNA interference specific for human HO-1 (HO-1i), Akt (Akti), Nrf2 (Nrf2i). Membranes were reprobed for β-actin, MAPK, and NF-κB.

Supplemental Figure 5. Model describing the potential interactions between Akt and HO-1 in cytoprotection against oxidative stress. Increased ROS activates Akt resulting in phosphorylation of Nrf2, which dissociates from its repressor Keap1 and translocates to the nucleus, where it binds to the antioxidant response element (ARE) and induces HO-1 gene transcription. The resultant increase in HO-1 activity leads to enhanced heme breakdown and increased production of bilirubin by biliverdin reductase (BVR). A positive feedback loop between Akt and HO-1 driven by ROS may operate at the post-translational level through reciprocal phosphorylation events between these two enzymes. Akt activity may be further enhanced via BVR-mediated phosphorylation. Termination of the positive feedback loop between Akt and HO-1 is probably mediated by bilirubin which decreases cytosolic ROS accumulation.