Platelet-Derived Growth Factor Stimulates Heme Oxygenase-1 Gene Expression and Carbon Monoxide Production in Vascular Smooth Muscle Cells

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Abstract—Recent studies indicate that vascular smooth muscle cells (VSMCs) generate CO from the degradation of heme by the enzyme heme oxygenase-1 (HO-1). Because platelet-derived growth factor (PDGF) modulates various responses of VSMCs, we examined whether this peptide regulates the expression of HO-1 and the production of CO by rat aortic SMCs. Treatment of SMCs with PDGF resulted in a time- and concentration-dependent increase in the levels of HO-1 mRNA and protein. Both actinomycin D and cycloheximide blocked PDGF-stimulated HO-1 mRNA and protein. In addition, PDGF stimulated the production of reactive oxygen species by SMCs. Both the PDGF-mediated generation of reactive oxygen species and the induction of HO-1 protein was inhibited by the antioxidant N-acetyl-L-cysteine.

Incubation of platelets with PDGF-treated SMCs resulted in a significant increase in platelet cGMP concentration that was reversed by treatment of SMCs with the HO-1 inhibitor tin protoporphyrin-IX or by addition of the CO scavenger hemoglobin to platelets. In contrast, the nitric oxide inhibitor methyl-L-arginine did not block the stimulatory effect of PDGF-treated SMCs on platelet cGMP. Finally, incubation of SMCs with the releasate from collagen-activated platelets induced HO-1 protein expression that was blocked by a neutralizing antibody to PDGF. These results demonstrate that either administered exogenously or released by platelets, PDGF stimulates HO-1 gene expression and CO synthesis in vascular smooth muscle. The ability of PDGF to induce HO-1-catalyzed CO release by VSMCs may represent a novel mechanism by which this growth factor regulates vascular cell and platelet function. (Arterioscler Thromb Vasc Biol. 1999;19:2666-2672.)

Key Words: platelet-derived growth factor ■ carbon monoxide ■ heme oxygenase

Heme oxygenase (HO) catalyzes the rate-limiting step in the oxidative degradation of heme to biliverdin, releasing equimolar amounts of carbon monoxide (CO) and iron.1 HO is a ubiquitous protein that exists in at least 3 different isoforms that are products of distinct genes.2,3 The HO-2 isofrom is constitutively expressed and is present at high levels in the brain and tests.4 In contrast, the HO-1 isotype is widely distributed and rapidly induced by several oxidants, including superoxide anion, hydrogen peroxide, ultraviolet radiation, and nitric oxide.5–8 More recently, a third HO protein, HO-3, that is closely related to HO-2 has been identified.9 The induction of HO may provide an important cellular defense mechanism against oxidative injury. HO-1 expression has been shown to be protective against ischemia-reperfusion and free radical damage in a number of tissues.7,10–12 These protective effects of HO-1 result from the conversion of pro-oxidant heme to the antioxidant bile pigments biliverdin and bilirubin.13,14 In addition, HO induction is accompanied by increased ferritin activity, which exerts an additional antioxidant effect by chelating free iron.15

More recently, HO-catalyzed CO release has been shown to play a significant physiological role in the circulation.16 Exogenous administration of CO relaxes isolated blood vessels from various vascular sources and animal species.17–20 Moreover, the 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 endogenous CO subserves a tonic vasodepressor function.21,22 CO also inhibits the synthesis of growth factors from vascular cells and directly blocks smooth muscle cell (SMC) growth, indicating a potentially important antiproliferative role for this gas.23,24 In addition to regulating SMC function, CO modulates platelet reactivity. Both exogenously administered and vascular cell–derived CO inhibit platelet aggregation.25,26 All these biological effects of CO are mediated via the activation of soluble guanylate cyclase and the consequent rise in intracellular cGMP levels in target tissues.17,19,24–26

Several studies have detected the presence of HO-1 and the release of CO by vascular smooth muscle cells (VSMCs).27–29 However, relatively little is known about the regulation of HO-1 gene expression and CO production by physiologically relevant stimuli. Platelet-derived growth factor (PDGF) is a
cationic peptide that is secreted by platelets, macrophages, and vascular cells at sites of inflammation and vascular damage.30 It has been implicated in the vascular response to injury and in the pathogenesis of atherosclerosis and hypertension.30–32 PDGF modulates numerous SMC responses, including growth, migration, and contraction.30,33 Recently, we found that PDGF also plays an important role in regulating the synthesis of another diatomic signaling gas, NO.34–36 Accordingly, the present study examined whether PDGF also regulates the synthesis of CO by VSMCs. We now report that PDGF, either exogenously administered or endogenously generated from activated platelets, induces HO-1 gene expression and CO release in VSMCs. The ability of PDGF to stimulate CO synthesis may provide a novel mechanism by which PDGF regulates SMC function.

Methods

Materials

Fatty acid–free albumin, FCS, l-glutamine, streptomycin, elastase, trypsin, collagenase, sodium citrate, sodium acetate, sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), 3-isobutyl-1-methylxanthine (IBMX), cycloheximide, EDTA, DTT, formaldehyde, tris(hydroxymethyl)aminomethane (Tris) base, N-acetyl-l-cysteine (NAC), creatine phosphate, creatine phosphokinase (HESPII), TES, sepharose 2B-300, Tween 20, and Eagle’s minimum essential medium (MEM) were from Sigma Chemical Co; guanidine isothiocyanate, sonicated salmon sperm, and CsCl were from Gibco; PDGF-AB (human recombinant), actinomycin D, ribonuclease A, and ribonuclease T1 were from Boehringer Mannheim; T7 RNA polymerase, RNA molecular weight markers, and antisense GAPDH template were from Ambion; 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was from Molecular Probes; anti-PDGF antibody was from StressGen; and [32P]UTP and radiolabeled ribonuclease were from Amersham.

Cell Culture

SMCs were isolated by elastase and collagenase digestion of rat thoracic aorta and were characterized according to morphological and immunological criteria, as previously described.37 Cells were propagated in MEM containing Earle’s balanced salts, 5.6 mmol/L glucose, 2 mmol/L l-glutamine, 20 mmol/L TES-NaOH, 20 mmol/L HEPES-NaOH, 10% (vol/vol) heat-inactivated FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin, and were passaged weekly by harvesting with trypsin/EDTA and seeded into 75-cm2 flasks. For experiments, subcultured confluent cells between passages 6 and 26 were used.

mRNA Analysis

Total cellular RNA was obtained by the guanidine isothiocyanate/CsCl procedure, and RNA concentration was determined by absorbance spectrophotometry at 260 nm.38 HO-1 mRNA levels were determined by solution hybridization/ribonuclease protection analysis, as previously described.4 In brief, total RNA (10 μg) was hybridized with 1×107 cpm [32P]UTP-labeled antisense HO-1 riboprobe and with antisense GAPDH (316-bp) RNA to control for GAPDH transcription. Protected RNA was analyzed by electrophoresis with 6% acrylamide/8 mmol/L urea gels. The gels were exposed overnight to x-ray film at −70°C in the presence of intensifying screens. The size of the predicted nucleotide-protected fragments was confirmed with a [32P]-labeled RNA molecular weight ladder. Relative mRNA levels were quantified by scanning densitometry (LKB 2222-020 Ultrascan XL, laser densitometer) and normalized with respect to GAPDH.

Protein Analysis

VSMCs were lysed in electrophoresis buffer (125 mmol/L Tris-HCl [pH 6.8], 12.5% glycerol, 2% SDS, and 2.5% DTT) and boiled for 10 minutes. The lysate was centrifuged at 14 000 g for 20 minutes at 4°C, the supernatant collected, and protein concentration determined by the bicinchoninic acid method with serum albumin as the standard.39 Proteins (20 μg) were separated on 10% polyacrylamide gels by SDS-PAGE and transferred to nitrocellulose membranes at 100 V for 1 hour. Membranes were blocked for 1 hour in PBS containing 0.1% Tween 20 and 3% nonfat milk and then incubated with the HO-1 antibody (1:500 dilution) in Tween 20 (0.1%) containing PBS for 1 hour. The membrane was then washed in PBS and incubated for 1 hour with anti-rabbit (1:7500 dilution) horseradish peroxidase–conjugated antibody. After further washing with PBS, blots were incubated in commercial chemiluminescence reagents (Amersham) and exposed to photographic film. Relative protein levels were determined by scanning densitometry.

Measurement of Reactive Oxygen Species

The intracellular production of reactive oxygen species was determined by measurement of the oxidation of CM-H2DCFDA to the fluorescent compound CM-H2DCF with a Cytofluor II multipurpose fluorimeter (Millipore) and calculated as a percentage of the releasate containing 0.1% Tween 20 and GAPDH. Fluorescence was monitored at excitation and emission wavelengths of 485 and 530 nm, respectively.

CO Detection System

CO release by VSMCs was determined by use of a previously described cointubation bioassay system,28,41 in which cGMP production in “detector” platelets layered in suspension over monolayers of SMCs reflects the activation of platelet soluble guanylate cyclase by SMC-derived CO. Before addition of platelets to SMCs, the medium was aspirated and the cells were thoroughly washed with PBS to ensure that platelets were not exposed to any of the treatment compounds. In some experiments, hemoglobin (50 μmol/L) was added to the platelet suspension. After 45 minutes of incubation with the SMCs, the platelet suspensions were collected in TCA (6% w/v), briefly sonicated, and pelleted in a microfuge. Platelet lysates were then extracted with 4 vol of water-saturated ether and assayed for cGMP with a commercially available radioimmunoassay kit (New England Nuclear-Dupont).

Platelet Preparation

Blood from drug-free donors was collected by antecubital vein phlebotomy into 15% (vol/vol) acid-citrate-dextrose and centrifuged at 220g for 14 minutes at 22°C. The platelet-rich plasma was collected and then adjusted to pH 6.5 with additional acid-citrate-dextrose, and creatine phosphate (5 mmol/L) and creatine phosphokinase (25 U/mL) were added. The platelet-rich plasma was layered over a BSA density gradient and centrifuged at 1620g for 16 minutes at 22°C. Interface platelets were collected and subjected to repeated BSA density gradient separation. Platelets were then gel-filtered through Sepharose 2B-300 and collected in Tyrode’s buffer (in mmol/L: NaCl 130, sodium citrate 10, Tris base 10, MgCl2 0.9, KH2PO4 0.8 [pH 7.35]) containing IBMX (0.1 mmol/L) when used in the CO detection experiments or in MEM when used to generate platelet releasates. Platelet releasates were generated by incubating suspensions of platelets with collagen (20 μg/mL) for 15 minutes. The platelets were then removed by centrifugation at 1620g for 16 minutes, and the releasate was collected. In some experiments, active PDGF present in the releasate was neutralized by incubating the platelet releasate with the IGF fraction (50 μg/mL) of a PDGF-neutralizing polyclonal antibody for 60 minutes at 22°C.
Statistics

Results are expressed as mean±SEM. Statistical analysis was performed with a Student’s two-tailed t test. Values of P<0.05 were considered statistically significant.

Results

Treatment of VSMCs with PDGF (30 ng/mL) resulted in the rapid induction of HO-1 mRNA and protein. PDGF markedly elevated HO-1 mRNA levels by nearly 9-fold within 1 hour of treatment, and HO-1 message gradually declined to basal levels after 8 hours of PDGF exposure (Figure 1). In contrast, the induction of HO-1 protein by PDGF was delayed, with a slight increase (1.4-fold) observed at 2 hours, followed by a maximum 4-fold elevation at 4 and 8 hours. HO-1 protein levels (1.7-fold) remained elevated 24 hours after PDGF stimulation (Figure 2). PDGF-mediated increases in HO-1 mRNA and protein were concentration-dependent (Figure 3) and were specific for the HO-1 isoform because PDGF failed to stimulate HO-2 expression (data not shown). Incubating SMCs with the protein synthesis inhibitor cycloheximide (5 μg/mL) or with the transcriptional inhibitor actinomycin D (2 μg/mL) blocked the basal expression of HO-1 mRNA and protein and prevented the PDGF-mediated induction of HO-1 mRNA and protein (Figure 4).

Treatment of VSMCs with PDGF (30 ng/mL) resulted in an immediate rise in the generation of reactive oxygen species that progressively increased over 30 minutes (Figure 5A). This PDGF-mediated increase in intracellular reactive oxygen species was markedly attenuated by the antioxidant NAC (3 mmol/L) (Figure 5A). In addition, NAC (3 mmol/L) inhibited the induction of HO-1 protein by PDGF (Figure 5B). In the absence of PDGF, NAC minimally affected reactive oxygen production (data not shown) or HO-1 protein expression (Figure 5B). Finally, incubation of SMCs with actinomycin D (2 μg/mL) resulted in a decay of HO-1 mRNA with a half-life of ≈90 minutes (Figure 6). PDGF failed to alter the stability of HO-1 message (Figure 6).

In subsequent experiments, HO activity was measured by monitoring SMC CO synthesis. Because CO is a readily diffusible, membrane-permeable gas that activates soluble guanylate cyclase,19,26–28 HO activity was determined by measurement of the intracellular concentration of cGMP in coincubated detector platelets. Incubating platelets with SMCs that had been treated with PDGF (30 ng/mL) for 6 hours resulted in a >3-fold greater increase in platelet cGMP concentration than that found in platelets exposed to untreated control SMCs (Figure 7). The stimulatory effect of platelet cGMP levels by PDGF-treated SMCs was inhibited by incubation of the SMCs with the HO inhibitor SnPP (20 μmol/L)41 or by addition of the CO scavenger hemoglobin (50 μmol/L) to platelets during their incubation with

Figure 1. Time course of HO-1 mRNA expression by PDGF in cultured VSMCs. A, Cells were treated with PDGF (30 ng/mL) and then analyzed for HO-1 mRNA expression at the indicated times. B, Quantification of relative HO-1 mRNA levels by laser densitometry after treatment with PDGF (30 ng/mL). Similar findings were observed in 4 separate experiments.

Figure 2. Time course of HO-1 protein expression by PDGF in cultured VSMCs. A, Cells were treated with PDGF (30 ng/mL) and then analyzed for HO-1 protein at the indicated times. B, Quantification of relative HO-1 protein by laser densitometry after treatment with PDGF (30 ng/mL). Similar findings were observed in 4 separate experiments.

Figure 3. Concentration-response of HO-1 mRNA (A) and protein (B) expression by PDGF in cultured vascular smooth muscle. Cells were stimulated with PDGF (1 to 30 ng/mL) for 1 hour for HO-1 mRNA analysis and for 6 hours for HO-1 protein expression. Similar findings were observed in 3 separate experiments.
PDGF-treated SMCs (Figure 7). In contrast, treatment of SMCs with methyl-L-arginine (L-NMA, 1 mmol/L) did not alter the stimulatory effect of PDGF-treated SMCs on platelet cGMP levels (Figure 7). In the absence of PDGF treatment, exposure of SMCs to SnPP (20 μmol/L) or hemoglobin (50 μmol/L) did not significantly affect the intracellular cGMP concentration of incubated platelets (data not shown).

PDGF is stored in platelet α-granules and is released on platelet activation by various stimuli, such as collagen. Treatment of SMCs with the releasate from collagen-activated platelets for 6 hours also stimulated HO-1 protein expression in a platelet concentration-dependent manner, with maximum induction observed at 2×10⁸ platelets/mL (Figure 8A). The addition of a PDGF-neutralizing antibody to the platelet releasate blocked the induction of HO-1 by the releasate (Figure 8B). In contrast, nonimmune IgG failed to modulate the stimulatory effect of the releasate (Figure 8B). The addition of the PDGF-neutralizing antibody or of the

Figure 4. Effect of cycloheximide (CX) and actinomycin D (Act D) on HO-1 mRNA (A) and protein (B) expression by PDGF in cultured VSMCs. Cells were treated with PDGF (30 ng/mL) in the presence or absence of CX (5 μg/mL) or Act D (2 μg/mL) for 1 hour for HO-1 mRNA analysis and for 6 hours for HO-1 protein expression. Similar findings were observed in 3 separate experiments.

Figure 5. Effect of NAC on reactive oxygen species production (A) and HO-1 protein expression (B) by PDGF in cultured VSMCs. For experiments measuring reactive oxygen species production, cells were preincubated in Hanks’ buffer containing CM-H₂DCFDA (5 μmol/L) for 20 minutes and then treated with PDGF (30 ng/mL) for 30 minutes in the absence (●) or presence (●) of NAC (3 mmol/L), and the fluorescence of CM-H₂DCF was monitored. For experiments measuring HO-1 protein expression, cells were treated with PDGF (30 ng/mL) for 6 hours in the absence or presence of NAC (3 mmol/L), and HO-1 protein levels were determined by Western blotting. Similar findings were observed in 3 separate experiments.

Figure 6. Effect of PDGF on HO-1 mRNA stability in cultured VSMCs. Cells were stimulated with PDGF (30 ng/mL) (●) or vehicle (●) for 1 hour, then actinomycin D (Act D; 2 μg/mL) was added to the cells, and relative HO-1 mRNA levels were determined by laser densitometry at the indicated times after Act D addition. Results are the mean±SEM of 4 separate experiments.

Figure 7. Regulation of platelet cGMP content by cultured SMC-derived CO. SMCs were treated with PDGF (30 ng/mL) for 6 hours in the presence or absence of SnPP (20 μmol/L) or L-NMA (1 mmol/L). After the exposure of SMCs to the various treatment regimens, media were removed, and cells were washed with PBS (pH 7.4) and then incubated with suspensions of IBMX (0.1 mmol/L)-treated platelets (2.0×10⁸ platelets/mL) for 30 minutes. Platelets were then collected, and intracellular cGMP concentration was measured as described under Methods. In some instances, hemoglobin (Hb; 50 μmol/L) was added to the platelet suspension. Results are the mean±SEM of 4 experiments, each performed in duplicate. *Statistically significant increase in platelet cGMP concentration compared with platelets exposed to control untreated SMCs.
sensitive transcription factor, raising the possibility that induction of HO-1 by PDGF. Interestingly, AP-1 is a redox-sensitive transcription factor,49 raising the possibility that induction of HO-1 by PDGF. Consistent with earlier studies, we found that PDGF stimulates a marked increase in production of reactive oxygen species. Consistent with earlier studies, we found that PDGF stimulates a marked increase in production of reactive oxygen species. Nonimmune IgG to untreated SMCs had no effect on HO-1 protein expression (Figure 8B).

Discussion

The present study demonstrates that PDGF induces the expression of the HO-1 gene and the synthesis of CO by VSMCs. PDGF stimulates HO-1 mRNA and protein production in a concentration- and time-dependent manner, with the elevation in HO-1 message preceding the increase in HO-1 protein. In addition, treatment of SMCs with PDGF stimulates the production and release of CO, as demonstrated by the CO-dependent increase in intracellular cGMP levels in cocultivated platelets. The PDGF-induced upregulation of HO-1 gene expression is dependent on de novo RNA synthesis and probably involves transcriptional activation of the gene, because PDGF does not alter the stability of HO-1 mRNA. Although the molecular mechanism by which PDGF stimulates HO-1 expression is not known, PDGF induces the expression of several genes by stimulating the formation of the activator protein-1 (AP-1) family of transcription factors.42,43 In this respect, promoter studies have identified functional AP-1-responsive elements in the 5′-flanking region of the HO-1 gene.44,45 The capacity of cycloheximide to inhibit PDGF-induced HO-1 mRNA expression suggests that de novo AP-1 synthesis may be required for HO-1 gene expression.

The induction of HO-1 by PDGF is dependent on the production of reactive oxygen species. Consistent with earlier studies, we found that PDGF stimulates a marked increase in the intracellular synthesis of reactive oxygen intermediates. Moreover, we observed that the antioxidant NAC inhibits both the PDGF-mediated production of reactive oxygen species and the induction of HO-1 in VSMCs. These findings indicate that reactive oxygen intermediates, which are well-established inducers of HO-1,5,6,48 mediate the induction of HO-1 by PDGF. Interestingly, AP-1 is a redox-sensitive transcription factor,49 raising the possibility that reactive oxygen species stimulate HO-1 expression via AP-1 activation.

The ability of PDGF to rapidly induce the expression of the antioxidant protein HO-1 may provide an important cellular defense mechanism against oxidative injury. The induction of HO-1 in vascular cells leads to an increased resistance to oxidative stress, whereas HO-1 deficiency results in enhanced vascular cell injury.7,12,50 The protective effect of HO-1 arises, in part, from the HO-mediated formation of biliverdin and its subsequent conversion to bilirubin by biliverdin reductase.7 Bilirubin is an efficient scavenger of reactive oxygen species and inhibits lipid peroxidation.13,51 Interestingly, recent studies have correlated elevations in serum bilirubin concentration with a marked reduction in the risk of coronary artery disease.52,53 Thus, the ability of PDGF to induce HO-1 and the formation of bilirubin by VSMCs may provide blood vessels with cytoprotection against oxidative tissue injury.

The PDGF-induced increase in HO-1 gene expression is associated with an increase in HO activity as measured by CO synthesis. Incubation of platelets with PDGF-treated SMCs results in a significantly greater increase in platelet cGMP concentration than that in platelets exposed to untreated control SMCs. The SMC-mediated rise in platelet cGMP results from increased HO activity, because the HO inhibitor SnPP abrogates the cGMP-elevating effect of PDGF-treated cells. Furthermore, the CO scavenger hemoglobin reverses the increase in platelet cGMP evoked by the PDGF-treated cells. In contrast, the NO synthase inhibitor L-NMA fails to modulate platelet cGMP levels during their incubation with SMCs. These results demonstrate that SMC-derived CO, not NO, is responsible for the elevation in platelet cGMP levels. The capacity of PDGF to induce CO release from SMCs may provide an important adaptive mechanism to maintain vascular homeostasis at sites of vascular injury. We have recently shown that SMC-derived CO inhibits platelet aggregation,25 indicating a potential role for this gas in the development of thromboreistance after blood vessel injury.54,55 In addition, the release of CO by SMCs may also serve to preserve blood flow at sites of vascular damage by reducing blood vessel spasm and SMC proliferation.24

The physiological significance of our finding is further suggested by the observation that the releasate from collagen-activated platelets stimulates HO-1 expression. SMCs may be exposed to products released by adherent, activated platelets at a site of vascular injury. This HO-1 stimulatory effect of the platelet releasate is mediated by PDGF, because a neutralizing antibody directed against PDGF reverses its induction of HO-1 protein. The ability of platelets to induce HO-1 expression provides a mechanism whereby antioxidant heme metabolites and CO are specifically induced at sites of vascular trauma. In this model, circulating platelets are recruited to sites of vascular injury, where they are activated by interaction with subendothelial collagen, resulting in the release of PDGF and the local expression of HO-1. This limited focal induction of HO-1 to sites of vascular damage may be of physiological significance, because a recent study demonstrated that the global induction of HO-1 in the vasculature and the subsequent release of large amounts of CO contribute to the severe hypotension associated with endotoxin shock.29

The ability of PDGF to stimulate HO-1 gene expression and CO synthesis contrasts with our earlier findings showing...
that PDGF blocks inducible NO synthase gene expression and NO formation in VSMCs.34–36 These results indicate that PDGF exerts a divergent regulatory effect on the production of biologically active gases by vascular cells. They further suggest that at sites of vascular injury, where endothelial cells are lost or damaged and PDGF is present, the predominant gaseous messenger released by the blood vessel is CO. Interestingly, Motterlini et al56 recently demonstrated that HO-1–derived CO is the principal in vivo gaseous modulator of blood pressure after vascular surgery. Similarly, Pannen et al57 showed that CO rather than NO serves as the primary regulator of hepatic perfusion after hemorrhagic shock. These findings indicate that during conditions of vascular stress, blood vessels switch gaseous monoxygenase production from the synthesis of highly reactive and potentially toxic NO to the nonreactive stable gas CO. This shift in diatomic gas formation during stress conditions may prevent the potentially harmful actions of NO while still retaining the beneficial effects associated with soluble guanylate cyclase activation.

In conclusion, the present study demonstrates that PDGF induces HO-1 gene expression and the generation of CO in VSMCs. The induction of HO-1 may play an important cytoprotective role by catalyzing heme and by generating antioxidant molecules. In addition, the HO-1–catalyzed production of guanylate cyclase–stimulatory CO may serve to promote blood flow and fluidity at sites of vascular injury.

Acknowledgments
This study was supported by National Heart, Lung, and Blood Institute grants HL-59976 and HL-36045, the Veterans Affairs Merit Review Board, and a Grant-in-Aid from the American Heart Association. The authors thank Dr Genevieve Sparagna for assistance with the measurement of reactive oxygen species and Lan Liao for providing the vascular smooth muscle cells.

References


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doi: 10.1161/01.ATV.19.11.2666

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