HO-1 and CO Decrease Platelet-Derived Growth Factor-Induced Vascular Smooth Muscle Cell Migration Via Inhibition of Nox1

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Objective—Heme oxygenase-1 (HO-1), via its enzymatic degradation products, exhibits cell and tissue protective effects in models of vascular injury and disease. The migration of vascular smooth muscle cells (VSMC) from the medial to the intimal layer of blood vessels plays an integral role in the development of a neointima in these models. Despite this, there are no studies addressing the effect of increased HO-1 expression on VSMC migration.

Results and Methods—The effects of increased HO-1 expression, as well as biliverdin, bilirubin, and carbon monoxide (CO), were studied in vitro models of VSMC migration. Induction of HO-1 or CO, but not biliverdin or bilirubin, inhibited VSMC migration. This effect was mediated by the inhibition of Nox1 as determined by a range of approaches, including detection of intracellular superoxide, nicotinamide adenine dinucleotide phosphate oxidase activity measurements, and siRNA experiments. Furthermore, CO decreased platelet-derived growth factor-stimulated, redox-sensitive signaling pathways.

Conclusion—Herein, we demonstrate that increased HO-1 expression and CO decreases platelet-derived growth factor-stimulated VSMC migration via inhibition of Nox1 enzymatic activity. These studies reveal a novel mechanism by which HO-1 and CO may mediate their beneficial effects in arterial inflammation and injury. (Arterioscler Thromb Vase Biol. 2010;30:98-104.)

Key Words: carbon monoxide ■ heme oxygenase-1 ■ NADPH oxidase ■ Nox1 ■ vascular smooth muscle
Materials
Tricarbonyldichlororuthenium (II) dimer (CORM-2), ruthenium (III) chloride hydrate (RuCl3), peg-SOD, platelet-derived growth factor BB (PDGF-BB), dihydroethidium, Triton X-100, dimethyl sulfoxide, and reduced NADPH were from Sigma (St. Louis, Mo). Diphenylene iodonium was from Calbiochem (San Diego, Calif). Lucigenin was from Alexis (San Diego, Calif). Antibodies against total and phospho-ERK, p42/p44, Jun NH2 terminal kinase, and p38 antibodies were from Cell Signaling Technology Inc. (Danvers, Mass). Co(III) protoporphyrin IX chloride was from Frontier Scientific (Logan, Utah).

Cell Culture
Rat aortic smooth muscle cells (RASMC) were isolated by collagenase/elastase digestion and maintained in DMEM with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. RASMC were used at passages 3 to 8.

Adenoviral Vectors
Adenovirus expressing recombinant β-galactosidase and recombinant rat HO-1 have been described elsewhere.19

HO Activity Assay
The activity of HO enzymes was determined in cell extracts by measuring conversion of hemin to bilirubin as previously described.20

Wound Migration Assay
PDGF-induced migration of RASMC was measured by wounding a monolayer of cells and monitoring the decrease in area after 18 hours.

Measurement of Intracellular \( \text{O}_2^\cdot \)
PDGF-induced intracellular \( \text{O}_2^\cdot \) production was evaluated by measuring the conversion of dihydroethidium to hydroxyethidium in a fluorometer (TECAN infinite M200).

Preparation of 28 000g Membrane Fraction
Membrane fractions were prepared as previously described.21

NADPH Oxidase Activity in Cell Culture
NADPH oxidase activity was measured by monitoring lucigenin (5 μmol/L) chemiluminescence or cytochrome c reduction.21

Preparation of Aorta for NADPH Oxidase Activity
The medial layers of aorta were isolated, membrane fractions were prepared, and NADPH oxidase activity was measured by lucigenin (5 μmol/L) chemiluminescence.21

Statistics
Statistics were performed using Graphpad Prizm software. Data were analyzed by 1-way ANOVA followed by Tukey test for multiple comparisons. For grouped analysis, data were analyzed by 2-way ANOVA followed by Bonferroni post hoc test.

Results
HO-1/CO Inhibits RASMC Migration
Increased expression of HO-1 has been shown to modulate VSMC proliferation,8 but not VSMC migration. Therefore, we examined PDGF-induced migration of RASMC with or without previous treatment with HO-1 adenovirus or control β-galactosidase adenovirus. In all cases, cells were treated with adenovirus 24 hours before stimulation with PDGF. As seen in Figure 1B, overexpression of HO-1 mediated by infection of cells with HO-1 adenovirus resulted in decreased RASMC migration. The inhibition of VSMC migration correlated with both increased HO-1 protein expression and activity (Figure 1A). Similar results were obtained using the chemical inducer of HO-1, Co(III) protoporphyrin IX chloride (supplemental Figure I).

Figure 1. Inhibition of PDGF-induced RASMC migration by HO-1 and CO. (A) Protein expression levels and HO activity in control, adenovirus expressing recombinant β-galactosidase, or HO-1 adenovirus-treated cells. (B), (C), (D) PDGF-induced (10 ng/mL) RASMC migration was measured by the wound assay. Cells were incubated with HO-1 adenovirus or adenovirus expressing recombinant β-galactosidase, or HO-1 adenovirus-treated cells. PDGF, Co(III) protoporphyrin IX chloride (supplemental Figure II).

HO-1 catalyzes the breakdown of heme into CO, free iron, and biliverdin (and subsequently bilirubin). To determine which of these products are involved in the regulation of PDGF-induced RASMC migration by HO-1, we treated cells with CO gas (250 ppm), biliverdin (30 μmol/L), or bilirubin (10 μmol/L). Only the addition of CO to the cells decreased PDGF-induced migration (Figure 1C), providing evidence that CO is responsible for the antimigratory effects seen with increased HO-1 expression. As an alternative to CO gas, we also tested whether a CO-releasing molecule, CORM-2, could inhibit RASMC migration. CORM-2 dose-dependently inhibited RASMC migration (Figure 1D). Importantly, RuCl3 did not affect PDGF-induced migration, demonstrating that the effect of CORM-2 was attributable to CO and not the presence of the ruthenium base compound. Similar results were obtained using the Boyden chamber assay (supplemental Figure II).
We hypothesized that the antimigratory effects of CO might be mediated via inhibition of NADPH oxidase-derived ROS. Using quantitative reverse-transcription polymerase chain reaction, we confirmed the expression of Nox1 and Nox4, but not Nox2, in our RAMSC cultures (supplemental Figure III). Our first approach to elucidate a possible role for ROS and NADPH oxidase in the CO-dependent inhibition of VSMC migration was to evaluate levels of $\text{O}_2^-$ in RASMC in response to PDGF. We observed that PDGF induced a rapid and sustained increase in $\text{O}_2^-$ levels (supplemental Figure IV) as measured by dihydroethidium fluorescence. To determine if the ROS production was NADPH oxidase-dependent, we preincubated the cells with the nonspecific flavo-protein inhibitor diphenylene iodonium, which is often used as a preliminary indicator of NADPH oxidase activity. Diphenylene iodonium abolished PDGF-induced increases in ROS levels, suggesting a possible role for NADPH oxidase in PDGF-induced $\text{O}_2^-$ production (Figure 2A). PEG-SOD (10 U/mL) was used to validate the specificity of the assay for $\text{O}_2^-$.

To determine whether CO gas (250 ppm) or CORM-2 could inhibit PDGF-stimulated increases in $\text{O}_2^-$, RASMC were treated with CO gas or CORM-2, and 30 minutes later RASMC were stimulated with PDGF. Superoxide production was monitored by dihydroethidium as described. Both CO gas and CORM-2, but not RuCl$_3$, decreased PDGF-induced $\text{O}_2^-$ production (Figure 2B).

**HO-1/CO Inhibits PDGF-Stimulated Increases in $\text{O}_2^-$**

We hypothesized that the antimigratory effects of CO might be mediated via inhibition of NADPH oxidase-derived ROS. Using quantitative reverse-transcription polymerase chain reaction, we confirmed the expression of Nox1 and Nox4, but not Nox2, in our RAMSC cultures (supplemental Figure III). Our first approach to elucidate a possible role for ROS and NADPH oxidase in the CO-dependent inhibition of VSMC migration was to evaluate levels of $\text{O}_2^-$ in RASMC in response to PDGF. We observed that PDGF induced a rapid and sustained increase in $\text{O}_2^-$ levels (supplemental Figure IV) as measured by dihydroethidium fluorescence. To determine if the ROS production was NADPH oxidase-dependent, we preincubated the cells with the nonspecific flavo-protein inhibitor diphenylene iodonium, which is often used as a preliminary indicator of NADPH oxidase activity. Diphenylene iodonium abolished PDGF-induced increases in ROS levels, suggesting a possible role for NADPH oxidase in PDGF-induced $\text{O}_2^-$ production (Figure 2A). PEG-SOD (10 U/mL) was used to validate the specificity of the assay for $\text{O}_2^-$.

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**HO-1/CO Inhibits PDGF-Stimulated NADPH Oxidase Activity**

To more directly test the hypothesis that HO-1/CO inhibits PDGF-induced NADPH oxidase activity, NADPH-dependent $\text{O}_2^-$ production was measured in 28 000g membrane fractions from treated cells using lucigenin-enhanced chemiluminescence. Treatment of RASMC with PDGF caused a 2-fold increase in NADPH-dependent $\text{O}_2^-$ production that was inhibited by diphenylene iodonium and scavenged by PEG-SOD (Figure 3A). Importantly, L-NAME or allopurinol had no effect on NADPH-dependent superoxide production, providing evidence that the superoxide measured was not from uncoupled nitric oxide synthase or xanthine oxidase activity. The effect of diphenylene iodonium or PEG-SOD on NADPH oxidase activity was further confirmed via measurement of $\text{O}_2^-$ using cytochrome $c$ reduction in similar experiments (Figure 3B). We next tested whether induction of HO-1 expression could inhibit PDGF-induced NADPH oxidase activity in RASMC. The increased HO-1 expression and activity attributable to HO-1 adenovirus resulted in significant inhibition of PDGF-induced NADPH oxidase activity (Figure 3C).

To determine whether the effect of HO-1 on NADPH oxidase activity was attributable to CO, RASMC were...
pretreated with different concentrations of CO gas (100 or 250 ppm) or 100 μmol/L CORM-2 and stimulated with PDGF. CO gas as well as CORM-2 decreased PDGF-induced NADPH oxidase activity (Figure 4A). In ex vivo experiments, strips of medial smooth muscle were preincubated with or without CORM-2 for 30 minutes, followed by an additional 45 minutes of incubation with PDGF. Membrane fractions from the treated medial strips were then prepared and NADPH oxidase activity measured. CORM-2, as well as peg-SOD, inhibited NADPH oxidase activity in the medial layer of the rat aorta (Figure 4B), providing evidence that CO could inhibit NADPH oxidase activity in an intact tissue.

We next sought to determine whether CO could directly inhibit NADPH oxidase activity. Figure 4C shows representative tracings of real-time lucigenin chemiluminescence from membrane fractions derived from control or PDGF-stimulated cells. Addition of NADPH to the membrane fraction from PDGF-stimulated cells results in a >2-fold increase in lucigenin chemiluminescence compared to membrane fraction from control cells. Addition of CO saturated buffer (final concentration of ~9 μmol/L based on the solubility of CO in water) to the PDGF-treated sample after NADPH addition caused a rapid decrease in lucigenin chemiluminescence.

**Effects of CO on PDGF-Induced Migration Are Mediated by Inhibition of Nox1**

To test whether the inhibition of migration by HO-1/CO is mediated by inhibition of Nox1, we transiently transfected RASMC with siRNA against Nox1, Nox4, or a nontargeting siRNA. Transfection of RASMC with Nox1 or Nox4 siRNA decreased their mRNA levels by ~85% and 60%, respectively, after 72 hours (supplemental Figure VA). Nox4, but not Nox1 or nontargeting siRNA, significantly inhibited basal NADPH oxidase activity measured in membrane fractions from unstimulated cells (Figure 5A). Alternatively, Nox1 siRNA, but not nontargeting or Nox4 siRNA, reduced PDGF-induced NADPH oxidase activity.
activity to basal levels as measured by lucigenin chemiluminescence in membrane fractions from PDGF-stimulated cells (Figure 5B). Importantly, CORM-2 inhibited PDGF-stimulated NADPH oxidase activity in Nox4, but not Nox1, siRNA-treated cells. Both Nox1 and Nox4 siRNA, but not nontargeting siRNA, significantly reduced PDGF-induced migration; however, only cells treated with nontargeting or Nox4 siRNA were still sensitive to inhibition of migration by CORM-2 (Figure 5C). Similar results were obtained using the Boyden chamber assay (supplemental Figure VB). These data strongly support the hypothesis that whereas Nox1 and Nox4 are both important in mediating RASMC migration, HO-1/CO inhibits RASMC migration via inhibition of Nox1.

**CO Inhibits PDGF-Stimulated Redox-Sensitive Signaling Pathways**

NADPH oxidase-derived ROS have been shown to activate pro-growth, pro-migratory pathways in RASMC. Serum-starved RASMC were stimulated with PDGF with or without previous treatment with CORM-2. Western blot analysis revealed that treatment of RASMC with CORM-2 resulted in decreased phosphorylation of ERK1/2, p38, Jun NH2 terminal kinase, and AKT when compared to control samples (Figure 6A–F). Each of these pathways is known to be involved in RASMC migration because specific inhibition of these pathways results in decreased migration. Finally, RuCl₃ had no effect on these redox-sensitive signaling pathways (supplemental Figure VI).

**Discussion**

The protective role of HO-1 has been studied in the context of various vascular diseases. In children lacking a functional HO-1 allele, atherosclerosis (hyperlipidemia, fatty streaks, and plaques) is increased. Moreover, HO-1 overexpression reduces lesional area in the aorta of apolipoprotein E⁻/⁻ mice. HO-1 is induced after balloon angioplasty in rats, and neointimal hyperplasia is exacerbated in HO-1–null mice. Additionally, both CO₈,₂⁷ and biliverdin²⁸ have been shown to inhibit neointima formation. Interestingly, to date, there exists no study exploring the effect of HO-1/CO on vascular smooth muscle cell migration, an integral process to the development of atherosclerosis and restenosis after angioplasty.

Herein, we provide evidence that induction of HO-1 expression in RASMC inhibits PDGF-induced migration. CO mediated the inhibition of migration by HO-1 because CO gas or the CO releasing molecule, CORM-2, but not biliverdin or bilirubin, was able to inhibit RASMC migration. Therefore, we focused on the mechanism by which CO inhibits PDGF-induced RASMC migration.

Many of the pro-migratory signaling pathways stimulated by PDGF are mediated by ROS. In VSMC, antioxidants block migration in response to PDGF. In contrast, VSMC extracted from Nox1 or p22phox-overexpressing mouse aortas exhibit an increase in PDGF-stimulated migration. These studies led us to hypothesize that CO inhibits RASMC migration via inhibition of NADPH oxidase activity. We demonstrate that CO inhibits PDGF-induced O₂⁻⁻ production in intact cells as well as NADPH oxidase activity in membrane fractions from PDGF-stimulated cells. By isolating the medial layer of rat aorta, we were likewise able to demonstrate the inhibition of PDGF-stimulated NADPH oxidase activity in an ex vivo setting. Furthermore, direct addition of CO to the membrane fraction isolated from PDGF-treated cells rapidly decreased NADPH-stimulated O₂⁻⁻ production. These data provide compelling evidence that CO inhibits NADPH oxidase activity in RASMC by directly interacting with the enzyme.

Aortic VSMC express both Nox1 and Nox4. Additionally, recent studies demonstrate an important role for either Nox1 or Nox4 in VSMC migration. In particular, Lee et al demonstrated that VSMC derived from Nox1-null mice exhibit decreased PDGF-induced migration, whereas VSMC derived from smooth muscle-specific Nox1-
overexpressing mice exhibit enhanced migratory responses. In this study, the specific involvement of Nox1 in PDGF-stimulated NADPH oxidase activity was revealed by experiments demonstrating that this activity could be prevented by Nox1 siRNA but not Nox4 siRNA. Both Nox1 siRNA and Nox4 siRNA were able to prevent PDGF-induced migration to various extents; however, cells treated with Nox1 siRNA were resistant to further inhibition by CO, whereas Nox4-treated cells were not. From these observations, we draw the conclusion that CO inhibits Nox1-dependent ROS production, leading to inhibition of VSMC migration.

Early studies on Nox2 (ie, gp91phox, cytochrome b558) used CO as a tool to study the 2 heme moieties within the protein. These in vitro studies using partially purified enzyme generally concluded that CO binds the heme group in Nox2 poorly, if at all. One inherent drawback of those studies, however, is that partially purified Nox2 was used, which likely contained only the membrane components of the enzyme complex. At the time, it was not known that additional cytosolic subunits, such as p47 and p67, are required in the enzymatic complex. This leaves open the possibility that the conformation of Nox2 may be altered when bound to these subunits, which could allow for interaction of CO with the heme group. More recent studies examining the effect of CO on Nox2 have demonstrated changes in the heme absorbance spectra in response to CO. Despite this demonstration, these studies did not address whether CO could directly inhibit NADPH oxidase enzymatic activity. Rather, these studies demonstrate decreased NADPH oxidase activity in intact cells treated with CO and thus could not distinguish whether CO was inhibiting signaling processes leading to the activation of Nox2 or directly inhibiting Nox2 activity. Our studies demonstrating a direct effect of CO on NADPH oxidase enzymatic activity are unique in this respect. Taken together, our studies along with those showing alteration of the heme spectra of Nox2 suggest that CO may inhibit NADPH oxidase enzymatic activity via binding to 1 or both of the heme groups in the Nox subunit of the enzyme complex.

As discussed, many of the pro-migratory pathways stimulated by PDGF are redox-sensitive. One important mediator of growth factor responses in VSMC is Akt. ROS sensitivity of Akt is conferred by the phosphorylation of mitogen-activated protein kinase APK-2 by p38, a redox-sensitive kinase. This leads to recruitment of mitogen-activated protein kinase APK-2 to an Akt–p38 mitogen-activated protein complex and phosphorylation of Akt. Besides p38 mitogen-activated protein kinase, other mitogen-activated protein kinases are sensitive to ROS. The Jun NH2 terminal kinase activation in response to angiotensin II is blocked by antioxidants. ERK1/2 was the earliest discovered redox-sensitive kinase having been shown to be activated by direct addition of hydrogen peroxide to cells. Additionally, Janus tyrosine kinases activate ERK1/2 in VSMC, and Janus tyrosine kinase-2 activation in response to angiotensin II was shown to be attenuated by NADPH oxidase inhibitors. In agreement with these findings, we were able to demonstrate that, in RASMNC, inhibition of Nox1 activity by CO correlates with decreased phosphorylation of AKT, as well as the mitogen-activated protein kinases, p38, ERK1/2, and Jun NH2 terminal kinase-1.

The use of CORM-2 in this study raises some question as to whether the effects seen are attributable to direct effects of CO or secondary effects of CORM-2. In particular, CORM-2 may induce HO-1 and thus contribute to the effects of CORM-2 in the wound migration assay. However, our studies show an effect of CO gas in this model of migration assay. Furthermore, CORM-2 also inhibits RASMNC migration in the Boyden chamber assay of migration. The time frame of this assay (4 hours) is such that one would not expect increased HO expression to be a factor. In terms of NADPH oxidase activity, these assays are all on a short time scale, and thus induction of HO-1 by CORM-2 cannot be a factor. Concerns that the ruthenium metal center might be mediating the effects of CORM-2 were assuaged by the demonstration that ruthenium chloride had no effects in the models studied.

In conclusion, our studies demonstrate that in RASMNC CO inhibits Nox1-dependent migration stimulated by PDGF. Furthermore, we show that CO inhibits Nox1 activity, likely via direct interaction with the enzyme complex. These studies reveal a novel mechanism by which increased HO-1 expression and activity and HO-1-derived CO may mediate their beneficial effects in arterial inflammation and injury.

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Disclosure
None.

References


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Expanded Methods

**Materials.** Tricarbonyldichlororuthenium (II) dimer (CORM-2), ruthenium (III) chloride hydrate (RuCl$_3$), peg-SOD, platelet-derived growth factor BB (PDGF-BB), dihydroethidium (DHE), Triton X-100, dimethyl sulfoxide, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were from Sigma (St. Louis, MO). Diphenylene iodonium (DPI) was from Calbiochem (San Diego, CA). Lucigenin was from Alexis (San Diego, CA). Antibodies against total and phospho-ERK, p42/p44, JNK and p38 antibodies were from Cell Signaling Technology Inc. (Danvers, MA). Co(III) Protoporphyrin IX chloride (CoPP) was from Frontier Scientific (Logan, UT).

**Cell Culture.** RASMC were isolated by collagenase/elastase digestion and maintained in DMEM with 10% FBS, 2mM L-glutamine, 100 units/ml penicillin, and 100µg/ml streptomycin. RASMC were used at passages 3-8.

**Adenoviral vectors.** Adenovirus expressing recombinant β-galactosidase (AdLacZ) and recombinant rat HO-1 have been described elsewhere. Recombinant adenoviruses were produced in human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA), extracted and purified through two cesium chloride gradients, and their titer determined.

**Heme oxygenase activity assay.** The activity of heme oxygenase enzymes was determined in cell extracts as previously described. Briefly, cell extracts were added to a reaction mixture containing potassium phosphate buffer (100 mM, pH 7.4) containing NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 units), 3 mg of rat liver cytosol, MgCl$_2$ (0.2 mM) and hemin (20 µM). This mixture was incubated in the dark at 37°C for one hour and then halted by the addition of 1 ml of chloroform and the concentration of bilirubin...
produced was calculated by the difference in absorption between 464 and 530 nm, with a molar extinction coefficient of 40 mM$^{-1}$cm$^{-1}$.

**Wound Migration Assay.** RASMC were grown to confluence and serum starved with 0.1% BSA for 48h (or 24h for siRNA experiments). In experiments using CoPP or adenovirus, the cells were treated with 1 µM CoPP, AdHO-1 or AdLacZ for the final 24h of serum starvation and washed before PDGF treatment. Treatment with CO gas, SnPP, biliverdin or bilirubin began 30 min prior to PDGF treatment. The concentration of CO chosen was based on previous reports demonstrating that 250ppm is within a therapeutic range$^3$. The concentration of biliverdin/bilirubin used was based on the highest dose that did not cause toxicity (data not shown). The cell monolayers were then wounded by scratching lengthwise with a sterile pipette tip. Images were obtained at time 0 and 18h after PDGF stimulation. Wound area was determined using Photoshop CS3 extended software (Adobe) and migration was calculated as percent decrease in wound area.

**Measurement of intracellular O$_2^-$**. RASMC were grown to confluence and serum starved with 0.1% BSA for 48h. Cells were pre-treated with test compounds as indicated in the figure legends followed by addition of PDGF. DHE (5µM) was added after 30 min and the cells were incubated for an additional 15 min. The cells were then washed 3x with cold PBS and lysed in 0.1% Triton X-100 plus protease inhibitors. O$_2^-$ was evaluated by measuring the conversion of DHE to hydroxyethidium in a fluorimeter (TECAN infinite M200) using an excitation and emission wavelengths of 495nm and 567nm, respectively.

**Preparation of 28,000-x g Membrane Fraction.** Membrane fractions were prepared as previously described$^4$. Treated cells were washed with PBS, harvested using 0.05% trypsin and collected by centrifugation for 5 min at 1000 x g. The supernatant was discarded and the pellet was resuspended in Tris-sucrose buffer (10mM Tris HCl, pH7.1, and 0.34M Sucrose) with
protease inhibitors and sonicated on ice 4x for 15 sec. The lysates were centrifuged twice at 28,000-x g at 4°C for 15 min, discarding the supernatant each time. The pellet was resuspended in 100µl of Tris-Sucrose buffer and assayed for NADPH oxidase activity.

**NADPH Oxidase Activity in Cell Culture.** NADPH oxidase activity was measured by monitoring lucigenin (5µM) chemiluminescence or cytochrome c reduction. Briefly, membrane fractions (10µg of protein) were diluted in Tris-sucrose buffer with protease inhibitors. Lucigenin (5µM) was added and the baseline luminescence measured in 30 sec intervals over 5 min. NADPH (100µM) was then added and the chemiluminescence measured again over the same interval. The specificity of O₂⁻ measured was confirmed by adding peg-SOD (10 units/ml). Alternatively, 10µM DPI was added to the membrane fraction to confirm NADPH oxidase activity. For cytochrome c reduction assays, membrane fractions (100µg protein) were diluted in Tris-sucrose buffer with protease inhibitors. Cytochrome c (50µM) and NADPH (100µM) were added in the presence or absence of peg-SOD (10units/ml) or DPI (10µM). Cytochrome c reduction was measured by reading absorbance at 550nm.

**Preparation of Aorta for NADPH Oxidase Activity.** The medial layers of aorta were isolated as previously described. Aorta were dissected from 300g Sprague Dawley rats and placed in ice-cold Krebs buffer (20mM HEPES, 119mM NaCl, 4.6mM KCl, 1.0mM MgSO₄-7H₂O, 0.4mM KH₂PO₄, 5mM NaHCO₃, 1.2mM CaCl₂ and 5.5mM Glucose). The adventitia was removed by peeling away from the medial layer, and the endothelium was removed by gently scraping with forceps. The medial layers were pretreated for 30 min with CORM-2 (100µM) and then stimulated with PDGF (25ng/ml) for 45 min. Finally, the tissue was homogenized in TRIS-Sucrose buffer containing protease inhibitors. Membrane fractions were prepared and NADPH oxidase activity was measured.
**Nox1 and Nox4 siRNA.** All siRNAs were designed and synthesized by Dharmaco. siRNA transfection was performed using a lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. Scrambled siRNAs were used as negative controls. To achieve optimal transfection efficiency, transfection reagent, siRNA, cell density, and time of transfection were optimized. Gene silencing was monitored by qRT-PCR 48h after transfection.

**Quantitative RT-PCR.** RNA was isolated using the RNeasy Mini Kit (Qiagen). After treating with DNase I, RNA was reverse transcribed using random hexamers and reverse transcriptase enzyme (Applied Biosystems). Primer/probe mix for Nox1, Nox2, or Nox4 was from Applied Biosystems. qPCR was run in the ABI PRISM 7700 Sequence Detection System. The threshold copy (CT) numbers for target and β2-microglobulin templates were calculated by the SDS software (Applied Biosystems). Genomic DNA contamination was assessed by omitting reverse transcriptase. Target gene expression was normalized to β2-microglobulin.

**Statistics.** Statistics were performed using Graphpad Prizm software. Data were analyzed by one-way ANOVA followed by Tukey’s test for multiple comparisons. For grouped analysis data were analyzed by two-way ANOVA followed by Bonferonni’s post hoc-test.

**Boyden Chamber Chemotaxis Assay:** Cell migration was assayed using a 48-well Boyden chamber (Neuro Probe, Inc., Gaithersburg MD) and collagen (1mg/ml) coated polycarbonate filters (8µm). Confluent cells were serum starved with DMEM with 0.1% BSA for 48h, trypsinized and resuspended at 4x10^5 cells/ml. The lower chamber was filled with or without chemoattractant (PDGF, 10ng/ml). The upper chamber was filled with 50ul of cell suspension. CORM-2 or RuCl₃ was added to the upper and lower chambers. After incubation at 37°C and 5%CO₂ for 4hrs, the cells were scrapped from upper surface. The membrane was stained with Hoechst stain (Sigma) to visualize nuclei. Images were acquired using a Nikon Eclipse
epifluorescent microscope. The number of migrated cells was determined by counting nuclei using Metamorph image analysis software.

References


Supplemental Figure 1. Chemical induction of HO-1 inhibits PDGF-stimulated RASMC migration and NADPH oxidase activity. (A) PDGF (10ng/ml)-induced RASMC migration was measured by the wound assay. Cells were incubated with CoPP (1µM) for 24h prior to PDGF stimulation. Data represent the mean +/- S.E.M. of three individual experiments. * = Statistically different (p<0.05) from PDGF alone. (C) Protein expression levels in control or CoPP treated cells.
Supplemental Figure 2. CO inhibits RASMC chemotaxis. Cells were placed in the top chamber of a 48 well Boyden Chamber and chemotaxis assessed in response to PDGF in the bottom chamber over 4h. In some wells CORM-2 or RuCl₃ were added to both sides of the Boyden chamber. Data represent mean +/- S.E.M. of 4 experiments. * = significantly different from PDGF alone (p<0.05).
Supplemental Figure 3. Expression of Nox homologues in RASMC. Expression of Nox1, Nox2, and Nox4 in RASMC was analyzed by qRT-PCR. Data are normalized to b-microglobulin and shown relative to Nox1 expression in quiescent RASMC and represent the mean +/- S.E.M. of three experiments.
Supplemental Figure 4. PDGF stimulation of RASMC induces rapid and sustained production of $\text{O}_2^-$. Serum starved RASMC were stimulated with PDGF for the indicated times and intracellular $\text{O}_2^-$ measured as described in materials and methods. * = Statistically different (p<0.05) from control (t=0) sample.
Supplemental Figure 5. Inhibition of RASMC chemotaxis by CO is mediated by inhibition of Nox1. (A) Knockdown of Nox1 and Nox4 by siRNA as assessed by qRT-PCR. (C) PDGF-induced chemotaxis in Nox1, Nox4 or NT siRNA transfected RASMC with or without CORM-2 (100µM) treatment. Data represent the mean +/- S.E.M. of 3 individual experiments. * = Statistically different (p<0.05) from NT siRNA w/o CORM-2 treatment. # = statistically different (p<0.05) from Nox4 siRNA w/o CORM-2 treatment.
Supplemental Figure 6. RuCl₃ does not inhibit redox-dependent signaling events in RASMC. Serum starved RASMC were stimulated with 10ng/ml PDGF for the indicated times with or without treatment with RuCl₃ and analyzed by Western analysis using antibodies against phospho- or total AKT, p38, JNK, or ERK1/2. Data are representative of 3 individual experiments.