Nitro-Fatty Acid Inhibition of Neointima Formation After Endoluminal Vessel Injury


Rationale: Fatty acid nitroalkenes are endogenously generated electrophilic byproducts of nitric oxide and nitrite-dependent oxidative inflammatory reactions. Existing evidence indicates nitroalkenes support posttranslational protein modifications and transcriptional activation that promote the resolution of inflammation.

Objective: The aim of this study was to assess whether in vivo administration of a synthetic nitroalkene could elicit antiinflammatory actions in vivo using a murine model of vascular injury.

Methods and Results: The in vivo administration (21 days) of nitro-oleic acid (OA-NO2) inhibited neointimal hyperplasia after wire injury of the femoral artery in a murine model (OA-NO2 treatment resulted in reduced intimal area and intima to media ratio versus vehicle- or oleic acid (OA)-treated animals, P < 0.0001). Increased heme oxygenase (HO)-1 expression accounted for much of the vascular protection induced by OA-NO2 in both cultured aortic smooth muscle cells and in vivo. Inhibition of HO by Sn(IV)-protoporphyrin or HO-1 small interfering RNA reversed OA-NO2-induced inhibition of platelet-derived growth factor-stimulated rat aortic smooth muscle cell migration. The upregulation of HO-1 expression also accounted for the antstenotic actions of OA-NO2 in vivo, because inhibition of neointimal hyperplasia following femoral artery injury was abolished in HO-1−/− mice (OA-NO2-treated wild-type versus HO-1−/− mice, P = 0.016).

Conclusions: In summary, electrophilic nitro-fatty acids induce salutary gene expression and cell functional responses that are manifested by a clinically significant outcome, inhibition of neointimal hyperplasia induced by arterial injury. (Circ Res. 2009;105:965-972.)

Key Words: fatty acids ▪ arteries ▪ stenosis ▪ nitric oxide

Basal and inflammatory redox signaling reactions are broadly regulated by NO. For example, secondary reactions of NO, promoted by a prooxidative inflammatory milieu, yield oxidizing, nitrosating and nitrating species that transduce NO signaling via cGMP-independent and -dependent mechanisms. Nitro-fatty acid (NO2-FA) derivatives are 1 class of lipid oxidation byproducts generated by NO-mediated inflammatory reactions. Existing insight indicates that the robust and reversible electrophilic reactivity of NO2-FA supports posttranslational protein modifications and transcriptional activation reactions that promote the resolution of inflammation. In this regard, in vitro studies reveal NO2-FA inhibit platelet aggregation, neutrophil activation, nuclear factor κB–mediated cytokine release and stimulate heme oxygenase (HO)-1 expression, all via cGMP-independent mechanisms. NO2-FA also serve as ligands for peroxisome proliferator-activated receptor γ, a nuclear lipid receptor that regulates the expression of cell differentiation, development, and inflammatory-related genes.

In the context of vascular responses to inflammation, NO2-FA may part inhibit vascular smooth muscle cell proliferation via activation of the Nrf2 (nuclear factor erythroid 2-related factor 2)/Keap 1 (Kelch-like ECH-associating protein) pathway. Under basal conditions, Keap1 represses nuclear translocation of Nrf2 and Nrf2-dependent transcription. When cells are exposed to reactive species, including thiol-reactive electrophiles such as NO2-FA, Nrf2 escapes Keap1-mediated repression to activate antioxidant responsive element-regulated gene expression. Expression of ARE-dependent gene products, including HO-1, attenuates inflammatory responses and maintains cellular redox homeostasis.

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HO-1 is the rate-limiting enzyme in the degradation of heme, yielding biliverdin, iron, and carbon monoxide. HO-1, especially when upregulated, limits vascular inflammatory injury via metabolic, vasodilatory, and immunemodulatory actions. Nitro-linoleic acid has recently been reported to transcriptionally activate cultured vascular endothelial HO-1 expression via peroxisome proliferator-activated receptor γ- and NO-independent mechanisms, but no phenotypic responses to elevated HO-1 expression via this mechanism have been observed either in vitro or in vivo. Herein, we reveal that the extended in vivo administration of the nitroalkene derivative of oleic acid at nanomolar concentrations potently inhibits neointimal hyperplasia after arterial injury via HO-1–dependent mechanisms, revealing the ability of endogenously produced inflammatory byproducts to limit the progression of vascular inflammatory injury.

Methods

OA-NO2 Synthesis
Nitro-oleic acid (OA-NO2) used in this study was synthesized via nitroselenation as previously described.

Wire-Mediated Vascular Injury
All animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Approval 0702181). Vehicle (V), OA (2 mg/kg per day), or OA-NO2 (2 mg/kg per day) were delivered by osmotic mini-pumps (21 days delivery, ALZET, Durect Corp). Sn(IV) protoporphyrin (SnPP) was administered to mice (IP, 50 μmol/L) immediately before unilateral femoral artery injury. Serum OA-NO2 levels in treated mice were quantified using 13C isotope dilution by reverse-phase high-performance liquid chromatography (HPLC) with electrospray ionization triple quadrupole mass spectrometry (ESI MS/MS) detection in the negative ion mode.

Detection and Quantitation of OA-NO2 in Serum
Serum OA-NO2 levels in treated mice were quantitated using 13C isotope dilution by reverse-phase high-performance liquid chromatography (HPLC) with electrospray ionization triple quadrupole mass spectrometry (ESI MS/MS) detection in the negative ion mode. Multiple reaction monitoring, following the transitions m/z = 326/46 (OA-NO2) and m/z = 344/46 ([13C]OA-NO2), was used to quantify serum OA-NO2 levels following [13C]OA-NO2 internal standard addition before serum lipid extraction.

Vessel Morphometry
Intimal and medial cross-sectional areas of injured and noninjured femoral arteries were measured in three sets of three serial 6 μm thick cross-sections of each artery, spaced at 300-μm intervals. Endothelial cells and smooth muscle cells were visualized by immunofluorescent staining against CD31 and smooth muscle α-actin respectively. Elastic lamina were visualized by autofluorescence.

Immunofluorescence
Cross-sections (6 μm thick) of injured femoral arteries were stained with antibodies against HO-1 or Ki67 followed by incubation with fluororecently labeled secondary antibodies. Images were obtained using a Zeiss confocal microscope. Nuclei were stained using Hoechst stain (10 mg/mL, Sigma-Aldrich Inc, St Louis, Mo). Quantitation of proliferating cells was achieved by dividing the number of Ki67-positive nuclei by the total number of nuclei.

Cells and Cell culture
Rat aortic smooth muscle cells (RASMCs) were isolated via explant and cultured in DMEM containing 10% FBS in 5% CO2 at 37°C. All experiments were performed using RASMCs between passage 3 and 8. Cell proliferation was assessed using the Cyquant NF proliferation assay as described by the manufacturer (Invitrogen, Carlsbad, Calif). Migration studies were performed using the wound assay as described in the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org.

For some experiments, RASMCs were transfected with 50 μmol/L small interfering (si)RNA against HO-1 or nontargeting control siRNA (Dharmacon Lafayette, Colo) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, Calif).

Real-Time Quantitative PCR
Total RNA from RASMCs and femoral artery tissue was isolated with TRIzol and further purified using the RNeasy Mini kit (Qiagen, Valencia, Calif). Complimentary DNA was obtained using iScript reagents (Bio-Rad Laboratories, Hercules, Calif) or SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, Calif) according to the manufacturer’s instructions. Quantitative mRNA expression was assessed using real-time PCR with Taqman Fast Universal PCR Master Mix or Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, Calif) using primers specific for HO-1, actin, or GAPDH. Samples were run in triplicate on the StepOne or Prism 7000 detection systems (Applied Biosystems, Foster City, Calif).

Western Blot Analysis
Protein preparation, SDS-PAGE, and Western analysis were performed as previously. Equal amounts of protein were loaded and both HO-1 (1:5000) and HO-2 (1:1000) were detected using Stressgen antibodies (Stressgen Biotechnologies, Ann Arbor, Mich).

HO Enzyme Activity
HO activity was measured by bilirubin generation in microsomal preparations from mouse liver as described previously.

Statistical Analysis
Results are expressed as means±SD or SEM. Statistical analysis was performed using 1-way ANOVA or unpaired Student t test as appropriate. Differences between groups were assessed by Bonferroni post hoc test. A value of P<0.05 was considered statistically significant. SPSS 15.0 was used for all calculations.

Results

In Vivo Delivery of OA-NO2
To test the effect of NO2-FA on intimal hyperplasia, C57BL/6 mice were administered vehicle (V), oleic acid (OA), or OA-NO2 via osmotic mini-pump implantation immediately before unilateral femoral artery injury. Serum OA-NO2 levels in treated mice were quantified using HPLC–mass spectrometry. Representative chromatograms of serum
lipid extracts reveal identical retention times for both OA-NO₂ administered in vivo and internal standard (Figure 1A). Serum OA-NO₂ levels were significantly greater in OA-NO₂–treated mice (6.21±0.60 nmol/L), compared to V- and OA-treated mice (1.43±0.02 and 1.36±0.08 nmol/L, respectively, P≤0.0001; Figure 1B).

**Inhibition of Neointimal Proliferation by OA-NO₂**

The influence of OA-NO₂ on neointimal formation was investigated in a murine model where endoluminal injury to the common left femoral artery was induced by an angioplasty guide wire. This injury induces a highly reproducible neointima that can be quantified after three to 4 weeks. Figure 2A through 2D shows representative micrographs of injured vessels from V-, OA-, or OA-NO₂–treated animals and the contralateral uninjured femoral artery from V-treated mice. Vessels were isolated and stained for smooth muscle α-actin (red) and endothelial CD31 (blue). Green fluorescence represents autofluorescence of the elastic lamina. Morphometric analysis of injured vessels from V- or OA-
treated animals revealed an intima to medial area ratio of >2, reflecting considerable neointimal hyperplasia. In contrast, injured vessels from OA-NO2–treated mice displayed a significantly reduced intimal area and intima to media ratio compared to V- or OA-treated animals (n=6 to 7 per group, P<0.0001; Figure 2E and 2F). Medial areas in all groups were not significantly different (Figure 2G).

**Induction of HO-1 Expression by OA-NO2**

To investigate whether OA-NO2 induces HO-1 expression in vascular smooth muscle cells in vitro, RASMCs were grown to 100% confluence and maintained in serum-free media. Two hours after incubation with OA-NO2 (50 to 1000 nmol/L), HO-1 mRNA levels increased in a dose-dependent fashion (P<0.01, Figure 3A). Western blot analysis also revealed increased expression of HO-1 protein, with no alterations in HO-2 occurring in response to OA-NO2 (100 to 1000 nmol/L; Figure 3B) after 24 hours. Administration of OA-NO2 in vivo increased HO enzyme activity by 2-fold in liver tissue (1.04±0.18 [V] versus 2.05±0.28 [OA-NO2] nmol bilirubin/mg protein per hour, P=0.02; n=6 animals per group). Furthermore, HO-1 expression was induced in vivo in the vasculature by OA-NO2 treatment. Following wire-induced injury (21 days), arterial segments immunostained for HO-1 reveal that HO-1 is abundantly expressed throughout the vascular wall in OA-NO2–treated mice. In contrast, there was a significantly lower extent of vessel wall HO-1 expression in OA- or V-treated mice (Figure 3C). In addition, quantitative real-time PCR revealed that HO-1 mRNA expression was increased in both injured and the contralateral uninjured femoral artery tissue 3 days after OA-NO2 treatment, compared with V-treated mice following femoral artery injury (Figure 3D). This supports that OA-NO2 is a potent inducer of HO-1 expression both in vitro and in vivo. Levels of HO-2 mRNA expression did not change in all femoral artery treatment groups (Figure 3E).

**Effects of OA-NO2 on Vascular Smooth Muscle Cell Proliferation and Migration**

Treatment of RASMCs with OA-NO2 significantly inhibited cell proliferation at a concentration of 2.5 μmol/L (P<0.001; Figure 4A). This antiproliferative effect of OA-NO2 in vitro
was not attenuated by either addition of the HO-1 inhibitor SnPP (50 μmol/L) or suppression of HO-1 expression by siRNA (50 μmol/L) treatment (Figure 4A and 4B). Effective inhibition of HO-1 expression by siRNA treatment was confirmed by western blotting, where HO-2 expression remained unaffected (Figure 4C). Of note, immunostaining of femoral artery sections with a Ki67 antibody revealed that OA-NO2 significantly inhibited smooth muscle cell proliferation in vivo (P<0.001; Figure 4D).

In addition to proliferation, the migration of vascular smooth muscle cells from the media to the intima after arterial injury is a key step in the development of neointimal hyperplasia. To define whether OA-NO2 influences neointimal formation by limiting vascular smooth muscle cell migration, RASMC monolayers were wounded by scratching. Images taken immediately after wounding and 18 hours later revealed that OA-NO2 (50 to 250 nmol/L) significantly inhibited RASMC migration in a dose-dependent manner, whereas OA had no effect (P<0.01; Figure 5A). These in vitro responses reveal that OA-NO2 preferentially inhibits RASMC migration rather than limiting cell proliferation, because OA-NO2 is at least a 20-fold more potent inhibitor of vascular smooth muscle cell migration.

To test the hypothesis that OA-NO2 inhibits vascular smooth muscle cell migration via induction of HO-1 expression and activity, RASMCs were coincubated with SnPP (50 μmol/L) or HO-1 siRNA and OA-NO2 (250 nmol/L). Both SnPP and HO-1 siRNA reversed the inhibitory effect of OA-NO2 on smooth muscle cell migration (Figure 5A and 5B), indicating a significant role for HO-1 in the inhibition of vascular smooth muscle cell migration by OA-NO2.

HO-1–Mediated Antistenotic Actions of OA-NO2 In Vivo

Two lines of evidence support that HO-1 expression and activity transduces OA-NO2 inhibition of wire-induced neointimal formation. (1) Administration of the HO-1 inhibitor SnPP, from the time of wire-induced injury until pathology evaluation at 21 days, significantly attenuated the antstenotic actions of OA-NO2 in OA-NO2–treated mice (SnPP: 2.20±0.32%, OA-NO2: 1.75±0.27%, P=0.023 versus OA-NO2–treated animals; n=6 to 7 per group). (2) OA-NO2–induced inhibition of neointimal hyperplasia was abolished in OA-NO2–treated HO-1−/− mice (P=0.016; Figure 6). In both SnPP-treated and in HO-1−/− mice, neointima formation was even more pronounced than in wild-type mice. These in vivo findings confirm that induction of HO-1 by NO2-FA predominantly mediates the protection of vessels from neointimal hyperplasia.

Discussion

This is the first report demonstrating that in vivo supplementation of nanomolar concentrations of an endogenous byproduct of nitro-oxidative inflammatory conditions induces tissue-protective actions. Electrophilic NO2-FAs are generated by NO and nitrite (NO2−)-dependent reactions that yield nitrogen dioxide (NO2) as the proximal instigator of fatty acid olefin nitration. Recent reports support that these reactions (1)
are accelerated in the hydrophobic milieu of membrane and lipoprotein compartments and (2) occur at accelerated rates in cells and organs exposed to inflammatory conditions.8,19,20 The addition of \(1^H28\)NO2 to the double bond of unsaturated fatty acids yields an array of regio- and stereoisomers detectable in vivo that display kinetically rapid and reversible Michael addition to proteins.1 Because of the unique physical characteristics of these derivatives, complex metabolic profiles, tissue and subcellular distribution and signaling actions are expected. In vitro studies indicate that NO2-FA will gain access to both the cytosol and nucleus to stimulate redox-dependent transcription factor and nuclear lipid receptor-dependent gene expression.4–6 Existing data support that NO2-FA (1) covalently adduct macromolecules containing nucleophilic centers (eg, thiol and histidine residues of proteins and glutathione)3; (2) reversibly react with water to form nitro-hydroxy derivatives; (3) react with coenzyme A and undergo \(1^H25\)-oxidation15; and (4) become esterified to complex lipids in membranes and lipoproteins.1 Because of these reactivities, the HPLC-MS-based detection of \(1^H25\)nmol/L “free” serum OA-NO2 on continuous osmotic minipump infusion over weeks in mice will underestimate the net pool of potentially bioactive OA-NO2-derived species that could manifest adaptive and antiinflammatory signaling actions.1

NO2-FA treatment in vitro induced HO-1 expression in cultured vascular endothelial cells and rat aortic segments.13 The increased gene expression of HO-1 is stimulated by a broad array of reactive inflammatory mediators and cytokines, leading to protection against vascular injury via multiple mechanisms including heme catabolism and the signaling actions of heme metabolites such as carbon monoxide.21 In this regard, increased expression of HO-1 attenuates intimal hyperplasia after arterial injury22 and reduces atherosclerotic lesion formation in low-density lipoprotein receptor– and apolipoprotein E–deficient mice.23,24 HO-1 and carbon monoxide can also inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia.25–27 Herein, we reveal that induction of HO-1 expression by OA-NO2 potently inhibits vascular smooth muscle cell migration. This occurs at much lower expression levels of HO-1 than required for the inhibition of vascular smooth muscle cell proliferation, because the concentrations of OA-NO2 that induced HO-1 and inhibited vascular smooth muscle cell migration had no effect on cell proliferation in vitro. This supports that the protective actions of HO-1 after endoluminal injury in vivo are mainly a consequence of the inhibition of cell migration, a key step in neointimal formation that is proximal to proliferation of vascular smooth muscle cells. In this context, it is noted that the serum OA-NO2 levels measured on chronic in vivo administration are not fully representative

**Figure 5.** OA-NO2 inhibits vascular smooth muscle cell migration and neointima formation via HO-1–dependent mechanisms. A and B, OA-NO2 inhibition of RASMC migration. After inducing monolayer wounding, cells maintained in serum-free media were treated with platelet-derived growth factor (PDGF) (20 ng/mL) and OA (250 nmol/L) or OA-NO2 (25, 50, 100, and 250 nmol/L), with SnPP (50 μmol/L) or HO-1 siRNA (50 μmol/L). Concentrations for HO-1 siRNA transfected experiments were as follows: OA (250 nmol/L), OA-NO2 (250 nmol/L), and PDGF (20 ng/mL). Quantitative image analysis was conducted 18 hours later to reveal extents of migration of RASMCs into the denuded area. Data are presented as means±SEM of 6 independent experiments (*P<0.01).

**Figure 6.** Protective actions of OA-NO2 are inhibited in HO-1–/– mice. Femoral artery tissue sections (HO-1–/– mice) from V (A) and OA-NO2 (B) treatment groups were labeled with anti–smooth muscle actin (red) and anti–CD31 (blue), with autofluorescence used to visualize the inner and outer elastic membrane (green) 21 days after wire-induced endoluminal injury (magnification, ×20; Olympus Provis I fluorescence microscope; scale bar=100 μm). C, Quantitative morphometric analysis of the intima to media ratio wild-type and HO-1–/– mice treated with or without OA-NO2 for 21 days. Data are expressed as means±SEM of 4 to 6 mice per group.
of the bioactive species that can accumulate. Electrophilic NO$_2$-FAs undergo protein adduction and partial $\beta$-oxidation to shorter chain metabolites that can retain signaling capabilities. Thus the higher concentrations of OA-NO$_2$ required for inhibition of smooth muscle cell proliferation in vitro may be a reflection of differences in model systems. The HO-1–mediated inhibition of smooth muscle cell migration is a significant mechanism underlying the antisenotic benefits of OA-NO$_2$. This property is further affirmed by the observation that siRNA inhibition of HO-1 expression or SNPP, a competitive inhibitor of HO, reversed the inhibition of RASMC migration but not the antiproliferative actions of OA-NO$_2$.

The induction of HO-1 expression by NO$_2$-FA is regulated by multiple signaling mechanisms. Initial HO-1 promoter activation analyses revealed a synergy between the cAMP-dependent response element CRE and activator protein-1 sequences in the $-4.5$Kb HO-1 promoter region in response to NO$_2$-FA exposure. More recently, chromatin structure analysis revealed that regulation of human HO-1 expression by NO$_2$-FA requires synergy between CRE, activator protein-1, and E-box sequences and involves the participation of CREB-1. Finally, NO$_2$-FA activate Nrf2/Keap1–dependent gene expression by electrophilic adduction of critical Keap1 thiol residues. In turn, activation of the Nrf2/Keap1 pathway mediates the induction of phase II genes, including HO-1.

The loss of OA-NO$_2$–dependent inhibition of wire-induced neointimal hyperplasia in both SnPP treated and HO-1–/– mice further supports the hypothesis that increased HO-1 expression in the vascular compartment accounts for a significant component of protection against intimal hyperplasia. This beneficial cardiovascular response to extended administration of fatty acid nitroalkene derivatives is likely to include additional signaling events, however. Mass spectrometric and gene expression analyses of cells exposed to electrophilic species reveals that $>300$ cellular proteins can be reproducibly posttranslationally modified, and the expression of a similar number of genes significantly affected (G Bonacci, F Schopfer, A Levonen, B Freeman, unpublished data, 2009). Characteristic cell and tissue responses are also expected for different electrophiles, with these events a consequence of the charge, size, and both the rate and reversibility of reaction with nucleophilic targets. Recent evidence indicates that reversibly reactive electrophiles may manifest little or no apparent cytotoxicity when administered at low concentrations. Moreover, the present data support that multiple transcription factors possess electrophile-reactive amino acids critical for the regulation of stress-related adaptive signaling reactions. These highly conserved genes and their allied signaling pathways promote adaptation to the myriad of electrophilic species present in the diet and those that are endogenously generated by toxin exposure, nitro-oxidative inflammatory conditions, and metabolic stress. In this regard, fatty acid nitroalkene derivatives are generated by inflammatory conditions, reversibly react via $S$-alkylation of protein thiols at kinetically rapid second order rate constants ($\approx 300$ M$^{-1}$ sec$^{-1}$) and activate multiple thiol-dependent transcriptional events. In summary, when administered in vivo for extended periods in low concentrations, a prototypic fatty acid nitration product induces antiinflammatory responses in both cell and animal models of vascular injury. This class of redox-derived electrophilic signaling mediators induces rapid adaptive signaling reactions, in response to changes in tissue metabolic, redox, and immune status, by modulating protein function and patterns of gene expression.

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Disclosures

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References


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Supplemental Material

Methods

**OA-NO₂ synthesis.** Nitro-oleic acid (OA-NO₂) used in this study was synthesized via nitroselenation. Oleic acid (OA) (NuCheck Prep, >99%) was converted to a nitro-phenylselenylated intermediate in the presence of mercuric salts, and then oxidized with hydrogen peroxide (30% aqueous) to yield the nitroalkene regioisomers of OA-NO₂. Product purification was performed by column chromatography on silica gel, with purity determined by ¹H NMR and HPLC-electrospray mass spectrometry. OA-NO₂ produced by this method yields an equimolar distribution of 9- and 10-nitro-octadec-9-enoic acid regioisomers.

**Wire-mediated vascular injury.** All animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Approval 0702181). Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg body weight; Aveco) and xylazine (5 mg/kg, Lloyd Laboratories) diluted 0.9% sodium chloride solution. Osmotic minipumps (21 day delivery, ALZET®, Durect Corporation, CA, USA) containing either vehicle (V), OA (2 mg/kg/d), or OA-NO₂ (2 mg/kg/d) were subcutaneously implanted in wildtype C57BL/6 male mice, 8-10 weeks of age (Jackson Laboratories, Bar Harbor, ME), immediately prior to left common femoral artery injury. OA-NO₂ was stabilized in mini-pumps by co-solvation in polyethylene glycol (PEG 400) containing 15% ethanol. In another cohort of C57BL/6 male mice, Sn(IV) protoporphyrin (SnPP) was administered to mice (i.p., 50 µmol/kg) one time immediately prior to mini-pump implantation and femoral wire injury and then every 3 days for 21 days.

Additionally, age-matched HO-1⁻ mice on a C57BL/6 background (kindly provided by Dr. Anupam Agarwal) were treated with either vehicle or OA-NO₂ and subjected to femoral artery injury.
To achieve unilateral femoral artery injury, the left common femoral artery was exposed using a midline leg incision, and blunt dissected to separate from the femoral vein and nerve. The femoral artery was clamped proximally and ligated distally with an 8-0 silk suture. An additional silk suture was looped around the femoral artery just proximal to the site of arteriotomy. A 0.014-inch flexible angioplasty guidewire was introduced into the femoral artery and the proximal clamp is removed. Endothelial denudation injury of the left common femoral artery is performed using wire withdrawal injury and three passes along the common femoral artery. Before removal of the guidewire, the femoral artery is clamped proximally. The wire is then removed and the femoral artery is ligated just proximal to the arteriotomy site.

At 21 days, mice received one intraperitoneal (i.p.) injection of Nembutal® sodium solution (65 mg/kg) (Abbott Laboratories, North Chicago, IL) for anesthesia and were perfused with PBS followed by 4% paraformaldehyde prior to removal of the femoral arteries. Femoral arteries were then post-fixed in 4% paraformaldehyde overnight and dehydrated in 30% sucrose for an additional 24 hours. Vessels were then embedded in OCT compound for sectioning. To obtain mRNA from femoral arteries, a cohort of mice were killed 3 days after femoral artery injury.

**Detection and quantitation of OA-NO₂ in serum.** Blood was removed from mice and transferred to a Microtainer® Brand Serum Separator Tube (Becton Dickinson and Company, Franklin Lakes, NJ) and allowed to clot for 2 hours at room temperature. The serum was obtained by centrifugation at 6000 x g at room temperature for 5 minutes. Serum was combined with cold (-20 °C) acetonitrile (1:4) and centrifuged at 2500 rpm for 15 minutes at 4 °C, to obtain lipid extracts. An internal standard ([1³C]OA-NO₂) was added during extraction to correct for losses due to sample preparation. A hybrid triple quadrupole/linear ion trap mass spectrometer (4000 Q-Trap LC/MS/MS; Applied Biosystems/MDS Sciex) was used to quantitate OA-NO₂ levels. The mass spectrometer interfaces with an HPLC system, enabling OA-NO₂ to be resolved via unique chromatographic properties and retention time. A C18 reverse phase
column was utilizing a gradient solvent system as follows: A (H₂O containing 0.1% formic acid) and B (CNCH₃ containing 0.1% formic acid) using the following conditions: 60% B (2 minutes); 60-95% B over 5 minutes; 95% B (3 minutes); 95-60% B over 0.1 minutes; and 60% B equilibration (4 minutes). Multiple reaction monitoring (MRM) scanning in the negative ion mode allows for transitions of intact and ionized species to be predicted and monitored. The MRM transitions used are based upon the precursor ion m/z and the most abundant product ion. Values for OA-NO₂, m/z 326/279, as well as [¹³C]OA-NO₂, m/z 344/279 are based upon the common loss of the nitro group which occurs during collision-induced dissociation of nitrated fatty acids. Following integration of the area under the peak(s), the ratios of analyte to internal standard areas are determined, permitting OA-NO₂ to be quantified using a standard curve with Analyst 1.4 software (Applied Biosystems/MDS Sciex).

**Vessel Morphometry.** To measure intimal and medial cross-sectional areas of injured and non-injured femoral arteries, three sets of three serial 6 μm thick cross-sections of each artery, spaced at 0.3 mm intervals, were cut. To visualize smooth muscle actin and endothelial cells, sections were permeabilized with 0.1% TritonX in phosphate buffered solution for 15 minutes, blocked for 45 minutes with 2% bovine serum albumin and incubated for 1 hour with a primary monoclonal anti-CD 31 antibody (BD Pharmingen, Franklin Lakes, NJ) and a monoclonal anti-α-smooth-muscle actin antibody conjugated with Cy3 (Sigma, Saint-Louis, MO). Subsequently, sections were incubated for 1 hour with a Cy5-conjugated Affini Pure antibody (Jackson ImmunoResearch, Baltimore, MD). Autofluorescence was used to visualize internal and external elastic membranes. Cross-sectional images were collected using an Olympus Provis I fluorescence microscope. Intimal and medial areas were quantified by image analysis (MetaMorph software, MDS, Toronto, Canada).
**Immunofluorescence.** Cross-sections (6 μm thick) of injured femoral arteries were blocked in 2% bovine serum albumin and incubated for 1 hour with a primary antibody to HO-1 (Stressgen, Victoria, Canada). Bound primary antibody was detected using a secondary Cy5-conjugated anti-rabbit antibody (Jackson ImmunoResearch, Baltimore, MD). Images were obtained using a Zeiss confocal microscope. To detect Ki67 positive cells femoral arteries were incubated with a primary antibody to Ki67 (Abcam, Cambridge, MA) followed by a secondary Alexa Fluor® 546 (Invitrogen, Carlsbad, CA) antibody. Nuclei were stained using Hoechst stain (10 mg/ml, Sigma-Aldrich, Inc, St Louis, MO).

**Cells and cell culture.** Rat aortic smooth muscle cells (RASMC) were isolated via explant and cultured in DMEM containing 10% FBS in 5% CO₂ at 37 °C. All experiments were performed using RASMC between passage 3 and 8. For cell proliferation studies, RASMC were plated onto 96 well plates at a density of 10,000 cells/cm² and allowed to attach overnight. For some experiments, RASMC were transfected with 50 μM siRNA against HO-1 or non-targeting control siRNA (Dharmacon Lafayette, CO) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). Transfections were performed using a reverse transfection protocol according to manufacturer´s instructions.

Cells were serum starved for 24 hours and then stimulated to proliferate with DMEM containing 2% serum and treated with 2.5 μM OA or 1 or 2.5 μM OA-NO₂. After 24 hours cell proliferation was assessed using the Cyquant NF proliferation assay as described by the manufacturer (Invitrogen, Carlsbad, CA).

For migration studies, RASMC were seeded into 6 well plates and grown to near confluence. RASMC were serum starved overnight and then scratched with a sterile pipette tip to produce a cell-free zone bordered by straight wound edges. Cells were stimulated to migrate with 20 ng/ml PDGF and concomitant addition of either 250 nM OA, or 25, 50, 100 or 250 nM OA-NO₂, with or without 50 μM SnPP (Frontier Scientific, Logan, UT). For some experiments RASMC were
transfected with non-targeting or 50 μM HO-1 siRNA as described above. At baseline as well as after 18h pictures were obtained with an IX-71 Olympus microscope and analyzed using Adobe Photoshop CS3 Extended software.

**Real-time quantitative PCR.** Total RNA from RASMC and femoral artery tissue was isolated with TRIzol® and/or further purified using the RNeasy Mini, RNA isolation kit (Qiagen, Valencia, CA). Complimentary DNA was obtained from 100 ng RNA using iScript reagents (Bio-Rad Laboratories, Hercules, CA) or SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Quantitative mRNA expression was assessed using real-time PCR with TaqMan Fast Universal PCR Master Mix or Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). Specific gene expression assays used are as follows: HO-1 (Rn00561387-m1), actin (4352340E) or primer sequences for mouse HO-1: forward, 5’-TCA GTC CCA AAC GTC GCG GT-3'; reverse, 5’-GCT GTG CAG GTG TTG AGC C-3’, HO-2: forward, 5’-ACT ACT CAG CCA CAA TGT CT-3'; reverse, 5’-GTG AAT CCG ATC CAC ATA CT-3’ and mouse GAPDH: forward, 5’TGA AGG TCG GTG TGA ACG GAT TT-3'; reverse, 5’CAC CAC CTG GAG TAC CGG AT G TAC-3’, where dissociation curves were observed for undesirable formation of primer-dimers. Samples were run in triplicate on the StepOne or Prism 7000 detection systems (Applied Biosystems, Foster City, CA).

**Western blot analysis.** Cells were washed twice with cold PBS and then lysed in buffer containing 50mM Tris, 1%NP-40, 1mM EDTA, 125mM NaCl, 20mM Deoxycholic Acid, 1mM Sodium Orthovanadate, 20mM Sodium Fluoride, 1mM Sodium Pyrophosphate, and Protease Inhibitors (Sigma cat: P8340). The lysates were slowly rotated at 4°C for 1 hour. The samples were centrifuged at 14,000 rpm for 5 minutes and the supernatant was collected and assayed for protein concentration. Equal amounts of protein were loaded onto an SDS page gel (4% stacking, 10% resolving) and separated by electrophoresis. Protein was transferred to a nitrocellulose membrane and the membrane blocked in tris buffered saline, 0.1% tween 20
(TBST) containing 5% dry milk (w/v). HO-1 (1:5,000) and HO-2 (1:1000) were detected using Stressgen antibodies (Stressgen Biotechnologies, Ann Arbor, MI) followed by the corresponding horse radish peroxidase linked secondary antibodies. Proteins were visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL).

**Heme Oxygenase Enzyme activity.** Heme oxygenase activity was measured by bilirubin generation in microsomal preparations from mouse liver as described previously\(^1\). Liver microsomes were incubated with rat liver cytosol, a source of bilirubin reductase (3 mg), hemin (20 µmol/L), glucose-6-phosphate (2 mmol/L), glucose-6-phosphate dehydrogenase (0.2 units), and NADPH (0.8 mmol/L) for 1 h at 37°C in the dark. The formed bilirubin was extracted with chloroform and the change in optical density, 464 to 530 nm, was measured (extinction coefficient, 40 mmol/L\(^{-1}\). cm\(^{-1}\) for bilirubin). Enzyme activity was expressed as nmol of bilirubin formed/mg protein/hour.

**Statistical analysis.** Results are expressed as mean ± SD or SEM. Statistical analysis was performed using one-way ANOVA and unpaired students t-test as appropriate. Differences between groups were assessed by Bonferroni post hoc test. A value of \(p < 0.05\) was considered statistically significant. SPSS 15.0 was used for all calculations.