Published in final edited form as:

Nitric Oxide. 2015 January 30; 44: 8–17. doi:10.1016/j.niox.2014.10.009.

Nitric oxide inhibits neointimal hyperplasia following vascular injury via differential, cell-specific modulation of SOD-1 in the arterial wall

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Abstract

Superoxide ($O_2^{\bullet-}$) promotes neointimal hyperplasia following arterial injury. Conversely, nitric oxide ($^*NO$) inhibits neointimal hyperplasia through various cell-specific mechanisms, including redox regulation. What remains unclear is whether $^*NO$ exerts cell-specific regulation of the vascular redox environment following arterial injury to inhibit neointimal hyperplasia. Therefore, the aim of the present study was to assess whether $^*NO$ exerts cell-specific, differential modulation of $O_2^{\bullet-}$ levels throughout the arterial wall, establish the mechanism of such modulation, and determine if it regulates $^*NO$ -dependent inhibition of neointimal hyperplasia. *In vivo*, $^*NO$ increased superoxide dismutase-1 (SOD-1) levels following carotid artery balloon injury in a rat model. *In vitro*, $^*NO$ increased SOD-1 levels in vascular smooth muscle cells (VSMC), but had no effect on SOD-1 in endothelial cells or adventitial fibroblasts. This SOD-1 increase was associated with an increase in *sod1* gene expression, increase in SOD-1 activity, and decrease in $O_2^{\bullet-}$ levels. Lastly, to determine the role of SOD-1 in $^*NO$ -mediated inhibition of neointimal hyperplasia, we performed the femoral artery wire injury model in wild type and SOD-1 knockout (KO) mice, with and without $^*NO$. Interestingly, $^*NO$ inhibited neointimal hyperplasia only in wild type mice, with no effect in SOD-1 KO mice. In conclusion, these data show the cell-specific modulation of $O_2^{\bullet-}$ by $^*NO$ through regulation of SOD-1 in the vasculature, highlighting its importance on the inhibition of neointimal hyperplasia. These results also shed light into the mechanism of $^*NO$ -dependent redox balance, and suggest a novel VSMC redox target to prevent neointimal hyperplasia.

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ESMB, NK, NCM, AYT, GEH, JMV, QJ, and EEK carried out the experiments. ESMB analyzed the data. ESMB, NCM, EEK, and MRK contributed to experimental design. ESMB and MRK wrote the manuscript. MRK contributed to the conception of the project and provided critical revision of the manuscript.

Keywords

Neointimal hyperplasia; Superoxide; Nitric oxide; Vascular; SOD1

1. Introduction

Atherosclerosis remains the leading cause of death and disability in the United States. Cardiovascular disease claims the lives of more than 2150 Americans each day [1]. Current interventions for the treatment of severe atherosclerosis include: balloon angioplasty with or without stenting, endarterectomy, or bypass grafting. However, vascular interventions continue to fail from restenosis secondary to neointimal hyperplasia. Neointimal hyperplasia is predominantly characterized by proliferation and migration of vascular smooth muscle cells (VSMC) and adventitial fibroblasts to the neointima [2–5]. We have demonstrated that local delivery of nitric oxide (NO) to the site of arterial injury effectively inhibits neointimal hyperplasia in different animal models [6–8]. The mechanisms by which NO accomplishes such inhibition are diverse, but include inhibition of VSMC proliferation and migration and promotion of endothelial proliferation [9–13]. We have recently focused our attention on the cell-specific effect of NO in the vascular wall as it is known that NO affects neighboring cell populations differently. In particular, we have focused on the effect of NO on superoxide ($\text{O}_2^{\bullet-}$) metabolism, since $\text{O}_2^{\bullet-}$ is integral to many of the cellular processes involved in the arterial injury response.

The role of reactive oxygen species (ROS) following vascular injury has been well-described. ROS, including $\text{O}_2^{\bullet-}$ and H_2O_2 , increase following arterial injury and stimulate proliferation and migration of VSMC and adventitial fibroblasts, resulting in neointimal hyperplasia [14–23]. Reducing ROS following arterial injury with different antioxidants, NOX inhibitors, genetic NOX deletions, or xanthine oxidase inhibitors has been shown to decrease the formation of neointimal hyperplasia [15,17,24–27]. Specifically demonstrating the importance of SOD to neointimal hyperplasia, overexpression of SOD or treatment with a SOD mimetic attenuated the formation of neointimal hyperplasia [28,29]. However, the data that exist on the effects of NO on $\text{O}_2^{\bullet-}$ and SOD-1 are conflicting. While NO can inhibit the mitochondrial respiratory chain and increase $\text{O}_2^{\bullet-}$, NO can both stimulate and inhibit different NOX subunits, thereby increasing and decreasing $\text{O}_2^{\bullet-}$ levels, in different cell types [30–32]. In particular, in pulmonary smooth muscle cells, Wedgwood et al. showed that NO decreases $\text{O}_2^{\bullet-}$ levels [33]. Furthermore, NO has been shown to increase SOD-1 in some cell types but inhibit SOD-1 in others [34–36]. Thus, while it is clear that both $\text{O}_2^{\bullet-}$ and SOD are intimately involved in regulating the arterial response to injury, little is known about how NO modulates SOD-1 and $\text{O}_2^{\bullet-}$ levels in the vasculature.

Here we explore the effect of NO on redox homeostasis in the cells that comprise the vascular wall *in vitro* and *in vivo*. We hypothesize that cell-specific, differential NO -mediated regulation of SOD-1 levels throughout the vascular wall mediates the ability of NO to prevent neointimal hyperplasia. Cell-specific modulation of SOD-1 levels by NO would represent a novel mechanism of NO -mediated redox modulation. Moreover, these findings could open the door for translational research focusing on cell-specific targeting of

antioxidant therapies with much promise to improve the outcomes of patients with vascular disease.

2. Methods

2.1. Chemicals and reagents

Disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazene-1-ium-1,2-diolate (PROLI/NO) and (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA/NO) were supplied by Dr. Larry Keefer (National Cancer Institute). SOD-1, SOD-2, p22phox, nitrotyrosine, and catalase antibodies were purchased from Abcam (Cambridge, MA). The detection reagents 5-(and 6)-chloromethyl-2',3'-dihydro-2',7'-dichlorofluorescein diacetate (CM-H2DCFDA, DCF) and dihydroethidium (DHE) were purchased from Invitrogen/Molecular Probes (Eugene, OR).

2.2. Animal surgery

All animal procedures were performed in accordance with principles outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 85-23, 1996) and approved by the Northwestern University Animal Care and Use Committee.

2.2.1. Rat carotid artery injury model—Adult male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 350–400 g were anesthetized with inhaled isoflurane (0.5–2%). Atropine was administered subcutaneously (0.1 mg/kg) to decrease airway secretions. The right common carotid artery for each rat served as the control group. After a sterile prep and midline neck incision, the left common, internal, and external carotid arteries were dissected and the internal and common carotid arteries were occluded. For the injury alone group, a No. 2 French arterial embolectomy catheter (Edwards Lifesciences, Irvine, CA) was inserted into the external carotid artery and advanced into the common carotid artery. Uniform injury was created by inflating the balloon to 5 atm of pressure for 5 minutes. After removal of the balloon, the external carotid artery was ligated and blood flow was restored. For the injury + •NO group, 10 mg of the •NO donor PROLI/NO was applied evenly to the external surface of the injured common carotid artery, as we have previously described [6,8]. The neck incision was closed. Treatment groups included control, injury, and injury + •NO ($n = 5$ rats/treatment group). Rats were sacrificed at 3 days at which time the carotid artery was harvested. The tissue was frozen in liquid nitrogen, ground with mortar and pestle, and homogenized in 20 mM Tris with PMSF (1 mM), leupeptin (1 µg/ml) and sodium orthovanadate (1 mM). Western blot analysis was performed as will be described later.

2.2.2. Mouse femoral artery injury model—Ten-week-old male SOD1 knockout (B6;129S7-Sod1^{tm1Leb/J}) mice and wild type litter mates were obtained from the Jackson Laboratory (Bar Harbor, ME). The mouse femoral artery wire injury model was performed in all mice as previously described by Sata et al. [37] Briefly, following a sterile prep, a small 1.5 cm groin incision was made directly overlying the femoral artery. The common femoral artery was dissected throughout its length, including the side branches. Vascular control was obtained proximally and distally with non-crushing vascular clamps. An

arteriotomy was made in the muscular side branch. A straight spring wire (0.38 mm diameter, No. C-SF-15-15, Cook Medical Inc., Bloomington, IN) was inserted through the arteriotomy into the common femoral artery where the guide wire was passed from the proximal to the distal common femoral artery three times. Following injury, the guide wire was removed and the branch artery was ligated proximal and distal to the arteriotomy. Blood flow to the common femoral artery was restored. For animals receiving $\bullet$ NO, PROLI/NO (1 mg) was administered in a powdered form directly to the external surface of the femoral artery following wire injury. The wound was closed in layers with an interrupted 4-0 vicryl followed by a running 4-0 silk suture for the skin. Treatment groups included control, injury, and injury + $\bullet$ NO ($n = 5-6$ mice/treatment group). Uninjured contralateral arteries served as controls. All procedures were performed by the same surgeon. Mice were sacrificed at 14 days at which time the femoral arteries were harvested.

2.3. Tissue processing

Prior to arterial harvest, mice underwent *in situ* perfusion fixation with phosphate buffer saline (PBS) followed by 2% paraformaldehyde. The harvested arteries were then fixed in 2% paraformaldehyde for an additional 1 h, and cryoprotected in 30% sucrose at 4 °C overnight. The tissue was quick-frozen in Optimum Cutting Temperature O.C.T.(TM) compound (Tissue Tek, Hatfield, PA). Arteries were cut into 5- μ m sections throughout the entire injured area, as previously described [8].

2.4. Histology

Mouse femoral arteries harvested at 14 days ($n = 5-6$ /group) were examined histologically for evidence of neointimal hyperplasia and vascular remodeling using routine hematoxylin and eosin (H&E) staining. Images of H&E-stained sections were collected with light microscopy using a Zeiss Imager.A2 microscope (Hallbergmoos, Germany). Morphometric analysis was performed by measuring lumen, intimal, and medial areas using ImageJ software (National Institutes of Health, Bethesda, MD).

2.5. Cell culture

Rat aortic VSMC and adventitial fibroblasts were isolated and cultured from the abdominal aorta of male Sprague–Dawley rats (Harlan; Indianapolis, IN) using methods described by Gunther et al. and Zhu et al., respectively [38,39]. Cells were maintained in media containing equal volumes of DMEM (low glucose) and Ham's F12 (JRH; Lenexa, KS) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 100 U/mL penicillin, 100 μ g/mL streptomycin and 4 mM L-glutamine, and incubated at 37 °C, 95% air and 5% CO₂. Rat aortic endothelial cells were obtained from Cell Applications (San Diego, CA). Cells used for experiments were between passages 4 and 9. Although PROLI/NO was used as an $\bullet$ NO donor in the *in vivo* experiments due to its efficacy, the short half-life of PROLI/NO makes it impractical to use for cell culture experiments *in vitro*. Hence, DETA/NO, which has a half-life of 24 h, was used as the $\bullet$ NO donor for all *in vitro* experiments.

2.6. *In vitro* ROS detection

For intracellular ROS detection dichlorofluorescein (DCF) and dihydroethidium (DHE) were used. For DCF detection, rat endothelial cells, adventitial fibroblasts, and VSMC were plated in 96-well plates at a density of 4000 cells per well and allowed to attach overnight. Cells were serum starved for 24 h and then treated with varying concentrations of DETA/NO in the presence of 5 μ M DCF. After 24 h, fluorescence was measured in a Molecular Devices (Sunnyvale, CA) Spectra Max M5 fluorescence plate reader using 485 nm excitation and 538 nm emission filters. For DHE detection of ROS, cells were treated with varying concentrations of DETA/NO. After 24 h the medium was changed and the cells were incubated with 10 μ M DHE for 1 h. Cells were washed and medium without phenol red added. Images of four different wells per treatment group were taken at 100 $\times$ magnification in an inverted Eclipse TE2000-U fluorescence microscope (Nikon, Tokyo, Japan) using a 500–550 nm excitation filter and a 550–600 emission filter. The fluorescence intensity was quantified using ImageJ and normalized by number of cells per field.

For $O_2^{\bullet-}$ detection, cells were plated in 100-mm dishes and allowed to attach overnight. When the cells were 80% confluent they were serum deprived overnight and treated with various concentrations of DETA/NO for 24 h. Some control groups also were incubated with 50 units (U) of pegylated (PEG)–SOD. After 24 h the cells were rinsed, the media changed, and 10 μ M DHE added for 1 h. Cells were then rinsed with cold PBS, spun and the cell pellet frozen and kept at -80°C until processing. The pellet was processed and analyzed according to published methods for HPLC/electrochemical detection of 2-hydroxyethidium (2-OH-E) [40]. In addition, verification and quantification of $O_2^{\bullet-}$ formation were assessed by electron paramagnetic resonance (EPR). Cells were exposed to the membrane-permeable EPR spin probe 1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) and analyzed at 37°C in a temperature- and O_2 -controlled Bruker (Bruker Corp, Billerica, MA) EPR [41]. Radical levels were quantified measuring the amplitude of the first peak of the $CM^{\bullet}$ radical. Exposure of cells to PEG–SOD was used to confirm $O_2^{\bullet-}$ as the initial radical reacting with the spin probe. To minimize the deleterious effects of contaminating metals, all buffers were treated with Chelex resin (Biorad, Hercules, CA) and contained 25 μ M deferoxamine.

2.7. Western blot analysis

Following exposure to the $^{\bullet}\text{NO}$ donor DETA/NO for 24 h, endothelial cells, VSMC or adventitial fibroblasts were rinsed with PBS, scraped and pelleted. The supernatant was removed and the pellet was resuspended in buffer (20mMTris, pH 7.4) with 1mM phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, MO), 2.3 mM leupeptin (1 mg/mL, Sigma) and 1 mM sodium orthovanadate (Sigma). Protein in the cell extracts or tissue homogenates was quantified with the bicinchoninic acid protein assay according to manufacturer's instructions (Pierce, Rockford, IL). Protein samples (20 μ g) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% or 13% gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were hybridized with rabbit polyclonal antibodies against SOD-1, SOD-2, p22-phox, catalase, or nitrotyrosine, followed by horse-radish peroxidase-linked secondary antibodies. Proteins were visualized using chemiluminescent reagents, according to the manufacturer's

instructions (Supersignal Substrate; Pierce), and the membranes were exposed to film and developed. Western blot films were scanned, and densitometry was performed on representative images using ImageJ.

2.8. Biochemical assays

For gene expression analysis, total RNA was extracted from fresh cell lysates using the RNeasy kit from Qiagen (Venlo, Limburg, Netherlands). *sod1* gene expression was assayed by qPCR using QuantiTect primers by Qiagen according to the manufacturer's instructions using an I-cycler iQ5 by Bio-Rad (Hercules, CA). SOD was increased in cells by incubating VSMC overnight with 50 units of pegylated (PEG)–SOD. For SOD-1 knockdown, VSMC were plated on day 1 at $\sim 2 \times 10^3$ cells/cm² (2×10^4 cells/well in 6 well plates, or 4×10^3 cells/well in 24 well plates). On day 2, cells were transfected using siRNA and HiPerFect transfection reagent by Qiagen (Venlo, The Netherlands) according to the manufacturer's instructions. VSMC were incubated with a mix of 2 siRNAs (Rn_Sod1_6 FlexiTube siRNA part#SI03028494, and Rn_Sod1_7 FlexiTube siRNA part#SI03079293, Qiagen, Venlo, The Netherlands) at a final total concentration of 120 nM, or with AllStar Negative siRNA labeled with AlexaFluor488 (Qiagen, Venlo, The Netherlands). Successful incorporation of the siRNA was verified through fluorescent microscopy imaging on days 3 and 4. On day 4 cells were either collected for WB analysis and SOD-1 activity determination, or a cell proliferation assay was performed. SOD-1 enzyme activity was assayed using a colorimetric kit by Cayman Chemicals (Ann Arbor, MI) according to the manufacturer's specifications. Potassium cyanide, a SOD-1 inhibitor, was used to differentiate SOD-1 from SOD-2 activity. Cell proliferation was assayed by ³H-thymidine incorporation. Cells were plated in 96-well plates at a density of 4000 cells/well, and allowed to attach overnight. Cultures were synchronized by serum deprivation overnight. Cells were treated with DETA/NO for 24 h in the presence of ³H-thymidine 5 μ Ci/ml. DNA was precipitated with addition of one volume of trichloroacetic acid (5%). Radioactivity was counted in a scintillation counter.

2.9. Statistical analysis

Results are expressed as mean $\pm$ SEM. Differences between multiple groups were analyzed using one-way analysis of variance with the Student–Newman–Keuls *post hoc* test for all pairwise comparisons. For studying the effect of SOD1 and *NO on VSMC proliferation (Fig. 8C) a two-way analysis of variance test was used. In the mouse femoral artery injury model (Fig. 9), experiments were analyzed with repeated-measures mixed-design analysis of variance. All statistical analyses were performed using either SigmaStat (Systat Software Inc. CA, USA), or SAS (SAS Institute Inc. NC, USA). Statistical significance was assumed when $p < 0.05$.

3. Results

3.1. Nitric oxide increases SOD-1 levels *in vivo* and *in vitro*

Three days following arterial injury, SOD-1 protein levels were diminished compared to control arteries (Fig. 1A). However, periadventitial delivery of the *NO donor PROLI/NO at the time of injury increased SOD-1 protein levels compared to injury alone (Fig. 1A and B).

To study what cell types are involved in this increase in SOD-1 levels by $\bullet$ NO, we treated endothelial cells, VSMC, and adventitial fibroblasts *in vitro* with the $\bullet$ NO donor DETA/NO for 24 h. Only VSMC showed a $\bullet$ NO-dependent increase in SOD-1 levels (Fig. 1C and D). Densitometry showed a twofold increase in SOD-1 protein levels in VSMC exposed to 1 mM DETA/NO (Fig. 1D). The increase in SOD-1 protein levels was preceded by a significant increase in *sod1* gene expression after 6 h of DETA/NO exposure, followed by a return to basal levels after 12 h (Fig. 2A). This DETA/NO-mediated increase in SOD-1 levels in VSMC corresponded with a DETA/NO-mediated 1.4-fold increase in SOD activity at 24 h (Fig. 2B). There was no increase in SOD activity at 6 or 12 h.

3.2. Nitric oxide regulates oxidative stress and decreases $O_2^{\bullet-}$ levels in VSMC

Treatment of VSMC with DETA/NO for 24 h increased DCF fluorescence but decreased DHE fluorescence (Fig. 3). The DHE fluorescence was also decreased by pre-treatment with PEG-SOD (50 U), suggesting the possible involvement of $O_2^{\bullet-}$ (Fig. 3). To further test the involvement of $O_2^{\bullet-}$, we used the cell-permeable spin probe CMH. Both DETA/NO and PEG-SOD (50 U) caused a decrease in the $CM^{\bullet}$ EPR signal (Fig. 4A). To unequivocally test if the increase in SOD-1 protein and SOD-1 activity had a direct impact on $O_2^{\bullet-}$ levels, we measured the DHE reaction-specific product 2-hydroxyethidium by HPLC, and saw a significant decrease in $O_2^{\bullet-}$ levels in VSMC treated with DETA/NO (Fig. 4B). We also evaluated total protein nitration and found an increase in VSMC treated with DETA/NO, suggesting ONOO $^-$ formation while $\bullet$ NO is present (Fig. 5). DETA/NO had no effect on the protein levels of the redox proteins SOD-2, catalase, or p22-phox (Fig. 6), suggesting that the SOD-1 increase is the specific redox regulation responsible for the decrease in $O_2^{\bullet-}$ levels. There was no decrease in $O_2^{\bullet-}$ levels in adventitial fibroblasts exposed to DETA/NO (Fig. 7A). However, there was a marked decrease in $O_2^{\bullet-}$ levels in endothelial cells exposed to DETA/NO (Fig. 7B).

3.3. DETA/NO inhibits VSMC proliferation more efficiently in the presence of SOD-1

To explore whether the ability of $\bullet$ NO to inhibit VSMC proliferation is related to the increase in SOD-1 protein, we knocked down SOD-1 with *sod1* siRNA, or increased SOD-1 activity by incubating VSMC with PEG-SOD. siRNA treatment resulted in a decrease in SOD-1 protein (Fig. 8A) concomitant with a 40% decrease in SOD activity (Fig. 8B). On the other hand, incubation of VSMC with PEG-SOD (50 units) for 24 h resulted in a 1.5-fold increase in SOD activity (Fig. 8B) similar to the increase induced by treatment with DETA/NO 1 mM (Fig. 2B). Moreover, this increase in activity observed with PEG-SOD treatment is also evidenced by a decrease in $O_2^{\bullet-}$ levels (Fig. 4B). After modifying SOD activity, we studied the antiproliferative effect of DETA/NO on VSMC with normal, increased, or decreased SOD. DETA/NO (0.5 mM) inhibited VSMC proliferation by 48% (Fig. 8C). However, DETA/NO was more effective at inhibiting VSMC proliferation in cells pre-treated with PEG-SOD compared to control cells (Fig. 8C, 48% inhibition vs 59% inhibition $P = 0.011$). On the other hand, after knocking down SOD-1, DETA/NO treatment only inhibited VSMC proliferation by 35% (Fig. 8C, 48% inhibition vs 35% inhibition $P = 0.049$). We also examined the effect of SOD on proliferation without DETA/NO treatment. Whereas PEG-SOD (50 units) did not cause a significant increase in basal VSMC

proliferation, knocking down SOD-1 with siRNA caused a 45% decrease in proliferation ($p < 0.05$).

3.4. SOD-1 deficiency blunts the ability of $\bullet$ NO to inhibit of neointimal formation *in vivo*

To evaluate the significance of the $\bullet$ NO-dependent increase in SOD-1, we evaluated the efficacy of the $\bullet$ NO donor PROLI/NO to inhibit neointimal formation in wild type and SOD-1 KO mice using the established femoral artery wire injury model [37]. Wire injury induced neointimal formation in both wild type and KO mice (Fig. 9A and B). Treatment with PROLI/NO resulted in reduced intima to media (I/M) area ratio in wild type mice but this reduction was not evident in SOD-1 KO mice (Fig. 9A and B). Specifically, morphometric analysis revealed a 47% reduction in the I/M area ratio by PROLI/NO following arterial injury in the wild type mice ($p = 0.01$, Fig. 9B). However, PROLI/NO had no effect on the I/M area ratio in the KO mice (Fig. 9B). In a similar fashion, wire injury caused 79% and 64% lumen occlusion in both wild type and KO animals, respectively. While PROLI/NO reduced lumen occlusion in wild type animals to 52% ($p = 0.006$), PROLI/NO did not reduce lumen occlusion in KO mice (Fig. 9C). Further, even though PROLI/NO did not cause a statistically significant increase in lumen area in the injured femoral arteries of wild type animals (Fig. 9D), it did show a trend for an increase (1.5-fold). However, this trend was not observed for the lumen area of femoral arteries from KO mice (Fig. 9D).

4. Discussion

Here we show a cell-specific effect of $\bullet$ NO on the redox environment of vascular cells, and offer mechanistic insight into how $\bullet$ NO alters $O_2^{\bullet-}$ levels via modulation of SOD-1. Nitric oxide caused a rapid increase in *sod1* gene expression that peaked at 6 h. This increase in expression lead to a later increase in SOD-1 protein and corresponding activity in VSMC at 24 h. However, this increase in SOD-1 protein levels was not observed in endothelial cells or adventitial fibroblasts. This cell-specific increase in SOD-1 was accompanied by a change in oxidative stress markers and a specific decrease in $O_2^{\bullet-}$ levels in VSMC. Increasing SOD activity in VSMC potentiated the anti-proliferative effects of $\bullet$ NO. Finally, the lack of SOD-1 *in vivo* prevented $\bullet$ NO from inhibiting neointimal formation. Thus, from these findings, we have identified a novel mechanism of $\bullet$ NO-mediated cell-specific modulation of ROS throughout the vascular wall and have linked this mechanism to $\bullet$ NO-mediated inhibition of neointimal hyperplasia.

The ability of $\bullet$ NO to inhibit neointimal hyperplasia is well established. We have shown that a high dose of NO at the time of injury provides protection well beyond the presence of the $\bullet$ NO donor in the vasculature [6,7]. We have shown that of a variety of donors applied periadventitially, the one with the shortest half-life provided the strongest inhibition of neointimal hyperplasia [6]. We have also shown that only 5 minutes of intraluminal $\bullet$ NO infusion at the time of injury also provided inhibition of neointimal growth at 2 weeks [7]. From these observations, it is clear that $\bullet$ NO inhibits neointimal hyperplasia through actions that occur early after arterial injury. By inhibiting the early events in the arterial injury response, the downstream cascade of events is durably prevented. Along these lines, we

show that *in vitro*, 24 h after exposure to $\bullet\text{NO}$, VSMC have increased SOD-1 protein and activity. We also showed that $\bullet\text{NO}$ applied at the time of injury induced an increase of SOD-1 in the injured artery after 3 days. These observations suggest that $\bullet\text{NO}$ at therapeutic doses exert redox regulation immediately after injury that result in a long-lasting effect.

ROS stimulate proliferation and migration of VSMC and adventitial fibroblasts by mechanisms that remain unclear [17]. After vascular injury the rate of ROS formation increases, with reports demonstrating increased levels of $\text{O}_2^{\bullet-}$, hydrogen peroxide, and ONOO^- [15]. Investigators have observed less neointimal formation upon treating arteries post injury with different antioxidants [16,17,24]. Though it is clear that increased ROS levels are crucial to stimulating VSMC proliferation and migration, there is debate as to whether $\text{O}_2^{\bullet-}$, H_2O_2 , or both are the primary species responsible. Yin and Huang suggested that increased H_2O_2 levels mediated by stimulation of both NOX1 and SOD accelerate VSMC proliferation [42]. However, other reports show that increasing $\text{O}_2^{\bullet-}$ production via stimulation of NOX1 is sufficient to drive VSMC proliferation [14,15,43]. In addition, decreasing $\text{O}_2^{\bullet-}$ levels through adenoviral-mediated SOD overexpression results in diminution of neointimal hyperplasia [28]. Both findings highlight the importance of $\text{O}_2^{\bullet-}$ as a key regulator in the development of neointimal hyperplasia. In addition, in our *in vivo* model, the presence of SOD-1 greatly affects the ability of $\bullet\text{NO}$ to inhibit of neointimal hyperplasia, highlighting the relevance of this differential modulation of the redox environment by $\bullet\text{NO}$. However, the importance of other reactive species cannot be dismissed. Moreover, our results show that DCF fluorescence increases with $\bullet\text{NO}$ treatment, which suggests that other peroxides might increase, including H_2O_2 and ONOO^- . However, the lack of specificity of DCF fluorescence does not allow us to make further conclusions. On the other hand, the direct opposition between the DCF and 2-OH-E results stresses the importance of the specific decrease in $\text{O}_2^{\bullet-}$ without a decrease in other oxidants.

$\bullet\text{NO}$ has been shown to inhibit $\text{O}_2^{\bullet-}$ -induced VSMC proliferation and to reduce ROS measured as DHE fluorescence levels. Wedgwood et al. observed a >80% decrease in ROS measured as DHE fluorescence in pulmonary artery smooth muscle cells (PASMC) treated with spermine NONOate [33]. We observe a more modest decrease in $\text{O}_2^{\bullet-}$ levels. However, Wedgwood et al. incubated the PASMC simultaneously with DHE and the $\bullet\text{NO}$ donor, which suggests that the decrease in fluorescence is due to a direct reaction of $\bullet\text{NO}$ with $\text{O}_2^{\bullet-}$. By contrast, we incubated the DHE 24 h after $\bullet\text{NO}$ treatment and with no NONOate donor in the media. Therefore, in our model the reduction in $\text{O}_2^{\bullet-}$ levels is most likely due to the increase in SOD. The *sod1* gene had previously been identified as a target for $\bullet\text{NO}$ regulation in rat glomerular mesangial cells [34]. However, we cannot discount a direct reaction between $\bullet\text{NO}$ and $\text{O}_2^{\bullet-}$ while the $\bullet\text{NO}$ donor is present. In fact, our findings agree with increased ONOO^- formation while $\bullet\text{NO}$ is present, evidenced by increased nitration in VSMC treated with DETA/NO.

Even though $\bullet\text{NO}$ increased SOD-1 levels only in VSMC and not in adventitial fibroblasts or endothelial cells, treatment with DETA/NO decreased $\text{O}_2^{\bullet-}$ levels in both VSMC and endothelial cells. $\bullet\text{NO}$ has been shown to inhibit $\text{O}_2^{\bullet-}$ production via a p47phox-dependent mechanism in the mouse aorta, or p22-phox-dependent mechanism in cardiac myocytes [30,31]. This suggests that $\bullet\text{NO}$ could be affecting endothelial cell $\text{O}_2^{\bullet-}$ levels through a

NOX-dependent mechanism in other cell types. This possibility remains to be explored in future work. However, as the endothelial cell layer gets depleted immediately after vascular injury, the regulation of the adventitial and medial redox environment takes a predominant role in the development of neointimal hyperplasia. Furthermore, in our animal model of vascular injury we use a short-lived $\bullet$ NO donor, so that by the time the endothelial cell layer gets repopulated, there may be little $\bullet$ NO left.

Our study is not without limitations. Our *in vitro* work focuses on the cell-specific effects of $\bullet$ NO on SOD-1. This situation could not be studied *in vivo* as our model utilized SOD-1 KO mice, which lack SOD-1 in all their cells and tissues. It would be preferable to study the efficacy of $\bullet$ NO at inhibiting neointimal hyperplasia in a smooth-muscle specific SOD-1 KO animal model; however, such a model is not available. Despite these limitations, we believe valuable information could be learned from our results. SOD-1 was not present in our KO model, and this resulted in a complete lack of efficacy of $\bullet$ NO at inhibiting neointimal hyperplasia. An unexpected finding from our work is that we did not observe more neointimal hyperplasia in the SOD-1 knockout mice following balloon arterial injury, as one might expect. The reason for this is unclear; however, this finding is consistent with published literature [44]. It is plausible that with the deletion of SOD-1, SOD-2 and/or SOD-3 may compensate for the loss of SOD-1 to maintain redox regulation.

5. Conclusions

In conclusion, we show that $\bullet$ NO regulates VSMC proliferation by differentially regulating the redox environment in these cells. Our findings highlight the role of $O_2^{\bullet-}$ as a signaling molecule driving neointimal growth and the importance of the ability of $\bullet$ NO to alter the redox balance through regulating SOD-1 in a cell-specific fashion to inhibit neointimal development. However, it is important to recognize that other regulatory redox mechanisms could also be involved. Moreover, $\bullet$ NO inhibits neointimal hyperplasia through diverse mechanisms with regulation of SOD-1 being one of them. These novel findings open a new translational avenue for the development of cell-specific redox therapies. Research efforts have been devoted to the development of antioxidants targeted to subcellular organelles such as mitochondria [45]. However, there is little research on the efficacy of antioxidant therapies targeted to specific cell-types or tissues [46]. Targeting redox modulation to vascular smooth muscle at the site of arterial injury might prove beneficial to prevent restenosis after vascular procedures.

Acknowledgments

The authors would like to acknowledge Dr. Nick Tsihlis for helpful discussions and suggestions, Mrs. Lynnette Dangerfield for her administrative assistance, Dr. Steve Miller for his expert statistics advice and assistance with SAS, and Natalie Fuller for her assistance with SAS coding.

Sources of funding

This work was supported in part by funding from the National Institutes of Health (1K08HL0842 and T32 HL094293), the U.S. Department of Veterans Affairs (VA Merit Review Grant), the Society of Vascular Surgery Foundation, the Northwestern Memorial Foundation Collaborative Development Initiative Center for Limb Preservation, and the American Heart Association Scientist Development Grant (10SDG3560005).

Abbreviations

CMH	1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrrolidine
DCF	dichlorofluorescein
DETA/NO	(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1,2-diolate
DHE	dihydroethidium
EPR	electron paramagnetic resonance
H&E	hematoxylin and eosin
I/M	intima/media
KO	knock out
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
[•]NO	nitric oxide
NOX	NADPH oxidase
O₂^{•-}	superoxide
OCT	optimum cutting temperature
[•]OH	hydroxyl radical
ONOO⁻	peroxynitrite
PASMC	pulmonary artery smooth muscle cells
PEG-SOD	pegylated superoxide dismutase
PROLI/NO	disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazene-1,2-diolate
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
SEM	standard error of the mean
VSMC	vascular smooth muscle cells
2-OH-E	2-hydroxyethidium

References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics – 2013 update: a report from the American Heart Association. *Circulation*. 2013; 127:e6–e245. [PubMed: 23239837]
2. Clowes AW, Clowes MM, Fingerle J, Reidy MA. Regulation of smooth muscle cell growth in injured artery. *J. Cardiovasc. Pharmacol.* 1989; 14(Suppl. 6):S12–S15. [PubMed: 2478818]
3. Clowes AW, Kirkman TR, Clowes MM. Mechanisms of arterial graft failure. II. Chronic endothelial and smooth muscle cell proliferation in healing polytetrafluoroethylene prostheses. *J. Vasc. Surg.* 1986; 3:877–884. [PubMed: 3712635]

4. Shi Y, Obrien JE, Fard A, Mannion JD, Wang D, Zalewski A. Adventitial myofibroblasts contribute to neointimal formation in injured porcine coronary arteries. *Circulation*. 1996; 94:1655–1664. [PubMed: 8840858]
5. Lindner V, Reidy MA. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc. Natl. Acad. Sci. U.S.A.* 1991; 88:3739–3743. [PubMed: 2023924]
6. Pearce CG, Najjar SF, Kapadia MR, Murar J, Eng J, Lyle B, et al. Beneficial effect of a short-acting NO donor for the prevention of neointimal hyperplasia. *Free Radic. Biol. Med.* 2008; 44:73–81. [PubMed: 18045549]
7. Havelka GE, Moreira ES, Rodriguez MP, Tsihlis ND, Wang Z, Martinez J, et al. Nitric oxide delivery via a permeable balloon catheter inhibits neointimal growth after arterial injury. *J. Surg. Res.* 2013; 180:35–42. [PubMed: 23164361]
8. Vavra AK, Havelka GE, Martinez J, Lee VR, Fu B, Jiang Q, et al. Insights into the effect of nitric oxide and its metabolites nitrite and nitrate at inhibiting neointimal hyperplasia. *Nitric Oxide*. 2011; 25:22–30. [PubMed: 21554972]
9. Ziche M, Morbidelli L, Masini E, Granger H, Geppetti P, Ledda F. Nitric oxide promotes DNA synthesis and cyclic GMP formation in endothelial cells from postcapillary venules. *Biochem. Biophys. Res. Commun.* 1993; 192:1198–1203. [PubMed: 8389543]
10. Guo JP, Panday MM, Consigny PM, Lefer AM. Mechanisms of vascular preservation by a novel NO donor following rat carotid artery intimal injury. *Am. J. Physiol.* 1995; 269:H1122–H1131. [PubMed: 7573510]
11. Sarkar R, Meinberg EG, Stanley JC, Gordon D, Webb RC. Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells. *Circ. Res.* 1996; 78:225–230. [PubMed: 8575065]
12. Tzeng E, Kim YM, Pitt BR, Lizonova A, Kovesdi I, Billiar TR. Adenoviral transfer of the inducible nitric oxide synthase gene blocks endothelial cell apoptosis. *Surgery*. 1997; 122:255–263. [PubMed: 9288130]
13. Kibbe MR, Nie S, Seol DW, Kovesdi I, Lizonova A, Makaroun M, et al. Nitric oxide prevents p21 degradation with the ubiquitin-proteasome pathway in vascular smooth muscle cells. *J. Vasc. Surg.* 2000; 31:364–374. [PubMed: 10664504]
14. Jeong HY, Jeong HY, Kim CD. p22phox-derived superoxide mediates enhanced proliferative capacity of diabetic vascular smooth muscle cells. *Diabetes Res. Clin. Pract.* 2004; 64:1–10. [PubMed: 15036821]
15. Dourron HM, Jacobson GM, Park JL, Liu J, Reddy DJ, Scheel ML, et al. Perivascular gene transfer of NADPH oxidase inhibitor suppresses angioplasty-induced neointimal proliferation of rat carotid artery. *Am. J. Physiol. Heart Circ. Physiol.* 2005; 288:H946–H953. [PubMed: 15388496]
16. Kennedy S, Preston AA, McPhaden AR, Miller AM, Wainwright CL, Wadsworth RM. Correlation of changes in nitric oxide synthase, superoxide dismutase and nitrotyrosine with endothelial regeneration and neointimal hyperplasia in the balloon-injured rabbit subclavian artery. *Coron. Artery Dis.* 2004; 15:337–346. [PubMed: 15346092]
17. Kanellakis P, Pomilio G, Walker C, Husband A, Huang JL, Nestel P, et al. A novel antioxidant 3,7-dihydroxy-isoflav-3-ene (DHIF) inhibits neointimal hyperplasia after vessel injury attenuating reactive oxygen species and nuclear factor-kappaB signaling. *Atherosclerosis*. 2009; 204:66–72. [PubMed: 18930230]
18. Callsen D, Sandau KB, Brune B. Nitric oxide and superoxide inhibit platelet-derived growth factor receptor phosphotyrosine phosphatases. *Free Radic. Biol. Med.* 1999; 26:1544–1553. [PubMed: 10401621]
19. Matesanz N, Lafuente N, Azcutia V, Martin D, Cuadrado A, Nevado J, et al. Xanthine oxidase-derived extracellular superoxide anions stimulate activator protein 1 activity and hypertrophy in human vascular smooth muscle via c-Jun N-terminal kinase and p38 mitogen-activated protein kinases. *J. Hypertens.* 2007; 25:609–618. [PubMed: 17278978]
20. Marumo T, Schini-Kerth VB, Fisslthaler B, Busse R. Platelet-derived growth factor-stimulated superoxide anion production modulates activation of transcription factor NF-kappaB and

- expression of monocyte chemoattractant protein 1 in human aortic smooth muscle cells. *Circulation*. 1997; 96:2361–2367. [PubMed: 9337212]
21. Miyake T, Aoki M, Shiraya S, Tanemoto K, Ogihara T, Kaneda Y, et al. Inhibitory effects of NFkappaB decoy oligodeoxynucleotides on neointimal hyperplasia in a rabbit vein graft model. *J. Mol. Cell. Cardiol*. 2006; 41:431–440. [PubMed: 16762361]
  22. Souza HP, Souza LC, Anastacio VM, Pereira AC, Junqueira ML, Krieger JE, et al. Vascular oxidant stress early after balloon injury: evidence for increased NAD(P)H oxidoreductase activity. *Free Radic. Biol. Med*. 2000; 28:1232–1242. [PubMed: 10889453]
  23. Azevedo LC, Pedro MA, Souza LC, de Souza HP, Janiszewski M, da Luz PL, et al. Oxidative stress as a signaling mechanism of the vascular response to injury: the redox hypothesis of restenosis. *Cardiovasc. Res*. 2000; 47:436–445. [PubMed: 10963717]
  24. Kanellakis P, Nestel P, Bobik A. Angioplasty-induced superoxide anions and neointimal hyperplasia in the rabbit carotid artery: suppression by the isoflavone trans-tetrahydrodaidzein. *Atherosclerosis*. 2004; 176:63–72. [PubMed: 15306176]
  25. Jacobson GM, Dourron HM, Liu J, Carretero OA, Reddy DJ, Andrzejewski T, et al. Novel NAD(P)H oxidase inhibitor suppresses angioplasty-induced superoxide and neointimal hyperplasia of rat carotid artery. *Circ. Res*. 2003; 92:637–643. [PubMed: 12609967]
  26. Lee MY, San MA, Mehta PK, Dikalova AE, Garrido AM, Datla SR, et al. Mechanisms of vascular smooth muscle NADPH oxidase 1 (Nox1) contribution to injury-induced neointimal formation. *Arterioscler. Thromb. Vasc. Biol*. 2009; 29:480–487. [PubMed: 19150879]
  27. Yamamoto Y, Ogino K, Igawa G, Matsuura T, Kaetsu Y, Sugihara S, et al. Allopurinol reduces neointimal hyperplasia in the carotid artery ligation model in spontaneously hypertensive rats. *Hypertens. Res*. 2006; 29:915–921. [PubMed: 17345792]
  28. Kuo MD, Bright IJ, Wang DS, Ghafouri P, Yuksel E, Hilfiker PR, et al. Local resistance to oxidative stress by overexpression of copper-zinc superoxide dismutase limits neointimal formation after angioplasty. *J. Endovasc. Ther*. 2004; 11:585–594. [PubMed: 15615548]
  29. Muscoli C, Sacco I, Alecce W, Palma E, Nistico R, Costa N, et al. The protective effect of superoxide dismutase mimetic M40401 on balloon injury-related neointima formation: role of the lectin-like oxidized low-density lipoprotein receptor-1. *J. Pharmacol. Exp. Ther*. 2004; 311:44–50. [PubMed: 15220383]
  30. Harrison CB, Drummond GR, Sobey CG, Selemidis S. Evidence that nitric oxide inhibits vascular inflammation and superoxide production via a p47phox-dependent mechanism in mice. *Clin. Exp. Pharmacol. Physiol*. 2010; 37:429–434. [PubMed: 19843095]
  31. Westermann D, Riad A, Richter U, Jager S, Savvatis K, Schuchardt M, et al. Enhancement of the endothelial NO synthase attenuates experimental diastolic heart failure. *Basic Res. Cardiol*. 2009; 104:499–509. [PubMed: 19255799]
  32. Owayed A, Dhaunsi GS, Al-Mukhaizeem F. Nitric oxide-mediated activation of NADPH oxidase by salbutamol during acute asthma in children. *Cell Biochem. Funct*. 2008; 26:603–608. [PubMed: 18508384]
  33. Wedgwood S, Black SM. Molecular mechanisms of nitric oxide-induced growth arrest and apoptosis in fetal pulmonary arterial smooth muscle cells. *Nitric Oxide*. 2003; 9:201–210. [PubMed: 14996427]
  34. Frank S, Zacharowski K, Wray GM, Thiernemann C, Pfeilschifter J. Identification of copper/zinc superoxide dismutase as a novel nitric oxide-regulated gene in rat glomerular mesangial cells and kidneys of endotoxemic rats. *FASEB J*. 1999; 13:869–882. [PubMed: 10224230]
  35. Baldelli S, Aquilano K, Rotilio G, Ciriolo MR. Neuronal nitric oxide synthase interacts with Sp1 through the PDZ domain inhibiting Sp1-mediated copper-zinc superoxide dismutase expression. *Int. J. Biochem. Cell Biol*. 2011; 43:163–169. [PubMed: 21056687]
  36. Kocaturk PA, Kavas GO. Effect of an inhibitor of nitric oxide production on Cu-Zn/SOD and its cofactors in diabetic rats. *Biol. Trace Elem. Res*. 2007; 115:59–65. [PubMed: 17406074]
  37. Sata M, Maejima Y, Adachi F, Fukino K, Saiura A, Sugiura S, et al. A mouse model of vascular injury that induces rapid onset of medial cell apoptosis followed by reproducible neointimal hyperplasia. *J. Mol. Cell. Cardiol*. 2000; 32:2097–2104. [PubMed: 11040113]

38. Gunther S, Alexander RW, Atkinson WJ, Gimbrone MA Jr. Functional angiotensin II receptors in cultured vascular smooth muscle cells. *J. Cell Biol.* 1982; 92:289–298. [PubMed: 6277961]
39. Zhu DL, Herembert T, Marche P. Increased proliferation of adventitial fibroblasts from spontaneously hypertensive rat aorta. *J. Hypertens.* 1991; 9:1161–1168. [PubMed: 1663971]
40. Zielonka J, Vasquez-Vivar J, Kalyanaraman B. Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. *Nat. Protoc.* 2008; 3:8–21. [PubMed: 18193017]
41. Dikalov SI, Li W, Mehranpour P, Wang SS, Zafari AM. Production of extracellular superoxide by human lymphoblast cell lines: comparison of electron spin resonance techniques and cytochrome C reduction assay. *Biochem. Pharmacol.* 2007; 73:972–980. [PubMed: 17222393]
42. Yin CC, Huang KT. H₂O₂ but not O₂- elevated by oxidized LDL enhances human aortic smooth muscle cell proliferation. *J. Biomed. Sci.* 2007; 14:245–254. [PubMed: 17124566]
43. Paravicini TM, Gulluyan LM, Dusting GJ, Drummond GR. Increased NADPH oxidase activity, gp91phox expression, and endothelium-dependent vasorelaxation during neointima formation in rabbits. *Circ. Res.* 2002; 91:54–61. [PubMed: 12114322]
44. Ishigami N, Isoda K, Adachi T, Niida T, Kujiraoka T, Hakuno D, et al. Deficiency of CuZn superoxide dismutase promotes inflammation and alters medial structure following vascular injury. *J. Atheroscler. Thromb.* 2011; 18:1009–1017. [PubMed: 21946535]
45. Smith RA, Murphy MP. Animal and human studies with the mitochondria-targeted antioxidant MitoQ. *Ann. N. Y. Acad. Sci.* 2010; 1201:96–103. [PubMed: 20649545]
46. de Haan JB, Cooper ME. Targeted antioxidant therapies in hyperglycemia-mediated endothelial dysfunction. *Front. Biosci. (Schol. Ed.)*. 2011; 3:709–729. [PubMed: 21196407]

Fig. 1. Nitric oxide donors increase superoxide dismutase (SOD)-1 levels *in vivo* and *in vitro*. (A) Western blot analysis of SOD-1 protein levels in whole carotid lysates 3 days after injury alone or injury and periadventitial application of PROLI/NO (10 mg). The carotid lysates were obtained by pooling carotid arteries from 5–6 animals. (B) Densitometry of carotid lysate Western blot ($N = 5–6$ rats; $*p < 0.05$). (C) Western blot analysis of SOD-1 protein levels in endothelial cells, vascular smooth muscle cells, and adventitial fibroblasts treated for 24 h with DETA/NO (0.5 and 1.0 mM). Images are representative of at least 3 separate

experiments. (D) Densitometry of Western blots analysis of SOD-1 in EC, VSMC, and AF treated with varying concentrations of DETA/NO for 24 h ($N = 3$; $*p < 0.05$).

Fig. 2.

Nitric oxide donor DETA/NO increases superoxide dismutase-1 transcription and activity in vascular smooth muscle cells (VSMC). (A) Gene expression analysis of *sod1* mRNA by qPCR in VSMC treated with DETA/NO (1.0 mM) for 6–24 h. (B) SOD-1 activity in lysates of VSMC treated with DETA/NO (1.0 mM) for 6–24 h. Data were pooled from 3 separate experiments (* $p < 0.05$).

Fig. 3.

Nitric oxide induces an increase in dichlorofluorescein (DCF) fluorescence but a decrease in dihydroethidium (DHE) fluorescence in VSMC. Vascular smooth muscle cells (VSMC) were treated with DETA/NO for 24 h. (A) VSMC were washed and incubated with 5 μ M DCF for 1 h and fluorescence was measured in a plate reader. (B) VSMC were washed and incubated with 10 μ M DHE for 1 h. Pictures were taken in an inverted fluorescent microscope. Fluorescence intensity was quantified using Image J. Both experiments were performed in triplicates. Data are pooled from 3 separate experiments (* $p < 0.05$). (C) VSMC were incubated in control media or media with 100 nM of PEG-SOD overnight. VSMC were further incubated with 10 μ M DHE for 1 h. Pictures were taken using an inverted fluorescent microscope. Fluorescent images are representative of 3 separate experiments.

Fig. 4.

Nitric oxide decreases superoxide levels in vascular smooth muscle cells (VSMC). (A) VSMC treated with DETA/NO for 24 h were washed and incubated with 200 mM 1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) for 30 minutes, then scraped and the electron paramagnetic resonance (EPR) signal of the $\text{CM}^\bullet$ radical measured. Control cells were pre-treated with pegylated superoxide dismutase (PEG-SOD) (50 units) for 24 h. (B) VSMC treated with DETA/NO for 24 h were washed and incubated with 10 μM DHE for 60 minutes. 2-hydroxyethidium was quantified by HPLC. Control cells were

pre-treated with PEG-SOD (50 units) for 24 h. Experiments were performed in triplicates. Data were pooled from 5 separate experiments (* $p < 0.05$).

Fig. 5. Nitric oxide induces an increase in protein nitration in vascular smooth muscle cells (VSMC). VSMC were incubated with various DETA/NO concentrations for 24 h. VSMC lysates were collected and analyzed by Western blot using a monoclonal mouse anti-nitrotyrosine antibody. (A) Representative Western blot of three separate experiments. (B) Densitometric quantification using Image J ($N = 3$, $*p < 0.05$).

Fig. 6. Nitric oxide does not affect other redox proteins in vascular cells. (A) Western blot analysis of catalase, p22phox, and superoxide dismutase (SOD)-2 protein levels in endothelial cells (EC), vascular smooth muscle cells (VSMC), and adventitial fibroblasts (AF) treated for 24 h with DETA/NO. Images are representative of at least 3 separate experiments. (B–D) Densitometry analysis of 3 or 4 different Western blots in EC, VSMC, and AF for (B) catalase, (C) p22-phox, and (D) SOD-2.

Fig. 7.

Nitric oxide affects superoxide levels differently in adventitial fibroblasts and endothelial cells. Nitric oxide does not affect superoxide levels in adventitial fibroblasts. (A) Adventitial fibroblasts or (B) endothelial cells treated with DETA/NO for 24 h were washed and incubated with 10 μ M DHE for 60 minutes. 2-hydroxyethidium was quantified by HPLC. Control cells were pre-treated with pegylated superoxide dismutase (PEG-SOD) (50 units) for 24 h. Experiments were performed in triplicates. Data were pooled from 5 separate experiments ($N = 5$, $*p < 0.05$).

Fig. 8.

Superoxide dismutase (SOD) enhances the antiproliferative effect of nitric oxide *in vitro*.

(A) Western blot showing successful knockdown of the SOD-1 protein with *sod1* siRNA.

(B) Assay of SOD activity in vascular smooth muscle cells after SOD-1 knockdown with *sod1* siRNA, and with pegylated (PEG)-SOD (50 units) treatment for 24 h ($N = 3$; $*p < 0.05$).

(C) VSMC proliferation exposed to DETA/NO for 24 h. Cells received no SOD pre-treatment, PEG-SOD treatment (50 units) for 24 h or SOD-1 knockdown with siRNA.

Proliferation was assayed by measuring the incorporation of [^{3} H]-thymidine. Data are

pooled from 3–6 separate experiments. Data in each group were normalized to their respective controls without DETA/NO. *Statistical significance for the percent decrease using a two-way ANOVA with the post-hoc test Student–Newman–Keuls for pairwise comparisons; 48% vs 59% $p = 0.029$; 59% vs 35% $p = 0.022$.

Fig. 9.

Nitric oxide (NO) is more effective at inhibiting neointimal hyperplasia in wild type mice vs superoxide dismutase (SOD)-1 knockout mice. The femoral wire injury model was performed in SOD-1 knockout mice and wild type (WT) litter mates. Both arteries were injured and one was treated with periadventitial application of disodium PROLI/NO (1 mg). Femoral arteries were harvested after 14 days. (A) Representative H&E stained sections were used to perform morphometric analysis of the (B) intima to media (I/M) area ratio, (C) percent lumen occlusion, and (D) lumen area. $N = 5-6$ animals per treatment group; *Statistical significance using a repeated-measures mixed-design ANOVA with the post-hoc test Student-Newman-Keuls for pairwise comparisons; (B) $*p = 0.011$; (C) $*p = 0.006$.