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Effect of Nitric Oxide on Neointimal Hyperplasia based on Sex and Hormone Status

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Abstract

Nitric oxide (NO)-based therapies decrease neointimal hyperplasia; however, studies have only been performed in male animal models. Thus, we sought to evaluate the effect of NO on vascular smooth muscle cells (VSMC) *in vitro* and neointimal hyperplasia *in vivo* based on sex and hormone status. In hormone-replete media, male VSMC proliferated at greater rates than female VSMC. In hormone-deplete media, female VSMC proliferated at greater rates than male VSMC. However, in both hormone environments, NO inhibited proliferation and migration to a greater extent in male versus female VSMC. These findings correlated with greater G_0/G_1 cell cycle arrest and changes in cell cycle protein expression in male versus female VSMC following exposure to NO. Next, the rat carotid artery injury model was performed to assess the effect of NO on neointimal hyperplasia *in vivo*. Consistent with the *in vitro* data, NO was significantly more effective at inhibiting neointimal hyperplasia in hormonally intact males versus females using weight-based dosing. An increased weight-based dose of NO in females was able to achieve efficacy equal to that in males. Surprisingly, NO was less effective at inhibiting neointimal hyperplasia in both sexes in castrated animals. In conclusion, these data suggest that NO inhibits neointimal hyperplasia more effectively in males than females and in hormonally-intact compared to castrated rats, indicating that the effect of NO in the vasculature may be sex- and hormone-dependent.

Keywords

neointimal hyperplasia; vascular smooth muscle proliferation; nitric oxide; hormones; cell cycle

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INTRODUCTION

Neointimal hyperplasia is caused by injury to the vascular wall, which occurs during every vascular intervention. Nitric oxide (NO) is a small gaseous molecule normally produced by endothelial cells that has many different vasoprotective properties, including inhibition of platelet aggregation, leukocyte adherence, vascular smooth muscle cell (VSMC) proliferation, VSMC migration, stimulation of VSMC apoptosis, and endothelial cell growth.[1-3] These vasoprotective properties have led many researchers to demonstrate the efficacy of NO at inhibiting neointimal hyperplasia in numerous small and large animal models of arterial injury and vein bypass grafting.2 However, all of these studies have been conducted in males. The efficacy of NO in female animal models remains unknown.

Women are a distinct subset of patients with cardiovascular disease (CVD). It has been shown that pre-menopausal women have a lower incidence of coronary artery disease, hyperlipidemia and hypertension compared to age-matched males and post-menopausal females.4, 5 In women with peripheral arterial disease (PAD), the prevalence increases with age and in post-menopausal women is equal to or greater than the prevalence in men.6 Women are also more likely to present with more advanced PAD and to have worse lower extremity function and worse outcomes following intervention for PAD than men.6-8 In spite of these findings, few large studies have adequately addressed potential gender disparities in response to medical or procedural interventions for PAD. Much research has evaluated the effect of estrogen on the cardiovascular system in a clinical arena9-11 as well as in cell culture[12, 13] and animal models.[14-17] While many vasoprotective properties of estrogen have been identified,[12, 13, 15] systemic therapy has fallen out of favor following the release of the Women's Health Initiative trial data.[18-20] Clearly hormones play a significant role in vasoprotection in the local, if not systemic environment.

Therefore, it is unclear, given the obvious sex and hormone differences that exist with respect to the cardiovascular system, if NO-based therapies will be equally effective in these two diverse populations. Since our overall goal is to develop a NO-based therapy that can be used in the clinical arena to improve outcomes following vascular interventions, and it is not yet established whether NO-based therapies will be efficacious in a female model, the purpose of this study is to evaluate the efficacy of NO at inhibiting neointimal hyperplasia *in vivo* as well as vascular smooth muscle cell (VSMC) proliferation, migration, and cell cycle progression *in vitro* based on sex and hormone status. We hypothesize that NO will be effective at inhibiting neointimal hyperplasia in a female rodent model of arterial injury, regardless of hormone status.

EXPERIMENTAL PROCEDURES

NO-releasing donors

The diazeniumdiolates evaluated in this study include: 1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO) for *in vitro* experiments and disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO) for *in vivo* experiments. Diazeniumdiolates were chosen as NO donors due to their predictable and spontaneous NO release rates of 2 moles of NO per mole of compound.[21-23] DETA/NO was chosen for the *in vitro* studies based on its half-life of 20 hours in an aqueous environment at 37°C and pH7.4, which is ideal for a 24-hour assay. PROLI/NO was chosen for the *in vivo* studies based on our prior publications demonstrating efficacy at inhibiting neointimal hyperplasia.[23-26] It is not possible to use PROLI/NO for the *in vitro* studies given that in an aqueous environment at pH 7.4 and temperature 37°C it has a half-life of approximately 30 seconds.[27] For the *in vitro* experiments, a 10 mmol/L stock solution of DETA/NO was prepared fresh immediately before use for each experiment. For *in vivo* experiments, the

PROLI/NO powder was applied directly to the periadventitial surface of the artery after injury and restoration of flow.

Cell culture

VSMC were isolated and cultured from the aortas of 8-week male and female Sprague-Dawley rats (Harlan, Indianapolis, IN) using the collagenase method and maintained as previously described.[3, 28, 29] Three different lots of VSMC were harvested for each sex on three different dates. Each lot was harvested from the aortas of three different animals for each sex. Purity of each lot of VSMC was confirmed using anti-smooth muscle α-actin monoclonal antibodies (Sigma; St. Louis, MO) and by specific morphology for VSMC. Cells were maintained in hormone replete medium (HRM) containing equal volumes of Dulbecco's Modified Eagle's Medium-low glucose (SAFC Biosciences; Lenexa, KS) and Ham's F12 (JRH; Lenexa, KS) supplemented with 10% fetal bovine serum (FBS, Invitrogen; Carlsbad, CA), 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen) and 4 mmol/L L-glutamine (VWR; West Chester, PA) and incubated at 37°C, 95% air an d 5% CO_2. In designated experiments, hormone deplete medium (HDM) was used containing 10% dextran-charcoal-stripped FBS (DCS-FBS, Invitrogen), a 1:1 mixture of DMEM-low glucose and Ham's F12 without phenol red (Invitrogen), which has been shown to be an estrogen receptor agonist, and penicillin, streptomycin, and L-glutamine as above.[30] VSMC were used between passages 3-8 for all experiments. Every experiment was conducted in male and female VSMC simultaneously under identifical conditions.

ELISA

Estrogen and testosterone levels were measured from FBS, DCS-FBS, HRM, HDM, and rat serum using commercially available ELISA kits (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Proliferation assay

Tritiated (^{3}H)-thymidine incorporation was assessed as a surrogate for cellular proliferation as previously described.[24] Male and female VSMC were plated in 12-well plates (4×10^4 cells/well) and were growth-arrested for 24 hours with media containing no FBS. Cells were then exposed to HRM and HDM ± DETA/NO (125 - 1000 mol/L) in the presence of ^{3}H-thymidine (5 mCi/L, PerkinElmer, Wellesley, MA) for an additional 24 hours. ^{3}H-thymidine incorporation into trichloroacetic acid–precipitated DNA was quantified by scintillation counting.

Migration

Male and female VSMC were plated in 6-well plates (1×10^5 cells/well) and were growth-arrested x 24 hours. Adherent cell monolayers were injured by a single scrape with a 1000 μL pipette tip along the transverse diameter of the plate then immediately photographed. Following treatment with media ± DETA/NO for 24 hours the cells were photographed again. Blinded counting of cell nuclei that migrated into the empty space created by the scrape was performed at both time points using Adobe Photoshop 8.0 (Adobe Systems Inc., San Jose, CA), and quantification was performed using ImageJ software (National Institutes of Health [NIH], Bethesda, MD).

Flow Cytometry

Aortic VSMC plated in 4 cm plates (9×10^5 cells/well) were assessed using flow cytometry to determine the percent of cells in each phase of the cell cycle, as previously described.[24] Cells were growth-arrested for 24 hours, after which they were exposed to media ± the DETA/NO (125-1000 μmol/L) for 24 hours. Trypsinized cells were re-suspended in 50 μL

PBS and fixed with 450 μL of ice-cold 70% ethanol, followed by re-suspension in a propidium iodide staining solution [1X PBS (pH 7.4), 50 mg/L PI (Invitrogen), 204 mg/L RNase A (Sigma), 0.1% Triton X-100 (Fisher Biotech; Fair Lakes, NJ)]. Samples were analyzed on a Coulter Epic XL flow cytometer gated to include debris. Analysis was performed using ModFit 3.1 LT (Verity; Topsham, ME).

Western blot analysis

VSMC were collected after 24 hours of exposure to the different treatment groups by scraping and were resuspended in 20 mmol/L Tris with 100 μmol/L phenylmethylsulfonylflouride (Sigma), 1 μmol/L leupeptin (Sigma), and 1 μmol/L sodium orthovanadate (Sigma). Protein was quantified with the bicinchoninic acid protein assay according to manufacturer's instructions (Pierce, Rockford, IL). Whole cell samples (20 μg) were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis on 8-13% gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were hybridized using antibodies to p21 (mouse monoclonal, 1:500), p27 (mouse monoclonal, 1:1000), cdc2 (rabbit polyclonal, 1:500), cyclin-dependent kinase (cdk) 2 (mouse monoclonal, 1:500), cdk4 (mouse monoclonal 1:500), cdk6 (mouse monoclonal, 1:750), cyclin D1 (mouse monoclonal, 1:500), cyclin D2 (rat monoclonal, 1:500), and cyclin D3 (rat monoclonal, 1:500) all from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibodies to cyclin A (mouse monoclonal 1:500) and cyclin B1 (mouse monoclonal, 1:500) were from Abcam (Cambridge, MA) for 1-2 hours followed by horseradish peroxidase-linked goat anti-rabbit secondary antibodies (1:10,000; Pierce) for 1 hour. 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 to JPEG images, and densitometry was performed on representative images using ImageJ software. Densitometries were calculated as a fold-induction standardized to male controls, after normalizing the data to the respective beta-actin loading control.

Animal surgery

All animal procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the NIH (NIH Publication 85-23, 1996) and approved by the Northwestern University Animal Care and Use Committee. For all surgeries, rats were anesthetized with inhaled isoflurane (0.5-3%), and weights were documented. Two weeks prior to conducting the rat carotid artery injury model, adult 10-week male and female Sprague-Dawley rats first underwent castration and ovariectomy, or a sham operation for the hormonally intact group. The rat carotid artery injury model was then performed as previously described.[23-25] Atropine was administered subcutaneously (0.1 mg/kg) to decrease airway secretions. Rimadyl (0.15 mg/kg) was given for pain control. Equaline eye ointment was applied to prevent dryness while anesthetized. Following 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 with non-crushing vascular clamps. A 2 French arterial embolectomy catheter (generously provided by 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 atmospheres of pressure for 5 minutes. After removal of the balloon, the external carotid artery was ligated and blood flow was restored. After injury and restoration of flow, PROLI/NO powder was applied to the external surface of the injured common carotid artery and the neck incision was closed. Because male rats weigh more than 100 grams heavier than the female rats (46% greater, $P<0.001$) and the carotid artery is larger at baseline in males than females (lumen area 122% greater, medial area 115% greater, circumference 115% greater, $P<0.05$ for all measurements), the animals were given weight-based dosing. Male intact and

castrated rats received 26 mg/kg of PROLI/NO, while female intact and castrated rats received 26 or 39 mg/kg of PROLI/NO. Alternatively, calculation of the absolute dose received according to the mean weight for the intact animals was 9.7 mg (males) and 6.6 and 10.0 mg (females). Absolute dose received for the castrated animals was 9.2 mg (males) and 6.9 and 10.4 mg (females). Rats were sacrificed at 14-day (n=6 per group) time points, and blood was collected to measure serum estrogen and testosterone levels using ELISA kits from Cayman Chemical (Ann Arbor, Michigan) according to manufacturer's instructions. One castrated female injured rat had an estrogen level > 300 pg/ml. This rat was excluded from morphometric analysis. All procedures were performed by the same surgeon.

Tissue processing

Carotid arteries were harvested following *in-situ* perfusion-fixation with PBS (300 mL) and 2% paraformaldehyde (300 mL). Tissue was processed as previously described.[24] Vessels were placed in paraformaldehyde at 4°C fo r 1 hour, then 30% sucrose in PBS at 4°C for cryo-protection. The tissue was qui ck-frozen in Optimal Cutting Temperature compound (Tissue Tek, Hatfield, PA) and 5-μm sections were cut throughout the entire injured segment of the common carotid artery.

Morphometric analysis

Carotid arteries harvested at 14 days were examined histologically for evidence of neointimal hyperplasia using routine hematoxylin and eosin staining. Digital images were collected with light microscopy using an Olympus BHT microscope (Melville, NY) with 4X, 10X and 40X objectives. Six evenly-spaced sections through each injured carotid artery were morphometrically analyzed. Luminal area and arterial circumference were measured. Intima area was measured as the area between the lumen and the internal elastic lamina. Media area was measured as the area between the internal and external elastic lamina. These values were measured using ImageJ software. All analysis was done by the same person. Because age-matched male rats weighed 46% more than female rats ($P<0.001$), and male arterial circumference, lumen area, and medial area were statistically significantly larger than female arterial circumference, lumen area, and medial area ($P<0.001$), morphometric measurements were normalized to arterial circumference to account for these differences in weight and arterial size.

Statistical analysis

Results are expressed as mean ± standard error of the mean. For baseline properties between male and female VSMC, the male control was compared to the female control. Each DETA/NO group was compared to the same-sex control group. In order to compare the effects of NO between the male and female groups, the male group was calculated as a percent of its control and the female group was calculated as a percent of its control. The percent reduction of each male DETA/NO group was compared to the female counterpart. Differences between two groups were analyzed using t-tests. Differences between multiple groups were analyzed using one-way analysis of variance with the Student-Newman-Keuls *post hoc* test for all pairwise comparisons (SigmaStat; SPSS, Chicago, IL). Statistical significance was assumed when $P<0.05$.

RESULTS

A hormone deficient environment was created using DCS-FBS

ELISA was performed to measure estrogen and testosterone concentrations in serum and media. DCS-FBS had a 24-fold decrease in estrogen levels (19.6 ± 0.33 pg/ml) and a 20-fold decrease in testosterone levels (10.1 ± 0.9 pg/ml) compared to standard FBS (476.6 ± 29.7

pg/ml and 202.0 ± 7.2 pg/ml, respectively, P<0.05). HDM with 10% DCS-FBS had a 4-fold reduction in estrogen (14.8 ± 6.1 pg/ml) and a 5-fold reduction in testosterone (5.4 ± 0.1 pg/ml) compared to HRM with 10% FBS (56.6 ± 5.4 pg/ml and 23.1 ± 0.5 pg/ml, respectively, P<0.05).

NO-mediated inhibition of VSMC proliferation based on sex and hormone status

In an effort to characterize the effects of DETA/NO on proliferation of VSMC based on sex and hormone status, both before and after exposure to NO, a ^{3}H-thymidine proliferation assay was conducted [Figure 1A-D]. In HRM, Male VSMC ($8.1\times10^{3}\pm433$ counts per minute [cpm]) proliferated more than female VSMC ($2.3\times10^{3}\pm86$ cpm, *P<0.05). Male and female VSMC demonstrated a dose-dependent inhibition of VSMC proliferation upon exposure to DETA/NO (125-1000 μmol/L; $^{\#}$P<0.05). However, at equivalent dosing, male VSMC exhibited a statistically significant greater reduction in proliferation upon exposure to similar concentrations of NO than female VSMC as can be seen by a shift in the dose-response curve to the right for females [Figure 1B, P<0.05 between the two curves]. In HDM, an opposite pattern was observed for baseline proliferation. Female VSMC (10.6×10^{3} ± 609 cpm) proliferated more than male VSMC (6.5×10^{3} ± 743 cpm, *P<0.05). Similar to that observed with HRM, male and female VSMC demonstrated a dose-dependent inhibition of VSMC proliferation with DETA/NO 125-1000 μM ($^{\#}$P<0.05) in HDM. Surprisingly, at equivalent dosing, male VSMC still exhibited a statistically significant greater reduction in proliferation upon exposure to NO even though baseline proliferation rates were lower [Figure 1D, P<0.05 between the two curves].

NO-mediated inhibition of migration in male and female VSMC

The scrape assay was used to delineate the effects of sex on VSMC migration at baseline and following exposure to NO in HRM [Figure2A-B]. Male VSMC migrated more (158±20 counts per high-power field [chpf]) than female VSMC (51±14 chpf, *P<0.05)[Figure 2A]. Male VSMC showed a statistically significant dose-dependent inhibition of migration with increasing concentrations of DETA/NO (125–1000 μmol/L). However, only the highest concentration of DETA/NO (1000 μmol/L) inhibited migration in female VSMC ($^{\#}$P<0.05). At equivalent dosing of DETA/NO, male VSMC exhibited greater reduction in migration than female VSMC (74% vs. 42% at 500 μmol/L; *P<0.05; Figure 2B).

NO-induced alterations in cell cycle progression in male and female VSMC

In order to further characterize the differences we observed between the sexes, the cell cycle was analyzed using flow cytometry with propidium iodide staining in HRM [Figures 3A-C]. All NO values for cell cycle analysis represent DETA/NO 500 μmol/L. More female VSMC were in G_0/G_1 than male VSMC under control conditions (70%±1.8% vs. 48%±1.7% respectively; *P<0.001). NO increased G_0/G_1 cell cycle arrest in male (90%±0.5%) and female (91%±0.2%) VSMC ($^{\#}$P<0.001). The percent increase was greater in males compared to females (88% vs. 30%, respectively, ^{t}P<0.001). More male VSMC were in G_2/M than female VSMC under control conditions (21%±1.8% and 11%±1.6% respectively; *P=0.002). NO decreased G_2/M in male and female VSMC (4.7%±0.2% vs. 3.5%±0.1% respectively; $^{\#}$P<0.001). After exposure to NO, more male VSMC were in G_2/M than female VSMC ($^{+}$P=0.008) and the percent decrease was greater in males compared to females (73% vs. 68%, respectively; $^{\tau}$P<0.05). More male VSMC were also in the S phase than female VSMC under control conditions (31%±1.3% vs. 19%±1.5% respectively; *P<0.001). NO decreased synthesis in male and female VSMC (4.8%±0.4% vs. 5.6%±0.3%, respectively; $^{\#}$P<0.001). However, the percent decrease was greater in males (84%) compared to females (70%, $^{\tau}$P<0.05). In summary, these differences between the sexes in cell cycle progression may in part explain the proliferation differences observed in male and female VSMC at baseline and following exposure to NO.

Cell cycle protein expression in male and female VSMC

Given the differences observed in the cell cycle based on sex and NO exposure, expression of cell cycle proteins was compared based on sex and after NO exposure using Western blot analysis in HRM [Figure 4A-E]. No differences were found in the protein expression of the cyclin-dependent kinase inhibitor p27 between male and female VSMC, but a small increase in expression was noted after NO exposure in females more than in males. Female VSMC exhibited a 6.5-fold increase in p21 expression compared to male VSMC at baseline [Figure 4B] but NO exposure resulted in a larger increase in p21 expression in males (13.4-fold, DETA/NO 500 μmol/L) compared to females (2.2-fold, DETA/NO 500 μmol/L). This increase in p21 expression at baseline in females, and greater increase in p21 expression following NO exposure in males, correlates with the proliferation and cell cycle analysis data, suggesting that female VSMC at baseline experience more arrest, but after NO exposure male VSMC are more responsive to cell cycle arrest than female VSMC.

Differences were observed between the sexes for cdc2 and cdk2. Males had a negligible increase in expression of cdc2 at baseline and following exposure to NO compared to females [Figure 4C]. NO caused a dose-dependent decrease in cdc2 expression in male (75% reduction, DETA/NO 500 μmol/L) and female VSMC (86% reduction, DETA/NO 500 μmol/L). Males exhibited a 2-fold greater expression of cdk2 than females [Figure 4D]. NO caused a dose-dependent decrease in cdk2 in males (80% reduction, DETA/NO 500 μmol/L), but no significant reduction was observed in females (26% reduction, DETA/NO 500 μmol/L). No differences based on sex or following NO exposure were found in the expression of cdk4; but, at baseline, female VSMC had a 1.5-fold greater expression of cdk6 compared to the male VSMC. However, no difference was observed in protein expression of cdk6 following NO exposure in male or female VSMC.

No differences based on sex or following NO exposure were found in the expression of cyclin A, cyclin D1, cyclin D2, and cyclin D3 in VSMC. Males had a negligible increase in expression of cyclin B compared to females [Figure 4E]. NO caused a dose-dependent decrease in cyclin B expression in males (58% reduction, DETA/NO 500 μmol/L) and females (71% reduction, DETA/NO 500 μmol/L). In summary, the expression of these cell cycle proteins correlated with the cell cycle data showing more males in G_2/M and S phases at baseline, and that NO caused a greater reduction in mitosis and synthesis phases in males compared to females. Cdk2 partners with cyclin E in the G_1 phase to regulate entry into the S phase. Cyclin B forms a complex with cdc2 to regulate entry into the M phase of the cell cycle. Thus, these subtle differences in expression of cdks may explain why male VSMC have a greater percentage of cells in G_2/M and S phases than females and why these cells proliferate and migrate more than female VSMC.

Inhibition of neointimal hyperplasia in hormonally intact and castrated male and female rodents

In an effort to characterize the effect of a NO-based therapy based on sex and hormone status, the rat carotid artery injury model was performed in intact and castrated male and female Sprague-Dawley rats. Serum was collected prior to sacrifice from all animals and estrogen and testosterone levels were measured to confirm the model [Figure 5]. In female rats, castration significantly decreased estrogen levels compared to intact animals in the injury [Figure 5A] (43.3±5.8 vs. 95.3±22.3 pg/ml, P=0.05) and injury plus NO treatment groups (40.9±8.6 vs. 100.8±16.8 pg/ml, P<0.05), but it did not affect estrogen levels in male rats. In male rats, castration significantly decreased testosterone levels compared to hormonally-intact animals in the injury [Figure 5B] (128±16.7 vs. 641±67.3 pg/ml, *P<0.05) and injury plus NO treatment groups (121±16.1 vs. 332±37.1 pg/ml, *P<0.05). In female rats, castration also reduced testosterone compared to intact animals in both the

injury (149±18.4 vs. 355±54.6 pg/ml, *P<0.05), and injury plus NO treatment groups (156±8.5 vs. 343±44.3 pg/ml, *P<0.05). Thus, castration was effective at significantly reducing estrogen and testosterone levels in females and testosterone levels in males.

In hormonally-intact animals, balloon injury produced reproducible neointimal hyperplasia at 14 days in both sexes [Table 1, Figure 6A and 6C]. With respect to neointimal hyperplasia, the intimal area and the intima/media area ratio (I/M) showed no differences between male and female rodents in the injury-alone treatment groups. In both sexes, NO significantly reduced intimal area and the I/M (P<0.05). To compare the efficacy of NO between the sexes, the percent reduction of neointima was compared. NO reduced intimal area to a greater extent in males versus females using equivalent weight-based dosing (65% vs. 53%, respectively, 26 mg/kg, P<0.05). NO resulted in a greater reduction in the I/M area ratio in males versus females at equivalent weight-based dosing (55% vs. 31%, respectively, 26 mg/kg, P<0.001) [Figure 6A and 6C]. However, when females received a higher weight-based dose of NO (39 mg/kg), they exhibited reductions similar to males in intimal area (68%) and I/M (52%).

With respect to luminal area, female rats had a 30% smaller luminal area than male rats in the injury-alone groups (P<0.05) [Table 1]. After NO therapy, female rodents experienced a significant enlargement in their luminal area for both NO doses, whereas males did not have enlargement in luminal area after NO therapy. With respect to medial remodeling, no differences were observed in the medial area between male and female rodents in the injury-alone groups. In both sexes, NO significantly reduced media area (P<0.05). However, no difference in percent reduction by NO was found in the medial area between male (28%) and female (28%) rodents.

In castrated animals, balloon injury produced reproducible neointimal hyperplasia at 14 days in both sexes [Table 2, Figure 6B and 6C]. Following arterial injury, males had a larger intimal area than females (17.6±0.92 vs. 13.0±0.68, P<0.05). In both sexes, NO significantly reduced intima area (7.3±0.25 males, 8.5±0.27 females [26 mg/kg], and 4.4±0.39 females [39 mg/kg], P<0.05). However, NO resulted in a greater reduction in intimal area in males than females at equivalent weight-based dosing (59% vs. 31%, 26 mg/kg, P<0.05). Females that received a higher weight-based dose of NO (39 mg/kg) experienced a greater reduction than males in intimal area (66%). Following arterial injury, males had a larger I/M than females (1.00±0.05 vs. 0.71±0.04, P<0.05). In male and high dose NO-treated female groups (39 mg/kg), NO significantly reduced the I/M (0.61±0.02 and 0.41±0.03, respectively, P<0.05). No reduction was observed in the I/M in the equivalent weight-based dosed female group (0.61±0.02, P=NS, 26 mg/kg). Thus, NO resulted in a significantly greater reduction in the I/M in males compared to females at equivalent weight-based dosing (39% vs. 15%, P<0.05). Females that received the higher dose of NO (39 mg/kg) achieved a reduction in the I/M equal to males that received 26 mg/kg.

With respect to luminal area, no significant differences were noted in the castrated rats between the sexes or after treatment with NO [Table 2]. With respect to medial area, male and female rats had equal media areas following arterial injury (17.6±0.61 and 17.7±0.81 respectively). In male and high dose NO-treated female groups, NO significantly reduced medial area (12.3±0.43 and 10.8±0.38 respectively, P<0.05). No reduction was observed in the equivalent-dosed female group (14.6±0.33). Thus, NO brought greater reduction in medial area in males than females at equivalent weight-based dosing (30% vs. 17%, P<0.05). Females with higher dosed NO had reduction equal to males in media area (39%) and greater than the other NO-treated female group (P<0.05).

Lastly, significant differences were noted between the hormonally-intact and castrated animals. Overall, castrated animals developed significantly less intimal area and I/M compared to hormonally-intact animals (Tables 1 and 2, Figure 6C). Furthermore, NO was found to be more effective at reducing neointimal hyperplasia in both sexes in hormonally-intact rats compared to castrated rats when evaluating intimal area and the I/M (Figure 6C).

DISCUSSION

In our experiments, we examined the effects of NO on VSMC proliferation, migration, and cell cycle regulation *in vitro* and neointimal hyperplasia *in vivo* based on sex and hormone status of the animal. As predicted by our hypothesis, the results of this study show that NO is effective at inhibiting these vascular processes in both sexes, however we found that NO appears to be more effective in males versus females and more effective in a hormonally intact versus deficient environment. At baseline in a hormone replete environment, male VSMC proliferated and migrated more and had less cell cycle arrest compared to female VSMC. After exposure to NO, male VSMC experienced greater inhibition of proliferation, migration, and cell cycle arrest than female VSMC. In a hormone deplete environment, while females proliferated more than males, NO was still more effective at inhibiting VSMC proliferation in males compared to females. In our *in vivo* model, when using weight-based dosing, NO therapy resulted in a greater reduction in neointimal hyperplasia in males than females, and in hormone intact versus castrated rats. In summary, this study suggests that VSMC growth and the efficacy of NO at inhibiting neointimal hyperplasia is sex- and hormone-dependent.

While we are unaware of any studies that have investigated the efficacy of NO-based therapies to inhibit neointimal hyperplasia in a female environment, our findings on the effects of NO on female VSMC are consistent with published literature for male systems. Inhibition of VSMC migration and proliferation and induction of cell cycle arrest after NO therapy have been well-established in previous publications.[3, 31, 32] Additionally, Ling et al evaluated mouse VSMC proliferation in male and female wild-type and aromatase-knockout mice.[33] While they did not evaluate the effects of NO, they found that in both wild-type and aromatase knockout mice male VSMC proliferated more than female VSMC.[33] Other studies exist that examine VSMC proliferation to assess sex-differences for alternative therapies, but frequently data were represented as a percentage of control, making comparisons between the sexes impossible.[10]

Multiple investigators have examined the effects of β-estradiol on VSMC migration and showed that this therapy inhibits migration of VSMC.[11-14] However, Dai-Do et al only examined cells from post-menopausal females and Kolodgie et al only examined female rat VSMC.[11, 12] Akishita et al examined male and female rat VSMC, but published results only include data on males, and Geraldes et al did not indicate the sex of the porcine VSMC evaluated for migration.[13, 14] Clearly, more sex-specific investigation into these processes is warranted given that VSMC proliferation and migration are the cornerstone of neointimal hyperplasia and this process occurs in both male and female patients.

Sex differences in the development of neointimal hyperplasia following arterial injury have been described. Chen et al in 1996 were the first to compare male and female neointimal hyperplasia after carotid balloon angioplasty.[15] Using the rat carotid artery injury model, they evaluated intact and castrated male and female rats, and administered back either estrogen or testosterone. Neointimal proliferation after injury was reduced in intact females compared to age-matched males. This did not hold true in castrated females, which developed equal injury compared to castrated male rats. Exogenous estrogen prevented neointimal formation in gonadectomized rats of both sexes, but greater in females compared

to males. We were surprised to find that our castrated female rats did not have greater injury than intact female rats or equal injury to male rats based on this previous study. Our data may be different for several reasons. First, the Sprague-Dawley rodents from the Chen et al study were obtained from Charles River Laboratories, which have been shown to develop a different injury pattern than Sprague-Dawley rats obtained from Harlan Laboratories.[34] Second, their injury technique was different from ours. Instead of advancing the balloon into the common carotid artery and inflating it once to a constant and controlled 5 atmospheres of pressure for 5 minutes, the balloon was inflated to unknown atmospheres, advanced into the thoracic aorta, and passed 6 times. Third, their rats were age-matched and the females weighed significantly less, and measurements were not controlled for the differences in arterial size due to weight differences[15]. In 2005, Kurumazuka et al compared male and female neointimal hyperplasia in Japanese rodents after carotid injury.[16] These authors evaluated atorvastatin as a therapy but found equivalent neointimal hyperplasia between male and female injury groups, similar to our study. Thus, studies have been conducted that demonstrate a difference in the development of neointimal hyperplasia based on sex and hormone status.

Our study does indicate a vasoprotective role for estrogen and NO in that the NO therapy reduced neointimal hyperplasia to a greater extent in hormonally intact versus castrated female rodents. The exact role, however, of estrogen and NO in this process is not fully understood and further experiments supplementing back estrogen and administering NO therapy need to be performed. Furthermore, it has been well-established that estrogen stimulates NO release in the vasculature.[35] But, it is not clear if the vasoprotective actions of NO are mediated through the estrogen-estrogen receptor signaling pathway. There is evidence to suggest the later may be the case. Both estrogen and NO share many similarities. They both inhibit VSMC proliferation, VSMC migration, leukocyte chemotaxis, and endothelial cell apoptosis, and stimulate endothelial cell proliferation and vasodilation.[36, 37] Thus, it is possible that NO may in fact function to stimulate or potentiate the estrogen-estrogen receptor signaling pathway to exert its vasoprotective effects and that when estrogen is deficient, the effects of NO are less potent. Alternatively, our *in vivo* data also show that NO is more effective at inhibiting neointimal hyperplasia in the presence of higher serum concentration of testosterone. This could potentially establish a role of testosterone mediating NO-induced vasoprotection. However, since testosterone is converted to estrogen locally via aromatase, serum testosterone levels may ultimately affect local estrogen levels. Neointimal hyperplasia and hormone pathways are complex, multifactorial processes. These data suggest that hormone status plays a role in mediating the efficacy of NO at inhibiting neointimal hyperplasia and VSMC proliferation, but more work needs to be conducted to evaluate the exact mechanisms.

This study has limitations. One inherent limitation is that controlling for every aspect of rodent characteristics other than sex is not possible. In order to mirror what has been demonstrated in the literature as well as the rat carotid artery model used in our laboratory, 12-week age-matched Sprague-Dawley rodents from Harlan were used. However, except in rare cases of retired breeders, age-matched female rodents are significantly smaller than male rodents. In our study, male rodents weighed on average 46% more than females, and the male carotid artery circumference, lumen area, and medial area were larger than in the females. Given this, we controlled dosing of PROLI/NO by weight, as is commonly done for many patient administered drugs. Analysis of the weight-based data suggests that NO inhibits VSMC proliferation and neointimal hyperplasia more in males than females. However, if analysis is conducted based on absolute dosing, few differences are observed between the sexes. For example, intact males that received 9.7 mg PROLI/NO (i.e. 26 mg/kg) experienced 55% reduction in neointimal hyperplasia while intact females that received 10.0 mg PROLI/NO (i.e. 39 mg/kg) experienced 52% reduction in neointimal hyperplasia. A

similar pattern is observed for the castrated male and female rodents that received 9.1 mg and 10.4 mg PROLI/NO (39% and 43% inhibition, respectively). Yet, the differences observed in the efficacy of NO based on hormone status persist, regardless of the type of analysis (i.e. weight-based vs. absolute dose). Even at the highest doses administered (ranging from 9.1-10.4 mg PROLI/NO), the NO-based therapy is more effective in hormonally intact males and females (55% and 52% inhibition, respectively) compared to hormonally castrated males and females (39% and 43% inhibition, respectively). With weight-based dosing, the differences are even greater. Thus, while NO may or may not have different efficacies based on sex, NO is clearly more effective in hormonally intact hormonally deprived environments.

Another inherent limitation of this study is that, in terms of castration, the animals were not deprived of their hormones chronically. Furthermore, the rats were not aged, thus making an exact comparison to post-menopausal females not possible. Also, the hormonally intact female group's estrous cycle was not controlled for, thus exact hormone levels were not controlled. Despite these limitations we wanted to simulate a hormone-deprived and hormone-rich environment for comparison purposes and this was achieved. Lastly, we did use different diazeniumdiolates for the *in vitro* work versus the *in vivo* work. PROLI/NO was chosen for the *in vivo* work due to our earlier work that has shown that PROLI/NO is the most efficacious diazeniumdiolate in this animal model.[23-26] Unfortunately, PROLI/NO cannot be used for the *in vitro* studies that were conducted over 24 hours due to its very short half-life in an aqueous environment at physiologic temperature and pH. However, given that the class of diazeniumdiolates release 2 moles of NO per mole of compound, we felt it was valid to use the diazeniumdiolate with the half-life idea for the assay being conducted, i.e. DETA/NO. This should have no bearing on the interpretation of our data, as our purpose was not to compare the *in vitro* studies to the *in vivo* studies, but to determine the effect of NO on VSMC *in vitro*, and also to determine the effect on neointimal hyperplasia *in vivo*, based on sex and hormone statues. We believe we accomplished this.

In conclusion, periadventitial application of NO inhibits neointimal hyperplasia in female rodents in a dose-dependent manner. The efficacy of NO at inhibiting neointimal hyperplasia is hormone-dependent, inhibiting neointimal hyperplasia to a greater extent in hormonally-intact versus castrated animals. Depending upon the method of analysis, the efficacy of NO at inhibiting neointimal hyperplasia may or may not be sex dependent. Since most female vascular patients are post-menopausal and most studies have shown that with regards to PAD women have an increased prevalence as they age, advanced disease at presentation, worse function from their disease, and worse outcomes after intervention for PAD than males, it is critical that specific attention be paid to this patient population when developing new therapies. Ultimately our goal is to develop a therapy that can be used in all patients to prevent restenosis following vascular interventions, and to be able to translate this therapy to every patient population that will benefit. Overall, NO-based therapies have promising clinical potential for an ever-growing population of patients with vascular disease, but more specific evaluation into mechanisms and efficacy of NO-based therapies is warranted.

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ABBREVIATIONS

NO	nitric oxide
VSMC	vascular smooth muscle cells
CVD	cardiovascular disease
PAD	peripheral arterial disease
DETA/NO	1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
PROLI/NO	disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate
HRM	hormone replete media
HDM	hormone deplete media
DCS-FBS	dextran-charcoal stripped FBS
^{3}H	tritiated
NIH	National Institutes of Health
H&E	hematoxylin-eosin
CPM	counts per minute
HPF	high power field
I/M	intimal to medial ratio

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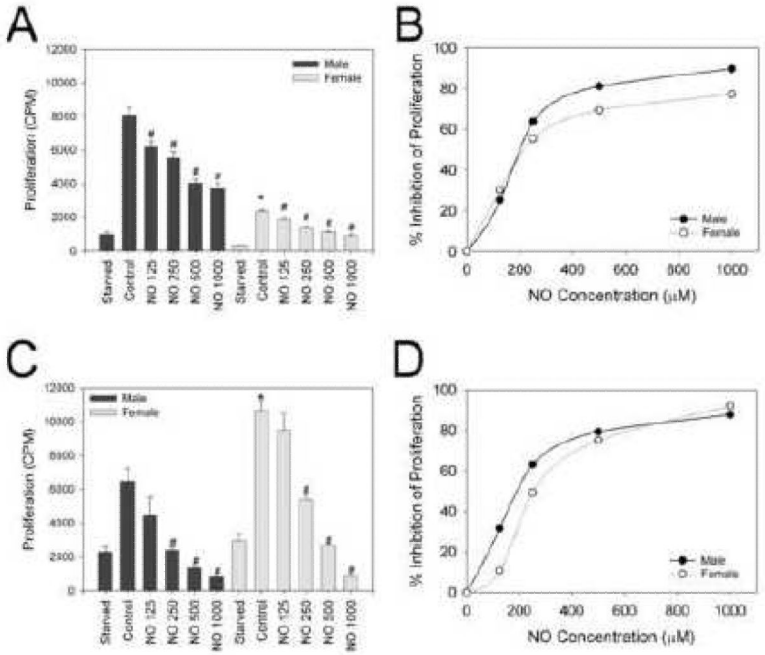

Figure 1.

Inhibition of proliferation by nitric oxide (NO) in male versus female vascular smooth muscle cells (VSMC) in hormone replete medium (HRM) and hormone deplete medium (HDM). VSMC proliferation was assessed using ^{3}H-thymidine incorporation at 24 hours ± exposure (CPM=counts per minute) to the NO donor DETA/NO (NO, units are mol/L). A) Male VSMC proliferated faster than female VSMC in HRM (*P<0.05 vs. male control). NO caused a dose-dependent inhibition of VSMC proliferation in both sexes ($^{\#}$P<0.05 vs. same-sex control). B) Dose response curve demonstrating the percent reduction of proliferation by a given concentration of NO in male and female VSMC (P<0.05 for male vs. female curve). C) Female VSMC proliferated faster than male VSMC in HDM (*P<0.05, compared to male control). NO caused a dose-dependent inhibition of VSMC cell proliferation in males and females ($^{\#}$P<0.05, compared to same-sex control). D) Dose response curve demonstrating the percent reduction of proliferation by a given concentration of NO in male and female VSMC in HDM (P<0.05 for male vs. female curve). Data shown are representative of more than five separate experiments; n = 3/treatment group.

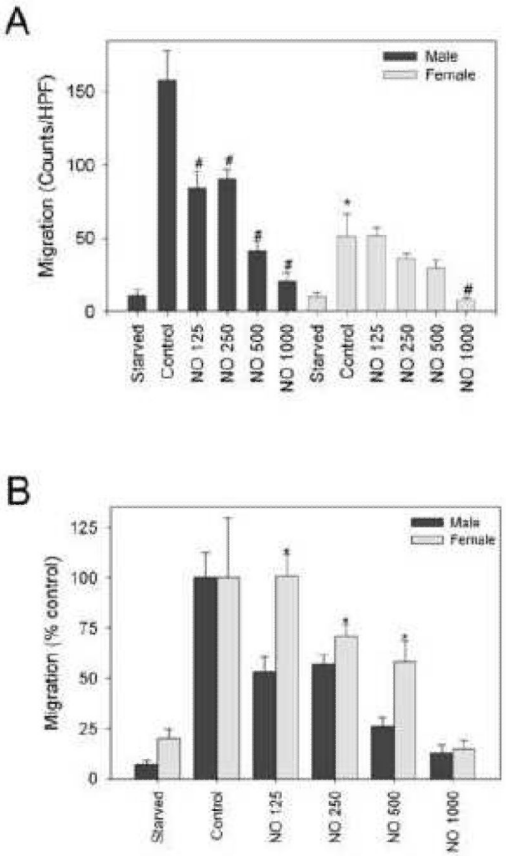

Figure 2.

Inhibition of migration by nitric oxide (NO) in male versus female vascular smooth muscle cells (VSMC). Migration was assessed using the scrape assay at 24 hours ± exposure (HPF=high power field) to the NO donor DETA/NO (NO, units are mol/L). A) Male VSMC migrate faster than female VSMC (*P<0.05, vs. male control). NO caused a dose-dependent inhibition of VSMC migration in males and decreased VSMC migration in females at the highest concentration ($^{\#}$P<0.05, vs. same-sex control). B) Graphical representation of the percent inhibition of migration by a given concentration of NO after adjusting the data to the control group (*P<0.05, vs. male NO counterpart). Data shown are representative of more than five separate experiments; n = 3/treatment group.

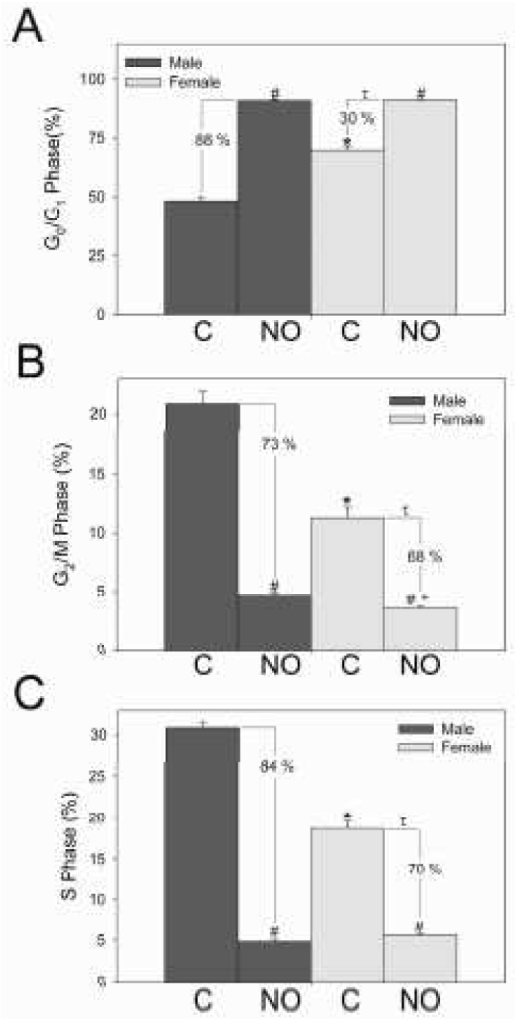

Figure 3.

Effect of nitric oxide (NO) on cell cycle progression in male and female vascular smooth muscle cells (VSMC). NO=DETA/NO 500 μmol/L and C=control. A-C) NO induced G_0/G_1 cell cycle arrest and decreased G_2/M and S phase in male and female VSMC ($^{\#}P<0.05$ vs. same sex control; $^{*}P<0.05$ vs. male control; $^{+}P<0.05$ vs. male NO). However, the percent change is greater in males compared to females ($^{\tau}P<0.05$ vs. percent reduction in males). Data shown are representative of five separate experiments; n = 3/treatment group.

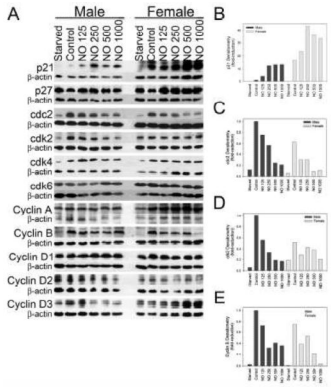

Figure 4.

Nitric oxide (NO) differentially effects the expression of cell cycle proteins based on sex. A) NO differentially effects the expression of cell cycle proteins based on sex by Western blot analysis. B-E) Densitometries of Western blots normalized by beta-actin loading controls and graphed as a percent of the male control. Female vascular smooth muscle cells (VSMC) expressed more p21 at baseline than male VSMC and NO increased p21 expression in males and females. NO decreased cdc2 expression in males and females. Male VSMC had a greater expression of cdk2 at baseline than female VSMC and that NO decreased cdk2 in male VSMC but not in female VSMC. NO decreased the expression of cyclin B in male and female VSMC, but to a slightly greater extent in females. NO=DETA/NO in mol/L. Data are representative of at least five separate experiments.

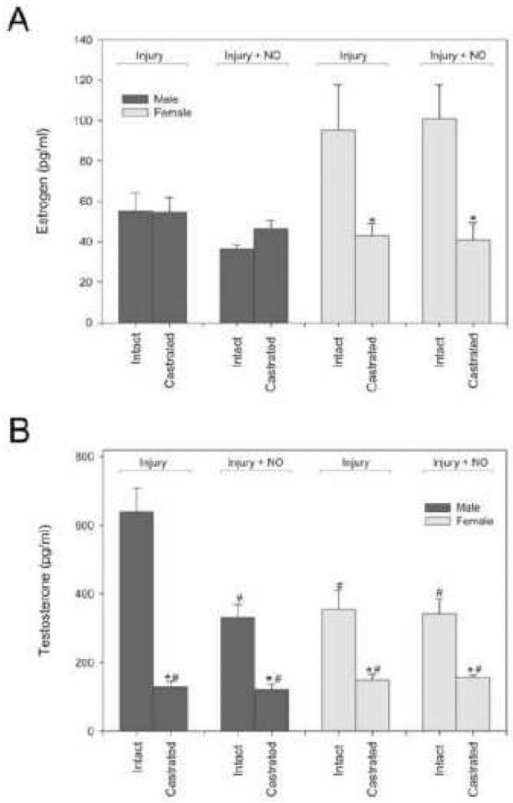

Figure 5.

A) Castration decreased estrogen levels in all female groups but did not affect estrogen levels in male rats (*P<0.05 compared to intact counterpart). B) Intact male injured rats had higher testosterone levels than any other rat groups ($^{\#}$P<0.05 compared to intact male injury). Castration significantly reduced testosterone levels in every group (*P<0.05 compared to intact counterpart). n=6/group.

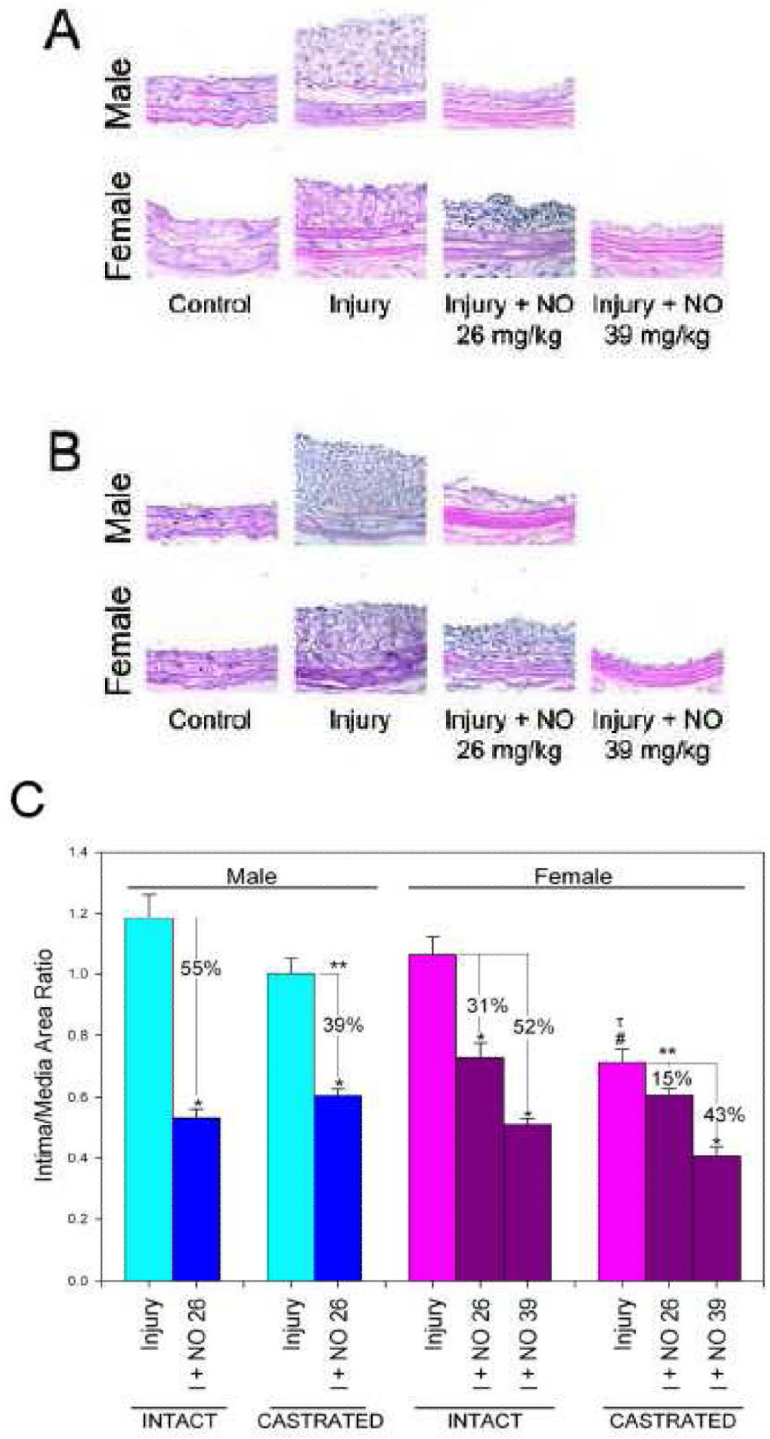

Figure 6.

Hematoxylin-eosin stained cross-sections of A) intact and B) castrated rat carotid arteries at 400X magnification. C) Morphometric analysis of the effect of NO on neointimal hyperplasia in male and female hormonally intact and castrated rodents (*P<0.05). NO=PROLI/NO (n=6/group).

Table 1

Carotid Artery Injury Model Morphometric Analysis in Hormonally Intact Rats

	Males		Females		
	Injury	**Injury + PROLI/NO 26 mg/kg**	**Injury**	**Injury + PROLI/NO 26 mg/kg**	**Injury + PROLI/NO 39 mg/kg**
Weight (grams)	371 ± 3	374 ± 4	269 ± 14 *b*	239 ± 7 *c*	257 ± 3 *c*
Circumference	1.71 ± 0.02	1.60 ± 0.02	1.44 ± 0.03 *b*	1.37 ± 0.02 *c*	1.41 ± 0.01 *c*
Luminal Area	8.33 ± 0.14	8.35 ± 0.20	5.82 ± 0.29 *b*	7.63 ± 0.17 *a,c*	7.90 ± 0.17*
Intima Area	21.96 ± 1.15	7.79 ± 0.44*a*	19.12 ± 0.97	9.76 ± 0.71 *a*	6.10 ± 0.23 *a*
Media Area	20.29 ± 0.80	14.69 ± 0.47*a*	18.00 ± 0.38	12.89 ± 0.37 *a*	12.37 ± 0.31 *a*
Intima/Media	1.18 ± 0.08	0.53 ± 0.03*a*	1.06 ± 0.06	0.73 ± 0.05 *a*	0.51 ± 0.02 *a*
Intima/(Intima + Media)	0.53 ± 0.01	0.35 ± 0.01*a*	0.51 ± 0.01	0.41 ± 0.02 *a,c*	0.33 ± 0.01 *a*

a P < 0.05 compared to same sex injury.

b P < 0.05 compared to male injury-alone.

c P < 0.05 compared to male injury + NO. n=6 per treatment group. All measured units of the artery are arbitrary units from ImageJ

Table 2

Carotid Artery Injury Model Morphometric Analysis in Hormonally Castrated Rats

	Males		Females		
	Injury	**Injury + PROLI/NO 26 mg/kg**	**Injury**	**Injury + PROLI/NO 26 mg/kg**	**Injury + PROLI/NO 39 mg/kg**
Weight (grams)	347 ± 5	357 ± 4	285 ± 5 *b*	243 ± 7 *c*	271 ± 6 *c*
Circumference	1.61 ± 0.01	1.56 ± 0.02	1.50 ± 0.02 *b*	1.51 ± 0.02	1.42 ± 0.02 *c*
Luminal Area	8.42 ± 0.14	8.86 ± 0.23	8.02 ± 0.24 *b*	7.97 ± 0.19 *c*	7.89 ± 0.24 *c*
Intima Area	17.6 ± 0.92	7.3 ± 0.25 *a*	13.0 ± 0.68 *b*	8.5 ± 0.27 *a*	4.4 ± 0.39 *a,c*
Media Area	17.6 ± 0.61	12.3 ± 0.43 *a*	17.7 ± 0.81	14.6 ± 0.33	10.8 ± 0.38 *a*
Intima/Media	1.00 ± 0.05	0.61 ± 0.02 *a*	0.71 ± 0.04 *b*	0.61 ± 0.02	0.41 ± 0.03 *a,c*
Intima/(Intima + Media)	0.50 ± 0.01	0.37 ± 0.01 *a*	0.44 ± 0.0 *b*	0.38 ± 0.01	0.27 ± 0.02 *a,c*

a P < 0.05 compared to same sex injury.

b P < 0.05 compared to male injury alone.

c P < 0.05 compared to male injury + NO. n=6 per treatment group. All measured units of the artery are arbitrary units from ImageJ