Redox Biology 4 (2015) 226–233

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Review Article

Sex-based differential regulation of oxidative stress in the vasculature by nitric oxide

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ARTICLE INFO

Article history:
Received 29 December 2014
Received in revised form
8 January 2015
Accepted 12 January 2015
Available online 13 January 2015

Keywords:
Neointimal hyperplasia
Superoxide
Nitric oxide
Vascular
Sex differences

ABSTRACT

Background: Nitric oxide ($^{\bullet}NO$) is more effective at inhibiting neointimal hyperplasia following arterial injury in male versus female rodents, though the etiology is unclear. Given that superoxide ($O_2^{\bullet-}$) regulates cellular proliferation, and $^{\bullet}NO$ regulates superoxide dismutase-1 (SOD-1) in the vasculature, we hypothesized that $^{\bullet}NO$ differentially regulates SOD-1 based on sex.

Materials and methods: Male and female vascular smooth muscle cells (VSMC) were harvested from the aortae of Sprague-Dawley rats. $O_2^{\bullet-}$ levels were quantified by electron paramagnetic resonance (EPR) and HPLC. *sod-1* gene expression was assayed by qPCR. SOD-1, SOD-2, and catalase protein levels were detected by Western blot. SOD-1 activity was measured via colorimetric assay. The rat carotid artery injury model was performed on Sprague-Dawley rats $\pm$ $^{\bullet}NO$ treatment and SOD-1 protein levels were examined by Western blot.

Results: *In vitro*, male VSMC have higher $O_2^{\bullet-}$ levels and lower SOD $-$ 1 activity at baseline compared to female VSMC ($P < 0.05$). $^{\bullet}NO$ decreased $O_2^{\bullet-}$ levels and increased SOD $-$ 1 activity in male ($P < 0.05$) but not female VSMC. $^{\bullet}NO$ also increased *sod* $-$ *1* gene expression and SOD $-$ 1 protein levels in male ($P < 0.05$) but not female VSMC. *In vivo*, SOD-1 levels were 3.7-fold higher in female versus male carotid arteries at baseline. After injury, SOD-1 levels decreased in both sexes, but $^{\bullet}NO$ increased SOD-1 levels 3-fold above controls in males, but returned to baseline in females.

Conclusions: Our results provide evidence that regulation of the redox environment at baseline and following exposure to $^{\bullet}NO$ is sex-dependent in the vasculature. These data suggest that sex-based differential redox regulation may be one mechanism by which $^{\bullet}NO$ is more effective at inhibiting neointimal hyperplasia in male versus female rodents.

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Introduction

Neointimal hyperplasia limits the long-term durability of vascular interventions such as balloon angioplasty, stenting, endarterectomy, and bypass grafting. Current FDA approved drug eluting stents, designed to prevent the development of neointimal hyperplasia, deliver derivatives of two different classes of drugs (i.e., rapamycin and paclitaxel), both of which indiscriminately inhibit all cellular proliferation, including endothelial cell proliferation. Thus, there is a great need to develop novel therapies that effectively prevent neointimal hyperplasia while also promoting vascular healing. Nitric oxide ($^{\bullet}NO$) is one such drug that possesses many different vasoprotective properties. $^{\bullet}NO$ is a small gaseous molecule that is known to inhibit platelet adherence and aggregation, mitigate leukocyte chemotaxis, and prevent vascular smooth muscle cell (VSMC) and adventitial fibroblast proliferation and migration [1–9]. Simultaneously, $^{\bullet}NO$ stimulates endothelial cell proliferation and prevents endothelial cell apoptosis [10,11]. Our laboratory and others, have demonstrated the beneficial effect of $^{\bullet}NO$ delivery to the vasculature to prevent neointimal hyperplasia in various different small and large animal models of arterial injury and bypass grafting [12–20]. However, our laboratory also demonstrated that $^{\bullet}NO$ has differential efficacy at inhibiting neointimal hyperplasia based on sex and hormone status [21]. The etiology for this difference, with $^{\bullet}NO$ being much more effective at inhibiting neointimal hyperplasia in males compared to females, and in hormonally intact versus castrated

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http://dx.doi.org/10.1016/j.redox.2015.01.007
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animals, remains unclear.

One possible explanation may reside in the regulation of oxidative stress between the sexes. Indeed, Vassalle et al. demonstrated differing levels of oxidative stress between men and postmenopausal women, with and without coronary artery disease (CAD). While postmenopausal females with CAD exhibited greater oxidative stress compared to men, they presented with less severe CAD [22]. Reactive oxygen species (ROS) have been found to extensively contribute to the severity of vascular disease and subsequent formation of neointimal hyperplasia [23–25]. Specifically, superoxide ($O_2^{\bullet -}$), one of the main ROS, has been shown to be elevated after vascular injury, resulting in the formation of neointimal hyperplasia through increased proliferation and migration of VSMC and adventitial fibroblasts [26–28]. Superoxide dismutases, modulate this response through dismutation of $O_2^{\bullet -}$ into oxygen and hydrogen peroxide, with the latter being enzymatically converted to water by catalase or other antioxidant peroxidases [29,30]. $^{\bullet}$NO can readily react with $O_2^{\bullet -}$ to form peroxynitrite, which can have detrimental effects in the vasculature. Our laboratory recently demonstrated that $^{\bullet}$NO regulates $O_2^{\bullet -}$ levels in a cell-specific manner in the vasculature through modulating SOD-1 levels [31]. Thus, given the role of $O_2^{\bullet -}$ in stimulating VSMC proliferation and migration and the role of $^{\bullet}$NO in modulating neointimal hyperplasia and SOD-1, we hypothesize that $^{\bullet}$NO differentially regulates SOD-1 levels based on sex. Here, we investigate the effect of $^{\bullet}$NO on $O_2^{\bullet -}$ generation, *sod-1 gene* expression, SOD-1 protein levels, and SOD activity *in vitro* and *in vivo* in male and female rodent models.

Materials and methods

$^{\bullet}$*NO-releasing donor*

1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO) and disodium 1-[(2-carboxylato)pyrrolidine-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO) were supplied by Dr. Larry Keefer (National Cancer Institute). Both DETA/NO and PROLI/NO are diazeniumdiolate $^{\bullet}$NO donors that release 2 mols of $^{\bullet}$NO per mole of compound at a predictable rate under physiologic conditions of pH 7 and temperature 37 °C [32]. Given that the duration of the *in vitro* experiments were up to 24 h, we used DETA/NO for these experiments since it has a half-life of 20 h. PROLI/NO was used for all animal studies given our prior work demonstrating superior efficacy of PROLI/NO in this animal model compared to other diazeniudiolate $^{\bullet}$NO donors [17,19,21,31].

Rat carotid artery injury model

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. Adult male and female Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing between 250–400 g were used for the study. Treatment groups included control, injury, and injury + $^{\bullet}$NO ($n=5$ rats/treatment group). Rats were anesthetized with inhaled isoflurane (0.5–2%), with subcutaneous atropine administration (0.1 mg/kg) to minimize airway secretions. The right common carotid artery for each rat served as the control group. Following sterile preparation, a midline neck incision was made. The left common, internal, and external carotid arteries were dissected, followed by occlusion of the internal and common carotid arteries. 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. The balloon was inflated to a pressure of 5 atm for 5 min to cause uniform injury. After the balloon was deflated and removed, the external carotid artery was ligated and blood flow restored. For the injury + $^{\bullet}$NO group, 10 mg of PROLI/NO was applied evenly to the external surface of the common carotid artery after balloon injury, as previously described [17,19,21,31]. Following injury and treatment, neck incisions were closed. Rats were sacrificed 3 days after treatment to harvest carotid arteries. Arteries within treatment groups were pooled, frozen in liquid nitrogen, powdered with mortar and pestle, and homogenized in 20 mM Tris (pH 7.4) with 1 μM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM sodium orthovanadate (Sigma, St. Louis, MO). Western blot analysis was performed as described below.

Cell culture

VSMC were harvested from the aortae of male and female Sprague-Dawley rats (Harlan) via methods as described by Gunther et al. [33] Male and female VSMC were confirmed via PCR (Supplementary Fig. 1) using SRY and GAPDH primers (IDT, Coralville, IA). Cells were maintained in media containing equal volumes of Ham's F12 and Dulbecco's modified Eagle's medium – low glucose (DMEM) (Invitrogen, Carlsbad, CA), complemented with 100 units/mL penicillin (Corning, Corning, NY), 100 μg/mL streptomycin (Corning), 4 mM L-glutamine (Corning), and 10% fetal bovine serum (FBS) (Invitrogen). Cells were incubated at 37 °C with 5% CO_2. To synchronize cells prior to DETA/NO treatment, cells were exposed to media lacking FBS for 24 h at 37 °C with 5% CO_2. VSMC used in this study were between passage 3 and 9.

EPR analysis

VSMC were plated on 100-mm dishes and allowed to attach overnight. Cells were serum starved for 24 h and exposed to DETA/NO (0.5–1.0 mM), pegylated (PEG)-SOD (50 U), or control media for 24 h. PEG-SOD was used as a control to ensure the signal measured was $O_2^{\bullet -}$-dependent. Cells were then washed with cold PBS, incubated with the cell-permeable spin probe, 1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethlpyrrolidine (CMH) (Enzo Life Sciences, Ann Arbor, MI) for 30 min, and subsequently analyzed in a temperature- and O_2-controlled Bruker EPR (Millerica, MA) at 37 °C, as described by Dikalov et al. [34]. The intensity of the first peak of the $^{\bullet}$CM radical spectrum was quantified. EPR signal intensity was normalized per mg of protein. All buffers contained 25 μM deferoxamine and were treated with Chelex resin from Biorad (Hercules, CA) to minimize deleterious effects of possible contaminating metals.

2-Hydroxyethidium analysis

VSMC were plated on 100-mm dishes and were incubated overnight to facilitate attachment. After cells reached 80% confluence, VSMC were exposed to DETA/NO (0.5–1.0 mM), pegylated PEG-SOD (50 U), or control media for 24 h. PEG-SOD was used as a control to ensure the changes measured were $O_2^{\bullet -}$-dependent. After 24 h of exposure to the various experimental conditions, VSMC were exposed to 10 μM of dihydroethidium (DHE) (Invitrogen) in the dark for 30 min. Subsequently, cells were washed with cold PBS, scraped, collected, protected from light, and stored at − 80 °C. $O_2^{\bullet -}$ levels were determined by quantifying the levels of 2-hydroxyethidium (2-OH-E) by HPLC with electrochemical detection according to Zielonka et al. [35]. The results were expressed as pmols of $O_2^{\bullet -}$ per mg of protein in total cell lysate.

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