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Nitric oxide is less effective at inhibiting neointimal hyperplasia in spontaneously hypertensive rats

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ABSTRACT

Exogenous administration of nitric oxide (NO) markedly decreases neointimal hyperplasia following arterial injury in several animal models. However, the effect of NO on neointimal hyperplasia in hypertension remains unknown. Here, we employ the spontaneously hypertensive rat (SHR) strain, inbred from Wistar Kyoto (WKY) rats, and the carotid artery balloon injury model to assess the effects of NO on neointimal hyperplasia development. 2 weeks after arterial injury, we showed that both rat strains developed similar levels of neointimal hyperplasia, but local administration of NO was less effective at inhibiting neointimal hyperplasia in the SHR compared to WKY rats (58% *vs.* 79%, $P < 0.001$). Interestingly, local administration of NO did not affect systemic blood pressure in either rat strain. Compared to WKY, the SHR displayed more proliferation in the media and adventitia following balloon injury, as measured by BrdU incorporation. The SHR also showed more inflammation in the adventitia after injury, as well as more vasa vasorum, than WKY rats. NO treatment reduced the vasa vasorum in the SHR but not WKY rats. Finally, while NO decreased both injury-induced proliferation and inflammation in the SHR, it did not return these parameters to levels seen in WKY rats. We conclude that NO is less effective at inhibiting neointimal hyperplasia in the SHR than WKY rats. This may be due to increased scavenging of NO in the SHR, leading to diminished bioavailability of NO. These data will help to develop novel NO-based therapies that will be equally effective in both normotensive and hypertensive patient populations.

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Introduction

Neointimal hyperplasia limits the long-term efficacy of cardiovascular interventions by leading to restenosis and ultimate occlusion of the artery or vein. Patients undergoing cardiovascular interventions typically have many co-morbidities, with one of the most common being hypertension. Hypertension is associated with endothelial dysfunction and vascular inflammation, both of which promote the development of medial wall thickening and perivascular fibrosis [1]. Previous studies have shown that hypertension is associated with increased development of neointimal hyperplasia in both vein and arterial bypass grafts [2,3]. In addition, a study of normotensive Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) showed an increase in wall thickness of arterial graft in the SHR compared to WKY rats [4]. Thus, hypertension appears to be an important contributor to the development of neointimal hyperplasia following cardiovascular interventions.

We have previously shown that local delivery of nitric oxide (NO) effectively inhibits neointimal hyperplasia following arterial injury in different animal models [5–9]. However, little is known about the effect of supplemental NO on the arterial wall in the setting of hypertension. Given that the overarching goal of our lab is to develop a clinically applicable NO-based therapy for patients with vascular disease, and that many patients with vascular disease have hypertension, the aim of the current paper is to understand the effects of NO on neointimal hyperplasia in the context of hypertension. To conduct this study, we used the SHR and its control, the WKY rat strain. The SHR becomes hypertensive at 4–6 weeks of age without any external intervention [10]. The adult SHR exhibits endothelial dysfunction and low bioavailability of NO, despite having high levels of endothelial nitric oxide synthase (eNOS) [11]. This finding may be due to the fact that the SHR also has high levels of superoxide [12], which can scavenge NO and limit its bioavailability. Given these findings of high superoxide levels and low NO bioavailability, we hypothesized that supplemental administration of NO will not be as effective in preventing neointimal hyperplasia in the SHR compared to WKY rats.

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Materials and methods

Diazeniumdiolated proline (PROLI/NO)

The NO donor (PROLI/NO) used in this study was kindly provided by Drs. Joseph Hrabie and Larry Keefer of NCI-Frederick. PROLI/NO was chosen based on its NO release rate ($t_{1/2}$ = 1.8 s) [13], safety profile, and superior efficacy in the prevention of neointimal hyperplasia when compared to other diazeniumdiolates [5,6].

Blood pressure and heart rate measurements

Blood pressure and heart rate measurements were collected using the CODA™ non-invasive blood pressure system (Kent Scientific; Torrington, CT). Measurements were taken prior to and following induction of anesthesia, and at 5 and 10 min following balloon injury (see below). For measurements taken prior to induction, animals were restrained in a Broome rat holder and blood pressure and heart rate measured with a large rat tail cuff. For each time point, a maximum of 20 measurement cycles were taken at 5 s intervals and all valid measurements were subsequently included in analysis. All animals were pre-conditioned for the Broome holder daily for several days prior to surgery. Results are reported as an average of the mean blood pressure and heart rate per animal in each treatment group.

Rat carotid artery injury model

All animal procedures were performed in accordance with the principles outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication National Academy Press, 1996) and approved by the Northwestern University Animal Care and Use Committee. Male, 10-week-old SHR or WKY rats (Charles River; Wilmington, MA) weighing 235–300 g were anesthetized with inhaled isoflurane (1.0–5.0%). Treatment groups for each rat strain were as follows: uninjured control (n = 3), injury (n = 8 for WKY and n = 9 for SHR), and injury + NO (10 mg PROLI/NO, n = 7 for WKY and n = 8 for SHR). Prior to the procedure, atropine (0.1 mg/kg) and carprofen (Rimadyl™, 0.15 mg/kg) were administered subcutaneously to decrease airway secretions and to control pain, respectively. Sterile lubricant (Equaline; Boise, ID) was applied to the animal's eyes. Following a midline neck incision, the left common, internal, and external carotid arteries were identified. After distal ligation of the external carotid artery, the internal and common carotid arteries were occluded with atraumatic clamps. A No. 2 French arterial embolectomy catheter (Edwards Lifesciences; Irvine, CA) was inserted through an arteriotomy 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 min. After removal of the balloon, the external carotid artery was ligated and blood flow restored. If applicable, powdered PROLI/NO was applied evenly to the external surface of the injured common carotid artery as we have previously described [6,8,9]. Briefly, the external surface of the artery was gently dried with sterile gauze, and an aliquot of PROLI/NO powder was sprinkled on the injured area. Forceps were used to ensure even coverage of the artery with PROLI/NO around its entire circumference, including underneath. The neck incision was closed in two layers. Rats were sacrificed at 2 weeks for morphometric, immunohistochemical, and immunofluorescence analysis.

Tissue processing

Carotid arteries were harvested following *in situ* perfusion-fixation with phosphate buffered saline (PBS) and 2% paraformaldehyde (w/v) in PBS. Vessels were placed in 2% paraformaldehyde at 4 °C for 1 h, then overnight in 30% sucrose (w/v) in PBS at 4 °C for cryoprotection. The tissue was quick-frozen in Tissue-Tek® Optimal Cutting Temperature compound (Sakura Finetek USA; Torrance, CA) and 5-μm sections were cut throughout the entire injured segment of the common carotid artery using a Microm HM 550 cryostat (Fisher Scientific; Pittsburgh, PA).

Morphometric analysis

Carotid arteries harvested at 2 weeks were examined histologically for evidence of neointimal hyperplasia using routine hematoxylin and eosin (H&E) staining. Digital images were collected with light microscopy using an Olympus BHT microscope (Melville, NY) with 4×, 10× and 40× objectives. Six evenly-spaced sections through each injured carotid artery were analyzed. ImageJ software (NIH; Bethesda, MD) was used to obtain area measurements of the lumen, intima, and media (mm^2), and all analysis was done by a single individual.

Immunohistochemistry

From each animal, three evenly-spaced carotid sections from the area of injury underwent immunohistochemical staining. To assess proliferation, rats received an intraperitoneal injection of bromodeoxyuridine (BrdU, 100 mg/kg) at 24 and 1 h prior to sacrifice. Frozen sections were fixed in acetone for 5 min, and rinsed in PBS–Tween 20 for 2 min. Sections were then blocked with horse serum (Sigma; St. Louis, MO) diluted 1:20 in 0.5% bovine serum albumin (BSA) for 30 min. Primary antibody against BrdU (ab8955, Abcam; Cambridge, MA) was diluted 1:200 in 0.5% BSA and applied for 1 h. Following two rinses in PBS-Tween 20, biotinylated anti-mouse IgG secondary antibody diluted 1:500 in BSA was applied for 30 min (Vector Labs; Burlingame, CA). The sections were then incubated in Vectastain ABC reagent for 30 min, and chromagen/substrate (DAB peroxidase substrate kit, Vector Labs) for 2 min. Following counterstaining with Gill's hematoxylin solution (Fisher Scientific) and dehydration, sections were coverslipped with Permount (Fisher Scientific). For negative controls, PBS was substituted for the primary antibody. Brightfield images were digitally acquired using an Olympus BHT microscope (Melville, NY) and SPOT Basic software (Diagnostic Instruments, Inc.; Sterling Heights, MI). Positively stained cells were counted by a blinded investigator in four high-power fields per arterial section and expressed as an average.

To assess damage caused by reactive oxygen species, sections were stained for nitrotyrosine. Frozen sections were fixed in acetone for 5 min, rinsed in PBS-Tween 20 for 2 min, and rinsed in PBS for 2 min. Endogenous peroxidases were blocked by incubation in a solution containing 2% H_2O_2 and 60% methanol for 30 min. Following rinses in PBS-Tween 20 and PBS, primary antibody raised against nitrotyrosine (ab7048, Abcam) was diluted 1:200 in IHC-Tek Antibody Diluent (1 W-1000, IHC World; Woodstock, MD), which also acts as a blocking agent, and applied to sections for 1 h. Following three rinses in PBS, biotinylated anti-mouse IgG secondary antibody diluted 1:500 in PBS was applied for 30 min (Vector Labs), and the rest of the procedure was completed as above. For negative controls, PBS was substituted for the primary antibody. Brightfield images were digitally acquired using a Zeiss Imager.A2 microscope (Hallbergmoos, Germany) and the 5× objective, and staining was quantified by 3 blinded investigators using a scale of 0–3.

Immunofluorescence

To assess eNOS, monocyte/macrophage, and soluble guanylyl cyclase (sGC) staining, sections were fixed in paraformaldehyde