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Insights into the Effect of Nitric Oxide and its Metabolites Nitrite and Nitrate at Inhibiting Neointimal Hyperplasia

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Abstract

Objective—Periadventitial delivery of the nitric oxide (NO) donor PROLI/NO following arterial injury effectively inhibits neointimal hyperplasia. Given the short half-life of NO release from PROLI/NO, our goal was to determine if inhibition of neointimal hyperplasia by PROLI/NO was due to NO, or its metabolites nitrite and nitrate.

Methods and Results—*In vitro*, the NO donor DETA/NO inhibited proliferation of rat aortic vascular smooth muscle cells (RASMC), but neither nitrite nor nitrate did. *In vivo*, following rat carotid artery balloon injury or injury plus the molar equivalents of PROLI/NO, nitrite, or nitrate (n=8–11/group), PROLI/NO was found to provide superior inhibition of neointimal hyperplasia (82% inhibition of intimal area, and 44% inhibition of medial area, $p<0.001$). Only modest inhibition was noted with nitrite or nitrate (45% and 41% inhibition of intimal area, and 31% and 29% inhibition of medial area, respectively, $p<0.001$). No effects on blood pressure were noted with any treatment groups. *In vivo*, only PROLI/NO inhibited cellular proliferation and increased arterial lumen area compared to injury alone ($p<0.001$). However, all three treatments inhibited inflammation ($p<0.001$).

Conclusions—PROLI/NO was more effective at inhibiting neointimal hyperplasia following arterial injury than nitrite or nitrate. However, modest inhibition of neointimal hyperplasia was observed with nitrite and nitrate, likely secondary to anti-inflammatory actions. In conclusion, we have demonstrated that the efficacy of NO donors is primarily due to NO production and not its metabolites, nitrite and nitrate.

Keywords

Peripheral Vascular Disease; Neointimal Hyperplasia; Nitric Oxide; Nitrite/Nitrate

INTRODUCTION

Peripheral arterial disease (PAD) is a major cause of morbidity and mortality in the United States and although both open and endovascular therapies are available to treat PAD, these

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treatment modalities are limited by high rates of restenosis secondary to neointimal hyperplasia.[1] Our lab and others have shown that periadventitial application of NO donors inhibits the development of neointimal hyperplasia in animal models of arterial injury.[2–7] Of the NO donors our laboratory has evaluated, the diazeniumdiolate disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO) has been the most effective.[2; 4] As a diazeniumdiolate, PROLI/NO provides a predictable release of 2 moles of NO per mole of compound and in powder form can be easily applied to the periadventitial surface of the artery. However, compared to other diazeniumdiolates, PROLI/NO has the shortest half-life (2 seconds) *in vitro* at a constant pH of 7.4 and temperature of 37°C.[8] Following the quick, high burst release from PROLI/NO, NO also has a very short half-life *in vivo* and is rapidly scavenged by hemoglobin or metabolized to the inorganic ions nitrite and nitrate, which have longer respective half-lives of approximately 45 minutes and up to 6 hours.[9] Given the very short half-life of NO release from PROLI/NO as well as NO itself, we considered if the inhibition in neointimal hyperplasia observed with PROLI/NO may be because of the metabolites nitrite and nitrate and not just to the initial release of NO from the donor PROLI/NO.

Inorganic nitrite and nitrate are no longer considered inert end products of NO metabolism but have been shown to be biologically active in the vasculature. Nitrite has been shown to have vasoprotective properties. For example, intravenous administration of nitrite *in vivo* has been shown to stimulate vasodilation in human and non-human primates and nitrite has also been shown to inhibit proliferation in smooth muscle cells from rat pulmonary arteries.[9; 10] However, these potentially therapeutic properties of nitrite and nitrate have largely been attributed to the ability of inorganic nitrite and nitrate to function as a reservoir for further NO production. Nitrate is reduced to nitrite, mostly by bacterial nitrate reductase, and then nitrite is converted back to NO through a variety of enzymatic and nonenzymatic mechanisms both in the circulation and in the tissues.[11] Therefore, it has been suggested that nitrite and nitrate may be potential therapeutic agents for pathology such as neointimal hyperplasia through the reconversion to NO which has many vasoprotective properties that inhibit neointima formation.

Thus, the aim of this study was to determine whether periadventitial application of the NO metabolites nitrite and nitrate following balloon angioplasty in the rat carotid injury model would be as efficacious as the molar equivalent of the NO donor PROL/INO for the prevention of neointimal hyperplasia. Our hypothesis is that the NO release from PROLI/NO will provide greater inhibition of neointimal formation than either inorganic nitrite or nitrate.

MATERIALS AND METHODS

NO donors

The NO donors used in this study are diazeniumdiolates that were generously supplied by Drs. Joseph Hrabie and Joseph Saavedra of NCI-Frederick. The diazeniumdiolates used in this study were chosen based on their NO release rates, their safety profiles, and for their efficacy in previously published experiments.[4; 8; 12] PROLI/NO was chosen for our *in vivo* experiments based on prior demonstration of its superior efficacy in the prevention of neointimal hyperplasia when compared to other diazeniumdiolates.[2; 4] For *in vitro* experiments, 1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO) was used because of its longer half-life of 20 hours, making this a more appropriate NO donor to use for 24-hour *in vitro* assays.[8; 12] Sodium nitrite [$NaNO_2$] and sodium nitrate [$NaNO_3$] were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell culture

Rat aortic smooth muscle cells (RASMC) and rat aortic endothelial cells (RAEC) were isolated and cultured from the aortas of 8-week male Sprague Dawley rats (Harlan, Indianapolis, IN) using the collagenase method and maintained as previously described.[13–15] RASMC and RAEC were characterized by anti-smooth muscle α-actin monoclonal antibodies (Sigma; St. Louis, MO) and von Willebrand factor (Dako Cytomation; Carpenteria, CA), respectively, and by cell-specific morphology. Cells were maintained in medium containing equal volumes of Dulbecco's Modified Eagle's Medium-low glucose (JRH; 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 mM L-glutamine (VWR; West Chester, PA) and incubated at 37°C, 95% air and 5% CO_2. RAEC were also supplemented with endothelial cell growth supplement (Millipore, Billerica, MA) and MEM non-essential amino acids (Invitrogen). For all experiments, cells used were between passages three and eight. After plating, cells were growth-arrested with media containing no FBS for 24 hours and then exposed to media containing DETA/NO or the molar equivalents of either nitrite or nitrate for an additional 24 hours before collection.

Proliferation assay

Tritiated (^{3}H) thymidine incorporation was assessed as a surrogate for cellular proliferation. Male RASMC and RAEC were plated in 12-well plates at 1×10^4 and 0.75×10^4 cells/well, respectively. In addition to DETA/NO, nitrite or nitrate, treatment media also contained ^{3}H-thymidine (5 μCi/mL, PerkinElmer, Wellesley, MA). ^{3}H-thymidine incorporation into trichloroacetic acid–precipitated DNA was quantified by scintillation counting.

Cell death assay

A trypan blue exclusion protocol was performed to assess overall cell death. RASMC and RAEC were plated in 6-well plates at 6×10^4 and 5×10^4 cells/well, respectively. Following starvation and treatment, cells were trypsinized, collected, and placed in suspension. Aliquots of cell suspension were exposed to an equal volume of 4% trypan blue and the percentage of blue (nonviable) cells was determined by counting on a hemocytometer.

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. Ten-week-old male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) weighing 300–400g were anesthetized with inhaled isoflurane (1.0–5.0%). Treatment groups included injury alone (n=11), injury+20 mg PROLI/NO (n=8), injury+11 mg sodium nitrite (n=8), or injury+13 mg sodium nitrate (n=8). Prior to the procedure, atropine (0.1 mg/kg) and carprofen (Rimadyl TM) (0.15 mg/kg) were administered subcutaneously to decrease airway secretions and for pain control, respectively. Equaline sterile lubricant (Boise, ID) was applied to the animal's eyes. Following a midline nick 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 and a No. 2-French arterial embolectomy catheter (Edwards Lifesciences, Irvine, CA, USA) 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 restored. PROLI/NO, nitrite, or nitrate were immediately applied evenly to the external surface of the injured

common carotid artery in their dry powder form. The neck incision was closed in two layers. Rats were sacrificed at 2 weeks for morphometric and immunohistochemical analysis.

Tissue processing

Carotid arteries were harvested following *in-situ* perfusion-fixation with phosphate buffered saline (PBS) and 2% paraformaldehyde. Vessels were placed in paraformaldehyde at 4°C for 1 hour, then overnight in 30% sucrose in PBS at 4°C for cryo-protection. The tissue was quick-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 using a Microm HM 550 cryostat.

Morphometric analysis

Carotid arteries harvested at 2 weeks were examined histologically for evidence of neointimal hyperplasia using routine hematoxylin-eosin (H&E) 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 analyzed. Intima area, media area, and luminal area were measured. These values were obtained (arbitrary units) using ImageJ software and all analysis was performed by a single individual.

Immunohistochemistry

From each animal, three evenly spaced carotid sections from the area of injury were stained and examined for evidence of proliferation and inflammation using immunohistochemical staining. For proliferation, rats received an intraperitoneal injection of bromodeoxyuridine (BrdU, 100 mg/kg) at 24 and 1 hour prior to sacrifice. For all immunohistochemistry, frozen sections were fixed in acetone for 5 minutes, rinsed in PBS-Tween 20 for 2 minutes, and blocked with horse serum (Sigma, St. Louis, MO, USA) in 0.5% bovine serum albumin (BSA) for 30 minutes. Primary antibody in BSA was applied for 1 hour: anti-ED1 (monocyte/macrophage, 1:200, Santa Cruz Biotechnology; Santa Cruz, CA), anti-CD45 (leukocyte, 1:200, AbD Serotec; Raleigh, NC) or anti-BrdU (1:200, Abcam; Cambridge, MA). Secondary antibody in BSA was applied for 30 minutes (horse anti-mouse biotinylated, affinity-purified anti-immunoglobulin, 1:500, Vector Labs; Burlingame, CA). The sections were incubated in Vectastain ABC reagent for 30 minutes, the chromagen/substrate (DAB peroxidase substrate kit, Vector Labs, Burlingame, CA) for 2 minutes, counterstained with Gill's hematoxylin solution (Fisher Scientific, Pittsburgh, PA), dehydrated, and coverslipped with mounting medium (Permount, Fisher Scientific, Pittsburgh, PA). For negative controls, PBS was substituted for the primary antibody. Digital images were taken with light microscopy using an Olympus BHT microscope (Melville, NY) and SPOT camera basic Software (Diagnostic Instruments, Inc, Sterling Heights, MI). For nuclear stains (i.e., CD45 and BrdU), cells with positive staining were counted by a blinded investigator in four high power fields per arterial section and expressed as an average. For cytoplasmic stains (i.e., ED1), staining was quantified by a blinded investigator using a scale of 0–4.

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 both prior to and following induction of anesthesia, and both 5 and 10 minutes following injury. For measurements taken prior to induction, animals were restrained in a Broome rodent restrainer (Harvard Apparatus; Holliston, MA) 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 second intervals and all valid measurements were subsequently included in analysis. Results are reported as an average of the mean blood pressure and heart rate per animal in each treatment group.

Statistical analysis

Results are expressed as mean ± standard error of the mean. Differences between multiple groups were analyzed using one-way analysis of variance (ANOVA) with the Student-Newman-Keuls *post hoc* test for all pair-wise comparisons (SigmaStat; SPSS, Chicago, IL). Statistical significance was assumed when $p<0.05$.

RESULTS

VSMC proliferation was inhibited by NO but not by nitrite or nitrate *in vitro*

To determine the effect of the metabolites nitrite and nitrate on proliferation and cell death *in vitro*, RASMC and RAEC were exposed to increasing concentrations of DETA/NO and molar equivalent concentrations of nitrite or nitrate (Figure 1A and 1C). Results were expressed as a percentage of the control group counts (i.e., proliferation) or total cells (i.e., cell death). For RASMC exposed to DETA/NO, proliferation was inhibited in a dose-dependent fashion and reached significance at the two highest concentrations, with 80% inhibition of proliferation observed with 1.0 mM DETA/NO ($p<0.001$; Figure 1A). RASMC exposed to the molar equivalent concentrations of nitrite or nitrate proliferated at rates similar to controls. We also evaluated the ability of nitrite and nitrate to inhibit proliferation of RASMC at very low concentrations, specifically at 50 nM, 125 nM, 250 nM, 500 nM, 1 μM, 5 μM, 10 μM, 25 μM, and 50 μM, and still observed no inhibition of proliferation. Last, we evaluated the effect of the parent compound, proline, on inhibition of RASMC proliferation and observed no inhibition over a range of concentrations (25–1000 μM).

Overall, there was less than 10% cell death in all treatment groups. As expected, there was a small increase in RASMC cell death following exposure to 0.5 and 1.0 mM DETA/NO, with a 3% and 2% increase in death compared to control respectively ($p<0.02$, Figure 1B). RASMC exposed to the molar equivalent concentrations of nitrite or nitrate experienced cell death similar to control.

Because RAEC *in vitro* have increased sensitivity to DETA/NO as compared to RASMC, lower concentrations of the NO donor and molar equivalent concentrations of nitrite and nitrate were evaluated. Although there was an overall increase in proliferation of RAEC exposed to DETA/NO, nitrite and nitrate, these results were not statistically significant (Figure 1C). Similar to RASMC, overall cell death in all treatment groups was less than 10% and although there was a trend toward decreased death in cells exposed to DETA/NO, nitrite or nitrate, these results did not reach statistical significance (Figure 1D).

Periadventitial delivery of PROLI/NO, nitrite, or nitrate does not cause hypotension

One limitation of NO-based therapies administered systemically is the development of unwanted side effects such as hypotension. Although NO is diffusible and can penetrate all layers of the artery wall when applied to the adventitia, it is our assertion that local periadventitial delivery does not produce enough systemic NO absorption to produce systemic side effects. To evaluate this definitively, we measured the blood pressure and heart rate at 4 time points before and after exposure to NO: 1) prior to anesthesia, 2) after induction of anesthesia, 3) 5 minutes after injury and NO exposure, and 4) 10 minutes after injury and NO exposure (Table 1). There were no significant changes in heart rate among any of the treatment groups for all time points measured. With regards to blood pressure, as anticipated, animals in all treatment groups experienced significant hypotension with

induction of anesthesia (mean Δ −41 mmHg for all treatment groups, p≤0.006). At 10 minutes following injury, mean blood pressure in the injury alone group decreased significantly compared to the post-anesthetic induction blood pressure (Δ −18 mmHg, p<0.001). However, treatment with PROLI/NO, nitrite or nitrate was associated with an increase in blood pressure (PROLI/NO Δ +17 mmHg, p<0.001; nitrite Δ +31 mmHg, p=0.218; nitrate Δ +7 mmHg, p<0.001). These data demonstrate that NO and its metabolic end products nitrite and nitrate do not cause hypotension when administered locally.

PROLI/NO inhibits neointimal hyperplasia more effectively than either nitrite or nitrate

The aim of our study was to compare the effect of PROLI/NO to the NO metabolites nitrite or nitrate *in vivo* on neointimal hyperplasia using the rat carotid injury model. A dose of 20 mg of PROLI/NO was chosen based on our previous studies.[2] Molar equivalent doses of nitrite and nitrate were calculated based on the predicted moles of NO that would be released from PROLI/NO. Analysis of our results indicates that although there was some inhibition of neointima formation following application of nitrite and nitrate, PROLI/NO was the most effective at inhibiting neointimal hyperplasia (Table 2 and Figure 2A–D). Compared to injury alone, PROLI/NO inhibited initmal area by 82% compared to only 45% and 41% observed with nitrite and nitrate, respectively (p<0.001 for PROLI/NO, nitrite, and nitrate vs. injury alone; p<0.05 for PROLI/NO vs. nitrite and nitrate, Figure 2A). However, only the PROLI/NO treated group resulted in a statistically significant decrease in the intima/media and intima/(intima+media) area ratios (63% and 48% reduction, respectively, p<0.001, Figure 2B, Table 2).

In addition to inhibition of neointimal formation, we have previously demonstrated that PROLI/NO affects arterial remodeling following injury.[2] Similar to previous results, PROLI/NO inhibited medial area in this study by 44% compared to injury alone (p<0.001, Figure 2C). Nitrite and nitrate also inhibited medial area but to a lesser extent with a 31% and 29% reduction compared to injury alone, respectively (p<0.001). The reduction in medial area induced by PROLI/NO was statistically greater than the reduction induced by nitrate (p<0.05) but not nitrite. With respect to lumen area, only the PROLI/NO-treated group resulted in a statistically significant increase in lumen area compared to injury alone (16% increase, p<0.001, Table 2).

PROLI/NO, but not nitrite or nitrate, inhibited cellular proliferation *in vitro*

Given that DETA/NO inhibited proliferation of RASMC but nitrite and nitrate did not, we wanted to evaluate the effect of PROLI/NO, nitrite, and nitrate on cellular proliferation following arterial injury *in vivo*. Interestingly, we observed a pattern similar to the observed *in vitro* results. PROLI/NO inhibited cellular proliferation by 59% compared to injury alone (p<0.001, Figure 3) whereas nitrite and nitrate did not affect cellular proliferation following arterial injury *in vivo*. In fact, compared to treatment with PROLI/NO, arteries exposed to nitrite or nitrate had significantly increased BrdU incorporation (3-fold, p<0.001 and 2-fold, p=0.012, respectively). This indicates that the efficacy of nitrite and nitrate *in vivo* is not secondary to effects on cellular proliferation.

PROLI/NO, nitrite, and nitrate inhibit the inflammatory response following arterial injury

We have previously demonstrated that NO inhibits monocyte/macrophage infiltrate following injury.[2] In this study, monocyte/macrophage and leukocyte infiltration were assessed with immunohistochemical staining for ED1 and CD45, respectively. PROLI/NO significantly inhibited monocyte/macrophage infiltrate by 28% compared to injury alone (p<0.001, Figure 4A and 4B). Monocyte/macrophage infiltration was also inhibited with nitrite and nitrate by 28% and 35%, respectively, compared to injury alone (p<0.001). The reduction in monocyte/macrophage infiltration was not statistically different among the

three groups. NO inhibited leukocyte infiltration by 44% compared to injury alone ($p<0.001$, Figure 4C and 4D). Nitrite inhibited leukocyte infiltration by 33% following injury ($p=0.001$), while nitrate inhibited leukocyte infiltration to an even greater extent following injury (61%, $p<0.001$). The reduction in CD45 infiltration by nitrate was significantly different than the reduction caused by nitrite ($p=0.035$), but not PROLI/NO. These data, together with the cellular proliferation data, provide a potential explanation for the differences observed in efficacy between PROLI/NO, nitrite, and nitrate at preventing neointimal formation. The efficacy of nitrite and nitrate may be due to their anti-inflammatory properties since they appear to have no antiproliferative properties in this animal model.

Discussion

In this paper we have shown that the NO donor PROLI/NO provides superior inhibition of neointimal hyperplasia when compared to either of its metabolites, nitrite or nitrate. Although less efficacious, nitrite and nitrate did provide modest inhibition of neointima formation and did affect arterial remodeling. Our data indicate that the efficacy of nitrite and nitrate at preventing neointimal hyperplasia appears to be related to its anti-inflammatory effects on monocyte/macrophage and CD45 leukocyte infiltration, since neither nitrite or nitrate inhibited proliferation *in vitro* or *in vivo*. Inflammation plays an important role in neointimal formation. Any therapy that has anti-inflammatory properties will affect the arterial injury response. Thus, our data suggest that although NO donors may provide superior efficacy for the prevention of neointimal hyperplasia *in vivo*, there is some therapeutic potential for nitrite and nitrate in the vasculature.

Nitrite and nitrate were once thought of as inert end products of NO metabolism. However, we now know that nitrite and nitrate serve as a storage reservoir for production of NO. Nitrate, either produced from NO metabolism or through dietary sources, can be converted to nitrite via nitrate reductase. Although this process is catalyzed primarily by bacterial nitrate reductase in the gut where nitrate serves as an alternative electron acceptor to oxygen, the enzyme xanthine oxidoreductase has also been shown to have nitrate reductase activity in rodents and humans, primarily in the liver and intestine, under acidic conditions.[16–19] Nitrite can be reduced to NO through several enzymatic and nonenzymatic mechanisms including interaction with deoxyhemoglobin, deoxymyoglobin, protons, and xanthine oxidoreductase.[11; 16; 18–22] In fact, in the vasculature conversion of nitrite to NO by deoxyhemoglobin is an important pathway for NO-mediated vasodilation under hypoxic conditions.[11] Nitrate reductase activity has even been identified in vascular smooth muscle cells, from homogenized rings of arterial wall, and even from mouse macrophage RAW264.7 cells.[23] Thus, these pathways serve as an important supplement to the production of NO from L-arginine by NO synthases (NOS).

Because the reduction of nitrite leads to the direct production of NO, in comparison with nitrate which must first be reduced to nitrite, most studies have focused on the therapeutic potential of nitrite. Several investigators have shown that nitrite causes vasodilation and a decrease in blood pressure in human and non-human primates.[9; 24–26] Furthermore, conversion of nitrite to NO has been proposed as a mechanism by which vasodilation occurs under hypoxic/acidic conditions.[26] In fact, the conversion of nitrite to NO by deoxyhemoglobin/myoglobin, the mitochondrial electron transport chain, and xanthine oxidoreductase are all dependent upon low oxygen tension. The increased reduction of nitrite to NO in hypoxic environments is important to the potential efficacy of nitrite for the prevention of tissue damage in ischemia reperfusion injury seen with myocardial infarction and post-subarachnoid hemorrhage vasospasm.[11; 27; 28] Nitrite has also been shown to affect cGMP production, cytochrome P450 activity, and heat shock protein 70 and heme

oxygenase-1 expression in several different tissues.[29] Thus, it is clear that nitrite has both direct and indirect biological activity *in vivo*.

In a recent study by Zuckerbraun et al, nitrite treatment inhibited proliferation in rat pulmonary artery vascular smooth muscle cells (VSMC) *in vitro* through the cyclin dependent kinase inhibitor p21.[10] Since NO-mediated inhibition of neointimal hyperplasia is mediated in part through its antiproliferative effects on VSMC,[30; 31] these findings support the assertion that nitrite and nitrate may be useful for the prevention of neointimal hyperplasia. Interestingly, our study did not reproduce the inhibitory effect of nitrite on proliferation reported by Zuckerbraun et al, even though the doses used in our study were significantly higher than those used to inhibit proliferation in the Zuckerbraun study. However, a distinct difference between our study and that of Zuckerbraun et al is that we studied the effects of nitrite and nitrate in a normoxic environment while Zuckerbraun et al studied the effects of nitrite and nitrate in a hypoxic environment. Since nitrite reduction to NO occurs at significantly higher rates under hypoxic conditions as discussed above,[32] it is possible that this preconditioning increased reduction of nitrite to NO, thereby accounting for the antiproliferative effects observed in the Zuckerbraun et al study. The modest inhibition in neointimal hyperplasia and anti-inflammatory effects of nitrite seen in our study may therefore be secondary to direct effects of the metabolites and not through conversion to NO.

A direct mechanism that may account for the modest efficacy of nitrite and nitrate at inhibiting neointimal hyperplasia in our study are their anti-inflammatory effects. It is well-known that inflammation plays an important role in stimulating proliferation following arterial injury.[33; 34] It is also known that NO inhibits inflammation following arterial injury.[2; 4; 35] Thus, our finding that both nitrite and nitrate inhibit infiltration of monocytes/macrophases and CD45 leukocytes in a normoxic environment is interesting and important. Stokes et al administered nitrite in the drinking water of mice on a hypercholesterolemic diet and found a decrease in leukocyte adhesion and improved vasorelaxation in the microvasculature.[36] This study was conducted in a normoxic environment, similar to our study. Thus, while our data support that of Stokes et al, we also report that nitrate has distinct biological activity against inflammatory cells, in fact greater than nitrite. Since our study as well as Stokes et al was conducted in a normoxic environment, these data suggest that nitrite and nitrate may inhibit inflammation through a direct mechanism, independent of conversion back to nitrite or NO.

Nitrite and nitrate may not have been as effective at inhibiting neointimal hyperplasia compared to PROLI/NO because of the delivery method. In our study, the NO donors and metabolites were applied periadventitially following arterial injury. We have found this method of delivery to result in consistent and reproducible effects of PROLI/NO on neointimal hyperplasia.[2; 37–39] Other studies that have examined the efficacy of nitrite or nitrate have focused on the intravenous, intraperitoneal or inhaled routes. Since NO is a highly diffusible molecule, it is possible that the efficacy of PROLI/NO is due to diffusion of NO throughout the arterial wall. Less is known about the diffusible nature of nitrite and nitrate. While nitrite and nitrate are small molecules, they are anions and may not be able to diffuse through all layers of the arterial wall to have direct effects on the cells in the media and intima when applied to the adventitia. However, since the adventitia is now recognized as a predominate source of cells that repopulates the media and contributes to neointimal development, application of any therapy to the adventitia is likely to be more effective than luminal delivery. Moreover, since many of the known pathways that convert nitrate to nitrite, or nitrite to NO exist in the vasculature,[11] and given the existence of the vaso vasorum in the adventitia, it is possible for these alternative routes of NO production to take

place in the adventitia. Thus, while our delivery method may have been a limitation of this study, it may also represent an advantageous method of delivery.

In conclusion, periadventitial delivery of PROLI/NO provides superior inhibition of neointimal hyperplasia following rat carotid artery injury when compared to the NO metabolites nitrite and nitrate. Although nitrite and nitrate did not demonstrate antiproliferative effects *in vitro* or *in vivo*, a modest inhibition of neointima formation was seen following treatment with these anions. This effect may be related to inhibition of the inflammatory response following injury observed with these anions. Overall, NO release from PROLI/NO is very effective at inhibiting neointimal hyperplasia by affecting multiple aspects of the arterial injury response (i.e., proliferation, inflammation, etc). The efficacy of PROLI/NO at inhibiting neointimal hyperplasia deserves to be studied in a preclinical large animal model.

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Abbreviations

PROLI/NO	the diazeniumdiolate disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate
DETA/NO	1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
RASMC	rat aortic smooth muscle cells
RAEC	rat aortic endothelial cells

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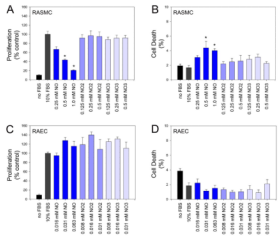

Figure 1.

Proliferation (A & C) and cell death (B & D) were assessed in rat aortic smooth muscle cells (RASMC) and rat aortic endothelial cells (RAEC) following exposure to increasing concentrations of DETA/NO or molar equivalent concentrations of nitrite and nitrate. Data representative of 3 separate experiments. n=3/treatment group. *p<0.05 compared to control; NO=nitric oxide, NO2=nitrite, NO3=nitrate.

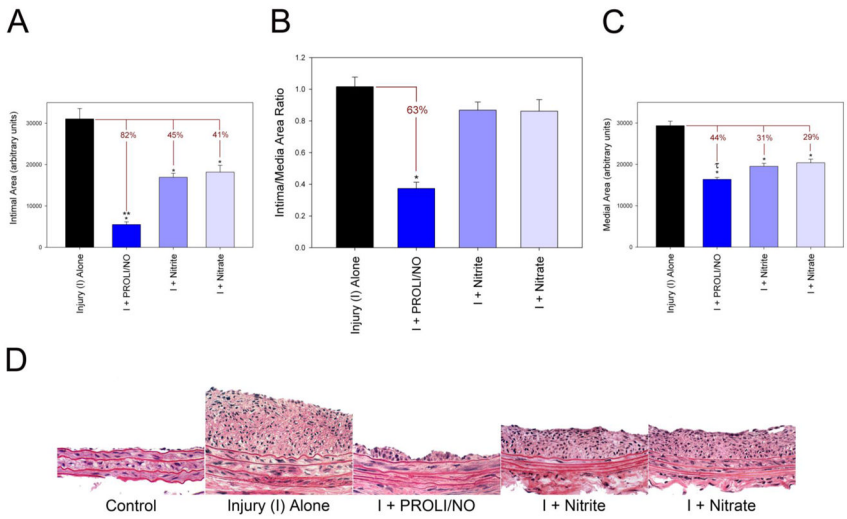

Figure 2.

Quantification of neointimal hyperplasia via morphometric analysis of rat carotid artery sections at 14 days: (A) intimal area, (B) intima/media area ratio and (C) medial area. Units are arbitrary (n=8–11). *p<0.05 vs. injury alone. **p<0.05 vs. nitrite or nitrate. τp<0.05 vs. nitrate. (D) H&E stained rat carotid artery sections from each treatment group (400x). I=injury.

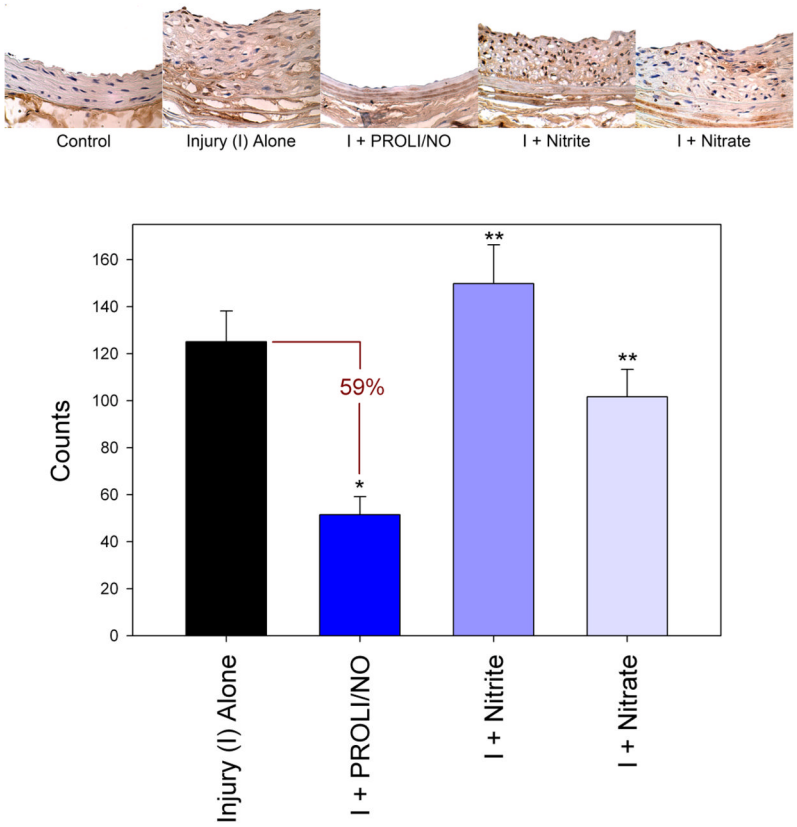

Figure 3.

Graphical and histologic representations of cellular proliferation in rat carotid artery sections at 14 days as indicated by immunohistochemical staining for bromodeoxyuridine (BrdU) DNA incorporation. *p<0.05 as compared to injury alone. **p<0.05 as compared to injury + PROLI/NO. I=injury, Counts=counts per high power field.

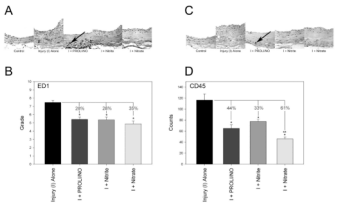

Figure 4.

Inflammation following rat carotid artery injury at 14 days: Representative sections and quantification of (A and B) monocyte/macrophage (ED1) and (C and D) leukocyte (CD45) infiltration, respectively. ED1 staining was quantified on a scale of 0–4 for the intima, media, and adventitia, and the sum of these grades is reported. CD45 staining was quantified as the number of positive staining cells (e.g., at arrow) per high power field. *$p<0.05$ vs. injury alone. **$p=0.035$ vs. nitrite. I=injury.

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Table 1

Mean Rat Blood Pressure (BP) and Heart Rate (HR)

Mean Systolic BP	**Before Anesthesia**	**After Anesthesia**	**5 min after Injury/Treatment**	**10 min after Injury/Treatment**
Injury Alone	115 ± 2	86 ± 1*	70 ± 2*#	68 ± 1*#
Injury + PROLI/NO	114 ± 9	64 ± 1*	85 ± 2#	81 ± 1#
Injury + Sodium Nitrite	94 ± 4	66 ± 2*	70 ± 3*	97 ± 4*
Injury + Sodium Nitrate	126 ± 5	68 ± 1	69 ± 2	75 ± 2
Mean HR	Before Anesthesia	After Anesthesia	5 min after Injury/Treatment	10 min after Injury/Treatment
Injury Alone	553 ± 14	530 ± 32	524 ± 44	469 ± 6
Injury + PROLI/NO	706 ± 127	607 ± 37	570 ± 31	489 ± 2
Injury + Sodium Nitrite	638 ± 45	603 ± 52	528 ± 12	515 ± 7
Injury + Sodium Nitrate	632 ± 48	405 ± 21	467 ± 14	492 ± 12

*$p<0.05$ compared to Before Anesthesia

#$p<0.05$ compared to After Anesthesia

Table 2

Morphometric analysis of the carotid arteries 14 days after balloon injury

Treatment Group	Lumen Area	Intima Area	Media Area	I/M	I/(I+M)
Injury alone	111387.3 ± 3622	34909.2 ± 3270	29652.0 ± 1099	1.11 ± 0.08	0.48 ± 0.01
Injury + PROLI/NO	129116.2 ± 2856*	8428.5 ± 1371*	16715.3 ± 577*	0.48 ± 0.06*	0.25 ± 0.02*
Injury + Sodium Nitrite	111530.3 ± 3756	17631.3 ± 1051*	20481.4 ± 973*	0.90 ± 0.60	0.45 ± 0.02
Injury + Sodium Nitrate	114674.1 ± 4032	18177.3 ± 1653*	21015.0 ± 935*	0.89 ± 0.07	0.43 ± 0.03

Abbreviations: I/M = intima/media area ratio; I/(I+M) = intima/(intima+media) area ratio.

*p<0.001 vs. injury alone

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