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NITRIC OXIDE DELIVERY VIA A PERMEABLE BALLOON CATHETER INHIBITS NEOINTIMAL GROWTH AFTER ARTERIAL INJURY

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Abstract

Background—Neointimal hyperplasia limits the longevity of vascular interventions. Nitric oxide (NO) is well known to inhibit neointimal hyperplasia. However, delivery of NO to the vasculature is challenging. Our study aims to evaluate the efficacy of delivering NO to the site of injury using a permeable balloon catheter. Our hypothesis is that ultra-short duration of NO delivery using a permeable balloon catheter will inhibit neointimal hyperplasia.

Materials and Methods—10-week-old male Sprague-Dawley rats underwent carotid artery balloon injury. Groups included: 1) control, 2) injury, 3) injury + periadventitial NO, and 4) injury + endoluminal NO via permeable balloon catheter. The catheter was inflated to 5 atmospheres of pressure for 5 minutes. Arteries were harvested 2 weeks following injury. Morphometric assessment for neointimal hyperplasia and immunohistochemical staining for inflammatory markers were performed.

Results—Injury increased neointimal hyperplasia compared to control (intima/media area [I/M] ratio 1.07 vs. 0.11, respectively, $p < 0.001$). Periadventitial delivery of NO reduced the I/M area ratio compared to injury alone (55% decrease, $p < 0.001$). Endoluminal delivery of NO also reduced the I/M area ratio compared to injury alone (65% decrease; $p < 0.001$). Both endoluminal and periadventitial NO affected the I/M ratio by reducing the intimal area (64% and 46%, respectively, $p < 0.001$) whereas neither affected the medial area. Periadventitial NO delivery increased lumen area ($p < 0.05$), whereas endoluminal NO delivery increased circumference ($p < 0.05$). Periadventitial NO delivery inhibited macrophage intimal infiltration compared to injury alone ($p < 0.05$).

Conclusion—These data demonstrate that short duration endoluminal NO delivery via permeable balloon catheters inhibits neointimal hyperplasia following arterial interventions. Endoluminal delivery of NO could become a focus for future clinical interventions.

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Keywords

Neointimal Hyperplasia; Restenosis; Nitric Oxide; Adventitia; Media; Intima; Inflammation; Macrophage; Endoluminal

INTRODUCTION

Cardiovascular disease affects over 70 million Americans, with atherosclerosis being responsible for the majority of morbidity and mortality in the United States.[1] Vascular intervention is often necessary to treat advanced atherosclerosis. Options include angioplasty, stenting, endarterectomy, and bypass grafting. The durability of these interventions is limited by the inevitable development of neointimal hyperplasia.[2;3]

Our lab and others have shown that nitric oxide (NO) is a potent inhibitor of neointimal hyperplasia.[4-11] Delivery methods have included inhalational, intraluminal, and periadventitial delivery; however, each of these delivery methods poses challenges for effective clinical use. Given that endoluminal therapies continue to evolve at a fast pace with many interventions requiring little, if any, open exposure, development of a simple methodology to deliver NO to the site of injury using current endoluminal technology would be advantageous. To date, little has been reported on the ability to successfully deliver NO to the vasculature using endoluminal methods. In one recent study, Harnek et al were able to inhibit neointimal growth by exposing porcine coronary arteries to the NO donor SIN-1 for 5 minutes, following injury using a double occlusion balloon.[12] However, this methodology requires that the arterial segment being treated has no branches.

The aim of this study was to investigate whether NO could successfully diffuse through the pores of a latex balloon catheter to effectively inhibit the development of neointimal hyperplasia after arterial injury, with only 5 minutes of delivery time. In addition, we sought to identify what differences, if any, would exist between intraluminal versus periadventitial delivery of NO with respect to neointimal hyperplasia, remodeling, and inflammation. Our hypothesis was that short duration endoluminal delivery of NO using a permeable balloon catheter to the site of injury is feasible and will effectively inhibit the development of neointimal hyperplasia.

MATERIALS AND METHODS

In Vitro Assessment of NO Release from the Latex Balloon Catheter

A 4 French arterial embolectomy balloon catheter was submerged in 1.5 ml PBS in a microfuge tube and insufflated to 5 atm of pressure with a 300 mM solution of 1-[(2-Carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO) until the sides of the balloon were approximating the tapered sides of the microfuge tube. The balloon was deflated after 5 minutes and the nitrite (NO_2^-) concentration was measured using the Griess reaction. A balloon filled with PBS was used as the negative control. Another balloon catheter was immersed in a vial containing 5 ml of PBS under constant stirring. The diffusion of NO through the balloon into the PBS was assessed using the Apollo 4000 free radical analyzer (World Precision Instruments, Sarasota, FL).

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. 10-week-old Sprague-

Dawley (Harlan, Indianapolis, IN) male rats were anesthetized using 2% inhaled isoflurane. The rat carotid artery injury model was performed in all rats as previously described, using a 2 French arterial embolectomy catheter inflated to 5 atmospheres of pressure for 5 minutes. [11;13;14] Subcutaneous atropine (0.1 mg/kg) was administered to decrease airway secretions. Rimadyl (0.15 mg/kg) was administered for analgesia and Equaline eye ointment was used to prevent eye dryness. All animals were weighed, and the results recorded, prior to surgery. Treatment groups included (n=6 rats per treatment group): 1) control, 2) injury, 3) injury + periadventitial NO delivery (PROLI/NO 26 mg/kg), and 4) injury + endoluminal NO delivery via permeable balloon catheter (PROLI/NO 300 mM). All procedures were performed by the same surgeon. Uninjured arteries served as controls.

NO Donors

For the animals that received periadventitial delivery of NO, PROLI/NO (26 mg/kg) was applied as a powder to the periadventitial surface of the artery after restoration of blood flow following balloon injury, as previously described.[11;15] For animals that received endoluminal delivery of NO, a solution of PROLI/NO (300 mM) dissolved in a phosphate buffer (0.75 M, pH 7.4) was made fresh immediately before creation of the arteriotomy on the external carotid artery. As soon as the PROLI/NO powder dissolved, the solution was used to insufflate the balloon catheter in the injury + NO diffusible balloon catheter group. Importantly, vigilant monitoring of the pressure in the balloon catheter was necessary as the NO gas released in the solution caused a steady rise in pressure. Adjustments in the insufflators were meticulously made to maintain a pressure at 5 atmospheres for 5 minutes. For all other treatment groups, deionized water was used to insufflate the balloon catheter.

Tissue Processing

All animals were sacrificed 14 days after injury. Carotid arteries were procured after *in situ* perfusion/fixation with cold PBS (300 mL) and 2% paraformaldehyde (300 mL) as previously described.[13] After procurement, arteries were preserved by submersion in 2% paraformaldehyde for 1 hour followed by cryoprotection with 30% sucrose. Each artery was then quick-frozen in Optimal Cutting Temperature medium (Tissue Tek, Hatfield, PA). Sections 5- μ m-thick were cut through the entire area of injury of the carotid artery specimen.

Morphometric Analysis

The resulting degree of neointimal hyperplasia was assessed histologically by performing standard hematoxylin and eosin staining. Light microscopy digital images, utilizing 4X, 10X and 40X objectives, of the stained sections were taken using an Olympus BHT microscope (Melville, NY). Morphometric analysis was performed on six evenly-spaced sections through the area of injury for each carotid artery. Data from the six sections per rat were averaged and statistical analysis performed on the means. ImageJ software (NIH, Bethesda, MD) was used to obtain measurements of lumen area, arterial circumference, intimal area (defined as the area between the lumen and internal elastic lamina) and medial area (defined as the area between the internal and external elastic lamina). The intima/media area ratio was subsequently derived. All measurements were performed by the same blinded investigator.

Immunofluorescence

Frozen sections were fixed in 2% paraformaldehyde, permeabilized with 0.3 % Triton X-100 in PBS for 10 minutes, blocked with serum (1:50 in BSA) for 30 minutes, exposed to the primary mouse monoclonal anti-ED1 antibody (1:50 in BSA, Santa Cruz Biotechnology, Santa Cruz, Ca) for 1 hour, and exposed to the secondary antibody AlexaFluor 555 (1:500 in

PBS, Invitrogen, Grand Island, NY) for 1 hour. Digital images of the sections were obtained and quantified by a blinded investigator with ImageJ on six evenly-spaced sections per artery. Quantification consisted of grading positive immunofluorescent staining on a scale of 0 (no staining) to 4 (maximum staining). The same blinded investigator performed all analyses. All technical replicates were pooled for statistical analysis.

Statistical Analysis

Data were reported using the mean $\pm$ the standard error of the mean. One-way analysis of variance was used to compare the differences between groups. The Student–Newman–Keuls post hoc test was then used for all pairwise comparisons (SigmaStat; SPSS, Chicago, IL, USA). Statistical significance was assumed for $P < 0.05$.

RESULTS

NO is able to diffuse through a latex balloon catheter

A 4F latex balloon catheter was insufflated with PROLI/NO (300 mM), and submerged for 5 minutes in a 5 ml vial of water. The initial rate of NO diffusion, was 17 ± 3 nmoles/min for the NO-filled balloon whereas no NO release was detected for the PBS-filled balloon (Figure 1). However, the diffusion rate slowed down after 1.5 to 2 minutes (Figure 1). A similar experiment was performed immersing a 4F latex balloon catheter insufflated with PROLI/NO (300 mM) in 1.5 ml of PBS in a microcentrifuge tube for 5 minutes. After the catheter was removed, the concentration of NO_2^- in the microcentrifuge tube measured by the Griess reaction, was 13 ± 1 μM , which shows that 19.5 nmoles of NO diffused across the latex membrane. Even though this result is slightly under the values obtained using the NO electrode, they are both in the same range of concentrations. NO_2^- was non-detectable for the PBS negative control (Griess limit of detection ~ 1 μM).

Short duration endoluminal delivery of NO effectively inhibits the development of neointimal hyperplasia

After documenting the capacity of NO to diffuse through a balloon catheter, we sought to identify whether this might translate into inhibition of neointimal hyperplasia in the rat carotid artery injury model. As expected, balloon injury caused a significant increase in neointimal hyperplasia compared to control arteries (Figure 2 and Figure 3), as assessed by the intima/media area ratio (I/M 1.07 vs. 0.11, respectively, $p < 0.001$). Periadventitial application of NO caused an expected reduction in the I/M area ratio compared to injury alone (55% decrease, $p < 0.001$ compared to injury alone). Interestingly, only 5 minutes of endoluminal delivery of NO through the balloon catheter resulted in a more profound decrease in the I/M area ratio compared to injury alone (65% decrease, $p < 0.001$ compared to injury alone). Similarly, both endoluminal and periadventitial delivery of NO resulted in a reduction of intimal area (64% vs. 46%, respectively, $p < 0.001$ compared to injury alone). Whereas periadventitial NO delivery increased luminal area (64% increase; $p < 0.001$ compared to injury alone), endoluminal delivery did not (10% increase, $p = \text{NS}$). On the contrary, whereas endoluminal delivery increased arterial circumference (6% increase; $p < 0.001$ compared to injury alone), periadventitial delivery did not (0.4% decrease, $p = \text{NS}$). Neither NO-based therapy affected medial area.

Periadventitial, but not endoluminal NO delivery mitigated macrophage infiltration following arterial balloon injury

Inflammation after arterial injury is important to the development of neointimal hyperplasia. NO inhibits the development of neointimal hyperplasia, in part, by reducing the inflammation caused by arterial injury.[16;17] We sought to quantify the effect of both

periadventitial and endoluminal delivery on injury-induced inflammation using immunofluorescent assessment for the macrophage marker, ED1. Injury predictably caused a significant increase in macrophage staining (Figure 4) in the adventitia (1.8-fold increase), the media (5.4-fold increase), and the neointima (9.3-fold increase) as compared to control ($p < 0.05$). Periadventitial application of NO reduced ED1 staining in the neointima (47% reduction, $p < 0.05$) compared to injury alone. There was no significant change in adventitial or medial ED1 staining after periadventitial delivery of NO compared to injury alone. Interestingly, endoluminal delivery of NO had no effect on medial and adventitial macrophage infiltration compared to injury alone. On the other hand, endoluminal delivery of NO resulted in an increased macrophage infiltration to the media compared to injury.

DISCUSSION

In this manuscript we report that an extremely short duration of balloon-mediated endoluminal delivery of NO is not only feasible but resulted in effective inhibition of neointimal hyperplasia following arterial injury. Numerous attempts have been made to develop an efficacious way to deliver NO to the site of arterial injury to prevent the development of neointimal hyperplasia. In 1996, Lee et al demonstrated a 43% reduction in neointimal growth in rats treated with inhalational NO for 2 weeks.[6] These results were questioned by Rich et al, when they found that NO levels were negligible in pulmonary arteries after inhalational therapy, presumably due to the inactivation of NO by hemoglobin.[18] McNamara et al and, separately, Chen et al found that the NO precursor L-arginine can inhibit the development of neointimal hyperplasia in animal models.[19;20] Unfortunately, no follow-up studies have proven the efficacy of systemic L-arginine in human subjects. Studies published by Groves et al and Napoli et al demonstrated that systemic NO donors can effectively inhibit neointimal hyperplasia in animal models.[21;22] Studies investigating the utility of systemic NO donors in humans have arrived at conflicting results.[23;24] In addition, systemic administration of NO donors can predispose recipients to numerous unwanted side effects, including vasodilation, headaches, hypotension and bleeding complications. Lastly, local periadventitial application of NO has been shown to reliably inhibit neointimal hyperplasia. Molsidomine delivered locally on a hydrogel-coated balloon in pigs, S-nitrosoacetylpenicillamine gel on vein grafts in rabbits, local infusion of 3-morpholino-syndonimine after PTCA in pigs as well as local periadventitial PROLI/NO application in rats, all have been shown to inhibit the subsequent development of neointimal hyperplasia after arterial injury.[11;25-27] However, no one has yet shown that ultra-short endoluminal delivery of NO has the ability to inhibit neointimal hyperplasia.

The realization that the endoluminal delivery of NO is efficacious has important clinical applications. Neointimal hyperplasia and restenosis are significant causes of long-term failure of vascular interventions. Endoluminal delivery is a simple and feasible mode of delivering NO to the areas of injury, thus delaying or preventing neointimal growth. This has profound implications for improving the longevity of all vascular interventions, but particularly endovascular interventions. There are many ways through which the neointima develops. Ross et al postulated that medial smooth muscle cells proliferate in response to arterial injury and migrate towards the arterial lumen, thus contributing to the neointima.[28] Perhaps the endoluminal delivery of NO is better able to limit the role that medial smooth muscle cells play in neointimal development compared to a periadventitial route, as the NO can more readily diffuse into the medial layer instead of having to traverse the adventitia to reach the media. Alternatively, endoluminal delivery of NO may have a greater effect on the circulating progenitor cells that contribute to the formation of neointimal hyperplasia.[29] Regardless, the endoluminal route of administration brings with it tremendous simplicity of use. Indeed, it is simpler than the drug-eluting balloon catheters being used in Europe, and in clinical trials in the United States, as all that would be required is to insufflate the balloon

catheters with a NO-donating solution instead of saline. This approach could be readily adapted in any clinical scenario.

An interesting finding from our data was that periadventitial NO caused a late increase in luminal area while endoluminal NO did not. The adventitia, we now know, is much more involved in the development of neointimal hyperplasia after arterial injury than was first thought.[30-32] The main cellular proponent of the adventitia, the fibroblast, is mainly a secretory cell. Arterial injury induces the fibroblast to differentiate into a myofibroblast, which has contractile, as well as migratory, properties. Shi et al, elegantly demonstrated the importance of adventitial myofibroblasts to neointimal development following adventitial proliferation, translocation to the neointima, and differentiation into myofibroblasts.[33] Preventing constrictive remodeling after injury may reflect an inhibition in either fibroblast differentiation into myofibroblasts or inhibition of the contractile properties of these cells. Periadventitial application of NO may be to be more efficacious than endoluminal delivery of NO at accomplishing this, thus leading to an increase in luminal area.

Surprisingly, periadventitial NO inhibited macrophage infiltration to the neointimal, whereas endoluminal NO did not. It is known that the adventitia serves as a nidus for the infiltration of inflammatory cells following arterial injury. In agreement with our results, Okamoto et al demonstrated in a porcine coronary artery injury model that the increase in adventitial macrophages outpaces the increase seen in either the media or intima.[34] Interestingly, while the absolute number of macrophages in the neointima is less than that seen in the adventitia, the relative increase in the neointima after injury is far greater than that seen in the adventitia.

The differences observed between the effect of endoluminal vs periadventitial delivery could be due to differential bioavailability of NO throughout the all at different time points. The periadventitial route is more likely to result in longer exposure times to NO. This may result in NO having an effect in signaling cascades that are triggered during the first 24h after injury. With endoluminal delivery, NO will only be present for the time of the intervention, given its short half-life. We have shown that this ultra-short exposure is enough to effectively inhibit neointimal hiperplaisa. However, this different temporal exposure to NO might account for the inability of NO to inhibit macrophage infiltration.

Our study did have limitations. By necessity, we used two different states of PROLI/NO. For the periadventitial application of PROLI/NO, a powder was applied at 26 mg/kg. For the endoluminal delivery of NO, a 300 mM solution of PROLI/NO in phosphate buffer was used to insufflate the balloon. Measuring nitrite by the Griess assay, the total amount of NO that diffused out of the balloon in 5 minutes was estimated to be 19.5 nmoles. Using the NO sensor and assuming a steady diffusion rate of 17 nmoles/min, the upper limit for the total amount of NO that diffused out of the balloon was estimated to be 85 nmoles. Assuming an average rat weight of 400 g, the total dose used for the endoluminal delivery of NO was between 6 and 27 μ g/kg. This means that similar inhibition of neointimal hyperplasia was achieved with an endoluminal dose 1,000 times smaller than that administered via the periadventitial route. However, we cannot estimate the effective concentrations of NO reached at the site of injury for either delivery method, which prevents us from drawing any further conclusions about which delivery method is most effective. Translating our data into the clinical setting will be impossible without testing the ability of NO to diffuse from an angioplasty, not an embolectomy, balloon catheter. Angioplasty catheter balloons are able to circumferentially dilate asymmetric, atherosclerotic arteries whereas embolectomy catheter balloons were never made for such a purpose. Angioplasty balloon catheters are much less compliant than embolectomy balloon catheters, allowing them to function effectively in the hostile environments of atherosclerotic arteries.

Our study focused on inhibiting neointimal hyperplasia induced in healthy arteries without evidence of atherosclerotic disease. It is too early in our investigation to draw conclusions on how the endoluminal delivery of NO would affect diseased, atherosclerotic lesions undergoing balloon angioplasty. These arteries are likely to exhibit asymmetric plaque burden. The inflammatory complement of the arterial wall will be much different than that seen in a previously healthy artery and what affect this may have on endoluminal NO's anti-neointimal effects is hard to say. We've successfully shown that NO can be delivered via an endoluminal route and, after diffusion through an embolectomy balloon catheter, can effectively inhibit neointimal hyperplasia. Additional studies need to be performed before our conclusions can be extrapolated to atherosclerotic arteries.

Although our data were consistent with published reports supporting the recruitment and subsequent migration of adventitial macrophages, our study did not seek to identify exactly how the increase in macrophages was occurring. Recruitment from circulating populations of monocytes or proliferation of resident macrophages could both explain the increases observed. An interesting follow-up to this project would be to evaluate the effect of endoluminal vs. periadventitial delivery of NO on macrophage activity. Furthermore, our study did not assess the concentration or activity level of other inflammatory cells. Neutrophils also exhibit early adventitial recruitment and activation after arterial injury. A full investigation of the range of inflammatory cells involved in neointimal hyperplasia would have to be performed before definite conclusions about the effect of NO delivery routes on neointimal inflammation can be made.

As endovascular interventions continue to evolve, the ability to deliver NO via an endoluminal route takes on increasingly significant clinical importance. Understanding the basis of the anti-proliferative properties of NO will ultimately lead to more targeted therapies to prevent neointimal hyperplasia.

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Figure 1. Nitric oxide diffusion across a latex balloon catheter

A NO analyzer was used to measure diffusion rates from a 4F latex balloon catheters filled with either PROLI/NO (300 mM) or PBS. The graph shows representative data from 4 separate experiments using 3 different balloon catheters.

Figure 2. Endoluminal and periadventitial delivery of NO effectively inhibit neointimal growth Hemotoxylin and eosin staining of rat common carotid arteries (50X) with magnified representative views (200X). Treatment groups (n=6/group) include control, injury, injury + periadventitial NO (PA NO), and injury + endoluminal NO (EL NO).

Figure 3. Endoluminal and periadventitial delivery of NO significantly reduce intimal area and intima to media area ratio after arterial injury

Graphical representation of A) intima/media (I/M) area ratio, B) lumen area, C) intimal area, D) medial area, and E) arterial circumference. Treatment groups (n=6/group) include control, injury, injury + periadventitial NO (PA NO), and injury + endoluminal NO (EL NO) (*p<0.001 vs control, **p<0.001 vs Injury).

Figure 4. Periadventitial NO but not endoluminal NO reduced inflammation after arterial injury

A) Representative images of immunofluorescent staining of rat common carotid arteries for the macrophage marker ED1 (50X). Treatment groups (n=6/group) include control, injury, injury + periadventitial NO (PA NO), and injury + endoluminal NO (EL NO). B) Quantification of 6 equally-spaced sections throughout the injured and treated segments was performed using a scale of 0-4 (0 representing minimal and 4 representing maximal staining) by the same blinded investigator. Macrophage infiltration of the intima, media, and adventitia are reported (*p<0.001 vs control, #p<0.001 vs Injury, †p<0.001 vs Injury + PA NO).