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Periadventitial adipose tissue modulates the effect of PROLI/NO on neointimal hyperplasia

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ARTICLE INFO

Article history:
Received 8 March 2016
Received in revised form
20 May 2016
Accepted 27 June 2016
Available online 5 July 2016

Keywords:
Neointimal hyperplasia
Nitric oxide
Periadventitial adipose tissue
Adipokines

ABSTRACT

Background: Periadventitial delivery of nitric oxide (NO) inhibits neointimal hyperplasia; however, the effect of periadventitial adipose tissue on the efficacy of NO at inhibiting neointimal hyperplasia has not been studied. The aim of our study was to assess the effect of NO in the presence and absence of periadventitial adipose tissue. We hypothesized that removal of periadventitial adipose tissue will increase neointimal formation and that NO will be more effective at inhibiting neointimal hyperplasia.

Methods: The effect of NO on 3T3 fibroblasts, adventitial fibroblast (AF), and vascular smooth muscle cell (VSMC) proliferation was assessed by ^{3}H-thymidine incorporation in adipocyte-conditioned or regular media. The rat carotid artery balloon injury model was performed on male Sprague–Dawley rats. Before balloon injury, periadventitial adipose tissue was removed (excised model) or remained intact (intact model). Treatment groups included injury or injury with periadventitial application of PROLI/NO. Adiponectin receptor (AR) levels were assessed via immunofluorescence.

Results: Adipocyte-conditioned media had an antiproliferative effect on 3T3 and AF and a pro-proliferative effect on VSMC *in vitro*. Interestingly, NO was less effective at inhibiting 3T3 and AF proliferation and more effective at inhibiting VSMC proliferation in adipocyte-conditioned media. *In vivo*, the excised group showed increased neointimal hyperplasia 2 wk after surgery compared with the intact group. NO reduced neointimal hyperplasia to a greater extent in the excised group compared with the intact group. Although NO inhibited or had no impact on AR levels in the intact group, NO increased AR levels in media and adventitia of the excised group.

Conclusions: These data show that periadventitial adipose tissue plays a role in regulating the arterial injury response and the efficacy of NO treatment in the vasculature.

Published by Elsevier Inc.

Introduction

Atherosclerosis is responsible for the majority of morbidity and mortality associated with cardiovascular disease in the United States.[1] Furthermore, the economic burden of surgical revascularization of severe atherosclerosis through angioplasty, stenting, endarterectomy, and bypass grafting continues to rise.[1,2] Compounding this problem, the durability of these interventions is limited due to negative remodeling and neointimal hyperplasia.[3] Neointimal hyperplasia is a multicomponent

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0022-4804/$ – see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.jss.2016.06.074

process triggered by endothelial cell injury that is characterized largely by inflammation, cellular proliferation, cellular migration, and extracellular matrix deposition.[4-7] Ultimately, this arterial injury response leads to restenosis and the need for subsequent revascularization or significant morbidity.

Evidence suggests that periadventitial adipose tissue modulates restenosis after arterial injury.[8-11] It has been shown that under physiological conditions, periadventitial adipose tissue is protective against restenosis.[10] Protective small molecules derived from periadventitial adipose tissue include nitric oxide (NO) and the anti-inflammatory adipokine adiponectin.[8] Adiponectin has been shown to negatively regulate neointimal growth, whereas pro-inflammatory adipokines such as leptin have been associated with increased neointimal hyperplasia.[9,10] Studies have shown that NO donors have great promise in limiting the development of neointimal hyperplasia.[12-16] However, the effect of periadventitial adipose tissue on the efficacy of NO donor treatment at inhibiting neointimal hyperplasia has not been studied.

The objective of this study was to assess the effect of NO donors in the presence and absence of periadventitial adipose tissue. We hypothesized that removal of periadventitial adipose will increase neointimal formation and that NO donors will be more effective at inhibiting neointimal hyperplasia in that setting.

Methods

Cell culture

3T3-L1 fibroblasts were purchased from ATCC (Manassas, VA) and cultured in 90% DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, ATCC; Manassas, VA). Primary rat vascular smooth muscle cells (VSMCs) and adventitial fibroblasts (AFs) were harvested and grown from the aortae of 10-wk-old male Sprague–Dawley rats and maintained in DMEM:Ham's F-12 (Invitrogen, Carlsbad, CA) media. Cells were used between passages 4 and 9.

Cellular proliferation

Adipocyte phenotype for 3T3-L1 fibroblasts was induced with 90% DMEM, 10% bovine calf serum, 1.0 μg/mL of insulin, 1.0 μM of dexamethasone, and 0.5 mM of methylisobutylxanthine, according to the ATCC (Manassas, VA) protocol. After 72 h, adipocytes were maintained in 90% DMEM, 10% FBS, and 1.0 μg/mL of insulin. Phenotype induction was confirmed by morphologic observation under the microscope. Media was changed and collected at days 9, 11, and 15 after induction of the adipocyte phenotype. This media was pooled, stored at 4°C protected from light, and used as conditioned media. VSMC, AF, or 3T3 fibroblasts were plated on day 1 in 24-well plates at a density of 26,000-30,000 cells/well. On day 2, cells were synchronized with serum-free media. On day 3, cells were exposed to media (DMEM + 10% FBS) or adipocyte-conditioned media, then treated with varying concentrations of the NO donor (Z)-1-[N-(2-Aminoethyl)-N-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate (DETA/NO) in the presence of ^{3}H-Thymidine (5 μM). After 24 h, incorporated ^{3}H-Thymidine was assessed using a scintillation counter, as previously described.[17,18] Although PROLI/NO was used as an NO donor in the *in vivo* experiments due to its efficacy, the short half-life of PROLI/NO makes it impractical to use for cell culture experiments *in vitro*. Hence, DETA/NO, which has a half-life of 24 h, was used as the NO donor for all *in vitro* experiments.

Animal surgery

All animal procedures adhered to the rules and regulations 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 all animal protocols were approved by the Northwestern University Animal Care and Use Committee. The carotid artery injury model was performed on 10-wk-old Sprague–Dawley rats. Removal of the periadventitial adipose tissue before the balloon injury was conducted in an atraumatic fashion using microforceps and microscissors with a dissecting microscope. Briefly, for the arteries in which the periadventitial adipose tissue was removed (excised group), we identified the fat loosely adherent to the surface of the artery, picked up the fat with curved microforceps, and excised the fat using microscissors. The entire dissection was conducted through a dissecting microscope. We took great care to not manipulate the artery in anyway. Proximal and distal control of the carotid artery was conducted similarly for both the treatment groups. For the group in which the periadventitial adipose tissue was left intact (intact group), we left all periadventitial adipose tissue on the artery. Treatment groups included control, injury, and injury plus the NO donor PROLI/NO (10 mg) for each model, $n = 5$ per treatment group. The NO donor was applied directly to the adventitia as previously described.[14-16] Arteries were harvested 2 wk after arterial injury after *in situ* perfusion with phosphate-buffered saline and 2% paraformaldehyde. The arteries were then fixed in 2% paraformaldehyde for 1 h, cryoprotected in 30% sucrose overnight, and frozen in OCT compound (Tissue-Tek, Torrance, CA).

Morphometric assessment

Arterial sections were stained with hematoxylin and eosin, and five evenly spaced sections, approximately 100 μm apart throughout the injured carotid artery, were used for morphometric analysis using ImageJ software (NIH, Bethesda, MD). The area and circumference of the lumen, internal elastic lamina, and external elastic lamina were manually traced for analysis of lumen area, circumference, intimal area, media area, and intima/media area ratio, using blinded digital images to limit bias.

Immunofluorescent staining

Arterial sections were fixed with 2% paraformaldehyde and permeabilized using 0.3% Triton-X. Arterial sections were incubated with an anti-adiponectin receptor (AR) primary antibody (1:1000; Santa Cruz, Dallas, TX) in IHC-Tek Antibody Diluent (IHC World, Woodstock, MD) for 1 h, washed in phosphate-buffered saline, and incubated with a Cy3-goat anti-rat secondary antibody (1:100; Invitrogen, Grand Island,