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Periadventitial Adipose Tissue Modulates the Effect of PROLI/NO on Neointimal Hyperplasia

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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 ³H-thymidine incorporation in adipocyte-conditioned or regular media. The rat carotid artery balloon injury model was performed on male Sprague Dawley rats. Prior to 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 was assessed via immunofluorescence.

Results—Adipocyte-conditioned media had an anti-proliferative 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 weeks after surgery compared to the intact group. NO reduced neointimal hyperplasia to a greater extent in the excised group compared to the intact group. While 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.

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

Keywords

neointimal hyperplasia; nitric oxide; periadventitial adipose tissue; adipokines

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 multi-component 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 physiologic 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 (VSMC) and adventitial fibroblasts (AF) were harvested and grown from the aortae of 10-week-old male Sprague Dawley rats and maintained in DMEM:Ham's F-12 (Invitrogen, Carlsbad, CA) media. Cells were used between passages 4–9.

Cellular Proliferation

Adipocyte phenotype for 3T3-L1 fibroblasts was induced with 90% DMEM, 10% bovine calf serum, 1.0 µg/ml Insulin, 1.0 µM Dexamethasone, and 0.5 mM Methylisobutylxanthine, according to the ATCC (Manassas, VA) protocol. After 72 hours, adipocytes were maintained in 90% DMEM, 10% FBS, and 1.0 µg/ml Insulin. Phenotype induction was confirmed by morphological observation under the microscope. Media was changed and collected at days 9, 11, and 15 post 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]diazene-1,2-diolate (DETA/NO) in the presence of ³H-Thymidine (5 µM). After 24 hours, incorporated ³H-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-week-old Sprague Dawley rats. Removal of the periadventitial adipose tissue prior to 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 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 (10mg) 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 weeks following arterial injury after *in situ* perfusion with phosphate buffered saline (PBS) and 2% paraformaldehyde. The arteries were then fixed in 2% paraformaldehyde for 1 hour, 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 5 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 hour, washed in PBS, and incubated with a Cy3-goat anti-rat secondary antibody (1:100; Invitrogen, Grand Island, NY). Sections were stained with DAPI and mounted with ProLong Gold antifade reagent (Invitrogen, Grand Island, NY).

Assessment of Immunofluorescent Staining

Pictures of arterial sections were captured using a 40X objective on a fluorescence microscope (Zeiss LSM-510 microscope; Hallbergmoos, Germany). Secondary antibody fluorescence was assessed with the HE CY3 filter (Zeiss filter #43; excitation: 550–575, and emission: 605–670 nm). Nuclei staining was observed with the DAPI filter (Zeiss filter #49; excitation: 365–395, and emission: 445–450 nm). Autofluorescence of the elastic lamina was observed with the green fluorescent protein filter (Zeiss filter #38; excitation: 470–495, and emission: 525–550 nm). Quantification of fluorescent staining was performed by layer of the arterial wall. Each layer was traced using the elastic lamina as reference, a threshold was applied on the red channel, and the red pixels quantified using ImageJ software. Data analysis was performed using SigmaPlot software (Systat Software Inc. CA, USA).

Statistical Analysis

Data are presented as mean $\pm$ SEM. Morphometric data and immunofluorescence data were analyzed by two-way analysis of variance (two-way ANOVA) or one-way ANOVA, with post hoc Tukey-Kramer correction for multiple comparisons. *In vitro* proliferation data were analyzed by bootstrapping the estimates of the main effects and interactions and constructing 95% confidence intervals. All data analysis was performed using SigmaPlot v10.0 (Systat Software Inc. CA, USA) or SAS (SAS Institute Inc., Cary, NC). Statistical significance was assumed for $P < 0.05$.

RESULTS

Differential Effect of Adipocyte Soluble Mediators on Fibroblast and VSMC Proliferation

To assess the effect of factors derived from periadventitial adipose tissue on fibroblast and VSMC proliferation *in vitro*, we incubated primary AF, primary VSMC, and 3T3 fibroblasts with conditioned media from adipocytes and evaluated cellular proliferation by ^3H -thymidine incorporation. Adipocyte conditioned media had a significant pro-proliferative effect in VSMC ($p < 0.05$) (Figure 1A) whereas it had a significant anti-proliferative effect in both primary AF ($p < 0.05$) and 3T3 fibroblasts ($p < 0.05$) (Figure 1B–C). Next, we exposed cells to increasing concentrations of DETA/NO and assessed how conditioned media influenced the known anti-proliferative effect of NO. NO inhibited cell proliferation in the

three cell types in a concentration-dependent fashion, as expected (Figure 1A–C). However, there was a significant interaction between NO and media type in all three cell types. Conditioned media caused a 5-fold increase in the anti-proliferative effect of NO on VSMC ($p<0.05$) (Figure 1A). On the other hand, it reduced the anti-proliferative effect of NO by 1.5-fold in AF and by 3-fold in 3T3 fibroblasts ($p<0.05$) (Figure 1B–C). These data suggest that soluble mediators produced by adipocytes affect the proliferation rates of fibroblasts and VSMC as well as the ability of NO to inhibit proliferation.

The Periadventitial Adipose Tissue Modulates Neointimal Hyperplasia

Next we assessed the *in vivo* implications of our findings by studying the effect of periadventitial adipose tissue removal on the development of neointimal hyperplasia and the ability of NO to inhibit this process following balloon injury. NO inhibited fibroblast proliferation *in vitro* more effectively without conditioned media. This implies that NO could be more effective inhibiting fibroblast proliferation in the absence of periadventitial adipose tissue. Removal of periadventitial adipose tissue has a significant negative effect in neointimal formation with a 27% increase in intimal area in the excised (periadventitial adipose tissue removed) versus intact (periadventitial adipose tissue present) group ($p<0.0001$). Examination of the impact of the periadventitial adipose tissue on the efficacy of NO at inhibiting neointimal hyperplasia revealed an interesting pattern. NO was more effective in the excised group, resulting in a 35% greater decrease in intimal area compared to the intact group ($p=0.0009$). The results were similar in the media, showing a significant interaction between NO and periadventitial adipose tissue removal. NO induced a 35% greater reduction in the medial area in excised versus intact group ($p<0.0001$). Similarly, NO reduced the intima/media area ratio in excised group by 86% and the intact group by only 65%, however this did not reach statistical significance ($p=0.067$). Lastly, analysis of percent occlusion revealed that NO reduced percent occlusion in both the excised and intact groups by 92% and 82%, respectively ($p<0.05$). These data suggest that periadventitial adipose tissue regulates the development of neointimal hyperplasia and its inhibition by NO.

PROLI/NO Increases Adiponectin Receptor (AR) Levels in the Absence of Periadventitial Adipose Tissue

Since adiponectin is an adipokine involved in regulation of intimal growth, we evaluated adiponectin receptor (AR) levels throughout the vascular wall after balloon injury in the presence or absence of NO in both the intact and excised groups. The results of the quantification of AR staining showed that balloon injury significantly increased AR levels in the intima compared to control artery ($p<0.05$), with no statistically significant increase in the media and adventitia ($p=NS$) (Figure 3 A–D). No significant differences in AR levels were noted between the intact and excised groups after balloon injury (Figure 3 A–C). However, after balloon injury and treatment with NO a significant decrease in AR staining was observed in the intima of the excised, and intact groups vs injury alone (60% reduction, $p=0.019$) (Figure 3 A, D). AR staining showed no significant difference in the media with the application of NO in the intact group (Figure 3 B, D). Application of NO to the excised group showed an increase trend that was not statistically significant (Figure 3 B, D). However, AR staining was significantly increased in the adventitia (2-fold increase, $p<0.05$), with the application of NO in the excised group compared to control (Figure 3C–D). These

data suggest a possible relationship between the periadventitial adipose tissue and regulation of AR levels throughout the arterial wall when NO is present.

DISCUSSION

Herein we show that soluble factors present in adipocyte-conditioned media regulated VSMC and fibroblast proliferation differently. Conditioned media had a pro-proliferative effect in VSMC but an anti-proliferative effect in AF and 3T3 fibroblasts. Moreover, conditioned media had the opposite effects on the anti-proliferative actions of NO on these cell types. While conditioned media potentiated the anti-proliferative effect of NO in VSMC, it did the opposite in AF and 3T3 fibroblasts. These findings on fibroblasts were paralleled *in vivo*. Removal of the source of periadventitial adipose tissue resulted in more neointimal hyperplasia following arterial injury. Short exposure to NO has been shown to provide long-term inhibition of neointimal hyperplasia.¹⁹ PROLI/NO, a NO donor with a very short half-life has been shown to cause early redox changes in the vascular wall that result in long-term inhibition of neointimal hyperplasia.¹⁴ Here, we also found a significant interaction between presence of periadventitial adipose tissue and effect of NO treatment, with NO being less effective in the presence of periadventitial adipose tissue. Together, these data suggest that periadventitial adipose tissue plays a role in the regulation of the arterial injury response and also affects the effectiveness of NO therapy administered periadventitally.

Periadventitial adipose tissue is a recognized source of mediators involved in vascular tissue. Adipocytes contained within the periadventitial adipose tissue are known to induce VSMC proliferation in a leptin-dependent manner.⁹ On the other hand, Chen et al. has shown that leptin has an inhibitory effect on fibroblast activation.²⁰ This differential effect of adipokines on VSMC and AF could explain our *in vitro* findings; VSMC and AF proliferation responded differently to adipocyte-conditioned media. However, *in vivo* there is a more complex interplay, and adipose-derived leptin has an overall effect that promotes neointimal hyperplasia.⁹ Conversely, adiponectin is the main anti-inflammatory adipokine with an inhibitory role on neointimal hyperplasia.^{8, 10, 21} Our *in vitro* data on fibroblasts correlate with our findings *in vivo*. NO was more effective inhibiting AF proliferation in the absence of adipocyte-derived mediators; in a similar fashion NO was more effective inhibiting neointimal hyperplasia in the absence of periadventitial adipose tissue. However, the data on VSMC show less efficacy of NO inhibiting proliferation in the absence of adipocyte-derived mediators. These findings highlight the role of the adventitia, and specifically AF, in the development of neointimal hyperplasia.

The periadventitial adipose tissue modulates the arterial injury response. Takaoka et al. showed that removal of periadventitial adipose tissue increased neointimal formation and that transplantation of subcutaneous adipose tissue of lean animals protects against vascular remodeling.¹⁰ We show similar results with enhanced neointimal formation after removal of periadventitial adipose tissue. This effect was attributed to a local deficiency in the anti-inflammatory adipokine adiponectin.¹⁰ Moreover, Takaoka et al. also showed that vascular injury induces a phenotypic change in periadventitial adipose reducing adiponectin levels

and induced a shift towards a pro-inflammatory state.¹¹ Thus, our work is consistent with published literature.

The adiponectin receptor is known to regulate cell proliferation. Cai et al. proposed that activation of the AR could inhibit fibroblast proliferation and phenotypic change to myofibroblasts.⁸ This correlates with our findings of increased AR staining in the adventitia of excised animals treated with PROLI/NO. Moreover, it has been shown that activation of the AR increases NO production.⁸ The effect of the interaction between the NO donor and the presence of periadventitial adipose tissue has not been previously studied. We report an increased NO-dependent inhibition of neointimal development in the excised group, suggesting that NO donors are more effective in the absence of periadventitial adipose tissue. The increase in AR signal in the presence of PROLI/NO in excised arteries, but not in the intact arteries suggests that adventitial-derived soluble mediators may mediate the efficacy of NO.

The main limitation of our study is that we cannot determine if the enhanced effect of PROLI/NO on neointimal development in excised arteries is due to an increase in the local effective concentration of the NO donor or due to an interaction with adventitial mediators. On the one hand, since NO is lipid soluble, removing the periadventitial adipose tissue could result in a higher effective dose of NO at the site of arterial injury. However, the effective concentration of NO species at the site of arterial injury are challenging to quantify locally. Moreover, we have performed studies using the carotid balloon injury model in male Sprague Dawley rats using 10 mg and 20 mg of PROLI/NO. In these studies, we found that there was no significant difference between the two doses, which suggests that a higher local concentration does not account for the greater inhibition of neointimal hyperplasia observed in this study. On the other hand, the adventitia and periadventitial adipose tissue act as processing centers for the production and secretion of key mediators of vessel function and injury response.²² Some of these mediators, such as adiponectin, could interplay with NO-dependent pathways.⁸

In conclusion, we report a significant increase in neointimal development in balloon injured arteries that underwent removal of the periadventitial adipose tissue. We also report an increased efficacy of NO treatment at inhibiting neointimal hyperplasia after removal of the periadventitial adipose tissue. These data suggest that soluble mediators in the periadventitial adipose tissue regulate the arterial injury response and the efficacy of NO. Further work is needed to identify what adipokines have a direct effect on cell proliferation *in vitro*, and *in vivo*. This work also highlights the importance of surgical technique when using the carotid balloon injury model as the presence of periadventitial adipose tissue has a significant effect on the endpoint outcome of neointimal hyperplasia.

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Figure 1. Interaction between DETA/NO and adipocyte-conditioned media on VSMC and fibroblast proliferation

Vascular smooth muscle cell (VSMC), adventitial fibroblasts (AF), or NIH/3T3 fibroblasts were seeded onto 12-well plates at a density of 25,000 cells/well. Cells were allowed to adhere overnight followed by serum starvation for 24 hours to induce cell synchronization. Cells were treated with regular media or adipocyte-conditioned media with or without different concentrations of DETA/NO for 24 hours. Proliferation was assessed by ^3H -thymidine incorporation in (A) Adventitial fibroblasts, (B) 3T3 Fibroblasts, and (C) VSMC. $N=3$, The solid and dotted lines represent the linear regression fit. The difference in the slopes of the dotted and solid lines are statistically significant at a $p < 0.05$ level.

Figure 3. NO decreased adiponectin receptor (AR) staining in the intima of both groups and increased AR staining in adventitia of the excised group

Quantification of adiponectin receptor (AR) staining from arterial sections in the (A) intima, (B) media, and (C) adventitia. (N=5 rats per treatment group) of control, injury, and injury + nitric oxide (NO). Injury increased AR staining in the intima of both groups (A). NO decreased adiponectin receptor (AR) staining in the (A) intima of both groups, but increased AR staining in the adventitia (C) of the excised group. (* $p < 0.05$ vs control, # $p < 0.05$ vs injury alone). (D) Representative images of AR staining in rat carotid arteries 14 days after balloon injury. Blue is nuclear staining (DAPI), green is auto-fluorescence of the internal elastic lamina, and red is AR staining.