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Adventitial Contributions of the ERK and Akt Pathways to Neointimal Hyperplasia

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Abstract

Background—We recently reported that the efficacy of nitric oxide (NO) appears to be based on both sex and hormone status. The mechanism responsible for this differential efficacy is unknown. The aim of this study is to characterize the effect of sex, hormones, and NO on the ERK and Akt signaling pathways following arterial injury.

Methods—Male and female Sprague Dawley rats underwent castration or sham surgery. Two weeks later, they underwent carotid artery balloon injury. Treatment groups included: control, injury, and injury+PROLI/NO (n=5/group). Arteries were harvested 2 weeks post-injury and assessed for pERK and pAkt expression.

Results—Following injury, more pERK and pAkt activity was seen in the adventitia than media in both sexes, regardless of hormone status ($P<0.05$). In hormonally-intact males, NO further increased pERK (44%) and pAkt (120%) after injury ($P<0.001$). Castration attenuated the effects of NO. In hormonally-intact females, NO caused the opposite pattern with pERK activity but did not affect pAkt activity.

Conclusion—Following arterial injury, ERK and Akt activity is significantly greater in the adventitia than the media, and depends on sex, hormone status, and NO. Understanding adventitial regulation of proliferative signaling pathways will allow the development of targeted therapies for neointimal hyperplasia.

Keywords

adventitia; ERK; Akt; neointimal hyperplasia

INTRODUCTION

Over 70 million Americans suffer from cardiovascular disease, with atherosclerosis accounting for the majority of morbidity and mortality in the United States.(1) Advanced atherosclerosis often requires surgical intervention such as angioplasty, stenting, endarterectomy, or bypass grafting. Unfortunately, these procedures fail at high rates due to the development of neointimal hyperplasia.(2;3) Our lab and others have shown that nitric oxide (NO) is a potent inhibitor of neointimal development. (4) We recently showed that NO

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is more efficacious in males than females at inhibiting neointimal hyperplasia following arterial injury.(5) Castration reduces the efficacy of NO in both sexes. Thus, the efficacy of NO appears to be based on both sex and hormone status.

The aim of this study was to investigate the mechanism by which NO imparts differential efficacy at inhibiting neointimal hyperplasia based on sex and hormone status. We focused on two signaling pathways, the ERK and Akt signaling pathways, since both pathways have been shown to regulate proliferation in vascular smooth muscle cells (VSMC) and adventitial fibroblasts (AF).(6–10) Both pathways become active upon phosphorylation and inactive upon dephosphorylation. The relative expression of the phosphorylated states allows for quantification of the activity of these signaling pathways. Therefore, we hypothesize that the differential efficacy of NO based on sex and hormones is mediated, in part, by modulation of the ERK and Akt pathways. Lastly, given the data on the emerging importance of the adventitia over the media in regulating early proliferation following arterial injury, we sought to evaluate the contributions of these pathways in the adventitia in addition to the media.(11)

METHODS

Animal Surgery

10 week old Sprague-Dawley (Harlan) male and female rats were anesthetized using 2% inhaled isoflourane. All rats underwent either gonadectomy or sham surgery as previously described.(5) Gonadectomy reduced the levels of testosterone and estrogen in male and female rats, respectively. Fourteen days after surgery, all rats underwent the carotid artery injury model as previously described.(5) Treatment groups (n=6/group) included control, injury, or injury ± periadventitial application of NO (PROLI/NO 26mg/kg). Uninjured arteries served as controls. Arteries were harvested 14 days after injury.

Tissue processing

Carotid arteries were procured after *in situ* perfusion/fixation with cold phosphate buffered saline (PBS) and 2% paraformaldehyde. After preservation in 2% paraformaldehyde at 4°C for 1 hour and 30% sucrose for 1 hour, the tissue was quick frozen and 5 µm sections were cut along the injured artery.

Immunohistochemistry

Frozen sections were fixed in 2% paraformaldehyde, permeabilized with 0.3 % Triton X-100 in PBS for 10 minutes, blocked with serum (1:20 in BSA) for 30 minutes, exposed to the primary antibody for either pERK or pAkt (1:50 in BSA, Santa Cruz) for 1 hour, and exposed to the secondary antibody AlexaFluor 555 (1:100 in PBS, Invitrogen) for 1 hour. After coverslipping, digital images of the sections were blinded by one investigator and quantified by a second, blinded investigator with Image J on six evenly-spaced sections per artery.

Data Analysis

Data were reported using the mean ± the standard error of the mean and analyzed by one-way analysis of variance. Statistical significance was assumed for P<0.05.

RESULTS

Arterial injury induces more adventitial than medial ERK and Akt activity, regardless of sex and hormone status

In light of recent advances in the understanding of the adventitia's role in neointima formation, we sought to identify whether ERK and Akt activity was more predominant in the adventitia or the media. Following arterial injury, ERK (Figure 1) and Akt (Figure 2) activity was greater in the adventitia than the media for all treatment groups ($P<0.05$). In intact males, ERK activity increased 10.6-fold in the adventitia after injury, but only 1.9-fold in the media ($P<0.001$). Castration blunted the injury-induced increase in ERK in the adventitia in males (only 8.7-fold, $P<0.0001$). In intact females, injury increased ERK activity 8.8-fold in the adventitia, but only 1.5-fold in the media ($P<0.001$). Castration also blunted the injury-induced increase in ERK in the adventitia in females (only 3.9-fold, $P<0.001$).

Akt activity followed a similar pattern to ERK in males, but an opposite pattern emerged in females. In intact males, injury increased Akt activity 6.9-fold in the adventitia, but only 1.3-fold in the media ($P=0.005$; Figure 2). Castration blunted the increase in Akt activity in the adventitia in males (only 5.6-fold, $P<0.001$). In intact females, Akt activity increased by 4.6-fold in the adventitia, but only 1.7-fold in the media ($P=0.001$). Surprisingly, castration had the opposite effect in females as compared to males and increased Akt activity in the adventitia to 13.7-fold ($P<0.001$).

NO modulation of ERK and Akt activity differs based on sex

Next, we evaluated ERK and Akt activity following injury and exposure to NO. In intact males, NO significantly increased adventitial ERK (44% increase; Figure 1) and Akt (120% increase; Figure 2) activity compared to injury alone ($P<0.001$). A different pattern was observed in females. NO significantly decreased adventitial ERK activity (57% decrease; Figure 1) and had no effect on Akt activity (Figure 2) compared to injury alone ($P<0.05$). No significant changes were observed in medial ERK or Akt activity with NO in either sex.

NO modulation of ERK and Akt activity differs based on hormones

Interestingly, the absence of hormones had a dramatic effect on the ability of NO to increase ERK and Akt activity in the adventitia after injury in males. After castration, NO was no longer able to effect an increase in adventitial ERK compared to injury alone ($P<0.05$), and its ability to cause an increase in adventitial Akt was significantly attenuated (32% increase; $P<0.05$). After castration in females, NO increased adventitial ERK activity compared to injury alone (90% increase, $P<0.001$), thus conferring on castrated females an ERK pattern similar to intact males. There was no significant change in adventitial Akt activity by NO in castrated females. No significant changes were observed in medial ERK or Akt activity with NO after castration.

The adventitial pERK:pAkt ratio is dependent on sex, hormone status and NO

Cross-talk between the ERK and Akt pathways is common. We sought to understand the effect of sex and hormones on the activity of the ERK pathway, relative to Akt pathway. To quantify this relationship in the adventitia, we reported the pERK:pAkt ratio. The pERK:pAkt ratio was 1.5 in both intact and castrated males following injury. NO equilibrated the ratio to 1.0 in both intact and castrated males. Interestingly, injury favored the ERK pathway in intact females with a ratio of 2.0, but favored the Akt pathway after castration with a ratio of 0.25. NO caused a shift to the Akt pathway in both intact and castrated females (ratio 0.6 and 0.5, respectively).

DISCUSSION

In this manuscript we report that the ERK and Akt pathways are important in the adventitial response to arterial injury and that modulation of these pathways by NO is dependent on sex and hormone status. Both pathways appear to be much more active in the adventitia than the media following injury, regardless of the application of NO. The ERK and Akt pathways are both upregulated by NO in males, but their activation appears to require the presence of hormones since castration resulted in a dramatic decrease in the effect of NO. An unexpected finding was the pattern of ERK activity exhibited by females. In striking contrast to the hormonally intact males, NO caused a decrease in adventitial ERK activity while castration caused an increase, thus conferring the intact male phenotype. The Akt pathway, interestingly, appears to function independently of NO in females, regardless of hormone status.

AF proliferate, differentiate into myofibroblasts, and subsequently migrate to the neointima after arterial injury. In a recent study by Couffignal et al, proliferation of AF was found to begin within hours of injury, much earlier than medial VSMC.(12) In addition, Wilcox et al demonstrated a 7-fold increase in adventitial versus medial cellularity soon after injury.(13) Data from our study indicate that arterial injury results in an increase of ERK and Akt activity, regardless of sex or hormone status. These data highlight the importance of the adventitia in the arterial response to injury and implicate the ERK and Akt pathways as key mechanisms through which increased adventitial activation and proliferation occur.

NO modulation of ERK and Akt pathway activity is dependent on sex and hormone status. What is unclear, is why NO, a vasoprotective molecule, increases proliferative signaling in hormonally intact males but not hormonally intact females. In hormonally intact males, NO may be inhibiting myofibroblast migration resulting in a relative increase in ERK and Akt activity as these cells accumulate in the adventitia. Alternatively, NO may be promoting the proliferation of vasoprotective adventitial cells, such as residential adventitial Sca1+ stem cells and vasa vasorum endothelial cells. Our lab has previously shown that NO is more efficacious at inhibiting neointimal hyperplasia in males than females.(5) This could explain why NO increases ERK and Akt activity in the adventitia of males but not females. However, in the male castrated model, the NO-induced ERK and Akt activity is lost. This correlates with data recently published by our laboratory that shows the ability of NO to inhibit neointimal hyperplasia in castrated males is significantly reduced.(5) To determine if ERK or Akt pathway inhibition modulates the downstream effects of NO, we are currently evaluating VSMC and AF proliferation in hormone replete and deplete media, with and without NO. These data will provide further insights into the role of the ERK and Akt pathways in these cells based on sex and hormone status.

A complex and interesting feature of the ERK and Akt pathways is their ability to engage in crosstalk, thereby influencing each other's activity. This balance between the pro-proliferative ERK pathway and the pro-cell survival Akt pathway have important implications for our understanding of the mechanisms through which NO exerts its vasoprotective effects. Jonas et al showed in a diabetic rat model that an increase in the pERK:pAkt ratio corresponded to an increase in neointimal hyperplasia after arterial injury.(14) In fact, our study supports this finding as injury in our models clearly favored adventitial activation of the ERK pathway. Interestingly, NO, in general, moves this relationship away from ERK dominance, thereby increasing the relative activity of the Akt pathway. Therefore, we are able to conclude that NO inhibits neointimal hyperplasia, in part, through inhibition of the adventitial ERK pathway. Our study is clinically relevant, given the dramatic increase in the incidence of coronary artery disease in women after menopause.

Comparing intact male to castrated female adventitial and medial ERK and Akt activity by cell type may show similarities in the pattern of cell type affected by injury and injury+NO.

There were limitations to our study. We evaluated the ERK and Akt pathways 2 weeks post-injury. Earlier time points may reveal a different pattern. Furthermore, earlier time points might allow identification of a potential target for future therapies. Adding back estrogen and testosterone to castrated animals and determining if ERK and Akt activity resumes a hormonally intact phenotype would help confirm our conclusions. Our study does not quantify ERK and Akt activity by cell type. The adventitia contains inflammatory cells, endothelial cells and stem cells. Any of these cell types may have ERK and Akt activity fluctuations after injury+/-NO. Double immunofluorescent staining for smooth muscle cells, endothelial cells, stem cells, or macrophages and ERK or Akt would help clarify what cell types exhibit changes in ERK and Akt pathway activity after injury and NO.

In summary, these data demonstrate the importance of the ERK and Akt pathways in the vessel wall following arterial injury. Their activation depends on sex, hormone status, and NO. Understanding how proliferation is regulated will allow the development of targeted therapies to prevent neointimal hyperplasia.

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Figure 1.

pERK immunofluorescent staining of rat common carotid arteries in hormonally intact and castrated males and females. Treatment groups (n=5/group) include control, injury and injury $\pm$ nitric oxide (NO). Staining intensity was analyzed by vessel wall layer (solid line = media; dashed line = adventitia) and expressed as 'fold increase' compared to control. Note the significant prevalence of pERK after injury and injury+NO in the adventitia versus the media (* $P < 0.001$). Also note the loss of NO-induced pERK increase after castration in males and the pattern of pERK expression with NO in the intact and castrated females ($\#P < 0.001$).

Figure 2.

pAKT immunofluorescent staining of rat common carotid arteries in hormonally intact and castrated males and females. Treatment groups (n=5/group) include control, injury and injury $\pm$ nitric oxide (NO). Staining intensity was analyzed by vessel wall layer (solid line = media; dashed line = adventitia) and expressed as 'fold increase' compared to control. Note the significant prevalence of pAKT after injury and injury+NO in the adventitia versus the media (* $P < 0.001$). Also note the attenuation of NO-induced pAKT increase after castration in males and the lack of effect NO has on the intact and castrated females (# = $P < 0.001$).