Mechanisms of Vascular Smooth Muscle NADPH Oxidase 1 (Nox1) Contribution to Injury-Induced Neointimal Formation


Objective—Vascular NADPH oxidases (Noxes) have been implicated in cardiovascular diseases; however, the importance of individual Nox homologues remains unclear. Here, the role of the vascular smooth muscle cell (VSMC) Nox1 in neointima formation was studied using genetically modified animal models.

Methods and Results—Wire injury–induced neointima formation in the femoral artery, along with proliferation and apoptosis, was reduced in Nox1−/− mice, but there was little difference in TgSMCnox1 mice compared with wild-type (WT) mice. Proliferation and migration were reduced in cultured Nox1−/− VSMCs and increased in TgSMCnox1 cells. TgSMCnox1 cells exhibited increased fibronectin secretion, but neither collagen I production nor cell adhesion was affected by alteration of Nox1. Using antibody microarray and Western blotting analysis, increased cofilin phosphorylation and mDia1 expression and decreased PAK1 expression were detected in Nox1−/− cells. Overexpression of S3A, a constitutively active cofilin mutant, partially recovered reduced migration of Nox1−/− cells, suggesting that reduction in cofilin activity contributes to impaired migration of Nox1−/− VSMCs.

Conclusions—These results indicate that Nox1 plays a critical role in neointima formation by mediating VSMC migration, proliferation, and extracellular matrix production, and that cofilin is a major effector of Nox1-mediated migration. Inhibition of Nox1 may be an efficient strategy to suppress neointimal formation. (Arterioscler Thromb Vasc Biol. 2009;29:00–00.)

Key Words: ●●●

The abnormal intimal growth of blood vessels as a “response to injury” is key in the development of vascular occlusive diseases such as in-stent restenosis, intimal proliferation after vein grafts, and atherosclerosis; hence, it is the major limitation for the efficacy of corrective surgery.1 The molecular mechanisms underlying these events are poorly understood.

Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide mediate signal transduction pathways that contribute to the pathophysiological responses of VSMCs including migration, proliferation, apoptosis, phenotypic modulation, and hypertrophy.3 Major sources of ROS in VSMCs, especially in pathological conditions, are the NADPH oxidase (Nox) family of enzymes. VSMCs from conduit arteries express Nox1 and Nox4,4 whereas those from resistance arteries express Nox2 and Nox4.5 These oxidases serve different functions within the cells,6 presumably owing to their distinct intracellular compartmentalization and different mechanism of regulation and activation.7 Of interest, studies have linked Nox1 to VSMC phenotypic changes including angiotensin II–induced hypertrophy,8 serum-induced proliferation,9 ECM production,9 and basic fibroblast growth factor (bFGF)-induced migration.10

In vivo studies of the role of specific Nox homologues in vascular lesions are limited. However, enhanced generation of superoxide and increased NADPH oxidase expression or activity are observed in rat balloon-injured carotid11 and coronary12 arteries and vein grafts.13 In accordance with these findings, antioxidant treatment with tempol or N-acetylcysteine reduces injury-induced restenosis.14 Furthermore, superoxide production is prominent in neointimal and medial SMCs after carotid injury,15 and intimal SMCs are predom-
inantly responsible for the elevated NADPH oxidase activity in venous bypass graft intimal hyperplasia. The expression of Nox1 increases early in the restenotic response and remains elevated during the growth phase of the lesion.

Based on these observations, we hypothesized that Nox1-derived ROS participate in neointimal formation by mediating PDGF-induced signaling. We tested this by subjecting Nox1 knockout (KO) and smooth muscle-specific, Nox1 overexpressing (OE) mice to wire injury of the femoral artery. Our data show that deletion of Nox1 indeed impairs the response to injury, support a role for Nox1 in proliferation, migration, and extracellular matrix secretion, and provide insight into the signaling that regulates such responses. Understanding the role of specific NADPH oxidases such as Nox1 will permit better design of therapies targeted to reducing oxidative stress in vascular disease.

Materials and Methods
An expanded Materials and Methods section is available in the online Data Supplement at http://atvb.ahajournals.org.

Reagents
All reagents and antibodies used here were purchased from standard suppliers. The coding sequence for the S3A cofilin mutant in pcDNA3 expression vector was kindly provided by Dr J.S. Condeelis (Albert Einstein College of Medicine, Bronx, NY). Anti-Nox4 rabbit polyclonal antibody was prepared as previously described.

Animals
Nox1−/− mice were generated by Dr K.H. Krause. TgSMCnox1 mice, transgenic mice overexpressing Nox1 in smooth muscle were also previously described. All mice are fully backcrossed onto a C57Bl/6 background.

Mouse Femoral Artery Injury Model
Transluminal mechanical injury of bilateral femoral arteries was induced by introducing a large wire as previously reported. At 21 days, the mice were euthanized and pressure-perfused at 100 mm Hg with 0.9% sodium chloride, followed by pressure-fixation with 10% formalin. Arteries were then carefully excised and embedded in paraffin. To assess the early apoptotic response to injury, arteries were obtained 2 hours after injury induction, as described previously.

Histological Analysis of Neointima
Hematoxylin and eosin (H&E) staining was used to assess morphological analysis. Proliferating cell nuclear antigen (PCNA) and TUNEL staining were performed to identify proliferating and apoptotic cells, respectively. Fibronectin and collagen were measured to determine matrix accumulation. Mac-3 was used to detect macrophages. Images were acquired with an Axioskop microscope and analyzed using NIH ImageJ.

Cell Culture
VSMCs were isolated from mouse aorta by enzymatic digestion and used between passages 3 to 10.

RNA Extraction and RT-PCR
Total RNA was extracted with the RNeasy kit (Qiagen). cDNA was synthesized using random primers and Superscript II (Invitrogen). For PCR, primers (forward: 5′-CTGAGGCGACCTGCTATT-3′; backward: 5′-CTGGAATTCTGACAGTAAGTTCAG-3′) were designed to amplify both mouse and human Nox1 cDNA, but not DNA from KO cells that lack the annealing site for the forward primer.

NADPH Oxidase Activity
NADPH oxidase activity was assayed in membrane preparations. Superoxide was quantified by NADPH-dependent, SOD-inhibitable formation of 3-carboxy-proxyl radical (CP*) from 1-hydroxy-3-carboxy-pyrrolidine (CPH), using ESR.

Cell Proliferation Assay
Proliferation was assessed by cell counting as previously described.

Migration Assay
Migration was measured using a modified Boyden chamber assay. Migration was stimulated by 10 ng/mL PDGF for 3 hours. Migrated cells were stained with DAPI. Four (×20) fields were visualized using Zeiss Axioskop and quantified with NIH ImageJ.

Cell Transfection
VSMCs were transfected with pcDNA3/S3Acofilin or pcDNA3 by electroporation using a Nucleofector (Amaxa Biosystems). The transfection was made 12 hours before a 48-hour serum deprivation before the experiments.

Extracellular Matrix Production
ECM production was measured by collagen I and fibronectin in the culture media. Serum-deprived VSMCs were maintained in media containing 50 μg/mL ascorbic acid and 50 μg/mL β-aminopropionitrile (β-APN) with or without 10 ng/mL PDGF. After 72 hours, media was collected and subjected to Western blot analysis. To correct for possible differences in ECM protein generation caused by differences in cell growth rate, gel loading was adjusted for total protein concentration of the media.

Cell Adhesion Assay
VSMCs were plated and allowed to adhere for 30, 60, and 90 minutes. Attached cells were fixed/stained with 0.5% crystal violet. Dye was extracted with 0.5 mL of 1% SDS, and absorbance was measured at 590 nm.

Antibody Microarray
Characterization of expression and phosphorylation of signaling molecules was performed by Kinexus (Vancouver, Canada) using the Kinex Antibody Microarray. All changes reported in Results were confirmed by Western analysis.

Western Blotting
VSMCs lysates were prepared and subjected to SDS-PAGE. Immunoreactive proteins were detected by chemiluminescence. Band intensity was quantified by densitometry using NIH ImageJ.

Statistical Analysis
Data are reported as mean±SE. Statistical significance was assessed by ANOVA on untransformed data, followed by contrast analysis. A P<0.05 was considered statistically significant.

Results
Analysis of Injury-Induced Neointima Formation and Histological Characteristics in Nox1−/− and TgSMCnox1 Mice
To assess the role of Nox1 in neointimal formation, a wire-injury model of the mouse femoral artery was used. Three weeks after injury, morphological analysis of the injured artery was performed. We confirmed that there was no increased intimal or medial thickness in sham-operated
mice. As shown in Figure 1, both intimal thickness and cross-sectional area of femoral arteries were significantly reduced in Nox1y/H11002 mice, but there was minimal change in TgSMCnox1 mice. No significant difference was found in medial thickness or area among WT, Nox1y/H11002, and TgSMCnox1 mice. Accordingly, the intima/media ratio and % stenosis were diminished in Nox1y/H11002 mice.

Cellular responses related with neointimal growth, proliferation, apoptosis, matrix accumulation were examined with histological methods such as PCNA, TUNEL, fibronectin, and collagen staining. Low levels of PCNA expression and TUNEL-positive cells were observed in neointima of Nox1y/H11002 arteries, whereas TgSMCnox1 arteries were not different from WT arteries (Figure 2A and 2B). Expression of fibronectin was lower in Nox1y/H11002 arteries; however, there was no significant difference in collagen among groups (Figure 2C). To investigate the involvement of inflammation in this injury model, we stained for macrophages using Mac3-conjugated quantum dots (supplemental Figure I). Macrophage distribution was not detected in the neointima, consistent with previous reports, and no difference was observed among groups. These results suggest that Nox1 is necessary for neointimal formation, but overexpression in smooth muscle itself does not augment the response to injury.

Characterization of VSMCs From Nox1y/H11002 and TgSMCnox1 Mice

To determine how Nox1 influences the cellular functions of smooth muscle that contribute to neointimal formation, we prepared VSMCs from Nox1y/H11002 or TgSMCnox1 animals. Cell identity was confirmed with smooth muscle α-actin and calponin staining (supplemental Figure II), and Nox1 expression was detected by RT-PCR. As predicted, Nox1 expression was undetectable in Nox1y/H11002 cells, whereas TgSMCnox1 cells exhibited a marked increase (Figure 3A). Because rodent aortic SMCs exclusively express Nox1 and Nox4 among gp91 homologues, we investigated a possible compensatory Nox4 upregulation by measuring Nox4 expression in Nox1y/H11002 and TgSMCnox1 cells. However, consistent with previous studies, we found no change in Nox4 basal expression (Figure 3B). This excludes a differential effect of Nox4 in the following cell function studies using Nox1y/H11002 and TgSMCnox1 cells.

To test whether alteration of Nox1 expression reflects a functional change in NADPH oxidase activity, NADPH oxidase-dependent superoxide production was measured in basal and PDGF-activated conditions. As shown in Figure 3C, WT superoxide production was increased by PDGF, which is consistent with previous reports demonstrating that Nox1 is PDGF-inducible. However, such an increase was not observed in Nox1y/H11002 cells, whereas TgSMCnox1 exhibited higher levels of superoxide compared with WT cells both basally and after treatment. Compared with Nox4, basal expression of Nox1 is remarkably low; therefore, basal production of superoxide was not different between Nox1y/H11002 and WT cells. These results confirm that manipulations of Nox1 expression are in fact functional.
Effect of Nox1 on VSMC Proliferation, Migration, Adhesion, and Extracellular Matrix Production

VSMC proliferation, adhesion, ECM production, and migration are major contributors to neointimal formation. Hence, the effect of Nox1 on these cellular functions was tested in Nox1-modified VSMCs. Nox1y/−/H11002 cells had a slightly, but significantly, reduced proliferative activity, whereas the growth rate of TgSmCnox1 cells was higher than that of WT cells (Figure 4A). Cellular migration was modified to a greater extent than proliferation. Compared to that in WT cells, PDGF-induced migration was inhibited by 35% ± 8% in Nox1y/− cells, and both basal and PDGF-stimulated migration was significantly increased in TgSmCnox1 cells (180% ± 17% and 180% ± 23% of WT cells, respectively; Figure 4B). This was not attributable to alterations in cell adhesion, because altering Nox1 expression did not induce any difference in adhesion capacity (supplemental Figure III). These results suggest that Nox1 contributes to the proliferative and migratory responses of VSMCs.

Figure 2. Histological analysis of proliferation, apoptosis, and extracellular matrix distribution in arterial cross-sections obtained from WT, Nox1y/−, and TgSmCnox1 mice. Arterial sections obtained on day 21 after injury induction were stained for proliferating cell nuclear antigen (PCNA; A), fibronectin (C, top panels), and collagen (C, bottom panels). TUNEL staining was performed after 2 hours of injury induction (B). PCNA and TUNEL-positive cells were stained red and dark brown, respectively (A and B). Red or orange color indicates fibronectin or collagen, respectively (C). Background images were obtained from WT arteries omitting primary antibody staining (A and C, upper panel), and nuclei were counterstained with hematoxylin (C, upper panel). Arrowhead indicates positive staining of PCNA (A), and neointimal area analyzed for fibronectin is indicated with a solid line in images (C, upper panel). Images for PCNA, TUNEL, collagen, and fibronectin staining are representative of sections from at least 3 animals of each genotype. Mean ± SE are provided to the right of each image. M and N denote media and neointima (A). Scale bar=30 (A), 20 (C, upper panel), 50 (B and C, lower panel) μm.
Figure 3. Characterization of primary-cultured aortic smooth muscle cells from Nox1−/− and TgSMCnox1 mice. A, PCR was conducted with primer pairs that can distinguish cDNA of Nox1−/− cells from that of WT or TgSMCnox1 cells. PCR products were detected on agarose gel electrophoresis. A representative gel image is presented. B, Nox4 protein expression was measured by Western blotting. CDK4 was measured as a loading control. Representative images are shown in upper panels. Values are means±SE from 5 independent experiments. C, Serum-starved VSMCs were treated with 25 ng/mL PDGF for 4 hours, which corresponds to maximal activation of NADPH oxidase. Membrane fractions were prepared, and NADPH-dependent SOD-inhibitable superoxide generation was measured with ESR using CPH. Values shown are means±SE from 3 independent experiments. *Significantly different from corresponding control (P<0.05).

The effect of Nox1 on ECM deposition was assessed by measuring the most abundant proteins present in ECM, collagen I, and fibronectin. Both basal and PDGF-stimulated fibronectin production were significantly enhanced in TgSMCnox1 compared with WT cells, whereas Nox1 knockout had no effect (Figure 4C). Neither Nox1−/− nor TgSMCnox1 cells exhibited significant changes in collagen I production. It thus appears that Nox1 is capable of inducing fibronectin production but may not be required for this response.

Mechanism of Nox1-Mediated VSMC Migration

The ROS-sensitive mechanisms underlying Nox1-mediated proliferation have been well studied, but those regulating migration are less understood. To clarify these mechanisms, we examined potential signaling molecules responsible for Nox1-mediated functional alterations of VSMCs. The goal was to identify targets that satisfied two criteria: relation with migration or proliferation, and regulation by ROS. Initially, we tested whether the activation of PDGF receptor itself is altered in Nox1−/− or TgSMCnox1 cells. Both the total expression and site-specific phosphorylation at Tyr716, an indicator of intrinsic receptor tyrosine kinase activity, were examined. Neither expressed protein nor activation by PDGF was changed by differential expression of Nox1 (supplemental Figure IV). Antibody microarray analysis was then performed with 4 samples, WT-untreated cell lysate, and lysates from WT, Nox1−/−, and TgSMCnox1 cells treated with 10 ng/mL PDGF for 5 minutes. Cogent results from the antibody microarray were confirmed with Western blotting. No significant difference was detected in total protein or phosphorylated levels of Src, PLCγ1, Erk1/2, p38, PRK1, p85Nck, PKCδ, PP2A, or vinculin (supplemental Table I). However, significant changes in protein expression or activation were detected in the cytoskeletal-associated proteins cofilin (a regulator of actin depolymerization), mDia1 (a RhoA adapter protein), and PAK1 (a serine/threonine kinase that promotes cytoskeletal reorganization; supplemental Table I and Figure 5). Nox1−/− cells exhibited more phosphorylated cofilin both basally and after PDGF stimulation, without alteration of cofilin expression. Expression of mDia1 was increased and PAK1 was decreased in Nox1−/− cells. Taken together, these data suggest that Nox1 may regulate migration by modulating the actin cytoskeleton.

Cofilin is known to be primarily involved in cytoskeletal reorganization by depolymerizing actin, which influences cell migration and growth. Phosphorylation of cofilin at Ser3 inhibits its activity. The observed increase in cofilin phosphorylation suggests that persistent inactivation of cofilin may explain the impaired migration in Nox1−/− cells. To examine this hypothesis, we attempted to reverse the reduced migration in Nox1−/− cells by expressing a constitutively active cofilin mutant, S3A. As shown in Figure 6, S3A recovered the impaired migration of Nox1−/− cells to the control level. This result suggests that decreased cofilin activity is mainly responsible for reduced migration of Nox1−/− cells.

Discussion

The results of this study support a role for Nox1 in the process of neointima formation induced by injury. Nox1 is required for the formation of neointima, but overexpression in VSMCs above the normal upregulation of Nox1 after injury does not significantly enhance injury-induced neointimal formation. In vitro studies reveal that both migration and growth of VSMCs are dependent on expression of Nox1. Regulation of cytoskeletal dynamics is presumably responsible for the effect of Nox1 on migration, and in particular, cofilin serves as Nox1 effector. Thus, suppression of Nox1 may be a promising strategy to treat vascular diseases associated with neointimal formation.

The process of neointimal formation is complex, involving endothelial injury, thrombosis, phenotypic transformation
followed by migration and proliferation of VSMCs, elaboration of ECM, redifferentiation of VSMCs and regrowth of the endothelium. For this reason, we studied the overall effect of Nox1y/-/H11002 or TgSMCnox1 in vivo, and used in vitro assays to probe those events related to VSMCs. The reduced neointima in Nox1y/- mice indicates that Nox1 is necessary for the neointimal response. In contrast, its overexpression does not induce a further response, because neointimal formation in TgSMCnox1 mice was similar to WT. In other words, additional activity of Nox1 does not evoke stronger signals, perhaps because Nox1 is already upregulated in injured WT arteries.11 It is also possible that because Nox1 mediates early proliferative and migratory responses, potential differences between TgSMCnox1 and WT mice were obscured by 3 weeks after injury. This view is well correlated with the finding that, in these experimental conditions, there was no prominent increase in proliferating cells in TgSMCnox1 (Figure 2A). Finally, non-VSMC components contribute to neointimal formation in vivo, making it difficult to quantify enhanced smooth muscle–specific responses in this setting. For these reasons, the reduction in neointimal formation in Nox1y/- animals gives a clearer picture of the importance of Nox1 in this response.

Our studies suggest several mechanisms by which Nox1 can affect lesion formation and healing in vivo. Previous studies showed that Nox1 mediates cell proliferation.20,27,28 Similarly, we found that overexpression of Nox1 enhanced, whereas knockout of Nox1 inhibited, proliferation. A similar pattern was seen for Nox1 in migration. Nox1 knockout also resulted in reduced apoptosis after injury, which is consistent with previous reports demonstrating the involvement of ROS signaling in apoptotic signals.30 The role of apoptosis in neointima formation is still controversial, but early apoptosis after injury is generally believed to induce a greater wound healing process.31 Therefore, the observed reduction of the apoptotic response in Nox1y/- arteries is consistent with subsequent decreased neointimal growth. However, we saw a limited effect of Nox1 on ECM production. Alteration of Nox1 had no effect on collagen in culture or in vivo. Fibronectin accumulation was reduced in the neointima of Nox1y/- arteries and augmented in TgSMCnox1 VSMCs. This suggests that Nox1 may differentially regulate matrix components, leading to enrichment of fibronectin. Taken together, our results indicate that Nox1 mediates multiple processes associated with the dedifferentiated synthetic VSMC phenotype.

Because others have delineated many of the growth-related signaling pathways associated with Nox1,27,28 we focused here on understanding the mechanisms by which Nox1 regulates cell migration. The Src/phosphoinositide-dependent kinase-1 (PDK1)/PAK signaling axis has been implicated as a major mediator of PDGF-induced, ROS- and NADPH oxidase-dependent migration of VSMCs,32,33 whereas thrombin-induced migration appears to depend on ROS-regulated p38 MAPK activation,34 and c-Jun-N-terminal kinase (JNK) regulates bFGF-induced Nox1-dependent migration of VSMCs.10 However, we found no changes in Src, p38 MAPK (supplemental Table I), or JNK (in antibody microarray, not shown), suggesting that migratory signaling may be agonist- and Nox-specific.
Migration is an integrated, dynamic, and cyclic process, dependent on a well-orchestrated regulation of the actin cytoskeleton. Important regulators of the actin cytoskeleton include cofilin, mDia1, and PAK1, all of which are effectors of the Rho GTPase. Cofilin is capable of disassembling and severing actin filaments. The activity of cofilin is controlled by its phosphorylation state: Enhancement of cofilin activity by dephosphorylation accelerates filamentous actin turnover and migration, whereas its phosphorylation impairs actin turnover. Our data revealed that Nox1y/−/H11002 cells possess less active cofilin than WT cells in both basal and PDGF-activated conditions. In addition, rescue of cofilin activity with a constitutively active mutant restored impaired migration of Nox1y/−/H11002 cells, suggesting that cofilin is responsible for the impaired migration of Nox1y/−/H11002 cells. These results are consistent with a previous report demonstrating that Nox1 mediates Ras-induced cofilin activation (dephosphorylation), and silencing of Nox1 by siRNA recovers phospho-cofilin levels in normal rat kidney fibroblasts.

The reason for the increase in cofilin phosphorylation in Nox1y/−/H11002 cells is unclear. PAK1 is upstream of cofilin, and activates the cofilin kinase LIMK, so one would expect its expression/activity to be increased, rather than decreased. The observed decrease in PAK expression may thus represent an attempt by the cell to compensate for persistent cofilin inactivation. In support of a compensatory effect, we also found an increase in mDia1 in Nox1y/−/H11002 cells. mDia contributes to cytoskeletal remodeling by regulating actin polymerization through profilin interaction, and promoting the stabilization and polarization of microtubules. Loss of mDia is associated with impaired T-lymphocyte trafficking, so increased mDia expression is unlikely to account for impaired migration in the Nox1y/−/H11002 cells. One other possibility is that Nox1 may influence slingshot (SSH) phosphatase, which mediates PDGF-induced cofilin phosphorylation. The redox-sensitivity of SSH is unknown; however, Nox1 may affect cofilin dephosphorylation by regulating SSH activation. Nonetheless, it is clear that Nox1 has a strong influence on cytoskeletal dynamics that will require further investigation.

In summary, Nox1 is a critical element of neointimal formation after vascular injury. It apparently exerts its effects in part by modulating VSMC growth and migration, and by influencing matrix accumulation. Because neointima formation is a complex and multistage process in which diverse cell types participate, the clear reduction in neointima in...
Figure 6. Recovery of impaired migration in Nox1+/− VSMCs by expression of constitutively active cofillin, S3A. pcDNA3/S3A or pcDNA3 was introduced into Nox1+/− or WT VSMCs by electroporation. After 12 hours, serum was removed for 48 hours, and migration induced by PDGF (10 ng/mL) was measured. Values are means±SE of 3 independent observations. *Significantly different from corresponding control, Nox1+/− cells transfected with pcDNA (P<0.05).

Nox1+/− mice suggests that the role of Nox1 should be investigated in other vascular cells as well. Meanwhile, these results identify Nox1 as a potentially novel target for therapy aimed at reduction of intimal hyperplasia.

Acknowledgments

We are grateful to Dr John Condeelis for providing the S3A plasmid.

Sources of Funding

This work was supported by NIH grants HL38206, HL093115, and HL058863, and American Heart Association fellowship #0525465B to A.S.M.

Disclosures

None.

References

18. Simmons S, Zebda N, Condeelis JS. Migration of smooth muscle cells transfected with Nox1+/− mice suggests that the role of Nox1 should be investigated in other vascular cells as well. Meanwhile, these results identify Nox1 as a potentially novel target for therapy aimed at reduction of intimal hyperplasia.

Sources of Funding

This work was supported by NIH grants HL38206, HL093115, and HL058863, and American Heart Association fellowship #0525465B to A.S.M.

Disclosures

None.
Mechanisms of Vascular Smooth Muscle NADPH Oxidase 1 (Nox1) Contribution to Injury-Induced Neointimal Formation


Arterioscler Thromb Vasc Biol. published online January 15, 2009;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2009/01/15/ATVBAHA.108.181925.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/01/27/ATVBAHA.108.181925.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
On-line Supplement

Contribution of Vascular Smooth Muscle NADPH oxidase 1 (Nox1) to Injury-induced Neointimal Formation

Moo Yeol Lee,1* Alejandra San Martin,1 Puja K. Mehta,1 Anna E. Dikalova,1 Abel Martin Garrido,1 Erin Lyons,1 Karl-Heinz Krause,3 Botond Banfi,4 J. David Lambeth,2 Bernard Lassègue,1 and Kathy K. Griendling1

1Department of Medicine, Division of Cardiology, and the 2Department of Pathology, Emory University, Atlanta, GA 30322, USA

3Department of Pathology and Immunology, University of Geneva, Geneva, Switzerland

4Department of Anatomy & Cell Biology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242

*Present address: College of Pharmacy, Chonnam National University, Gwangju, 500-757, Korea

Materials and Methods

Reagents

Recombinant human PDGF-BB was purchased from R&D Systems, Inc (Minneapolis, MN). Superoxide dismutase (SOD), β-aminopropionitrile, ascorbic acid, 4,6-diamidino-2-phenylindole (DAPI), anti-vinculin (Clone VIN-11-5), anti-smooth muscle α-actin (Clone 1A4), anti-calponin (Clone hCP), anti-fibronectin (Clone FN-3E2), and anti-β-tubulin (Clone TUB 2.1) antibodies were from Sigma-
Aldrich (St. Louis, MO). 1-Hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CPH) was purchased from Alexis Biochemicals (San Diego, CA), and protease inhibitor cocktail tablet and phosphatase inhibitor cocktail were from Roche Diagnostic Corporation (Indianapolis, IN) and Calbiochem (San Diego, CA), respectively. The coding sequence for the S3A cofilin mutant in pcDNA3 expression vector was kindly provided by Dr. J. S. Condeelis (Albert Einstein College of Medicine, Bronx, New York).\(^1\) Other antibodies and sources were as follows: anti-cofilin, anti-phospho-cofilin (Ser3), anti-PAK1, anti-phospho-PRK1(Thr774)/PRK2(Thr816), anti-p70S6K, anti-phospho-p70S6K(Thr421/Ser424), anti-p38, anti-phospho-p38(Thr180/Tyr182), anti-Erk1&2, anti-phospho-Erk1&2(Thr202/Tyr204), anti-PLC\(\gamma\)1, anti-phospho-PLC\(\gamma\)1(Tyr783), anti-Src, anti-phospho-Src(Tyr416), and anti-phospho-PKC\(\delta/\theta\)(Ser643/676) antibodies (Cell Signaling Technology, Beverly, MA); anti-MHC (Clone G-4), anti-CDK4 (Clone C-22) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); anti-PDGFR-\(\beta\), anti-phospho-PDGFR-\(\beta\)(Tyr716) antibodies (Upstate Biotechnology, Lake Placid, NY); anti-collagen I antibody (RDI Research Diagnostics, Flanders, NJ); anti-PKC\(\delta\), anti-PRK1, anti-mDia1 antibodies (BD Transduction Laboratories, Lexington, KY); HRP-conjugated anti-rabbit IgG antibody (Bio-Rad Laboratories, Hercules, CA); HRP-conjugated anti-mouse IgG antibody (Amersham Biosciences, Piscataway, NJ); Rhodamine Red-X-conjugated anti-mouse IgG antibody (Molecular Probes, Eugene, OR). Anti-Nox4 rabbit polyclonal antibody was prepared as previously described.\(^2\) All the other reagents not described here were from standard suppliers and of the highest grade available.

**Animals**
Nox1\(^{-/-}\) mice were generated by Dr. K. H. Krause and have been reported previously.\(^3,4\) To remove the neomycin cassette in the Nox1\(^{-/-}\), males were crossed with a female Flp deleter strain in the C57BL/6 background (Jackson stock number 003800). Pups in which the neomycin cassette had been successfully deleted, as determined by PCR, were selected for further back-crossing with C57BL/6 mice for seven successive generations. For genotyping, genomic DNA was extracted from tail snips using DirectPCR Lysis Reagent (Viagen Biotech, Los Angeles, CA). PCR was performed with a mixture of three primers: sense (intron 2) 5\'-ACGGGCACATGTGTAAGACTCACC-3' (500 nM), first antisense (intron 2) 5\'-CTACCAGGCCAATCTCTCTGTTTCCA-3' (500 nM) and second antisense (exon 7) 5\'-GCCTGCAACTCCCCTTATGGTCA-3' (150 nM), with annealing at 61°C. PCR products of 806 and 667 bp were generated from the wild-type (first antisense primer) and knock-out (second antisense primer) alleles, respectively. Tg\(^{SMCnox1}\) mice, transgenic mice overexpressing Nox1 in smooth muscle, were also previously described.\(^5\) For arterial injury experiments, male mice weighing 25-35 g were used, and 9-10 week old male mice were used for primary culture of aortic smooth muscle cells. For surgical procedures, the mice were anesthetized by intraperitoneal injection of 3 mg/kg acepromazine, 10 mg/kg xylazine, and 80 mg/kg ketamine diluted in 0.9% sodium chloride solution. All procedures for animal handling and experiments were performed in accordance with protocols approved by the Emory University Institutional Animal Care and Use Committee.

**Mouse femoral artery injury model**

Transluminal mechanical injury of bilateral femoral arteries was induced by introducing a large wire as previously reported.\(^6\) In brief, the left femoral artery was
exposed by blunt dissection, and was looped proximally and distally with 6-0 silk suture for temporal control of blood flow during the procedure. A straight spring wire, 0.38 mm in diameter (Cook, Bloomington, IN), was carefully inserted into the femoral artery toward the iliac artery via a small branch between the rectus femoris and vastus medialis muscles. The wire was left in place for 1 minute to denude and dilate the artery. Then the wire was removed, and the silk suture at the proximal portion of the muscular branch artery was secured. Blood flow in femoral artery was restored by releasing the sutures placed in the proximal and distal femoral portions. Skin incision was closed with a 6-0 silk suture. At 21 days after injury, the mice were sacrificed by CO₂ inhalation, and were pressure-perfused at 100 mm Hg with 0.9% sodium chloride solution, followed by pressure-fixation with a 4% paraformaldehyde solution. The femoral artery was then carefully excised and embedded in paraffin. To assess early apoptotic response to injury, arteries were obtained 2 hrs after injury induction.

Histological staining and assessment of neointimal formation

Femoral cross sections of 7 µm thickness were cut in a proximal direction starting 0.7 mm from the arterial branch. Standard hematoxylin and eosin (H&E) staining was used for assessment of neointimal formation. To examine the proliferating cells and apoptosis, proliferating cell nuclear antigen (PCNA) and TUNEL staining were performed, respectively. Matrix accumulation was measured by using fibronectin and collagen as markers. PCNA and fibronectin were immunostained with anti-PCNA and anti-fibronectin antibodies and visualized with a biotinylated secondary antibody and the ABC alkaline phosphatase (Vector Laboratory). In Situ Cell Death Detection Kit (Roche Diagnostic Corporation) was
used for TUNEL staining, and collagen was stained with picro-sirius red.

Background images for PCNA and fibronectin were obtained from WT arteries omitting primary antibody incubation, and nuclei were counterstained with hematoxylin in fibrinogen staining. Sections were mounted with Permount mounting media (Sigma-Aldrich) and images were acquired with an Axioskop microscope and Axiocam CCD camera (Carl Zeiss, Inc, Göttingen, Germany). Collagen was visualized under the circularly polarized light. To compare the intensity of each stain, images were obtained under the same optic conditions in each experiment. To quantify neointima formation, cross-sectional area and thickness of the intima and media were measured in two serial sections and averaged. To measure wall thickness, perpendicular lines were drawn to determine the distance from internal elastic lamina to the external lamina at 4 locations per aortic section and a mean value was calculated. Percent stenosis was determined as the ratio of the intimal area and the area inside the internal elastic lamina x 100. For quantification of fibronectin or collagen accumulation, distribution of pixels in the red channel normalized to total pixel number in the designated area (fibronectin) or averaged intensity of each pixel was analyzed in neointima of arteries (collagen). Pixel distribution in fibronectin staining was presented after subtraction of background and normalization of pixel number. NIH ImageJ software (Ver. 1.62) or MetaMorph (Molecular Devices, West Chester, PA) software was used for image analysis.

**Cell culture**

VSMCs were isolated from mouse thoracic aorta by enzymatic digestion as described previously. Briefly, the aortas were removed, cut open longitudinally, cleaned of connective tissue, fat and endothelium, and digested with collagenase.
and elastase to remove the adventitia and to dissociate the VSMCs. Individual cells were plated in a culture flask, and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The identity of VSMCs was confirmed by immunostaining with an antibody to smooth muscle α-actin and calponin. Cells were passaged by trypsinization, and passages between 3 to 10 were used for experiments.

**RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from cells with the RNeasy kit (Qiagen, Valencia, CA) as recommended by the manufacturer. cDNA was obtained from 5 µg of total RNA by reverse transcription employing the Superscript II enzyme (Invitrogen, Carlsbad, CA) and random nanomer primers as previously described. For PCR, primers (forward: 5'-CTGAGGGGCACCTGCTCATT-3', backward: 5'-CTGGAATTTGTACCAGATAGATTTCAAG-3') were designed to amplify both mouse and human Nox1 cDNA, but not DNA from Nox1−/− cells that lack the annealing site for the forward primer.

**NADPH oxidase activity**

NADPH oxidase activity was assayed by measuring NADPH-dependent superoxide production from membrane preparations of VSMCs as described previously. VSMCs were made quiescent in 0.1% FBS-containing DMEM for 48 hours, and further incubated with or without 25 ng/ml PDGF for 4 hours, which corresponds to maximal activation of NADPH oxidase. Cells were harvested in lysis buffer (50 mM phosphate, 10 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, and 0.5 mM PMSF, pH 7.4), ruptured by sonication, and then, subjected to
centrifugation at 28,000 g for 15 min at 4°C. The membrane pellet was resuspended in lysis buffer, and protein concentration was measured by the Bradford assay (Bio-Rad Laboratories). A reaction mixture was prepared containing 20 μg protein, 1 mM 1-hydroxy-3-carboxy-pyrrolidine (CPH), 200 μM NADPH, and 0.1 mM diethylenetriaminepentaacetic acid (DTPA) in 100μL of Chelex-treated PBS. Superoxide formation was quantified by NADPH-dependent, SOD-inhibitable formation of 3-carboxy-proxyl radical (CP•) using an EMX ESR spectrometer (Bruker BioSpin Corp., Billerica, MA) and a super-high Q microwave cavity. Superoxide dismutase (50 U/ml) inhibited 95-98% of CP• production.

**Cell proliferation assay**

Proliferation was assessed by following the change of cell number as previously described. VSMCs were plated at the density of 5X10⁴ cells per a 35 mm dish. Growth curves were obtained by counting cell number with a Coulter counter (Beckman Coulter, Inc., Fullerton, CA) every 24 hours up to 6 days.

**Migration assay**

Migration was assessed using a modified Boyden chamber assay in Transwell plates with 6.5 mm diameter and 8 μm pore-size (Costar, Lowell, MA). Serum-starved VSMCs were added to the upper chamber of the Transwell (5x10⁴/well), and exposed to 10 ng/ml PDGF in the lower chamber for 3 hours. Non-migrated cells were removed from the upper surface of the membrane using a cotton swab. The cells remaining on the inserts were fluorescently stained with DAPI and visualized using an Axioskop microscope equipped with an Axiocam CCD camera (Carl Zeiss,
Inc). Four (×20) images of separate fields were taken from each insert, and migrated cell number was quantified using NIH ImageJ software.

**Cell transfection**

VSMCs were transfected with pcDNA3/S3A or pcDNA3 by electroporation using a Nucleofector (Amaxa Biosystems, Gaithersburg, MD) set to the P24 program. The transfection was made 12 hours before a 48 hour serum deprivation prior to the experiments.

**Extracellular matrix (ECM) production**

Generation of ECM was assessed by measuring collagen I and fibronectin in culture media. VSMCs were seeded on culture dishes at 5×10^5 cells/60 mm plate. After 48 hours, cells were made quiescent, and maintained in 5 ml of media containing 50 µg/ml ascorbic acid and 50 µg/ml β-aminopropionitrile (β-APN), an inhibitor of collagen cross-linking, with or without 10 ng/ml PDGF. After 72 hours of treatment, media was collected and subjected to Western blot analysis. To correct for possible differences in ECM generation caused by differences in cell growth rate, gel loading was adjusted for total protein concentration of the media as determined by Bradford assay (Bio-Rad Laboratories).

**Cell adhesion assay**

VSMCs were seeded at 2×10^5 cells per well of a 24-well plate, and allowed to adhere for 30, 60 and 90 min. After washing with PBS, attached cells were fixed with 4% paraformaldehyde (pH 7.5) and stained with 0.5% crystal violet in 2% ethanol for
10 min. The dye was extracted with 0.5 ml of 1% SDS for 30 min with mild shaking, and absorbance was measured at 590 nm.

**Antibody microarray**

Expression and phosphorylation of signaling molecules were characterized by the Kinex Antibody Microarray (Kinexus, Vancouver, Canada). This microarray tracked 623 cell signaling proteins in duplicate for 253 different phospho-sites, 241 protein kinases and 129 other cell signaling proteins. Cell lysates for analysis were prepared according to the Kinexus protocol. In brief, WT, Nox1<sup>−/−</sup>, and Tg<sup>SMCnox1</sup> VSMCs were treated with 10 ng/ml PDGF for 5 min and harvested with ice-cold lysis buffer containing 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 3 mM bezamidine, 5 µM pepstatin A, 10 µM leupeptin, 1% Triton X-100, and 1 mM dithiothreitl (pH 7.2). Cells were then ruptured by sonication and subjected to centrifugation at 90,000 x g for 30 min at 4 °C. Supernatants were collected and protein concentrations were determined by Bradford assay (Bio-Rad Laboratories). Antibody microarray analysis was performed by Kinexus. All changes in phosphorylated proteins reported in Results were confirmed by Western analysis.

**Western blotting**

VSMCs lysates were prepared and subjected to SDS-PAGE as described previously. Proteins were detected by horseradish peroxidase (HRP)-conjugated secondary antibody and ECL advance (Amersham Biosciences). Band intensity was quantified by densitometry of immunoblots using NIH ImageJ.
Statistical Analysis

Data are reported as mean±SE. Statistical significance was assessed by ANOVA on untransformed data, followed by comparison of group averages by contrast analysis, with the use of the SuperANOVA statistical program (Abacus Concepts, Berkeley, CA). A probability value <0.05 was considered statistically significant.

References


**Supplementary Figure Legends**

**Figure S1.** Distribution of macrophage in neointima of injured femoral arteries from Nox1<sup>y/-</sup> and Tg<sup>SMcnox1</sup> mice. Arterial sections were obtained on 21 day after injury induction, and were stained for macrophages. Macrophages were visualized with Mac3-conjugated quantum dots. Red fluorescence indicates macrophage. Scale bar=50 µm.

**Figure S2.** Identification of cultured VSMCs by immunocytochemistry. Primary cultured VSMCs were fixed with 4% formaldehyde and immunostained using anti-smooth muscle α-actin or anti-calponin antibodies and Rhodamine Red-X-conjugated secondary antibodies. The exclusion of endothelial cell contamination was confirmed by negative staining for vWF. The background image was obtained by omitting primary antibody. DAPI, a DNA stain, was used for counterstaining. Representative images are presented. Scale bar=30 µm.

**Figure S3.** Effect of Nox1 on VSMC adhesion. Cells were seeded at a density of 2×10⁵ cell per well in 24-well plates, and allowed to adhere for 30, 60 and 90 min. After washing, attached cells were stained with 0.5% crystal violet. Dye was extracted with 1% SDS, and absorbance was measured at 590 nm. Values are mean±S.E. of 3 independent experiments.

**Figure S4.** Effect of Nox1 on expression and phosphorylation-mediated activation of the PDGF receptor (PDGFR)-β. Quiescent VSMCs were treated with 10 ng/ml
PDGF for 5 min. Cells were harvested and lysates were prepared. Relative PDGFR-β expression was normalized to CDK4. Phosphorylation was detected with a phospho-specific antibody against tyrosine 716, and normalized to the amount of total PDGFR-β. Values are means±SE of 3 independent experiments.

**Table S1.** Expression and phosphorylated levels of signaling molecules in VSMCs.
Figure S1
Figure S2

α-actin  calponin  vWF  background
Figure S3
Figure S4
TABLE S1. Expression and Phosphorylated Levels of Signaling Molecules in VSMCs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Phospho-site</th>
<th>Total protein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phospho-protein&lt;sup&gt;b&lt;/sup&gt;</th>
<th>n=3</th>
<th>PDGF-stimulated&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nox1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Tg&lt;sup&gt;SMCnox1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Src</td>
<td>Y416</td>
<td>0.99 ± 0.07</td>
<td>0.95 ± 0.10</td>
<td>6</td>
<td>1.09 ± 0.08</td>
</tr>
<tr>
<td>PLCγ1</td>
<td>Y783</td>
<td>1.05 ± 0.12</td>
<td>0.91 ± 0.07</td>
<td>6</td>
<td>1.31 ± 0.15</td>
</tr>
<tr>
<td>Erk1&amp;2</td>
<td>T202/Y204</td>
<td>1.05 ± 0.05</td>
<td>0.95 ± 0.13</td>
<td>6</td>
<td>0.85 ± 0.05</td>
</tr>
<tr>
<td>p38</td>
<td>T180/Y182</td>
<td>1.11 ± 0.07</td>
<td>1.21 ± 0.06</td>
<td>3</td>
<td>1.08 ± 0.28</td>
</tr>
<tr>
<td>PRK1</td>
<td>T774</td>
<td>1.04 ± 0.11</td>
<td>1.06 ± 0.09</td>
<td>3</td>
<td>1.26 ± 0.28</td>
</tr>
<tr>
<td>p85S6K&lt;sup&gt;c&lt;/sup&gt;</td>
<td>T444/S447</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>1.07 ± 0.18</td>
</tr>
<tr>
<td>p85S6K</td>
<td>T421/S424</td>
<td>1.06 ± 0.02</td>
<td>1.14 ± 0.07</td>
<td>4</td>
<td>0.97 ± 0.15</td>
</tr>
<tr>
<td>PKCδ</td>
<td>S643</td>
<td>1.01 ± 0.08</td>
<td>1.06 ± 0.11</td>
<td>3</td>
<td>1.04 ± 0.18</td>
</tr>
<tr>
<td>Cofilin</td>
<td>S3</td>
<td>0.96 ± 0.06</td>
<td>1.05 ± 0.07</td>
<td>4</td>
<td>1.94 ± 0.32</td>
</tr>
<tr>
<td>mDia1</td>
<td>-</td>
<td>1.56 ± 0.11</td>
<td>0.73 ± 0.09</td>
<td>3</td>
<td>---</td>
</tr>
<tr>
<td>PAK1</td>
<td>-</td>
<td>0.68 ± 0.10</td>
<td>1.02 ± 0.11</td>
<td>6</td>
<td>---</td>
</tr>
<tr>
<td>PP2A</td>
<td>-</td>
<td>1.01 ± 0.07</td>
<td>1.04 ± 0.07</td>
<td>5</td>
<td>---</td>
</tr>
<tr>
<td>Vinculin</td>
<td>-</td>
<td>1.14 ± 0.04</td>
<td>1.03 ± 0.12</td>
<td>3</td>
<td>---</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE.

<sup>a</sup> Band density of each protein was normalized with that of β-tubulin or CDK4, loading controls.

<sup>b</sup> Phosphorylated protein level is the ratio of band densities obtained with phospho- and total-antibodies, and normalized to WT control. Data are relative levels compared with unstimulated WT cells. n=3 for all experiments.

<sup>c</sup> Cells were treated with PDGF (10 ng/ml) for 5 min.

<sup>d</sup> Phosphorylation ratios were calculated with CDK4 instead of total p85S6K.

* Significantly different from WT value.