MEF2B-Nox1 Signaling Is Critical for Stretch-Induced Phenotypic Modulation of Vascular Smooth Muscle Cells


Objective—Blood vessel hemodynamics have profound influences on function and structure of vascular cells. One of the main mechanical forces influencing vascular smooth muscle cells (VSMC) is cyclic stretch (CS). Increased CS stimulates reactive oxygen species (ROS) production in VSMC, leading to their dedifferentiation, yet the mechanisms involved are poorly understood. This study was designed to test the hypothesis that pathological CS stimulates NADPH oxidase isoform 1 (Nox1)–derived ROS via MEF2B, leading to VSMC dysfunction via a switch from a contractile to a synthetic phenotype.

Approach and Results—Using a newly developed isoform-specific Nox1 inhibitor and gene silencing technology, we demonstrate that a novel pathway, including MEF2B-Nox1-ROS, is upregulated under pathological stretch conditions, and this pathway promotes a VSMC phenotypic switch from a contractile to a synthetic phenotype. We observed that CS (10% at 1 Hz) mimicking systemic hypertension in humans increased Nox1 mRNA, protein levels, and enzymatic activity in a time-dependent manner, and this upregulation was mediated by MEF2B. Furthermore, we show that stretch-induced Nox1-derived ROS upregulated a specific marker for synthetic phenotype (osteopontin), whereas it downregulated classical markers for contractile phenotype (calponin1 and smoothelin B). In addition, our data demonstrated that stretch-induced Nox1 activation decreases actin fiber density and augments matrix metalloproteinase 9 activity, VSMC migration, and vectorial alignment.

Conclusions—These results suggest that CS initiates a signal through MEF2B that potentiates Nox1-mediated ROS production and causes VSMC to switch to a synthetic phenotype. The data also characterize a new Nox1 inhibitor as a potential therapy for treatment of vascular dysfunction in hypertension. (Arterioscler Thromb Vase Biol. 2015;35:430-438. DOI: 10.1161/ATVBAHA.114.304936.)

Key Words: MEF2B ■ Nox1 ■ oxidative stress ■ vascular remodeling

Differentiated vascular smooth muscle cells (VSMC) are major constituents of the blood vessel wall and play a vital role in the maintenance of vessel homeostasis. These highly specialized cells regulate vessel tone, blood pressure, and blood flow distribution.1,2 Mature VSMC that are fully differentiated exhibit a contractile phenotype characterized by low protein: DNA synthetic activity, reduced proliferation rate, and a unique set of contractile proteins and signaling molecules.3,4 Unlike skeletal and cardiac muscle cells, mature VSMC retain a remarkable ability to modulate their phenotype and dedifferentiate into a synthetic phenotype in response to changes in local environmental cues.5,6 The synthetic phenotype is characterized by increased VSMC migration, loss of contractility, and abnormal extracellular matrix production. These hallmarks have been observed clinically and in animal models of vascular injury and diseases, including systemic hypertension, angioplasty-induced restenosis, atherosclerosis, and aortic aneurysm formation.7,8

VSMC phenotype is influenced by diverse hormonal and environmental cues, including cytokine stimulation, cell–cell contact, cellular adhesions, vascular injury, and increased mechanical force. In vivo, VSMC are constantly subjected to mechanical forces as a consequence of pulsatile blood flow and shear stress. Among multiple hemodynamic forces, VSMC are primarily subjected to pulsatile cyclic stretch (CS) in response to systolic–diastolic fluctuations in pressure. As such, in vitro CS serves as a model of pressure fluctuations in the vasculature with 10%, 1 Hz stretch mimicking hypertension.9 Indeed, CS is a well-established stimulus for VSMC dedifferentiation and a switch to the synthetic phenotype,10-12 yet the mechanism involved is incompletely understood.
NADPH oxidase (Nox) isozymes are expressed in vascular endothelial cells, smooth muscle cells, and adventitial fibroblasts, in which they are major reactive oxygen species (ROS) producers and mediators of cardiovascular physiology and pathophysiology.\(^{13}\) Consistent with a role for Nox in stretch-induced dedifferentiation, smooth muscle Nox isoform 1 (Nox1)-derived superoxide anion (O\(_2^−\)) production is elevated in neointimal growth in response to balloon angioplasty.\(^{14,15}\)

Myocyte-enhanced factor 2 (MEF2) proteins are a family of transcription factors that play a pivotal role in the transduction of extracellular signals to the genome that control cell differentiation, proliferation, morphogenesis, survival, and apoptosis.\(^{16}\) MEF2s are evolutionarily conserved and serve as lynchpins in the transcriptional circuits that control cell differentiation and organogenesis of an ancient regulatory differentiation network. Importantly, MEF2s are involved in morphogenesis and myogenesis of skeletal, cardiac, and smooth muscle cells.\(^{17,18}\) Moreover, MEF2s are established contributors to growth, proliferation, and hypertrophy of multiple cell types.\(^{19}\) However, their role in VSMCs is less clear.

MEF2s are subject to multiple positive and negative control mechanisms, which serve to fine-tune the diverse transcriptional circuits in which these factors participate. In adult rat aortic VSMC (RASMC), 3 MEF2 isoforms (MEF2A, MEF2B, and MEF2D) are expressed, whose levels are increased in vascular injury.\(^{19,20}\) Interestingly, studies using RT-PCR (5′RACE) suggest that the Nox1 promoter region possesses a cis-regulatory element that is a consensus site for MEF2B.\(^{21}\) Despite the evidence for a role of MEF2s in developmental myogenesis and their upregulation in vessel injury, the role of MEF2s and their link to Nox/ROS and adult smooth muscle cell differentiation in vascular disease are entirely unknown.

We postulated that CS via induction of MEF2B activity stimulates Nox1-derived O\(_2^−\) production, leading to a switch from a contractile to synthetic smooth muscle cell phenotype. This includes marked changes in phenotypic markers, including calponin 1 (CNN1), smoothelin B, and osteopontin, in concert with a decrease in F-actin fiber density, enhanced matrix metalloproteinase (MMP) activity, cell migration, and aberrant vectorial cell alignment.

**Results**

**Uniaxial CS Induces Nox1 Expression and Activity**

To determine the effect of uniaxial mechanical CS on Nox1 mRNA, protein levels, and activity in RASMC, cells were subjected to 10% CS (1 Hz) for different time periods. Nox1 mRNA expression increased in a time-dependent response, reaching a plateau of 3.8-fold versus static after 3 hours of stimulation (Figure 1A). Likewise, Nox1 protein levels increased in a time-dependent manner, yielding a maximum signal of 1.45-fold versus static after 24 hours of stimulation (Figure 1B). As 24 hours yielded maximum Nox1 protein upregulation, we chose this time point for the remainder of experiments in this study. After 24 hours of mechanical stretch, we observed an ≈2-fold increase in O\(_2^−\) production in stretched cells versus static control. Preincubation of RASMC with a recently developed isozyme-specific Nox1 peptidic inhibitor, NoxA1ds,\(^{22}\) completely inhibited CS-induced O\(_2^−\) generation to values observed in static conditions (Figure 1C).

Additionally, a time course of optimal NoxA1ds effectiveness was tested (Figure 1 in the online-only Data Supplement), showing that administration of NoxA1ds 4 hours before the end of CS was maximally effective. The inhibitory effect of NoxA1ds in RASMC under control conditions and in response to classical Nox agonists, such as phorbol myristate acetate and platelet-derived growth factor, is shown in Figure II in the online-only Data Supplement. For detailed evidence of NoxA1ds isoform-specificity, efficacy, and mechanism of action, refer Ranayhossaini et al.\(^{22}\)

**Uniaxial CS Induces MEF2B Promoter and Nox1 Expression**

To evaluate whether MEF2B is activated under stretch conditions, cells were cotransfected with MEF2B firefly luciferase promoter (pMEF2B-pGL3) and activity was compared with control *Renilla* luciferase (pRL-CMV) promoter activity. pMEF2B-pGL3 promoter activity was increased ≈2-fold after 1 hour CS (Figure 2A). In separate experiments, RASMCs were subjected to a time course of CS (1, 3, 6, 9, and 24 hours), and MEF2B protein expression was investigated by Western blot. CS gradually increased MEF2B protein expression over the course of 24 hours (Figure 2B). To investigate whether MEF2B activity regulates Nox1 protein expression, cells were transfected with siRNA against MEF2B or scrambled siRNA (Scr); after 24 hours of CS stimulation, Nox1 protein levels were evaluated by Western blot. We observed that under stretch conditions, cells that were treated with Scr siRNA displayed a 1.8-fold increase in Nox1. Gene silencing of MEF2B by 60% (data not shown) reverted Nox1 protein expression to static levels (Figure 2C). MEF2B siRNA showed no effect on basal Nox1 levels.

**Nox1 Mediates a Decrease in RASMC Contractile Marker Expression in Response to Uniaxial CS**

First, we tested whether the RASMC used for the experiments are homogeneous and express markers of fully differentiated SMCs using confocal microscopy. The representative confocal images demonstrate that virtually all cells express smooth muscle α-actin, smoothelin, smooth muscle myosin heavy chain, and SM22α (Figure IIIA–IID in the online-only Data Supplement).

In response to CS, expression of contractile phenotype markers CNN1 and smoothelin B were decreased (42% and
and subjected to CS. Confirming the effectiveness of Nox1 siRNA, quantitative polymerase chain reaction showed that levels of Nox1 mRNA were decreased by ≈65% in Nox1 siRNA–treated versus Scr control–treated cells. In contrast, we observed no change in Nox4 mRNA levels. Nox1 siRNA had no effect on CNN1 or smoothelin B levels under static conditions. CS induced a significant decrease in both markers in cells treated with Scr siRNA (Figures 3B and 3D). On the other hand, Nox1 siRNA abolished the decrease in both markers (Figure 3B and 3D). In contrast, CS (24 hours) had no effect on myosin heavy chain expression in RASMC (myosin heavy chain/β-actin ratio: 0.39±0.1 and 0.46±0.2 for static control and stretch, respectively, n = 3), perhaps suggesting that 24 hours CS is not sufficient to effect a change in myosin heavy chain.

Separate experiments were performed to investigate whether Nox4 (Nox2 and Nox5 are not expressed in RASMC) is also involved in CS-induced changes in contractile proteins. Nox4 was gene silenced in RASMC using siRNA, cells exposed to CS for 24 hours, and CNN1 and osteopontin expression were analyzed by Western blot. Our data showed that gene silencing Nox4 in RASMC did not reverse CS-induced changes in CNN1 and osteopontin protein levels (Figure IVA and IVB in the online-only Data Supplement). In the case of CNN1, Nox4 silencing had an effect to augment the reduction in CNN1 in response to stretch, perhaps suggesting that Nox4 plays a role in attenuating this phenotypic change.

**Nox1 Mediates a Shift in RASMC Cell Alignment in Response to Uniaxial CS**

In cell culture, CS causes a shift in the total population of cells toward perpendicular realignment of RASMC (higher cell percentage closer to 90°) relative to the stretch axis direction. As measured by nuclear orientation, radial histograms show a greater distribution of cells shifted toward 90° under CS. This effect was ablated by NoxA1ds but not scrambled control (Figure 3E and 3F). Neither NoxA1ds nor its Scr control had an effect on cell alignment under static conditions.

**Nox1 Mediates an Increase in RASMC Synthetic Phenotype Markers in Response to Uniaxial CS**

To further assess the role of Nox1 in stretch-induced synthetic phenotype, osteopontin protein expression was measured after 24 hours of CS. CS increased osteopontin protein levels by 1.5-fold versus static conditions, which was inhibited by NoxA1ds-pretreatment and Nox1 gene silencing but not scrambled controls (Figure 4A and 4B).

**Nox1 Increases MMP9 Activity and Stimulates Migration in Response to CS**

Increased MMP expression contributes to the dedifferentiation process and plays a role in the migration of synthetic SMC. Thus, we determined whether Nox1 participates in CS-induced MMP activity and VSMC migration. CS stimulated MMP9 activity by ≈2.5-fold versus static control (Figure 4C). NoxA1ds, but not Scr control, treatment reduced MMP9 activity to static levels (Figure 4C). Finally, CS induced a significant increase in RASMC migration compared to static control.
with static, which was reduced after pharmacological inhibition of Nox1 (Figure 4D and 4E).

Finally, we tested whether CS induces changes in the architecture of F-actin network in SMC. CS significantly increased the density of actin filaments in SMC in a Nox1-dependent manner (Figure 4F). In contrast, quantitative analysis of fiber thickness showed no significant actin thickening in response to CS.

Discussion

The present study illustrates for the first time that uniaxial stretch-induced phenotypic transitioning of vascular smooth muscle cells from a contractile to synthetic phenotype, as detected by changes in cytoskeletal proteins, F-actin density, MMP9 activity, cell migration, and cell orientation, is mediated by an early increase in MEF2B transcription and protein levels, upregulation of Nox1 expression, and increased Nox1-derived O$_2^-$ production. These novel findings indicate that MEF2B to Nox1 signaling causing alterations in cytoskeletal proteins and MMP9 activation are pivotal for the synthetic and hyperproliferative/promigratory VSMCs in response to CS.

Blood vessels are continuously subjected to hemodynamic mechanical forces, including CS and shear stress, and these forces are highly dependent on the fluid dynamics of the blood, in particular, flow and viscosity. Increases in any of these conditions concomitantly lead to increased CS and shear stress. Under physiological conditions, as in early vascular development, blood pressure and thus mechanical stress in the arterial wall regulate critical parameters of vascular function and maintain the balance between blood supply and tissue oxygen demand. In contrast to these physiological processes, sustained or chronic elevations in blood pressure and flow lead to phenotypic changes of the vascular wall and vascular remodeling.

NADPH oxidases (Noxes) are well established as major sources of ROS in the vasculature, as well as significant contributors to vascular pathologies, including neointima formation. A previous study demonstrated that p22phox-expressing smooth muscle cells in the neointima, that have greater capability to produce ROS, are positive for SMemb but not for SM2, suggesting that ROS-producing cells could possess a synthetic rather than a contractile phenotype. The manner and means by which MEF-induced Nox-derived ROS induces a molecular shift away from contractile proteins and activity to a highly proliferative and synthetic phenotype are unknown.

We observed that uniaxial CS (10%, 1 Hz, conditions mimicking hypertension in humans) increased Nox1 mRNA, protein expression, and activity in smooth muscle cells. Our findings illustrate an early upregulation of Nox1 mRNA at 3 to 4 hours of stretch followed by increased protein expression at 12 to 24 hours. A previous report suggested that Nox1 can be upregulated in smooth muscle cells under in vitro CS conditions. Our data confirm this finding and go further in demonstrating a rise in Nox1 mRNA, protein expression, and specific activity in response to CS. On establishing optimal conditions for Nox1 upregulation, we measured O$_2^-$ production (via cyt c reduction assay) and observed a robust increase in SOD-inhibitable O$_2^-$ production after 24 hours of CS. Previous reports suggested that CS induces O$_2^-$ production.
or implicated a CS-induced O$_2^-$ effect on transduction pathways using broad-range flavoprotein inhibitor DPI or apocynin.37 Nevertheless, those data pointed to a role for O$_2^-$ in CS.

Our findings are the first to our knowledge to identify the functional involvement of Nox1, or any Nox for that matter, in the stretch response of VSMC (using 2 approaches: gene silencing and pharmacological inhibition using an isoform-specific Nox1 inhibitor, NoxA1ds).22 Previous work in our laboratory confirmed siRNA efficacy to inhibit Nox1 by 60% to 70% in this assay.38 Moreover, NoxA1ds was able to completely blunt Nox1 activity in RASMC. Taken together, these data strongly support that mimicking hypertensive conditions via CS causes an increase in Nox1 expression and Nox1-derived O$_2^-$ production. The time frame of our results suggest a tightly regulated signaling role for Nox1.

Accumulating evidence suggests that the family of MEF2 transcription factors is involved in VSMC differentiation and disease.17,19 It has been proposed that Nox1 promoter region

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**Figure 3.** NADPH oxidase isoform 1 (Nox1) mediates a decreased contractile phenotype in response to uniaxial cyclic stretch. Rat aortic vascular smooth muscle cells (RASMCs) were subjected for 24 hours to stretch or static conditions. A and B, Calponin 1 (CNN1) protein expression measured by Western blot (n=4). C and D, Smoothelin B protein expression measured by Western blot (n=4). E, Cell nuclear perpendicular alignment vs the direction of the stretch vector represented by radial histograms (n=5, over 130 cells). F, Summary of the average alignment angles (n=5 over 130 cells). A, C, E, and F, Cells were pretreated with NADPH oxidase 1 inhibitor (NoxA1ds) or scrambled peptide (10 μmol/L). B and D, Cells were pretreated with Nox1 siRNA or scrambled (Scr) siRNA. Data are expressed as means±SEM. *P<0.05 vs static control.
Figure 4. NADPH oxidase isoform 1 (Nox1)–mediated increase in synthetic phenotype in response to uniaxial cyclic stretch. Rat aortic vascular smooth muscle cells (RASMCs) were subjected for 24 hours to stretch or static conditions. A and B, Osteopontin (OPN) was measured by Western blot (n=4). C, Extracellular matrix metalloproteinase 9 (MMP9) activity was measured by zymography (n=5). D and E, Cell migration was measured by wound healing assay (n=8). F, Uniaxial cyclic stretch increases actin fiber density via Nox1. Fluorescence microscopy images of phalloidin-stained RASMC. Fiber density was assessed using ImageJ software and with the aid of the Hessian matrix plug-in. The graph depicts mean values of 8 cells from 4 independent experiments. The green bars represent 20 μm. A, C, D, E, and F, Cells were treated with NADPH oxidase 1 inhibitor (NoxA1ds) or scrambled (Scr) peptide (10 μmol/L). B, Cells were pretreated with Nox1 siRNA or scrambled siRNA (5 pmol/mL) for 48 hours. Data are expressed as means±SEM. *P<0.05 vs static control. #P<0.05 vs stretch scrmb.

possesses a cis-regulatory element that is a consensus site for MEF2B binding. To corroborate the existence of this MEF2B regulatory element in the Nox1 gene, we evaluated the Nox1 promoter region using Ensembl Genome Browser and found a consensus sequence for MEF2B binding located at −438 bp upstream of the Nox1 transcription initiation codon (Figure VI in the online-only Data Supplement), demonstrating a putative association between MEF2B and Nox1.
Furthermore, our results showed that there is a rapid increase in MEF2B promoter activity in stretch-stimulated RASMC and that inhibition of MEF2B expression using siRNA attenuates stretch-induced Nox1 expression, supporting the link between MEF2B and Nox1. These data suggest for the first time a tightly regulated, time-dependent response to CS that involves MEF2B, Nox1, and O$_2^-$ production.

VSMC phenotypic switching is a varied and complex process. Although numerous reports have addressed diverse molecular mechanisms behind VSMC lineage determination and differentiation, there is a lack of information on how Nox-derived O$_2^-$ affects these processes. VSMC cytoskeletal proteins are commonly used to define contractile or synthetic phenotypes, which each exhibit distinct proliferative and migratory manifestations.\textsuperscript{36–41} Generally, synthetic VSMC exhibit higher growth rates and higher migratory activity than contractile VSMC and are identified in part by detection of reduced contractile proteins.\textsuperscript{42} Our observations demonstrate that stretch-induced Nox1 activity leads to a major decrease in 2 of the classical contractile proteins: CNN1\textsuperscript{43} and smoothelin B.\textsuperscript{44} as well as changes in F-actin fiber density. The observed stretch-induced reduction in CNN1 and smoothelin B expression are reversed by an isoform-specific Nox1 inhibitor (Nox1A1ds) and Nox1 siRNA. Osteopontin and fiber density, on the other hand, were increased in a Nox1-dependent manner. Taken together, these results are indicative of a less contractile and more synthetic VSMC phenotype.\textsuperscript{45}

Although these data indicate that Nox1 plays a major role in CS-induced phenotypic changes of smooth muscle cells, it is likely that other Nox isoforms and ROS-generating enzymes or even ROS-independent processes are involved in the transition of SMC into the proliferative, synthetic phenotype. A variety of signaling mediators have been associated with the phenotypic change of SMC, some of which happen to be redox-sensitive (ie, protein phosphatases and MMP). In addition, the role of microRNAs and intracellular Ca\textsuperscript{2+} signaling have recently been demonstrated in VSMC phenotype switching.\textsuperscript{46–48} Other Nox-independent pathways initiated by CS (ie, basic fibroblast growth factor, insulin-like growth factors, epidermal growth factor, etc.) are also expected to contribute to CS-induced phenotypic changes.\textsuperscript{49} Future studies are required to investigate whether one or more of these factors elicit phenotypic changes involving changes in the redox status of the cell. In the present study, we investigated whether Nox4 (Nox2 and Nox3 are not expressed in RASMC) contributes to CS-induced changes in contractile proteins. Our data demonstrated that gene silencing Nox4 in RASMC does not rescue CS-induced changes in CNN1 and osteopontin protein levels.

To further validate our observations of a decreased VSMC contractile phenotype, we proceeded to measure VSMC orientation with respect to the direction of the stretch stimulus. In vivo, arterial smooth muscle cells are aligned primarily in the circumferential direction in the media of the artery. The circumferential orientation and structural network of the VSMC layers are key to maintaining mechanical strength and function of the arterial wall in response to increased wall stress and also provide the flexibility required for pulsatile blood flow.\textsuperscript{50} This effect can be evaluated in cell culture probing an alignment response to persistent mechanical force. It has been reported that, in response to uniaxial CS, VSMC rapidly realign in an orientation perpendicular (90°) to the axis of the strain.\textsuperscript{36,51} We postulated that under CS conditions, there is a Nox1-dependent decrease in contractile proteins in concert with increased RASMC perpendicular realignment. Indeed, both effects were mediated by Nox1 and are indicative of an impaired contractile VSMC state.

One of several structural proteins shown to be increased in RASMC in a synthetic phenotype is osteopontin. Osteopontin is a cytokine upregulated in diabetes mellitus, which augments MMP activation, promoting migration in vascular cells.\textsuperscript{52} Overexpression of MMPs in the aortic wall is believed to play an important role in dilative vascular pathologies. MMP2 and MMP9 upregulation has been observed in aortic walls from patients with thoracic aortic dissection and thoracic aortic aneurysm.\textsuperscript{53} In addition, in vitro cultured VSMC derived from abdominal aortic aneurysmal wall exhibits an increased synthesis of MMP2 and MMP9.\textsuperscript{54} Our results show that osteopontin expression, MMP9 activation, and migration were increased during CS versus static conditions. All of these cell changes were suppressed by Nox1 inhibition. Interestingly, CS did not increase MMP2 activation (Figure V in the online-only Data Supplement). Incidentally, these results are consistent with previous observations in vivo in which after 2 weeks of balloon angioplasty, there is an increased Nox1 expression within the neointima associated with MMP9 activation.\textsuperscript{55}

In summary, these findings highlight the involvement of a new signaling pathway originating at mechanical stretch, stimulating MEF2B activity, and upregulating a Nox1-mediated shift to a synthetic and migratory VSMC phenotype. This includes marked Nox1-mediated shifts in CNN1, smoothelin B and osteopontin, and MMP9 activity along with Nox1-enhanced F-actin density, cell migration, and aberrant cell orientation. VSMC migration and proliferation are key processes in neointima formation in multiple vascular diseases, including atherosclerosis, restenosis, and vein graft failure,\textsuperscript{56} suggesting that our findings have broad implications for hyperproliferative vascular diseases. Moreover, the data provide new insight into mechanisms controlling vascular dysfunction initiated by hemodynamic alterations and are expected to elucidate the mechanisms regulating vascular responses to elevations in mean arterial blood pressure and pulse pressure.\textsuperscript{57,58} The findings also further support MEF2B and Nox1 as therapeutic targets in ameliorating vascular dysfunction in hypertension and multiple other cardiovascular disorders.

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**Significance**

This is the first study to establish a link between MEF2B and Nox1 and identify both factors as mediators of vascular smooth muscle cell phenotypic changes during mechanical stretch. Our findings illustrate that mechanical stretch stimulates MEF2B transcription, leading to Nox1 upregulation and increased reactive species production. The data also demonstrate that under pathological cyclic stretch, Nox1 mediates a vascular smooth muscle cell contractile to synthetic phenotypic switch manifested by alterations in key cytoskeletal proteins, matrix metalloproteinase 9 activation, disorientation of cell alignment, and augmented cell migration. Furthermore, the data demonstrate the feasibility of a novel isoform-specific inhibitor to ameliorate these phenotypic changes and provide additional proof of its viability as a therapeutic agent in vascular disease (see appendix Ranayhossaini et al). 22
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Material and Methods

MEF2B - Nox1 Signaling is Critical for Stretch-Induced Phenotypic Modulation of Vascular Smooth Muscle Cells

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Reagents

Cytochrome c, catalase, and superoxide dismutase (SOD), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin and Dulbecco’s Modified Eagle’s medium (DMEM) were purchased from Mediatech (Mediatech Inc., Manassas, VA). Optimem, Lipofectamine 2000, Nox1, MEF2B and scrambled Stealth siRNA, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Protease inhibitor cocktail was purchased from Roche Diagnostics GmbH (Mannheim, Germany). NoxA1ds and scrambled NoxA1ds were synthesized by the Tufts University Core Facility (Boston, MA, USA). The sequence of human NoxA1ds is as follows: [NH₃]-E-P-V-D-A-L-G-K-A-K-V-[CONH₂]. The scrambled NoxA1ds sequence (Scr) is as follows: [NH₃]-L-V-K-G-P-D-A-E-K-V-A-[CONH₂]. In both cases the [NH₃] group represents the amino end and [CONH₂] represents the amide of the carboxy terminus, a consequence of the synthetic procedure. Each peptide was prepared in several batches, with no batch having a purity of less than 90%.

Cell Culture

Rat aortic smooth muscle cells (RASMC) (Lonza, Walkersville, MD, USA) were grown in DMEM (Cellgro) with 4.5 g/l glucose, L-glutamine, and sodium pyruvate containing 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were employed at passages 3–6. Cells were grown to 80% confluence and serum starved with 0.1% FBS for 24 hours before any experimental procedure.

Membrane Fractions

RASMC cells were suspended to a concentration of 5 × 10⁷ cells/ml in ice-cold disruption buffer (8 mM potassium, sodium phosphate buffer, pH 7.0, 131 mM NaCl, 340 mM sucrose, 2 mM NaN₃, 5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, and protease inhibitor cocktail [Roche
Diagnostics, 11697498001}). Lysates were subjected to five freeze/thaw cycles and passed through a 30-gauge needle five times to further lyse the cells. Cell disruption was confirmed by phase-contrast microscopy. The cell lysate was first centrifuged at 1000 g for 10 min at 4°C to remove unbroken cells, nuclei, and debris. The supernatant was then centrifuged at 28,000 xg for 15 min and membrane fractions were pelleted from cytosol.

**O$_2^-$ Detection by Cytochrome Complex (Cyt. c.) reduction Assay**

Particulate membrane fractions (20 μg/ml) were suspended in Oxidase Assay Buffer (OAB, 65 mM sodium phosphate buffer (pH 7.0), 1 mM EGTA, 10 μM FAD, 1 mM MgCl$_2$, 2 mM Na$_3$, 100 U/ml catalase, and 0.2 mM cyt c). O$_2^-$ production was measured from the initial linear rate (over 10 min) of SOD-inhibitable cytochrome c reduction quantified at 550 nm using the extinction coefficient of 21.1 mM$^{-1}$ cm$^{-1}$ (Biotek Synergy 4 hybrid multimode microplate reader). O$_2^-$ production was initiated by the addition of 180 μM NADPH.

**Western Blot**

Following stimulation, cells were washed with ice cold PBS (1x) and lysed with RIPA® buffer (Pierce, #89900) supplemented with protease and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Cells were centrifuged at 1000 x g for 10 min at 4°C, and the supernatant collected. Protein concentration was measured using the Bradford method (Thermo Scientific, Rockford, IL). Samples were prepared with Tris-Glycine SDS sample buffer, resolved by SDS–PAGE along with a molecular weight standard (Bio-Rad Laboratories, Hercules, CA), and transferred onto Trans Blot nitrocellulose membranes (Bio-Rad). Membranes were blocked with the Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) and incubated with rabbit polyclonal Nox1 (Santa Cruz Biotechnology, sc-25545), rabbit polyclonal calponin (CNN1) (Santa Cruz Biotechnology, sc-16604), rabbit polyclonal smooth muscle myosin heavy chain (abcam, ab53219), mouse monoclonal smoothelin (Santa Cruz Biotechnology, sc-376902), and mouse monoclonal osteopontin (OPN) (Santa Cruz Biotechnology, sc-21742) antibodies. Membranes were probed with their respective secondary antibodies (1:10,000 dilution, Li-Cor Biosciences). Protein loading was assessed by reprobing the membranes with a mouse monoclonal β-actin (Santa Cruz Biotechnology, sc-47778) antibody. Blots were scanned using the Odyssey infrared imaging system (Li-Cor Biotechnology).
Quantitative PCR (qPCR)

RASMC were lysed in RLT® buffer and RNA was purified using RNasey-mini kit (Qiagen). RNA (1 µg) was retro-transcribed to cDNA using Superscript First-Strand Synthesis System (Invitrogen) and qPCR was performed using TaqMan Universal PCR Master Mix (Applied Biosciences). Samples were mixed with primer/probe for Nox1 or 18S and qPCR performed in a 7900HT Fast Real-Time PCR System (ABI) for 40 cycles. Relative quantification was obtained using the Ct (threshold cycle) method:

\[ \Delta C_t = \Delta C_{t\text{Nox1}} - C_{t18S}; \]
\[ \Delta\Delta C_t = \Delta C_{t\text{Nox1 siRNA transfected sample}} - \Delta C_{t\text{scrambled transfected sample}} \]

Relative expression was calculated as \( 2^{-\Delta\Delta C_t} \).

Characterization of RASMC by immunostaining

Cells were fixed in 2% paraformaldehyde and stained with monoclonal antibodies specific for SM α-actin, smoothelin, myosin heavy chain, and SM22α. Primary antibodies were visualized by staining with Cy3-conjugated secondary antibodies. Nuclei were stained by Hoechst and F-actin was labeled by 488 phalloidin. Images were taken with an Olympus FluoView™ FV1000 confocal microscope. Unstained controls were performed omitting the primary antibody in each case.

Cell Flex Stretch System

RASMC between passages 3 and 5 were detached from plates with 0.01% EDTA-0.02% trypsin and transferred to a 6 well BioFlex® Culture Plates (CellFlex International) coated with type I collagen at a density of 2.0×10^4 cells per well for 24-36 hr (75-80% confluence). Twelve hours prior to stretching, cells were brought to a quiescent state by incubation in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) with 0.1% serum. A uniaxial sinusoidal stretch of 10% in strain at 1 Hz was applied using stretching apparatus driven by a computer-controlled stepping motor Flexcell® FX-5000™ Tension System in an atmosphere of
5% CO₂ and 95% air at 37°C. These cyclic stretch conditions mimic the cyclic pressure observed in patients with chronic hypertension. Cells incubated under static conditions on BioFlex plates were used as time-matched controls.

RASMC Gene Silencing

RASMC were grown to 30–50% confluence on 6-well plates and were transfected with scrambled siRNA or siRNA (5 pmol) against Nox1 or MEF2B (Stealth RNAi™, Invitrogen) using the transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Cells were assayed 48 h later. To control for possible non-specific effects of siRNA, Stealth RNAi™ siRNA negative controls matched by GC content were used to confirm the effect of Nox1 or MEF2B siRNA.

Cell Alignment

One Hz uniaxial cyclic stretching of 10% elongation was applied to RASMCs in the culture environment over 24 h. After the mechanical force stimulation the cells were stained with nuclear dye DAPI (4’,6-diamidino-2-phenylindole, Vector Laboratories H-1500) and images were taken at 20x magnification using a Nikon Eclipse 800 microscope. Nuclear angle orientation of static vs. stretched cells was measured against the mechanical stretch direction using Image J software (http://rsbweb.nih.gov/ij/) with Orientation J plugin. The cell angles were sorted into nine groups based on degree angle deviation from the axis of stretch: 0-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90. The total cell number was set as 100% and the number in each group was expressed relative to this value.

Zymography

The MMP activities were assessed using gelatin zymography. Equivalent protein amounts (by BCA methods) from concentrated cultured media were loaded on SDS-acrylamide precast gel with 0.28 % w/v gelatin (type A) (Biorad # 161-1167). After electrophoresis in a buffer containing 25 mM Tris–HCl, 250 mM glycine, and 1% SDS, the gel was washed at room temperature with 2.5% Triton X-100, 5 mM CaCl₂, 50 mM Tris–HCl (pH 7.5) and incubated 3 times in the same buffer for 15 min each, followed by incubation overnight (16 h) at 37 °C in 5 mM CaCl₂, 1 μM
ZnCl$_2$, 50 mM Tris–HCl (pH 7.5). The gel was stained with Coomassie Brilliant Blue G (0.5%, w/v) and destained with methanol/acetic acid/water (45:10:45). The area of gelatin degradation (gelatinase activity) on the gel zymograph was depicted as clear bands against a blue background of undegraded gelatin. Gelatinolytic bands were measured densitometrically with Image J software. MMP2 and MMP9 specific bands were determined by loading pure enzyme extracts into the gel.

**Luciferase promoter assay**

MEF2B firefly luciferase reporter plasmid (pMEF2-pGL3) was a kind gift from Dr. Joseph Miano (University of Rochester Medical Center). *Renilla* luciferase control reporter plasmid (pRL-CMV) was purchased from Promega. Expression of the firefly luciferase driven by the clone’s MEF2B promoter DNA fragment was correlated with a co-transfected control reporter expressing *Renilla* luciferase under the control of the cytomegalovirus (CMV) promoter. This protocol allows normalization of activity of the experimental reporter to an internal control, which minimizes experimental variability. Briefly, pMEF2-pGL3 (1μg/well) and pRL-CMV (0.5μg/well) were co-transfected into RASMC with lipofectamine LTX reagent as per the manufacturer’s protocol (Invitrogen). Cells were cultured in Optimem with 10% serum for 24 hours, and then starved overnight. They were subsequently stimulated for 1, 3 or 6 hours with 10% cyclic stretch at 1 Hz. MEF2B and *Renilla* promoter activity were determined using the Dual-Luciferase® Reporter Assay System.

**Wound Migration Assay**

Monolayer RASMC were scraped in a straight line to create a “scratch” across the bottom of the BioFlex plate well using a sterile p-200 pipette tip. Cells were subjected to 24 hr uniaxial sinusoidal stretch of 10% in strain at 1 Hz; respective static plates were used as time controls. At time 0 and after 24 hr, cells were imaged at 20x magnifications using a Nikon Eclipse 800 microscope. To quantify migration of wound healing the area of the gap across the bottom of the dish was quantified using Image J software. After each treatment, the area of the same gap was measured again. The difference between initial and final areas was calculated. Larger deltas represent enhanced migration and were expressed as percent wound closure.
Measurement of F-actin density

Fiber analysis was performed as described previously. Briefly, confocal fluorescence images were taken using a ZEISS LSM 510 Meta / UV microscope (Belo Horizonte, Minas Gerais, Brazil). Fluorescence images for phalloidin-labeled actin fibers were analyzed through a Hessian-based filter in NIH ImageJ (version 1.44) software plugin, FeatureJ. The following parameter options were selected: “Largest eigenvalue of Hessian tensor” option, “Absolute eigenvalue comparison” option, and the “Smoothing scale” factor was set to 0.5. Region of interest (ROI) was defined using ImageJ’s “line tool”. The pixel intensities along the line were plotted using the “Plot Profile” tool. Cells were subjected to two scans, measuring the larger and smaller cell axis. All line scan data was imported into Microsoft Excel (version 15.0.4551.1512, Microsoft, Redmond, WA) for further analysis. The intensity matrices were processed in Microsoft Excel through a slope peak-detection formula. The total number of peaks was then divided by the length of each line to yield the average number of peaks per mm scan.

Statistical Analysis

All results are expressed as mean ± SEM. Significance of the differences were assessed by 2-way ANOVA followed by Bonferroni post hoc test. A value of $P<0.05$ was considered to be statistically significant.

Supplemental References

Supplemental Material

MEF2B - Nox1 Signaling is Critical for Stretch-Induced Phenotypic Modulation of Vascular Smooth Muscle Cells

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Supplemental Figure Legends

Supplemental Figure I. Time response of Nox1 activity in response to varied incubation times of NoxA1ds. RASMC were subjected to 24 hr of cyclic stretch and NoxA1ds (5 µM) was added at 1, 4, 12, & 24 hr before the end of cyclic stretch. SOD-inhibitable O$_2^-$ was measured by cytochrome c reduction ($n = 7$). Data are expressed as means + SEM. *$P<0.05$ versus static vehicle. #$P<0.05$ versus cyclic stretch.

Supplemental Figure II. NoxA1ds inhibits agonist-induced O$_2^-$ production in RASMC. RASMC were stimulated by classical Nox agonist for 1 hr PMA (20 µM) and PDGF (5 ng/ml). SOD-inhibitable O$_2^-$ was measured by cytochrome c ($n = 6$). RASMC were preincubated with NoxA1ds or scrambled peptide (10 µM). Data are expressed as means + SEM. *$P<0.05$ versus static control.

Supplemental Figure III. Characterization of RASMC by immunostaining. RASMC were fixed in 2% paraformaldehyde and stained with monoclonal antibodies specific for SM α-actin (A), smoothelin (B), myosin heavy chain (C), and SM22α (D). Primary antibodies were visualized by staining with Cy3-conjugated secondary antibodies (red). Nuclei were stained by Hoechst (blue) and F-actin was labeled by 488 phalloidin (green). Images were taken with an Olympus FluoView™ FV1000 confocal microscope. Unstained controls were performed omitting the primary antibody in each case.

Supplemental Figure IV. Nox4 siRNA does not reverse CS-induced decreases in CNN1 (A) and increases in OPN (B) levels but augments the reduction in CNN1. RASMCs were pretreated with scrambled (Scr) siRNA or Nox4 siRNA (48 hrs) and then subjected to CS for 24 hr. CNN1 and OPN protein expression was investigated by Western blot ($n = 3$). Data are expressed as means + SEM. *$P<0.05$ versus static control.
Supplemental Figure V. Cyclic Stretch causes no change in MMP2 activity. RASMC were subjected for 24 hr to stretch or static conditions. Extracellular MMP2 activity was measured by zymography \((n = 5)\). RASMC were preincubated with NoxA1ds or scrambled peptide (10 µM).

Supplemental Figure VI. MEF2B consensus sequence site is located at -438 bp upstream of the transcription initiation codon (ATG) within the Nox1 promoter region. Analyzed by ENSEMBL genome browser (www.ensembl.org) in Rattus Norvegicus (Rat): Chromosome X: 104,742,297-104,765,479 (reverse strand).
Supplemental Figures

Supplemental Figure I

Static  □  Stretch  ■

nmol O₂⁻/min/mg prot

Veh  NoxA1ds 4hr (5 µM)  Control  NoxA1ds 1hr (5 µM)  NoxA1ds 4hr (5 µM)  NoxA1ds 12hr (5 µM)  NoxA1ds 24hr (5 µM)
Supplemental Figure II

- Veh
- NoxA1 ds Scrm (5 μM)
- NoxA1 ds peptide (5 μM)
- PMA (20 μM) 60 min
- PMA + NoxA1 ds Scrm (5 μM)
- PMA + NoxA1 ds (5 μM)
- PDGF (5 ng/ml)
- PDGF + NoxA1 ds (5 μM)

nmol O2⁻/min/mg prot
Supplemental Figure III

A) Unstained control

B) Smoothelin
C) Hoechst Phalloidin Cy3
Unstained control
Myosin heavy chain

D) Hoechst Phalloidin Cy3
Unstained control
SM22α
Supplemental Figure IV

A)

Static Stretch

β-actin 42 kD

CNN1 36 kD

siRNA
Scr Nox4 Scr Nox4

Static Stretch

CNN1/β-actin

Scr siRNA Nox4 siRNA

* *

B)
**Graphical Representation**

- **Static Stretch**
  - **OPN 66 kD**
  - **β-actin 42 kD**

**siRNA**
- **Scr**
- **Nox4**

**Bar Graph**

- **Scr siRNA**
- **Nox4 siRNA**

**Y-axis**: OPN/β-actin

**X-axis**: Static, Stretch

*Significant difference indicated by *
Supplemental Figure V
Supplemental Figure VI

-468  AAAAAGATAAATAAATAAAGAATTAAA  AAATAAGAAAACATGCTCCACTCTTACTTCTATT
  TATGTTTCTCAGCTTAATTACGTTGCTTTCAAAACTTCCTAACATAATGCTATCAAGGAAAGCAGTGCTATATGAAAT
  CACATTGTGAAAGATTATCAGAGAGACTATTTTGACATAAAGAAGACTGATATTTCCTGAAGGGGTGAAAAACCTCAG
  CAGGGAAAACCCCCTGTTGTGTCATAGGCAGGCTTGTCCCACCTAAGCTGATAAAAAGTTCCGTCCTACACGAGGAA
  GCCAGAGTACACTTTATCTATCTTTTGAAGGCTTTTCTGAGTGGTAGTGTCATTTGAGTGTCATAAAGACATATATCTTTGAG
  GCTAGACAGAAGTTTCTATCCTGAAGGATGCCCATCAGAGAAAACAGATTGCCTCCAAAGAGAGGCTCCAGACCTCATT
  TGACAGATG

-8  TGACAACTGGCTGTTAACCACCTGGCTCAGTTGGTTTGATGAGGGCTTTTTCTG

MEF2B recognition site

Nox1 promoter region

Initiation Codon

Nox1 first exon

Nox1 intron

-391

-69