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: $[NH_3]$-E-P-V-D-A-L-G-K-A-K-V-$[CONH_2]$. The scrambled NoxA1ds sequence (Scr) is as follows: $[NH_3]$-L-V-K-G-P-D-A-E-K-V-A-$[CONH_2]$. In both cases the $[NH_3]$ group represents the amino end and $[CONH_2]$ 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 5×10^7 cells/ml in ice-cold disruption buffer (8 mM potassium, sodium phosphate buffer, pH 7.0, 131 mM NaCl, 340 mM sucrose, 2 mM NaN_3, 5 mM $MgCl_2$, 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.

$O_2^{\bullet-}$ 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 NaN_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). $O_2^{\bullet-}$ 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 × 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 RNeasy-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:

$\Delta Ct = \Delta Ct_{Nox1} - Ct_{18S}$;

$\Delta\Delta Ct = \Delta Ct_{\text{Nox1 siRNA transfected sample}} - \Delta Ct_{\text{scrambled transfected sample}}$

Relative expression was calculated as $2^{-\Delta\Delta Ct}$.

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% CO_2 and 95% air at 37°C. These cyclic stretch conditions mimic the cyclic pressure observed in patients with chronic hypertension.[1] 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 RNAiTM, 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 $CaCl_2$, 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 $CaCl_2$, 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 (1ug/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.[2]

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.[3] 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

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2. Solberg, N. & Krauss. Luciferase assay to study the activity of a cloned promoter DNA fragment.Methods Mol Biol.2013;977:65-78
3. Sathyanesan A., Ogura T., Lin W. Automated Measurement of Nerve Fiber Density Using Line Intensity Scan Analysis J Neurosci Methods. 2012; 206:165–175