Supplemental Material

NOX4 and Poldip2 induce F-actin oxidation and promote its interaction with vinculin during integrin-mediated cell adhesion

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Materials and methods

Chemicals

Unless otherwise indicated, chemicals of analytical grade were purchased from Millipore Sigma, USA or Thermo Fisher Scientific, USA.

Cell culture, antibodies and plasmids/adenoviruses

Human aortic smooth muscle cells (HASMs) were obtained from Clonetics and cultured in Media 231 (Catalog No. M231500 Cascade Biologics, Portland, OR) supplemented with 5% smooth muscle growth supplement (SMG), 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Primary rat aortic smooth muscle cells (RASMs) were isolated from Sprague Dawley rat aortas as previously reported[1] and grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cell identity was verified with staining for smooth muscle α-actin. Cells between passages 5 and 12 were used in experiments. All cells were cultured at 37°C in a 5% CO_2 incubator.

Anti-NOX4 rabbit antibody was a gift from Dr. David Lambeth (Emory University, GA, USA) and has been previously reported.[2] We utilized commercially available anti-poldip2 rabbit antibody (Catalog No. ab68663, Abcam, Cambridge, UK), mouse anti-β-actin (Catalog No. A5441, Sigma, MO, USA), mouse anti-myc-tag (Catalog No. 2276s, Cell Signaling, CA, USA), mouse anti-paxillin (Catalog No. 05-417, Millipore, USA), rabbit anti-vinculin (Catalog No. ab73412, Abcam, Cambridge, UK or rabbit anti-vinculin (Catalog No. V4139, Sigma, USA), rabbit anti-zyxin (Catalog No. ab109316, Abcam, Cambridge, UK) and secondary antibodies were goat anti-rabbit (Catalog No. 7074s, Cell Signaling Technology, CA, USA), sheep anti-mouse (Catalog No. NA931V, GE healthcare, USA), Alexa Fluor 488 goat anti-mouse IgG (Catalog No. A11001, Thermo Fisher Scientific, USA), Alexa Fluor 546 goat anti-Rabbit IgG (H+L) (Catalog No. A11010, Thermo Fisher Scientific, USA), Alexa Fluor 633 goat anti-Rabbit IgG (H+L) (Catalog No. A21070, Thermo Fisher Scientific, USA), and Alexa Fluor™ 568 Phalloidin (Catalog No. A12380, Thermo Fisher Scientific, USA).

The plasmids used in this study include pC1-HyPer-3 (gift from Dr. Vsevolod Belousov) (Addgene plasmid # 42131)[3] and wild type human beta-actin cDNA in a bacterial expression plasmid (GeneCopoeia, Rockville, MD, USA). A beta-actin construct with mutations C272A and C374A was prepared using the QuikChange

Lightning kit (Catalog No. 210515, Agilent, CA, USA). Wild type and mutant inserts were subsequently subcloned into the pcDNA3.1 mammalian expression vector using PCR and digestion with *Xba* I and *Bam*H I enzymes to produce wild type and mutant beta actin constructs with an N-terminal Kozak consensus and C-terminal myc and 6xHis tags. All plasmids were verified by sequencing. Adenoviruses were prepared using the AdEasy System with either no insert (AdGFP) or C-terminal myc-tagged rat Poldip2 (AdPoldip2). Vascular smooth muscle cells (VSMCs) were transduced with virus as described previously.[4]

Transfection and electroporation

HASMs were transfected with plasmid DNA by electroporation using an Amaxa Nucleofector (Amaxa Biosystems, Koln, Germany). Cells were grown to 70-80% confluency prior to transfection. A total of 1 x 10^6 cells were electroporated with 5 µg DNA mixed with 100 µl Ingenio electroporation solution (Catalog No. MIR 50114, Mirus Bio LLC, USA) per electroporation cuvette using the optimized program U-25. Transfected cells were then plated on culture dishes for further analysis. Media was changed on the following day and the experiments were performed 72 hours after transfection.

Western blotting

VSMCs were lysed in 1% Triton lysis buffer, pH 7.4 (25 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1 mM Na-orthovanadate, 1% Na deoxycholate, 0.1% SDS, 10% glycerol) containing protease inhibitors (10 µg/ml aprotinin, 1 mM phenylmethanesulfonyl fluoride, 10 µg/ml leupeptin). Samples were placed on ice for 30 minutes, and sonicated for 10 seconds. After protein quantification, the protein lysates were separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 5% non-fat dry milk and incubated with mouse anti-β-actin antibody (0.1 µg/ml) or rabbit anti-poldip2 antibody (0.5 µg/ml) overnight at 4°C. Subsequent incubation with sheep anti-mouse secondary antibody (0.2 µg/ml) or goat anti-rabbit secondary antibody (0.5 µg/ml) was performed for 1 hour at room temperature. Proteins were detected by ECL chemiluminescence (Thermo Fisher Scientific, USA). Band intensity was quantified by densitometry using ImageJ software.

Reverse transcription and quantitative PCR

Total RNA was extracted from cultured HASMs using the RNeasy Plus kit (Qiagen, Chatsworth, CA, USA). cDNA was made from of 1 µg of extracted RNA utilizing the High Capacity cDNA Reverse Transcription Kit (Catalog No. 4368814, Thermo Fisher, USA) with RNase Inhibitor Murine (Catalog No. M0314L, New England BioLabs, USA) and was purified with QIAquick kit (Qiagen, CA, USA). Quantitative PCR was performed in LightCycler capillaries (Catalog No. 4929292001 Roche Applied Science, IN, USA), using Platinum Taq DNA polymerase and SYBR green (Invitrogen, Carlsbad, CA, USA). Nox4 expression was measured using primers 5'-CTGGAGGAGCTGGCTCGCCAACGAAG-3' and 5'-GTGATCATGAGGAATAGCACCACCACCATGCAG-3' in the presence of 4 mM $MgCl_2$, and annealing temperature set at 68°C. Poldip2 was measured using primers 5'-

TGCAGCTCCAGAAAAAGCAGAGAACC-3' and 5'-CTGACATAGTCCAAGCCTGGGATG-3', in the presence of 3 mM $MgCl_2$, and annealing temperature set at 60°C. The housekeeping gene RPL was used for normalization and was measured using primers 5'-CCTGGAGGAGAAGAGGAAAGAGA-3'and 5'-TTGAGGACCTCTGTGTATTTGTCAA-3' in the presence of 4 mM $MgCl_2$ with annealing temperature set at 55°C. Data analysis was performed using the mak3i module of the qpcR software in the R environment, as described previously.[5]

Immunofluorescence staining

HASMs grown on non-coated coverslips were fixed with 4% paraformaldehyde (PFA) at room temperature for 10 minutes, and then permeabilized in 0.1% Triton in phosphate-buffered saline (PBS) for 8 minutes. After incubation with blocking buffer (3% BSA, 0.4% fish gelatin in PBS) for 1 hour, cells were incubated with mouse anti myc-tag primary antibody (0.2 µg/ml) overnight at 4°C. Following incubation with Alexa Fluor 488 goat anti-mouse IgG diluted to 10 µg/ml and Alexa Fluor™ 568 Phalloidin diluted to 1 U/ml for 1 hour at room temperature, slides were mounted with Vectashield (Catalog No. H-1200, Vector Laboratories, Burlingame, CA, USA) and observed on a Zeiss LSM 510 META Laser Scanning Confocal Microscope (Zeiss, Oberkochen, Germany) using the 488-nm and 543-nm laser lines, respectively. Negative control samples were incubated with secondary antibodies only.

siRNA knockdown

Small interfering RNAs (siRNAs) against human Nox4 (sense 5'-GCAUCUGUUCUUAACCUCA[dTdT]-3', antisense 5'-UGAGGUUAAGAACAGAUGC[dTdG]-3') and human Poldip2 (sense 5'-CGUGAGGUUUGAUCAGUAA[dTdT]-3', antisense 5'-UUACUGAUCAAACCUCACG[dTdG]-3') were obtained from Sigma. All-Stars negative control (Catalog No. SI03650318, Qiagen, Hilden, Germany) was used in experiments with human cells. A stealth siRNA against Poldip2 (sense 5'-GCCCACAUAUAUCUCAGAGAUCUCA-3', antisense 5'-UGAGAUCUCUGAGAUAUAUGUGGGC-3') and a stealth control siRNA of the corresponding GC content (Invitrogen, Carlsbad, CA, USA) were used with rat cells in cysteine sulfenic acid detection experiments. In the proximity ligation assay, fluorescent inactive siRNA (siGLO, Catalog No. D-001630-02-05, Dharmacon, USA) was used to identify transfected cells. VSMCs were grown to 70-80% confluency before transfection with 25 nM siRNA using Lipofectamine RNAiMAX (Catalog No. 13778150, Thermo Fisher Scientific, USA) according to the manufacturer's manual. Media was changed after 24 hours and further analysis was performed at 72 hours post-transfection.

Real-time imaging of intracellular H_2O_2

To measure intracellular ROS production, we utilized a genetically encoded, highly specific H_2O_2 sensor, cyto-HyPer3.[3] This fluorescent probe consists of the bacterial H_2O_2-sensitive transcription factor OxyR fused to circularly permuted yellow fluorescent protein (YFP), which allows dynamic monitoring of intracellular H_2O_2 concentration.[3] Single cell images of HASMs transfected with cyto-Hyper3 plasmid

were acquired in a live cell chamber at 37°C with 5% CO_2 using a Leica confocal system TCS SP5 II with a 63x (1.4 NA) oil-immersion objective. Cyto-Hyper3 transfected cells were sequentially excited with a 405-nm diode and with an argon laser at 488 nm, while emissions were recorded with a HyD3 detector at 500-530 nm. The ratio of fluorescence intensities following sequential excitations at these two different wavelengths was calculated with background subtraction. We verified that increases in ratio with increasing H_2O_2 were due to an increase in signal following excitation at 488 nm and a decrease in signal following excitation at 405 nm. A 1 mM H_2O_2 solution was used as positive control (data not shown). For the attachment experiment, transfected cells were re-plated in 35-mm glass-bottom dishes (MatTek, USA) and the cyto-HyPer3 fluorescence ratio was recorded at appropriate intervals for 6 hours. For the integrin activation experiment, cells expressing cyto-Hyper3 were selected to record the fluorescence ratio after 30 minutes of treatment with 50 μM RGDS (Catalog No. A9041, Sigma, USA) or RGES peptides (Catalog No. A5686, Sigma, USA). A single cell was imaged in each experiment. All images were obtained at the last detectable optical section with LAS AF Version 2.60 software and analyzed with Photoshop and Image J software.

NADPH-dependent H_2O_2 production measurement by electron spin resonance

RASMs were harvested in lysis buffer containing protease inhibitors as above. Cells were sonicated and the membrane pellet was re-suspended in electron spin resonance (ESR) buffer (50 U/ml SOD, 1 U/ml HRP, 1 mM AAP, 100 μM NADPH, 0.2 mM DTPA, and 1 mM CPH in Chelex-treated PBS). The production of H_2O_2 in membrane fractions was measured by ESR spectroscopy using 1-hydroxy-3-carboxy-pyrrolidine (CPH) as a spin probe in the presence of horseradish peroxidase (HRP) and acetamidophenol (AAP), as previously described.[6, 7] Detection of H_2O_2 was confirmed by inhibition with 50 μg/ml catalase. Quantitative measurement of H_2O_2 was verified by analysis of superoxide production generated by the xanthine and xanthine oxidase system.

Transmission electron microscopy

Early passage RASMs (P2) grown on 35-mm diameter plastic Petri dishes (Catalog No. 430165, Corning, Midland, Michigan) were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 hour, rinsed in 0.1 M sodium cacodylate buffer 3 times, postfixed in 1% osmium tetroxide in cacodylate buffer for 1 hour, then treated with 1% tannic acid[8] in cacodylate buffer for 30 minutes for enhanced stability and contrast of membranes and vesicles.[9] After rinsing 3 times in cacodylate buffer, samples were dehydrated in a graded ethanol series, embedded in resin, sectioned and poststained with uranyl acetate and lead citrate. Images of the samples were acquired by using a Hitachi H-7500 electron microscope operated at 75 kV. Images were prepared for publication using the FigureJ plugin in Fiji (ImageJ) and Photoshop CS6 Extended.

For TEM immunoperoxidase experiments, RASMs grown to 75% confluency as above were fixed in 4% PFA overnight at room temperature. After blocking in a buffer containing 5% BSA, 0.1% cold water fish skin gelatin and 5% normal serum for 60 minutes at 4°C, cells were incubated with primary anti-NOX4 rabbit antibody (gift from D.

Lambeth,[2] 5 µg/ml) at 4°C overnight. After rinsing 6 times with 0.1% BSA in PBS, cells were incubated with biotinylated goat anti-rabbit secondary antibody in 0.1% BSA in PBS overnight at 4°C. After rinsing with 0.1% BSA in PBS 6 times and PBS 3 times, cells were incubated with a drop each of Solutions A and B of the ABC kit (Catalog No. PK6100, Vector ABC Elite kit) in 5 ml PBS for 4 hours at 4°C, followed by reaction with 0.05% DAB/0.003% H_2O_2 for 10 minutes. After rinsing with PBS 4 times for 10 minutes each, cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer for 2 hours at 4°C, followed by post-fixation in 1.0% osmium tetroxide in phosphate buffer for 30 minutes at room temperature. Samples were dehydrated in a graded ethanol series, embedded in fresh resin, sectioned and post stained with lead citrate. Images of the samples were acquired by using a Hitachi H-7500 electron microscope operated at 75 kV. Images were acquired and prepared for publication as above.

Cysteine sulfenic acid detection

Cysteine thiol group oxidation to sulfenic acid was determined using a DCP-Bio1-based assay.[10, 11] At 100% confluency, RASMs were washed with PBS and lysed in 1% Triton lysis buffer containing 0.1 mM DCP-Bio1 (Catalog No. EE0028, Kerafast, Boston, MA), 100 µM DTPA, 10 mM NEM, 10 mM iodoacetamide, protease inhibitor cocktail, and 200 U/ml catalase at room temperature for 15 minutes, followed by addition of β-mercaptoethanol (0.1 mM). Cell lysates were centrifuged at 25,000 x g at 4°C for 10 minutes. Supernatants including G-actin were collected. Pellets including F-actin were re-suspended with lysis buffer containing 0.1 mM β-mercaptoethanol, 100 µM DTPA,10 mM NEM, 10 mM iodoacetamide, and 200U/ml catalase. F-actin samples were vortexed with cytochalasin D at 4°C for 1 hour and supernatants were collected after centrifugation at the same speed. After protein measurement, samples (200 µg) were incubated with 30 µl precleared Pierce streptavidin agarose resins (Catalog No. 20347, Thermo Fisher Scientific, USA) in 1 ml lysis buffer overnight at 4°C. Samples were then subjected to 3 stringent washes (1% Triton lysis buffer containing 100 µM DTPA, 0.1 mM β-mercaptoethanol and 200 U/ml catalase) with centrifugations at 5,000 x g for 2 minutes and re-suspended in 30 µl 1 X SDS loading buffer, followed by boiling for 10 minutes. Cysteine-oxidized actin was detected by western blotting using mouse anti-β-actin antibody (0.1 µg/ml) and sheep anti-mouse secondary antibody (0.2 µg/ml). Quantification was performed by densitometry using ImageJ software.

Proximity ligation assay

For assessing protein-protein interaction, we performed a proximity ligation assay with the Duolink kit (DUO92005, DUO92001, DUO92014, DUO92013, Millipore Sigma, USA or Axxora, Farmingdale, NY). Briefly, HASMs grown on coverslips were fixed with 4% PFA for 10 minutes, permeabilized with 0.1% Triton X-100 buffer, blocked with 3% BSA, and then incubated with primary antibodies (rabbit anti-vinculin (5 µg/ml), mouse anti-β-actin (2 µg/ml) or mouse myc-tag antibody (0.2 µg/ml)) overnight at 4°C. After incubation with PLA PLUS and/or MINUS probes conjugated with oligonucleotide for 2 hours, assays were performed according to the manufacturer's instructions. Images were acquired with a Zeiss LSM 510 META Laser Scanning Confocal Microscope System using Plan-Apo 40x oil and Plan-Apo 63x oil objective lens and ZEN acquisition software, followed by analysis using Image J software.

Measurement of focal adhesion assembly

Transfected HASMs were replated on coverslips and fixed as above after incubation for 1, 3, and 6 hours. Fixed and permealized cells were incubated with rabbit zyxin primary antibody (5 µg/ml), mouse paxillin primary antibody (4 µg/ml) overnight at 4°C and then incubated with secondary antibodies (goat anti-rabbit IgG labeled with Alexa Fluor 633 and goat anti-mouse IgG labeled with Alexa Fluor 488) diluted to 10 µg/ml. Images were obtained by Zeiss LSM 510 META Laser Scanning Confocal Microscope (Zeiss, Oberkochen, Germany). Zyxin/paxillin ratio was used for assessment of focal adhesion assembly during cell attachment.[12, 13]

Wound healing assay

Transfected HASMs were seeded on coverslips in 24-well plates (Corning, USA). After 3 days, when cells reached 90% confluence, a straight scratch wound was made utilizing a 200 µl pipette tip. Detached cells were washed away with PBS. Subsequently, migration was stimulated by treating cells with 10 ng/ml PDGF (Catalog No. 220-BB-050, R&D Systems, Minneapolis, MN). After 6 hours, cells were fixed and then stained using Phalloidin and DAPI as described above. Myc-tagged cells were stained with primary mouse anti-myc-tag antibody (0.2 µg/ml) at 4°C overnight and then incubated with goat anti-rabbit IgG labeled with Alexa Fluor 546 antibody diluted to 10 µg/ml. Images were obtained with a Zeiss LSM 510 META Laser Scanning Confocal Microscope System using 5x or Plan-Apo 40x oil objective lens and ZEN acquisition software, followed by analysis using Image J software.

Statistical analysis

Results are presented as mean ± SEM for the indicated number of cells or experiments. Statistical calculations were performed using GraphPad Prism version 6.0c. We tested for normality and equal variance with Shapiro-Wilk and Brown-Forsythe tests, respectively, before deciding to utilize ANOVA. In each figure, the statistical significance was assessed by one or two-way ANOVA for repeated experiments followed by Dunnett's multiple comparison tests. A p value of <0.05 was considered to be statistically significant. The n value is specified in the legends.

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