MATERIALS AND METHODS

Reagents and Antibodies: Recombinant human NEP (rNEP) was provided by Dr. Catherine Magill, AXYS Pharmaceuticals, Inc (San Francisco, CA). Lentivirus expressing full length human NEP was obtained from Dr. Louis Hersh (University of Kentucky). PDGF-BB and neuropeptides were obtained from Bachem (Torrance, CA). Other chemicals and all siRNA and shRNA used were from Sigma (St. Louis, MO) unless specified. Recombinant human NEP (rNEP) was provided by Dr. Catherine Magill, AXYS Pharmaceuticals, Inc (San Francisco, CA). Lentivirus expressing full length human was obtained from Dr. Louis Hersh (University of Kentucky). PDGF-BB and neuropeptides were obtained from Bachem (Torrance, CA). Other chemicals and all siRNA and shRNA used were from Sigma (St. Louis, MO) unless specified. Antibodies used were as follows: α-SMA, p190RhoGAP ^{Y1105}, RhoGDISer^{164} and total RhoGDI Abs antibody were from Sigma, mouse NEP Ab from R&D Systems; Phospho and total Abs for Cofilin, Mlc, p190RhoGAP, p-Tyr, p-Thr and p-ser from Cell Signal Technology (Danvers, MA).RhoA, myocardin, RhoGDI antibody were from Santa Cruz and Rac from Upstate Biologicals, Calponin, SM-22 and CRBP-1 were from Abcam. SM-myosin ab was a gift from Dr R.S Adelstein NIH MD[1] and NM-myosin antibody was from biolegend.

Isolation and characterization of PASMC from adult C57BL/6 mice: PASMCs were isolated from proximal medial tissue of the pulmonary artery from individual age matched 13-17wk old NEP+/+ and NEP-/- littermate mice as described [2]. SMCs were characterized by positive staining for SM-actin and SM-myosin and negative for Factor

VIII. Cells were quiesced in serum 0.2% for 24h prior to treatment with agonists; (Control=0% serum, Serum=0.5%, ET1=100nMoles/l, PDGF 10ng/ml).

Human Lung Tissue: COPD lung tissue with FEV1 <50 % and >80% were obtained from National Institutes of Health (NIH) Lung Tissue Research Consortium (LTRC; www.ltrcpublic.com. Studies on de-identified human tissue were approved by the Colorado Multiple Institution Review Board (COMIRB), exemption #16–0075 obtained for VJK.

Migration and proliferation: The effect of growth factors and neuropeptides on migration and DNA synthesis was evaluated as previously described[3]. Average from 3-6 different populations was used for statistical analysis.

Subcellular fractionation: Adherent PASMC lysates were treated with ice-cold hypotonic buffer containing 10 mM HEPES, pH 7.9, with 1.5 mM $MgCl_2$ and 10 mM KCl, 1 mM PMSF, and 10 mM aprotinin and leupeptin. Cells were incubated on ice for 30 min and homogenized.

Cytosol Fraction: The cytosol-containing supernatant was obtained by centrifugation at 20,000 rpm for 30 min.

Membrane Fraction: Pellets were gently washed twice by the same lysis buffer and resuspended in 100 ul of lysis buffer supplemented with 1% Triton X-100 and 0.1% SDS. Cell debris was separated by centrifugation (14,000 rpm at 4 °C for 20 min), and the supernatant was saved as particulate fraction.

Nuclear Fraction: Cells were incubated in the hypotonic buffer and pelleted at 2000 rpm for 10 min. The pellets were extracted with buffer containing Extraction Buffer: 20 mM HEPES, pH 7.9, with 1.5 mM MgCl2, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) Glycerol to obtain the nuclear protein. Cell fractions were then assayed for total protein. Equal amounts were analyzed by Western blotting. Typically, 10 μg of protein was analyzed representing 1–2% of the cytoplasmic fraction and 10–20% of the membrane and nuclear fraction.

Rac and Rho GTPase assay: Rac1 and Rho GTP bound forms were affinity purified using kits from Cytoskeleton Inc as per manufacturer's protocol. Briefly cells were stimulated with agonists and lysed. 200 μg of protein was incubated with PAK PBD agarose beads to selectively isolate Rac, and Rhotekin-agarose beads for Rho. The precipitated GTP-Rac and GTP-Rho is detected by Western blot analysis using an anti-Rac1/Rho specific monoclonal antibody.

Western Blotting: Lung lysates were prepared buffer (20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40; 1 mM dithiothreitol, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). Lysates were cleared by centrifugation for 10 min at 10,000 rpm. The protein concentration was determined using the BCA protein assay, and separated on SDS-PAGE and transferred onto nitrocellulose membranes (GE health care). Membranes were blocked in PBS containing 0.1% Tween 20 and 1% BSA for 1h at RT. Membranes

were incubated with primary antibodies overnight at 4 °C, and with secondary antibodies for 1 h at room temperature. All antibodies were diluted in PBS containing 0.1% Tween 20 and 1% BSA, and ECL (Perkin Elmer Inc) was used for immunodetection.

[3]. GAPDH was used as a loading control. A Bio-Rad gel scanner and densitometer (Gel DocXR with Quantity 1 program) were used to assess the intensity of the bands obtained by Western blots.

Phalloidin and DNase I staining: Cells were fixed with 3.7% (final concentration) fresh paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS for 5 min and incubated with FITC-labeled phalloidin (1 μg/ml) to label F-actin and Texas red-labeled DNase I (10 μ/ml) to label G-actin in 1% BSA respectively. The wells were washed thrice with PBS, and mounted with Vectashield H-1000 to prevent rapid photo bleaching.

Differential ultracentrifugation of homogenates for ratios of filamentous- and globular-actin. Cells were homogenized 200 µl of lysis buffer (37°C); 50 mM PIPES (pH 6.9), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 5% (vol/vol) glycerol, 0.1% Nonidet P-40 (NP-40), 0.1% Triton X-100, 0.1% Tween 20, 0.1% 2-mercapto-ethanol, 0.001% antifoam C with 100 mM ATP and a cocktail of protease inhibitors. Cell homogenates were incubated at 37°C for 10 min and spun at 150,000 *g* for 1 h at 37°C to separate the G-actin (supernatant) and F-actin fractions (pellet). The pellets were resuspended with the same amount of volume of ice-cooled distilled water containing 10 µM cytochalasin D. The pellets were dissolved by trituration with a pipette and left on ice for 1 h, vortexing every 15 min for 1 min to dissociate F-actin. Resuspended solutions were centrifuged at

2,300 *g* for 5 min at 4°C. The second supernatants (F-actin) were collected, and all samples were diluted with appropriate loading buffer and boiled for 5 min. The samples were stored at −80°C until further separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Lentivirus infection: PASMCs were transfected with lentivirus expressing human full length NEP at an MOI of 10 as described previously[2]. ShRNA infections are performed at MOI of 10 and cells were selected with 10μg/ml of puromycin. ShRNA for Rac and RhoA, were obtained from Sigma. Cells were used 48h after transfection for migration and proliferation assays.

SiRNA transfection: We tested three different siRNA (Novus, Santa Cruz and Sigma) for knockdown of NEP. NEP siRNA from Sigma decreased protein expression by > 90% at 48h. PASMC were transfected with mouse specific siRNA for NEP or universal siRNA from Sigma Aldrich, using Dharmafect Reagent® from Dharmacon (Denver, CO) as per manufacturer recommendations. SiRNA for NEP was obtained from Sigma. The final concentration of siRNA was 10nM. Universal siRNA was used as a negative control. Cells were used 48h after transfection for migration and proliferation assays. Protein lysates were analyzed by Western blotting to determine efficiency of knockdown.

Statistical analysis: Data were analyzed using GraphPad Prism 4.02 for Windows (GraphPad Software for Science Inc., San Diego, CA). Results are presented as mean

± SEM. The significance of differences between two measurements was determined by unpaired, two-tailed *t*-tests; one-way analysis of variance was used for multiple comparisons followed by Fishers least significance analyses. $P<0.05$ was considered statistically significant. In all experiments with PASMCs 'n' represents the number of cell populations each isolated from different matched NEP+/+ and NEP-/mice or the number of matched pairs of wt and NEP-/- mice.

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