Online supplement

Materials and Methods

Reagents

Recombinant human PDGF-BB was purchased from BioVision (Mountain View, CA). Cell lysis buffer, rabbit monoclonal anti-PDGFβ-R, anti-p44/42 MAPK (ERK1/2), anti-phospho-44/42 MAPK (Thr202/Tyr204), anti-SAPK/JNK MAPK (JNK1/2), anti-phospho-SAPK/JNK MAPK (Thr183/Tyr185), anti-p38 MAPK, anti-phospho-p38 MAPK, anti-c-jun, anti-phospho-c-jun, anti-mouse IgG alkaline phosphatase-conjugated antibodies were from Cell Signaling Technology (Danves, MA).

Rabbit polyclonal anti-phospho-PDGFβ-R (Tyr579) antibody was purchased from Abcam (Cambridge, MA). Anti-β-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO). Alkaline phosphatase-conjugated goat anti-rabbit secondary antibody was from Upstate Biotechnology (Lake Placid, NY). siRNAs against bovine JNK1 (5'-GGAGCUAGAUCAUGAAAGAUU-3') and JNK2 (5'-GGAAAGAGCUAAUUUACAAUU-3'), on-target plus control siRNA (5'-UGGUUUACAUGUCGACUAA-3'), transfection indicator siGLO Red were purchased from Thermo Scientific (Rockford, IL). All the other reagents not described here were from standard suppliers and of the highest grade available.

Isolation and growth of neonatal bovine main pulmonary artery adventitial fibroblasts:

Pulmonary artery adventitia was harvested from 15-day old normoxic and hypoxic

neonatal calves. Normoxic calves were born and remained at Fort Collins, CO (1,524 m altitude, barometric pressure (P_B) = 650 Torr). Hypoxic calves were born at Fort Collins altitude, but 1 day after their birth, they were placed into a chamber at simulated altitude (4,570 m, P_B = 445 Torr), where they remained for 2 weeks (Stenmark KR, Fasules J, Voelkel NF, Henson J, Tucker A, Wilson H, and Reeves JT. Severe pulmonary hypertension and arterial adventitial changes in newborn calves at 4300 m. *J Appl Physiol* 1987;62:821-830). At postmortem examination, adventitial tissue from main pulmonary artery was isolated, carefully dissected free of blood vessels and fat under a dissecting microscope, and cut into small pieces. Fibroblasts were then isolated according to our previously described method (Das M, Dempsey EC, Reeves JT, and Stenmark KR. Selective expansion of fibroblast subpopulations from pulmonary artery adventitia in response to hypoxia. Am J Physiol Lung Cell Mol Physiol 2002;282:L976-L986). Cells were maintained in culture in MEM supplemented with L-glutamine, penicillin/streptomycin, non-essential amino acids and 10% FBS. Fibroblasts were passaged by trypsinization, and passages between 3 and 7 were used for experiments.

Immunohistochemistry:

Formalin-fixed, paraffin-embedded tissue sections (5 μm) were baked overnight at 37ºC, deparaffinized, and rehydrated. For antigen retrieval, tissues were rinsed in water and boiled in 0.01 M sodium citrate (pH 6.0) for 10 min. The sodium citrate bath was returned to room temperature (RT) before the sections were rinsed in water. Endogenous peroxidase activity was quenched by treating sections with 0.3% H_2O_2 for 10 min. Sections were then washed in water and blocked for 1 hr at RT with 1% horse serum.

Tissue sections were incubated with primary antibodies (1:100) overnight at 4ºC, washed with PBS, and incubated with biotinylated secondary antibody (Vector Laboratories, CA) for 1 hr at RT. Bound antibodies were detected by treating sections with streptavidin-horseradish peroxidase complex (Vector Laboratories, CA) for 30 min. Peroxidase activity was visualized with 3, 3' diaminobenzidine, and sections were counterstained with hematoxylin. Primary antibody was omitted from staining reactions of lung sections to serve as a negative control. Sections were mounted with Permount mounting media (Sigma-Aldrich) and images were acquired with Leica DMI 3000B (Leica, Bannockburn, IL) microscope at 200X magnification using SPOT CCD camera (SPOT Diagnostics, Sterling Heights, MI). To compare the intensity of each stain, images were obtained under the same optic conditions in each experiment.

Western blotting:

Cells were harvested on ice with cell lysis buffer supplemented with phosphatase inhibitor (Thermo Scientific, IL) and protease inhibitor (Sigma, St. Lois, MO). Lysates were centrifuged at 10,000 X g at 4ºC for 10 min to remove cell debris. The protein concentration of the resulting supernatant was determined using Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Proteins (5 μg for ERK1/2, 25 μg for JNK1/2 and p38 MAP kinase, 40 μg for PDGFβ-R) were electrophoresed on 4 – 12% gradient Bis-Tris gels in MOPS SDS running buffer (Invitrogen, CA) and transferred to PVDF membranes (GE Water & Process Technologies Trevose, PA). Pre-stained molecular weight marker proteins (Bio-Rad Laboratories) were used. PVDF membranes were blocked with 5% BSA in Tris-Buffered Saline containing 0.05% Tween-20 (TBST) for 1 hr at RT, washed

with TBST, and probed with primary antibody diluted in blocking buffer overnight at 4ºC. The membrane was subsequently washed, incubated with secondary antibody diluted in blocking buffer for 1 hr at RT. Antibody labeling was visualized using chemiluminescence Lumi-Phos WB reagent (Thermo Scientific). β-actin protein served as an internal loading control.

Proliferation assays:

Cells were plated in 96-well plates in the concentration of 4×10^3 cells/well in 10% FBS containing media. After 72 hrs of growth arrest in 0.1% FBS containing media, cells were stimulated with PDGF-BB (25 ng/ml). For experiments where ROS scavengers were used, cells were pre-treated with NAC (Sigma-Aldrich) or superoxide dismutase mimetic TEMPOL (Invitrogen, Carlsbad, CA) for 1 hr at the concentration of 5×10^{-6}mol/L. Cell numbers were determined using Cell Titer 96 Aqueous One solution Cell Proliferation Assay (Promega, Madison, WI) according to manufacture's protocol. For experiments with NAC pre-treatment, cell numbers were determined by counting the cells with hemocytometer. Chemical inhibitors of MEK1/2 (U0126) and JNK1/2 (SP600125) (Calbiochem, San Diego, CA) were used to determine the role of MAPKs in PDGF-BB induced cell proliferation. Cells were treated with either U0126 or SP600125 (10×10^{-6}mol/L) for 1 hr and then stimulated with PDGF-BB. Selectivity of MAPK inhibition was confirmed by immunoblotting the cell lysates using antibodies against phospho-ERK1/2 and phospho-c-jun.

Intracellular ROS measurement:

Intracellular hydrogen peroxide (H_2O_2) and superoxide (O_2^-) levels were evaluated using dichlorofluorescein diacetate (DCFDA) and dihydroethidine (DHE) (Invitrogen, Carlsbad, CA), respectively, at the concentration of 5×10^{-6}mol/L. Cells were incubated with either DCFDA or DHE for 30 min and then washed twice with PBS. Fluorescence was recorded using a SpectraMax Gemini XS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 498 nm and an emission wavelength of 522 nm for DCFDA, and 520/610 nm for DHE.

Fibroblast transfection:

Cells (4×10^3 cells/well) were plated in 96-well plates with media containing 10% FBS. After overnight incubation, culture media was replaced with 0.1% FBS containing media for 24 hrs. PA adventitial fibroblasts were then transfected using TransIT-TKO transfection reagent (Mirus, Madison, WI) and siRNA (25 nM) according to manufacturer's protocol. Cells were allowed to recover from transfection for 48 hrs and proliferation assay was performed as described above. To confirm specificity of siRNA, cell lysates were collected after 48 hrs of transfection and immunoblotted against JNK1/2 and ERK1/2.

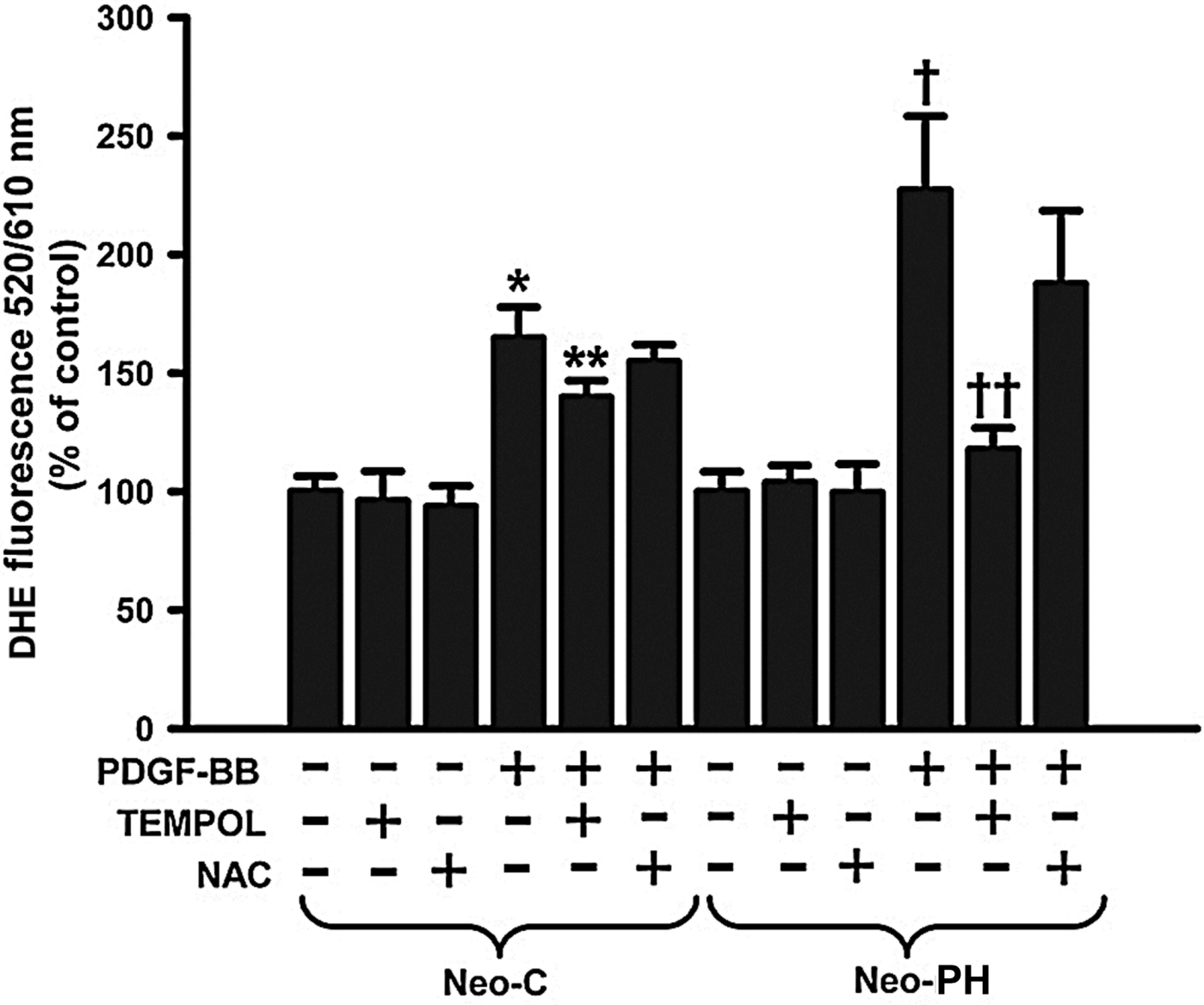
Online Figure Legends

Supplemental Figure 1. PDGF-BB induces an increase in intracellular superoxide (O_2^-) production in PA adventitial fibroblasts. Growth arrested Neo-C and Neo-HA cells were stimulated with PDGF-BB for 2 hrs and O_2^- levels were measured using DHE fluorescent dye. Intracellular O_2^- levels are represented as % of untreated cells. SOD mimetic, TEMPOL, or general antioxidant NAC (a precursor of glutathione) was used to reduce O_2^- levels. * $p < 0.001$ *vs* untreated Neo-C. ** $p < 0.01$ *vs* PDGF-BB treated Neo-C cells. † $p < 0.01$ *vs* untreated Neo-HA cells. †† $p < 0.01$ *vs* PDGF-BB treated Neo-HA cells.

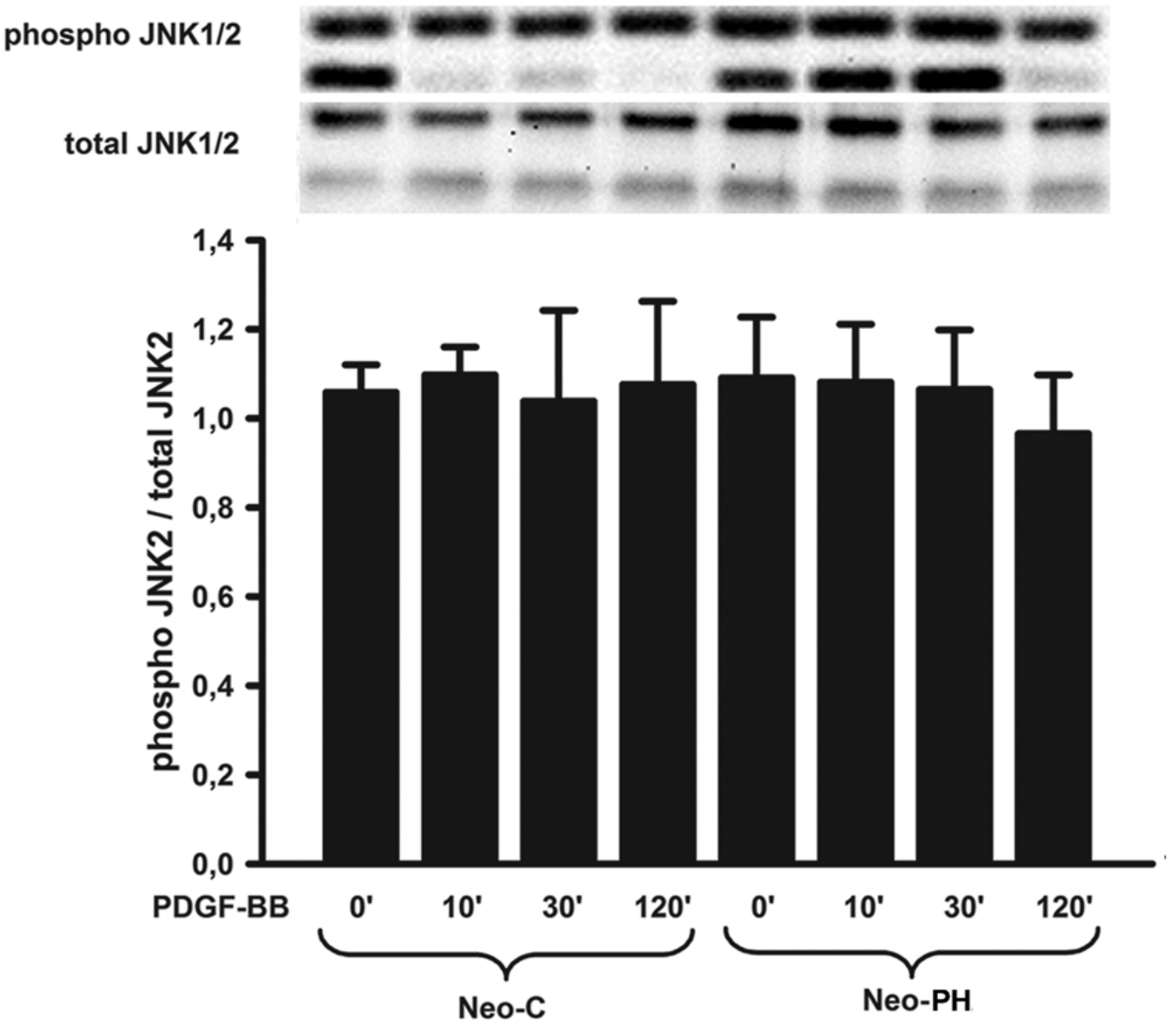
Supplemental Figure 2. JNK2 phosphorylation is unchanged in PDGF-BB-stimulated PA adventitial fibroblasts. Quiescent Neo-C and Neo-HA cells were stimulated with PDGF-BB for different lengths of time. Cell lysates were immunoblotted using anti-phosphoJNK1/2 and anti-totalJNK1/2 antibodies. PhosphoJNK2 (upper bands) were quantified using NIH Image J program and expressed as a ration of phosphoJNK2 to total JNK2.

Supplemental Figure 3. siRNA transfection efficiency with TransIT-TKO in PA adventitial fibroblasts. Neo-C and Neo-HA cells were transiently transfected with siGLO Red indicator using TransIT-TKO reagent according to manufacturer's protocol. Twenty four hrs after transfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X100 in PBS. Images of Neo-C and Neo-HA cells were acquired with Leica DMI 3000 B microscope at 200X magnification using SPOT CCD camera. PA adventitial fibroblast transfection was evaluated with siGLO Red (red).

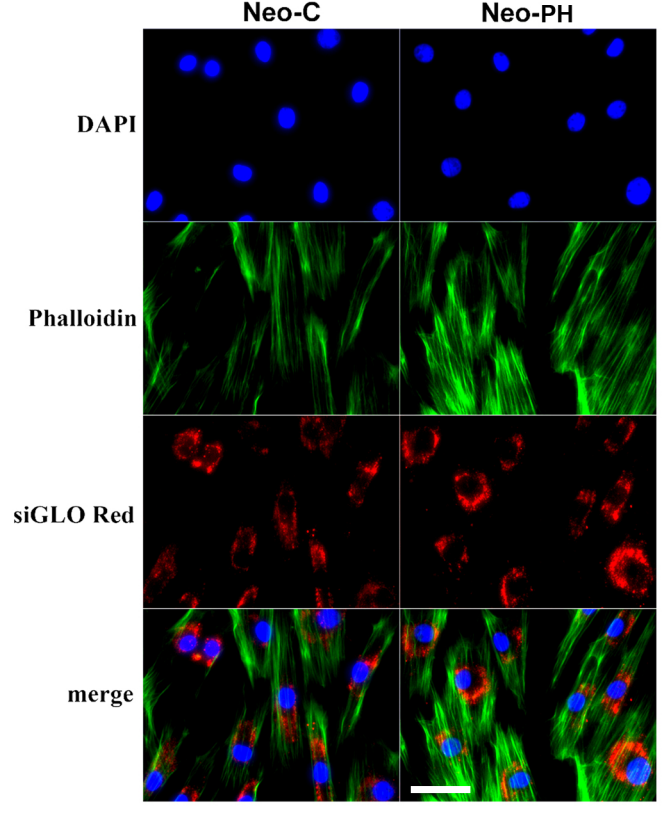
Alexa488-phalloidin conjugate was used to stain F-actin fibers for outline the cell contours (green). Hoechst dye was used to stain nuclei (blue). Scale bar equals to 75 microns.



Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3