SUPPLEMENTAL MATERIALS

METHODS

Amplex Red Activity Assay. Hydrogen peroxide levels were measured in cell lysates using the horseradish peroxidase-linked Amplex Red assay (Invitrogen) as described previously.[1]

Aldosterone and ET-1 levels. Cells were treated with ET-1 (1-100 nM), pioglitazone (50 μM/l), or angiotensin II (10 μM) for 24 h (all from Sigma-Aldrich). In selected experiments, cells were pre-treated for 6 h with BQ-788 (1.5 μM) (Sigma-Aldrich) to inhibit the ET_B receptor. Aldosterone levels were measured in the medium of cells grown in phenol-free EGM-2 medium supplemented with charcoal-stripped serum, by enzyme immunoassay according to the manufacturer's instructions (Cayman). Results were standardized to cell protein concentration. Levels of aldosterone and ET-1 from plasma and whole lung tissue were measured by enzyme immunoassay according to the manufacturer's instructions (Cayman).

$NO^{\bullet}$ metabolites. Nitrite (NO_2^-) and nitrate (NO_3^-) were measured in cell culture medium containing 2% fetal bovine serum and L-arginine (1 mmol/L) (Sigma-Aldrich) by 1(H)-naphthotriazole fluorescence (Cayman) as previously reported.[1] To measure NO_2^- levels in whole lung tissue, lung specimens were harvested from rats and snap frozen in liquid nitrogen. Samples were thawed, homogenized in PBS (pH 7.4), and centrifuged at 14,000 x *g* at 4ºC for 20 min. The supernatant was ultrafiltered using a 30 kDa molecular weight filter (Millpore), and the eluant was used to measure NO_2^- levels according to manufacturer's instructions (Cayman).

Immunoblotting. Proteins were size-fractionated electrophoretically using SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were incubated with anti-ET_A (Santa Cruz), anti-ET_B (Santa Cruz), anti-NOX4 (Santa Cruz), anti-$p22^{phox}$ (Santa Cruz), anti-eNOS (Cell Signaling), anti-PGC-1α (Santa Cruz), and anti-SF (Santa Cruz) antibodies overnight at 4°C and visualized using the ECL detection system (Amersham Biosciences). In experiments to assess ET_A expression, purified ET_A protein (Novus Biological) was loaded to serve as an internal control.

Co-immunoprecipitation of proteins. Cell monolayers were washed twice with ice-cold PBS and incubated on ice with RIPA buffer supplemented with various protease inhibitors (Millpore). Cells were scraped with a rubber policeman and samples were rotated at 4 °C for 15 min. Lysates were centrifuged at 14,000 x *g* at 4 °C for 15 min and the then pre-cleared with a 50% slurry of Protein G agarose beads (Santa Cruz Biotechnology) mixed with PBS. Following removal of the beads by centrifugation, cell lysates were incubated with an anti-ET_B or anti-PGC-1α antibody (Santa Cruz Biotechnology) overnight at 4 °C. The immunocomplex was captured by incubating lysates with 50% Protein G agarose bead slurry at 4 °C for 1 h. Beads were collected by pulse centrifugation, resuspended in non-reducing sample buffer, and then boiled for 10 min to dissociate the immunocomplex from the beads. Western analysis was performed with an anti-SF (Santa Cruz) or anti-sulfenic acid (R-SOH) antibody (Millpore) as described above.

Chromatin immunopercipitation assay. Chromatin immunoprecipitation assays were performed using the QuikChIP assay (Imgenex) according to the manufacturer's instructions. PCR amplification was performed on the proximal region of the *CYP11B2*

promoter region containing the gonadotrope-specific element.[2] The primers used were: forward 5′-GAGAAAGGAGAGGCCAGGTC-3′ and reverse 5′-CAGGAACCTGCTCTGGAAAC-3′. *CYP11B2* primers used for PCR were forward: 5′ GAGAAAGGAGAGGCCAGGTC-3′ and reverse: 5′-CAGGAACCTGCTCTGGAAAC-3′.

eNOS activity. eNOS activity was measured using the NOS activity kit (Cayman) according to the manufacturer's instructions with some modifications. Cells were washed with PBS containing 1 mM EDTA, transferred to a microcentrifuge tube, and centrifuged at 14,000 x *g* at 4 ºC for 2 min. The supernatant was decanted and homogenization buffer (250 mM Tris-HCl, pH 7.4, 6 μM BH_4, 2 μM flavin adenine dinucleotide, and 2 μM flavin adenine mononucleotide) was added to the cell pellets. The cells were lysed and exposed to [^{14}C] arginine (100 μCi/ml) for 2 min prior to incubation with ET-1 (10 nM) or PBS as vehicle control for 30 min at room 25 ºC. The samples were then centrifuged at 14,000 x *g* for 30 seconds and radioactivity of the eluant was quantified in a liquid scintillation counter (Beckman-Coulter).

3-Nitrotyrosine immunohistochemistry. Cells grown to confluence on glass chamber slides were fixed following treatments and anti-3-nitrotyrosine immunohistochemistry (Santa Cruz) was performed using the 3,3′-diaminobenzidine substrate method (Vector laboratories) as described previously.[1]

Site-directed mutagenesis and transfection. cDNAs encoding wild type (WT) eNOS and WT-ET_B from human were cloned into the mammalian expression vector pCMV6 (Origene). The C405A-ET_B mutant was purchased from Genewiz (South Plainfield, NJ). COS-7 cells, which do not express endogenous eNOS or ET_B, were plated in P100 tissue

culture dishes and transfected with 10 μg of WT-eNOS and WT-ET_B or C405A-ET_B DNA for 4.5 h with Lipofectamine 2000™ in OptiMEM medium (Invitrogen). After this time, the medium was replaced with Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% FBS, and experiments were performed after 24 h.

Lung tissue histology. Rat lung vessels were perfused with saline through the pulmonary artery and inflated with 10% phosphate-buffered formalin at a pressure of 20 cm H_2O through the trachea as described previously.[3] After fixation for 24 h at 4°C, the lung tissue was processed and embedded in paraffin using a Hypercenter XP System and Embedding Center (Shandon, Pittsburg, PA). The paraffin-embedded lung tissue was cut into 5-μm sections. Hematoxylin and eosin staining was performed according to methods published previously.[4] The 3,3′-diaminobenzidine substrate method was used for smooth muscle α-actin immunohistochemical staining. The number of muscularized arteries with a diameter of 20–50 μm located distal to terminal bronchioles were counted in 20 consecutive fields (100X) per section,[3] and the cross-sectional area was assessed using Image J software (NIH).

Sections were stained with a Gomori's Trichrome Staining Kit according to the manufacturer's instruction (Fischer Scientific). Image J software (NIH) was used to measure the per cent perivascular collagen deposition of muscularized arterioles with a diameter of 20–50 μm by subtracting the area of the lesser curvature from the greater curvature and dividing by the lesser curvature x 100. Collagen was also assessed in tissue sections using Picrosirius Red Stain Kit according to the manufacturer's instruction (Polysciences). Lung sections were visualized under polarized light using an Olympus BX51™ microscope and images were acquired by the Picture Taker™ software package.

Echocardiography. Transthoracic two-dimensional, M-mode, and Doppler imaging were performed in rats using a Vevo 2010 ultrasonographic system with a 15-MHz transducer. M-mode and Doppler tracings were acquired at a sweep speed of 200 mm/s following optimization of endocardial visualization and spectral display of Doppler profiles as described previously.[4] M-mode measurements of the right ventricular free-wall thickness were measured in the parasternal short-axis view just below the levels of the aortic valve, as described previously and in accordance to recommendations on M-mode measurement of the RV by the American Society of Echocardiography.[5] All studies were performed by a cardiologist and experienced sonographer who was blinded to the treatment group and was responsible for image analysis.

Right heart catheterization. An incision was made in the anterior triangle of the right neck, and a dissection was performed to expose the right internal jugular vein. A 0.04 x 0.023 in.-sized polyvinylchloridine catheter with a curved end was flushed with heparinized saline and connected to a Grass pressure transducer and Grass model 79 polygraph. A 4.0-proline suture was used to achieve hemostasis at the distal end of the jugular vein prior to insertion of the catheter. The tube was advanced and RV systolic pressure was recorded, which was assumed to be equal to PASP in the setting of a normal pulmonic valve.[4] All right heart catheterizations were performed within 10 min of echocardiography and both procedures were completed within 30 min.

Left heart catheterization and hemodynamics. Following completion of the right heart catheterization, a deep neck dissection was performed to identify the right carotid artery. Without disrupting the carotid sinus or vagus nerve, a cross-clamp was applied to the proximal and distal aspects of the carotid artery. A high fidelity Millar catheter (Millar

Instruments, Inc.) was inserted into the carotid artery, the distal clamp was released, and the catheter was advanced past the aortic arch to record central aortic blood pressure. The catheter was then advanced across the aortic valve and left ventricular end-diastolic pressure (LVEDP) were recorded. Cardiac index (CI) was derived from pressure-volume loop analysis as described previously.[6] The pulmonary vascular resistance index was calculated as [(mean pulmonary artery pressure-LVEDP)/CI)] and systemic vascular resistance index was calculated as [(mean arterial pressure-mean right atrial pressure)/CI].

Right ventricular weight. After sacrifice, the heart was dissected immediately. A 2-cm incision was made in the anterior aspect of the RV and LV and the residual intracavitary blood volume was exsanguinated prior to weighing the RV. Data are expressed as the ratio of RV weight (mg)/LV + septum weight (g).

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FIGURES

Supplemental Figure 1. PAH is associated with increased lung tissue CYP11B2 protein levels. Lung tissue was isolated from male Sprague-Dawley rats treated with vehicle control (V) or monocrotaline (MCT) (50 mg/kg) for 25 days to induce PAH and CYP11B2 (aldosterone synthase) expression was examined in homogenates by Western analysis (n=4). Arb. units, arbitrary units. Data are expressed as mean ± S.E.M. Representative blots are shown.

Supplemental Figure 2. Aldosterone does not affect systemic blood pressure or left ventricular hemodynamics in PAH. Male Sprague-Dawley rats were treated with vehicle control (V) or monocrotaline (MCT) (50 mg/kg) and randomized immediately to

V or spironolactone (25 mg/kg/d) in the drinking water. Following treatment for 25 days, the contribution of aldosterone to changes in **(a)** central aortic mean arterial pressure (MAP) and **(b)** left ventricular end-diastolic pressure (LVEDP) were assessed by cardiac catheterization (n=3-4 rats per condition). SP, spironolactone. Data are expressed as mean ± S.E.M.

Supplemental Figure 3. Spironolactone decreases right ventricular weight in PAH. Hearts from rats with and without monocrotaline (MCT)-induced PAH and treatment with vehicle control or spironolactone (25 mg/kg/d) for 25 days were dissected immediately after sacrifice. The weights of the right ventricle (RV) and left ventricle (LV), which included the interventricular septum, were recorded. *$p<0.01$ vs. vehicle control, n=4-5 rats condition. MCT, monocrotaline; SP, spironolactone. Data are presented as mean ± S.E.M.

Supplemental Figure 4. Aldosterone increases pulmonary vascular fibrillar collagen in PAH. Paraffin-embedded lung sections obtained from rats with and without monocrotaline (MCT)-induced PAH and treated with vehicle control or spironolactone (25 mg/kg/d) for 25 days were stained with picrosirius red and analyzed by polarized light microscopy. Levels of fibrillar collagen (red, yellow) were assessed in the wall of pulmonary arterioles measuring 20-50 μm and located distal to terminal bronchioles. (400x magnification). MCT, monocrotaline; SPIRO, spironolactone.

Supplemental Figure 5. Pulmonary vascular injury is evident at 14 days following administration of monocrotaline. Perivascular inflammatory cell infiltrate was assessed by hematoxylin and eosin staining of distal pulmonary arterioles harvested from Sprague-

Dawley rats 0, 7, and 14 days following injection of monocrotaline (MCT)(50 mg/kg) (n=3 rats per time point). Representative photomicrographs shown (400x magnification).

Supplemental Figure 6. ET-1 increases aldosterone synthase and aldosterone levels in HPAECs. (a) CYP11B2 (aldosterone synthase) protein expression was assessed by Western analysis in HPAECs exposed to vehicle control (V) or ET-1 (1, 10, 100 nM) for 24 h (n=3). **(b)** The effect of ET-1 on aldosterone (ALDO) levels in the cell culture medium was assessed by EIA (n=4). Data are expressed as mean ± S.E.M. A representative blot is shown.

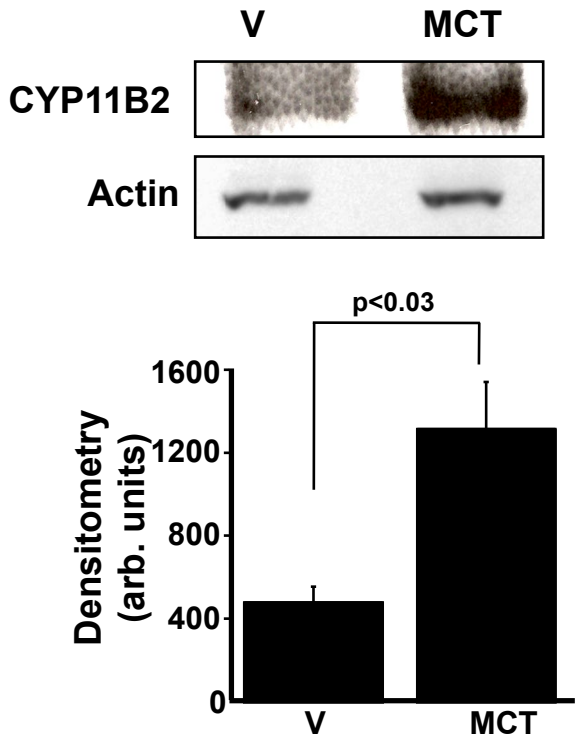
Supplemental Figure 7. ET-1 increases aldosterone in an ET_B-dependent manner. HPAECs were exposed to vehicle control (V) or ET-1 (10 nM) for 24 h in the presence or absence of the selective ET_B antagonist BQ-788 (1.5 μM), and aldosterone (ALDO) levels were measured in the culture medium (n=4). Data are presented as mean ± S.E.M.

Supplemental Figure 8. Aldosterone increases NOX4 expression to increase oxidant stress in HPAECs. (a) HPAECs were exposed to vehicle control (V) or aldosterone (ALDO) (10^{-7} mol/L) in the presence or absence of spironolactone (SP) (10 μM) for 24 h, and hydrogen peroxide (H_2O_2) levels were assessed by measuring Amplex Red fluorescence (n=3). To determine a potential source of H_2O_2 in ALDO (10^{-9}-10^{-7} mol/L)-treated cells, Western analysis was performed to assess protein expression levels of **(b)** NOX4 and **(c)** the NOX4 subunit $p22^{phox}$ (n=3). Data are presented as mean ± S.E.M. Representative blots are shown.

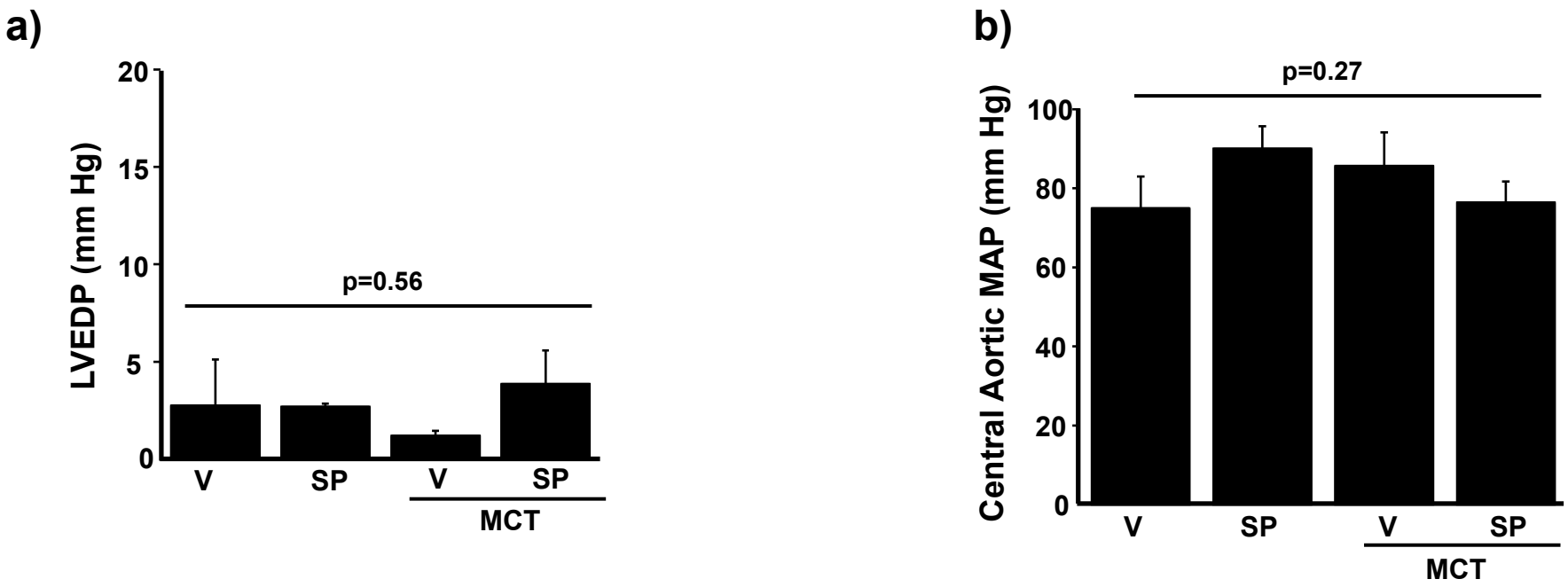
Supplemental Figure 9. Aldosterone does not influence ET_A or ET_B protein levels in HPAECs. HPAECs were exposed to vehicle control (V) or aldosterone (ALDO)(10^{-7}

mol/l) for 24 h and Western analysis (n=3) was performed to monitor for changes in protein expression levels of ET_A and ET_B. For ET_A analyses, purified recombinant ET_A receptor protein was used as a positive control. Representative blots are shown.

Supplemental Figure 10. Aldosterone increases peroxynitrite formation to decrease NO_2^-/NO_3^-. (a) HPAECs were treated with vehicle control (V) or aldosterone (ALDO) (10^{-7} mol/L) for 24 h, and stimulated with ET-1 (10 nM) for 10 min immediately prior to measuring NO_2^-/NO_3^-. The contribution of ALDO to changes in NO_2^-/NO_3^- was confirmed by co-incubation of ALDO-treated cells with spironolactone (SP)(10 μM)(n=3). **(b)** Peroxynitrite formation was assessed by 3-nitrotryosine immunohistochemistry (n=3). arb. units, arbitrary units. Data are expressed as mean ± S.E.M. Representative photomicrographs are shown.



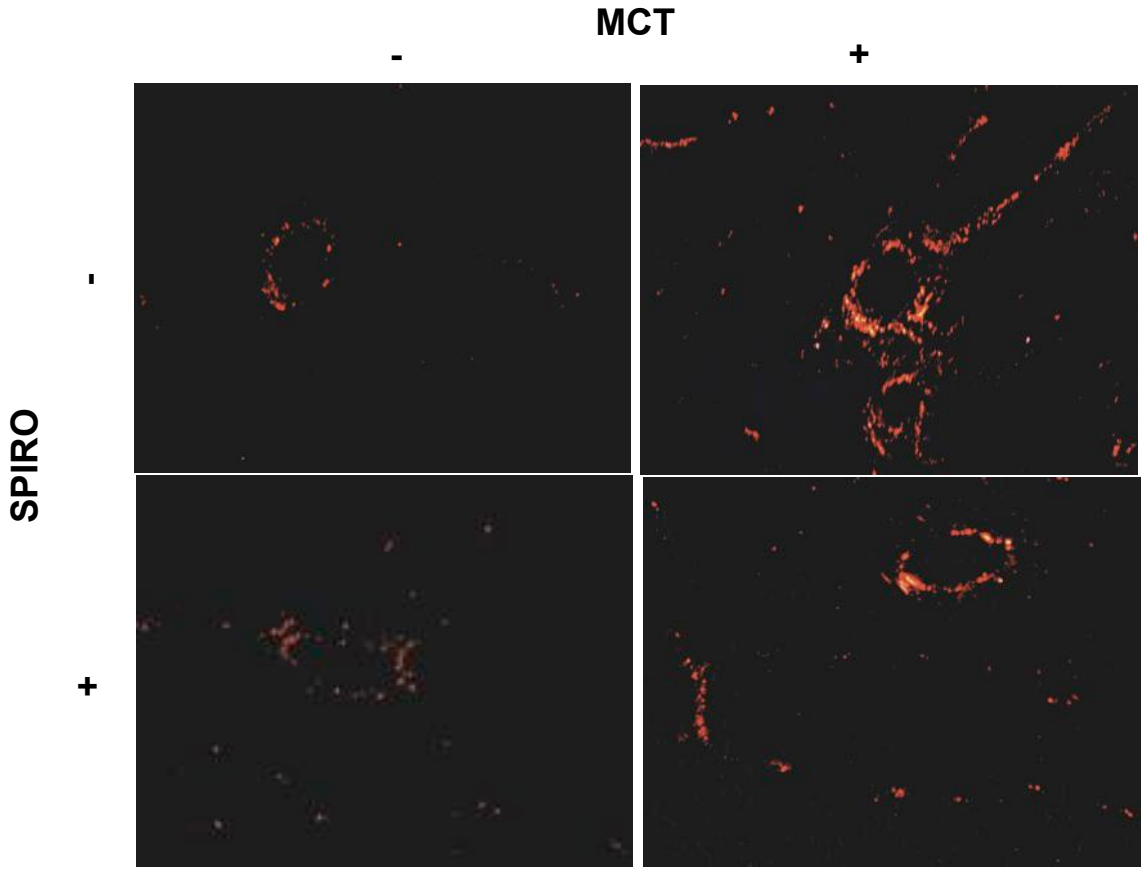
Supplemental Figure 1



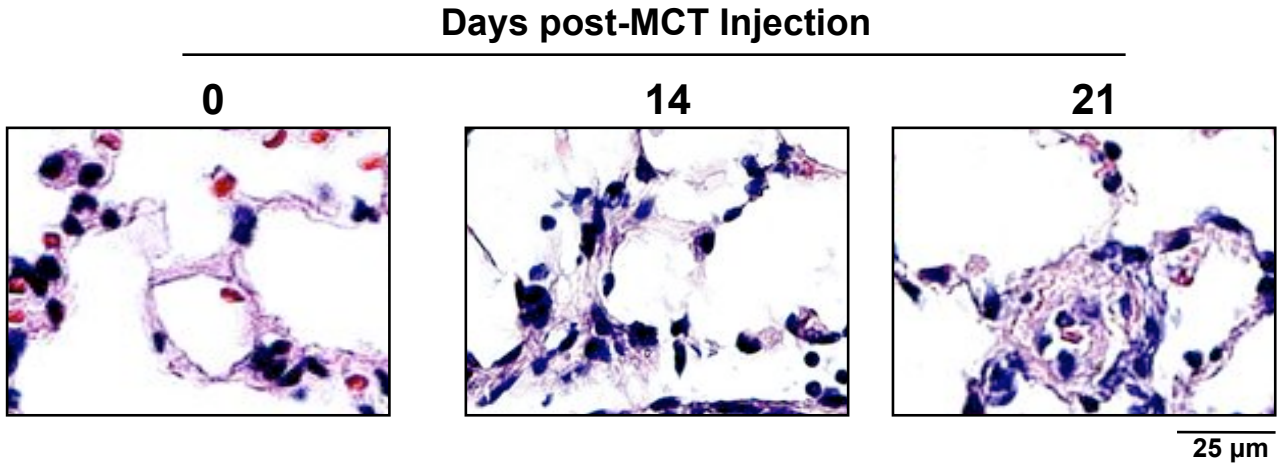
Supplemental Figure 2

Condition	RV/LV+Septum Weight
Vehicle	0.22 ± 0.05
SP	0.26 ± 0.02
MCT	0.43 ± 0.07*
MCT+SP	0.35 ± 0.04

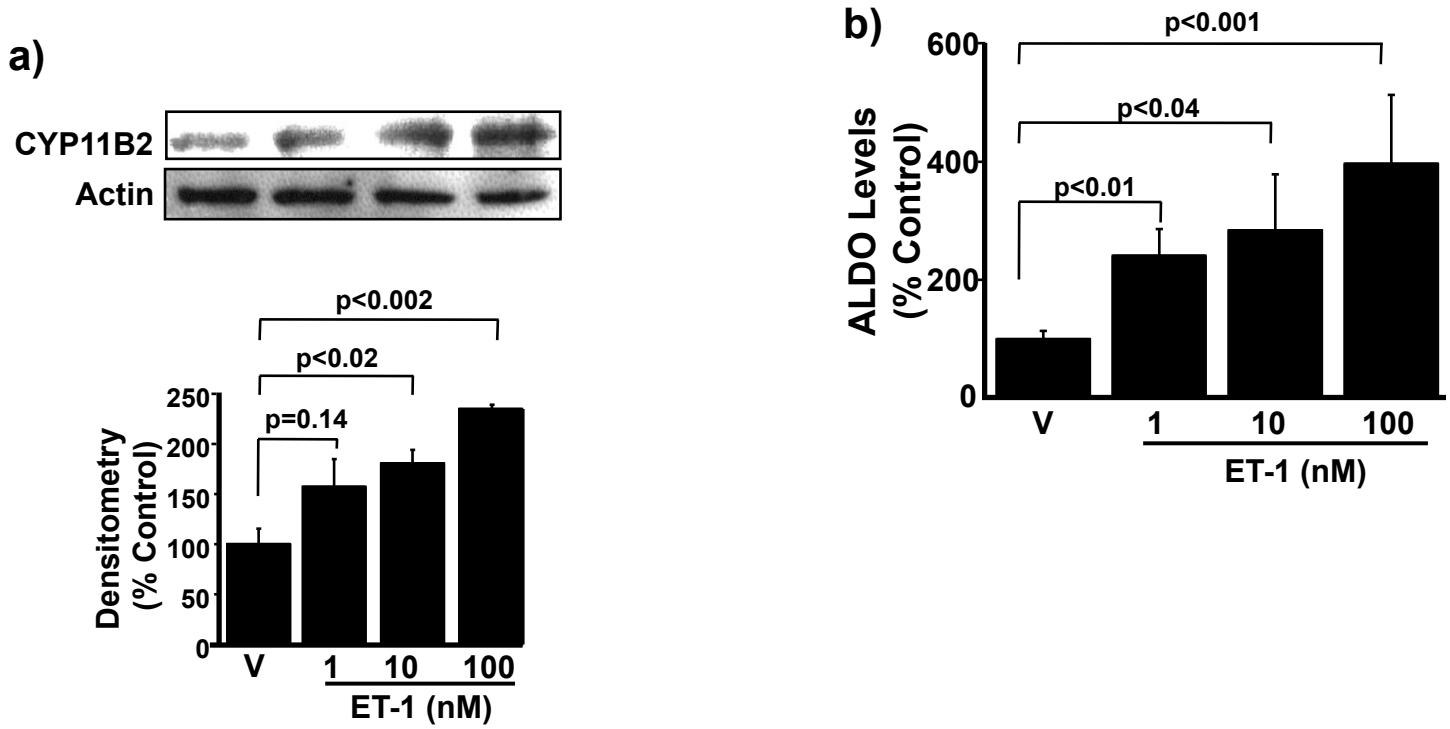
Supplemental Figure 3



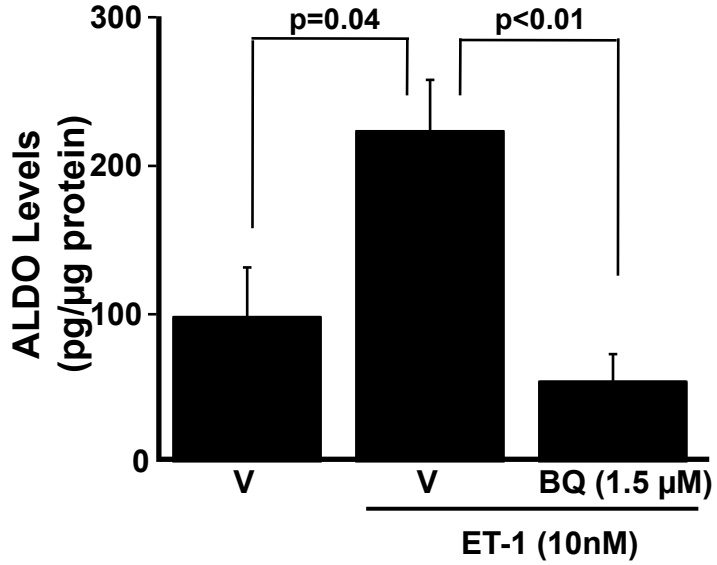
Supplemental Figure 4



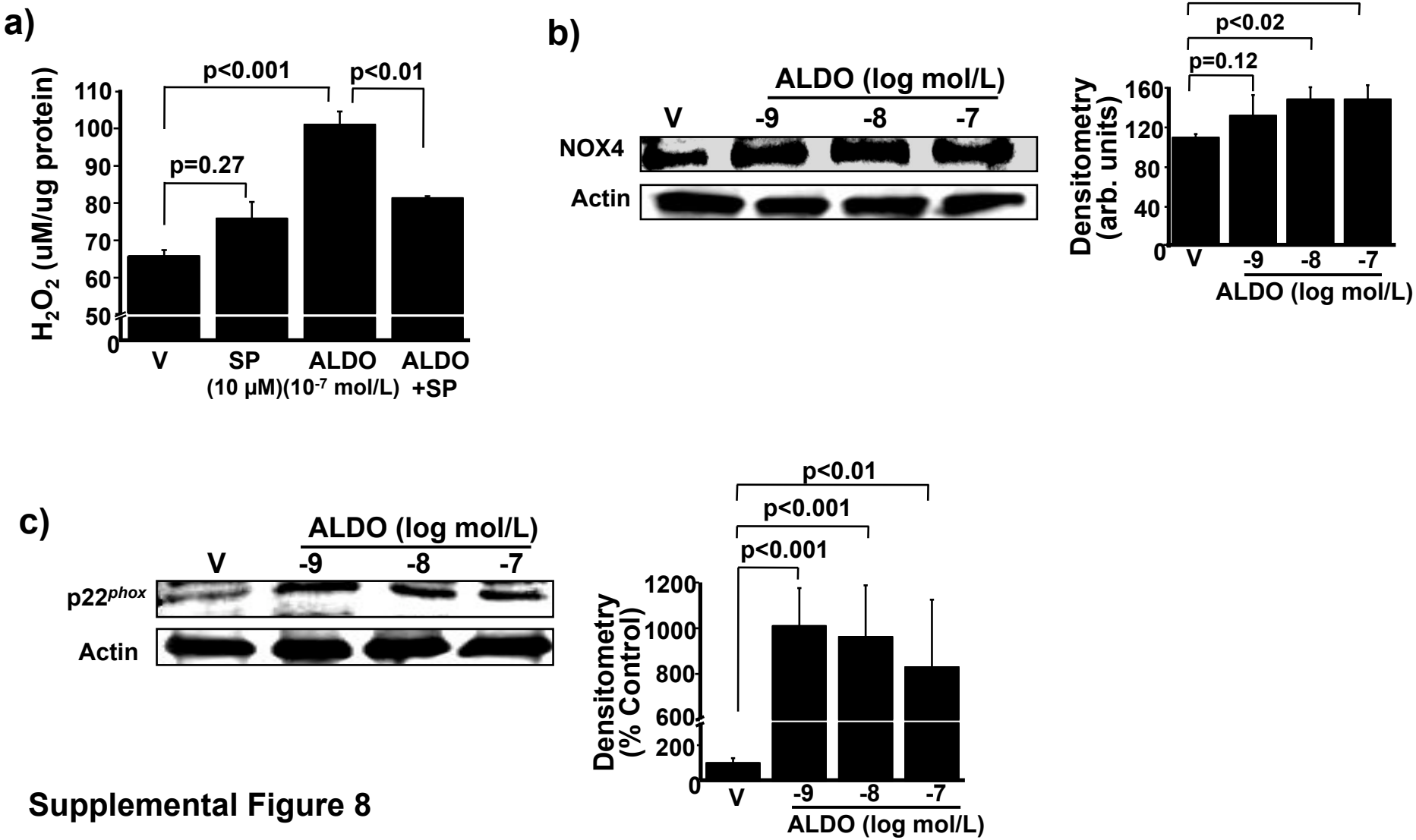
Supplemental Figure 5



Supplemental Figure 6

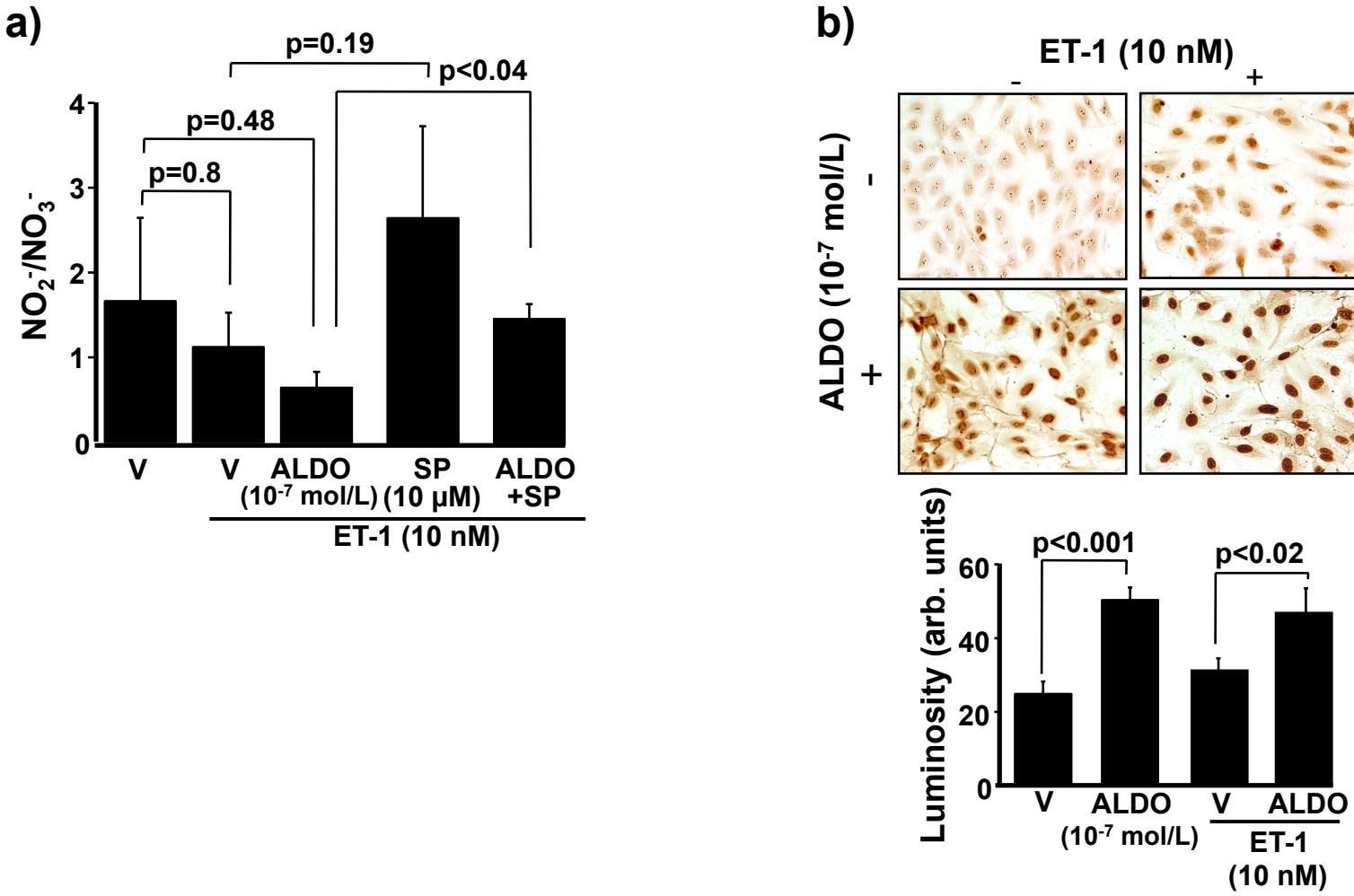


Supplemental Figure 7



Supplemental Figure 8

Supplemental Figure 9



Supplemental Figure 10