SUPPLEMENTAL MATERIAL

Supplemental Methods

Aldosterone and progesterone level measurements by enzyme immunoassay. Cells were treated with normoxia or hypoxia for 24 hr and progesterone or aldosterone levels were measured from the cell culture medium. Prior experiments by our laboratory demonstrated no significant difference between levels of aldosterone or progesterone in medium supplemented with regular FBS vs. serum stripped with charcoal. Nevertheless, hormone levels for HPAECs and HPASMCs were measured in culture medium supplemented with serum stripped with charcoal by enzyme immunoassay (EIA) according to the manufacturer’s instructions (Cayman). Levels of aldosterone from plasma and whole lung tissue were measured by enzyme immunoassay according to the manufacturer’s instructions (Cayman).

In select experiments, cells were treated with 20-α-hydroxycholesterol (20-α-OH-C) (5 µg/ml)(Sigma) at the time of exposure to normoxia/hypoxia in order to assess the effect of maximal StAR activity on hormone synthesis as reported previously.\(^1,2\) In other experiments, cells exposed to normoxia/hypoxia were co-incubated with the antagonist to AP-1 transcription factor activity SR-11302 (1 µM)\(^3\) to assess further the role of c-Fos/c-Jun-StAR interaction to aldosterone synthesis in HPAECs. Results were normalized to cell protein concentration.

Immunoblotting. Proteins were size-fractionated electrophoretically using SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were incubated with anti-c-Fos (Cell Signaling), anti-c-Jun (Cell Signaling), anti-StAR (Santa Cruz
Biotechnology), anti-collagen III (Novus Biological), anti-CTGF (Cell Signaling), anti-MMP-2 (Santa Cruz Biotechnology), anti-MMP-9 (Santa Cruz Biotechnology), or anti-HIF-1α (Novus Biologicals) antibodies overnight at 4°C and visualized using the ECL detection system (Amersham Biosciences).

*StAR siRNA transfection.* Cells were transfected with StAR siRNA (20 or 40 nM) or scrambled (negative) control siRNAs (Santa Cruz Biotechnology) using Lipofectamine™ 2000 (Invitrogen) for 5 h in OptiMEM I media, which also served as vehicle control. Cells were then placed in full growth media for 24 h before treatment with hypoxia for 24 h. The StAR siRNA sequences used for transfection were: sense 5´-CCAAUGUCAAGGAGAUCAAtt-3´ and antisense: 5´-UUGAUCUCCUUGACAUUGGtt3´.

**Quantitative real time PCR.** Total RNA was isolated from cells with the RNeasy Mini Kit (Qiagen) using DNase I and cDNA was synthesized with the Advantage RT for PCR kit (Clontech) using oligo-dT primers. RT-qPCR analysis was performed with an Applied Biosystems 7500 Fast Real Time PCR system with pre-designed TaqMan gene expression assays (Applied Biosystems). Relative *VEGFA* (probe Hs00900055_m1, Life Technologies) expression was calculated using the comparative cycle threshold method referenced to *POLR2A* (probe Hs00172187_m1, Life Technologies) mRNA.

Assessment of mitochondrial content and membrane potential (*ψ_m*). Mitochondrial content and membrane potential were assessed using Mito Tracker® Red FM and the Mitoprobe JC-1 assay kit (Invitrogen), respectively, according to the manufacturer’s
instructions. Cells were exposed to normoxia or hypoxia (2.0% FiO₂) in the presence or absence of spironolactone (10 µM) for 24 hour and then mitochondria were labeled with 25 nM Mito Tracker® Red FM for 45 min or 2 µM JC-1 for 30 min as reported previously.⁴ Cells were imaged using an inverted Nikon TE300 fluorescence microscope. A minimum of 20 fields were randomly selected and imaged in each condition. The cytosol area in cells was selected and the average fluorescence intensity was measured with the aid of ImageJ (NIH).⁴ The ratio of green to red fluorescence intensity was measured to assess for a change in mitochondrial membrane potential between normoxia and hypoxia.

Animal Histology. Rat lung vessels were perfused with saline through the pulmonary artery and the lungs were inflated with 10% phosphate-buffered formalin at a pressure of 20 cm H₂O through the trachea as described previously.⁵ After fixation for 24 h, the lung tissue was processed and paraffin-embedded using a Hypercenter XP System and Embedding Center (Shandon, Pittsburg, PA). The paraffin-embedded lung tissue was cut into 5-µm sections. The 3,3’-diaminobenzidine substrate was method used for anti-StAR, anti-CTGF, and anti-collagen III immunohistochemical staining. Lung sections were visualized under polarized light using an Olympus BX51™ microscope and images were acquired by the Picture Taker™ software package. Image J software (NIH) was used to measure the luminal area of arterioles by subtracting the area of the lesser curvature from the greater curvature and dividing by the lesser curvature x 100.
Animal hemodynamic and structural analyses. All cardiac catheterization and gross tissue experiments (described below) were performed according to methods published previously by our laboratory.5,6

Right heart catheterization. An incision was made in the lateral aspect of the anterior triangle of the right neck, and a deep 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.5,6 The catheter was connected to a Grass pressure transducer and Grass model 79 polygraph and leveled. A 4.0-proline suture was tied at the distal end of the jugular vein to maintain hemostasis, and the catheter was then advanced into the jugular vein to the RV. The RV systolic pressure was recorded, which was assumed to equal PASP in the setting of a normal pulmonic valve.5 All right heart catheterizations were completed within 60 min.

Left heart catheterization and hemodynamics. Following completion of the right heart catheterization, a medial deep neck dissection was performed to identify the right vagus nerve and right carotid artery.5 Without disrupting the key nerve structures, a cross-clamp was applied to the distal aspects of the carotid artery and the proximal aspect of the carotid artery was ligated using a prolene suture. 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.5 The catheter was then advanced across the aortic valve into the left ventricle and pressure-volume loops were recorded to derive cardiac index (CI) as described previously.5,7 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].

**Gross anatomic evaluation.** After exsanguination and animal sacrifice, each rat’s heart was immediately dissected. A 2.5-cm incision was made in the anterior aspect of the RV and LV, and the residual intracavitary blood volume was removed by gauze absorption or needle aspiration prior to weighing the RV and LV + septum. Data are expressed as the ratio of RV weight (mg)/LV + septum weight (g).

**Immunohistochemistry in vitro.** Cells grown to confluence on glass chamber slides were fixed following treatments and anti-StAR immunohistochemistry (Santa Cruz Biotechnology) was performed using the 3,3'-diaminobenzidine substrate method (Vector laboratories) as described previously.5,6

**Human Subjects.** The four control patients included 2 males and 2 females without pulmonary hypertension who ranged in age from 6 to 13 years (mean, 10.5 years). Each was an oncology patient who underwent staging wedge biopsy of a lung nodule that proved histologically to represent a benign lymph node; none had been previously treated with chemotherapy or radiation (Supplemental Table 2). The four patients with pulmonary hypertension ranged in age from 9 to 16 years (mean, 11.5 years). Their clinical conditions are summarized in Supplemental Table 3; lung tissue was resected diagnostically or therapeutically in the setting of clinically diagnosed pulmonary hypertension. All lung biopsy tissue was fixed in formalin, embedded in paraffin, and sectioned at 5 µm. Histologic examination confirmed severe pulmonary arterial
hypertensive remodeling in all pulmonary hypertension patients (with plexiform change
seen in 3 of the 4 patients) and normal pulmonary vasculature in the control patients.

Supplemental References


SUPPLEMENTAL FIGURES

Supplemental Figure 1. Hypoxia increases aldosterone levels selectively in pulmonary artery endothelial cells. (A) Human pulmonary artery endothelial cells (HPAECs) were exposed to normoxia (21% FiO\textsubscript{2}) or hypoxia (2.0% FiO\textsubscript{2}) for 0, 6, or 24 hr and aldosterone (ALDO) levels were measured in the culture medium by enzyme immunoassay (n=3). (B) In contrast to our observations in HPAECs, no significant effect of hypoxia on ALDO levels was observed in normal human lung fibroblasts (NHLFs)(n=3), (C) human pulmonary artery smooth muscle cells (HPASMCs)(n=3), or (D) human coronary artery smooth muscle cells (HCSMCs)(n=3). ND, not detectable. Data are presented as mean ± S.E.

Supplemental Figure 2. The effect of hypoxia on HIF-1\alpha and VEGF-A in various cells involved in pulmonary vascular fibrosis. (A) Human pulmonary artery endothelial cells (HPAECs)(n=3) and normal human lung fibroblasts (NHLFs)(n=3) were exposed to normoxia or hypoxia (0.2% FiO\textsubscript{2}) for 24 h and expression levels of HIF-1\alpha were assessed by immunoblot. (B) The effect of normoxia or hypoxia (0.2% FiO\textsubscript{2}) for 24 h on VEGF-A mRNA levels was performed by quantitative real-time PCR in HPAECs (n=4), NHLFs (n=7), and human pulmonary artery smooth muscle cells (HPASMCs)(n=4). For PCR results, data are expressed as fold-change over normoxia. *p<0.05 vs. normoxia. HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; a.u., arbitrary units. Representative immunoblots shown. Data are presented as mean ± S.E.
Supplemental Figure 3. The hypoxic dose-response effect on c-Fos expression and aldosterone levels in pulmonary artery endothelial cells. (A) Human pulmonary artery endothelial cells were exposed to normoxia (21% FiO₂) or varying degrees of hypoxia (5.0%, 2.0%, 0.2% FiO₂) and c-Fos expression levels (n=3) were assessed by immunoblot, and (B) aldosterone (ALDO) levels were measured in the culture medium by enzyme immunoassay (n=5). a.u., arbitrary units. Data are presented as mean ± S.E. Representative blots are shown.

Supplemental Figure 4. Hypoxia does not influence c-Fos or c-Jun levels in human pulmonary artery smooth muscle cells (HPASMCs) or coronary artery smooth muscle cells (HCSMCs). (A) Cultured HPASMCs and (B) HCSMCs were exposed to hypoxia (2.0% FiO₂) for 15, 30, or 60 min and protein levels of c-Fos (n=3) and c-Jun (n=3) were assessed by immunoblot. NS, not statistically significant. a.u., arbitrary units. Data are presented as mean ± S.E. Representative blots are shown.

Supplemental Figure 5. The effect of hypoxia and hypoxia-induced aldosterone on mitochondrial content in pulmonary artery endothelial cells. Human pulmonary artery endothelial cells (n=3) were treated with normoxia (21% FiO₂) or hypoxia (2.0% FiO₂) in the presence or absence of the mineralocorticoid receptor antagonist spironolactone (SP)(10 µM) for 24 hr, and cells were incubated with red fluorescence dye Mito Tracker® to assess changes in mitochondrial content. a.u., arbitrary units. Data are presented as mean ± S.E. Representative photomicrographs (at 400x) are shown.
Supplemental Figure 6. The effect of hypoxia on mitochondrial membrane potential in pulmonary artery endothelial cells. Human pulmonary artery endothelial cells (n=3) were treated with normoxia or hypoxia (2.0% FiO2) for 24 hr, and changes in mitochondrial membrane potential was assessed by measuring differences in the accumulation of red to green fluorescence of JC-1. a.u., arbitrary units. Data are presented as mean ± S.E. Representative photomicrographs (at 400x) are shown. ND, not detectable.

Supplemental Figure 7. Aldosterone increases levels of proteins associated with hypoxia-induced pulmonary vascular remodeling and fibrosis. Human pulmonary artery endothelial cells were treated with vehicle control (V) or aldosterone (ALDO) (10^{-9}-10^{-7} mol/L) for 24 hr and expression levels of collagen III (n=3) and connective tissue growth factor (CTGF) (n=3), which are associated with hypoxia-mediated vascular fibrosis, and matrix-metalloproteinase (MMP)-2 (n=4) and MMP-9 (n=3), which are associated with hypoxia-mediated vascular remodeling, were assessed by Western immunoblot. a.u., arbitrary units. Data are presented as mean ± S.E. Representative blots are shown.

Supplemental Figure 8. Remodeled pulmonary arterioles express increased StAR levels in a murine model of hypoxia-induced pulmonary arterial hypertension (PAH). Eight-week-old male mice were exposed to normoxia (21% FiO2) or normobaric hypoxia (10% FiO2) for 7 or 28 days. The Vector Red substrate method used to perform anti-StAR immunohistochemical staining on paraffin-embedded distal pulmonary blood
vessels. Compared to normoxia, StAR expression levels were increased in mice exposed to hypoxia for 7 d or 28 days. n=3 mice/condition. a.u., arbitrary units. Data are presented as mean ± S.E. Representative photomicrographs (at 400x) are shown.

**Supplemental Figure 9. Pulmonary hypertension is evident 14 days following initiation of the SU-5416/hypoxia experimental model of PAH.** Male Sprague-Dawley rats were treated with vehicle control or administered Sugen-5416 (20 mg/kg) and treated with chronic hypoxia for 14 days (SU-5416/hypoxia). (A) Right heart catheterization demonstrated that compared to control, pulmonary hypertension was present in a (B) SU-5416/hypoxia-treated rat at day 14. n=1/condition. PASP, pulmonary artery systolic pressure. Representative hemodynamic tracings are shown.

**Supplemental Figure 10. Plasma and lung homogenate levels of aldosterone are increased in an experimental model of severe PAH involving hypoxia.** Male Sprague-Dawley rats were treated with vehicle control or administered Sugen-5416 (20 mg/kg) and treated with chronic hypoxia for 3 weeks followed by exposure to normoxia for 16-17 d (SU-5416/hypoxia-normoxia). (A) Plasma and (B) lung homogenate aldosterone (ALDO) levels were measured by enzyme immunoassay. n=6-7 rats/condition. Horizontal line represents median for each condition.
## Supplemental Tables

<table>
<thead>
<tr>
<th>PAH Protocol</th>
<th>Species</th>
<th>Duration</th>
<th>Design</th>
<th>Treatment</th>
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</table>
| SU-5416/Hypoxia    | Male SD rat   | 21 d     | SU-5416 (20 mg/kg) administration followed by chronic hypoxia until study completion | Drug: Eplerenone (EPL)  
Dose: 0.6mg/1g chow  
Protocol: Prevention  
EPL first dose at time of study initiation |
| SU-5416/Hypoxia-Norm | Male SD rat   | 37-38 d  | SU-5416 (20 mg/kg) administration followed by treatment with chronic hypoxia for 21 d, then exposure to normoxia for 16-17 d until study completion | Drug: Spironolactone (SP)  
Dose: 25/mg/kg/d  
Protocol: Reversal; SP first dose at study day 14 |
| Chronic Hypoxia    | C57 mouse     | 7 or 28 d | Exposure to chronic hypoxia (10% FiO₂) for study duration              | No treatment                                                              |

Table 1. Experimental models of pulmonary arterial hypertension (PAH). SD, Male Sprague Dawley; Norm, normoxia.
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<th>Sex</th>
<th>Diagnosis</th>
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<th>Echocardiography Data</th>
<th>Hemodynamics</th>
<th>Pathology</th>
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**Supplemental Table 2. Clinical characteristics of control patients.**

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<td>repair of complete AV canal/LVOT</td>
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<td>Clinical Pathology</td>
<td>Bilateral lung transplant for PAH</td>
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**Supplemental Table 3. Clinical and histopathological characteristics of patients with pulmonary arterial hypertension (PAH).** M, male; F, female; RVSP, right ventricular systolic pressure; RA, right atrium; RV, right ventricle; LV, left ventricle; CI, cardiac index; mPAP, mean pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; W.U., Wood units; RVH, right ventricular hypertrophy; AV, atrial-ventricular; LVOT, left ventricular outflow tract; SBp, systolic blood pressure; iNO, inhaled nitric oxide vasodilator trial.