Upregulation of Steroidogenic Acute Regulatory Protein by Hypoxia Stimulates Aldosterone Synthesis in Pulmonary Artery Endothelial Cells to Promote Pulmonary Vascular Fibrosis

Running title: Maron et al.; Hypoxia increases pulmonary vascular aldosterone levels

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Abstract

**Background**—The molecular mechanism(s) regulating hypoxia-induced vascular fibrosis are unresolved. Hyperaldosteronism correlates positively with vascular remodeling in pulmonary arterial hypertension (PAH), suggesting that aldosterone may contribute to the pulmonary vasculopathy of hypoxia. The hypoxia-sensitive transcription factors c-Fos/c-Jun regulate steroidogenic acute regulatory protein (StAR), which facilitates the rate-limiting step of aldosterone steroidogenesis. We hypothesized that c-Fos/c-Jun upregulation by hypoxia activates StAR-dependent aldosterone synthesis in human pulmonary artery endothelial cells (HPAECs) to promote vascular fibrosis in PAH.

**Methods and Results**—Patients with PAH, rats with Sugen/hypoxia-PAH, and mice exposed to chronic hypoxia expressed increased StAR in remodeled pulmonary arterioles, providing a basis for investigating hypoxia-StAR signaling in HPAECs. Hypoxia (2.0% FiO2) increased aldosterone levels selectively in HPAECs, which was confirmed by liquid chromatography-mass spectrometry. Increased aldosterone by hypoxia resulted from enhanced c-Fos/c-Jun binding to the proximal activator protein (AP-1) site of the StAR promoter in HPAECs, which increased StAR expression and activity. In HPAECs transfected with StAR-siRNA or treated with the AP-1 inhibitor, SR-11302, hypoxia failed to increase aldosterone, confirming that aldosterone biosynthesis required StAR activation by c-Fos/c-Jun. The functional consequences of aldosterone were confirmed by pharmacological inhibition of the mineralocorticoid receptor with spironolactone or eplerenone, which attenuated hypoxia-induced upregulation of the fibrogenic protein connective tissue growth factor and collagen III in vitro, and decreased pulmonary vascular fibrosis to improve pulmonary hypertension in vivo.

**Conclusions**—Our findings identify autonomous aldosterone synthesis in HPAECs due to hypoxia-mediated upregulation of StAR as a novel molecular mechanism that promotes pulmonary vascular remodeling and fibrosis.

**Key words:** hypoxia, aldosterone, pulmonary hypertension, fibrosis
Introduction

The molecular mechanism(s) that regulate pulmonary vascular collagen deposition and fibrosis are incompletely characterized. In systemic blood vessels, matrix metalloproteinase (MMP)-2, MMP-9, and the fibrogenic protein connective tissue growth factor (CTGF) promote the maladaptive remodeling response to vascular injury that includes fibrosis. Hypoxia is a key risk factor for pulmonary vascular diseases characterized by fibrosis, and is observed concomitantly with elevated plasma levels of the pro-fibrotic hormone aldosterone in experimental animal models of hypoxic pulmonary vascular dysfunction as well as pulmonary arterial hypertension (PAH). Aldosterone, in turn, is a bona fide activator of CTGF in vascular cells in vitro, and hyperaldosteronism correlates with adverse pulmonary vascular remodeling in PAH in vivo. Despite these collective observations, the contribution of aldosterone to hypoxia-induced pulmonary vascular fibrosis is not known.

Local aldosterone synthesis in human pulmonary artery endothelial cells (HPAECs) has been reported, although the relevance of aldosterone synthesized in cardiovascular tissue is controversial. Earlier studies examining extra-adrenal aldosterone synthesis tested factors that upregulate aldosterone synthase, which catalyzes the later steps of aldosterone biosynthesis from cholesterol. We hypothesized that factors affecting steroidogenic acute regulatory protein (StAR), which transports cholesterol into the inner mitochondrial membrane and facilitates the first and rate-limiting step in aldosterone biosynthesis, may generate aldosterone levels in PAECs that are sufficient to induce pulmonary remodeling and fibrosis.

The proximal region of the StAR promoter contains an activator protein-1 (AP-1) site that is required for StAR expression. The transcription factors c-Fos and c-Jun, which are activated by hypoxia in HPAECs, bind the AP-1 site in adrenal cells to induce aldosterone synthesis.
Based on these observations, we hypothesized that hypoxia increases StAR expression and activity in HPAECs to stimulate extra-adrenal aldosterone synthesis, which, in turn, promotes adverse pulmonary vascular remodeling, including fibrosis.

**Methods**

An expanded Methods section is located in the supplement.

**Cell culture and treatments**

HPAECs (Lonza) (male donors), normal human lung fibroblasts (NHLFs) (Lonza), human pulmonary artery smooth muscle cells (HPASMCs) (Lonza), and human coronary smooth muscle cells (HCSMCs) (Lonza) were grown to confluence using phenol red EGM-2, SmGM-2, and FGM-2 medium for endothelial cells, smooth muscle cells, and fibroblasts, respectively. The medium was supplemented with 5.0% fetal bovine serum, and cells were incubated at 37°C, 5.0% CO₂. Cells were passaged twice-weekly using 0.5.0% trypsin/EDTA, and experiments were performed on cells from passages 4-10. Aldosterone (Steraloids) was dissolved in dimethylsulfoxide (10 nmol/L), which served as a vehicle control. Cells were treated with aldosterone (10⁻⁹–10⁻⁷ mol/L) for 24 h and in selected experiments co-incubated with the mineralocorticoid receptor inhibitor spironolactone (10 μM) (Sigma).

**Exposure to hypoxia**

HPAECs were exposed for various time points as indicated to standard nonhypoxic cell-culture conditions (21% O₂, 5.0% CO₂, with N₂ balance at 37 °C) or to varying degrees of hypoxia (0.2%, 2.0%, or 5.0% O₂, 5.0% CO₂, with N₂ balance at 37 °C), in either a modular hypoxia chamber or a tissue culture incubator. These conditions were based on prior studies assessing the effects of hypoxia on HPAECs.¹⁶
**Aldosterone levels measured by liquid chromatography-mass spectrometry (LC-MS)**

Samples were prepared by diluting 1 mL of spent cell culture medium with 4 mL of methanol (MeOH). Precipitated proteins and cell debris were removed by centrifugation at 14,000 x g at 4 °C for 20 min. The supernatant was evaporated to dryness under a stream of N₂, then resuspended in 100 μL 5 mM ammonium acetate (mobile phase). LC-MS was performed using a Surveyor MS Pump Plus HPLC and Finnigan LTQ linear ion trap mass spectrometer (Thermo) as follows: separation of 75 μL of sample was performed with a linear gradient from 50% to 95.0% MeOH over 4 min after an initial run-in at 50% MeOH for 1 min on a 100 mm x 2 mm, 2.5 μm Synergi Hydro-RP reverse phase column (Phenomenex) with a flow rate of 200 μL·min⁻¹. The HPLC was next coupled to MS by negative electrospray ionization (-4.5 kV), and ion optics, gas flows, and capillary temperature were optimized using a direct injection of an aldosterone standard. The m/z transitions monitored were 359 > 189, 297, 315, and 331. Integrated peak areas were normalized to cellular protein content for analysis.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assays were performed on cells exposed to normoxia or hypoxia for 1 hr using the QuikChIP assay (Imagenex) according to the manufacturer’s instructions.⁷ PCR amplification was performed on the proximal region of the StAR promoter region containing the AP-1 elements. The primers used for PCR were forward: 5´AGCAGCCTCAAGGTACTGT-3´ and reverse: 5´-TTGGGTTCTACCACTAGTC-3´, and ChIP-grade anti-c-Fos and anti-c-Jun antibodies (Abcam) were used to assess protein binding to the StAR promoter, while IgG (Abcam) served as a negative control.

**Human PAH and normal control lung specimen histology**

To assess the *in vivo* relevance of our observations involving fibrosis in pulmonary vascular cells
in vitro, we performed experiments using lung tissue harvested from patients with PAH owing to the central role of pulmonary arteriole fibrosis to pulmonary vascular dysfunction in this disease. Immunohistochemistry was performed on distal pulmonary arterioles from formalin-fixed, paraffin-embedded archival specimens acquired at the time of surgery in PAH patients (n=4) or age-matched non-PAH patients (n=4). The experimental protocol was approved by the institutional review board at Boston Children’s Hospital.

Animal models of pulmonary vascular fibrosis.

Sugen-5416/hypoxia rat model of PAH

Male Sprague-Dawley rats (age 12-14 weeks; Charles River Laboratories) were handled in accordance with US National Institutes of Health guidelines, and all procedures were approved by the local committee at Brigham and Women’s Hospital, Harvard Medical School. All surgeries were performed under ketamine/xylazine anesthesia. For SU-5416/hypoxia-induced PAH, rats (~225 g) were administered a single subcutaneous injection of the vascular endothelial growth factor (VEGF)-2 inhibitor SU-5416 (20 mg/kg; Sigma) and exposed immediately to chronic hypoxia (barometric pressure, 410 mm Hg; inspired O₂ tension 76 mm Hg) for 21 days as described previously. This experimental model of PAH was selected for analysis (rather than a model of hypoxia alone) due to the fact that pulmonary hypertension in SU-5416/hypoxia treated rats is more severe and recapitulates a vasculopathy that is similar to that in patients with PAH, including substantial vascular fibrosis.

For the prevention study, rats were randomized to standard chow or chow supplemented with eplerenone (0.6 mg/1g chow) beginning immediately following administration of SU-5416 and continued until hemodynamic and tissue analyses were performed at day 21 (SU-5416/hypoxia). For the reversal study, treatment with spironolactone (25 mg/kg/d) (Henry
Schein) or vehicle added to the drinking water was initiated 14 days following administration of SU-5416 and chronic hypoxia exposure. However, in this protocol, rats were removed from hypoxia at day 21 and exposed to normoxia for 16-17 days (SU-5416/hypoxia-normoxia) and then hemodynamic and aldosterone experiments were performed (Supplemental Table 1).

**Murine hypoxia model of pulmonary hypertension**

Eight week-old male C-57 background mice were exposed to normoxia (21% FiO₂) or normobaric hypoxia (10% O₂; OxyCycler chamber, Biospherix Ltd, Redfield, NY) for 7 days or 28 days, as described previously.¹⁷

**Statistical analysis**

The normality of sample distribution was confirmed using the Shapiro-Wilk test. When samples were distributed normally, data are expressed as mean ± S.E.M. Comparison between groups was performed by the Student’s unpaired two-tailed t-test. One-way analysis of variance (ANOVA) was used to examine differences in response to treatments between groups, with *post-hoc* analysis performed by the method of Tukey. Data were presented as median [interquartile range] for aldosterone levels *in vivo*, and, when data were not normally distributed comparisons between groups were performed by the Mann-Whitney test. For all analyses, P<0.05 was considered significant.

**Results**

**Hypoxia increases aldosterone levels selectively in pulmonary artery endothelial cells**

We first explored the possibility that hypoxia (2.0% FiO₂) is an unrecognized stimulator of extra-adrenal aldosterone synthesis in HPAECs. This level of hypoxia was selected owing to previous reports indicating that adverse pulmonary vascular remodeling and pulmonary artery endothelial
dysfunction is observed under these conditions.\textsuperscript{16} Compared to normoxia-treated cells, hypoxia for 24 h increased aldosterone levels significantly in the cell culture medium as assessed by enzyme immunoassay (EIA) (187.2 ± 86.0 vs. 376.0 ± 94.0 pg/µg protein, P=0.02, n=5) (Figure 1A). A time course analysis revealed that no greater aldosterone levels were observed following shorter durations of hypoxia (Supplemental Figure 1A), and, therefore, subsequent experiments were performed using the 24 h time point for hypoxia exposure.

In adrenal cells, others have observed an inhibitory effect of hypoxia on aldosterone synthesis;\textsuperscript{18-20} thus, cell culture medium from HPAECs exposed to normoxia or hypoxia for 24 hr was next analyzed using liquid chromatography mass spectrometry (LC-MS) as a second methodology to confirm our observations by EIA. At a retention time of 3.68 minutes (Figure 1B), a peak was observed that corresponded to the expected MS-MS spectrum of aldosterone identifying the aldosterone parent ion at 359.2 m/z [M-H\textsuperscript{-1}], as well as the aldosterone daughter ions at 189 m/z, 297 m/z, and 331 m/z (Figure 1C). Semi-quantitative analysis of the integrated peak area demonstrated that akin to our data obtained by EIA, a 2.1-fold increase in aldosterone levels was detected in the culture medium of hypoxia-treated cells compared to normoxia.

To determine if the effect of hypoxia on aldosterone was selective to HPAECs, culture medium samples from other cell types that could be involved in the pathogenesis of pulmonary vascular fibrosis were analyzed by EIA. However, compared to normoxia, we observed no significant difference in aldosterone levels in hypoxia-treated NHLFs (44.4 ± 44.4 vs. 33.0 ± 33.0 pg/µg protein, P=0.85, n=3), HPASMCs (80.2 ± 40.2 vs. 34.6 ± 60.2 pg/µg protein, P=0.44, n=3), or HCSMCs (0.0 ± 0.0 vs. 0.0 ± 0.0 pg/µg protein, P=1.0, n=3) (Supplemental Figure 1B-D).

To confirm that this effect was not due to a global difference in the response to hypoxia
in HPAECs as compared to the other studied cell types, we next measured expression levels of HIF-1α and VEGF-A, which are established signaling mediators involved in hypoxia-induced lung/pulmonary vascular injury.\textsuperscript{4,5} Compared to normoxia, treatment for 24 h at a level of hypoxia reported previously\textsuperscript{16} to upregulate these mediators in vascular cells (0.2% FiO\textsubscript{2}) increased HIF-1α protein levels similarly in HPAECs and NHLFs by 309% (P<0.04, n=3,) and 310% (P<0.001, n=3), respectively (\textbf{Supplemental Figure 2A}). Moreover, VEGF mRNA levels were also increased by hypoxia in HPAECs, PASMCs, and NHLFs by 9.1-fold (P<0.02, n=4), 11.4-fold (P<0.02, n=3), and 15.1-fold (P<0.01, n=7), respectively (\textbf{Supplemental Figure 2B}).

**Hypoxia-induced aldosterone synthesis in pulmonary artery endothelial cells is StAR-dependent**

As a means by which to account for our observation that aldosterone levels were increased in hypoxia-treated HPAECs, we next monitored changes in protein levels of CYP11B2 (aldosterone synthase) and endothelin-1, which are hypoxia-sensitive\textsuperscript{21} proteins involved in the late-steps of aldosterone biosynthesis. Compared to normoxia, hypoxia for 24 h did not significantly influence expression levels of aldosterone synthase (78.0 ± 19.6 vs. 74.7 ± 23.2 arb. units, P=0.91, n=3) or endothelin-1 (43.0 ± 3.4 vs. 50.7 ± 6.7 arb. units, P=0.34, n=3). However, compared to normoxia, we observed a significant increase in StAR levels in hypoxia-treated cells assessed by immunoblotting (28.1 ± 0.1 vs. 41.6 ± 0.1 arb. units, P<0.01, n=3) (\textbf{Figure 2A}) and anti-StAR immunohistochemistry (56.5 ± 2.7 vs. 87.8 ± 9.9 arb. units, P<0.04, n=3) (\textbf{Figure 2B}). Importantly, we observed no effect of hypoxia on protein expression levels of aldosterone synthase (95.7 ± 5.1 vs. 96.2 ± 2.7 arb. units, P=0.94, n=3) or StAR (77.8 ± 6.5 vs. 71.3 ± 1.2 arb. units, P=0.38, n=3) in HCSMCs, supporting our earlier observation that hypoxia-induced increases in aldosterone was selective for HPAECs.
We next sought to determine the mechanism by which hypoxia increased StAR expression in HPAECs. In human Leydig cells, the transcription factors c-Fos and c-Jun interact with the AP-1 site in the proximal promoter region of StAR to upregulate StAR expression and increase aldosterone synthesis. Therefore, to determine if hypoxia increased StAR protein levels by this mechanism in HPAECs, we first explored the effect of hypoxia on c-Fos/c-Jun protein expression in these cells. Compared to time point 0, hypoxia for 15, 30, and 60 min induced an increase in expression levels of c-Fos (31.5 ± 5.7 vs. 69.6 ± 10.5 vs. 66.7 ± 4.9 vs. 76.0 ± 13.7 arb. units, P<0.03, n=3) and c-Jun (14.1 ± 10.6 vs. 38.2 ± 16.2 vs. 73.1 ± 9.1 vs. 99.1 ± 6.9 arb. units, P<0.02 for 30 and 60 min, n=3) in HPAECs (Figure 3A). To determine the minimum level of hypoxia sufficient to stimulate c-Fos signaling, a hypoxic dose-response analysis was next performed. Cells were treated with varying levels of hypoxia (5.0%, 2.0%, 0.2% FiO₂) for 60 min, and c-Fos expression was measured by immunoblotting. Compared to normoxia, we observed a significant increase in protein levels in HPAECs exposed only to 2.0% or 0.2% FiO₂ ([Normoxia] 0.62 ± 0.17 vs. [5.0% FiO₂] 1.5 ± 0.6 vs. [2.0% FiO₂] 2.08 ± 0.47 vs. [0.2%] 3.47 ± 0.31 arb. units, P<0.05 for 2.0% and 0.2%, n=3) (Supplemental Figure 3A), which was associated with an attendant significant increase in aldosterone at those levels of hypoxia only ([5.0%] 150.3 ± 40.0 vs. [2.0%] 200.2 ± 19.3 vs. [0.2%] 182.0 ± 19.7 % normoxia, P<0.05 for 2.0% and 5.0% FiO₂, n=5) (Supplemental Figure 3B). Consistent with the HPAEC-specific effect of hypoxia on aldosterone levels, we also observed no significant effect of hypoxia on c-Fos/c-Jun expression in HPASMCs or HCSMCs (Supplemental Figure 4).

Next, we performed a chromatin immunoprecipitation assay to determine if hypoxia-induced upregulation of c-Fos/c-Jun expression was associated with an increase in their association with the StAR promoter. Compared to normoxia, hypoxia for 1 h increased
significantly association with the StAR promoter for c-Fos (32.0 ± 20.5 vs. 63.9 ± 18.8 arb. units, P<0.04, n=3), while a directionally similar effect was observed for c-Jun (64.4 ± 27.2 vs. 84.8 ± 37.4 arb. units, P=0.28, n=3) and (Figure 3B). The functional relevance of c-Fos/c-Jun association with the StAR promoter to changes in aldosterone levels mediated by hypoxia in HPAECs was assessed by two methods. First, AP-1 transcription factor activity was inhibited by incubation of cells with SR-11302 (1 μM), which decreased aldosterone levels by 61.9% (P=0.06, n=3-4) in hypoxia-treated cells. Next, StAR protein expression was inhibited using StAR siRNA (40 nM) (Si-StAR). Compared to hypoxia alone, si-StAR-transfected cells demonstrated a significant decrease in StAR protein levels (160.4 ± 18.6 vs. 44. ± 5.8 arb. units, P<0.01, n=3) that was not observed in cells transfected with a negative (scrambled) control siRNA (Figure 3C). Molecular inhibition of StAR was associated with a decrease in aldosterone levels in normoxia-treated cells to undetectable levels (59.4 ± 28.8 vs. 0.0 ± 0.0, pg/μg protein, P=0.09, n=3-6), as well as an 88.7% decrease in aldosterone levels in hypoxia-stimulated cells (152.0 ± 30.8 vs. 17.2 ± 16.4 pg/μg protein, P<0.05, n=6) (Figure 3D).

**Cholesterol augments aldosterone biosynthesis in hypoxia-treated pulmonary artery endothelial cells**

By facilitating sterol substrate translocation into the inner mitochondria, StAR regulates the conversion of cholesterol to pregnenolone, which is the primary precursor to the formation of aldosterone. Owing to our observation that hypoxia increased StAR expression, we next assessed the effect of hypoxia on StAR activity. To accomplish this goal, cells were treated at the time of normoxia/hypoxia for 24 h with 20-α-OH-cholesterol (20-α-OH-C) (5 μg/ml), which compared to cholesterol is (a more) soluble intermediate in the cholesterol side-chain cleavage reaction, and, thus, enables assessment of maximal StAR activity. We observed that compared to
vehicle (V) control, treatment with 20-α-OH-C increased levels of progesterone in cells exposed
to normoxia (0.0 ± 0.0 vs. 258.0 ± 65.8 pg/μg protein, n=4) and hypoxia (82.4 ± 53.6 vs. 672.8 ±
81.4 pg/μg protein, P<0.05, n=4). Moreover, 20-α-OH-C augmented hypoxia-stimulated
progesterone levels by 160% (P<0.05, n=3) compared to 20-α-OH-C-treated cells exposed to
normoxia (Figure 4A). The effect of 20-α-OH-C on hypoxia-stimulated aldosterone production
paralleled these findings: compared to V-treated cells, 20-α-OH-C increased aldosterone levels
in cells exposed to normoxia (50.6 ± 8.0 vs. 293.8 ± 11.2 pg/μg protein, P<0.05, n=4) and
hypoxia (111.0 ± 36.2 vs. 454.2 ± 16.2 pg/μg protein, P<0.05, n=4), and 20-α-OH-C increased
aldosterone levels in hypoxia-stimulated cells by 54% (P<0.05, n=4) compared to 20-α-OH-C-
cells exposed to normoxia (Figure 4B).

We next determined if changes in cellular mitochondrial content resulted from hypoxia or
hypoxia-induced aldosterone synthesis to influence StAR expression/activity in HPAECs.
Compared to normoxia, no significant difference was observed in mitochondrial content as
assessed by Mito Tracker® Red FM fluorescence in hypoxia-treated cells or in hypoxia-treated
cells co-incubated with spironolactone (4225 ± 1145 vs. 5140 ± 1136 vs. 6695 ± 456 arb. units,
P=0.27 by ANOVA, n=3) (Supplemental Figure 5). Similarly, there was no evidence of
damage to the mitochondrial membrane potential in HPAECs (n=3) subjected to normoxia or
hypoxia (Supplemental Figure 6). Taken together, these data illustrate the role of cholesterol as
a substrate required for optimizing StAR-activity by hypoxia and that hypoxia-induced StAR
upregulation is not contingent on changes in cellular mitochondrial content or membrane
potential.

**Aldosterone induces remodeling and fibrosis in pulmonary artery endothelial cells in vitro**

Hypoxia promotes adverse remodeling and fibrosis of distal pulmonary arterioles in PAH in
vivo,6,7 although the contribution of aldosterone to this effect is not known. To explore this issue further, we first monitored changes in expression levels of various proteins associated with hypoxia-mediated vascular fibrosis (CTGF and collagen III) and remodeling (MMP-2 and MMP-9) in HAPECs treated for 24 h with vehicle (V) control or concentrations of aldosterone relevant to PAH (10⁻⁹-10⁻⁷ mol/l). Compared to V-treated cells, aldosterone (10⁻⁷ mol/l) induced a significant increase in levels of: CTGF (3.4 ± 0.2 vs. 11.6 ± 1.9 arb. units, P<0.01, n=3); collagen III (118.0 ± 28.1 vs. 234.9 ± 58.4 arb. units, P<0.04, n=3) (Pearson’s r=0.86, P<0.001 for CTGF vs. Collagen III); MMP-2 (99.2 ± 11.1 vs. 144.5 ± 13.5 % control, P<0.05, n=4); and, MMP-9 (100.0 ± 22.8 vs. 199.3 ± 9.9 % control, P<0.05, n=3) (Supplemental Figure 7).

Aldosterone antagonism attenuates hypoxia-mediated vascular remodeling and fibrosis in pulmonary artery endothelial cells in vitro

We next assessed the contribution of aldosterone to changes in expression levels of remodeling/fibrosis proteins in hypoxia-treated cells. Compared to normoxia, hypoxia increased levels of CTGF, collagen III, MMP-2, and MMP-9 by 27.9% (P<0.001, n=3), 40.9% (P<0.03, n=3), 114% (P=0.05, n=3), and 40.3% (P<0.01, n=3), respectively. However, treatment with the mineralocorticoid receptor inhibitor spironolactone (10 μM) attenuated hypoxia-stimulated increases in CTGF (127.2 ± 9.4 vs. 74.9 ± 8.0 arb. units, P<0.02, n=3), collagen III (100.2 ± 6.1 vs. 30.0 ± 3.4 arb. units, P<0.001, n=3), MMP-2 (77.3 ± 14.4 vs. 27.2 ± 8.4 arb. units, P<0.05, n=3), and MMP-9 (140.3 ± 8.6 vs. 101.7 ± 8.7 % control, P<0.04, n=3) (Figure 5). Collectively, these data suggest that mineralocorticoid receptor activation by aldosterone is involved in the remodeling/fibrosis response to hypoxia in HPAECs in vitro.

Increased aldosterone stimulated by hypoxia in HPAECs are sufficient to promote CTGF upregulation in PASMCs in vitro
Owing to the observation that pulmonary arterioles harvested from patients with pulmonary vascular fibrosis demonstrate intimal collagen deposition that is concomitant with endothelial dysfunction, we next determined if hypoxia-induced increases in aldosterone levels in HPAECs are sufficient to activate CTGF in PASMCs (Figure 6A). Compared to normoxia-treated PASMCs, no difference in CTGF expression levels was observed in hypoxia-treated PASMCs (34.9 ± 2.3 vs. 37.4 ± 15.3 arb. units, P=0.9, n=3). However, PASMCs exposed for 24 hr to conditioned medium from hypoxia-treated HPAECs expressed a significant increase in CTGF levels compared to normoxia-treated PASMCs (76.6 ± 5.8 vs. 37.3 ± 15.3 arb. units, P<0.003, n=3) as well as compared to PASMCs exposed to culture medium from normoxia-treated HPAECs (76.6 ± 5.8 vs. 43.1 ± 9.4 arb. units, P<0.04, n=3) (Figure 6B).

**StAR is increased in experimental PAH and in PAH patients in vivo**

To support our observations in HPAECs in vitro, we next explored the potential involvement of StAR in a disease characterized by pulmonary vascular fibrosis in vivo. Anti-StAR immunohistochemical staining with Vector red substrate was performed on pulmonary arterioles [located distal to terminal bronchioles with diameters 20-50 μm] harvested from rats treated with SU-5416 (20 mg/kg) and exposed to hypoxia for 21 days (SU-5416/hypoxia).26 Compared to control rats, SU-5416/hypoxia rats with confirmed PAH and elevated aldosterone levels7 demonstrated a significant increase in StAR expression levels (19.2 ± 0.5 vs. 30.2 ± 0.5 arb. units, P<0.001, n=4-6 rats/condition) (Figure 7A). Although treatment with the selective mineralocorticoid receptor antagonist eplerenone (0.6mg/1g) attenuated pulmonary hypertension severity and pulmonary vascular fibrosis significantly,7 this treatment had no effect on StAR expression levels in SU-5416/hypoxia rats (30.2 ± 0.5 vs. 27.9 ± 2.2 arb. units, P=0.29, n=4-6 rats/condition). In a second animal model of pulmonary vascular fibrosis, mice (n=3/condition)
exposed to chronic hypoxia for 7 or 28 days, expressed a 23% (P<0.03) and 66% (P<0.02) increase in pulmonary arterial StAR levels, respectively, compared to mice exposed to normoxia (Supplemental Figure 8).

We have demonstrated previously that pulmonary arteriolar levels of aldosterone are increased in patients with PAH compared to controls. Thus, to determine if increased StAR expression is observed in patients with PAH, Vector red anti-StAR immunohistochemistry was performed on distal pulmonary arterioles harvested from normal controls or a cohort of patients with severe PAH (Supplemental Tables 2 and 3). Compared to controls, a 40.0% increase in StAR expression was observed in PAH patients (12.0 ± 0.86 vs. 16.8 ± 0.88 arb. units, P<0.01, n=4 patients/condition) (Figure 7A). This finding is consistent with our observation in experimental SU-5416/hypoxia-PAH, and, overall, links hypoxia with StAR upregulation and hyperaldosteronism in the pulmonary vasculature in PAH.

**StAR upregulation associates with aldosterone-induced vascular fibrosis in PAH in vivo**

We next determined if StAR activation is associated with aldosterone-dependent increases in fibrosis in distal pulmonary arterioles in PAH in vivo. Compared to controls, rats with SU-5416/hypoxia-PAH and expressing increased StAR in the pulmonary vasculature demonstrated increased pulmonary arteriolar levels of CTGF (88.4 ± 11.5 vs. 171.8 ± 5.9 arb. units, P<0.05, n=6 rats/condition) and collagen III (61.5 ± 18.8 vs. 126.5 ± 6.3, arb. units, P<0.05, n=6 rats/condition) assessed by immunohistochemical analysis. In turn, eplerenone (0.6 mg/1g chow) decreased CTGF and collagen III levels in SU-5416/hypoxia PAH by 81.8% (P<0.05, n=6 rats/condition) and 42.8% (P<0.05, n=6) (Figure 7B), respectively, which was associated with a significant increase in the cross-sectional luminal area of vessels (21.5 ± 2.7 vs. 44.9 ± 4.5 % cross sectional area, P<0.01, n=6 rats/condition) as well as substantial improvements to
pulmonary vascular resistance, pulmonary artery pressure, and right ventricular remodeling, as shown previously.\(^7\)

**Aldosterone inhibition with spironolactone reverses experimental PAH involving hypoxia in vivo**

The effect of mineralocorticoid receptor antagonist therapy on reversing severe experimental PAH was assessed next. Rats were administered SU-5416 and treated with hypoxia for 21 d followed by exposure to normoxia for two weeks (SU-5416/hypoxia-normoxia). In a reversal study, rats were randomized to receive vehicle control or spironolactone (25 mg/kg/d) in the drinking water beginning at day 14, which was confirmed to be a time point following the development of pulmonary hypertension in this model (Supplemental Figure 9). Compared to controls, SU-5416/hypoxia-normoxia rats demonstrated elevated aldosterone levels in plasma (352.7 [331.1 – 548.0] vs. 1659.3 [1342.5 – 2990.0] pg/ml, n=6, P<0.001) and lung homogenates (115.1 [88.6 – 201.7] vs. 434.3 [160.4 – 863.1] pg/µg protein, n=6-7 rats/condition, P<0.04) (Supplemental Figure 10), which was associated with severe pulmonary hypertension (Table 1). We observed that without influencing body weight, mean arterial pressure, or indexed systemic vascular resistance significantly, spironolactone decreased in SU-5416/hypoxia-normoxia rats the mean right atrial pressure (5.3 ± 1.1 vs. 2.9 ± 1.9 mm Hg, P<0.05, n=6), mean pulmonary artery pressure (40.1 ± 6.9 vs. 26.5 ± 3.9 mm Hg, P<0.05, n=6), pulmonary artery systolic pressure (116.6 ± 20.7 vs. 84.5 ± 25.8 mm Hg, P<0.05, n=6), pulmonary vascular resistance index (46.2 ± 12.9 vs. 15.4 ± 3.6 mm Hg*min*g/ml, P<0.01, n=6), and right ventricular (RV) weight/left ventricular + septum weight (0.54 ± 0.08 vs. 0.41 ± 0.07, n=6, P<0.05) (Table 1).
Discussion

Our findings demonstrate that StAR-dependent synthesis of aldosterone is a key mediator of the pulmonary vascular injury response to hypoxia. In HPAECs, hypoxia induced binding of c-Fos/c-Jun to the promoter region of StAR, which activated StAR in a substrate-dependent manner, as increased bioavailable levels of cholesterol augmented StAR-dependent biosynthesis of aldosterone. We used a cell co-culture method to demonstrate that increased pulmonary endothelial aldosterone synthesis due to hypoxia is sufficient to upregulate CTGF in pulmonary artery smooth muscle cells, providing for the first time evidence in support of cross-talk between fibrosis signaling pathways in different pulmonary vascular cells stimulated by extraadrenal aldosterone biosynthesis. The possibility that extra-adrenal aldosterone is functionally relevant to cardiovascular disease was supported further by our observation that pharmacological inhibition of aldosterone action attenuated hypoxia-induced increases in CTGF, collagen III, MMP-2, and MMP-9 in HPAECs in vitro, which was associated with decreased pulmonary vascular fibrosis and remodeling of distal pulmonary arterioles, and reversed experimental PAH in vivo.

Our findings are in agreement with previous reports indicating that upregulation of classical hypoxic signaling pathways, such as HIF-1α and VEGF-A, occurs similarly in HPAECs as compared to other lung/vascular cell types in vitro.27 However, hypoxia increased aldosterone levels in HPAECs selectively at 2.0% FiO₂, and, therefore, this level of hypoxia was used to study c-Fos-StAR-aldosterone signaling. Thus, the possibility that autonomous aldosterone synthesis is induced in other lung/vascular cells by a greater level of hypoxia cannot be excluded by data presented in the current study.

Our observation that hypoxia induced aldosterone synthesis selectively in HPAECs parallels previous reports indicating suppressed aldosterone steroidogenesis in cultured adrenal
cortical cells under similar experimental (hypoxic) condition. Variability in hypoxia-aldosterone signaling across cell types may be attributed to differences in the effect of hypoxia on proteins that affect steroidogenesis. For example, hypoxia is associated with decreased aldosterone synthase expression and inhibition of aldosterone synthesis in adrenal cells, whereas no difference in aldosterone synthase expression was observed between normoxia and hypoxia in any of the cell types (e.g., HPAECs, HCMSCs) analyzed in our study. Additionally, the functional effect of hypoxia-sensitive transcription factors known to regulate proteins involved in steroidogenesis, such as c-Fos/c-Jun, appears to vary according to cell type: our observation that c-Fos/c-Jun upregulation is associated with increased StAR activity is in agreement with other reports in adrenal glomerulosa, although c-Fos/c-Jun-mediated inhibition of StAR expression has been reported in Y1 adrenal tumor cells. In light of these cell-specific differences, LC-MS, which is the gold-standard method for hormone detection, was used to confirm our findings that hypoxia induces aldosterone synthesis in HPAECs.

The precise mechanism(s) by which hypoxia promotes pulmonary vascular fibrosis is unresolved. For example, hypoxia has been proposed as a stimulator of the endothelial-mesenchymal transition in cardiovascular cells, which promotes fibrosis through transforming growth factor-β (TGF-β) signaling. Although we did not measure TGF-β or cell markers necessary to confirm a phenotype switch in hypoxia-treated pulmonary vascular cells per se, our findings implicate an alternative but overlapping mechanism by which to account for hypoxia-induced fibrosis: hormonal regulation of CTGF, which is an established downstream mediator of TGF-β-dependent fibrosis in vascular and non-vascular cells.

We observed that mineralocorticoid receptor inhibition reversed pulmonary hypertension severity and RV hypertrophy in an experimental model of severe PAH involving hypoxia in vivo.
The potential contribution of angiotensin-aldosterone signaling to hypoxia-mediated lung injury, pulmonary fibrosis, and pulmonary vascular disease is gaining increasing attention. Angiotensin is a modifiable contributor disease severity in experimental models of PAH. Hyperaldosteronism was identified in the clinical profile of patients with high-altitude pulmonary edema and aldosterone inhibition is associated with improved outcome in PAH patients. Our findings demonstrate the functional consequences of mineralocorticoid receptor stimulation by aldosterone on collagen deposition in three experimental animal models of hypoxic pulmonary vascular disease in vivo. Although the precise mechanism by which the mineralocorticoid receptor mediates pulmonary vascular fibrosis due to hypoxia was not examined in the current work, this observation is in concert with previous reports from our group and others in systemic vascular cells indicating that mineralocorticoid receptor stimulation is associated with increased oxidant stress, NF-κB signaling, and mitogen activated protein kinase signaling, which are established stimulators of pathologic collagen deposition. To that end, the pharmacotherapeutic role of mineralocorticoid receptor antagonism in patients with PAH is the subject of ongoing clinical trials (NCT01468571; NCT01712620 at clinicaltrials.gov accessed March 5, 2014).

We observed that plasma and lung homogenate aldosterone levels were elevated in experimental PAH in vivo despite removal of animals from hypoxia for two weeks. This finding raises the possibility that factors other than to hypoxia may regulate pulmonary vascular aldosterone synthesis in PAH, such as elevated levels of endothelin-1 due to increased pulmonary artery pressure, which has been reported previously to mediate aldosterone synthase expression and aldosterone synthesis in HPAECs. Along these lines, it is important to note that extra-adrenal steroidogenesis alone is unlikely to account for levels of aldosterone observed in
experimental models of pulmonary vascular fibrosis or PAH patients, and that delineating the contribution of adrenal aldosterone from extra-adrenal aldosterone to pulmonary vascular fibrosis requires further investigation.

In summary, our findings demonstrate upregulation of c-Fos/c-Jun-StAR signaling by hypoxia as a novel molecular mechanism underpinning autonomous aldosterone stimulation in HPAECs, which, in turn, increases CTGF to promote pulmonary vascular fibrosis. Our observations demonstrate the relevance of this signaling pathway to pulmonary vascular disease: StAR is increased in remodeled pulmonary arterioles of PAH patients and in experimental models of PAH, which is associated with mineralocorticoid receptor-dependent upregulation of collagen and pulmonary vascular fibrosis in PAH in vivo. Identifying the involvement of StAR and the mineralocorticoid receptor in modulating adverse pulmonary vascular remodeling may have important pharmacotherapeutic implications for patients with PAH and other diseases characterized by pulmonary vascular fibrosis.

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Conflict of Interest Disclosures: B.A.M. and S.Y.C receive funding to research pulmonary hypertension from Gilead Sciences Inc.
References:


2011;11:72.


Table 1. The effect of mineralocorticoid receptor antagonism on the reversal of adverse cardiopulmonary hemodynamics in experimental PAH.

<table>
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<tr>
<th></th>
<th>Control</th>
<th>SU-5416/hypoxia-normoxia</th>
<th>SU-5416/hypoxia-normoxia</th>
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<tr>
<td></td>
<td>-SP</td>
<td>+SP</td>
<td></td>
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<tr>
<td>HR (bpm)</td>
<td>263 ± 27</td>
<td>256 ± 46.8</td>
<td>283 ± 35.4</td>
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<td>RV/LV</td>
<td>0.25 ± 0.03</td>
<td>0.54 ± 0.08*</td>
<td>0.41 ± 0.07**</td>
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<tr>
<td>Mean RAP (mmHg)</td>
<td>3.5 ± 1.5</td>
<td>5.3 ± 1.1*</td>
<td>2.9 ± 1.9**</td>
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<tr>
<td>Mean PAP (mmHg)</td>
<td>11.4 ± 1.1</td>
<td>40.1 ± 6.9*</td>
<td>26.5 ± 3.9**</td>
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<td>PASP (mmHg)</td>
<td>34.0 ± 4.0</td>
<td>116.6 ± 20.7*</td>
<td>84.5 ± 25.8**</td>
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<tr>
<td>MAP (mmHg)</td>
<td>58.1 ± 3.7</td>
<td>71.1 ± 18.2</td>
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<tr>
<td>CI (ml/min/g)</td>
<td>121.0 ± 33.9</td>
<td>72.6 ± 41.5</td>
<td>119 ± 40.1</td>
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<tr>
<td>PVRi (mmHg<em>min</em>g/ml)</td>
<td>7.6 ± 3.6</td>
<td>46.2 ± 12.9*</td>
<td>15.4 ± 3.6*</td>
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<tr>
<td>SVRi (mmHg<em>min</em>g/ml)</td>
<td>49.4 ± 13.4</td>
<td>74.9 ± 26.1</td>
<td>57.6 ± 21.8</td>
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In a reversal study, Sprague-Dawley rats were treated with vehicle control or Sugen-5416 (20 mg/kg) and exposed to chronic hypoxia for 3 weeks followed by normoxia for 16-17 days (SU-5416/hypoxia-normoxia). The effect of spironolactone (SP) (25 mg/kg/d) on pulmonary hypertension was assessed by invasive cardiac catheterization (n=5-6 rats per condition). *P<0.05 vs. Control; **P<0.05 vs. SU-5416/hypoxia. bpm, beats per minute; RV, right ventricle; LV, left ventricle; RAP, right atrial pressure; PAP, pulmonary artery pressure; PASP, pulmonary artery systolic pressure; MAP, mean arterial pressure; CI, cardiac index; PVRi, indexed pulmonary vascular resistance; SVRi, indexed systemic vascular resistance.

Figure Legends:

Figure 1. Hypoxia increases aldosterone levels in human pulmonary artery endothelial cells. (A)

Human pulmonary artery endothelial cells (HPAECs) were exposed to normoxia (21% FiO₂) or hypoxia (2.0% FiO₂) for 24 h (n=5), and enzyme immunoassay was performed to quantify aldosterone (ALDO) levels in the culture medium. (B) Liquid chromatography-mass spectrometry (LC-MS) was performed as a second methodology to confirm our findings that hypoxia increased ALDO levels in HPAECs. The total ion chromatogram with peak intensities normalized to protein content demonstrated a peak at a retention time of 3.68 min that corresponds to (C) the correct MS-MS spectrum identifying the aldosterone parent ion at 359 m/z [M-H⁻], as well as the three daughter ions at m/z 189, 297, and 331. This peak was not observed in unconditioned medium (i.e., culture medium not used to treat HPAECs). a.u.,
arbitrary units. Data are presented as mean ± S.E..

**Figure 2.** Hypoxia increases StAR protein expression in pulmonary artery endothelial cells. (A) Human pulmonary artery endothelial cells were exposed to normoxia (21% FiO₂) or hypoxia (2.0% FiO₂) for 24 h and protein levels of StAR were assessed by immunoblotting (n=3) and (B) anti-StAR immunohistochemistry (n=3). Data are presented as mean ± S.E. a.u., arbitrary units. Representative blots and photomicrographs (at 400x magnification) are shown.

**Figure 3.** Hypoxia increases StAR activity to promote aldosterone synthesis in pulmonary artery endothelial cells (HAPECs). (A) HPAECs were exposed to normoxia (21% FiO₂) or hypoxia (2.0% FiO₂) for 15, 30, or 60 min and protein levels of c-Fos (n=3) and c-Jun (n=3), which are known to influence StAR activity in adrenal cells, were assessed by immunoblotting. (B) The effect of hypoxia on c-Fos/c-Jun association with the *StAR* promoter was assessed by chromatin immunoprecipitation (n=3). Compared to normoxia, association with *StAR* was increased by hypoxia for 1 hr for c-Jun and c-Fos. IgG served as negative control. (C) The functional effect of increased c-Fos/c-Jun association with the *StAR* promoter to changes in aldosterone (ALDO) levels mediated by hypoxia was assessed next. To accomplish this, StAR expression was inhibited using StAR siRNA (40 nM)(Si-StAR). Cells transfected with si-StAR and stimulated with hypoxia demonstrated decreased StAR expression compared to hypoxia-stimulated cells treated with vehicle control (n=3), which was associated with (D) a significant decrease in ALDO levels in normoxia-treated cells and in cells stimulated with hypoxia (n=6). a.u., arbitrary units. NS, not statistically significant; V, vehicle control, which was OptiMEM I media; SSc, negative (scrambled) control siRNA; Lipo, Lipofectamine™ 2000; ANOVA, analysis of
variance. Data are presented as mean ± S.E.; Representative blots are shown.

**Figure 4.** Hypoxia increases StAR activity to increase progesterone and aldosterone levels in pulmonary artery endothelial cells. Human pulmonary artery endothelial cells were treated with vehicle (V) control or 20-α-hydroxycholesterol (20-α-OH-C) (5 μg/ml) at the time of exposure to normoxia (21% FiO₂) or hypoxia (2.0% FiO₂) for 24 h. As a soluble cholesterol derivative, 20-α-OH-C is a bioavailable substrate for steroidogenic acute regulatory protein (StAR) activation; thus, supplementing cells with 20-α-OH-C affords assessment of maximal StAR activity. The effect of 20-α-OH-C and hypoxia on levels of (A) progesterone (n=4) and (B) aldosterone (ALDO)(n=3) in the cell culture medium was assessed by enzyme immunoassay. ANOVA, analysis of variance. Data are presented as mean ± S.E.

**Figure 5.** Aldosterone promotes hypoxia-induced pulmonary vascular remodeling and fibrosis. Human pulmonary artery endothelial cells were treated with vehicle (V) control or the mineralocorticoid receptor antagonist spironolactone (SP)(10μM) at the time of exposure to normoxia (21% FiO₂) or hypoxia (2.0% FiO₂) for 24 h, and expression levels of collagen III and connective tissue growth factor (CTGF), which are associated with hypoxia-mediated vascular fibrosis, and matrix-metalloproteinase (MMP)-2 and MMP-9, which are associated with hypoxia-mediated vascular remodeling, were assessed by Western immunoblot. n=3 for each immunoblot. Data are presented as mean ± S.E. Representative blots are shown.

**Figure 6.** Increased aldosterone levels stimulated by hypoxia in HPAECs are sufficient to promote CTGF upregulation in pulmonary artery smooth muscle cells (PASMCs) *in vitro.* (A)
Cultured human PASMCs were exposed for 24 hr to normoxia (21% \( \text{FiO}_2 \)) or hypoxia (2.0% \( \text{FiO}_2 \)) in the presence or absence of standard culture medium or conditioned medium (CM) from HPAECs treated with normoxia or hypoxia for 24 hr, and (B) connective tissue growth factor (CTGF) expression levels were assessed by Western immunoblot (IB) \((n=3)\). Data are presented as mean \( \pm \) S.E. Representative blots are shown.

**Figure 7.** StAR is increased in experimental pulmonary arterial hypertension (PAH) and in PAH patients *in vivo.* (A) Paraffin-embedded lung sections were harvested from PAH patients \((n=4)\) or age-matched controls \((n=4)\) and anti-StAR Vector red immunohistochemical staining was performed on pulmonary arterioles. (B) In a prevention study, Sugen-5416/hypoxia3-treated rats were randomized to receive standard chow or the mineralocorticoid receptor antagonist eplerenone (EPL) \((0.6 \text{ mg/1g chow})\) immediately following exposure to hypoxia until completion of the study 21 days later. Severe pulmonary hypertension and hyperaldosteronism in lung homogenates was confirmed \((\text{ref. 7})\), and the effect of EPL on vascular fibrosis in paraffin-embedded pulmonary arterioles specimens \((20-50 \mu \text{m in diameter})\) was assessed using anti-connective tissue growth factor (CTGF) and anti-collagen III immunohistochemistry \((n=4-6 \text{ rats/condition})\). a.u., arbitrary units. Data are presented as mean \( \pm \) S.E. Representative photomicrographs \((400x \text{ magnification})\) are shown.
Upregulation of Steroidogenic Acute Regulatory Protein by Hypoxia Stimulates Aldosterone Synthesis in Pulmonary Artery Endothelial Cells to Promote Pulmonary Vascular Fibrosis
Bradley A. Maron, William M. Oldham, Stephen Y. Chan, Sara O. Vargas, Elena Arons, Ying-Yi Zhang, Joseph Loscalzo and Jane A. Leopold

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Supplemental Figure 1

A) ALDO Levels (% Normoxia) over time for HPAEC with a *p<0.001 vs. Time 0 hr.

B) NHLF ALDO levels (pg/μg protein) at 21% and 2.0% FiO₂, with P=0.85.

C) HPASMC ALDO levels (pg/μg protein) at 21% and 2.0% FiO₂, with P=0.44.

D) HCSMC ALDO levels (pg/μg protein) at 21% and 2.0% FiO₂, with ND for both conditions.

*P<0.001 vs. Time 0 hr.
Supplemental Figure 2
Supplemental Figure 3

A) 

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Densitometry (a.u.)

B) 

![Bar chart showing ALDO (% Normoxia) vs. FiO₂]

ALDO (% Normoxia)

- 21%: P=0.24
- 5.0%: P<0.0001
- 2.0%: P<0.0001
- 0.2%: P=0.53

FiO₂
A)

2.0% FiO₂ (min)

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P=NS

c-Fos Densitometry (a.u.)

B)

2.0% FiO₂ (min)

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</table>

P=NS

c-Fos Densitometry (a.u.)

Supplemental Figure 4
Supplemental Figure 5

Fluorescence Intensity (a.u.)

-SP  +SP  -SP  +SP

21% FiO₂  2.0% FiO₂

P=0.29 by ANOVA

2000  4000  6000  8000  10000

400x
Supplemental Figure 6

Membrane Potential

Red Green

21% FiO₂

2% FiO₂

Green Fluorescence Intensity (a.u.)

ND ND

21% 2.0%

FiO₂

400x
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</table>

Supplemental Figure 7
Supplemental Figure 8
Supplemental Figure 9
Supplemental Figure 10

A) Plasma ALDO (pg/ml) comparison between Control and SU-5416/hypoxia-normoxia. The panels show a significant difference (P<0.001).

B) Lung Homogenate ALDO (pg/ml) comparison between Control and SU-5416/hypoxia-normoxia. The panels show a significant difference (P<0.04).
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 (*ψₘ*).** 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 \([\text{mean} \ldots]^{\text{...}}\).
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 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$_2$) or hypoxia (2.0% FiO$_2$) 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-1α 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$_2$) for 24 h and expression levels of HIF-1α were assessed by immunoblot. (B) The effect of normoxia or hypoxia (0.2% FiO$_2$) 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% FiO\(_2\)) or varying degrees of hypoxia (5.0%, 2.0%, 0.2% FiO\(_2\)) 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% FiO\(_2\)) 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% FiO\(_2\)) or hypoxia (2.0% FiO\(_2\)) 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>
</tr>
</thead>
</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.
<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Surgery/Indication</th>
<th>Echocardiography Data</th>
<th>Hemodynamics</th>
<th>Pathology</th>
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<td>Right apical lung nodule resection</td>
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<td>Pulmonary nodule resection</td>
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<td>13</td>
<td>Male</td>
<td>Non-metastatic synovial cell sarcoma</td>
<td>Lung biopsy (benign pulmonary nodule)</td>
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<td>No prior chemotherapy</td>
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**Supplemental Table 2. Clinical characteristics of control patients.**

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<th>Method of Diagnosis</th>
<th>Surgery/Indication</th>
<th>Echocardiography Data</th>
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<th>Pathology</th>
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<tbody>
<tr>
<td>9</td>
<td>F</td>
<td>Clinical Pathology</td>
<td>Wedge biopsy in setting of cardiac repair of complete AV canal/LVOT membrane to evaluate for PAH</td>
<td>Severe PH Normal LV function</td>
<td>RVSP:SBp= ~100% iNO: RVSP:SBp= 75%</td>
<td>Plexogenic arteriopathy</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>Clinical Pathology</td>
<td>Bilateral lung transplant for PAH</td>
<td>RVSP: 135 mmHg Dilated RA/RV RVH</td>
<td>CI: 3.0 L/min/m² mPAP: 65 mmHg PCWP: 9 mmHg PVR: 14.7 W.U.</td>
<td>PAH remodeling; Marked medial hypertrophy</td>
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<tr>
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<td>M</td>
<td>Clinical Pathology</td>
<td>Bilateral lung transplant for PAH</td>
<td>RVSP: 120 mmHg Dilated RA/RV Normal LV function</td>
<td>CI: 2.5 L/min/m² mPAP: 90 mmHg PCWP: 8 mmHg PVR: 32 W.U.</td>
<td>PAH remodeling; Plexiform change</td>
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<tr>
<td>16</td>
<td>M</td>
<td>Pathology</td>
<td>Wedge biopsy for interstitial lung disease in the setting of connective tissue disease</td>
<td>Not available</td>
<td>Not available</td>
<td>Severe PAH remodeling; Plexiform and angiomatoid change</td>
</tr>
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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.