Aldosterone Inactivates the Endothelin-B Receptor via a Cysteinyl Thiol Redox Switch to Decrease Pulmonary Endothelial Nitric Oxide Levels and Modulate Pulmonary Arterial Hypertension

Running title: Maron et al.; Aldosterone promotes pulmonary arterial hypertension

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Abstract:

**Background** - Pulmonary arterial hypertension (PAH) is characterized, in part, by decreased endothelial nitric oxide (NO’) production and elevated levels of endothelin-1. Endothelin-1 is known to stimulate endothelial nitric oxide synthase (eNOS) via the endothelin-B receptor (ET_{B}), suggesting that this signaling pathway is perturbed in PAH. Endothelin-1 also stimulates adrenal aldosterone synthesis; in systemic blood vessels, hyperaldosteronism induces vascular dysfunction by increasing endothelial reactive oxygen species (ROS) generation and decreasing NO’ levels. We hypothesized that aldosterone modulates PAH by disrupting ET_{B}-eNOS signaling through a mechanism involving increased pulmonary endothelial oxidant stress.

**Methods and Results** - In rats with PAH, elevated endothelin-1 levels were associated with elevated aldosterone levels in plasma and lung tissue and decreased lung NO’ metabolites in the absence of left heart failure. In human pulmonary artery endothelial cells (HPAECs), endothelin-1 increased aldosterone levels via PGC-1α/steroidogenesis factor-1-dependent upregulation of aldosterone synthase. Aldosterone also increased ROS production, which oxidatively modified cysteinyi thiols in the eNOS-activating region of ET_{B} to decrease endothelin-1-stimulated eNOS activity. Substitution of ET_{B}-Cys405 with alanine improved ET_{B}-dependent NO’ synthesis under conditions of oxidant stress, confirming that Cys405 is a redox sensitive thiol that is necessary for ET_{B}-eNOS signaling. In HPAECs, mineralocorticoid receptor antagonism with spironolactone decreased aldosterone-mediated ROS generation and restored ET_{B}-dependent NO’ production. Spironolactone or eplerenone prevented or reversed pulmonary vascular remodeling and improved cardiopulmonary hemodynamics in two animal models of PAH in vivo.

**Conclusions** - Our findings demonstrate that aldosterone modulates an ET_{B} cysteinyi thiol redox switch to decrease pulmonary endothelium-derived NO’ and promote PAH.

**Key words:** endothelin; nitric oxide; pulmonary heart disease; aldosterone; redox biochemistry
Introduction

Pulmonary endothelial reactive oxygen species (ROS) have been implicated in the pathobiology of pulmonary arterial hypertension (PAH) and have been shown to disrupt nitric oxide (NO\(^{-}\))-dependent vasodilatory signaling pathways to promote pulmonary vasoconstriction, muscularization of pulmonary arterioles, and perivascular fibrosis.\(^{1,2}\) However, contemporary PAH pharmacotherapies that aim to restore pulmonary vascular NO\(^{-}\) levels have waning long-term efficacy and do not maintain normal pulmonary vascular tone and pulmonary hemodynamics.\(^{3}\) This observation suggests that in PAH, perturbations to the redox milieu of pulmonary vascular tissue is sufficient to offset the vasodilatory effects of NO\(^{-}\), although the factor(s) that modulate this effect have not been fully elucidated.

Elevated levels of the mineralocorticoid hormone aldosterone are associated with a vasculopathy in systemic blood vessels that is characterized by mineralocorticoid receptor-dependent increases in endothelial ROS generation that decreases levels of bioavailable NO\(^{+}\) resulting in vascular endothelial dysfunction, vascular fibrosis, and decreased vascular compliance.\(^{4}\) In patients with hyperaldosteronism and hypertension or congestive heart failure, mineralocorticoid receptor antagonism with spironolactone or eplerenone improves vascular reactivity and attenuates the adverse effects of aldosterone on blood vessel function and architecture.\(^{5}\) We hypothesized that hyperaldosteronism is present in PAH owing to increased circulating levels of endothelin-1 (ET-1), which is a potent stimulus of adrenal aldosterone synthesis,\(^{6}\) and/or overactivation of the renin-angiotensin-aldosterone axis. Together, these observations and the derivative hypothesis suggest the possibility that by increasing pulmonary endothelial ROS levels, hyperaldosteronism is an unrecognized contributor to the pathobiology of PAH.
The mechanism(s) by which ROS decreases pulmonary endothelial NO\(^{•}\) levels in PAH is unresolved. In the systemic vasculature, ROS has been implicated in the oxidative modification of redox-sensitive cysteinytl thiols in regulatory proteins involved in NO\(^{•}\)-dependent vasodilatory signaling to decrease NO\(^{•}\) bioactivity.\(^7\) A key source of endogenous NO\(^{•}\) generation in pulmonary endothelial cells is via endothelin type B receptor (ET\(_B\))-mediated activation of endothelial nitric oxide synthase (eNOS).\(^8\) ET\(_B\) contains an intracellular cysteine-rich region near its carboxyterminal domain that includes Cys405, demonstrated previously to be a cysteinytl thiol that regulates ET\(_B\) signal transduction.\(^9\) Taken together, we hypothesized that oxidative modification of ET\(_B\) Cys405 by aldosterone-induced ROS serves as a redox switch that disables ET\(_B\)-dependent synthesis of NO\(^{•}\) to promote pulmonary vascular dysfunction and negative remodeling of pulmonary arterioles in PAH.

**Methods**

An expanded Methods section is located in the online supplement

**Cell culture and treatments**

Human pulmonary artery endothelial cells (HPAECs) (Lonza) (male donors) were grown to confluence using phenol-free EGM-2 medium supplemented with 5% fetal bovine serum at 37\(^\circ\)C, 5% CO\(_2\). Cells were passaged twice-weekly using 0.5% trypsin/EDTA, and experiments were performed on cells from passages 4-10. Aldosterone (Steraloids) and ET-1 (1-100 nM) (Sigma-Aldrich) were dissolved in dimethylsulfoxide (10 nmol/L) and deoxygenated water, respectively, which served as vehicle controls. 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 \(\mu\)M) (Sigma-Aldrich).

**Western analysis to detect ET\(_B\) disulfide bond formation**
Western analysis to detect ET\(_B\) disulfide bond formation was performed as described previously.\(^7\) Briefly, protein extracts from cells were lysed in alkylating buffer containing 0.1 M Tris-HCl, pH 6.8, 1% SDS, 100 mM iodoacetamide, and 100 mM N-ethylmaleimide, and sonicated on ice for 5 min followed by a 30-min incubation at 25 °C. Alkylated proteins were then precipitated with acetone. Proteins were resuspended in 50 μl of 0.1 M Tris-HCl, pH 7.4, 1% SDS; and disulfides were reduced with 5mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Following a 20-min incubation at 25 °C, TCEP was removed with a Micro Bio-Spin column 6 (Bio-Rad), and 1% SDS was added to the eluant. The cysteines previously participating in a disulfide bond, now reduced, were labeled with 1 mM polyethylene glycol-conjugated maleimide (molecular mass 10 kDa) (Fluka). After a 1-h incubation at 25 °C, proteins were precipitated with acetone, resuspended in 50 μl of non-reducing SDS electrophoresis buffer, and boiled for 10 min. Protein samples were then size-fractionated electrophoretically using SDS-PAGE, and transferred to a polyvinylidene fluoride membrane. The membrane was immunoblotted with an anti-ET\(_B\) antibody to the region of ET\(_B\) that contains Cys405 (amino acid sequence to which ET\(_B\) antibody was raised:

CLCCWCQSFEEEKQSLEEKQSCLKFKANDHGYDNFRSSNKYSSS) (Santa Cruz Biotechnology). Bands were visualized using the ECL detection method.\(^4\)

**Animal 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 the monocrotaline (MCT) model of PAH, rats were fed standard chow and treated with a 0.5 ml intraperitoneal injection of
MCT (50 mg/ml) (Sigma-Aldrich) or 0.9% saline as control. Rats were randomized to spironolactone (25 mg/kg/d) (Henry Schein) or vehicle added to the drinking water. For the prevention study, treatment with spironolactone began immediately following administration of MCT and continued for 23-25 days until hemodynamic and tissue analyses were performed. For the reversal study, a second experiment was performed in which rats were randomized to spironolactone or vehicle that was initiated 14 days following the administration of MCT and continued until hemodynamic and tissue analyses were performed 10 days later.

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) as described previously. Rats were randomized to either the selective mineralocorticoid receptor antagonist eplerenone (0.6 mg/1 gm standard chow; Test Diet Inc.) or standard chow as a control. Hemodynamic and tissue analyses were performed on all rats 21 days following exposure to chronic hypoxia.

**Statistical analysis**

Normality was tested using the Shapiro-Wilk test. When samples were normally distributed, results are expressed as mean ± SEM, and an unpaired t-test was used to compare two independent groups. Comparisons between multiple groups were made using a one-way analysis of variance (ANOVA) with *post-hoc* analysis performed using the protected Fisher LSD test. When data were not normally distributed, data are presented as median and range, and comparisons between two groups were made using the Mann-Whitney test. P <0.05 was considered significant.
Results

PAH is associated with increased plasma and lung tissue levels of ET-1 and aldosterone.

The Sprague Dawley rat monocrotaline (MCT) model of PAH was selected initially to test the hypothesis that hyperaldosteronism is present in PAH in vivo as MCT is believed to induce pulmonary hypertension through a mechanism that involves elevated levels of the aldosterone secretagogue ET-1.\textsuperscript{12} Transthoracic echocardiography demonstrated that compared to vehicle control (V)-treated rats, MCT decreased the pulmonary artery (PA) flow acceleration time (PAAT) (35.4 ± 2.6 vs. 14.1 ± 1.2 msec, \textit{p}<0.005, \textit{n}=6) and increased right ventricular (RV) free-wall thickness (0.58 ± 0.05 vs. 1.1 ± 0.05 mm, \textit{p}<0.03, \textit{n}=6). Right heart catheterization confirmed that MCT increased significantly pulmonary artery systolic pressure (PASP) (assumed to be equivalent to RV systolic pressure in the setting of a normal pulmonic valve) (28.3 ± 2.7 vs. 89.3 ± 5.3 mm Hg, \textit{p}<0.01, \textit{n}=6). In rats with PAH, there was a 274\% increase in ET-1 levels in plasma (1.76 [non-detectable-4.82] vs. 4.83 [2.3-12.6] pg/ml, \textit{p}<0.04, \textit{n}=6) and a 183\% increase in lung homogenates (335.5 [279.8-453.6] vs. 615.3 [458.4-806.5] pg/\mu g protein, \textit{p}=0.03, \textit{n}=4) (Figure 1a,b), which correlated with an increase in aldosterone levels of 442\% (357.5 [223.0-784.0] vs. 1580.4 [611.4-2790.5] pg/ml, \textit{p}<0.001, \textit{n}=7-8 rats per condition) and 183\% (100.0 [87.3-113.4] vs. 183.1 [126.1-197.4] pg/\mu g protein, \textit{p}<0.04, \textit{n}=4) in plasma and lung tissue, respectively (Figure 1c,d).

The finding of increased aldosterone levels in lung tissue suggested that PAH may be associated with extraadrenal aldosterone synthesis. To determine if this occurred, we examined lungs for expression of the enzyme CYP11B2 (aldosterone synthase), which catalyzes the final and rate-limiting step in aldosterone steroidogenesis. Following saline perfusion of lungs prior to organ harvest, protein levels of CYP11B2 were increased significantly in lung tissue of rats with...
PAH compared to controls (483 ± 75 vs. 1319 ± 226 arb. units, p<0.03, n=4) (Supplemental Figure 1), indicating that it is plausible that elevated levels of aldosterone in lung tissue may also result from local synthesis of aldosterone in PAH.

**Aldosterone increases pulmonary artery pressure and pulmonary vascular remodeling in PAH in vivo.**

To determine if hyperaldosteronism contributes to increased pulmonary artery pressure in PAH in vivo and if mineralocorticoid receptor antagonism could prevent PAH, rats were treated with spironolactone (25 mg/kg/d) or V starting at the time of MCT injection. We observed that without significantly decreasing plasma ET-1 levels or influencing body weight, mean arterial pressure (MAP), or left ventricular end-diastolic pressure (LVEDP) (Supplemental Figure 2), spironolactone decreased PASP significantly in PAH (89.3 ± 5.2 vs. 69.5 ± 5.4 mm Hg, p<0.01, n=6) (Figure 2a), which was confirmed by an increase in PAAT (14.1 ± 1.2 vs. 22.3 ± 2.2 ms, p<0.005, n=6) (Figure 2b). Spironolactone also decreased RV free-wall thickness (1.07 ± 0.05 vs. 0.86 ± 0.03 mm, p<0.03, n=6) (Figure 2c) and RV weight (0.43 ± 0.07 vs. 0.35 ± 0.04 RV weight/LV septum weight, p=0.22, n=5) (Supplemental Figure 3). Notably, these findings were associated with increased levels of the stable NO• metabolite, nitrite (NO2•), in lung tissue specimens harvested from spironolactone-treated rats with PAH as compared to V-treated rats with PAH (759 ± 55 vs. 506 ± 86 µM/µg protein, p=0.048, n=4) indicating that spironolactone improved NO• bioavailability (Figure 3a).

Spironolactone also prevented pathophenotypic changes to distal pulmonary arterioles [located distal to terminal bronchioles with diameters 20-50 µm13] as demonstrated by immunohistochemical staining for smooth muscle α-actin. Compared to V-treated rats with PAH, spironolactone decreased the number of α-actin-stained muscularized distal pulmonary
arterioles (76.0 [64-95] vs. 59.5 [59-61] musclelarized pulmonary arterioles/20 high powered fields, p<0.005, n=5) (**Figure 3b**), and increased significantly the cross-sectional luminal area of vessels (13.7 [12.7-16.1] vs. 36.8 [30.1-38.0] % cross sectional area, p<0.02, n=5). Furthermore, Gomori’s trichrome staining of rat lung sections revealed that, compared to V-treated rats with PAH, spironolactone decreased perivascular collagen deposition by 77% (p<0.001, n=4-5 rats per condition), similar to levels observed in rats without PAH (**Figure 3c**). Analysis using picrosirius red staining paralleled these findings, indicating that hyperaldosteronism contributed to perivascular collagen deposition (i.e., fibrillar collagen), which, in turn, is strongly associated with impaired vascular compliance in PAH (**Supplemental Figure 4**).1

To determine if aldosterone antagonism reverses established PAH, a second study was performed in which V or spironolactone (25 mg/kg/d) was initiated 14 days following administration of MCT, a time point associated with histological evidence of MCT-induced inflammatory injury to distal pulmonary arterioles (**Supplemental Figure 5**). Compared to V-treated rats with PAH, spironolactone decreased levels of perivascular collagen by 71% (p=0.03, n=6), which was associated with a significant decrease in indexed pulmonary vascular resistance (PVRi) (35.9 ± 3.2 vs. 21.5 ± 3.2 mm Hg*min*g/ml, n=4, p<0.02) and PASP (60.3 ± 5.2 vs. 39.5 ± 4.1 mm Hg, n=6, p<0.005) without changes to heart rate, cardiac index (CI), LVEDP, MAP, or indexed systemic vascular resistance (SVRi) (**Figure 4a**).

Next, to confirm the role of aldosterone in a second animal model of PAH and to determine if there was a class effect for mineralocorticoid receptor antagonists, we studied the preventive effects of eplerenone on the development of abnormal cardiopulmonary hemodynamics in rats administered SU-5416 and exposed to chronic hypoxia for 21 days. Compared to normal rats, plasma aldosterone levels were increased by 397% in SU-
5416/hypoxia-induced PAH (352.7 [223.0-557.2] vs. 1402.5 [542.3-2620.1], p<0.02, n=5). Eplerenone decreased perivascular collagen in SU-5416/hypoxia-induced PAH by 67% (n=5, p<0.02), which was associated with a decrease in PVRi (64.6 ± 21.4 vs. 43.9 ± 8.7 mm Hg/min/gm/ml, n=3-4 rats/condition, p=0.18) and PASP (80.5 ± 4.9 vs. 61.5 ± 6.5 mm Hg, p=0.048, n=5) (Figure 4b) without significantly influencing body weight, heart rate, MAP, CI, LVEDP, or SVRi. Collectively, our findings demonstrate that hyperaldosteronism modulates PAH and that a class effect exists among mineralocorticoid receptor antagonists for abrogating the adverse consequences of aldosterone on pulmonary vascular remodeling, PVRi, and PASP in two animal models of PAH in vivo.

ET-1 increases aldosterone levels in pulmonary artery endothelial cells.

As ET-1 levels associated positively with lung CYP11B2 protein expression and aldosterone levels in MCT-induced PAH in vivo, we explored the possibility that ET-1 is an unrecognized stimulus of extraadrenal aldosterone synthesis in HPAECs in vitro. We first confirmed that compared to V-treated cells, ET-1 (1, 10, 100 nM) increased CYP11B2 protein expression levels (157.3 ± 27.5 vs. 180.4 ± 13.4 vs. 234.8 ± 4.3 % control, respectively, p<0.02, n=3) (Supplemental Figure 6a), which correlated with a concentration-dependent increase in aldosterone levels detected in the cell culture medium (241.1 ± 44.8 vs. 283.5 ± 94.7 vs. 396.0 ± 116.5 % control, respectively, p<0.04, n=4) (Supplemental Figure 6b). Consistent with prior reports in dispersed adrenal cortical cells,6,14 ET-1 increased aldosterone levels via activation of the ETB receptor in HPAECs (Supplemental Figure 7).

We next sought to determine the mechanism by which ET-1 increases aldosterone levels in HPAECs. In adrenal cortical Y-1 cells, the transcription factor PPAR-γ co-activator-1α (PGC-1α) interacts with the nuclear receptor protein steroidogenesis factor-1 (SF) to regulate CYP11B2
gene transcription and induce aldosterone synthesis. Therefore, to determine if ET-1 increased aldosterone synthase protein levels by this mechanism in HPAECs, we first explored the effect of ET-1 on PGC-1α and SF protein expression levels in these cells. Compared to V-treated cells, exposure to ET-1 (1, 10, 100 nM) for 24 h induced a concentration-dependent increase in PGC-1α protein expression levels (176.5 ± 52.8 vs. 224.7 ± 68.1 vs. 296.7 ± 145.8 % control, respectively, p<0.02, n=3) (Figure 5a). ET-1 had no effect on SF protein levels; however, ET-1 did increase the association between PGC-1α and SF as demonstrated by co-immunoprecipitation (1260 ± 104 vs. 160 ± 71 arb. units, p<0.001, n=3) (Figure 5b).

We next performed a chromatin immunoprecipitation assay to assess the effect of ET-1 (10 nM) for 24 h on PGC-1α and/or SF association with the CYP11B2 promoter. PGC-1α alone did not bind to the CYP11B2 promoter in cells treated with either V or ET-1; however, compared to V, ET-1 induced a significant increase in SF binding to the CYP11B2 promoter (16.3 ± 9.8 vs. 61.6 ± 9.3 arb. units, p<0.03, n=3) (Figure 5c). Collectively, these data indicate that ET-1 stimulates PGC-1α binding with SF, which, in turn, promotes the association of SF to the promoter region of CYP11B2 to upregulate CYP11B2 protein expression levels. We confirmed that PGC-1α stimulation is linked functionally to aldosterone synthesis in cells treated with the selective PGC-1α agonist pioglitazone (50 μM) for 24 h, which, compared to V, increased aldosterone levels by 365% (p<0.001, n=3) (Figure 5d). Thus, ET-1 increases extraadrenal aldosterone synthesis in endothelial cells via upregulation of CYP11B2 in a PGC1-α/SF-dependent manner.

**Aldosterone increases oxidant stress in HPAECs.**

Next, to determine if hyperaldosteronism in PAH could contribute to pulmonary vascular dysfunction akin to what we observed previously in the systemic vasculature, we investigated
the effect of aldosterone on ROS levels in HPAECs. Cells were exposed to increasing concentrations of aldosterone (10⁻⁹, 10⁻⁸, 10⁻⁷ mol/L) for 12-36 h and H₂O₂ levels were measured by Amplex Red assay. Compared to V-treated cells, maximal H₂O₂ accumulation was observed in cells treated with aldosterone (10⁻⁷ mol/L) for 24 h (65.4 ± 1.6 vs. 100.6 ± 3.5 μM/mg protein, p<0.001, n=3); this effect was abrogated by 56% in aldosterone-treated cells coincubated with spironolactone (p<0.01, n=3), indicating that a majority of aldosterone-induced H₂O₂ formation was due to mineralocorticoid receptor activation (Supplemental Figure 8a). As no further H₂O₂ generation was observed in aldosterone-treated cells beyond 24 h, subsequent experiments were performed at this time point using (patho)physiologically relevant levels of aldosterone similar to those observed in MCT- or SU-5416/hypoxia-treated rats with PAH in vivo. Furthermore, the observed increase in ROS was due to aldosterone, and not ET-1, as ET-1 (10 nM) had no effect on H₂O₂ levels compared to V-treated cells (p=0.43, n=4).

NADPH oxidase type 4 (NOX4) is implicated as a key source of vascular ROS generation in pulmonary hypertension and human vascular endothelial cells exposed to pathophysiologic concentrations of aldosterone.¹⁶,¹⁷ The primary product of NOX4 activation is H₂O₂, and its formation is closely aligned to changes in NOX4 protein expression.¹⁷ Therefore, we examined the effect of aldosterone on NOX4 expression in HPAECs as a potential mechanism to explain the aldosterone-mediated increase in H₂O₂ formation. Compared to V-treated cells, aldosterone (10⁻⁹, 10⁻⁸, 10⁻⁷ mol/L) increased protein levels of NOX4 (134.6 ± 16.5 vs. 146.3 ± 12.4 ± vs. 157.0 ± 4.4 % control, respectively, p<0.02, n=3) and of p22phox (1009.4 ± 167.0 vs. 961 ± 226.2 vs. 829.5 ± 295.6 % control, respectively, p<0.01, n=3), a NOX4 subunit that is required for NOX4-mediated H₂O₂ formation, in a concentration-dependent manner (Supplemental Figure 8b,c).
Aldosterone decreases ET<sub>B</sub>-dependent activation of eNOS and NO<sup>•</sup> levels.

We next investigated the effect of ET<sub>B</sub> receptor activation by ET-1 (10 nM) on levels of the NO<sup>•</sup> metabolite nitrite (NO<sub>2</sub>•). Compared to V-treated cells, ET-1 increased NO<sub>2</sub>• generation with a maximum effect observed at 10 min (29.4 ± 3.6 vs. 139.4 ± 31.8 μM/μg protein, p<0.001, n=3). We then evaluated the effect of aldosterone on ET<sub>B</sub>-stimulated NO<sup>•</sup> levels. Without influencing protein expression of ET<sub>B</sub>, or inducing expression of ET<sub>A</sub> (which is not constitutively expressed in HPAECs)<sup>18</sup> (Supplemental Figure 9), exposure to aldosterone (10<sup>-7</sup> mol/L) for 24 h decreased ET<sub>B</sub>-mediated NO<sub>2</sub>• levels by 60.3% (p<0.01, n=3). Coincubation with spironolactone (10 μM) restored NO<sub>2</sub>• levels to those observed in cells stimulated with ET-1 in the absence of aldosterone (Figure 6a).

We and others have demonstrated previously that in the absence of oxidant stress, NO<sup>•</sup> metabolism to NO<sub>2</sub>• and nitrate (NO<sub>3</sub>•) occurs in a ratio that favors NO<sub>2</sub>• by approximately 2:1, but that this ratio shifts in favor of increased NO<sub>3</sub>• formation in the presence of superoxide anion (•O<sub>2</sub>•), owing to the interaction of NO<sub>2</sub>• with •O<sub>2</sub>• to generate peroxynitrate (O<sub>2</sub>NOO<sup>−</sup>)<sup>7</sup> or via tautomerization of peroxynitrite (ONOO<sup>−</sup>) to NO<sub>3</sub>•<sup>19</sup>. In HPAECs, ET-1 alone did not affect the NO<sub>2</sub>•/NO<sub>3</sub>• ratio significantly compared to V. In contrast, exposure to aldosterone decreased the NO<sub>2</sub>•/NO<sub>3</sub>• ratio by 62% in ET-1-stimulated cells, which was restored fully by coincubation of aldosterone with spironolactone (p<0.04, n=3) (Supplemental Figure 10a). This effect was likely mediated by increased ONOO<sup>−</sup> formation as aldosterone-treated HPAECs had increased levels of 3-nitrotyrosine, a marker of ONOO<sup>−</sup>, compared to cells stimulated with V or ET-1 alone (24.1 ± 3.3 vs. 31.2 ± 3.2 vs. 46.8 ± 6.6 arb. units, p<0.02, n=5) (Supplemental Figure 10b).

We also examined the effect of aldosterone on ET-1-stimulated eNOS activity. Without influencing eNOS protein levels, aldosterone decreased eNOS activity in ET-1 (10 nM)
stimulated cells (18.5 ± 3.5 vs. 7.6 ± 2.4 [14C] L-citrulline c.p.m./mg protein, n=3, p<0.02) (Figure 6b), leading to a decrease in total NO• metabolite (NOx: NO2- + NO3- ) formation (157.9 ± 12.7 vs. 103.4 ± 12.2 μM/μg protein, p<0.01, n=3). Coincubation with spironolactone increased NOx levels in aldosterone-treated cells stimulated with ET-1 by 87% (p<0.02, n=3) (Figure 6c). Taken together, these data demonstrate that aldosterone diminished levels of bioavailable NO• in ET-1-stimulated cells by decreasing ET-1-mediated eNOS activity to limit NO• generation, increasing ONOO• formation, and by oxidizing NO2- to NO3•.

Aldosterone decreases ETB-dependent NO• levels by oxidative modification of Cys405.

Given that aldosterone decreased ET-1-stimulated eNOS activity and NO• generation, we postulated that aldosterone affected ETB receptor function. As aldosterone induced H2O2 formation and ETB contains functionally essential cysteiny thiol residues in its eNOS-activating region, it is plausible that aldosterone may induce an oxidative post-translational modification of ETB that influences receptor function. To examine ETB for oxidation of cysteinyl thiols, protein extracts from HPAECs were treated with V, aldosterone (10−7 mol/L) for 24 h, or H2O2 (200 μmol/L) for 20 min, and free thiols were blocked with iodoacetamide and N-ethylmaleimide. Disulfides were reduced with TCEP hydrochloride, and previously oxidized (now reduced) cysteines were labeled with PEG-conjugated maleimide (molecular mass 10 kDa). In this way, each reduced disulfide bond yields a shift in the apparent molecular mass of the reduced protein by 20 kDa. Western analysis using an antibody specific to the region of ETB containing Cys405 revealed that only the reduced form of ETB was present (50 kDa) in V-treated cells; however, bands at 70 kDa and 90 kDa were evident in cells treated with H2O2 or aldosterone, indicating the de novo formation of one or two disulfide bonds under these conditions of increased oxidant stress (Figure 6d).
To support these findings, we determined if aldosterone modulates the formation of other higher oxidative intermediates of ET_{B} Cys405. Cells were treated V or aldosterone (10^{-7} \text{ mol/L}) for 24 h and the region of ET_{B} containing Cys405 was immunoprecipitated using the specific ET_{B} containing Cys405 antibody (Santa Cruz). Western analysis using an anti-sulfenic acid (RSOH) antibody (derivatized with dimedone)\textsuperscript{20} (Millipore) revealed that compared to V-treated cells, aldosterone increased ET_{B}-SOH protein expression levels by 639\% (p=0.04, n=3)(Figure 6e).

To confirm that oxidative modification of Cys405 has functional implications for ET_{B}-dependent NO\textsuperscript{·} generation, we transiently transfected COS-7 cells with human DNAs coding for wild type (WT)-eNOS and WT-ET_{B} or a mutant ET_{B} containing a substitution of cysteine with alanine, which is insensitive to oxidant stress, at position 405 (C405A-ET_{B}). Expression of transiently transfected WT-eNOS and WT-ET_{B} or C405A-ET_{B} DNA was established by immunoblotting (Figure 7a). Additionally, immunoblotting of PEG-conjugated maleimide-labeled extracts confirmed that compared to WT-ET_{B}, in which H_{2}O_{2} (200 \mu\text{mol/L} for 20 min) induced the formation of one or two disulfide bonds, C405A-ET_{B} was resistant to the formation of disulfide bonds (Figure 7b). Next, COS-7 cells expressing eNOS and WT-ET_{B} or C405A-ET_{B} were exposed to H_{2}O_{2} (200 \mu\text{mol/L}) for 60 min and ET_{B}-dependent NO\textsuperscript{·} synthesis was assessed. This treatment time point was selected because activation of eNOS by H_{2}O_{2} is time-dependent and attenuated fully within 60 min following exposure of eNOS to H_{2}O_{2}.\textsuperscript{21} After this time, the medium was replaced and cells were treated with ET-1 (10 nM) for 10 min to stimulate ET_{B} signal transduction. Although exposure to H_{2}O_{2} decreased ET-1-stimulated NO\textsuperscript{2−} formation by 78.0\% in WT-ET_{B} transfected cells compared to V-treated cells (p<0.005, n=4), this effect was attenuated significantly in C405A-ET_{B}-transfected cells in which H_{2}O_{2} decreased nitrite.
levels by only 45.0% compared to V-treated cells (p=0.07, n=4) (Figure 7c). Taken together, these data confirm that Cys405 is a redox sensitive, functional cysteinyi thiol whose oxidation to sulfenic acid impairs ETB-dependent NO’ generation.

Discussion

In this study, we found that elevated levels of ET-1 in PAH are associated with increased plasma and lung tissue levels of aldosterone, indicating that the pathophysiological effects attributed to ET-1 may, in part, occur as a result of systemic and local hyperaldosteronism. This conclusion was confirmed in vivo by demonstrating that the mineralocorticoid receptor antagonists spironolactone or eplerenone, given in the absence of ET-1 blockade, decreased PASP, RV hypertrophy, PVRi, and pulmonary vascular remodeling. These effects did not occur as a result of changes in left-sided hemodynamics or differences in plasma ET-1 levels as a result of mineralocorticoid receptor blockade. We demonstrated that ET-1 increases aldosterone levels through a mechanism that involves upregulation of CYP11B2, the rate-limiting enzyme in aldosterone synthesis, in a PGC-1α/SF-dependent manner. The functional consequences of elevated aldosterone levels include increased oxidant stress and decreased bioavailable NO*.

Although diminished NO* levels resulted, in part, from its consumption by ROS as demonstrated by an increase in ONOO- formation, we also found a novel mechanism to explain the aldosterone-mediated decrease in ET-1-stimulated NO* formation: oxidation of cysteinyi thiols (Cys405) in the eNOS-activating region of the ETB receptor (to sulfenic acid and the disulfide form) (Figure 8). Thus, aldosterone contributes to high pulmonary vascular tone by oxidizing cysteinyi thiols in ETB, which, in turn, acts as a redox switch to impair ET-1-stimulated endothelial NO* generation.
Other studies have linked hyperaldosteronism to end-stage disease in idiopathic pulmonary hypertension.\textsuperscript{22} Although studies to date examining the role of mineralocorticoid receptor antagonism in PAH are limited to case reports,\textsuperscript{23} recently a clinical trial was announced to examine the hypothesis that secondary hyperaldosteronism modulates the adverse effects of PAH leading to RV failure. In this study, patients will be treated with spironolactone and the effect of mineralocorticoid receptor blockade on pulmonary hemodynamics and World Health Organization functional class will be examined.\textsuperscript{24} While this study focuses on the efficacy of aldosterone antagonism once PAH is established, our data suggest that aldosterone antagonism may also have benefit when started early in the disease course.

We implicate ET-1 as the stimulus for increased lung tissue and plasma aldosterone levels in PAH. Using the MCT rat model of PAH, we confirmed a 3-fold increase in plasma ET-1 levels, which supports prior studies that reported an increase in ET-1 levels and showed that ET-1 contributed to the pathogenesis of PAH.\textsuperscript{12} The levels of ET-1 that we observed were 1000-fold higher than that required to stimulate aldosterone secretion from adrenocortical cells \textit{in vitro}.\textsuperscript{5} Furthermore, the levels of plasma ET-1 measured in this study, akin to those observed in patients with PAH,\textsuperscript{25} were sufficient to increase plasma aldosterone levels by 442\%. These plasma aldosterone levels are similar to what have been observed in patients with left-sided congestive heart failure and secondary pulmonary hypertension.\textsuperscript{26,27} Moreover, our study likely underestimated the maximal level of hyperaldosteronism achieved in PAH as we measured plasma levels antecedent to advanced stage disease, which is associated with decreased cardiac output \textit{vis-à-vis} cor pulmonale that results in a decline in PASP and compensatory \textit{(over)activation} of the renin-angiotensin-aldosterone system.\textsuperscript{27}

The mechanism by which ET-1 stimulates aldosterone secretion in HPAECs involved
upregulation of the expression of CYP11B2, the rate-limiting enzyme in aldosterone biosynthesis. The concept of extraadrenal aldosterone synthesis by the vascular endothelium remains controversial. CYP11B2 expression in human pulmonary vascular endothelial and smooth muscle cells has been demonstrated and shown to be responsive to angiotensin II or potassium resulting in an increase in local aldosterone production. In contrast, other studies performed in HPAECs failed to show an effect of angiotensin II on CYP11B2 transcription or aldosterone production; however, these studies were performed on cells at passage 14 or older, which may adversely affect global vascular endothelial mRNA and protein expression levels. Moreover, this earlier study measured aldosterone production using an assay with a lower limit of detection reported to be 20 pg/ml. Our study utilized a more sensitive assay with a lower limit of detection of 7 pg/ml. Our observation that CYP11B2 expression was increased via upregulation of PGC-1α and its association with SF at the promoter region of the CYP11B2 gene confirms prior work in adrenal cortex-derived Y1 cells that demonstrated a similar mechanism of CYP11B2 upregulation. We were also able to provide additional evidence for this mechanism by PGC-1α agonism with the thiazolidinedione, pioglitazone. Notwithstanding this finding, the relationship between thioglitazone/PGC-1α and aldosterone remains unresolved. In one study performed in healthy volunteers, pioglitazone treatment for 6 weeks increased circulating aldosterone levels, whereas rosiglitazone has been linked to aldosterone-independent plasma volume expansion through inhibition of sodium transport in the renal collecting duct. More recently, studies performed in adrenocortical H295R cells demonstrated that pioglitazone suppressed CYP11B2 expression in angiotensin II-stimulated cells, and this effect was associated with a modest decrease in aldosterone secretion. Although we now report differing results, we believe they may be attributable to the cell types studied as well as the duration of exposure to
pioglitazone and angiotensin II.

We and others have shown previously that the adverse effects of aldosterone on the systemic vasculature include increased oxidant stress and decreased bioavailable NO\textsuperscript* that promotes endothelial dysfunction and impairs vascular reactivity.\textsuperscript{4, 7, 26, 33} Our finding of increased pulmonary endothelial oxidant stress is not surprising as others have reported an increase in reactive oxygen species production owing to increased NOX1 expression in the small muscularized arteries isolated from the MCT-rat model of PAH.\textsuperscript{34} Here, we focused selectively on oxidant stress in the endothelium and found an increase in expression of NOX4 and the NOX4 subunit p22\textsubscript{phox}, indicating that both NOX1 and NOX4 systems may be operative in PAH. Furthermore, our in vitro studies attribute this increase in NADPH oxidase activity to aldosterone and not to ET-1. Conversely, other studies have reported that ET-1 decreased H\textsubscript{2}O\textsubscript{2} production in fetal pulmonary artery endothelial cells in an ET\textsubscript{B}-dependent manner;\textsuperscript{35} however, these studies were not performed in a timeframe that would afford upregulation of aldosterone synthesis by ET-1.

While there is a consensus opinion that PAH is associated with a decrease in eNOS activity and bioavailable NO\textsuperscript*, several mechanisms have been demonstrated to explain this phenomenon. In the setting of increased oxidant stress, NO\textsuperscript* reacts with superoxide to form ONOO\textsuperscript{•}, which we observed in our study. Other mechanisms include uncoupling of eNOS to form superoxide in preference to NO\textsuperscript*, upregulation of arginase II,\textsuperscript{36} oxidation of tetrahydrobiopterin,\textsuperscript{37} altered S-nitrosoglutathione reductase activity,\textsuperscript{38} and caveolin-1 deficiency.\textsuperscript{39}

We now identify an additional mechanism to explain the decrease in eNOS activity and bioavailable NO\textsuperscript*: dysfunctional ET-1/ET\textsubscript{B}-eNOS signaling in the setting of elevated aldosterone...
levels owing to oxidative posttranslational modification of redox-sensitive cysteinyli thiol(s) in the ET\textsubscript{B} receptor. Oxidation of cysteine residues to form higher oxidative intermediates of cysteine, including sulfinic acid and the disulfide form, is known to occur under conditions of oxidant stress and to regulate protein function.\textsuperscript{40} The ET\textsubscript{B} receptor is a 7 transmembrane domain G-coupled protein receptor with a carboxy-terminal cytoplasmic tail that contains 3 functional cysteine residues: Cys402, Cys403, and Cys405.\textsuperscript{9} Here, we report that these cysteines are oxidatively modified, which is associated with functional consequences for ET\textsubscript{B}-dependent eNOS activity. It is known that these cysteines are subject to posttranslational modification, such as palmitoylation, and site-directed mutagenesis has revealed that palmitoylation is required for coupling with G\textsubscript{i} but not G\textsubscript{q} subunits.\textsuperscript{41} In the current work, the relationship between ET\textsubscript{B} cytoplasmic tail cysteines, palmitoylation, and eNOS was not explored. Interestingly, an \textit{in vitro} study using a synthetic peptide of ET\textsubscript{B} constructed to contain residues 390-409, and, therefore, including the 3 cytoplasmic tail cysteines, was shown to bind eNOS and inhibit its activity with an EC\textsubscript{50} of 3 ± 1.8 \textmu M.\textsuperscript{42} While that study did not examine the cysteinyli residues directly for posttranslational modification(s), our observation that oxidative posttranslational modification of ET\textsubscript{B} Cys405 is associated with impaired ET\textsubscript{B}-dependent NO\textsuperscript{•} generation suggests that this cysteine functions as a redox switch to modulate eNOS activity. In support of this concept is our observation that site-directed mutagenesis of Cys405 rendered ET\textsubscript{B} resistant to oxidant stress-induced sulfinic acid and disulfide formation, and, as a result, improved redox-sensitive signaling. It is, however, important to acknowledge that our methods did not utilize liquid chromatography-mass spectrometry, which is required to definitively characterize this effect. Moreover, our observation that site-directed mutagenesis of Cys405 alone restored ET\textsubscript{B}-dependent NO\textsuperscript{•} generation incompletely in the presence of pathological concentrations of H\textsubscript{2}O\textsubscript{2}.
suggests further that Cys402 and Cys403 may also be redox sensitive cysteiny1 thiols involved in 
ETB-eNOS signal transduction. Our finding that cysteines in the ETB cytoplasmic tail are 
oxidatively modified is also supported by the observation that global sulphydryl (-SH) levels are 
decreased in lung tissue isolated from rats with MCT-induced PAH compared to controls.43

Our results may account, in part, for limitations in the clinical efficacy of endothelin 
receptor antagonism for patients with PAH, viz., currently available ET receptor antagonists are 
believed to improve pulmonary vascular tone primarily by attenuating ETA-mediated pulmonary 
vasoconstriction in pulmonary vascular smooth muscle cells, and, therefore, these drugs do not 
address the potential contribution of abnormal ETB signal transduction in HPAECs to pulmonary 
vascular dysfunction in PAH. Along these lines, our findings suggest that by preventing 
aldosterone-induced oxidation of ETB, mineralocorticoid receptor antagonism preserves normal 
ET-1-ETB vasodilatory signaling to maintain levels of NO\(^{-}\) in HPAECs and attenuate pulmonary 
vascular remodeling in PAH in vivo (Figure 8). Importantly, however, the effect of 
spironolactone/eplerenone on the development of plexiform lesions was not specifically 
addressed in this study. Thus, the role of mineralocorticoid receptor antagonists in modulating 
cardiopulmonary hemodynamic improvements in forms of PAH that are characterized primarily 
by the plexiform arteriopathy remains unknown.

In summary, we identify aldosterone as an unrecognized biological intermediate that 
modulates the adverse vascular effects of ET-1 in PAH. We describe a novel mechanism by 
which to explain the defect in ET-1/ETB-eNOS signaling associated with PAH: oxidative 
posttranslational modification of the ETB receptor. Our observations demonstrate further that a 
class effect exists for mineralocorticoid receptor antagonists and that these agents ameliorate the 
PAH phenotype by improving pulmonary hemodynamics and (mal)adaptive pulmonary vascular
remodeling. Collectively, these findings suggest that mineralocorticoid receptor antagonism in PAH may represent a novel pharmacotherapeutic strategy to improve pulmonary vascular dysfunction and its attendant sequelae in patients with PAH.

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Conflict of Interest Disclosures: None.

References:


21. Hu Z, Chen J, Wei Q, Xia Y. Bidirectional actions of hydrogen peroxide on endothelial nitric-oxide synthase phosphorylation and function: co-commitment and interplay of Akt and...


**Figure Legends:**

**Figure 1.** Elevated levels of ET-1 are associated with hyperaldosteronism in PAH. (a,b) Levels of ET-1 (n=4-6) and (c,d) aldosterone (ALDO) (n=4-8) were measured in plasma and lung tissue
homogenates of Sprague-Dawley rats 25 days following treatment with vehicle control (V) or monocrotaline (MCT) (50 mg/kg). Horizontal line represents the median for each condition.

**Figure 2.** Aldosterone promotes PAH *in vivo*. Sprague-Dawley rats were treated with vehicle control (V) or monocrotaline (MCT) (50 mg/kg) and randomized immediately to V or spironolactone (SP) (25 mg/kg/d) for 25 days (n=6 rats per condition). The contribution of aldosterone to PAH was assessed by (a) right heart catheterization to measure pulmonary artery (assumed to be equivalent to right ventricular) systolic pressure (PASP); echocardiography to assess changes in (b) pulmonary artery acceleration time (PAAT); and, (c) right ventricular (RV) free-wall thickness. Horizontal line represents the mean for each condition.

**Figure 3.** Spironolactone increases pulmonary vascular NO* levels and attenuates pulmonary vascular remodeling in PAH. (a) The effect of spironolactone (SP)(25 mg/kg/d) on pulmonary vascular NO* levels in PAH was assessed by measuring nitrite (NO2) in lung tissue homogenates from Sprague-Dawley rats treated with vehicle control (V) or monocrotaline (MCT) (50 mg/kg) (n=4). (b) Tissue sections were stained with anti-smooth muscle cell α-actin antibody and the number of muscularized distal pulmonary arterioles (red arrows) was counted in 20 consecutive fields per section (100x magnification). Compared to V-treated rats with PAH, spironolactone decreased significantly the number of α-actin-stained muscularized distal pulmonary arterioles (76.0 [64-95] vs. 59.5 [59-61] muscularized pulmonary arterioles/20 high powered fields, p<0.005, n=5). (c) Gomori’s trichrome stain was performed on paraffin-embedded lung sections and perivascular collagen deposition in pulmonary arterioles measuring 20-50 μm located distal to terminal bronchioles (400x magnification) was measured. Compared to V-treated rats with PAH, spironolactone decreased perivascular collagen deposition by 77% (p<0.001, n=4-5 rats
per condition). Representative photomicrographs are shown.

**Figure 4.** The effect of mineralocorticoid receptor antagonism on reversal or prevention of adverse cardiopulmonary hemodynamics in two models of experimental PAH. (a) In a reversal study, Sprague-Dawley rats were randomized to receive vehicle control (V) or spironolactone (SP) (25 mg/kg/d) 14 days following the administration of V or monocrotaline (MCT) (50 mg/kg), and cardiopulmonary hemodynamics were assessed by cardiac catheterization 10 days later. *p<0.02 vs. MCT, n=6 rats per condition; **p<0.04 vs. V, n=4 rats per condition. Data are presented as mean ± S.E. (b) In a prevention study, Sprague-Dawley rats were injected with SU-5416 and exposed to chronic hypoxia for 21 days. Immediately following exposure to hypoxia, rats were randomized to receive standard chow or eplerenone (0.6 gm/1 gm chow) until completion of the study. The effect of eplerenone on pulmonary artery systolic pressure (PASP) was assessed by cardiac catheterization (n=5 rats per condition). HR, heart rate; CI, cardiac index; LVEDP, left ventricular end-diastolic pressure; PVRI, pulmonary vascular resistance index; SVRI, systemic vascular resistance index.

**Figure 5.** ET-1 stimulates PGC-1α-dependent association of SF with CYP11B2 to increase aldosterone levels. (a) The effect of ET-1 on PGC-1α expression was assessed by Western analysis (n=4). (b) Co-immunoprecipitation experiments demonstrated that incubation of HPAECs with ET-1 (10 nM) for 24 h induced the association of PGC-1α with steroidogenesis factor-1 (SF) (n=3). (c) Chromatin immunoprecipitation (n=3) of cell lysates using antibodies to PGC-1α, SF, and immunoglobulin-G (IgG) as a negative control was followed by PCR amplification of the proximal region of the CYP11B2 promoter region containing the gonadotrope-specific element. (d) The functional effect of PGC-1α stimulation on aldosterone
production was assessed in cells treated with the selective PGC-1α agonist pioglitazone (50 μM) for 24 h (n=4), or with ET-1 (10 nM) or angiotensin II (ANG)(10 μM) for 24 h as positive controls. PGC-1α, PPAR-γ co-activator-1α; arb. units, arbitrary units; IP, immunoprecipitation, IB; immunoblot. Data are presented as mean ± S.E.M. Representative blots are shown.

**Figure 6.** Aldosterone decreases ET<sub>B</sub>-dependent synthesis of NO•. (a) HPAECs were exposed to vehicle (V) or aldosterone (ALDO) (10<sup>-7</sup> mol/L) for 24 h in the presence or absence of spironolactone (SP) (10 μM) and NO<sub>2</sub>⁻ formation was assessed. Prior to analysis, cells were exposed to ET-1 (10 nM) for 10 min to stimulate ET<sub>B</sub> signaling (n=4). (b) The effect of ALDO on ET<sub>B</sub>-dependent activation of eNOS was determined (n=4). c.p.m., counts per minute. (c) The effect of ALDO on ET<sub>B</sub>-dependent NO• generation was assessed by measuring total NO• metabolite levels (NOx: NO<sub>2</sub>⁻ + NO<sub>3</sub>⁻) (n=3). (d) HPAECs were exposed to V, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (200 μM) for 20 min, or ALDO (10<sup>-7</sup> mol/L) for 24 h to assess changes to the redox status and *de novo* disulfide bond formation by ET<sub>B</sub> cysteiny1 thiols. For each disulfide formed, a 20-kDa shift in band location of the reduced ET<sub>B</sub> protein occurs on the Western blot using an antibody specific to the region of ET<sub>B</sub> containing Cys405 (n=4). Cyss, disulfide bond. A representative blot is shown. (e) The region of ET<sub>B</sub> containing Cys405 was immunoprecipitated from cells treated with V or ALDO (10<sup>-7</sup> mol/L) for 24 h and immunoblotting was performed to detect differences in protein sulfenic acid levels (R-SOH) (n=3). IP, immunoprecipitation, IB; immunoblot. Data are presented as mean ± S.E.M. Representative blots are shown.

**Figure 7.** Oxidation of Cys405 impairs ET<sub>B</sub>-dependent NO• generation. (a) COS-7 cells were transiently transected with wild type (WT)-eNOS and WT-ET<sub>B</sub> or mutant ET<sub>B</sub> DNA containing a substitution of alanine for cysteine at position 405 (C405A-ET<sub>B</sub>) and protein expression was
confirmed. No Tx, untransfected. (b) Disulfide bond formation was assessed by Western immunoblotting of PEG-conjugated maleimide-labeled cell extracts exposed to H$_2$O$_2$ (200 μmol/L for 20 min). Compared to WT-ET$_B$-transfected cells, in which H$_2$O$_2$ (200 μmol/L for 20 min) induced the formation of 1 or 2 disulfide bonds, C405A-ET$_B$ was resistant to disulfide bond formation (n=4) Cyss, disulfide bond. (c) COS-7 cells expressing WT-eNOS and WT-ET$_B$ or C405A-ET$_B$ were exposed to vehicle (V) control or hydrogen peroxide (H$_2$O$_2$) (200 μmol/L) for 60 min. After that time, the cell culture medium was replaced and cells were treated with ET-1 (10 nM) for 10 min. and nitrite (NO$_2^-$) levels were measured (n=4). Data are presented as mean ± S.E.M. Representative blots are shown.

**Figure 8.** A proposed mechanism by which hyperaldosteronism decreases pulmonary endothelial eNOS activation and NO$^+$ generation in PAH. Hyperaldosteronism (ALDO) in pulmonary arterial hypertension (PAH) may occur via i) endothelin-1 (ET-1)-mediated activation of PPARγ coactivator-1α (PGC-1α)/steroidogenesis factor-1 (SF) to increase CYP11B2 (aldosterone synthase) gene transcription in HPAECs, and/or ii) upregulation of adrenal ALDO synthesis via ET-1 and/or overactivation of the renin-angiotensin pathway. Stimulation of the mineralocorticoid receptor (MR) in HPAECs by ALDO activates NADPH oxidase type 4 (NOX4) to increase levels of hydrogen peroxide (H$_2$O$_2$), which, in turn, oxidatively modifies redox sensitive, functional cysteiny1 thiol(s) in the ET$_B$ receptor (Cys405) to impair ET$_B$-dependent activation of eNOS and decrease synthesis of nitric oxide (NO$^+$). eNOS, endothelial nitric oxide synthase; R-SO$_X$H, higher oxidative intermediaries of cysteine.
Lung Homogenate NO$_2^-$ (μM/ug protein)

- MCT
- MCT+SP

p = 0.048

V
MCT
MCT+SP

SPIRO
-
+

- MCT

40 μm

50 μm
<table>
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<th></th>
<th>V</th>
<th>MCT</th>
<th>MCT+ Sp</th>
</tr>
</thead>
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<tr>
<td>HR (beats/min)</td>
<td>255 ± 12</td>
<td>261 ± 8</td>
<td>259 ± 14</td>
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<td>MAP (mmHg)</td>
<td>67.1 ± 2.9</td>
<td>62.5 ± 2.2</td>
<td>60.7 ± 5.4</td>
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<tr>
<td>CI (ml/min/g)</td>
<td>138.3 ± 39.3</td>
<td>86.9 ± 8.5</td>
<td>94.3 ± 15.3</td>
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<tr>
<td>LVEDP (mmHg)</td>
<td>3.2 ± 0.6</td>
<td>1.8 ± 0.5</td>
<td>2.8 ± 0.9</td>
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<tr>
<td>PASP (mmHg)</td>
<td>26.1 ± 2.2</td>
<td>60.3 ± 5.2</td>
<td>39.5 ± 4.1*</td>
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<td>PVRi (mmHg<em>min</em>g/ml)</td>
<td>10.4 ± 3.0</td>
<td>35.9 ± 3.2**</td>
<td>21.5 ± 3.2*</td>
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<td>SVRi (mmHg<em>min</em>g/ml)</td>
<td>73.8 ± 7.2</td>
<td>68.4 ± 6.0</td>
<td>66.4 ± 10.7</td>
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**PASP**

- Eplerenone
- +Eplerenone

SU-5416/Hypoxia

p=0.048

Normal PASP
a) PGC-1α and Actin Densitometry

- Densitometry (% Control)
  - V
  - 1
  - 10
  - 100

b) IP: PGC-1α

- Densitometry (arb. units)
  - p<0.01

b) ET-1 (10 nM)

- V
  - ET-1 (10 nM)
  - PIO (50 μM)
  - ANG (10 μM)

- ALDO Levels (pg/μg protein)
  - p<0.01
  - p<0.02
  - p<0.001
a) 

\[ \text{NO}_2^- \text{ (\% Control)} \]

\[ \text{ALDO (10}^{-7} \text{ mol/L)} (10 \mu\text{M}) \]

\[ \text{ET-1 (10 nM)} \]

\[ \text{SP} \]

\[ \text{ALDO +SP} \]

b) 

\[ \text{\[^{14}\text{C} \] L-Citrulline} \text{ (c.p.m./mg protein)} \]

\[ \text{V} \]

\[ \text{ALDO} \]

\[ \text{ET-1 (10 nM)} \]

\[ \text{ET-1 (10 nM) +ALDO} \]

c) 

\[ \text{NOx (\muM/\mug protein)} \]

\[ \text{V} \]

\[ \text{ALDO (10}^{-7} \text{ mol/L)} (10 \mu\text{M}) \]

\[ \text{SP} \]

\[ \text{ALDO +SP} \]

d) 

\[ \text{CySS} \]

\[ \text{CySS} \]

\[ \text{Reduced} \]

e) 

\[ \text{ET}_B-\text{SO}_2 \]

\[ \text{IP: ET}_B \]

\[ \text{IB: Anti-cysteine sulfinic acid} \]
a) 

Input 

\[ \begin{array}{l}
\text{eNOS} \\
\text{ET}_B
\end{array} \]

\[ \begin{array}{l}
\text{No Tx} \\
\text{WT-ET}_B \\
\text{C405A-ET}_B
\end{array} \]

b) 

\[ \begin{array}{l}
\text{C405A} \\
\text{WT}
\end{array} \]

\[ \begin{array}{l}
\text{H}_2\text{O}_2^- \\
\text{+H}_2\text{O}_2
\end{array} \]

\[ \begin{array}{l}
\text{kDa} \\
100 \\
75 \\
50
\end{array} \]

\[ \begin{array}{l}
\text{Reduced} \\
\text{CySS} \\
\text{CySS}
\end{array} \]

c) 

\[ \begin{array}{l}
\text{NO}_2^- (\% \text{ Control}) \\
\text{WT} \\
\text{C405A}
\end{array} \]

\[ \begin{array}{l}
\text{V} \\
\text{H}_2\text{O}_2 (200 \mu\text{M})
\end{array} \]

\[ \begin{array}{l}
p<0.005 \\
p=0.02 \\
p=0.07
\end{array} \]
Aldosterone Inactivates the Endothelin-B Receptor via a Cysteinyl Thiol Redox Switch to Decrease Pulmonary Endothelial Nitric Oxide Levels and Modulate Pulmonary Arterial Hypertension

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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 \(\mu\)M/l), or angiotensin II (10 \(\mu\)M) for 24 h (all from Sigma-Aldrich). In selected experiments, cells were pre-treated for 6 h with BQ-788 (1.5 \(\mu\)M) (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\(^\cdot\) metabolites. Nitrite (NO\(_2^\cdot\)) 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 \(\times\) \(g\) at 4°C for 20 min. The supernatant was ultrafiltered using a 30 kDa molecular weight filter (Millipore), 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<sub>A</sub> (Santa Cruz), anti-ET<sub>B</sub> (Santa Cruz), anti-NOX4 (Santa Cruz), anti-p22<sup>phox</sup> (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<sub>A</sub> expression, purified ET<sub>A</sub> 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<sub>B</sub> 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 immunoprecipitation 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. The primers used were:
forward 5´-GAGAAAGGAGAGGCCAGGTC-3´ and reverse 5´-CAGGAACCTGCTCTGGAAAC-3´. CYP11B2 primers used for PCR were forward: 5´GAGAAAAGGAGAGGCCAGGTC-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 BH4, 2 µM flavin adenine dinucleotide, and 2 µM flavin adenine mononucleotide) was added to the cell pellets. The cells were lysed and exposed to [14C] 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.

**Site-directed mutagenesis and transfection.** cDNAs encoding wild type (WT) eNOS and WT-ETB from human were cloned into the mammalian expression vector pCMV6 (Origene). The C405A-ETB mutant was purchased from Genewiz (South Plainfield, NJ). COS-7 cells, which do not express endogenous eNOS or ETB, were plated in P100 tissue
culture dishes and transfected with 10 μg of WT-eNOS and WT-ET<sub>B</sub> or C405A-ET<sub>B</sub> DNA for 4.5 h with Lipofectamine 2000<sup>TM</sup> 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<sub>2</sub>O through the trachea as described previously.<sup>3</sup> 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.<sup>4</sup> 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,<sup>3</sup> 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<sup>TM</sup> microscope and images were acquired by the Picture Taker<sup>TM</sup> 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. 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. 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. 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 \([\text{mean pulmonary artery pressure} - \text{LVEDP}] / \text{CI}\) and systemic vascular resistance index was calculated as \([\text{mean arterial pressure} - \text{mean right atrial pressure}] / \text{CI}\).

*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).

REFERENCES


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 ETB-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 ETB 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_{2}O_{2}) levels were assessed by measuring Amplex Red fluorescence (n=3). To determine a potential source of H_{2}O_{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 ETA or ETB 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\textsubscript{A} and ET\textsubscript{B}. For ET\textsubscript{A} analyses, purified recombinant ET\textsubscript{A} receptor protein was used as a positive control. Representative blots are shown.

**Supplemental Figure 10. Aldosterone increases peroxynitrite formation to decrease NO\textsubscript{2}/NO\textsubscript{3}.** (a) HPAECs were treated with vehicle control (V) or aldosterone (ALDO) (10\textsuperscript{-7} mol/L) for 24 h, and stimulated with ET-1 (10 nM) for 10 min immediately prior to measuring NO\textsubscript{2}/NO\textsubscript{3}. The contribution of ALDO to changes in NO\textsubscript{2}/NO\textsubscript{3} was confirmed by co-incubation of ALDO-treated cells with spironolactone (SP)(10 \textmu M)(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

- **CYP11B2**
- **Actin**

The bar graph shows a densitometry assay comparing V and MCT conditions. The densitometry values (arb. units) are significantly different with a p-value of <0.03.
Supplemental Figure 2
<table>
<thead>
<tr>
<th>Condition</th>
<th>RV/LV+Septum Weight</th>
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</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>SP</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>MCT</td>
<td>0.43 ± 0.07*</td>
</tr>
<tr>
<td>MCT+SP</td>
<td>0.35 ± 0.04</td>
</tr>
</tbody>
</table>

Supplemental Figure 3
Supplemental Figure 5
Supplemental Figure 6
Supplemental Figure 7
Supplemental Figure 8

(a) 

(b) 

(c) 

p<0.001

p<0.01

p=0.12

p<0.02

p<0.001

p<0.001
Supplemental Figure 9

ALDO
(10^{-7} \text{ mol/l})
Recombinant ET_{A}
Supplemental Figure 10

**a)**

- **NO$_2^-$/NO$_3^-$ Levels:
  - V: 1
  - V (ALDO $10^{-7}$ mol/L): 2
  - SP: 3
  - ALDO + SP: 4

  - p-values: p=0.8, p=0.48, p=0.19, p<0.04

**b)**

- Luminosity (arb. units):
  - ALDO (10$^{-7}$ mol/L) + ET-1 (10 nM): 60
  - V: 30
  - ALDO: 40
  - ET-1 (10 nM): 50

- p-values: p<0.001, p<0.02
L’aldostérone inactive le récepteur à l’endothéline B par l’intermédiaire d’une substitution redox des cysténil-thiols, ce qui diminue la teneur de l’endothélium pulmonaire en monoxyde d’azote et favorise l’hypertension artérielle pulmonaire

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**Contexte**—L’hypertension artérielle pulmonaire (HAP) se caractérise notamment par une diminution de la production endothéliale de monoxyde d’azote (NO) et par une élévation du taux d’endothéline 1. Sachant que celle-ci stimulate la monoxyde d’azote synthétisée endothéliale (eNOS) par l’intermédiaire des récepteurs à l’endothéline B (ETb), il est permis de penser que cette voie de signalisation est altérée dans l’HAP. L’endothéline 1 stimule également la synthèse surnéanaillée de l’aldostérone ; au niveau des vaisseaux périphériques, l’hyperaldostéronisme induit une dysfonction vasculaire en augmentant la formation endothéliale d’espèces réactives de l’oxygène et en diminuant les taux de NO. Nous avons donc formulé l’hypothèse selon laquelle l’aldostérone favoriserait l’HAP en perturbant la signalisation de l’ETb et de l’eNOS par un mécanisme consistant à accroître le stress oxydatif au sein de l’endothélium pulmonaire.

**Méthodes et résultats**—Chez le rat atteint d’HAP, l’élévation du taux d’endothéline 1 va de pair avec l’augmentation des concentrations en aldostérone dans le sang et les tissus pulmonaires et avec la diminution des taux pulmonaires de métabolites du NO, cela en l’absence de toute insuffisance cardiaque gauche. Nous montrons que, dans les cellules endothéliales d’artères pulmonaires humaines, l’endothéline 1 augmente le taux d’aldostérone par un processus de stimulation de l’aldostérone synthétase qui est médie à la fois par le co-activateur IκB des récepteurs gamma activés par les proliférations de peroxyxomes et par le facteur stéroidogénique de type 1. L’aldostérone augmente également la production d’espèces réactives de l’oxygène, ce qui, en oxydant les cysténil-thiols au sein de la région de l’ETb, qui régit l’activation de l’eNOS, diminue l’activité de cette enzyme médiate par l’endothéline 1. Le remplacement de la Cys405 de l’ETb par une alanine a amélioré la synthèse du NO dépendante de ce médiateur en situation de stress oxydatif, ce qui confirme que la Cys405 est un thiol redox-sensible qui est indispensable à la voie de signalisation de l’ETb-eNOS. Dans les cellules endothéliales d’artères pulmonaires humaines, l’inhibition des récepteurs aux minéralocorticoides par la spironolactone a diminué la libération d’espèces réactives de l’oxygène médieée par l’aldostérone et restauré la production de NO dépendante de l’ETb. Dans deux modèles animaux d’HAP in vivo, l’administration de spironolactone ou d’éplérénone a prévenu ou aboli le remodelage vasculaire pulmonaire et amélioré l’hémodynamique cardio-pulmonaire.

**Conclusions**—Nos observations démontrent que l’aldostérone induit une modification de l’oxydoréduction des cysténil-thiols de l’ETb qui a pour effet de diminuer la formation de NO à partir de l’endothélium pulmonaire et de favoriser l’HAP. (Traduit de l’anglais : Aldosterone Inactivates the Endothelin-B Receptor via a Cysteinyli Thiol Redox Switch to Decrease Pulmonary Endothelial Nitric Oxide Levels and Modulate Pulmonary Arterial Hypertension. Circulation. 2012;126:963–974.)

**Mots clés** : endothéline ■ monoxyde d’azote ■ cardiopathie pulmonaire ■ aldostérone ■ processus biochimiques d’oxydoréduction

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**Risque cardiovasculaire associé à l’entraînement aérobie chez les patients coronariens qu’il est d’intensité élevée ou modérée**

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**Contexte**—Il a été établi que, lorsqu’il est pratiqué de façon relativement énergique, l’entraînement physique augmente fortement la capacité aérobie et a un effet cardioprotecteur plus marqué que lorsqu’il est effectué à un rythme modéré. Une corrélation inverse a également été mise en évidence entre le risque d’événement coronaire et l’intensité relative de l’activité physique, indépendamment du volume total de cette dernière. Bien que la pratique d’un entraînement physique d’intensité supérieure constitue un moyen efficace de réduire l’incidence des événements cardiovasculaires, il semblerait toutefois qu’une activité physique trop énergique ait pour effet d’augmenter fortement les risques aigus de mort subite et d’infarctus du myocarde chez les individus prédisposés. Cette éventualité pourrait justifier de reconsidérer les programmes de réhabilitation cardiaque.

**Méthodes et résultats**—Nous avons évalué le risque d’événement cardiovasculaire encouru du fait de la mise en application d’un programme d’entraînement fractionné selon qu’il était d’intensité élevée ou modérée chez 4 846 patients coronariens pris en charge dans trois centres de réhabilitation cardiaque norvégiens. Sur une durée totale de 175 820 heures d’entraînement physique pendant lesquelles tous les patients avaient effectué les deux types d’exercices, nous avons recensé un arrêt cardiaque fatal survenu lors d’une période d’entraînement d’intensité modérée (129 456 heures d’exercice physique) et deux arrêts...