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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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 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-sided heart failure. In human pulmonary artery endothelial cells, endothelin-1 increased aldosterone levels via peroxisome proliferator-activated receptor gamma coactivator-1α/steroidogenesis factor-1–dependent upregulation of aldosterone synthase. Aldosterone also increased reactive oxygen species production, which oxidatively modified cysteinyl 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 human pulmonary artery endothelial cells, mineralocorticoid receptor antagonism with spironolactone decreased aldosterone-mediated reactive oxygen species generation and restored ET_B-dependent NO· production. Spironolactone or eplerenone prevented or reversed pulmonary vascular remodeling and improved cardiopulmonary hemodynamics in 2 animal models of PAH in vivo.

Conclusions—Our findings demonstrate that aldosterone modulates an ET_B cysteinyl thiol redox switch to decrease pulmonary endothelium-derived NO· and promote PAH. (Circulation. 2012;126:963-974.)

Key Words: endothelin ■ nitric oxide ■ pulmonary heart disease ■ aldosterone ■ redox biochemistry

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 vascular constriction, muscularization of pulmonary arterioles, and perivascular fibrosis. 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. 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.

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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 de-
creases 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 cysteinyl 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 cysteinyl 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-only Data Supplement.

**Cell Culture and Treatments**

Human pulmonary artery endothelial cells (HPAECs) (Lonza) (male donors) were grown to confluence by using phenol-free EGM-2 medium supplemented with 5% fetal bovine serum at 37°C, 5% CO\(_2\). Cells were passaged twice weekly by using 0.5% trypsin/EDTA, and experiments were performed on cells from passages 4 to 10. Aldosterone (Steroloids) and ET-1 (1–100 nmol/L) (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (10 nmol/L) and deoxygenated water, respectively, which served as vehicle controls. Cells were dissolved in dimethyl sulfoxide (10 nmol/L) and deoxygenated water, respectively, which served as vehicle additions. Aldosterone (10\(^{-9}\)–10\(^{-7}\) mol/L) for 24 hours and, in selected experiments, coinubated with the mineralocorticoid receptor inhibitor spironolactone (10 \(\mu\)mol/L) (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.\(^7\) In brief, protein extracts from cells were lysed in alkylyating buffer containing 0.1 mol/L Tris-HCl, pH 6.8, 1% SDS, 100 mmol/L iodoacetamide, and 100 mmol/L N-ethylmaleimide, and sonicated on ice for 5 minutes followed by a 30-minute incubation at 25°C. Alkylyated proteins were then precipitated with acetone. Proteins were resuspended in 50 \(\mu\)L of 0.1 mol/L Tris-HCl, pH 7.4, 1% SDS, and disulfides were reduced with 5 mmol/L tris(2-carboxyethyl)phosphine hydrochloride. After a 20-minute incubation at 25°C, tris(2-carboxyethyl)phosphine hydrochloride was removed with a Micro Bio-Spin column 6 (Bio-Rad), and 1% SDS was added to the eluent. The cysteines previously participating in a disulfide bond, now reduced, were labeled with 1 mmol/L polyethylene glycol–conjugated maleimide (molecular mass 10 kDa) (Fluka). After a 1-hour incubation at 25°C, proteins were precipitated with acetone, resuspended in 50 \(\mu\)L of nonreducing SDS electrophoresis buffer, and boiled for 10 minutes. Protein samples were then size-fractionated electrophoretically by 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: CCWQCQSFEEKQSLEREQSCLFK-14.1 KANDHGYDNFRRSNKYYSSS (Santa Cruz Biotechnology). Bands were visualized by using the enhanced chemiluminescence detection method.\(^4\)

**Animal Models 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 randomly assigned to spironolactone (25 mg/kg per day) (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 to 25 days until hemodynamic and tissue analyses were performed. For the reversal study, a second experiment was performed in which rats were randomly assigned 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-2 inhibitor SU-5416 (20 mg/kg; Sigma) and exposed immediately to chronic hypoxia (barometric pressure, 410 mm Hg; inspired O\(_2\) tension 76 mm Hg; inspired O\(_2\) tension 76 mm Hg) as described previously.\(^10\) Rats were randomly assigned to either the selective mineralocorticoid receptor antagonist eplerenone (0.6 mg/L standard chow; Test Diet Inc) or standard chow as a control.\(^11\) Hemodynamic and tissue analyses were performed on all rats 21 days following exposure to chronic hypoxia.

**Statistical Analysis**

Normality was tested by 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 2 independent groups. Comparisons between multiple groups were made by using a 1-way ANOVA with post hoc analysis performed using the protected Fisher least-significant difference test. When data were not normally distributed, data were presented as median and range, and comparisons between 2 groups were made with use of 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 MCT model of PAH was selected initially to test the hypothesis that hyperaldosteronism is present in PAH in vivo, because MCT is believed to induce pulmonary hypertension through a mechanism that involves elevated levels of the aldosterone secretagogue ET-1.\(^12\) Transthoracic echocardiography demonstrated that, in comparison with vehicle control (V)–treated rats, MCT decreased the pulmonary artery flow acceleration time (35.4±2.6 versus 14.1±1.2 ms, \(P<0.005\), \(n=6\)) and increased right ventricular...
(RV) free-wall thickness (0.58±0.05 versus 1.1±0.05 mm, \(P<0.03\), \(n=6\)). 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 versus 89.3±5.3 mm Hg, \(P<0.01\), \(n=6\)). In rats with PAH, there was a 274% increase in ET-1 levels in plasma (1.76 ng/mL versus 89.3 ng/mL, \(P<0.01\), \(n=6\)) and an increase in aldosterone (ALDO) (nondetectable to 4.82 versus 4.83 pg/µg protein, \(P=0.03\), \(n=4\)) (Figure 1A and 1B), which correlated with an increase in aldosterone levels of 442% (357.5 pg/µg protein versus 1831 pg/µg protein, \(P<0.04\), \(n=4\)) in plasma and lung tissue, respectively (Figure 1C and 1D).

The finding of increased aldosterone levels in lung tissue suggested that PAH may be associated with extra-adrenal aldosterone synthesis. To determine whether 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 before organ harvest, protein levels of CYP11B2 were increased significantly in lung tissue of rats with PAH in comparison with controls (483±75 versus 1319±226 arbitrary units, \(P<0.03\), \(n=4\)) (online-only Data Supplement 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.

Figure 1. (A, B) Elevated levels of ET-1 are associated with hyperaldosteronism in PAH. 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. PAH indicates pulmonary arterial hypertension; ET-1, endothelin-1.

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

Aldosterone Increases Pulmonary Artery Pressure and Pulmonary Vascular Remodeling in PAH in Vivo

To determine whether hyperaldosteronism contributes to increased pulmonary artery pressure in PAH in vivo and mineralocorticoid receptor antagonism could prevent PAH, rats were treated with spironolactone (25 mg/kg per day) 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 or left ventricular end-diastolic pressure (online-only Data Supplement Figure II), spironolactone decreased PASP significantly in PAH (89.3±5.2 versus 69.5±5.4 mm Hg, \(P<0.01\), \(n=6\)) (Figure 2A), which was confirmed by an increase in pulmonary artery flow acceleration time (14.1±1.2 versus 22.3±2.2 ms, \(P<0.005\), \(n=6\)) (Figure 2B). Spironolactone also decreased RV free-wall thickness (1.07±0.05 versus 0.86±0.03 mm, \(P<0.03\), \(n=6\)) (Figure 2C) and RV weight (0.43±0.07 versus 0.35±0.04 RV weight/LV septum weight, \(P=0.22\), \(n=5\)) (online-only Data Supplement Figure III). Notably, these findings were associated with increased levels of the stable NO metabolite, nitrite (NO\(_2^–\)), in lung tissue specimens harvested from spironolactone-treated rats with PAH in comparison with V-treated rats with PAH (759±55 versus 506±86 µmol/L/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 μm) as demonstrated by immunohistochemical staining for smooth muscle α-actin. In comparison with V-treated rats with PAH, spironolactone decreased the number of α-actin-stained muscularized distal pulmonary arterioles (76.0 [64–95] versus 59.5 [59–61] muscularized pulmonary arterioles per 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] versus 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, in comparison with 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. PAH indicates pulmonary arteriolar hypertension; SPIRO, spironolactone.

To determine whether aldosterone antagonism reverses established PAH, a second study was performed in which V or spironolactone (25 mg/kg per day) was initiated 14 days following administration of MCT, a time point associated with histological evidence of MCT-induced inflammatory injury to distal pulmonary arterioles (online-only Data Supplement Figure V). In comparison with 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 (35.9 ± 3.2 versus 21.5 ± 3.2 mm Hg × min × g/mL, n = 4, \( P < 0.02 \)) and PASP (60.3 ± 5.2 versus 39.5 ± 4.1 mm Hg, n = 6, \( P < 0.005 \)) without changes to heart rate, cardiac index, left ventricular end-diastolic pressure, mean arterial pressure, or indexed systemic vascular resistance (Figure 4A).

Next, to confirm the role of aldosterone in a second animal model of PAH and to determine whether 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. In comparison with normal rats, plasma aldosterone levels were increased by 397% in SU-5416/hypoxia-induced PAH (352.7 [223.0–557.2] versus 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 indexed pulmonary vascular resistance (64.6 ± 21.4 versus 43.9 ± 8.7 mm Hg × min × g/mL, online-only Data Supplement Figure V).
ET-1 Increases Aldosterone Levels in Pulmonary Artery Endothelial Cells

Because 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 extra-adrenal aldosterone synthesis in HPAECs in vitro. We first confirmed that, in comparison with V-treated cells, ET-1 (1, 10, 100 nmol/L) increased CYP11B2 protein expression levels (157.3 ± 27.5 versus 180.4 ± 13.4 versus 234.8 ± 4.3% control, respectively, \( P < 0.02, n = 3 \)) (online-only Data Supplement Figure VIA), which correlated with a concentration-dependent increase in aldosterone levels detected in the cell culture medium (241.1 ± 44.8 versus 283.5 ± 94.7 versus 396.0 ± 116.5% control, respectively, \( P < 0.04, n = 4 \)) (online-only Data Supplement Figure VIB). Consistent with previous reports in dispersed adrenal cortical cells,\(^6,14\) ET-1 increased aldosterone levels via activation of the ET\(_B\) receptor in HPAECs (online-only Data Supplement Figure VII).

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 peroxisome proliferator-activated receptor gamma coactivator-1\(\alpha\) (PGC-1\(\alpha\)) interacts with the nuclear receptor protein steroidogenesis factor-1 (SF) to regulate CYP11B2 gene transcription and induce aldosterone synthesis.\(^15\) Therefore, to determine whether ET-1 increased aldosterone synthase protein levels by this mechanism in HPAECs, we first explored the effect of ET-1 on PGC-1\(\alpha\) and SF protein expression levels in these cells. In comparison with V-treated cells, exposure to ET-1 (1, 10, 100 nmol/L) for 24 hours induced a concentration-dependent increase in PGC-1\(\alpha\) protein expression levels (176.5 ± 52.8 versus 224.7 ± 68.1 versus 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-1\(\alpha\) and SF as demonstrated by coimmunoprecipitation (1260 ± 104 versus 160 ± 71 arbitrary units, \( P < 0.001, n = 3 \)) (Figure 5B).

We next performed a chromatin immunoprecipitation assay to assess the effect of ET-1 (10 nmol/L) for 24 hours on PGC-1\(\alpha\) and/or SF association with the CYP11B2 promoter. PGC-1\(\alpha\) alone did not bind to the CYP11B2 promoter in cells treated with either V or ET-1; however, in comparison with V, ET-1 induced a significant increase in SF binding to the CYP11B2 promoter (16.3 ± 9.8 versus 61.6 ± 3.9 arbitrary units, \( P < 0.03, n = 3 \)) (Figure 5C). Collectively, these data indicate that ET-1 stimulates PGC-1\(\alpha\) 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-1\(\alpha\) stimulation is linked functionally to aldosterone synthesis in cells treated with the selective PGC-1\(\alpha\) agonist pioglitazone (50 \(\mu\)mol/L) for 24 hours, which, in comparison with V, increased aldosterone levels by 365\% (\( P < 0.001, n = 3 \)) (Figure 5D). Thus, ET-1 increases extra-adrenal aldosterone synthesis in endothelial cells via upregulation of CYP11B2 in a PGC1-\(\alpha\)/SF–dependent manner.

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Figure 4. The effect of mineralocorticoid receptor antagonism on reversal or prevention of adverse cardiopulmonary hemodynamics in 2 models of experimental PAH. A, In a reversal study, Sprague-Dawley rats were randomly assigned to receive vehicle control (V) or spironolactone (SP) (25 mg/kg per day) 14 days after the administration of V or monocrotaline (MCT) (50 mg/kg), and cardiopulmonary hemodynamics were assessed by cardiac catheterization 10 days later. \(^{P < 0.02}\) versus MCT, \( n = 6 \) rats per condition; \(^{*P < 0.04}\) versus V, \( n = 4 \) rats per condition. Data are presented as mean ± SE. 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 randomly assigned to receive standard chow or eplerenone (0.6 mg/1 g 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 indicates heart rate; MAP, mean arterial pressure; CI, cardiac index; LVEDP, left ventricular end-diastolic pressure; PVRI, pulmonary vascular resistance index; and SVRI, systemic vascular resistance index.
**Aldosterone Increases Oxidant Stress in HPAECs**

Next, to determine whether 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 to 36 hours and H_{2}O_{2} levels were measured by Amplex Red assay. In comparison with V-treated cells, maximal H_{2}O_{2} accumulation was observed in cells treated with aldosterone (10^{-7} mol/L) for 24 hours (65.4±1.6 versus 100.6±3.5 μmol/L/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 H_{2}O_{2} formation was due to mineralocorticoid receptor activation (online-only Data Supplement Figure VIII A).

Because no further H_{2}O_{2} generation was observed in aldosterone-treated cells beyond 24 hours, 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; because ET-1 (10 nmol/L) had no effect on H_{2}O_{2} levels in comparison with 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. The primary product of NOX4 activation is H_{2}O_{2}, and its formation is closely aligned to changes in NOX4 protein expression. Therefore, we examined the effect of aldosterone on NOX4 expression in HPAECs as a potential mechanism by which to explain the aldosterone-mediated increase in H_{2}O_{2} formation. In comparison with V-treated cells, aldosterone (10^{-7}, 10^{-8}, 10^{-7} mol/L) increased protein levels of NOX4 (134.6±16.5 versus 146.3±12.4 ± versus 157.0±4.4% control, respectively, P<0.02, n=3) and of p22phox (1009.4±167.0 versus 961±226.2 versus 829.5±295.6% control, respectively, P<0.01, n=3), a NOX4 subunit that is required for NOX4-mediated H_{2}O_{2} formation, in a concentration-dependent manner (online-only Data Supplement Figure VIII B and VIII C).

**Aldosterone Decreases ET_{B}-Dependent Activation of eNOS and NO\textsuperscript{-} Levels**

We next investigated the effect of ET_{B} receptor activation by ET-1 (10 nmol/L) on levels of the NO metabolite nitrite (NO\textsuperscript{-}). In comparison with V-treated cells, ET-1 increased NO\textsuperscript{-} generation with a maximum effect observed at 10 minutes (29.4±3.6 versus 139.4±31.8 μmol/L/μg protein, P<0.001, n=3). We then evaluated the effect of aldosterone on ET_{B}-stimulated NO levels. Without influencing protein expression of ET_{B}, or inducing expression of ET_{A} (which is not constitutively expressed in HPAECs), exposure to aldosterone (10^{-7} mol/L) for 24 hours decreased ET_{B}-mediated NO\textsuperscript{-} levels by 60.3% (P<0.01, n=3). Coincubation with spironolactone (10 μmol/L) restored NO\textsuperscript{-} levels to those observed in cells stimulated with ET-1 in the absence of aldosterone (Figure 6A).
Figure 6. Aldosterone decreases ET_{B}-dependent synthesis of nitric oxide (NO). A, HPAECs were exposed to vehicle (V) or aldosterone (ALDO) (10^{-7} mol/L) for 24 hours in the presence or absence of spironolactone (SP) (10 µmol/L) and NO\textsubscript{2}⁻ formation was assessed. Before analysis, cells were exposed to ET-1 (10 nmol/L) for 10 minutes to stimulate ET\textsubscript{B} signaling (n=4). B, The effect of aldosterone (ALDO) on ET\textsubscript{B}-dependent activation of endothelial nitric oxide synthase (eNOS) was determined (n=4). C, The effect of ALDO on ET\textsubscript{B}-dependent NO· generation was assessed by measuring total NO· metabolite levels (NO\textsubscript{x}: NO\textsubscript{2}⁻ + NO\textsubscript{3}⁻) (n=3). D, HPAECs were exposed to V, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) (200 µmol/L) for 20 minutes, or ALDO (10^{-7} mol/L) for 24 hours to assess changes to the redox status and de novo disulfide bond formation by ET\textsubscript{B} cysteinyll thiols. For each disulfide formed, a 20-kDa shift in band location of the reduced ET\textsubscript{B} protein occurs on the Western blot using an antibody specific to the region of ET\textsubscript{B} containing Cys405 (n=4). A representative blot is shown. E, The region of ET\textsubscript{B} containing Cys405 was immunoprecipitated from cells treated with V or ALDO (10^{-7} mol/L) for 24 hours, and immunoblotting was performed to detect differences in protein sulfenic acid levels (R-SOH) (n=3). Data are presented as mean±SEM. Representative blots are shown. HPAECs indicates human pulmonary artery endothelial cells; ET\textsubscript{B}, endothelin-B receptor; ET-1, endothelin-1; c.p.m., counts per minute; Cyss, disulfide bond; IP, immunoprecipitation, and IB; immunoblot.

We and others have demonstrated previously that, in the absence of oxidant stress, NO metabolism to NO\textsubscript{3}⁻ and nitrate (NO\textsubscript{3}⁻) occurs in a ratio that favors NO\textsubscript{2}⁻ by approximately 2:1, but that this ratio shifts in favor of increased NO\textsubscript{3}⁻ formation in the presence of the superoxide anion, owing to the interaction of NO\textsubscript{2}⁻ with the superoxide anion to generate peroxynitrite (O\textsubscript{2}⁻NO\textsubscript{2}⁻) or via tautomerization of peroxynitrite (ONOO\textsuperscript{−}) to NO\textsubscript{3}⁻.\textsuperscript{19} In HPAECs, ET-1 alone did not affect the NO\textsubscript{2}⁻/NO\textsubscript{3}⁻ ratio significantly compared with V. In contrast, exposure to aldosterone decreased the NO\textsubscript{2}⁻/NO\textsubscript{3}⁻ ratio by 62% in ET-1–stimulated cells, which was restored fully by coinoculation of aldosterone with spironolactone (P<0.04, n=3) (online-only Data Supplement Figure XA). This effect was likely mediated by increased ONOO\textsuperscript{−} formation as aldosterone-treated HPAECs had increased levels of 3-nitrotyrosine, a marker of ONOO\textsuperscript{−}, in comparison with cells stimulated with V or ET-1 alone (24.1±3.3 versus 31.2±3.2 versus 46.8±6.6 arbitrary units, P<0.02, n=5) (online-only Data Supplement Figure XB).

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 mmol/L)–stimulated cells (18.5±3.5 versus 7.6±2.4 [14C]-L-citrulline cpm/mg protein, n=3, P<0.02) (Figure 6B), leading to a decrease in total NO· metabolite (NO\textsubscript{x}: NO\textsubscript{2}⁻ + NO\textsubscript{3}⁻) formation (157.9±12.7 versus 103.4±12.2 µmol/L per µg protein, P<0.01, n=3). Coinoculation with spironolactone increased NO· metabolite 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\textsuperscript{−} formation, and by oxi-dizing NO\textsubscript{2}⁻ to NO\textsubscript{3}⁻.

**Aldosterone Decreases ET\textsubscript{B}-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 ET\textsubscript{B} receptor function. Because aldosterone induced H\textsubscript{2}O\textsubscript{2} formation and ET\textsubscript{B} contains functionally essential cysteinyll thiols resides in its eNOS-activating region, it is plausible that aldosterone may induce an oxidative posttranslational modification of ET\textsubscript{B} that influences receptor func-
To examine ETB for oxidation of cysteinyl thiols, protein extracts from HPAECs were treated with V, aldosterone (10^{-7} mol/L) for 24 hours, or H_{2}O_{2} (200 μmol/L) for 20 minutes, and free thiols were blocked with iodoacetamide and N-ethylmaleimide. Disulfides were reduced with tris(2-carboxyethyl)phosphine hydrochloride, and previously oxidized (now reduced) cysteines were labeled with polyethylene glycol–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 H_{2}O_{2}, or aldosterone, indicating the de novo formation of 1 or 2 disulfide bonds under these conditions of increased oxidant stress (Figure 6D).

To confirm that oxidative modification of Cys405 has functional implications for ETB-dependent NO generation, we transiently transfected COS-7 cells with human DNAs coding for wild-type (WT) eNOS and WT-ETB or a mutant ETB containing a substitution of cysteine with alanine, which is insensitive to oxidant stress, at position 405 (C405A-ETB). Expression of transiently transfected WT-eNOS and WT-ETB or C405A-ETB DNA was established by immunoblotting (Figure 7A). Additionally, immunoblotting of polyethylene glycol–conjugated maleimide-labeled extracts confirmed that, in comparison with WT-ETB, in which H_{2}O_{2} (200 μmol/L for 20 minutes) induced the formation of 1 or 2 disulfide bonds, C405A-ETB was resistant to the formation of disulfide bonds (Figure 7B). Next, COS-7 cells expressing eNOS and WT-ETB or C405A-ETB were exposed to H_{2}O_{2} (200 μmol/L) for 60 minutes, and ETB-dependent NO 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 minutes 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 nmol/L) for 10 minutes to stimulate ETB signal transduction. Although exposure to H_{2}O_{2} decreased ET-1–stimulated NO\textsubscript{2}^{-} formation by 78.0% in WT-ETB–transfected cells in comparison with V-treated cells (P<0.005, n=4), this effect was attenuated significantly in C405A-ETB–transfected cells in which H_{2}O_{2} decreased nitrite levels by only 45.0% in comparison with V-treated cells (P=0.07, n=4) (Figure 7C). Taken together, these data
confirm that Cys405 is a redox-sensitive, functional cysteiny1 thiol whose oxidation to sulfenic acid impairs ET_B-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 occur, in part, 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, pulmonary vascular resistance, 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 cysteiny1 thiol(s) (Cys405) in the eNOS-activating region of the ET_B receptor (to sulfenic acid and the disulfide form) (Figure 8). Thus, aldosterone contributes to high pulmonary vascular tone by oxidizing cysteiny1 thiol(s) in ET_B, which, in turn, acts as a redox switch to impair ET_B-dependent endothelial NO generation.

Other studies have linked hyperaldosteronism to end-stage disease in idiopathic pulmonary hypertension. Although studies to date examining the role of mineralocorticoid receptor antagonism in PAH are limited to case reports, a clinical trial was recently 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. Although 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 previous studies that reported an increase in ET-1 levels and showed that ET-1 contributed to the pathogenesis of PAH. The levels of ET-1 that we observed were 1000-fold higher than the level required to stimulate aldosterone secretion from adrenocortical cells in vitro. Furthermore, the levels of plasma ET-1 measured in this study, akin to those observed in patients with PAH, 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. Moreover, our study likely underestimated the maximal level of hyperaldosteronism achieved in PAH, because we measured plasma levels antecedent to advanced stage disease, which is associated with decreased cardiac output vis-à-vis cor pulmonary that results in a decline in PASP and compensatory (over)activation of the renin-angiotensin-aldosterone system.

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 extra-adrenal 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 with the use of an assay with a lower limit of detection reported to be 20 pg/mL. Our study used 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 previous work in adrenal cortex–derived Y1 cells that demonstrated a similar mechanism of CYP11B2 upregulation.15 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 thioctilazone/PGC-1α and aldosterone remains unresolved.

In one study performed in healthy volunteers, pioglitazone treatment for 6 weeks increased circulating aldosterone levels,36 whereas rosiglitazone has been linked to aldosterone-independent plasma volume expansion through inhibition of sodium transport in the renal collecting duct.31 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.32 Although we now report differing results, we believe they may be attributable to the cell types studied, and the duration of exposure to pioglitazone and angiotensin II, as well.

We and others have shown previously that the adverse effects of aldosterone on the systemic vasculature include increased oxidant stress and decreased bioavailable NO that promotes endothelial dysfunction and impairs vascular reactivity.4,7,26,33 Our finding of increased pulmonary endothelial oxidant stress is not surprising, because others have reported an increase in ROS production owing to increased NOX1 expression in the small muscularized arteries isolated from the MCT rat model of PAH.34 Here, we focused selectively on oxidant stress in the endothelium and found an increase in expression of NOX4 and the NOX4 subunit p22phox, 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 H2O2 production in fetal pulmonary artery endothelial cells in an ETβ-dependent manner18; however, these studies were not performed in a time frame that would afford upregulation of aldosterone synthesis by ET-1.

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

We now identify an additional mechanism to explain the decrease in eNOS activity and bioavailable NO: dysfunctional ET-1/ETβ-eNOS signaling in the setting of elevated aldosterone levels owing to oxidative posttranslational modification of redox-sensitive cysteiny1 thiol(s) in the ETβ receptor. Oxidation of cysteine residues to form higher oxidative intermediates of cysteine, including sulfenic acid and the disulfide form, is known to occur under conditions of oxidant stress and to regulate protein function.40 The ETβ receptor is a 7 transmembrane domain G-coupled protein receptor with a carboxyterminal cytoplasmic tail that contains 3 functional cysteine residues: Cys402, Cys403, and Cys405.9 Here, we report that these cysteines are oxidatively modified, which is associated with functional consequences for ETβ-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 G1, but not Gq subunits.41 In the current work, the relationship between ETβ cytoplasmic tail cysteines, palmitoylation, and eNOS was not explored. Interestingly, an in vitro study using a synthetic peptide of ETβ constructed to contain residues 390 to 409, and, therefore, including the 3 cytoplasmic tail cysteines, was shown to bind eNOS and inhibit its activity with an EC50 of 3 ± 1.8 μmol/L.42 Although that study did not examine the cysteine residues directly for posttranslational modification(s), our observation that oxidative posttranslational modification of ETβ Cys405 is associated with impaired ETβ-dependent NO 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 ETβ resistant to oxidant stress–induced sulfenic acid and disulfide formation, and, as a result, improved redox-sensitive signaling. It is, however, important to acknowledge that our methods did not use liquid chromatography-mass spectrometry, which is required to definitively characterize this effect. Moreover, our observation that site-directed mutagenesis of Cys405 alone restored ETβ-dependent NO generation incompletely in the presence of pathological concentrations of H2O2 suggests further that Cys402 and Cys403 may also be redox-sensitive cysteine thiols involved in ETβ-eNOS signal transduction. Our finding that cysteines in the ETβ cytoplasmic tail are oxidatively modified is also supported by the observation that global sulfhydryl levels are decreased in lung tissue isolated from rats with MCT-induced PAH in comparison with controls.43

Our results may account, in part, for limitations in the clinical efficacy of endothelin receptor antagonism for patients with PAH, namely, currently available ET receptor antagonists are believed to improve pulmonary vascular tone primarily by attenuating ETα-mediated pulmonary vasoconstriction in pulmonary vascular smooth muscle cells, and, therefore, these drugs do not address the potential contribution of abnormal ETβ signal transduction in HPACs to pulmonary vascular dysfunction in PAH. Along these lines, our findings suggest that by preventing aldosterone-induced oxidation of ETβ, mineralocorticoid receptor antagonism preserves normal ET1–ETβ vasodilatory signaling to maintain levels of NO in HPACs and attenuate pulmonary vascular remodeling in PAH in vivo (Figure 8). Importantly, however, the effect of spironolactone/epیرeronene on the development of plexiform lesions was not specifically ad-
dressed 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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Disclosures

None.

References

Despite recent advances in diagnosis and treatment, pulmonary arterial hypertension (PAH) remains a devastating disease that is associated with a 10% mortality rate within the first year of diagnosis. Currently available pharmacotherapies based on known biological mediators of the disease are limited and, in certain cases, have waning long-term efficacy. In this study, we identify aldosterone as a novel contributor to the pathobiology of PAH and demonstrate that mineralocorticoid receptor antagonism is efficacious in the prevention and reversal of experimental PAH. We describe a novel mechanism for the increase in pulmonary aldosterone levels whereby elevated levels of endothelin-1, which have been observed in PAH, function as a potent stimulator of adrenal and extra-adrenal aldosterone synthesis to modulate pulmonary vascular dysfunction. Our findings demonstrate that aldosterone-induced oxidant stress impairs endothelin-B receptor signal transduction to diminish endothelin-B–dependent nitric oxide synthesis in pulmonary artery endothelial cells in vitro and promote negative remodeling of pulmonary arterioles and pulmonary vascular dysfunction in 2 experimental rat models of PAH in vivo. Importantly, mineralocorticoid receptor antagonism with spironolactone or eplerenone prevented or reversed the adverse effects of hyperaldosteronism on pulmonary vascular remodeling and improved pulmonary vascular resistance, pulmonary artery pressure, and remodeling of the right ventricle. Moreover, our findings relating to the potential benefit of spironolactone or eplerenone in attenuating pulmonary vascular dysfunction in PAH may support future clinical trials and/or repurposing of mineralocorticoid receptor antagonists, which are already an accepted medical therapy in patients with certain cardiovascular diseases, to those patients with PAH and other pulmonary vascular diseases with similar pathobiology.
Aldosterone Inactivates the Endothelin-B Receptor via a Cysteiny1 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\(^*\) metabolites. Nitrite (NO\(_2^-\)) and nitrate (NO\(_3^-\)) were measured in cell culture medium containing 2% fetal bovine serum and L-arginine (1 mmol/L) (Sigma-Aldrich) by 1(H)-naphthotriazole fluorescence (Cayman) as previously reported.\(^1\) To measure NO\(_2^-\) levels in whole lung tissue, lung specimens were harvested from rats and snap frozen in liquid nitrogen. Samples were thawed, homogenized in PBS (pH 7.4), and centrifuged at 14,000 x g at 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\(_A\) (Santa Cruz), anti-ET\(_B\) (Santa Cruz), anti-NOX4 (Santa Cruz), anti-p22\(^{phox}\) (Santa Cruz), anti-eNOS (Cell Signaling), anti-PGC-1\(\alpha\) (Santa Cruz), and anti-SF (Santa Cruz) antibodies overnight at 4\(^\circ\)C and visualized using the ECL detection system (Amersham Biosciences). In experiments to assess ET\(_A\) expression, purified ET\(_A\) protein (Novus Biological) was loaded to serve as an internal control.

**Co-immunoprecipitation of proteins.** Cell monolayers were washed twice with ice-cold PBS and incubated on ice with RIPA buffer supplemented with various protease inhibitors (Millipore). Cells were scraped with a rubber policeman and samples were rotated at 4 \(^\circ\)C for 15 min. Lysates were centrifuged at 14,000 x g at 4 \(^\circ\)C for 15 min and the then pre-cleared with a 50% slurry of Protein G agarose beads (Santa Cruz Biotechnology) mixed with PBS. Following removal of the beads by centrifugation, cell lysates were incubated with an anti-ET\(_B\) or anti-PGC-1\(\alpha\) antibody (Santa Cruz Biotechnology) overnight at 4 \(^\circ\)C. The immunocomplex was captured by incubating lysates with 50% Protein G agarose bead slurry at 4 \(^\circ\)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' GAGAAAGGAGAGGCCAGGTC-3' and reverse: 5'-CAGGAACCTGCTCTGGAAAC-3'.

eNOS activity. eNOS activity was measured using the NOS activity kit (Cayman) according to the manufacturer’s instructions with some modifications. Cells were washed with PBS containing 1 mM EDTA, transferred to a microcentrifuge tube, and centrifuged at 14,000 x g at 4 ºC for 2 min. The supernatant was decanted and homogenization buffer (250 mM Tris-HCl, pH 7.4, 6 µM 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-ET₄ from human were cloned into the mammalian expression vector pCMV6 (Origene). The C405A-ET₄ mutant was purchased from Genewiz (South Plainfield, NJ). COS-7 cells, which do not express endogenous eNOS or ET₄, were plated in P100 tissue
culture dishes and transfected with 10 µg of WT-eNOS and WT-ET_{B} or C405A-ET_{B} DNA for 4.5 h with Lipofectamine 2000\textsuperscript{TM} 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\textsubscript{2}O through the trachea as described previously.\textsuperscript{3} After fixation for 24 h at 4°C, the lung tissue was processed and embedded in paraffin using a Hypercenter XP System and Embedding Center (Shandon, Pittsburg, PA). The paraffin-embedded lung tissue was cut into 5-µm sections. Hematoxylin and eosin staining was performed according to methods published previously.\textsuperscript{4} The 3,3’-diaminobenzidine substrate method was used for smooth muscle α-actin immunohistochemical staining. The number of muscularized arteries with a diameter of 20–50 µm located distal to terminal bronchioles were counted in 20 consecutive fields (100X) per section,\textsuperscript{3} and the cross-sectional area was assessed using Image J software (NIH).

Sections were stained with a Gomori’s Trichrome Staining Kit according to the manufacturer's instruction (Fischer Scientific). Image J software (NIH) was used to measure the per cent perivascular collagen deposition of muscularized arterioles with a diameter of 20–50 µm by subtracting the area of the lesser curvature from the greater curvature and dividing by the lesser curvature x 100. Collagen was also assessed in tissue sections using Picrosirius Red Stain Kit according to the manufacturer's instruction (Polysciences). Lung sections were visualized under polarized light using an Olympus BX51\textsuperscript{TM} microscope and images were acquired by the Picture Taker\textsuperscript{TM} 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. The pulmonary vascular resistance index was calculated as [(mean pulmonary artery pressure-LVEDP)/CI] and systemic vascular resistance index was calculated as [(mean arterial pressure-mean right atrial pressure)/CI].

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

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 ET₄B-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 ET₄B 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 (H₂O₂) levels were assessed by measuring Amplex Red fluorescence (n=3). To determine a potential source of H₂O₂ 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 p22phox (n=3). Data are presented as mean ± S.E.M. Representative blots are shown.

Supplemental Figure 9. Aldosterone does not influence ET₄A or ET₄B protein levels in HPAECs. HPAECs were exposed to vehicle control (V) or aldosterone (ALDO)(10⁻⁷ mol/L)
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 μM)(n=3). (b) Peroxynitrite formation was assessed by 3-nitrotryosine immunohistochemistry (n=3). arb. units, arbitrary units. Data are expressed as mean ± S.E.M. Representative photomicrographs are shown.
Supplemental Figure 1
Supplemental Figure 2
<table>
<thead>
<tr>
<th>Condition</th>
<th>RV/LV+Septum Weight</th>
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<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

Days post-MCT Injection

0  14  21

25 µm
Supplemental Figure 6
Supplemental Figure 7

ET-1 (10nM)

V

BQ (1.5 μM)

ALDO Levels (pg/μg protein)

V

V

0 100 200 300

p=0.04

p<0.01
Supplemental Figure 8
Supplemental Figure 9
Supplemental Figure 10
L’aldostérone inactive le récepteur à l’endothéline B par l’intermédiaire d’une substitution redox des cystéinyl-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 synthétase 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 surrénaïlienne de l’aldostérone ; au niveau des vaisseaux périphériques, l’hyperlaldostérionisme 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 médié à la fois par le co-activateur 1α des récepteurs gamma activés par les proliférateurs de peroxysones et par le facteur stéroïdogenique de type 1. L’aldostérone augmente également la production d’espèces réactives de l’oxygène, ce qui, en oxydant les cystéinyl-thiols au sein de la région de l’ETB, qui régule l’activation de l’eNOS, diminue l’activité de cette enzyme médiée 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 minéralocorticoïdes par la spironolactone a diminué la libération d’espèces réactives de l’oxygène médiée 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’éplérène 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 la configuration d’oxydréduction des cystéinyl-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’oxydréduction

Risque cardiovasculaire associé à l’entraînement aérobie chez les patients coronariens associé 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 plus 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 coronarien 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 cardiaco.

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 cardiaco fatal survenu lors d’une période d’entraînement d’intensité modérée (129 456 heures d’exercice physique) et deux arrêts