Mineralocorticoid receptor antagonism attenuates experimental pulmonary hypertension

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ABSTRACT

Mineralocorticoid receptor (MR) activation stimulates systemic vascular and left ventricular remodeling. We hypothesized that MR contributes to pulmonary vascular and right ventricular (RV) remodeling of pulmonary hypertension (PH). We evaluated the efficacy of MR antagonism by spironolactone in two experimental PH models; mouse chronic hypoxia-induced PH (prevention model) and rat monocrotaline-induced PH (prevention and treatment models). Lastly, the biological function of the MR was analyzed in cultured distal pulmonary artery smooth muscle cells (PASMCs). In hypoxic PH mice, spironolactone attenuated the increase in RV systolic pressure, pulmonary arterial muscularization, and RV fibrosis. In rat monocrotaline-induced PH (prevention arm), spironolactone attenuated pulmonary vascular resistance and pulmonary vascular remodeling. In the established disease (treatment arm), spironolactone decreased RV systolic pressure and pulmonary vascular resistance with no significant effect on histologic measures of pulmonary vascular remodeling, or RV fibrosis. Spironolactone decreased RV cardiomyocyte size modestly with no significant effect on RV mass, systemic blood pressure, cardiac output, or body weight, suggesting a predominantly local pulmonary vascular effect. In distal PASMCs, MR was expressed and localized diffusely. Treatment with the MR agonist aldosterone, hypoxia, or platelet-derived growth factor promoted MR translocation to the nucleus, activated MR transcriptional function and stimulated PASMC proliferation, while spironolactone blocked these effects. In summary, MR is active in distal PASMCs and its antagonism prevents PASMC proliferation and attenuates experimental PH. These data suggest that MR is involved in the pathogenesis of PH via effects on PASMCs and that MR antagonism may represent a novel therapeutic target for this disease.

Keywords: aldosterone, hypoxia, monocrotaline, pulmonary vascular remodeling, spironolactone.
INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive, fatal disease. In its idiopathic form, mortality is 30 to 50% at 5 years, even with recent advances in available therapies. In its secondary forms, pulmonary hypertension (PH) contributes significantly to the morbidity and mortality of chronic lung and heart diseases. The pathological changes include medial thickening of the pulmonary vasculature due to smooth muscle cell hyperplasia and hypertrophy (12), muscularization of distal nonmuscular arteries, neointimal thickening composed of smooth muscle cells or myofibroblasts, and the occurrence of plexiform lesions due to endothelial and smooth muscle cell proliferation (41). Various altered pathways including the platelet derived growth factor (PDGF) signaling pathway may be involved in the abnormal vascular proliferation (28). Pulmonary vascular remodeling has become a key target for therapy. A treatment protocol that can decrease the number or size of the pulmonary artery smooth muscle cells (PASMCs) is the goal of many ongoing experimental and clinical therapeutic approaches. Although current therapies improve symptoms and hemodynamics, true reversal of pulmonary vascular remodeling is rarely achieved. Therefore, there is need to develop more effective therapies to target the abnormal pulmonary vasculature and, at the same time, to improve right ventricular function.

The mineralocorticoid receptor (MR), a member of the steroid receptor family, regulates systemic blood pressure by mediating the effects of the hormone aldosterone on renal sodium handling. Recent studies show that the MR also regulates systemic vascular function and contributes to cardiovascular disease through mechanisms independent of its actions on the renal system (14, 15, 21). MR activation may alter vascular function via genomic mechanisms, in which MR functions as a ligand-activated transcription factor to modulate vascular gene expression, and by non-genomic, rapid effects of MR that intersect with multiple important vascular signaling pathways such as those of epidermal growth factor, PDGF, insulin-like
growth factor, and angiotensin 2 (20). MR is expressed in the vasculature in endothelial cells (6) and vascular SMCs (14) where it promotes proliferation and fibrosis after carotid artery injury (15). MR also promotes generation of reactive oxygen species and vascular oxidative stress, which in turn stimulates vascular cell proliferation (24).

A potential role for the MR pathway in PH has recently been suggested in animal models and in humans. The role of MR was recently explored in two rat models of PH, focusing on its effects on pulmonary endothelial cells (19). Maron et al. showed in rats that MR antagonism partially reversed right ventricular (RV) pressure elevation and collagen deposition in the pulmonary vasculature of monocrotaline (MCT) and Sugen-hypoxia-induced PH and, in pulmonary artery endothelial cells, that aldosterone modulated endothelin-induced endothelial nitric oxide synthase (eNOS) and promoted production of reactive oxygen species. Another recent study found that plasma aldosterone levels were elevated in a small cohort of PAH patients compared with controls and correlated with markers of disease severity (18). Lastly, enhanced activation of the renin-angiotensin-aldosterone axis has been described in patients with idiopathic PAH (9).

We hypothesized that the MR contributes to the remodeling process in experimental PH by participating in the proliferative process of PASMCs, thereby contributing to pulmonary vascular and cardiac remodeling. In this study, we explore the effects of the MR antagonist on disease severity in vivo in two different PH models in two rodent species and on cell biology in vitro in distal bovine PASMCs.
MATERIALS AND METHODS

Animals and Experimental Design
Animal studies were approved by the IACUC at Tufts Medical Center and were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. Pellets containing sustained release spironolactone (15 mg/kg/day) or vehicle (Innovative Research of America) were implanted in mice a day before exposure to 5 weeks of normobaric hypoxia (10% O₂) or control normoxia. Rats were injected with 60 mg/kg MCT subcutaneously. Pellets containing sustained release spironolactone 40 mg/kg/day or vehicle were implanted either a day before MCT for 21 days (prevention arm), or at day 21 after MCT injection for 14 days release (treatment arm). The doses of spironolactone chosen have been previously shown to have no effect on systemic blood pressure while modulating systemic vascular function in rodent models of systemic vascular disorders (4, 27).

Hemodynamic Measurements
At the end of the exposure period (hypoxia or MCT), animals were anesthetized with pentobarbital (20 mg/kg ip) and ketamine (60 mg/kg im). The trachea was cannulated and lungs were ventilated with a rodent ventilator using room air. The left carotid artery was isolated and cannulated with PE-60 tubing connected to a fluid-filled force transducer (grass PT23) and the systemic blood pressure (SBP) was measured. For rats, a catheter was advanced into the RV via the right jugular vein for continuous right ventricular systolic pressure (RVSP) recording. Cardiac output was measured, as previously described (25). Cardiac index was calculated as the ratio between the cardiac output and body weight. Pulmonary vascular resistance index was calculated as the ratio between RVSP and cardiac index. For RVSP measurements in mice, a 25-gauge needle attached to a Statham P23-G pressure transducer by a short segment of P-50
tubing was inserted directly into the RV using a transthoracic approach. At the end of the experiment, animals were euthanized with a pentobarbital injection (120 mg/kg ip). The thorax was opened immediately. Lungs were inflated for histological preparation, as previously described (25). Heart chambers were weighed and the Fulton index \([\text{RV/Left ventricle (LV)+Septum (S)}]\) was determined as a measure of RV hypertrophy (11).

Lung histology for pulmonary vascular morphometry

Paraffin-embedded lung sections were stained with Verhoeff-VanGieson (VVG) for elastin followed by morphometric analysis of the vessels by light microscopy (Zeiss, Thornwood, NY). In each animal, 80–100 intraacinar arteries (20–80 µm diameter) were categorized as muscular (>75% of the circumference of the vessel), partially muscular (25-75%), or nonmuscular (<25%). All histological evaluations and measurements were performed by treatment blinded investigators and data analyzed after all measurements were completed.

Lung histology for analysis of inflammation

Hematoxilin and eosin stained lungs were assessed for the degree of inflammation, as previously described (33). Absence of inflammation was assigned a score 0; discrete inflammatory infiltrate consisted of scanty cells identified only at high-power magnification (40x) (score 1); moderate inflammation defined by cells identified in eccentric perivascular location, at medium-power magnification (20x) (score 2); abundant perivascular inflammation defined by recognition of inflammatory cells at low magnification (10x) (score 3).

Assessment of RV cardiomyocyte cross-sectional area and RV collagen deposition

Sections of the free wall of the right ventricle were fixed in 10% neutral buffered formalin. Paraffin imbedded RV sections were then stained with hematoxylin and eosin for myocyte size
154 (8) and with mason trichrome for collagen deposition (32). 12-15 regions of photomicrographs covering the whole section were obtained and scanned for myocytes cut in cross section and exposing the nucleus centrally. Cross-sectional area was measured using an Olympus CH2 microscope with a DP25 camera and DP2-BSW software (Tokyo, Japan). For interstitial collagen fraction quantification, blue-stained areas and myocyte areas from each section were determined using color-based thresholding (39). The total fibrosis area was calculated as a percentage of total surface area, using image software (Image-Pro Plus 7.0), as the summed blue-stained areas divided by total ventricular area.

164 Isolation and culture of distal PASMCs

165 Freshly excised bovine pulmonary arteries less than 1.5 mm in diameter were obtained from new born calves and stripped of adventitia and peripheral PASMCs were isolated, as previously described (2). SMC phenotype was assessed by the hill and valley morphology (1). Cells were grown in DMEM supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 units/ml), and fungizone (1.25 μg/ml) and were passaged every 1-2 weeks at a 1:3 ratio using trypsin. Medium was changed every 2-3 days. Passage 2-4 cells at 80% confluence were used for experiments.

174 Treatment of PASMCs and assessment of proliferation

175 Prior to experiments, cells were starved for 48 hours in 1% FBS DMEM (for hypoxia exposure), or 0.1% FBS DMEM (for various treatments), with antibiotics. PASMCs were exposed to hypoxia (3% oxygen) in a humidified modular incubator chamber (Billups-Rothenberg, Del Mar, CA) that was maintained at 37°C (26). The incubator chamber was sealed and purged with 3% O₂, 5% CO₂, balanced N₂ for 15 minutes. Normoxic control PASMCs were exposed to 95%
ambient air, 5% CO₂ for the entire incubation period. A portable gas analyzer (Hudson Ventronics Division) was used to ensure that the O₂ concentration inside the chamber was 3%. Cells were exposed to hypoxia or PDGF 1nM (Sigma Aldrich) in the presence of vehicle or spironolactone 1 µM (Sigma Aldrich) or treated with aldosterone 10 nM (6) (Sigma Aldrich) or vehicle in 0.1% FBS DMEM with antibiotics. Cell proliferation was assessed by the rate of [³H]thymidine incorporation, as previously described (26).

**Immunofluorescence of PASMCs**

PASMCs were grown to 75% confluence on glass cover slips. After 24 hours of serum starvation, cells were pretreated with vehicle or spironolactone (1 µM) one hour before overnight (18 hours) stimulation with vehicle, or aldosterone (10 nM). The cells were then fixed with 3.7% paraformaldehyde for 10 minutes and immunostained with a polyclonal anti-MR antibody (Santa Cruz) at 1:50 dilution in 10% donkey serum for 1 hour. Cells were then washed and incubated with donkey-anti-rabbit rhodamine conjugated secondary antibody for 1 hour (14), then counterstained with 4',6-diamidino-2-phenylindole (DAPI) for nuclear localization prior to mounting. The specificity of the MR antibody has been previously validated in HEK 293 cells, which lack endogenous MR (14) and reactivity with bovine MR has been previously confirmed (16). Negative control sections were stained with an isotype rabbit IgG antibody and the secondary antibody, then counterstained with DAPI.

**Immunoblotting**

Cell lysates were prepared and protein concentrations were determined as previously described (26). 50 µg of protein were electrophoresed through 12% SDS-polyacrylamide gel and electroblotted onto a polyvinylidine difluoride membrane. The membrane was blocked and
incubated with the polyclonal MR primary antibody (1:200) (14) or actin (Santa Cruz Biotechnology, 1:1000) and visualized by chemiluminescence techniques.

Transfection and Luciferase Assay

Cells were transfected with a plasmid containing a MR-response element (MRE) reporter driving expression of the luciferase gene or control reporter containing an estrogen receptor response element (ERE), as previously described (22). After 24 hours of serum starvation, indicated concentrations of vehicle, ligand, and inhibitor or its vehicle were then added for 18 hours. Cells were lysed and luciferase activity was determined in triplicate, as described (3, 14).

Statistical Analysis

Data are expressed as mean ± SEM, % of total vessels (for lung morphometry), or % of total area analyzed (for collagen deposition). Two treatment groups were compared by Student t-test. More than two experimental groups were analyzed by one-way analysis of variance and Student-Newman-Keuls post hoc test for multiple comparisons. Calculations were performed using SigmaStat 3.1 software (Systat Software, CA). A P value of < 0.05 was considered statistically significant.

RESULTS

MR antagonism alleviates chronic hypoxia-induced PH in mice

Spironolactone treatment started from the beginning of hypoxia significantly lowered the hypoxia-induced increase in RVSP compared with the vehicle-treated group (Figure 1A) without
significantly changing systemic blood pressure (SBP), or body weight (Figures 1B and 1C). As expected, hypoxic animals had lower body weights than normoxic mice. The lack of effect on SBP suggests that the dose of spironolactone was low enough to avoid significant systemic diuretic effects. In addition to the hemodynamic effects, spironolactone reduced pulmonary vascular remodeling as demonstrated by a significant reduction in the number of fully muscularized pulmonary arteries compared to vehicle-treated hypoxic controls (Figures 1D and 1E).

Effects of MR antagonism on RV structure in hypoxia-induced PH

The fewer muscularized vessels and lower RVSP were not accompanied by a decrease in overall RV mass in hypoxic mice treated with spironolactone, as measured by the Fulton index (Figure 2A). While the increase in right ventricular cardiomyocyte cross sectional area with hypoxia was significant in vehicle-treated animals, there was no significant difference in the average cardiomyocyte size between normoxic- and hypoxic–spironolactone treated animals (Figure 2B), suggesting that spironolactone had a modest blunting effect on the degree of cardiomyocyte hypertrophy. MR activation stimulates collagen deposition in systemic arteries (15) and its blockade decreases collagen deposition (35). Therefore, to better understand the changes in RV structure in the presence or absence of MR antagonism, we assessed the percent collagen deposition in the RV. RV fibrosis was predominantly perivascular, but also interstitial in this model and the percent collagen deposition increased significantly with hypoxia and this increase was completely prevented by spironolactone (Figures 2C and 2D).
MR antagonism has beneficial effects in MCT-induced PH in rats

We next assessed whether spironolactone impacts the degree of PH induced by MCT in rats using two protocols (Figure 3A). Spironolactone was either started the day of MCT injection and continued for 21 days until PH is fully developed (17) (prevention arm), or started 21 days after the MCT injection, at which time PH in untreated animals is severe (17), and then continued for 14 days (treatment arm). As expected, in control rats, RVSP increased significantly at 21 days after MCT, and more severely after 35 days (Figure 3B). Spironolactone treatment tended to decrease the MCT-induced RVSP elevation compared to controls in the prevention group (p = 0.051) and significantly inhibited the RVSP increase in the treatment group (Figure 3B), without affecting SBP, body weight (Figures 3C and 3D), or cardiac index (Figure 3E). Pulmonary vascular resistance was elevated at 21 days and increased further at 35 days in the MCT-vehicle groups. Spironolactone attenuated MCT-induced elevation of pulmonary vascular resistance compared with vehicle both in the prevention and in the treatment arms (Figure 3F).

Effects of MR antagonism on pulmonary vasculature in MCT-induced PH

Morphometric analysis of the degree of remodeling of peripheral pulmonary arteries revealed that spironolactone completely prevented muscularization in the prevention arm, while in the treatment arm spironolactone given for 2 weeks did not appreciably affect the degree of muscularization (Figures 4A and 4B). Of note, compared to the 21 day MCT-vehicle group, the 35 day MCT-vehicle group had a significantly increased RVSP (Figure 3B) and pulmonary vascular resistance (Figure 3F), but there was no difference in pulmonary vascular muscularization, suggesting that the majority of the pulmonary vascular remodeling occurred at an earlier stage of the disease while pressure continued to rise (Figures 4A and 4B).
Because MCT is known to produce a greater inflammatory reaction than hypoxia (38) and MR antagonism is known to attenuate inflammation in various tissues (10, 31), we assessed the degree of lung inflammation. Semiquantitative analysis of lung sections by inflammation score showed that MCT significantly increased the accumulation of inflammatory cells only at 35 days and spironolactone treatment did not significantly affect the degree of inflammation (Figures 4C and 4D).

**Effects of MR antagonism on RV structure in MCT-induced PH**

RV hypertrophy was significantly increased 21 days after MCT injection and increased further at 35 days (Figure 5A). Spironolactone treatment significantly decreased right ventricular cardiomyocyte size at both 21 and 35 days (Figures 5B and 5C) but this did not translate into a significant change in overall RV weight (Figure 5A). The MCT model produced only a trend toward increased perivascular RV collagen deposition at 21 and 35 days which was not influenced by spironolactone (Figures 5C and 5D).

**MR expression and function in distal PASMCs**

MR is present in vascular smooth muscle cells of the systemic vasculature and its activity has been associated with cell proliferation (23). Because of the beneficial effects observed with MR antagonism on pulmonary vascular remodeling in both PH models *in vivo*, we aimed to determine if MR is functional in distal PASMC *in vitro*. Since remodeling in PH takes place in the peripheral pulmonary arterioles, rather than the main pulmonary artery, we first examined MR expression and activity in bovine PASMCs from distal, intraacinar vessels. MR is expressed and localizes by immunofluorescence predominantly in a diffuse pattern in the cytoplasm of quiescent PASMCs (Figure 6A). When non-specific control IgG antibody was used, there was
no staining of PASMCs (Figure 6B). Stimulation with aldosterone, hypoxia, or PDGF shifted MR localization to mainly an intra- and peri- nuclear distribution (Figure 6C, 6E and 6G, respectively), which was prevented by spironolactone administered one hour prior to stimulation. (Figure 6D, 6F, and 6H, respectively). Protein quantification via immunoblotting showed no change in overall MR protein content with the three stimulants, or with spironolactone (Figure 6I). Consistent with its role as a hormone-activated transcription factor (14), aldosterone activated MR transcriptional activity in bovine distal PASMC in a dose-dependent manner, as measured by luciferase activity of an MR responsive reporter gene (Figure 6J). Aldosterone did not activate the estrogen response element reporter supporting the specificity of the MR binding site. Spironolactone had no effect on basal MR reporter activity, but it effectively blocked aldosterone-induced MR transcriptional activation.

To gain insight into the cellular mechanisms of action of the MR in experimental PH, we examined the effects of aldosterone on proliferation of cultured pulmonary arteriolar SMCs. PASMCs were treated with aldosterone at 10 nM, a pathologically relevant concentration found in patients with heart failure and that activates only the MR. Aldosterone simulated cell proliferation in distal PASMC (Figure 7A). PASMC proliferation was also enhanced by exposure to hypoxia and PDGF, and this was prevented by co-treatment with the MR antagonist, spironolactone (Figures 7B and 7C). These data taken together suggest that MR functions as hormone-activated transcription factor in distal PASMCs and contributes to the proliferative response of these cells in response to hypoxia and PDGF, two important factors in the pathophysiology of PAH.
We have demonstrated that MR antagonism by spironolactone attenuates the effects of hypoxia-induced PH in mice by preventing pulmonary vascular remodeling and the rise in RV pressure, without detrimental systemic effects. Although RV hypertrophy was not influenced, RV fibrosis was significantly reduced in this model. Similarly, in the MCT model of PH in rats, MR antagonism blunted the increase in RV pressures, pulmonary vascular resistance, and pulmonary vascular remodeling. Cardiomyocyte hypertrophy was modestly attenuated but collagen deposition and inflammation were unaffected by spironolactone in this model. The protection from MCT-induced PH by MR inhibition could be demonstrated even when the drug was started after PH was well established, virtually arresting the progression of the disease.

Supportive of a mineralocorticoid effect on the pulmonary vasculature, we also demonstrated in vitro that SMCs from peripheral pulmonary arteries express functional MRs that mediate aldosterone-induced gene transcription and PASMC proliferation. PASMC MRs translocate to the nucleus in response to hypoxia or PDGF, potential drivers of PH, and MR antagonism attenuates hypoxia- and PDGF-induced MR nuclear translocation and PASMC proliferation. These studies support a novel role of pulmonary arteriolar SMC MR in the regulation of vascular proliferation and remodeling in response to PH stimuli and provide further support for the potential role of MR antagonism as a therapy for PAH.

Our experiments confirm and expand upon another recent report demonstrating a role for aldosterone and MR in experimental PH (19). The study by Maron et al. demonstrated that aldosterone and endothelin levels are elevated in the MCT-rat model of PH and that MR antagonism with spironolactone or eplerenone prevents or attenuates PH in two rat models of PH. Maron et al. focused mechanistically on the role of the endothelial cell, demonstrating that aldosterone enhances reactive oxygen species production and prevents nitric oxide production in PA endothelial cells. The current study adds novel findings supporting the concept that MR
antagonism affects pulmonary vascular structure by directly inhibiting SMC proliferation in response to PH-associated stimuli and preventing lung vessel muscularization in two models of experimental PH. In addition, the demonstration of a role for MR in a mouse model of PH used in our experiments will encourage future studies using genetically altered mice to better define the molecular pathways involved.

The involvement of MR in development of PH-associated RV fibrosis is also a novel finding and will warrant further exploration to determine whether MR antagonism in more advanced RV failure has beneficial effects on RV function via inhibition of cardiac fibrosis. Lastly, while the effects of aldosterone were previously described in pulmonary artery endothelial cells (19), we showed for the first time that MR is functional as a transcription factor in distal PASMCs and its blockade attenuates effects of stimuli that are known to be activated in PAH. Further exploration of the genes regulated by MR in distal PASMC and their role in proliferation and vasoconstriction could yield novel targets to treat PAH patients.

In models of systemic hypertension, myocardial hypertrophy is related to ventricular systolic pressure work. Myocardial fibrosis, on the other hand, is not related to hemodynamic workload, but rather to activation of pro-fibrogenic pathways by multiple factors, including mineralocorticoid excess and cardiac MR activation (36, 37). Therefore, it was of interest to assess the effect of MR antagonism on RV fibrosis. In the hypoxic model, which is associated with significant perivascular and interstitial RV collagen deposition, spironolactone completely prevented the increase in collagen deposition. Conversely, in the MCT model, which produced a mild and nonsignificant increase in RV collagen deposition, spironolactone had no effect on collagen deposition. These findings indicate that MR antagonism can inhibit development of RV fibrosis, even when having no significant effect on RV hypertrophy overall. In our study, the effect of spironolactone on RV cardiomyocyte size, although statistically significant, was modest
enough that did not translate into a significant reduction in overall RV size. Whether the effects
of the MR antagonist on RV hypertrophy and fibrosis have beneficial effects on RV function
cannot be ascertained from our current data, but deserves attention in future studies.

There is considerable current interest in the role of inflammatory mechanisms in the
pathogenesis of PAH (30). At the same time, the role of the MR in inducing local tissue
inflammation in the systemic vasculature is well established (McGraw AP et al, JAHA 2013,
accepted)(20). In the MCT model, a PH model with a significant inflammatory component, the
accumulation of inflammatory cells was not significantly reduced by spironolactone. The lack of
effect of MR antagonism on inflammation in the MCT model may indicate that the inflammatory
pathways involved in this model are not under the influence of the MR, or alternatively, that the
dose used in these experiments was insufficient to inhibit inflammation.

In both models of PH used in our studies, MR inhibition had a more profound effect on
preventing pulmonary vascular remodeling and the rise in pulmonary pressure than on changes
in RV structure and inflammation, suggesting a more direct pulmonary vascular effect.
Interestingly, in vehicle treated animals, pulmonary pressures continued to rise from 21 to 35
days after MCT treatment, while the degree of pulmonary vascular remodeling did not progress
after 21 days, as measured by the degree of vessel muscularization. The more severe PH at 35
days could be due to continued RV maladaptation to the fixed PA remodeling or to progressive
PA vasoconstriction. MR antagonism effectively improved hemodynamics at both time points by
blunting the RV pressure elevation and decreasing the pulmonary vascular resistance, even
when started after PH was established, without changing pulmonary vascular histology. Since
MR has been shown to modulate systemic SMC Rho signaling, L-type calcium-channel function
and arteriolar contractile responses (5, 29) (21), it is possible that it also affected these
pathways in PASMCs by enhancing pulmonary vasoconstriction, thereby contributing to the rise
in RVSP after pulmonary vascular remodeling is complete. The role of SMC MR in modulating pulmonary vascular contraction in PAH warrants further investigation, as this component may explain the hemodynamic worsening late in the disease course in our model and suggests that MR antagonism may be beneficial even at late stages in the disease.

Our findings showing anti-proliferative effects of MR antagonism in PASMCs are consistent with those in systemic vascular SMCs and with a prior report demonstrating that aldosterone has proliferative effects on human PASMCs (40). Various pathways have been implicated in MR-induced SMC proliferation \textit{in vitro} and \textit{in vivo} including MAPK signaling, endothelin, placental growth factor, PDGF, and epidermal growth factor (20). Our data derived from distal PASMCs in culture support the concept that MR antagonism blocks multiple pathways that are responsible for abnormal PASMC proliferation in PH, such as those involving hypoxia and PDGF activation.

It is unclear whether the contribution of the MR pathway to pulmonary vascular remodeling in PH is mainly an effect on the endothelial cells (19), SMCs (as we demonstrated here), or both, or whether this contribution varies with the type of PH (idiopathic PAH versus PH secondary to left heart failure). While MR inhibition did not show beneficial effects on secondary PH and RV dysfunction in rats with LV failure (7), new clinical data suggest a potential role for aldosterone in human PAH. Aldosterone levels have been shown to be elevated in patients with PAH in whom levels correlated with disease activity (18). In addition, the renin-angiotensin activities are upregulated systemically and in the lungs of PAH patients (9). Our preclinical study provides additional support and guidance for clinical studies of MR antagonism in PAH, currently under way (13). The beneficial effects of spironolactone in animal models of PH occurred with maintenance of systemic blood pressure and cardiac output suggesting that these drugs act locally on the pulmonary vasculature and could be used safely in PAH. In addition,
spironolactone entirely prevented pulmonary vascular remodeling at 21 days and arrested the
disease progression during the late treatment phase (21 to 35 days) in the MCT treated rat.
These data suggest that spironolactone started early in the disease process may have
significant beneficial impact both hemodynamically and histologically, while when started late in
the disease course, it prevents the progression of disease severity.

There are several limitations of our study that should be considered. We did not achieve
complete resolution of experimental PH in either model. This might be due to submaximal
doses of spironolactone used in order to determine if MR blockade can have a selective effect
on the pulmonary circulation; higher doses may have produced greater effects on systemic
pressures (4) and/or diuresis and might have been more effective in preventing or reversing RV
and lung remodeling. Considering that MR deletion in macrophages protects against cardiac
hypertrophy, fibrosis, and vascular damage (34), higher doses of spironolactone that could
inhibit cortisol-induced MR activation in macrophages may also have been more effective for
inhibiting RV inflammation and fibrosis and perhaps better preserving RV function. Finally, all
preclinical studies share the limitation that animal models may not recapitulate human disease.
This is particularly true for PH in which the individual animal models do not recapitulate all
aspects of human disease. For this reason, we tested the effects of spironolactone in two
different well-established models of PH in two distinct species. These data, combined with the
recent study demonstrating a beneficial role for MR antagonism by both spironolactone and
eplerenone in two rat models of PH add substantially to the notion that this pathway may be an
important therapeutic target in this otherwise lethal disease.

In conclusion, we present novel data indicating that the aldosterone-MR pathway influences
proliferation of PASMCs, vascular remodeling and pulmonary hemodynamics in experimental
PH. We show that MR antagonism in doses that do not cause systemic effects is beneficial in preventing and treating experimental PH through blockade of MR actions that contribute to elevated RV pressures, PASMC proliferation, RV fibrosis, and pulmonary vascular remodeling. Lastly, we show that MR is active in distal PASMCs and contributes to their proliferative response to hypoxia and PDGF. Since MR antagonists are already available and their safety profile is well characterized, even in patients with advanced heart failure, our results have potential for rapid translational applicability.


Figure 1. Effect of mineralocorticoid receptor (MR) antagonism on chronic hypoxia-induced pulmonary hypertension (PH) in mice. Mice exposed to normoxia or hypoxia were treated with vehicle (black) or spironolactone (SP; white) and A. Right ventricular systolic pressure (RVSP). B. Systemic blood pressure (SBP) and C. Body weights were measured. D. Lung morphometry. Proportion of nonmuscularized, partially muscularized, and fully muscularized pulmonary arteries. E. Representative sections of lungs from normoxic, hypoxic, vehicle-, and spironolactone-treated mice; visualization by light microscopy (20x). N = 6-8 per group. *P < 0.05, ***P < 0.001 vehicle-treated, hypoxia versus normoxia; §P < 0.05, §§§P < 0.001 spironolactone-treated, hypoxia versus normoxia; ††P < 0.01, †††P < 0.001 hypoxia, vehicle versus spironolactone treatment.

Figure 2. Effect of mineralocorticoid receptor (MR) antagonism on right ventricular (RV) structure in hypoxia-induced pulmonary hypertension (PH) in mice. Mice exposed to normoxia or hypoxia were treated with vehicle (black) or spironolactone (white) and A. Fulton index: (right ventricle to left ventricle plus septum weight ratio (RV/LV+S)) was measured. B. Quantification of RV myocyte size by cardiomyocyte cross sectional area. C. Quantification of RV collagen deposition. D. Representative RV sections stained with mason trichrome for collagen assessment; visualization by light microscopy (40x). N = 8-10 per group for Fulton index measurements and 3-4 per group for B and C. *P < 0.05, ***P < 0.001 vehicle-treated, hypoxia versus normoxia; §§§P < 0.001 spironolactone-treated, hypoxia versus normoxia; †††P < 0.001 hypoxia, vehicle versus spironolactone treatment.
Figure 3. Effect of mineralocorticoid receptor (MR) antagonism on monocrotaline (MCT)-
induced pulmonary hypertension (PH) in rats. A. Treatment scheme. Effect of
Spironolactone (SP) compared to placebo given for prevention (D0-21) or treatment (D21-35) of
MCT-induced PH on B. Right ventricular systolic pressure (RVSP). C. Systemic blood pressure
(SBP). D. Body weights. E. Cardiac index (CI) as defined by the cardiac output to body weight
ratio, and F. Pulmonary vascular resistance index (PVRI), as defined by the ratio between
RVSP and cardiac index. N = 8-10 per group. **P < 0.01, ***P < 0.001 MCT versus vehicle; ‡‡‡
P < 0.001 MCT 35 days-spironolactone versus spironolactone-only; §P < 0.05, §§P < 0.01, §§§P <
0.001 MCT-spironolactone versus MCT-vehicle; ††P < 0.01 MCT 21 days-vehicle versus MCT
35 days-vehicle.

Figure 4. Effect of mineralocorticoid receptor (MR) antagonism on lung remodeling and
inflammation in monocrotaline (MCT)-induced pulmonary hypertension (PH) in rats. A.
Lung vascular morphometry: Proportion of nonmuscularized, partially muscularized, and fully
muscularized pulmonary arteries in MCT-induced PH rats treated with vehicle or spironolactone
(SP). B. Representative sections of lungs from control, prevention (21 days) and treatment (35
days) arms visualization by light microscopy (20x). C. Lung inflammation: Lung inflammation
score in MCT-induced PH rats treated with vehicle or spironolactone (SP). D. Representative
lung sections stained with hematoxylyn and eosin for assessment of inflammation; visualization
by light microscopy (20x). N = 3-4 per group. *P < 0.05, **P < 0.01, ***P < 0.001 MCT versus
vehicle; ‡P < 0.05, ‡‡P < 0.01, ‡‡‡P < 0.001 MCT-spironolactone versus spironolactone-only; †P
< 0.05, ††P < 0.01 MCT 21 days versus MCT 35 days; §§P < 0.05, §§§P < 0.01, §§§§P < 0.001 MCT-
spironolactone versus MCT-vehicle.

Figure 5. Effect of mineralocorticoid receptor (MR) antagonism on right ventricular (RV)
structure in monocrotaline (MCT)-induced pulmonary hypertension (PH) in rats. Effect of
Figure 6. Assessment of mineralocorticoid receptor (MR) in distal pulmonary artery smooth muscle cells (PASMCs). (A-H) MR localization in cultured distal PASMCs by immunofluorescence microscopy (40x), nuclear counterstaining with DAPI. A. Intracellular and cytoplasmic localization of the receptor in vehicle-treated cells. B. Negative control sections stained with isotype primary antibody (rabbit IgG) and secondary antibody. MR localization in cells stimulated overnight with (C) aldosterone (10 nM), (E) hypoxia (3% O₂), or (G) platelet derived growth factor (PDGF, 1 nM) alone or with spironolactone (SP) 1 µM treatment prior to aldosterone (F), hypoxia (G), or PDGF (H). I. Representative immunoblot studies of MR protein content after 24 hours of treatment with aldosterone (10 nM), hypoxia (3% O₂), or PDGF (1 nM) with or without SP pretreatment (N = 3 experiments). J. Functional MR in distal PASMCs. Distal PASMCs were transfected with a plasmid containing a MR-response element (MRE) reporter driving expression of the luciferase gene or control reporter containing an estrogen receptor response element (ERE). The indicated concentrations of aldosterone or SP were added and luciferase activity measured. Bars represent fold-activation of luciferase activity relative to...
control cells exposed to vehicle alone. (N = 3 experiments). ***$P < 0.001$ aldosterone-stimulation vs. MRE-control; †$P < 0.01$ aldosterone-SP treatment vs. aldosterone-vehicle.

Figure 7. Assessment of PASMC proliferation.

A. Proliferation of distal PASMC by thymidine incorporation after treatment with aldosterone 10 nM. B. Hypoxia-induced PASMC proliferation is inhibited by spironolactone (SP) 1µM. C. Platelet derived growth factor (PDGF)-induced cell proliferation is inhibited by SP. N = 48 wells per treatment group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ hypoxia, PDGF, or aldosterone versus vehicle-control; †††$P < 0.001$ hypoxia or PDGF-SP versus vehicle-control; §§$P < 0.01$ hypoxia or PDGF-SP versus hypoxia or PDGF-vehicle, respectively.