Mineralocorticoid receptor antagonism attenuates experimental pulmonary hypertension

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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). Last, 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 histological 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.

aldosterone; hypoxia; monocrotaline; pulmonary vascular remodeling; spironolactone

PULMONARY ARTERIAL HYPERTENSION (PAH) is a progressive, fatal disease. In its idiopathic form, mortality is 30–50% at five 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 (SMC) hyperplasia and hypertrophy (12), muscularization of distal nonmuscular arteries, neointimal thickening composed of SMCs or myofibroblasts, and the occurrence of plexiform lesions due to endothelial and SMC 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 (RV) function.

The mineralocorticoid receptor (MR), a member of the steroid receptor family, regulates systemic blood pressure (SBP) 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 nongenomic, 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 II (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 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 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). Last, 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 vas-
cular 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 Institutional Animal Care and Use Committee 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⁻¹ · day⁻¹) or vehicle (Innovative Research of America) were implanted in mice a day before exposure to 5 wk of normobaric hypoxia (10% O₂) or control normoxia. Rats were injected with 60 mg/kg MCT subcutaneously. Pellets containing sustained-release 40 mg · kg⁻¹ · day⁻¹ spironolactone 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 SBP 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 SBP was measured. For rats, a catheter was advanced in 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 in the RV using a transthoracic approach. At the end of the experiment, animals were killed 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 [RV/left ventricle (LV) + septum] was determined as a measure of RV hypertrophy (11).

**Lang histology for pulmonary vascular morphometry.** Paraffin-embedded lung sections were stained with Verhoeff-VanGieson for elastin followed by morphometric analysis of the vessels by light microscopy. Embedded lung sections were stained with Verhoeff-VanGieson for elastin followed by morphometric analysis of the vessels by light microscopy. Sections of the free wall of the RV were fixed in 10% neutral buffered formalin. Paraffin-embedded RV sections were then stained with hematoxylin and eosin for myocyte size (8) and with mason trichrome for collagen deposition (32). Twelve to 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.

**Isolation and culture of distal PASMCs.** Freshly excised bovine pulmonary arteries <1.5 mm in diameter were obtained from newborn 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 U/ml), streptomycin (100 U/ml), and fungizone (1.25 μg/ml) and were passaged every 1–2 wk 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.

**Treatment of PASMCs and assessment of proliferation.** Before experiments, cells were starved for 48 h 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, balance N₂ for 15 min. Normoxic control PASMCs were exposed to 95% ambient air, 5% CO₂ for the entire incubation period. A portable gas analyzer (Hudson Ventritions Division) was used to ensure that the O₂ concentration inside the chamber was 3%. Cells were exposed to hypoxia or 1 nM PDGF (Sigma Aldrich) in the presence of vehicle or 1 μM spironolactone (Sigma Aldrich) or treated with 10 nM aldosterone (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 h of serum starvation, cells were pretreated with vehicle or spironolactone (1 μM) 1 h before overnight (18 h) stimulation with vehicle, or aldosterone (10 nM). The cells were then fixed with 3.7% paraformaldehyde for 10 min and immunostained with a polyclonal anti-MR antibody (Santa Cruz) at 1:50 dilution in 10% donkey serum for 1 h. Cells were then washed and incubated with donkey-anti-rabbit rhodamine-conjugated secondary antibody for 1 h (14) and then counterstained with 4,6-diamidino-2-phenylindole (DAPI) for nuclear localization before 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 and then counterstained with DAPI.

**Immunoblotting.** Cell lysates were prepared, and protein concentrations were determined as previously described (26). Protein (50 μg) was electrophoresed through 12% SDS-polyacrylamide gel and electroblotted on a polyvinylidine difluoride membrane. The membrane was blocked and incubated with the polyclonal MR primary antibody (1:200) (14) or actin (1:1,000; Santa Cruz Biotechnology) and visualized by chemiluminescence techniques.

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

**Statistical analysis.** Data are expressed as means ± SE, percent of total vessels (for lung morphometry), or percent of total area analyzed (for collagen deposition). Two treatment groups were compared by Student’s t-test. More than two experimental groups were analyzed by Student's t-test.
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). 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 (Fig. 1A) without significantly changing SBP or body weight (Fig. 1, B and C). 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 with vehicle-treated hypoxic controls (Fig. 1, D and E).

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 (Fig. 2A). While the increase in RV 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 (Fig. 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. This increase was completely prevented by spironolactone (Fig. 2, C and D).

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 (Fig. 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 and continued for 21 days until PH is fully developed (17) (prevention arm).
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 (Fig. 3B). Spironolactone treatment tended to decrease the MCT-induced RVSP elevation compared with controls in the prevention group (*P < 0.051) and significantly inhibited the RVSP increase in the treatment group (Fig. 3B), without affecting SBP, body weight (Fig. 3, C and D), or cardiac index (Fig. 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 (Fig. 3F).

Effects of MR antagonism on pulmonary vascularity 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, whereas in the treatment arm spironolactone given for 2 wk did not appreciably affect the degree of muscularization (Fig. 4, A and B). Of note, compared with the 21-day MCT-vehicle group, the 35-day MCT-vehicle group had a significantly increased RVSP (Fig. 3B) and pulmonary vascular resistance (Fig. 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 (Fig. 4, A and B).

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 (Fig. 4, C and D).

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

**MR expression and function in distal PASMCs.** MR is present in vascular SMCs 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. Because 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, intra-acinar vessels. MR is expressed and localizes by immunofluorescence predominantly in a diffuse pattern in the cytoplasm of quiescent PASMCs (Fig. 6A). When nonspecific control IgG antibody was used, there was no staining of PASMCs (Fig. 6B). Stimulation with aldosterone, hypoxia, or PDGF shifted MR localization to mainly an intracellular and perinuclear distribution (Fig. 6, C, E, and G, respectively), which was prevented by spironolactone administered 1 h before stimulation (Fig. 6, D, F, and H, respectively). Protein quantification via immunoblotting showed no change in overall MR protein content with the three stimulants, or with spironolactone (Fig. 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 (Fig. 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 stimulated cell proliferation in distal PASMC (Fig. 7A). PASMC proliferation was also enhanced by exposure to hypoxia and PDGF, and this was prevented by cotreatment with the MR antagonist spironolactone (Fig. 7, B and C). These data taken together suggest
that MR functions as a 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.

DISCUSSION

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 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.

Fig. 4. Effect of MR antagonism on lung remodeling and inflammation in MCT-induced 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 SP. B: representative sections of lungs from control, prevention [21 days (d)], and treatment (35 days) arms visualized by light microscopy (×20). C: lung inflammation score in MCT-induced PH rats treated with vehicle or SP. D: representative lung sections stained with hematoxylin and eosin for assessment of inflammation; visualization by light microscopy (×20); n = 3–4/group. *P < 0.05, **P < 0.01, and ***P < 0.001, MCT vs. vehicle. †P < 0.05, ††P < 0.01, and †††P < 0.001, MCT-spironolactone vs. spironolactone only. ‡P < 0.05 and ‡‡P < 0.01, MCT 21 days vs. MCT 35 days. §P < 0.05, §§P < 0.01, and §§§P < 0.001, MCT-spironolactone vs. MCT-vehicle.
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 overall. In our study, the effect of spironolactone on RV hypertrophy and fibrosis has beneficial effects on RV function that can be ascertained from our current data, but deserves attention in future studies.

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 profibrogenic 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 it 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 (20, 21a). 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, whereas 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 the result of 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. Because MR has been shown to modulate systemic SMC Rho signaling, L-type calcium channel function, and arteriolar contractile responses (5, 21, 29), 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, since 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 in vitro and 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 vs. PH secondary to left heart failure). Whereas 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 SBP 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 (21–35 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, whereas, 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 because of submaximal doses of spironolactone used 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 in inhibiting RV inflammation and fibrosis and perhaps better preserving RV function. Finally, all preclinical studies share the limitation that animal models may 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 exper-
imantal 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. Last, we show that MR is active in distal PASMCs and contributes to their proliferative response to hypoxia and PDGF. Because 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.

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DISCLOSURES
No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

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