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Dysregulated Renin-Angiotensin-Aldosterone System Contributes to Pulmonary Arterial Hypertension

Frances S. de Man^{1,2}, Ly Tu¹, M. Louis Handoko^{2,3}, Silvia Rain², Gerrina Ruiter², Charlène François¹, Ingrid Schalijs², Peter Dorfmueller¹, Gérald Simonneau¹, Elie Fadel¹, Frederic Perros¹, Anco Boonstra², Piet E. Postmus², Jolanda van der Velden³, Anton Vonk-Noordegraaf², Marc Humbert¹, Saadia Eddahibi¹, Christophe Guignabert¹

¹INSERM U999, Le Plessis-Robinson, France; ²Department of Pulmonology and ³Physiology, VU University Medical Center / Institute of Cardiovascular Research, Amsterdam, The Netherlands

CORRESPONDING AUTHOR

Frances S. de Man, PhD; VU University Medical Center; Department of Pulmonology; De Boelelaan 1117; 1081 HV Amsterdam; The Netherlands; Tel: +31 20 44 44782; Fax: +31 20 44 44328; Email: fs.deman@vumc.nl

AUTHORS CONTRIBUTION

FSdM, LT, MLH, AVN, MH, SE and CG contributed to conception and design of the present study. FSdM, LT, SR, GR, CF, IS, PD, GS, EF, FP, AB and CG contributed to the acquisition of data. FSdM, LT, MLH, PEP, JvdV, AVN, MH, SE and CG contributed to analysis or interpretation of the data. FSdM, LT, MLH, and CG drafted the manuscript, and SR, GR, CF, IS, PD, GS, EF, FP, AB, PEP, JvdV, AVN, MH, and SE subsequently revised the article critically for intellectual content. All authors gave their final approval of this version of the manuscript to be published.

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SHORT RUNNING HEAD

RAAS-activity in iPAH-patients

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Accumulating evidences suggest increased renin-angiotensin-aldosterone system (RAAS)-activity in idiopathic pulmonary arterial hypertension (iPAH). However, the functional consequences of these abnormalities to iPAH pathogenesis remains obscure.

What This Study Adds to the Field

In this translational study we have demonstrated that:

- 1) Systemic RAAS activation is increased in iPAH patients and associated with worse prognosis;
- 2) Due to the increased ACE-activity, pulmonary endothelial cells of iPAH-patients produces more angiotensin II, which induces significant pulmonary artery smooth muscle cell proliferation of iPAH-patients via AT₁-receptor signalling;
- 3) AT₁-receptor expression and signalling is upregulated in iPAH-patients, without changes in AT₂-receptor expression;

4) The use of an AT₁-receptor blocker could have potential clinical implications due to its positive effects on pulmonary vascular remodeling.

This article has an Online Data Supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

For Review Only

ABSTRACT

Rationale – Patients with idiopathic pulmonary arterial hypertension (iPAH) often have a low cardiac output. To compensate, neurohormonal systems like renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system are upregulated but this may have long-term negative effects on the progression of iPAH.

Objectives – Assess systemic and pulmonary RAAS-activity in iPAH-patients and determine the efficacy of chronic RAAS-inhibition in experimental PAH.

Measurements and Main Results – We collected 79 blood samples from 58 iPAH-patients in the VU University Medical Center Amsterdam (between 2004-2010), to determine systemic RAAS-activity. We observed increased levels of renin, angiotensin (Ang) I and AngII, which was associated with disease progression ($p < 0.05$) and mortality ($p < 0.05$). To determine pulmonary RAAS-activity, lung specimens were obtained from iPAH-patients (during lung transplantation, $n=13$) and controls (during lobectomy or pneumonectomy for cancer, $n=14$). Local RAAS-activity in pulmonary arteries of iPAH-patients was increased, demonstrated by elevated ACE-activity in pulmonary endothelial cells and increased AngII type 1 (AT_1) receptor expression and signaling. In addition, local RAAS-upregulation was associated with increased pulmonary artery smooth muscle cell proliferation via enhanced AT_1 -receptor signaling in iPAH-patients compared to controls. Finally, to determine the therapeutic potential of RAAS-activity, we assessed the chronic effects of an AT_1 -receptor antagonist (losartan) in the monocrotaline PAH-rat model (60 mg/kg). Losartan delayed disease progression, decreased RV afterload and pulmonary vascular remodeling and restored right ventricular-arterial coupling in PAH-rats.

Conclusions – Systemic and pulmonary RAAS-activities are increased in iPAH-patients and associated with increased pulmonary vascular remodeling. Chronic inhibition of RAAS by losartan is beneficial in experimental PAH.

WORDS: 247

KEYWORDS: idiopathic pulmonary arterial hypertension, renin angiotensin system, endothelial cells, smooth muscle cells, monocrotaline.

For Review Only

INTRODUCTION

Idiopathic pulmonary arterial hypertension (iPAH) is a fatal disease with an average mortality rate of 58% within 3 years after diagnosis.(1) The disease is characterized by excessive pulmonary vascular remodeling, leading to elevated pressures in the pulmonary arterial system and the right heart.(2) As a consequence, iPAH-patients often have a low cardiac output.(2) To compensate, neurohormonal systems like renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system (SNS) are upregulated but this may have long-term negative effects on the progression of iPAH.(3)

In contrast to the increasing knowledge on the role of altered SNS signaling in the development of iPAH,(4-7) only little is known about alterations in RAAS signaling in iPAH. Two different polymorphisms in the angiotensin II type 1 (AT₁) receptor and angiotensin converting enzyme (ACE) are associated with disease progression and age-at-diagnosis in iPAH-patients.(8, 9) In addition, increased expression of pulmonary vascular ACE has been observed in patients with primary and secondary plexiform pulmonary hypertension.(10) Nevertheless, it is still unclear whether systemic and local RAAS-activity is associated with disease progression and pulmonary vascular remodeling.

There are several therapeutic strategies to interfere in the RAAS-signaling: renin inhibitors reduce the conversion of angiotensinogen into angiotensin (Ang) I, ACE-inhibitors reduce the conversion of AngI into AngII, and AT₁-receptor antagonists inhibit binding of AngII to AT₁-receptor.(11) Of these three, AT₁-receptor antagonists are the most specific, targeting only the AT₁-receptor and keeping AT₂-receptor signaling intact.(12, 13) However, due to the fear of inducing systemic hypotension and the unsatisfactory acute reductions in pulmonary vascular resistance by ACE-inhibitors, studies to the role of RAAS-activity and the therapeutic potential of RAAS-inhibitors in iPAH have not yet been performed.

Recently, the discovery of angiotensin converting enzyme 2 (ACE2) lead to renewed interest in the RAAS-signaling in PAH.(14, 15) ACE2 functions as a carboxypeptidase and cleaves a single carboxylate-terminal residue from AngII to form Ang (1-7).(16) Ang (1-7) is known to have vasodilatory effects via binding to the MAS-receptor and antagonizes the actions of AngII.(16)

Interestingly however, molecular analyses of the lungs of experimental PAH demonstrated mainly increased renin, angiotensinogen, ACE and AT₁-receptor expression, but no changes in MAS-receptor or ACE2.(14) Therefore, we hypothesize that AT₁-receptor antagonists will be a more relevant therapy for PAH.

The goal of this study was: 1) To determine whether RAAS-activity is increased in iPAH-patients, both systemically in serum and locally in pulmonary arteries (PA) and pulmonary endothelial cells (P-EC) of iPAH-patients and controls; 2) To investigate whether local RAAS-activity could induce proliferation of human cultured pulmonary artery smooth muscle cell (PA-SMC) derived from iPAH-patients and controls; 3) To elucidate the therapeutic potential of RAAS, by evaluating the effects of chronic AT₁-receptor inhibition in an experimental PAH model.

Some of the results of this study have been previously reported in the form of an abstract.(17, 18)

METHODS

Part I – Systemic RAAS-activity

Between 2004 and 2010, 879 blood samples were collected from patients admitted to the Pulmonology Department of the VU University Medical Center Amsterdam for standard clinical care. From these samples, 79 blood samples were obtained from iPAH-patients with no treatment history with beta-blockers or RAAS-inhibitors. Measurements of serum RAAS were performed in the IJssellandziekenhuis (Capelle a/d IJssel, The Netherlands) as described in the Online Data Supplement.

Part II – Local RAAS-activity in pulmonary vasculature of iPAH-patients

This part of the study was approved by the local ethics committee of the CPP Ile-de France VII, Le Kremlin-Bicêtre (France). All patients gave informed consent before the study. Lung specimens were obtained during lung transplantation (iPAH, n=13) and during lobectomy or pneumonectomy for cancer (controls, n=14). In controls, pulmonary cells were isolated distant from tumor areas. Pulmonary arteries (PA), P-ECs and PA-SMCs of iPAH-patients and controls were isolated, as described before.^(19, 20) PAs were used for protein analyses of AT₁-receptor, AT₂-receptor expression, SRC- and ERK-activation. Localization of AT₁-receptor, AngII production by P-ECs and PA-SMC proliferation after exposure of AngII were assessed as described in the Online Data Supplement.

Part III – Effects of chronic AT₁-receptor inhibition in experimental PAH

All animal experiments were approved by the Institutional Animal Care and Use Committee of the VU University Amsterdam (the Netherlands).

Prior to the efficacy study, we performed a dose-finding study to determine the maximum tolerated dose of losartan, as described before.⁽⁵⁾ The maximum tolerated dose was defined as less than 10% reduction in systemic blood pressure.

Subsequently, efficacy of chronic losartan treatment was tested in 27 rats (no telemetry): 9 control and 18 PAH-rats (monocrotaline 60 mg/kg). Ten days after monocrotaline-injection (when rats have developed PAH), (5) PAH-rats were randomized for losartan treatment (20 mg/kg dissolved in vanilla pudding) or vehicle (vanilla pudding) once daily (n=9/group). Rats were treated for maximal 25 days. For hemodynamic assessment, longitudinal echocardiography and pressure-volume analyses were performed. (5, 21) To determine morphological changes in heart and lungs, RV hypertrophy and muscularization of pulmonary arterioles were assessed. (5, 21)

Statistical analyses

All data were verified for normal distribution and log transformed when necessary. Data are presented as mean $\pm$ SEM, unless stated otherwise. A p-value of $p < 0.05$ was considered statistically significant. Comparisons of serum renin, AngI and AngII with their upper limit of normal reference values were performed by one-sample t-test. Prognostic relevance of serum renin, AngI and AngII was determined by univariate Cox-regression analyses and subsequent correction for confounding effects of gender, age and time-to-diagnosis. All-cause mortality and lung transplantation was recorded as an event in the Cox-regression analyses. Subgroup analyses of serum follow-up measurements were performed by 2-way ANOVA for repeated measurements. AT₁-receptor expression and activity was analyzed by unpaired t-test. P-EC AngII production, PA-SMC proliferation, the effects of losartan on disease progression and pressure-volume analyses were analyzed by one-way ANOVA with bonferroni post-hoc analyses. Histological data were analyzed using multilevel analysis to correct for non-independence of successive measurements per animal (MLwiN 2.02.03, Center for Multilevel Modeling, Bristol, UK). (5, 21-23)

RESULTS

Part I – Systemic RAAS-activity

Systemic RAAS-activity was measured in 79 serum samples of 58 iPAH-patients, 9 patients were treatment naïve on the day of sampling, and from 21 patients follow-up serum samples were available with a median follow up of 39 months (interquartile range 19–48 months). Patient characteristics are shown in Table 1.

Serum levels of renin-activity, AngI and AngII were significantly increased in comparison to the upper-limit of normal (dotted line; Fig.1A-C). In addition, increased AngII and renin-activity were significantly associated with an increase risk of death or lung transplantation, even after correction for age, gender and time-to-diagnosis (Table 3).

Subgroup analyses of the 21 patients with follow-up measurements of serum RAAS revealed that renin-activity, AngI and AngII serum levels were associated with worsening of iPAH. For this purpose, we divided patients in two groups: stable iPAH-patients with <10% decrease in 6MWD and progressive iPAH-patients with >10% decrease in 6MWD. Renin-activity, AngI and AngII serum levels were unaltered in stable iPAH-patients, whereas they were significantly increased over time in progressive iPAH-patients (Fig.1D-F).

These findings indicate that systemic RAAS-activity is elevated and associated with disease progression and mortality.

Part II – Local RAAS-activity in pulmonary vasculature of iPAH-patients

Increased AngII production via elevated ACE-activity in pulmonary endothelial cells of iPAH-patients

To investigate whether RAAS-activity is also locally upregulated in the pulmonary vasculature of iPAH-patients, we isolated P-ECs of lung specimens of iPAH-patients and control subjects. We measured AngII levels in media of isolated P-ECs of iPAH-patients and controls with and without exposure to AngI. Both in controls and iPAH-patients, AngI exposure induced an increase in AngII production. However, P-ECs of iPAH-patients produced significantly more AngII after AngI exposure than P-ECs of control subjects ($p < 0.01$; Fig.2). Conversion of AngI to AngII is regulated via ACE. To investigate whether the increased AngII production in P-ECs of iPAH-patients is caused by elevated

ACE-activity, we subsequently exposed P-ECs of iPAH-patients and controls to AngI in combination with the ACE inhibitor enalapril. Interestingly, co-incubation with enalapril totally abolished the observed difference in AngII production between P-ECs of iPAH-patients and control subjects ($p < 0.001$; Fig.2). This indicates that P-ECs of iPAH-patients produce significantly more AngII upon AngI incubation via elevated ACE-activity.

Elevated expression and signaling of the AT₁-receptor in pulmonary arteries of iPAH-patients

AngII has binding affinity for two receptors: the AT₁-receptor and the AT₂-receptor.(24) To investigate whether protein levels of AngII receptors are altered in the pulmonary vasculature of iPAH-patients, we performed immunohistochemical localization of proteins *in situ* and Western blot analysis of lung specimens from iPAH-patients and controls. Interestingly, we found a marked increased AT₁-receptor within walls of distal pulmonary arteries from iPAH-patients as shown by immunohistology (Fig. 3A). Consistent with these *in situ* findings, Western blot analyses of the AT₁- and AT₂-receptor, revealed a more than two fold increase in expression of AT₁-receptor (Fig.3B). To test whether the observed elevation in AT₁-receptor expression also resulted in increased receptor signaling, we measured the activity of downstream targets of AT₁-receptor, the tyrosine kinase SRC and extracellular regulated kinase (ERK). As can be observed in Fig.3C, SRC- and ERK-activity were significantly increased in iPAH-patients in comparison to controls. These findings indicate that in iPAH-patients the AT₁-receptor expression and signaling are increased.

AngII induces proliferation of pulmonary artery smooth muscle cells of iPAH-patients via AT₁-receptor signaling

To investigate whether AngII induce PA-SMC proliferation, we isolated and cultured PA-SMCs of iPAH-patients and controls. Incubation with 10% fetal calf serum confirmed the previously found pro-proliferative state of iPAH PA-SMCs.(19) AngII incubation induced specific proliferation of the PA-SMCs of iPAH-patients, while no change in proliferation of control PA-SMCs was observed (Fig.4). To

test whether AngII exerts its proliferative effect on PA-SMCs via binding to the AT₁-receptor, PA-SMCs were exposed to AngII in combination with the AT₁-receptor antagonist losartan. Interestingly, the difference in PA-SMC proliferation between control and iPAH was completely abolished after co-incubation with losartan. These findings indicate that AngII can induce PA-SMC proliferation in iPAH via AT₁-receptor signaling.

Part III – Effects of chronic AT₁-receptor inhibition in experimental PAH

To investigate whether chronic inhibition of AT₁-receptor signaling could have therapeutic potential for the treatment of PAH, we performed a pre-clinical study in a well-established rat-model with PAH induced by high dose of monocrotaline.(21) First, we performed a dose-finding study to determine the maximum tolerated dose of losartan, which was defined as less than 10% reduction in systemic blood pressure (Fig.5; dotted line). A clear dose response was observed in control rats, where systemic blood pressure gradually decreased with higher dosages of losartan. A dose of 40 mg/kg induced >10% reduction in systemic blood pressure, and was therefore not repeated in PAH-rats. No difference in change in systemic blood pressure was observed in PAH-rats between a dose of 5 mg/kg, 10 mg/kg and 20 mg/kg losartan. Since all dosages were tolerated by PAH-rats, we investigated the chronic effect of 20 mg/kg losartan in the main pre-clinical study.

Losartan reduces pulmonary vascular remodeling in PAH-rats

Ten days after injection, echocardiographic analyses revealed clear signs of PAH in all monocrotaline-treated rats. Estimated right ventricular (RV) systolic pressures (PAAT/cl and eRVSP) and pulmonary vascular remodeling (PVR) were significantly increased in comparison to controls, and early signs of RV hypertrophy and dilatation were observed in all 18 PAH-rats (Table E1).

Serial echocardiography (at day 10 and at end-of-study) demonstrated a significantly delayed disease progression in losartan treated rats (Fig.6). The increment in pulmonary vascular remodeling was reduced in losartan treated PAH-rats in comparison to vehicle-treated PAH-rats (Fig. 6A,B;

supplement Table E2). No changes were observed in RV function (Fig. 6C,D) or hypertrophy (Fig. 6E) after losartan treatment, but a reduced RV dilation was noted (Fig. 6F).

RV pressure-volume measurements at end-of-study (Fig. 7A-C), revealed that losartan was able to significantly reduced RV afterload (arterial elastance; Fig. 7D), without affecting RV contractility (end-systolic elastance; Fig. 7E). This resulted in improved RV ventricular-arterial coupling in losartan-treated rats (E_{es}/E_a : PAH 0.74 ± 0.11 vs. PAH+losartan 1.32 ± 0.20 ; $p < 0.05$). In addition, RV relaxation was improved, as demonstrated by reduced RV diastolic elastance (Fig. 7F).

Histological analyses of PA-wall thickness and cross-sectional area of RV cardiomyocytes demonstrated a clear increase in PAH-rats in comparison to controls (both $p < 0.001$; Fig. 8). Losartan significantly reduced PA wall thickness in PAH-rats, which could explain the reduction in RV afterload by losartan. Although RV afterload was reduced in losartan treated PAH-rats, no differences were observed between losartan and vehicle-treated PAH-rats in RV hypertrophy, whether expressed as RV mass, RV/LV+septum or RV cross sectional area (Fig. 8B, supplement Table E3).

These findings indicate that chronic inhibition of AT_1 -receptor signaling delayed disease progression, reduced RV afterload and pulmonary vascular remodeling, restored right ventricular-arterial coupling and improved RV diastolic function.

DISCUSSION

To the best of our knowledge, this is the first study to demonstrate the effects of increased systemic and local RAAS-activity in iPAH-patients. Using a translational approach with a set of physiological and pathological endpoints, we have demonstrated that:

1. Systemic RAAS-activity is increased in iPAH-patients and associated with disease progression and the risk of death or lung transplantation.
2. Due to increased ACE-activity, pulmonary endothelial cells of iPAH-patients produce more AngII, which induces significant pulmonary artery smooth muscle cell proliferation of iPAH-patients via AT₁-receptor signaling;
3. Upregulated AT₁-receptor expression and signaling, without changes in AT₂-receptor expression
4. Chronic inhibition of AT₁-receptor signaling by losartan delays disease progression, reduces RV afterload and pulmonary vascular remodeling, restores right ventricular-arterial coupling and improves RV diastolic function in experimental PAH.

Our data demonstrate the importance of AT₁-receptor signaling on pulmonary vascular remodeling in PAH and provide sufficient evidence to validate further clinical investigation to the therapeutic potential of AT₁-receptor blockers in PAH.

Elevated systemic and local RAAS-activity

RAAS-activity is regulated systemically and locally in a variety of tissues (brain, skin, digestive organs, lymphatic tissue, adipose tissue, vasculature and heart).(25) Systemic RAAS-activity is mainly controlled via the release of renin, secreted by the juxtaglomerular cells of the kidney, cleaving angiotensinogen into AngI, which is subsequently cleaved by ACE to form AngII.(26) In left heart failure, the prognostic importance of increased systemic RAAS-activity has been recognized for a long time.(27) In PAH, it is suggested that systemic RAAS-activity was upregulated, based on the findings of upregulated SNS-activity (4-7) and the prognostic importance of hyponatremia.(28) However,

parameters that more directly measure RAAS-activity have not been systematically investigated in PAH-patients until now.(2) We observed a close relation between systemic RAAS-activity with disease progression and mortality, which suggest that RAAS-activity could be an important factor in PAH.

Besides the conventional systemic RAAS-activity, recent findings have revealed local RAAS-activity in several organs.(25) Especially the local cardiac and vascular RAAS-activity are interesting in the setting of iPAH. In this article, we have mainly focussed on the vascular RAAS-activity. Local vascular RAAS-activity is characterized by the presence of ACE, AT₁-, AT₂-receptor on the surface of vascular endothelial cells, and AT₁-receptor on vascular smooth muscle cells.(25) In this study, we observed that iPAH-patients had an increased ACE-activity in their pulmonary endothelial cells and elevated expression and signaling of the AT₁-receptor in the pulmonary vasculature. Interestingly, no alterations were observed in AT₂-receptor expression. AngII can bind to both the AT₁- and AT₂-receptors, however binding of AngII to the different receptors results in opposing effects.(25) Binding of AngII to AT₁-receptor can result in increased vasoconstriction via upregulation of endothelin or decreasing NO-availability via increased oxidative stress, whereas binding of AngII to the AT₂-receptor results in vasodilation via activation of protein phosphatase and increased NO bio-availability via NO/cGMP activation.(25) Besides vasomotor effects, stimulation of the AT₁-receptor can result in migration and proliferation of vascular smooth muscle cells via activation of intracellular tyrosine kinases.(29) Downstream tyrosine kinases of the AT₁-receptor are SRC and ERK,(30) which are important regulators of cell proliferation.(31) In our study we were able to demonstrate that besides elevated AT₁-receptor expression, phosphorylation of SRC and ERK was increased in iPAH-patients, indicating for increased AT₁-receptor activity. The finding that PA-SMC proliferation is restored after incubation with an AT₁-receptor antagonist underlines the causal relation between AT₁-receptor activity and PA-SMC proliferation.

Whether the systemic changes in RAAS-activity override the local changes in the lungs is unclear. However, it is expected that increased systemic RAAS-activity would further exaggerate the local

changes in RAAS-activity in the lungs. In our study, we used similar concentrations of AngI for both PAH-patients and controls, whereas in reality P-ECs of iPAH-patients are exposed to even higher AngI concentrations. The observed changes in local RAAS-activity may therefore even be underestimated.

Therapeutic potential of RAAS

In the nineties, two small case series testing the short-term effectiveness of ACE-inhibitors in iPAH-patients and patients with connective tissue disease have been performed.(32, 33) However, due to the fear of inducing systemic hypotension and the unsatisfactory acute reductions in pulmonary vascular resistance by ACE-inhibitors, studies to the role of RAAS-activity and the therapeutic potential of other RAAS-inhibitors have not yet been performed.

We therefore used in the current study an AT₁-receptor antagonist, which specifically inhibit AT₁-receptor signaling, thereby keeping AT₂-receptor and ACE2 signaling intact.(12) Our results demonstrate the therapeutic potential of AT₁-receptor blockers in experimental PAH. We observed significant reductions in disease progression, RV afterload and pulmonary vascular remodeling. Our findings confirm the previous reported preventive effects of losartan in a shunt-induced PAH-model.(34) In addition, two other pre-clinical studies have investigated the effect of an AT₁-receptor antagonist on RV morphology and hypertrophy. In contrast to our findings, they observed a significant reduction in RV hypertrophy.(35, 36) This might be explained by differences in type and dosage of the used AT₁-receptor antagonist and the difference in follow-up time.

Mixed effects of AT₁-receptor antagonist on cardiac function were observed in our pre-clinical model: we found no changes in stroke volume/cardiac output and RV hypertrophy, but reduced RV dilatation and diastolic elastance. It can be speculated that chronic AT₁-receptor blockade exerts pulmonary effects rather than cardiac effects. The changes in RV dilatation and diastolic elastance would then be a mere consequence of reduced RV afterload due to reduced pulmonary vascular remodeling. Nevertheless, then still a reduction in RV wall thickness would be expected. Probably,

reversion of RV hypertrophy takes more time and the relative short treatment period of the PAH-model could have prohibited a significant reduction in RV hypertrophy.

Furthermore, although losartan was able to reduce RV afterload, RV afterload was not normalized.

We hypothesize that the reduction in RV afterload was only sufficient to exert partial normalization of RV function and morphology. We did similar observations in clinical PAH,(37) where reduction in PVR not always translated into improvement in RV ejection fraction.

Limitations

In this study, we only used patient samples with iPAH to prevent confounding effects of other co-morbidities as for instance inflammation in scleroderma-associated PAH. Based on the previous finding of increased sympathetic system activity in PAH and the close association between sympathetic system and RAAS activity, we would suspect that systemic and local RAAS-activation are also increased in other forms of PAH. However, at this point we cannot conclude this with firm certainty.

To assess AT₁-receptor activity, we measured activity of the tyrosine kinase SRC and ERK. However, other upstream pathways could also affect phosphorylation of these kinases. Nevertheless, the abolishing effect of AT₁-receptor antagonist on PA-SMC proliferation, indicates that increased AT₁-receptor activity is important in the pathophysiology of iPAH.

In our study we could not observe a significant reduction in AT₂-receptor expression in pulmonary arteries. This may be due to a low power of the analyses. Although, the difference between control and iPAH is small, we cannot exclude that reduced AT₂-receptor expression could further exaggerate the proliferative effects on PA-SMCs. However, this further underlines the importance of selective AT₁-receptor blockers, since ACE-inhibitors would affect both AT₁ as well as AT₂-receptor expression. Our pressure-volume analyses do not allow us to determine whether treatment has altered diastolic and systolic volumes. However, in combination with our echocardiographic derived RV end-diastolic

diameters, we were able to conclude that AT₁-receptor antagonist could reduce RV dilatation in PAH-treated rats.

Conclusions

In this translational study, we have demonstrated systemic and local upregulation of RAAS-activity in pulmonary arteries of iPAH-patients. In addition, local RAAS upregulation was associated with increased pulmonary artery smooth muscle cell proliferation via enhanced angiotensin II type 1 (AT₁) receptor signaling in iPAH-patients compared to controls. Chronic treatment with an AT₁-receptor antagonist in experimental PAH revealed reduced disease progression, decreased RV afterload and pulmonary vascular remodeling and restored right ventricular-arterial coupling. Our findings warrant further clinical investigation and re-evaluation of the use of RAAS-inhibitors for treatment of iPAH-patients.

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TABLES

Table 1 – Baseline characteristic iPAH-patients for serum RAAS measurements

iPAH-patients (n=58)	
Age (years)	49±16
Gender	
Male	17 (29%)
Female	41 (71%)
NYHA	
II	35 (60%)
III	18 (31%)
IV	5 (9%)
6MWD (m)	478±134
NT-proBNP (pg/ml)	1171±1747
mPAP (mmHg)	47±14
PVR (dynes.s/cm ⁵)	637±308
CO (l/min)	5.5±1.6
HR (beats/min)	79±14
RAP (mmHg)	6±5
PCWP (mmHg)	7±4
Sodium (mmol/l)	139±3
Potassium (mmol/l)	3.8±0.3
BUN (mmol/l)	5.9±1.8
Creatinin (mg/dl)	0.9±0.2
eGFR (ml/min/1.73 m ²)	77±16
Treatment	
Naive	9 (16%)
Mono	33 (57%)
Duo	12 (20%)
Triple	4 (7%)
Treatment strategies	
Lisdiuretics	30
Spironolacton	26
Bosentan	18
Sitaxentan	7
Ambrisentan	2
Sildenafil	21
Treprostenil	5
Epoprostenol	11
Iloprost	1
Ca ²⁺ -blocker	3
Simvastatin	1

Data presented as mean±SD. NYHA, New York Heart Association class; 6MWD, six minute walk distance; NT-proBNP, N-terminal pro brain natriuretic peptide; mPAP, mean pulmonary artery pressure; PVR, pulmonary vascular resistance; CO, cardiac output; HR, heart rate; RAP, right atrial pressure; PCWP, pulmonary capillary wedge pressure; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate.

Table 2 – Patients characteristics tissue samples

	iPAH	Controls
	N=13	N=14
Age (yrs)	34 ±13	62 ±13
Sex, M/F (ratio)	5/8	7/7
mPAP (mmHg)	70 ±14	
CO (l/min)	4.0 ±1.1	
PVR (mmHg/l/min)	1302.8 ±333.6	
PCWP (mmHg)	7.4 ±1.1	

Data presented as mean±SD. mPAP=mean pulmonary artery pressure; CO=cardiac output; PVR=pulmonary vascular resistance; PCWP=pulmonary capillary wedge pressure

Table 3 – Cox regression analyses serum RAAS-activity

Variables	Hazards Ratio	95% CI	p-value
<i>Univariate Cox-regression analyses</i>			
LN_renin	1.651	1.026-2.658	0.039
LN_Angiotensin I	0.949	0.601-1.496	0.821
LN_Angiotensin II	2.883	1.358-6.122	0.006
<i>Cox regression analyses corrected for age, gender and time from diagnosis</i>			
LN_renin	1.793	1.129-2.846	0.013
LN_Angiotensin I	0.971	0.576-1.636	0.911
LN_Angiotensin II	3.017	1.404-6.484	0.005

Abbreviations: 95% CI, 95% confidence interval; ln, logarithmic transformation.

FIGURE LEGENDS**Figure 1 – Serum RAAS in iPAH-patients**

Serum levels of renin, AngI and AngII were increased in the majority of patients in comparison to upper limit of normal (dotted line; A-C). Follow-up measurements in a subgroup of patients (n=21) revealed that increased serum levels of renin, AngI and AngII were associated with progressive iPAH (D-F).

Data presented as mean $\pm$ SEM, n=58 iPAH-patients. Dotted line represents the upper limit of reference. Light blue bars represent baseline, dark blue bars represent follow up. iPAH, idiopathic pulmonary arterial hypertension.

Figure 2 – Pulmonary endothelial cells of iPAH-patients produce more angiotensin II

Exposure of pulmonary endothelial cells to AngI revealed significant higher production of AngII in pulmonary endothelial cells of iPAH-patients than controls. Co-incubation with ACE-inhibitor enalapril totally abolished this effect, indicating that ACE-activity in pulmonary endothelial cells of iPAH-patients is increased.

** $p < 0.01$, *** $p < 0.001$ vs. values of unstimulated cells (Base). Data presented as mean $\pm$ SEM, n=3 per group. CON, control; iPAH, idiopathic pulmonary arterial hypertension; Base, unstimulated condition; AngI, angiotensin I; Enalapril, ACE-inhibitor.

Figure 3 – Angiotensin II type 1 receptor expression and signaling is increased in pulmonary arteries of iPAH-patients

Typical examples of histological sections of lung specimens are shown of a control and iPAH-patient (A), stained for angiotensin II type 1 receptor (AT₁-receptor). Western blot analyses revealed significant upregulation of AT₁-receptor expression in pulmonary arteries of iPAH-patients in comparison to control; no changes in AT₂-receptor expression were observed (B). In addition,

tyrosine kinase SRC-activity and ERK-activity (downstream targets of AT₁-receptor) were significantly increased, suggesting increased signaling activity of the AT₁-receptor (C).

Data presented as mean $\pm$ SEM, n=5 per group. AT₁-receptor, angiotensin II type 1 receptor; p-SRC, expression phosphorylated form of tyrosine kinase SRC; t-SRC, total protein expression of tyrosine kinase SRC; p-ERK, expression phosphorylated form of extracellular regulated kinase; t-ERK, total protein expression of extracellular regulated kinase; P, idiopathic pulmonary arterial hypertension; C, control; M, marker.

Figure 4 – Angiotensin II incubation induces selective proliferation of the pulmonary artery smooth muscle cells of iPAH-patients via AT₁-receptor signaling

Exposure of pulmonary artery smooth muscle cells (PA-SMC) to angiotensin II induced significantly more PA-SMC proliferation in iPAH-patients in comparison to controls. Co-incubation with an AT₁-receptor antagonist (losartan) abolished this effect completely. This indicates that angiotensin II exerts its proliferative effect in PA-SMC of iPAH-patients via AT₁-receptor signaling.

** p<0.01; *** p<0.001 vs. values of unstimulated cells (Base). Data presented as mean $\pm$ SEM, n=4 per group. iPAH, idiopathic pulmonary arterial hypertension; PA-SMC, pulmonary artery smooth muscle cells; Base, unstimulated condition; FCS, fetal calf serum; AngII, angiotensin II; losartan, AT₁-receptor antagonist

Figure 5 – Losartan dose finding in control and PAH rats

Maximal tolerated dosage (<10% reduction in aortic pressure; dotted line) of losartan was tested by 48-hours telemetry registration. Four different dosages of losartan were tested in control rats (total n=9): 5 mg/kg (n=2); 10 mg/kg (n=3); 20 mg/kg (n=2); 40 mg/kg (n=2). After MCT-injection, dose finding was repeated with three different dosages: 5 mg/kg (n=3); 10 mg/kg (n=3); 20 mg/kg (n=3). A dose of 40 mg/kg was not tested in PAH-rats due to >10% reduction in aortic pressure in control rats.

All dosages tested in PAH-rats did not induce >10% reduction in aortic pressure. A dose of 20 mg/kg was therefore used to test the chronic effects of losartan in PAH-rats.

Data presented as mean $\pm$ SEM, n=2/3 per group. PAH, pulmonary arterial hypertension; Δ P aorta, change in aortic pressure.

Figure 6 – Losartan significantly delayed disease progression in PAH-treated rats

Losartan treatment delayed the progression of pulmonary vascular remodeling (A, B), and delayed RV dilation (F). No changes were observed in cardiac function (C,D) or RV wall thickness (E).

Data presented as mean $\pm$ SEM, n=9 per group. ### p<0.01, #### p<0.001 PAH/PAH+losartan vs. control, * p<0.05, ** p<0.01 PAH vs. PAH+losartan. RV, right ventricle; TAPSE, tricuspid annular plane systolic excursion; MCT, monocrotaline; Con, control; PAH, pulmonary arterial hypertension.

Figure 7 – Losartan significantly reduced RV afterload, restore ventricular-arterial coupling and improved RV diastolic function

Typical examples of pressure-volume relation are shown for control, PAH and PAH+losartan (A-C). Losartan reduced RV arterial elastance (D) without affecting RV contractility (E). In addition, RV diastolic function improved significantly after losartan treatment illustrated by reduced RV end-diastolic elastance (F). For measurements of RV dilatation, see Figure 6.

Data presented as mean $\pm$ SEM, n=9 per group. Con, control; PAH, pulmonary arterial hypertension.

Figure 8 – Losartan treatment reduced muscularization of the pulmonary arterioles without changing RV hypertrophy

Representative images of histological sections of lung specimens are shown of control, PAH and PAH+losartan rats (A), stained for WGA (glycocalyx, red). Losartan significantly reduced pulmonary artery wall thickness in PAH rats (B). RV cross sectional area was equally increased in both PAH and PAH+losartan rats (C).

Data presented as mean $\pm$ SEM, n=9 per group. PA, pulmonary artery; CSA, cross sectional area; CON, control; PAH, pulmonary arterial hypertension.

For Review Only

SUPPLEMENT**Dysregulated Renin-Angiotensin-Aldosterone System Contributes to Pulmonary Arterial Hypertension**

Frances S. de Man, PhD^{1,2}, Ly Tu, MSc¹, M. Louis Handoko, MD PhD^{2,3}, Silvia Rain, MD², Gerrina Ruiter, MD², Charlene François, BSc¹, Ingrid Schali, BSc², Peter Dorfmueller, MD PhD¹, Gérald Simonneau, MD PhD¹, Elie Fadel, MD PhD¹, Frederic Perros, PhD¹, Anco Boonstra, MD PhD², Piet E. Postmus, MD PhD², Jolanda van der Velden, PhD³, Anton Vonk-Noordegraaf, MD PhD², Marc Humbert, MD PhD¹, Saadia Eddahibi PhD¹, Christophe Guignabert, PhD¹

¹INSERM U999, Le Plessis-Robinson, France; ²Department of Pulmonology and ³Physiology, VU University Medical Center / Institute of Cardiovascular Research, Amsterdam, The Netherlands

Expanded METHODS

Part I – Systemic RAAS activity

We collected 79 blood samples from 58 iPAH-patients between 2004 and 2010 in the VU University Medical Center Amsterdam. Of these samples, 9 were obtained in treatment naïve patients; from 21 patients follow-up plasma samples were available, with a median follow up of 39 months (IQR 19–48 months). In parallel, right heart catheterization and MRI measurements were performed in all patients. Estimated glomerular fraction rate (eGFR) was calculated according to the modification of diet in renal disease (MDRD) formula^{E1}: $eGFR = 186 \times \text{serum creatinin}^{-1.154} \times \text{Age}^{-0.203} \times [0.742 \text{ if female}]$. All blood samples were immediately cooled on ice, centrifuged at 4°C and stored at -20°C for further analysis.

Laboratory analyses

Renin activity (n=79 samples) was determined using the Renin III generation assay kit (Cisbio, Codolet, France). This assay consists of a pair of antibodies: first monoclonal antibody recognizes both the active and inactive form of renin, second monoclonal antibody specifically recognizes the active form of renin. In addition, serum levels of Ang I (n=73 samples) by a home-brew radioimmunometric assay (IJssellandziekenhuis) and Ang II (n=79 samples) by using a radioimmunoassay kit (RB320, Euro-diagnostica, Malmö, Sweden).

Part II – Local RAAS activity in pulmonary vasculature of iPAH-patients

Measurement of Ang II production by P-ECs

Medium of P-EC was used to measure Ang II levels upon Ang I incubation. For this purpose, medium was obtained from serum-starved P-ECs in MCDB 131 medium (0% FCS) for 24 hours with or without 10^{-10} M Ang I or 10^{-10} M Ang I in combination with 50 µM enalapril. Ang II levels in P-EC medium were determined by ELISA (R&D systems, Lille, France).

Assessment of PA-SMC proliferation after Ang II exposure

Cell proliferation was determined by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporation into cellular DNA using the BrdU cell proliferation assay kit (Cell signaling technology, Danvers, USA). PA-SMC proliferation was assessed with or without 10% fetal calf serum, 100 μ M Ang II or 100 μ M Ang II in combination with 10^{-6} M losartan (AT_1 -receptor antagonist).

Immunohistological and protein analyses of AT_1 -receptor expression and activity

Pulmonary arteries (PA) were homogenized in RIPA buffer containing phosphatase and protease inhibitors (Sigma-Aldrich, St. Louis, USA). Fifty μ g of protein extract was used to detect AT_1 -receptor expression (1:500; Ab9391, Abcam, Camebridge, UK), phospho-(Tyr 416)-SRC (1:1000; 2101, Cell signaling technology, Danvers, USA) and total-SRC (1:1000; 2109, Cell signaling technology, Danvers, USA) by SDS-PAGE, as previously described.^{E2} Protein levels were corrected for differences in protein loading by β -actin (1:5000; A5316, Sigma-Aldrich, St. Louis, USA).

For the localization of the AT_1 -receptor expression, paraffin-embedded sections were incubated overnight at 4°C with primary AT_1 -receptor antibody (1:100; sc-1173, Santa Cruz Biotechnology, Santa Cruz, USA), as previously described.^{E2}

Part III – Beneficial effects of chronic AT_1 -receptor inhibition in experimental PAH

Nine rats were equipped with an implantable telemetric pressure-transmitter placed in the abdominal aorta as previously described.^{E3} First, baseline aortic pressures were measured over 3 consecutive days. Subsequently, different dosages of losartan dissolved in vanilla pudding were administered for 3 consecutive days: 2 rats received 40 mg/kg losartan, 2 rats received 20 mg/kg losartan, 3 rats received 10 mg/kg losartan and 2 rats received 5 mg/kg losartan. After dose-finding in control rats, we induced pulmonary hypertension by a single injection of a high dose of monocrotaline (MCT 60 mg/kg). Two weeks after the MCT-injection, PAH was developed and we repeated baseline measurements for 2 consecutive days in the PAH rats. Subsequently, different

dosages of losartan dissolved in vanilla pudding were administered for 3 consecutive days: 3 rats received 20 mg/kg losartan, 3 rats received 10 mg/kg losartan and 3 rats received 5 mg/kg losartan. The dosage of 40 mg/kg losartan was not repeated in PAH-rats, since it induced >10% reduction in systemic blood pressure in the control rats. The effect of losartan on systemic blood pressure was analyzed off-line, using Dataquest A.R.T. Analysis software (version 4.2, DSI). After these experiments, all rats were euthanized and their organs examined. No additional measurements were performed.

Assessment of disease progression by longitudinal echocardiography after losartan

Rats were evaluated by echocardiography 10 days after (monocrotaline-)injection and at end-of-study (when manifest right heart failure developed, or 35 days after injection). When rats developed clinical signs of manifest right heart failure (defined as >10% loss in body mass and/or respiratory distress, cyanosis, lethargy) were euthanized earlier, in keeping with the protocol of the local animal care and use committee.^{E3-7}

Transthoracic echocardiographic measurements (ProSound SSD-4000 system equipped with a 13-MHz linear transducer (UST-5542), Aloka, Tokyo, Japan) were performed on anesthetized but spontaneously breathing rats (isoflurane 2.0% in 1:1 O₂/air mix; Pharmachemie, Haarlem, The Netherlands),^{E3;5;7} which allows for serial assessment of cardiac dimensions and hemodynamics in rats with equal diagnostic accuracy as cardiac MRI, due to its high temporal resolution.^{E6} Analyses were performed off-line (Image-Arena 2.9.1, TomTec Imaging Systems, Unterschleissheim/Munich, Germany). Measured parameters for RV function were: cardiac output (Doppler-derived stroke volume, heart rate), and tricuspid annular plane systolic excursion (TAPSE). Parameters for RV remodeling were: RV end-diastolic diameter (RVEDD) and RV wall thickness. Pulmonary artery acceleration time normalized for cardiac cycle length (PAAT/cl) was used to a non-invasive estimate for RV systolic pressure (PAAT/cl and RVSP are inversely correlated). Disease progression of PAH during treatment-period was expressed as percentage changes in hemodynamics over time,^{E7} e.g.

change in cardiac output: $\Delta \text{cardiac output} = [(cardiac\ output_{end} - cardiac\ output_{start}) / cardiac\ output_{start}] / \text{days-of-treatment} * 100\%$. Other parameters for disease progression were calculated similarly.

Measurement of RV function and ventricular-arterial coupling after losartan

At the end of study protocol, all rats underwent open-chest RV catheterization (Millar Instruments, Houston TX). The rats were sedated by inhalation of isoflurane (induction: 4.0% in 1:1 O₂/air mix; maintenance: 2.0% in 1:1 O₂/air mix), intubated (16 G Teflon tube) and attached to a mechanical ventilator (Micro-Ventilator, UNO, Zevenaar, The Netherlands; ventilator settings: breathing frequency 75/min, pressures 9/0 cmH₂O, inspiratory/expiratory ratio 1:1). The rats were placed on a warming pad to maintain body temperature.

After opening of the thorax, a temporal ligature was placed around the inferior vena cava. Following an apical stab (23G), a combined pressure-volume catheter (SPR-869, Millar Instruments, Houston TX) was inserted into the right ventricle and positioned along its long axis. The signals (processed by MPVS-400, Millar Instruments), obtained at steady state (at least 10s) and during transient vena cava occlusion were digitally recorded (2.0 kHz sampling rate; Chart 5.5.6, AD Instruments, Sydney, Australia) and analyzed off-line. Stroke volume (in RVU) derived from the conductance signal was calibrated, using stroke volume (in ml) derived from echo-Doppler as external reference in PVAN 3.6 (Millar instruments). Using custom-made algorithms (programmed in MATLAB 2007b, The Math Works, Natick MA) RV (peak-)systolic pressures and RV end-diastolic pressures were automatically determined from steady-state measurements, as well as arterial elastance (Ea), a measure for RV afterload.^{E8;9} From occlusion-data, end-systolic elastance (Ees; contractility) and end-diastolic elastance (Eed; filling) were determined.^{E9;10} The ratio Ees/Ea was calculated as an estimate for ventricular-arterial coupling (cardiac adaptation in relation to its load).^{E8;9}

Assessment of RV hypertrophy and muscularization of pulmonary arterioles

After the final hemodynamic assessment, all 27 rats were euthanized (by exsanguination under isoflurane), and heart, lungs and other major organs were harvested. Lungs were weighed and the left lobe was subsequently filled by 1:1 mix of saline and cryofixative (Tissue-Tek O.C.T. compound, Sakura, Fintek, Europe, Zoeterwolde, The Netherlands), and snapfrozen in liquid nitrogen. The right lobe was used to measure wet/dry lung mass ratio. The heart was perfused, weighted, dissected and snap-frozen in liquid nitrogen.

Images were collected by the use of a Leica DMRB microscope (Wetzlar, Germany), a Sony XC-77CE camera (Towada, Japan) and an LG-3 frame grabber (Scion, Frederick MD) ImageJ for Windows 1.42 software (National Institutes of Health, Bethesda MD) was used for image analysis, taking the pixel-to-aspect ratio into account.

Cardiomyocyte cross-sectional area. Haematoxylin & eosin (HE)-stained cardiac cryosections (5 µm) were used to determine RV cardiomyocyte cross-sectional area (CSA). Cardiomyocyte size for each ventricle was expressed as the average CSA of minimally twenty transversally cut cardiomyocytes at the level of the nucleus, randomly distributed over the ventricles.

Relative wall thickness of pulmonary arterioles. Pulmonary sections (5 µm) were stained with Elastica von Giesson for morphometric analysis of vascular dimensions. Minimally fifty transversally cut pulmonary arterioles, with an outer diameter between 25 and 100 µm, randomly distributed over the lungs, were measured. Relative wall thickness of pulmonary arterioles (PA) was calculated as:

$$PA \text{ wall thickness} = 2 * \text{medial wall thickness} / \text{outer diameter} * 100\% .$$

Immunofluorescence imaging AT₁-receptor

To determine changes in AT₁-receptor expression after chronic losartan treatment, we performed immunofluorescence imaging. Lung cryosections (5 µm) were incubated overnight at 4°C with primary AT₁-receptor antibody (1:20, ab9391, abcam, Cambridge, UK), followed by appropriate secondary antibody staining as well as WGA (glycocalyx) and DAPI (nuclei) counterstaining. Image

acquisition was performed on a Marianas digital imaging microscopy workstation (Intelligent Imaging Innovations (3i), Denver CO) and SlideBook imaging analysis software (SlideBook 4.2, 3i).

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Table E1 – Baseline characteristics control and PAH-rats

	Control (n=9)	PAH (n=9)	PAH+losartan (n=9)
Cardiac output (ml/min)	98±9	78±4	88±6
Stroke volume (ml)	0.25±0.02	0.19±0.01	0.23±0.01
Heart rate (bpm)	401±9	410±9	392±4
TAPSE (mm)	2.6±0.1	2.7±0.1	2.6±0.2
RV wall thickness (mm)	0.99±0.03	1.20±0.04***	1.20±0.03***
RVEDD (mm)	3.0±0.1	3.9±0.3*	4.0±0.2**
PAAT/cl (*100)	18.1±0.5	13.3±0.3***	12.6±0.3***
eRVSP (mmHg)	20±1	32.9±3***	35.7±1***
PVR (mmHg/ml*min)	0.15±0.02	0.28±0.02*	0.29±0.04**

PAAT/cl, pulmonary acceleration time divided by cyclus length (inversely related to RVSP); eRVSP, estimated right ventricular systolic pressure; PVR, pulmonary vascular resistance; SV, stroke volume; HR, heart rate; CO, cardiac output; RVWT, right ventricular wall thickness; RVEDD, right ventricular end-diastolic diameter.

*: p<0.05 vs. control

Table E2 – Disease progression in experimental PAH, assessed by serial echocardiography

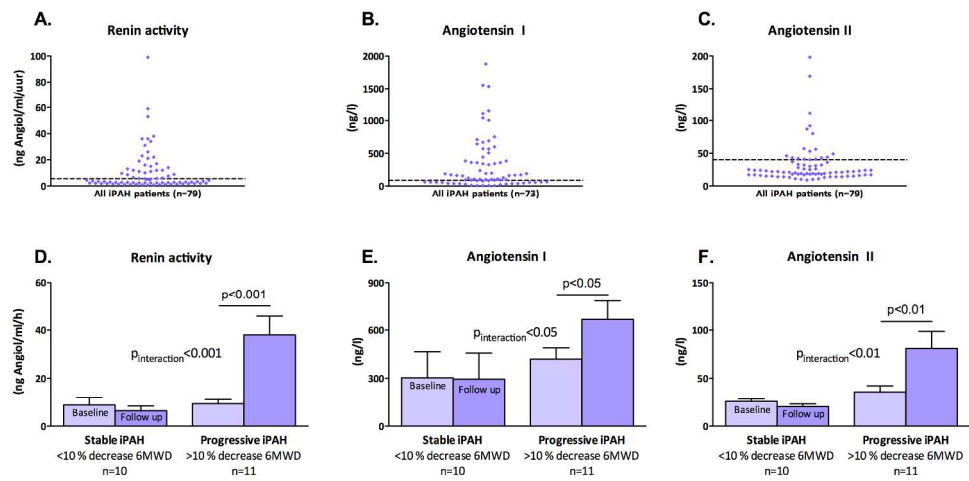
	Control	PAH	PAH+losartan	p-value	
	(n=9)	(n=9)	(n=9)	Control vs. PAH	PAH vs. PAH+losartan
ΔPAAT/cl (%/day)	0.3 ± 0.2	-2.3 ± 0.2	-1.4 ± 0.2	<0.001	0.004
ΔeRVSP (%/day)	-0.4 ± 0.2	5.6 ± 0.8	2.6 ± 0.4	<0.001	<0.001
ΔPVR (%/day)	-0.7 ± 0.3	19.3 ± 2.8	12.4 ± 2.2	<0.001	0.024
ΔRV wall thickness (%/day)	0.3 ± 0.1	1.6 ± 0.3	1.8 ± 0.2	<0.001	0.651
ΔRV end-diastolic diameter (%/day)	0.5 ± 0.1	4.5 ± 0.6	2.2 ± 0.4	<0.001	0.001
ΔTAPSE (%/day)	0.3 ± 0.1	-1.7 ± 0.4	-1.3 ± 0.4	<0.001	0.410
ΔCardiac output (%/day)	0.5 ± 0.2	-2.1 ± 0.1	-2.0 ± 0.3	<0.001	0.677
ΔHeart rate (%/day)	0.1 ± 0.1	-0.6 ± 0.1	-0.6 ± 0.1	<0.001	0.744
ΔStroke volume (%/day)	0.4 ± 0.1	-1.8 ± 0.1	-1.7 ± 0.2	<0.001	0.622

ΔPAAT/cl, daily percentage change in pulmonary artery acceleration time normalized for cardiac cycle length; ΔeRVSP, daily percentage change in estimated right ventricular systolic pressure, ΔPVR, daily percentage change in estimated pulmonary vascular resistance; ΔRV wall thickness, daily percentage change in right ventricular wall thickness; ΔRV end-diastolic diameter, daily percentage change in right ventricular end-diastolic diameter; ΔTAPSE, daily percentage change in tricuspid annular plane systolic excursion; Δcardiac output, daily percentage change in cardiac output; ΔHeart rate, daily percentage change in heart rate; ΔStroke volume, daily percentage change in stroke volume; PAH, PAH-rats (treated with vehicle); PAH+losartan: PAH-rats treated with 20 mg/kg losartan once daily from day 10.

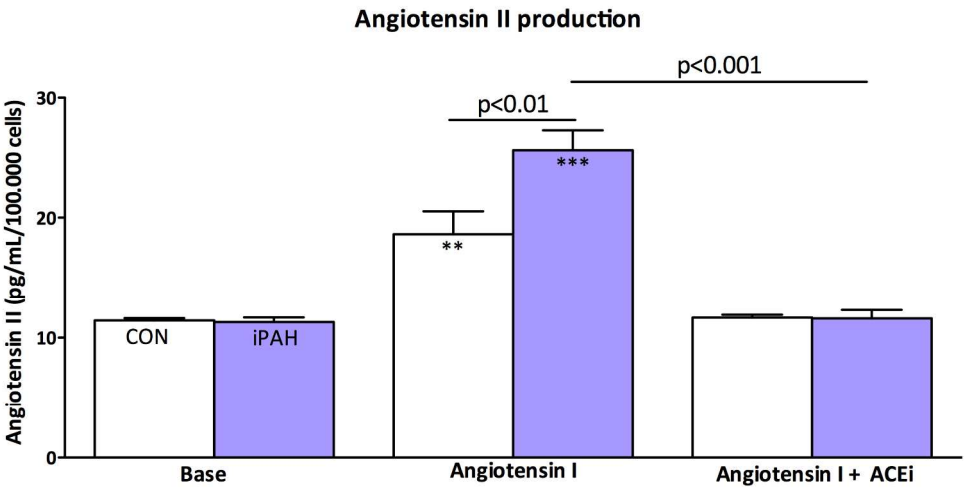
Table E3 – Autopsy data

	Control	PAH	PAH+losartan	p-value	
	(n=9)	(n=9)	(n=9)	Control vs. PAH	PAH vs. PAH+losartan
Body mass (g)	338 ± 12	250 ± 6	257 ± 8	<0.001	0.592
BMchange (%/2d)	0.8 ± 0.4	-3.0 ± 0.8	-2.6 ± 0.7	<0.001	0.616
Tibia length	36.7 ± 0.3	34.3 ± 0.1	34.4 ± 0.1	<0.001	0.695
Lungs/tl (g/mm*1000)	33.6 ± 0.8	52.4 ± 2.6	49.4 ± 1.8	<0.001	0.249
Lung wet/dry ratio	5.0 ± 0.1	5.4 ± 0.2	5.6 ± 0.3	0.139	0.516
Heart/tl (g/mm*1000)	33.2 ± 0.7	43.6 ± 3.6	44.0 ± 1.7	0.002	0.867
RV mass/tl (g/mm*1000)	5.3 ± 0.4	8.7 ± 0.6	9.1 ± 0.8	0.001	0.641
LV mass/tl (g/mm*1000)	8.5 ± 0.6	6.0 ± 0.5	6.9 ± 0.8	0.002	0.335
RV/(LV+S)	0.31 ± 0.03	0.78 ± 0.02	0.81 ± 0.07	<0,001	0.729
Liver/tl (g/mm*1000)	335 ± 4.1	228 ± 9	250 ± 14	<0.001	0.162
Spleen/tl (g/mm*1000)	16.4 ± 0.3	16.0 ± 0.8	15.2 ± 0.6	0.651	0.395
Kidneys/tl (g/mm*1000)	66.4 ± 2.2	49.2 ± 3.4	57.6 ± 1.8	<0.001	0.023

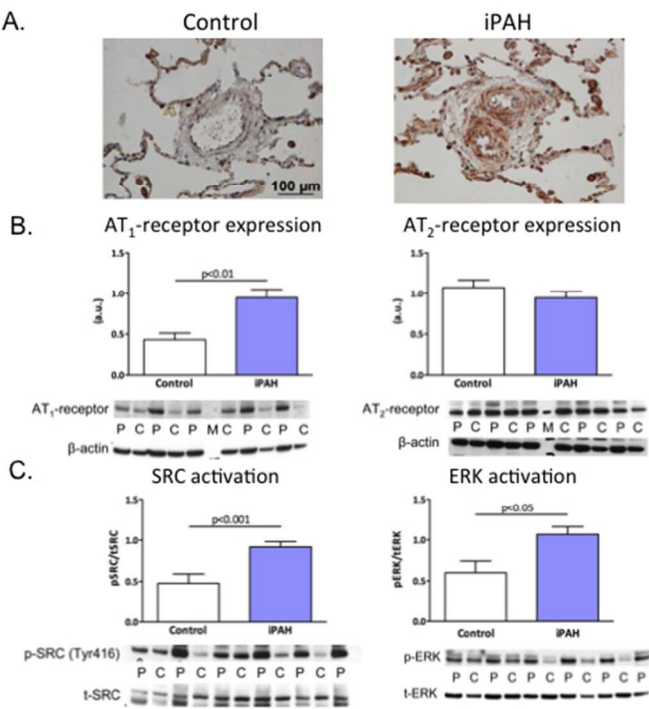
BMchange, percentage change in body mass of the last 2 days; .../tl, organ mass normalized for tibia length; RV / (LV+S), RV-to-LV (including septum) mass ratio



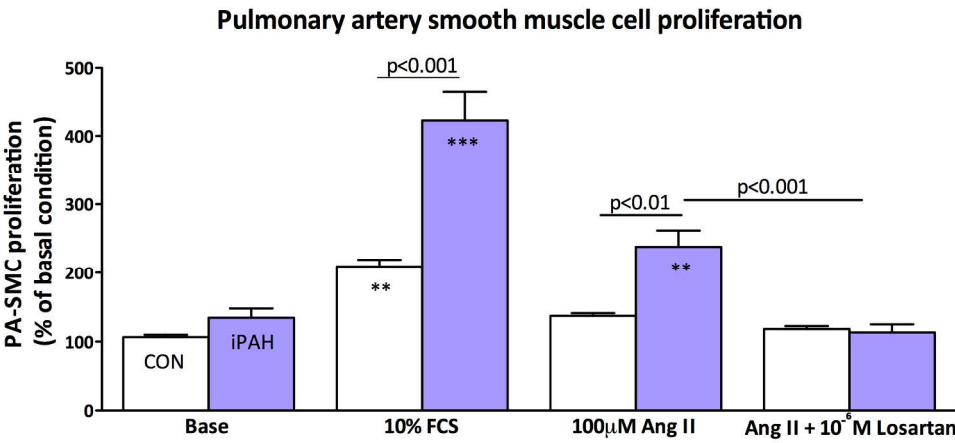
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210x111mm (300 x 300 DPI)

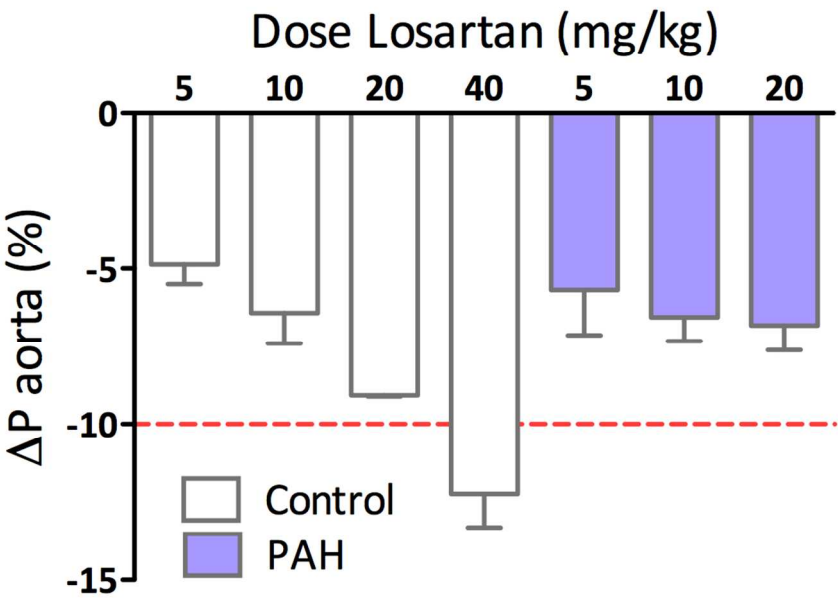


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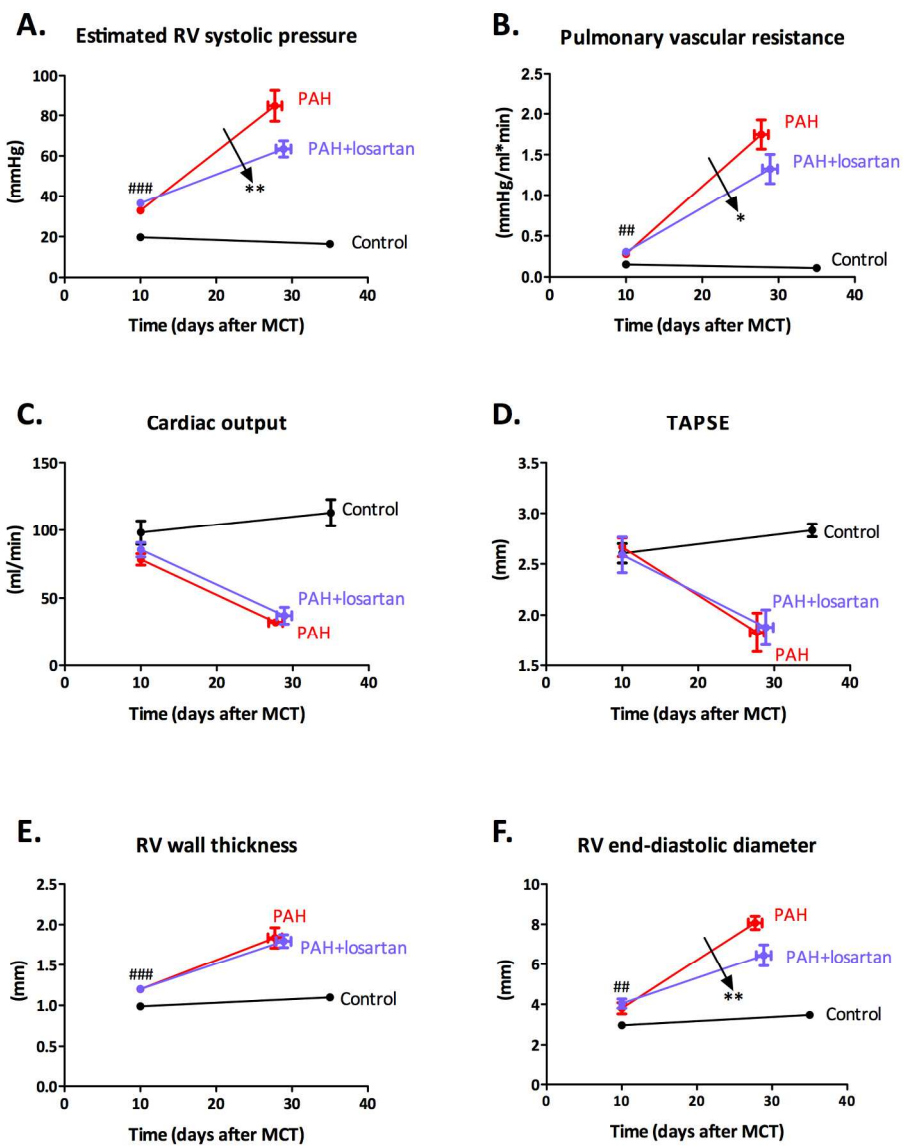


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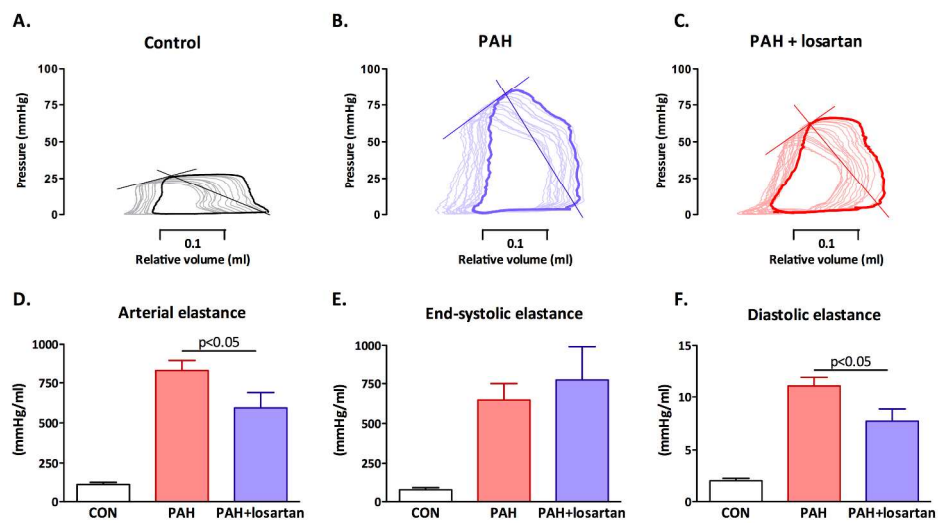
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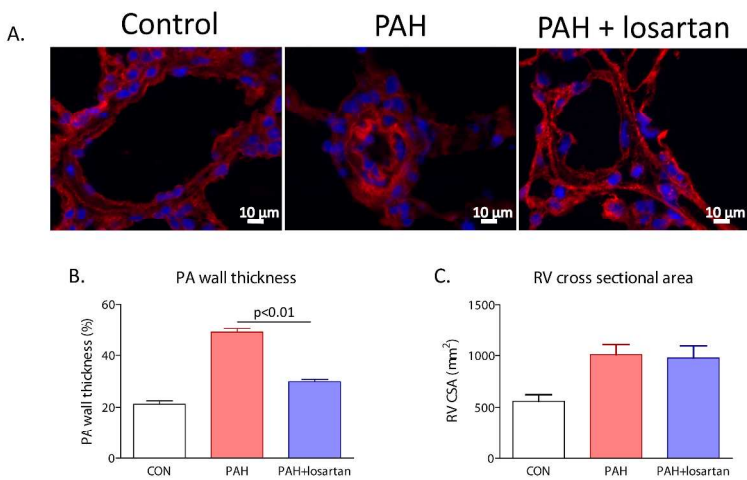
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179x223mm (300 x 300 DPI)



278x160mm (300 x 300 DPI)



387x205mm (300 x 300 DPI)