The elusive heart
Borgdorff, Reinout

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ACORNERSTONE OF HEART FAILURE TREATMENT IS NOT EFFECTIVE IN EXPERIMENTAL RIGHT VENTRICULAR FAILURE

MAJ Borgdorff, B Bartelds, MG Dickinson, P Steendijk, RMF Berger

ABSTRACT

Background
Right ventricular (RV) failure due to increased pressure load causes significant morbidity and mortality in patients with congenital heart diseases and pulmonary arterial hypertension. It is unknown whether renin-angiotensin-aldosterone-system (RAAS) inhibition (the cornerstone of left ventricular failure-treatment) is effective in RV failure. We investigated the effects of combination treatment of aldosterone-blocker eplerenone + angiotensin II-receptor blocker losartan (Ep/Lo) on RV remodeling and function in a model of RV failure due to increased pressure load.

Methods and Results
Rats (n=48) were randomized for pulmonary artery banding (PAB) or sham surgery and for losartan (20mg/kg/d)+eplerenone (100mg/kg/d) treatment (Ep/Lo) or vehicle (VEH). RV function was assessed by echocardiography and pressure-volume analysis at 5 and 11 weeks, or at the occurrence of clinical RV failure symptoms necessitating termination.

PAB resulted in RV failure in all rats, as defined by reduced cardiac output, RV stroke volume, increased RV end diastolic pressure and liver congestion as well as RV fibrosis, hypertrophy and reduced capillary density. Clinical RV failure necessitated termination in 5/12 PAB-VEH rats. Angiotensin II type 1-receptor expression in the RV was reduced in PAB rats indicating local RAAS activation. Treatment of PAB rats with Ep/Lo significantly lowered arterial pressures, but had no significant effect on RV function, remodeling or survival compared to PAB-VEH rats.

Conclusions
RAAS-inhibition does not beneficially affect experimental RV failure due to chronic pressure load. This is of high clinical relevance, because it indicates that the RV might respond fundamentally different to RAAS-inhibition than the LV.
Right ventricular (RV) failure due to pressure load is a primary risk factor for early mortality and morbidity in patients with congenital heart diseases and the main cause of death in pulmonary arterial hypertension (1-4). Despite the recognized clinical importance of preserving RV function, the mechanisms of RV dysfunction and failure are yet unknown and as a consequence there are no clinically established treatments for RV failure (5). This is in sharp contrast with left ventricular (LV) failure (6,7) and it is tempting to extrapolate proven treatment strategies for LV failure to the RV. This is, however, associated with a number of potential hazards. The RV differs functionally and morphologically from the LV (8,9) and the RV derives embryologically from a distinct set of precursor cells (10), implicating that RV cardiomyocytes might respond fundamentally different to stress (5,11,12). Furthermore, the RV is coupled to the pulmonary circulation, physiologically a low pressure, high compliance circulation with different properties than the systemic circulation, which might affect the RV response to commonly used LV drugs (13). However, the implications of these differences between RV and LV for the treatment of RV failure remain largely speculative and the effects on RV failure of proven treatment strategies for LV failure are insufficiently studied (14).

One of the cornerstones in the treatment of LV failure is inhibition of an over-activated renin-angiotensin-aldosterone-system (RAAS) with an angiotensin II converting enzyme-inhibitors (ACEi) or angiotensin II receptor-blocker (ARB) combined with an aldosterone receptor-blocker (6,7). RAAS inhibition has been shown to improve LV function and attenuate adverse remodeling (fibrosis, hypertrophy, ventricular dilatation) in (pre)clinical studies of LV failure (15-19). Clinical studies suggest that RAAS over-activation might also play a role in RV adaptation to various forms of abnormal loading and RV failure (20). Such data suggest patients with a systemic RV to be prime candidates for RAAS inhibiting treatment, as their RV is chronically pressure loaded and prone to failure. However, trials of ACEi (21,22) or ARBs (23-25) in this patient group, reported negative results. Most of these studies were limited by insufficient power, short-follow up or retrospective set-up (26).

Preclinical data could provide a proof-of-principle that RAAS inhibition is beneficial for the chronically pressure loaded RV. Unfortunately, data regarding the in vivo functional effects of RAAS-inhibition on the pressure loaded RV are
lacking. Therefore, we tested both functional and histological effects of long-term combined pharmacological inhibition of the angiotensin II receptor (type 1) and the aldosterone receptor by losartan/eplerenone treatment in a model of pressure load induced RV failure. We hypothesized that this clinically applicable treatment would attenuate remodeling and, as a consequence, sustain RV function and prevent RV failure.

MATERIALS AND METHODS

Animal model and study design
Animal care and experiments were conducted according to the Dutch Animal Experimental Act and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The Animal Experiments Committee of the University of Groningen, the Netherlands approved the experimental protocol. Wistar rats (n=48; male; 160-180g; Charles River, the Netherlands) were randomized to pulmonary artery banding (PAB) or sham surgery. PAB (n=33) was performed to induce RV pressure overload, as described previously(27), except that for this study we used a tighter PAB size (19G; 1.1.mm instead of 1.3mm). Three rats died during PAB surgery (1 severe bleeding, 1 acute RVF, 1 trachea perforation). The remaining 30 animals with PAB were randomly assigned to a group of 15 rats to receive vehicle or a group of 15 rats to receive eplerenone and losartan. Inadvertedly, the last three animals assigned to the vehicle-group received eplo-treatment from the start, resulting in a vehicle-treated group (PAB, n=12) and an eplerenone and losartan treated group (PAB-eplo, n=18). After randomization, pairs were made between both groups, so that each PAB rat had a paired PAB-eplo rat, which made it possible to terminate the rats pairwise in case of clinical deterioration. Sham operated animals (n=15) underwent the PAB procedure without the actual banding of the pulmonary artery and served as control (CON), and were randomized into vehicle-treated (CON, n=7) and treated (CON-eplo, n=8) groups.

Termination
Rats were terminated when clinical RVF (see Definition of clinical RV failure below) developed. Along with the failing rat, the paired rat was terminated at the same time point. At 11 weeks after surgery, all remaining rats (all CON(-eplo) rats and the remaining PAB(-eplo) rats) were terminated.
One rat (in the PAB-eplo group) developed an abdominal tumor within two weeks after PAB and was terminated. This animal had severe unilateral hydroureteronephrosis and was excluded from further analysis.

**Definition of clinical RV failure**

Rats were examined daily for clinical signs of RV failure according to a previously described checklist examining for appearance, activity, bodyweight changes, peripheral circulation, cyanosis, dyspnea/tachypnea and edema/effusions. Clinical RV failure was defined as the presence of at least: inactivity, ruffled fur, severe dyspnea and palpable ascites. The decision whether RV failure was present or not was made by 2 experienced observers who were blinded to the experimental group of the rats(27).

**RAAS-inhibiting treatment**

Treatment was given from the moment of surgery onward and consisted of the combination of angiotensin II receptor (type 1) blocker losartan (20mg/kg BW/d)(28,29) via the drinking water and mineralo-corticoid receptor blocker eplerenone (100mg/kg BW/d)(15,30) mixed in conventional rat chow, which have been previously shown to be effective dosages in models of LV disease(15,28-30). The untreated groups received conventional rat chow and regular drinking water throughout the experiment.

**Echocardiography**

Transthoracal echocardiography was performed under general anesthesia (isoflurane/air mixture: 5% induction, 2-3% maintenance) in all animals at 5 weeks after surgery and at termination as described previously (27) using a Vivid Dimension 7 system and 10S-transducer (GE Healthcare, Waukesha, WI, USA). We used apical 3- and 4- chamber views and parasternal short and long axis views to measure RV and right atrial dimensions, tricuspid insufficiency, tricuspid annular plane systolic excursion (TAPSE), and continuous wave Doppler for the gradient across the PAB. Cardiac output was calculated as (aorta diameter)$^2 \times 3.14 \times$ velocity time integral $\times$ heart rate, using systolic aorta diameter and pulsed wave Doppler measurements of aorta flow. Measurements from 6-12 consecutive beats were used to average out beat-to-beat variation.
Heart catheterization

Hemodynamic assessment of the RV was performed by pressure-volume analysis, obtained at termination by RV catheterization according to a previously described protocol(27).

Briefly, rats were anesthetized (isoflurane/air mixture, 5% induction; 2-3% maintenance), intubated and ventilated. Following bilateral thoracotomy and pericardiotomy a pressure-conductance catheter (SPR-869, Millar Instruments Inc., Houston, TX, USA) was introduced into the RV apically and positioned in the RV outflow tract. RV pressures and conductance were recorded using a MPVS 400 processor at a sample rate of 1.000 Hz with Chart 5 (Millar Instruments Inc., Houston, TX, USA). Analyses were performed offline using custom-made software (CircLab 2012, P. Steendijk). Stroke volume (in mL) measured by echocardiography was used to calibrate stroke volume (in arbitrary units) derived from the conductance signal. End systolic and end diastolic elastance were determined using the single-beat method(31). Following the RV measurements, the catheter was introduced in the aorta and the LV via the right carotid artery to measure systemic pressures.

Organ weights, hypertrophy and fibrosis

After heart catheterization, the rats were terminated by excising the heart-lung block from the thorax. Heart, lungs and liver were dissected. RV, interventricular septum, LV and both atria were separated and weighed. The liver lobe and lung lobe were weighed, dried overnight at 65°C and weighed again to determine wet weight/dry weight ratio. Midventricular RV sections were fixated (formalin) and stained to assess cardiomyocyte cross-sectional area (wheat germ agglutinin), fibrosis (Masson Tri-chrome) and capillary density (lectin) as described previously(27,32).

Gene expression of RAAS and remodeling

To assess activation of the local RAAS, mRNA expression of the angiotensin II receptors type 1 and 2 (AT1R and AT2R) were measured. To study the underlying mechanisms of putative effects of Ep/Lo treatment the expression of key markers of the fetal gene program (myosin heavy chain isoforms, natriuretic pro peptides type A and B) were measured, as well as genes involved in myocardial remodeling: hypertrophy (ACTA, RCAN1), fibrosis (TGFβ-1, OPN-1, Col1A2, Col3A1) and
oxidative stress (HO-1, NOX-4). RV (free wall) tissue was snap-frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA); high quality was confirmed (RQI 9.3) using Experion (Bio-Rad, Veenendaal, the Netherlands), before conversion to cDNA by QuantTitect Reverse Transcription (Qiagen, Venlo, the Netherlands). Gene expression was measured with Absolute QPCR SYBR Green ROX mix (Abgene, Epsom, UK) in the presence of 7.5ng cDNA and 200nM forward and reverse primers. qRT-PCR was carried out on the Biorad CFX384 (Bio-Rad, Veenendaal, the Netherlands) using a standard protocol of maximally 35 cycles. Primer sequences are available upon request. mRNA levels are expressed in relative units based on a standard curve obtained by a calibrator cDNA mixture. All mRNA levels were corrected for 36B4 reference gene expression.

Statistical analysis
Quantitative data are expressed as mean±standard error of the mean (SEM). CON versus PAB differences were evaluated using Students t-test or Mann-Whitney U test as appropriate. Treatment effects were tested by ANOVA with Bonferroni post-hoc testing for multiple comparisons or Fisher’s Exact Test as appropriate. Group sizes were 7 (CON); 8 (CON-eplo); 12 (PAB); 17 (PAB-eplo), unless specified otherwise. P<0.05 was considered significant (PASW Statistics 18 for Windows, SPSS, Chicago, Illinois).

RESULTS

Model characterization: Pulmonary artery banding induces RV failure in vehicle-treated rats
In vehicle-treated rats, pulmonary artery banding resulted in severe pressure overload which induced (sub)clinical RV failure, characterized by reduced cardiac index (Fig 1A) and stroke volume (Fig 1B), reduced TAPSE (Fig 1C), RV dilatation (Fig 1D), tricuspid insufficiency (Fig 1E), right atrial enlargement (Fig 1F), increased RV end diastolic pressure (Fig 1G) and liver congestion (Fig 1H). Five of the 12 untreated rats developed overt clinical RVF within 11 weeks after PAB surgery, which necessitated termination. Local RAAS was activated in the RV, indicated by downregulation of AT1-receptor mRNA (Fig 2). AT2-receptor mRNA expression was not detectable at the maximum of 35 PCR cycles (Fig 2).
Figure 1. Model characterization: Pulmonary artery banding induces RV failure in vehicle-treated rats

A Cardiac index B RV stroke volume C tricuspid annular plane systolic excursion (TAPSE) D RV end diastolic diameter (RVEDD) E percentage of rats with tricuspid insufficiency (TI) F right atrial diameter (RA) G RV end diastolic pressure (EDP) H liver wet weight: dry weight ratio. All parameters measured by echocardiography, except EDP, which was measured by catheterization. Mean±SEM. * indicates p<0.05 between groups. CON= control, PAB= pulmonary artery banding, both vehicle-treated

Figure 2. mRNA expression of angiotensin II receptor type 1 (AT1R) and 2 (AT2R)

AT1R (left panel) was downregulated in PAB. AT2R (right panel) was not detectable (nd) at the 35th PCR cycle. Mean±SEM. * indicates p<0.05 between groups. CON= control, PAB= pulmonary artery banding
This RV failure phenotype was accompanied by pathological remodeling, including threefold increment of myocardial fibrosis (Fig 3A), RV hypertrophy (expressed as RV weight/tibia length (Fig 3B) or cardiomyocyte cross-sectional area (Fig 3C), reduced capillary density (Fig 3B-D) and upregulation of hypertrophy related genes and the fetal gene program (Table 2).

**Figure 3. Fibrosis, hypertrophy and capillary density**

A RV fibrosis (representative images in two top rows of pictures: Masson-Trichrome stained RVs, ruler is 1mm, black box width 1.3mm) B RV free wall weight normalized for tibia length (measure of RV hypertrophy) C RV cardiomyocyte cross-sectional area (third row of pictures: representative images of RV sections stained with a membrane marker (wheat germ agglutinin, green), ruler is 125μm. D RV capillary density, expressed as number of capillaries per 100*100μm (bottom row: representative images of RV sections stained with capillary-marker lectin, ruler is 125μm). Mean±SEM. * indicates p<0.05 vs. CON. CON= control, PAB= pulmonary artery banding (untreated), PAB-eplo= PAB treated with eplerenone/losartan.
**Eplerenone/Losartan treatment effects**

Ep/Lo significantly reduced left ventricular peak pressure and aortic systolic and diastolic blood pressure in PAB (Fig 4A-C). However, Ep/Lo treatment did not have any significant effect on RV hemodynamics (Table 1, S1), representative pressure-volume loops in Fig 5A-C. Contractility (end systolic elastance), active relaxation (tau) and passive diastolic properties (end diastolic elastance, end diastolic pressure) were unaffected by Ep/Lo (Table 1). Neither did Ep/Lo prevent dilation of the RV and RA, tricuspid insufficiency or liver congestion (Table 1). In line with this lack of hemodynamic benefit, Ep/Lo treatment did not delay (Fig 5D) or prevent development of RV failure (5/17 vs. 5/12, PAB vs. PAB-eplo, p=0.494).

Myocardial fibrosis and RV hypertrophy were not prevented by Ep/Lo treatment (Fig 3A-C). Ep/Lo also did not affect capillary density (Fig 3D). In line with this, expression of genes of the fetal gene program and genes related to hypertrophy, fibrosis and oxidative stress were unaffected by Ep/Lo treatment (Table 2). All parameters in CON and CON-eplo were equal (p=ns, data not shown).

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**Figure 4. Effects of treatment on systemic pressures**

A left ventricular (LV) peak pressure B aorta maximum pressure C aorta minimum pressure. Mean±SEM. * indicates p<0.05 vs. CON; † indicates p<0.05 vs. PAB. CON= control, PAB= pulmonary artery banding (untreated), PAB-eplo= PAB treated with eplerenone/losartan.
A cornerstone of heart failure treatment is not effective in RV failure.

**DISCUSSION**

In this study we assessed the effects of proven LV failure treatment, the combination of eplerenone and losartan, in a rat model of RV failure due to chronic pressure load. We found that Ep/Lo neither prevented adverse remodeling, nor clinical RV failure nor affected RV function. Our findings show that RAAS-inhibition does not beneficially affect experimental RV failure due to chronic pressure load, which is in strong contrast to previous
findings in the left ventricle\(15,33-37\). These data indicate that response of the pressure loaded RV to RAAS-inhibition might differ fundamentally from that of the (pressure loaded) LV, which may have highly relevant clinical consequences.

**Eplerenone and losartan: works in the LV, not in the RV?**

Pharmacotherapeutic guidelines developed for treating LV failure serve as a roadmap in the search for effective treatments for RV failure. Even though an increasing catalog of clinical studies of ACEi (21,22) or ARBs (23-25) in patients with a pressure loaded RV has reported negative results, the paradigm remains that RAAS inhibition should work in RV dysfunction. The negative results of the studies are assumed to be attributable to insufficient power, short-follow up or retrospective set-up of these studies(26). Although the putative benefits of RAAS inhibition in this population certainly should not dismissed at this point, the results of our study suggest a more fundamental explanation for the lack of clinical effect.

A distinctive characteristic of the current study is the severity of the PAB model which, in contrast to previously described PAB models(38-40), induces a clear phenotype of clinical and functional RV failure. Previous studies of ACEi and ARB in PAB models showed no effect on RV hypertrophy(41-44) in compensated RV pressure loading. Our study adds the clinically relevant notion that Ep/Lo treatment does not affect RV remodeling nor function in severe pressure load induced RV failure. PAB rats had reduced cardiac output, activation of the systemic RAAS (confirmed by blood pressure effect of Ep/Lo), and activation of local RAAS (confirmed by downregulation of the AT1-receptor(45)). The dosages of Ep/Lo and the administration regimens that were used in the current study have been shown to effectively target LV disease(15,16,28,29). The lack of treatment effects then, suggests that the RV responds differently to RAAS inhibition than the LV.

The contribution of the RAAS to LV remodeling and function in fixed LV pressure load is well established(46,47). Multiple studies show that RAAS inhibition with losartan or eplerenone can attenuate remodeling and improve function in the aortic constriction model(15,17,33-37). In contrast, the contribution of RAAS to RV remodeling and function is insufficiently studied. RV pressure load
activates local RAAS, even in the absence of systemic RAAS activation in mild PAB[41]. However, blocking local RAAS activation by losartan did not prevent remodeling or improve papillary length-tension relationship[41]. The current study shows that it also does not affect RV function in vivo. One explanation might be that the local RAAS system of the RV functions differently than that of the LV. This is supported by our observation that the ‘beneficial’ AT2-receptor, which is upregulated in the pressure loaded LV[48], was not upregulated in the pressure loaded RV. Additionally, in RV pressure load, the AT1-receptor has been shown to be functionally uncoupled from its downstream effectors[42] and protein kinase C isozymes[44], which are important regulators of remodeling and function in the LV. This could explain why RAAS inhibition, as employed in the current study, does not work in the RV. We added an aldosterone-receptor blocker, eplerenone, to the losartan treatment to circumvent the possibility that compensatory activation of the aldosterone-pathway (partially) negates the inhibitory effects on AT1-receptor[15,49]. Indeed, in the LV, pharmacological inhibition on both levels of the RAAS resulted in more pronounced improvement of remodeling and function than monotherapy[16].

Taken together, these data indicate important differences between the RV and LV with regard to RAAS activation due to increased pressure load and the response to RAAS inhibiting therapy. From the currently available data it is not clear whether these different responses are caused by physiological differences between the RV and LV, of by fundamental differences between right and left ventricular cardiomyocytes, which embryologically derive from distinct precursor cells.

Either way, the differences in both RAAS activation and response to therapy might explain why clinical studies of RAAS inhibition in systemic RVs have failed to show positive results[22,23,25]. These studies certainly do not close the book on RAAS inhibition in RV failure. A recent preliminary study in a murine PAB model, has suggested that stimulation of the alternative ACE2-Ang-(1-7) pathway might be beneficial for RV function[50]. To take the exploration of RAAS inhibition as a treatment strategy for RV failure a step further, the local RV RAAS activity and its differences with local LV RAAS should be further unraveled.

Importantly, RAAS-inhibition has been reported to have beneficial effects in models of pressure-loaded RV associated with pulmonary hypertension. Studies
in experimental pulmonary hypertension have described beneficial effects of losartan/telmisartan treatment on RV function and remodeling (51-53). It is important to realize that increased local RAAS-activity has been demonstrated in pulmonary arteries of patients with pulmonary arterial hypertension (52). Therefore, the described beneficial effects of RAAS-inhibition in these models are not necessarily direct myocardial effects in the pressure loaded RV, but may also be secondary to AT1R-inhibiting effects on the pulmonary vasculature, leading to decreased RV-afterload and thereby secondary to improved RV performance and remodeling. Effects of RAAS inhibition on the pulmonary vasculature have been reported: losartan prevented pulmonary vascular remodeling and decreased BMPR-2 expression in a model of shunt-induced pulmonary hypertension (52, 53). The current PAB model, with a fixed afterload, excludes such ‘confounding’ pulmonary vascular effects and thus allows assessing direct effects of RAAS-inhibition on the RV-myocardium (54). The lack of losartan effect in our study therefore indicates that RV effects of losartan in (experimental) PH are secondary to the pulmonary vascular effects. These experimental data suggest that RAAS-inhibition may be beneficial in patients with RV-failure associated with pulmonary vascular disease, but not in patients with RV-failure in the setting of CHD, in which RV-afterload is not determined by pulmonary vascular resistance, including systemic RV, pulmonary branch stenosis or other RV outflow tract obstructions.

**Limitations**

The current experiments were designed to test the preventive effects of a ‘clinical’ Ep/Lo treatment strategy on RV remodeling and function. In the clinical setting, therapeutic effects that reverse established remodeling/dysfunction are of high importance. However, given the lack of preventive benefits, it is unlikely Ep/Lo would have therapeutic effects in the pressure loaded RV. Secondly, we did not include monotherapy groups treated with losartan or eplerenone. Although, in light of the lack of effects of the combination treatment, it seems unlikely that monotherapy would have beneficial effects in PAB, we cannot conclude this firmly based on the present study. As in all preclinical studies, caution is required when data from experimental models are extrapolated to the clinical setting.
Conclusion

Combination treatment with eplerenone and losartan did not prevent adverse remodeling, clinical RV failure or benefit RV function in a model of pressure load induced RV failure.

Our findings indicate that local RAAS activation in the pressure loaded RV and its response to effective RAAS inhibition differ from that in the LV. This is of high clinical relevance when treating patients with RV dysfunction due to abnormal loading conditions. To further explore a potential role for RAAS inhibition in the treatment of RV failure, the local RV RAAS activity and its differences with local LV RAAS should be unraveled.

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REFERENCES


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Supplemental table 1

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<th>PAB-eplo</th>
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Table S1. Additional pressure-volume parameters and echocardiographic parameters at 5 weeks

dPdmax indexed= dPdmax/ peak pressure, dPdmin indexed= dPdmin/ end systolic pressure, PAB= pulmonary artery banding, TAPSE= tricuspid annular plane systolic excursion, RVEDD= RV end diastolic diameter, RA= right atrium. Means±SEM. p-values for eplerenone/losartan effect in right-hand column.