Endothelin Axis Is Upregulated in Human and Rat Right Ventricular Hypertrophy

Jayan Nagendran, Gopinath Sutendra, Ian Paterson, Hunter C. Champion, Linda Webster, Brian Chiu, Al Haromy, Ivan M. Rebeyka, David B. Ross, Evangelos D. Michelakis

Rationale: Right ventricular (RV) function is the most important determinant of morbidity and mortality in pulmonary arterial hypertension (PAH). Endothelin (ET)-1 receptor antagonists (ERAs) are approved therapies for PAH. It is not known whether ERAs have effects on the RV, in addition to their vasodilating/antiproliferative effects in pulmonary arteries.

Objective: We hypothesized that the ET axis is upregulated in RV hypertrophy (RVH) and that ERAs have direct effects on the RV myocardium.

Methods and Results: RV myocardial samples from 34 patients with RVH were compared with 16 nonhypertrophied RV samples, and from rats with normal RV versus RVH attributable to PAH. Confocal immunohistochemistry showed that RVH myocardial ET type A (but not type B) receptor and ET-1 protein levels were increased compared with the nonhypertrophied RVs and positively correlated with the degree of RVH (RV thickness/body surface area; \( r^2 = 0.838 \) and \( r^2 = 0.818 \), respectively; \( P < 0.01 \)). These results were recapitulated in the rat model. In modified Langendorff perfusions, ERAs (BQ-123 and bosentan \( 10^{-5}, 10^{-4}, 10^{-5} \) mol/L) decreased contractility in the hypertrophied, but not normal RV, in a dose-dependent manner (\( P < 0.01 \)).

Conclusions: Patients and rats with PAH have an upregulation of the myocardial ET axis in RVH. This might be a compensatory mechanism to preserve RV contractility, as the afterload increases. ERAs use potentially worsen RV function, and this could explain some of the peripheral edema noted clinically with these agents. Further studies are required to evaluate the effects of ERAs on the RV in patients with RVH and PAH. (Circ Res. 2013;112:347-354.)

Key Words: contractility • endothelin-1 • endothelin receptor antagonists • endothelin receptors • hypotrophy • pulmonary hypertension • right ventricle • right ventricular failure • right ventricular function

The increased levels of endothelin (ET)-1 in the pulmonary arteries and serum of patients with pulmonary arterial hypertension (PAH) contribute to the vasoconstriction and proliferative vascular remodeling that characterize PAH. Several ET receptor antagonists (ERAs) are now either approved or under clinical trials for PAH. The initial enthusiasm after the recognition of the role of ET-1 in PAH and the beneficial pulmonary vascular effects in animal models was limited by the realization that these drugs can offer only a small, but potentially important, benefit in PAH patients. PAH remains an untreatable disease, leading to progressive worsening of right ventricular (RV) function, heart failure, and death.

Although the most important determinant of morbidity and mortality in PAH is RV function, the effects of most PAH therapies on RV function are unknown. In atrial or left ventricular (LV) myocardium from animals or humans, ET-1 has direct positive inotropic effects mediated by the ET type A receptor (ET\(_{\text{A}}\)-A) and ERAs decrease contractility. In neonatal mouse RV cardiomyocytes, ET-1 increased contractility via an increase in intracellular Ca\(^{2+}\) transients through activation of the ET\(_{\text{A}}\)-A receptor and an increase in Ca\(^{2+}\) influx via the Na\(^{+}-\text{Ca}\(^{2+}\) exchanger during the action potential. In clinical trials of LV failure, where circulating levels of ET-1 are also increased like in PAH, ERAs worsened outcomes (in part as a result of early worsening of heart failure and fluid retention), prompting a premature ending of trials and the development of these drugs for congestive heart failure. It is thus surprising...
that the possibility of direct effects on RV function in ERA-treated PAH patients is not typically considered. Several trials showed a decrease in pulmonary artery pressure, but lack of the expected improvement in cardiac index, with either ET-A-selective or nonselective ERAs. Furthermore, several ERAs, including ET-A antagonists, are associated with increased incidence of edema. Although this seems to be a class effect, additional postmarketing reports of edema to the Food and Drug Administration (at times requiring hospitalization for decompensated heart failure) within a few weeks from ambrisentan initiation, led to a warning for significant edema in ERA labels. In clinical practice, edema is assumed to be attributable to renal effects of these drugs on sodium reabsorption and may not lead to discontinuation of ERA therapy. However, it is known that such effects are mediated by the ET type B receptor, not the ET-A. We speculated that such cases could represent potential early reduction of RV contractility. Indeed, there is recent evidence to suggest that a group of PAH patients who were treated with therapies including ERAs, despite an early decrease in pulmonary vascular resistance, progress with worsening of RV function (measured by MRI) and poor outcomes; supporting the concept that a therapy like ERAs may adversely worsen RV function in parallel to beneficial effects on the pulmonary arteries.

Nonetheless, negative acute inotropy is not necessarily a weakness of PAH drugs in the long term. For example, β-receptor antagonists have negative inotropy on the LV myocardium but unquestionable benefits in the long-term treatment of LV dysfunction. However, trials with β-receptor antagonists were never terminated as a result of early worsening of heart failure, as seen with ERAs and heart failure.

We hypothesized that the ET-1 axis is upregulated in the hypertrophied RV, potentially as part of a compensatory response, promoting hypertrophy and increased contractility, as the RV afterload increases in PAH. We also hypothesized that ERAs will have negative inotropic effects directly on the hypertrophied RV myocardium. Protein and mRNA expression of ET-1 and ET receptors were measured in RV tissues (hypertrophied versus nonhypertrophied) from 50 patients and from rats, and also the direct effects of ET-1 and ERAs on RV myocardium were studied in a modified RV-Langendorff model.

**Methods**

All experiments on human tissues and rats were performed with permission from the University of Alberta committees on human ethics and animal policy and welfare, respectively. Please see the expanded Methods section in the Online Data Supplement.

**Human Specimens**

We prospectively collected and studied RV specimens from 50 patients (hypertrophied RV=34, nonhypertrophied RV=16; Online Table I). The RV tissue came from surgery or transplant materials. The quantification of RV hypertrophy (RVH) was made by echocardiography in the perioperative period, measuring RV free-wall thickness in at least 2 standard views by blinded cardiologists.

**Immunohistochemistry and Confocal Microscopy**

Confocal microscopy with multiple-staining technique and time-coursed image acquisition was used to quantify the expression of ET-1 and ET receptors in RV myocardium, as previously described.

**Isolated RV-Langendorff Perfusion**

A modified Langendorff perfusion model was adapted to measure developed pressure in the RV, and was performed as previously described. The ex vivo perfusion of the heart is critical to separate the concomitant effects ERAs would have on the pulmonary vasculature during in vivo studies, where a decrease in pulmonary vascular resistance may mask direct effects on RV myocardium.

**ET Immunoassay**

A commercially available ELISA kit (Cayman Chemicals, Ann Arbor, MI) with a double-antibody sandwich technique was used.

**Data Analysis**

Data were expressed as mean±SEM, and differences were evaluated using the Student t test for unpaired data or 1-way ANOVA followed by post hoc Bonferroni correction. Linear regression was performed on human ET-A and ET-1 expression versus a standard RVH index (RV free-wall thickness divided by body surface area). Significance was defined as P<0.05 (SPSS 17.0, SPSS Inc, Chicago, IL).

**Results**

There was a significant increase in the levels of both ET-1 (+240%) and ET-A protein (+125%) in the RV myocardium of patients with RVH, compared with those with nonhypertrophied RVs, measured by immunohistochemistry and confirmed with immunohistochemistry (Figure 1). Colocalization with the cardiomyocyte marker myosin heavy chain showed that this upregulation occurred in the myocardium. The ET receptor type B was only observed in the coronary vasculature (stained with smooth muscle actin) of both hypertrophied and nonhypertrophied RV, and not in the myocardium. Overall, the levels of ET receptor type B protein did not differ between the nonhypertrophied and hypertrophied RV myocardium (Online Figure I).

To determine whether there is a relation between the degree of RVH and the upregulation of the ET-1 axis in RV myocardium we plotted the levels of ET-1 or ET-A against each
There was a strong positive correlation between the degree of RVH and the amount of both ET-1 and ETR-A. The more hypertrophied the RV, the stronger the ET axis upregulation was (Figure 2A).

To determine whether ET-1 and ETR-A mRNA were also increased in the RV myocardium, we used qRT-PCR and laser-captured microdissection. This technique allows for cuts of either muscle or vessel, determined visually and confirmed by the relative amount of myosin heavy chain and smooth muscle actin mRNA that amplifies within the cut. There was increased expression of both ET-1 and ETR-A mRNA in the hypertrophied compared with the nonhypertrophied RV myocardium (Figure 2B). The mRNA increase in the hypertrophied versus nonhypertrophied tissues was similar to the increase in the protein level, that is, +230% for the ET-1 and +100% for the ETR-A. This upregulation was specific for the myocardium cuts (high ratio of myosin heavy chain to smooth muscle actin) because the mRNA levels did not differ in the vessel cuts (low ratio).

The human tissue immunohistochemistry and laser-captured microdissection data were recapitulated in a standard rat model of RVH secondary to monocrotaline-induced PAH. Hypertrophied rat RVs (high “RV/LV+septum” weight ratio and histological evidence of hypertrophied cardiomyocytes) had increased myocardial expression of both ET-1 and ETR-A protein and mRNA, compared with normal RVs from vehicle-treated rats (Figure 3, Online Figures II and III). Studies on the LV myocardium from the same animals showed that the ETR-A protein levels did not differ between the LVs of the same animals (Online Figure IV). This suggested that the up-regulation of the ETR-A was intrinsic to the hypertrophic process in the RV and not due to a circulating factor.

We then used a model in which RV contractility could be studied directly in isolated perfused rat hearts while preload and afterload remain constant, as we have previously described. Both hypertrophied and normal RV hearts showed an increase in developed pressure from baseline when treated with the β-agonist isoproterenol or ET-1 (Figure 4A and 4B). Two different ERAs (the ETR-A antagonist BQ-123 and bosentan, a nonselective ERA used in PAH) caused a significant and reversible decrease in RV developed pressure in hypertrophied, but not normal RVs (Figure 4A and 4B, Online Figure V). The negative RV inotropic effect of bosentan was dose-dependent. The exogenous positive inotropic effect of ET-1 was effectively reversed when bosentan was added to the perfusate (Online Figure VI). Bosentan decreased both positive and negative delta pressure/delta time (dp/dt), suggesting negative effects in both contractility and lucitropy (Online Figure VII). Heart rates of the hypertrophied RVs remained similar during perfusion with ERAs (194±16.8 versus 182±19.3 at baseline and during bosentan infusion, respectively). There were also no significant changes in coronary blood flow during bosentan infusion (Online Figure VIII).
The ET-1 concentration in the coronary sinus effluent in hearts with hypertrophied RV ex vivo was higher compared with the normal RV at baseline and increased further by isoproterenol (Figure 4C). Although the coronary sinus effluent reflects blood returning from both ventricles, the increase in ET-1 is likely a result of its upregulation in the hypertrophied RV. β-agonist spillover of ET-1 has previously been described, suggesting that the upregulated ET-1 axis in the hypertrophied RV could have important autocrine/paracrine effects, particularly in response to sympathetic activation.

Figure 2. Endothelin type A receptor (ET<sub>A</sub>-A) and Endothelin (ET)-1 expression is proportional to the amount of right ventricular (RV) hypertrophy, and the increased expression is also seen at the level of mRNA. A, Linear regression analysis of RV wall thickness indexed to body surface area (RVWTi) vs immunofluorescence (fluorescence units [FU]) shows a strong correlation for both ET<sub>A</sub>-A and ET-1 expression. Patient characteristics are described in Online Table I and Method sections. B, Laser-captured microdissection (LCM) and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) were applied on human RV tissue. LCM was used to isolate myocardium vs coronary arteries in normal and hypertrophied human RV tissue, which was then studied with qRT-PCR. A myocardium sample was characterized by a high myosin heavy chain (MHC) to smooth muscle actin (SMA) ratio (black bars), whereas a coronary artery sample was characterized by a low MHC to SMA ratio. Endothelin-1 (white bars) and ET<sub>A</sub>-A (gray bars) mRNA is markedly increased in RV hypertrophy (RVH) compared with normal RV myocardium, whereas it is similarly expressed in the coronary vessels, in agreement with the immunohistochemistry data in Figure 1.

The ET-1 concentration in the coronary sinus effluent in hearts with hypertrophied RV ex vivo was higher compared with the normal RV at baseline and increased further by isoproterenol (Figure 4C). Although the coronary sinus effluent reflects blood returning from both ventricles, the increase in ET-1 is likely a result of its upregulation in the hypertrophied RV. β-agonist spillover of ET-1 has previously been described, suggesting that the upregulated ET-1 axis in the hypertrophied RV could have important autocrine/paracrine effects, particularly in response to sympathetic activation.

Figure 3. Expression of Endothelin type A receptor (ET<sub>A</sub>-A) and endothelin-1 (ET-1) expression is increased in the hypertrophied but not the normal rat right ventricular (RV). A, Multiple-staining immunohistochemistry technique and multiphoton confocal microscopy show that ET<sub>A</sub>-A and endothelin-1 protein is expressed in low levels in the normal RV and both are markedly upregulated in the myocardium (colocalization with myosin heavy chain [MHC]) of the hypertrophied RVs. The left column (green) shows ET<sub>A</sub>-A or ET-1. The middle column (red) shows expression of either smooth muscle actin (SMA) or MHC expression on the same slide. The third column is a merged picture of the red and green channels plus a nuclear stain with DAPI (blue). B, Ratio of RV dry weight to left ventricle (LV)+Septum, showing significant RV hypertrophy in rats 3 weeks postmonocrotaline injection (*P<0.01). C, Mean immunofluorescence data for the expression of ET<sub>A</sub>-A and ET-1 in normal RV (n=15) and RV hypertrophy (n=11; *P<0.01 vs normal RV). DIC indicates differential interface contrast; and FU, fluorescence units.
venous blood from rats with PAH and RVH had increased concentrations of ET-1, compared with the control rats; and these levels were ≈50 times higher than the levels of ET-1 in the coronary sinus effluent (Figure 4D). Thus, the increased production of ET-1 by the RVH hearts may have autocrine and paracrine effects (Figure 4E), though it may not contribute significantly to the circulating ET-1 levels.

Discussion

We show that the ET axis is upregulated in the human hypertrophied RV myocardium (both at the protein and mRNA levels), and that there is a strong positive correlation between the expression of ET_{R-A} and ET-1 and the degree of hypertrophy. The same upregulation also occurs in hypertrophied rat RVs, but not in the LVs of the same animals (which are not hypertrophied). We also show that ERAs have direct effects only on the hypertrophied rat RV, decreasing contractility and lucitropy. The induction of ET-1 and ET_{R-A} in the hypertrophied RV in PAH, might serve as an early adaptive mechanism that would promote hypertrophy and maintain contractility as the RV afterload increases. The increased sympathetic drive from the upregulation of the neurohormonal axis in PAH could be responsible for the increased production of ET-1 by the RVH myocardium. β-agonists cause a calcineurin-mediated increase in the ET-1 promoter activity in cardiomyocytes. The paracrine/autocrine effects of increased RVH production of ET-1 can also help maintain cardiomyocyte survival in the environment of sustained increase in β-adrenergic stimulation in PAH. ET-1 is known to abate the proapoptotic effects of β-agonists on cardiomyocytes.

The fact that the myocardial ET-1 axis is upregulated in exercise-induced LV hypertrophy suggests that it plays a role in the adaptive and physiological hypertrophy process. The RV ET-1 can have paracrine or autocrine inotropic effects, and the presence of ET-converting enzyme in myocardial cells suggests that the cardiomyocyte ET-1 is biologically active.

It is unlikely that the upregulation of the ET-1 axis in RVH is attributable to a circulating factor, like an inflammation mediator, for example. The fact that in the monocrotaline model (characterized by a strong inflammatory environment) this upregulation does not take place in the LV (which is exposed to the same circulating factors) suggests that it is intrinsic to the RVH process (Online Figure IV). Nevertheless, our study cannot rule out the intriguing possibility that the ET-1 axis upregulation occurs in parallel to (rather than following) the increase in afterload, independently driving the hypertrophy process, at least in part.
Although advanced PAH is essentially a heart failure syndrome, despite the heavy clinical use of ERAs, the possibility that these drugs have direct effects on RV function is currently not considered.\(^4\) Both nonselective (bosentan) or ET\(_A\) selective ERAs (ambrisentan, sitaxsentan) are often associated with significant edema, early after the initiation of therapy. These effects could be attributable to a negative inotropic effect on the RV. Previous data\(^4\) as well as the current work showing that the ET-1 axis is upregulated in RVH, suggest that ERAs could blunt this potentially beneficial response to hypertrophy. In published hemodynamic clinical studies, an ERA-induced decrease in pulmonary artery pressure is not always associated with evidence of improved RV function as one would expect.

Ambrisentan,\(^22\) at the approved dose of 10 mg, and sitaxsentan\(^22\) decreased the pulmonary artery pressure by 13 and 11 mm Hg, respectively (\(P<0.05\) for both), but the right atrial pressure and cardiac index did not improve. In fact, in the sitaxsentan trial, where individual patient responses were shown, 37% of patients had an actual decrease in the cardiac index.\(^2\) Edema developed in 45% of the sitaxsentan-treated patients in that trial and in 25% of the patients in the ambrisentan trial.\(^2,22\) In patients with PAH resulting from Eisenmenger syndrome, bosentan decreased the pulmonary artery pressure, yet edema developed in 19% of the cohort.\(^21\) In another study, a direct, blinded comparison between sildenafil and bosentan showed that although the 2 drugs improved the pulmonary artery pressure similarly, RV ejection fraction (measured by MRI) improved in the sildenafil but not the bosentan group.\(^45\) Recently, van de Veerdonk et al\(^1\) studied a cohort of PAH patients on therapy (75% using ERAs) for >12 months. Despite decreases in pulmonary vascular resistance there was a progression of RV deterioration from baseline in 25% of treated patients, which was associated with poor outcomes. Taken together, these data suggest that ERAs may decrease RV function in some patients with PAH, blunting any beneficial effects that these drugs may have by the decrease in PA pressure.

Several studies on human cardiomyocytes,\(^14-18\) have shown that ET-1 increases inotropy, and in that sense its upregulation in the diseased LV might be beneficial. For example, in mouse hypertrophied LV there is significant upregulation of the ET\(_A\)-ET\(_A\)-ET-1 compared with normal LV and bosentan inhibits contractility in response to increasing end-diastolic pressures (Frank Starling mechanism) by 52% in the hypertrophied but not the normal LV.\(^10\) Inhibition of a potentially adaptive upregulation of the ET axis could explain the increased morbidity shown in patients with LV failure treated with ERAs.\(^20,21\)

Potential effects on the RV need to be studied in both animal, human tissues, and clinical trials in all therapies used to treat PAH patients. For example, although phosphodiesterase type 5 is not significantly expressed in the normal RV, its expression is induced in RVH and phosphodiesterase type 5 inhibitors (the other class of approved oral drugs in PAH) can have positive inotropic effects in the hypertrophied RV myocardium.\(^29,46\)

We propose that the beneficial effects of ERAs in decreasing the RV afterload, by reversing pulmonary artery vasoconstriction and remodeling, may be limited by a potential negative inotropic effect on the RV, at least in some patients. This negative effect on contractility may be small and might not take place in all patients treated. For example, we only studied patients and rats with hypertrophied and compensated RVs; not patients and rats with dilated and decompensated ventricles, where ET receptors may be downregulated. In such patients ERAs may not have negative inotropy.\(^18\) Such patients likely have symptoms of World Health Organization functional class III and IV. Clinically, once patients have transitioned to decompensated RV failure, the prognosis is exceedingly poor and the opportunity to positively impact survival is low, which is why we have focused this study on patients and animals with compensated RVH. Also, there is now a tendency to expand the use of ERAs in patients with World Health Organization functional class II.\(^47\) In such patients with compensated RVH, ERAs may adversely affect the RV function acutely. Nevertheless, long-term effects cannot be extrapolated from our data.

The dose-dependent effects of bosentan on RV contractility/lucinority at doses within the range of bosentan serum levels in treated patients,\(^23,38\) suggest that they are clinically relevant. Because BQ-123 had the same effects, this is likely an ERA class effect. The presence of early edema in patients treated with ERAs might be an indication to discontinue the ERA therapy, rather than just adding diuretics, as is currently the practice. Future trials of ERAs in PAH should study their effects in prospectively identified subgroups, based on RV size and function, allowing for improved outcomes in the subgroups that do not show evidence of negative RV inotropy and avoiding deterioration in those patients in whom the ET-1 axis was contributing to a beneficial compensation against the increasing RV afterload.

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### Disclosures

None.

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**Novelty and Significance**

**What Is Known?**
- Endothelin-1 (ET-1) is a potent vasoconstrictor that is expressed along with ET receptor type A in the pulmonary vasculature of patients with pulmonary arterial hypertension (PAH).
- Endothelin receptor antagonists (ERAs) have shown important, though modest, benefits in patients with PAH.
- The most important determinant of morbidity and mortality in PAH is not the degree of pulmonary hypertension, but the function of the right ventricle (RV).

**What New Information Does This Article Contribute?**
- Both the mRNA and protein content of ET-1 and ET receptor type A are increased in the myocardium of rats and patients with RV hypertrophy.

- The upregulation of the ET-1 axis correlates positively with the degree of RV hypertrophy.
- ERA treatment of perfused hearts with RV hypertrophy decreased RV contractility.

ERAs are approved for PAH but their effects on the RV have not been studied. Because endothelin is a positive inotrope, we hypothesized that a potential upregulation of the endothelin axis may be a compensatory mechanism in the RV hypertrophy and that ERAs may be negative inotropes. We found that the endothelin axis is upregulated in RV myocardium of rats and humans with RVH and that ERAs decrease contractility in RVH rat hearts ex vivo. Therefore, the possibility of direct effects of ERAs on the diseased RV must be considered when interpreting clinical trials or treating PAH patients.
Endothelin Axis Is Upregulated in Human and Rat Right Ventricular Hypertrophy
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An erratum has been published regarding this article. Please see the attached page for:
/content/114/6/e32.full.pdf

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/12/05/CIRCRESAHA.111.300448.DC1
In the *Circulation Research* article by Nagendran et al (Endothelin axis is upregulated in human and rat right ventricular hypertrophy. *Circ Res*. 2013;112:347–354. DOI: 10.1161/CIRCRESAHA.111.300448), the text incorrectly referred to a Food and Drug Administration “black box warning” for ambrisentan; the text should have simply listed it as a “warning”.

The error has been corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/112/2/347.
Human Specimens: All samples were handled in an identical manner, minimizing the variability of fixation artifacts, immediately flash frozen in liquid nitrogen and stored in a -80°C freezer.

The hypertrophied RV tissues were obtained from patients diagnosed with:
a) single ventricle undergoing the Norwood procedure (n=5), b) RV pressure overload syndromes that included Tetralogy of Fallot, Pulmonary Atresia+Ventricular Septal Defect, Double Outlet RV, Truncus Arteriosus (n=12), c) Volume+Pressure overload syndromes that included Atrial/Ventricular Septal Defects with RV outflow obstruction (n=10) d) idiopathic PAH (n=7).

The non-hypertrophied RV tissues came from patients undergoing the Ross procedure, or autopsy, or unused transplant material or from patients undergoing transplant surgery for diseases not affecting the RV like large atrial sarcoma (n=16).

Since in many cases at the time of surgery the ET receptor antagonists were not approved for use in patients with congenital heart disease, overall only 5 of the 34 RVH patients had been exposed to these drugs at the time the tissues were removed

Immunohistochemistry and Confocal Microscopy: Staining was performed on sections after heat mediated antigen retrieval with citrate/citric acid buffer (pH 6). Image enhancer IT (Invitrogen Canada Inc., Burlington, ON, Canada) was used for blocking, followed by Super Block Buffer + 0.05% Tween20 (Pierce, Rockford II. U.S.A). Primary antibodies used included: goat anti-human Myosin Heavy Chain (Y-20) #sc-12117 (dilution 1:50), Santa Cruz Biotechnology Inc., Santa Cruz,CA); goat anti-human Endothelin-1 #sc-21625 (dilution 1:100), Santa Cruz Biotechnology Inc., Santa Cruz, CA); rabbit anti-rat Endothelin Receptor-A #AB3260-50uL (dilution 1:50) (Chemicon International). Secondary antibodies included: donkey anti goat tritc 1:100 for MHC (Molecular Probes, Invitrogen); goat anti-rabbit fitc 1:100 for ERA (Molecular Probes, Invitrogen); donkey anti goat fitc 1:100 for ET-1 (Molecular Probes, Invitrogen); goat anti rabbit tritc 1:100 for ERA (Molecular Probes, Invitrogen). Antibodies were applied for 1 hr at 37 C. Lack of nonspecific staining for the antibodies used was confirmed by application of secondary antibody only. All slides were also stained with a nuclear stain, DAPI #D21490 (Invitrogen) 1 μM for 10 minutes at room temperature. Slides were imaged on a Zeiss LSM 510 confocal microscope (FITC: 488nm excitation, 505-530 nm emission; TRITC: 543nm excitation, 565-615nm emission; DAPI: 740 nm two photon excitation, 390-465nm emission. Lack of nonspecific staining for the antibodies used was confirmed by application of only secondary antibody. Densitometry analysis was performed with the Zeiss Image Examiner software. Fluorescence intensity of ET-1 or Endothelin Receptor A was performed by measuring fluorescence units (FU) in circular regions of interest (0.126mm²). A minimum of five random regions of interest were drawn into each field of view, where the circle encompassed myocardial tissue only and not coronary vessels, identified as the regions of tissue stained with myosin heavy chain. Once regions were encompassed, the myosin heavy chain channel (stained red) was turned off, such that only fluorescence from ET-1 or endothelin receptor A (green) was measured. At least three regions were studied per slide and at least three slides
per patient were quantified. The mean fluorescence intensity from each patient was plotted against the RV free wall thickness from the same patient.

**Laser captured microdissection and quantitative RT-PCR:** RNA extraction for human and rat ventricular tissue was performed using a kit (Qiagen, Mississauga, ON, Canada). Samples were added to a microwell plate, along with TaqMan probes, endothelin primers and reagents (all from Applied Biosystems, Foster City, CA). 18S mRNA was used as the housekeeping gene and data were quantified by the $2^{DDC_{t}}$ technique, as previously described$^{1-5}$.

**Animal Model of RVH:**
We studied RVH using a model of experimental PAH by injecting monocrotaline, an alkaloid from crotalaria spectabilis, an established rat PAH model. Monocrotaline’s metabolites from the liver are toxic to the pulmonary arterial endothelium and induce an inflammatory environment, both of which result in marked pulmonary vascular remodeling, that peaks 3-4 weeks post-injection at which time RVH is present. The presence of pulmonary hypertension and RVH were confirmed prior to euthanasia with echocardiography (Doppler ultrasound and 2-D echo respectively)$^{3,6-11}$.

**References:**


Online Table I:

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<thead>
<tr>
<th>RV status</th>
<th>n</th>
<th>Age (years±SD)</th>
<th>Gender (male%)</th>
<th>RVWTi (cm/m²±SEM)</th>
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<tr>
<td>Hypertrophied RV (congenital heart disease undergoing open heart surgery, heart/lung transplant recipients due to PAH)</td>
<td>34</td>
<td>13.2±12.7</td>
<td>76%</td>
<td>1.55±0.26</td>
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<tr>
<td>Non-hypertrophied RV (Ross procedures, transplant recipients due to atrial sarcomas, unused donor transplant material)</td>
<td>16</td>
<td>31.7±17.1</td>
<td>44%</td>
<td>0.23±0.02</td>
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Online Figure I. Mean immunofluorescence data for the expression of Endothelin Receptor-B I in the right ventricles shown in Figure 1. There is no significant difference in expression of the receptor (FU=fluorescence units).
Online Figure II. Representative H&E stained slides of normal rat right ventricles and hypertrophied right ventricles from rats with monocrotaline induced PAH. There is an obvious cardiomyocyte hypertrophy seen in these transverse sections.
Online Figure III: Laser-captured microdissection (LCM) and qRT-PCR were applied on rat RV tissue. LCM was used to isolate myocardium vs coronary arteries in normal and hypertrophied human RV for qRT-PCR. A myocardium sample was characterized by a high MHC to SMA ratio. In contrast, a coronary artery sample was characterized by a low MHC to SMA ratio. Endothelin type A receptor and ET-1 mRNA is markedly increased in RVH (n=7) compared to normal RV myocardium (n=6), whereas it is expressed similarly in the coronary vessels, in agreement with our immunohistochemistry data (Figure 3). These data recapitulate the findings in human tissue (Figure 2).
**ET<sub>R</sub>-A: Rat RV and LV**

Online Figure IV. Mean immunofluorescence data for the expression of Endothelin Receptor-A in right and left ventricles of control rats and rats with monocrotaline-induced PAH which have right ventricular hypertrophy. There is a significant increase in the expression of the receptor in the hypertrophied RVs (compared to the non-hypertrophied RVs) but not in the LV of the same animals (FU=fluorescence units,* p<0.001 versus normal RV).
Online Figure V. Mean data showing that the RV systolic pressure (RVSP) is decreased in response to an ET$_{R}$-A antagonist (BQ-123) and a non-selective ERA (bosentan), in the hypertrophied (RVH, n=11) but not the normal RV (n=9). Both normal and hypertrophied RVs responded similarly to isoproterenol and ET-1 (*p<0.01 versus baseline).
Online Figure VI. Mean data of change in right ventricular developed pressure from baseline in control (n=6) and right ventricular hypertrophy (RVH, n=7) rat hearts. The increase in developed pressure caused by Endothelin-1 is blunted by bosentan (*p<0.01 versus RVH without bosentan).
Online Figure VII: Mean data showing that bosentan decreased both contractility and lucitropy (positive and negative dP/dt respectively) in the hypertrophied (n=11) but not the normal RV (n=9) (*p<0.01 versus baseline).
Online Figure VIII. Mean coronary flow of control rat hearts (n=9) and hearts with right ventricular hypertrophy (n=11). The addition of bosentan did not cause any significant change in coronary flow.