Prevention of pulmonary vascular remodeling and of decreased BMPR-2 expression by losartan therapy in shunt-induced pulmonary hypertension

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THE PATHOGENESIS of pulmonary arterial hypertension (PAH) remains unknown. A series of biological abnormalities have been identified in all compartments of the pulmonary arteriolar wall, and current views focus on possible interactions between an endothelium-derived vasoconstrictors/vasodilators imbalance and abnormal bone morphogenetic protein receptor-2 (BMPR-2) signaling (7, 10). The renin-ANG system is not usually mentioned among the possible mechanistic pathways promoting PAH (7, 10). This is surprising because the expression of ANG converting enzyme (ACE) has been reported to be increased in pulmonary arteries of PAH patients (19, 26) with a functional predominance at the site of arteriolar remodeling (17). Furthermore, it has been reported that increased binding of ANG II to ANG II type 1 receptors (AT1) in pulmonary arteries from PAH patients, together with ANG II and AT1 receptor-mediated activation of mitogen-activated protein kinase and increased DNA and protein synthesis in human pulmonary artery smooth muscle cells (17). It is thus possible that ANG II contributes to abnormal pulmonary vascular tone and remodeling in PAH.

PAH is either idiopathic or occurs in association with a variety of conditions that include congenital heart disease with left-to-right shunting (27). Early stages of shunt-induced PAH can be reproduced by the anastomosis of the systemic to the pulmonary circulation in growing animals (21, 22, 32). This is actually the only experimental animal model that exactly reproduces clinical PAH (27), and thus it is of particular interest to investigate the pathobiology of the disease. We previously reported on the anastomosis of the left innominate artery to the pulmonary arterial trunk (modified Blalock-Taussig procedure) to reproduce in a 3-mo period of time significant pulmonary hypertension with medial hypertrophy corresponding to early PAH (24). We showed that this PAH model is associated with the overexpressions of endothelin-1 (ET-1), phosphodiesterase-5 (PDE-5), and angiopoietin-1, together with a decreased expression of BMPR-2 (24, 25). Interestingly, we also found increased expressions of ANG II and both the AT1 and AT2 receptors (25). In these studies, pretreatment with the dual ET-1 receptor blocker bosentan or the PDE-5 inhibitor sildenafil largely prevented the increase of pulmonary vascular resistance (PVR) but only partially prevented pulmonary arteriolar remodeling (24, 25), raising the question about the contribution of alternative or parallel pathways, such as the renin-ANG system.

In the present study, we pretreated piglets with the modified Blalock-Taussig procedure with the specific AT1 receptor blocker losartan to test the hypothesis that ANG II and AT1 signaling might contribute to the biological derangements of early cardiac shunt-induced PAH.

MATERIALS AND METHODS

Twenty-six piglets (18 ± 1 days old and 5.5 ± 0.2 kg body wt) were included in the present study, which was approved by the institutional Committee on Animal Welfare from the School of Medicine of the Free University of Brussels. The animals were randomized to a sham operation (n = 8 piglets) or to an anastomosis between the left innominate artery and the pulmonary arterial trunk as
nary arterial flow, and blood gases were measured as previously reported (24). Losartan was a gift of Therabel (Brussels, Belgium).

**Hemodynamic evaluation.** After an observation period (90 ± 1 days), the animals were anesthetized, paralyzed, ventilated, and equipped with fluid-filled pulmonary and systemic artery catheters, an inferior vena cava balloon catheter, and an ultrasonic flow probe on the pulmonary arterial trunk as previously described (24) with a 5-Fr high-fidelity manometer-tipped catheter (SPC 350, Millar, Houston, TX) in the right ventricle. Heart rate (HR), mean pulmonary arterial pressure (Ppam), occluded Ppa (Ppao), systemic arterial pressure (Psa), and blood gases were measured as previously reported (24). PVR was defined by multipoint Ppam-to-Q plots obtained by rapid inflation of the inferior vena cava balloon (24).

The decrease of right ventricular volume during systole was computed by the integration of the instantaneous pulmonary arterial flow. The systolic portion of the right ventricular pressure-volume loop was constructed from instantaneous right ventricular pressures and volumes, as reported by Brimioulle et al. (3) to compute end-systolic elastance (Ees) and arterial elastance (Ea). Right ventricular systolic function was also estimated by the computation of the maximum rate of change in pressure per unit of time (dP/dtmax).

Hemodynamic and blood gas measurements were obtained after ensuring steady-state conditions (stable HR, Ppam, and Psa) for 60 min after shunt closure in the shunted animals. After the measurements, the animals were euthanized with an anesthetic overdose.

**Morphometry.** Pulmonary arterial morphometry was performed as reported previously (24). Only arteries with an external diameter (ED) of <500 μm and a complete muscular coat were measured and assigned to five groups according to ED: 0 to 75 μm, 76 to 150 μm, 151 to 225 μm, 226 to 300 μm, and 300 to 500 μm. Medial thickness (MT) was related to arterial size with the following formula: %MT = 2 × MT/ED × 100.

**RIA.** Systemic arterial plasma ET-1 and ANG II were measured by RIA after extraction as previously described (24) with commercially available antibodies and standard (ET-1 RAS 6901 and ANG II RAS-7002 from Peninsula). The tracers were iodinated in our laboratory and purified by high-performance liquid chromatography. The samples displaced the tracer parallel to the standard curve.

**RTQ-PCR.** Pulmonary tissue mRNA levels were measured by SYBR Green real-time quantification (RTQ-PCR) as previously described (24). Primers for the report gene hypoxanthine-guanine phosphoribosyl transferase (HPRT), angiotensinogen, AT1, and AT2 receptors, ET-1, ETα, and ETβ, and ET-converting enzyme (EC-1), angiopoietin-1, BMPR-1A, and BMPR-2 have already been used in our laboratory (24, 25). The sequences reported elsewhere for angiopeptin-2 (GenBank accession no. NM213808) and angiopoietin receptor tyrosine kinase with immunoglobulin and EGF homology domains-2 (Tie-2) (GenBank accession no. AF251494) were used to design specific primers on Primer Express software (Applied Biosystems), porcine-specific primers adapted to RTQ-PCR conditions (Table 1). The primers were produced on an automated synthesizer (Applied Biosystems) according to the manufacturer’s protocol. SYBR Green RTQ-PCR analysis was performed with the GeneAmp 5700 (Applied Biosystems) as previously reported (24). To ensure the quality of the measurements, both negative and positive controls were systematically included (in double) in each plate. The statistical analysis of the RTQ-PCR results was calculated by using the ΔCt value (Ct, gene of interest – Ct reporter gene). Relative gene expression was obtained by ΔΔCt methods (ΔCt sample – ΔCt calibrator), with the use of the sham-operated group as a calibrator for comparison of all unknown sample gene expression levels. The conversion between ΔΔCt and relative gene expression levels is as follows: fold induction = 2−ΔΔCt, where 2−ΔΔCt is relative gene expression (34).

**Immunohistochemistry.** The immunohistochemistry analysis was performed as previously reported (24) with rabbit monoclonal antibody to ET-1 (1:100 dilution) prepared in our laboratory and with commercial rabbit polyclonal antibody against ANG I and ANG II (H-300-SC-20717, 1:100 dilution; Santa Cruz Biotechnology). Quantitative immunohistochemical assessments were performed as previously reported (24). A mean optical density, which relates to immunohistochemical staining intensity, was calculated in the vessel wall of 20 pulmonary arteries of <500 μm. This mean optical density value was obtained by dividing the integrated optical density value for the immunohistochemical staining by the area of tissue covered by this staining.

**Statistical analysis.** Values are reported as means ± SE. Multipoint pressure-flow relations were submitted to linear regression analysis, and standardized pressure values were calculated from individual regressions at the Q of 2 and 5 l·min−1·m−2 (33). The effects of the shunt and drugs were analyzed by a repeated measures ANOVA. When the F ratio of the ANOVA reached a P < 0.05 critical value, Scheffé’s post hoc tests were performed to compare specific situations (33). Correlations were calculated via a linear regression analysis (33).

**RESULTS**

Weight gain averaged 40 kg and was not different in the three study groups. Arterial blood gases and hematocrits were normal and not different in the three study groups. The ratio of pulmonary to systemic flow before closure of the shunt was 4.3 ± 0.4 during the period of study (from 20 to 150 days). The hemodynamics of the three study groups are presented in Table 1.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers Sequences</th>
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<tbody>
<tr>
<td>Ang-2</td>
<td>Sense: 5’-GGC-AGG-TTG-TTT-TCT-TGG-CT-3’; Antisense: 5’-CTG-ATA-GCT-CTT-GTC-CGC-3’</td>
</tr>
<tr>
<td>Tie-2</td>
<td>Sense: 5’-TGA-GCC-TTA-CCT-TGG-GGA-TG-3’; Antisense: 5’-TGG-AGG-GAG-AGG-TCA-TAT-TC-3’</td>
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Losartan therapy prevented the increases in Ppam, Ppao, dp/dtmax, Ees, and Ea without change in HR, Q, Psa, or the ratio of Ees to Ea (Table 2). The Ppam-to-Q relationships were shifted to higher pressures (Fig. 1). There was an increase in pulmonary arterial MT, and this effect was most pronounced in the smallest arterioles (Fig. 2). Plasma ET-1 increased from 2.0 ± 0.1 pg/ml in the sham-operated controls to 2.4 ± 0.1 pg/ml in the placebo group (P < 0.05) without changes in circulating ANG II (sham-operated group, 25.2 ± 0.5 pg/ml; placebo group, 24.5 ± 0.6 pg/ml).

Losartan therapy increased gene expressions for AGT II, the AT1, and AT2 receptors tyrosine kinase with immunoglobulin and EGF homology domains-2 (Tie-2) and partially prevented the shift to higher pressure of Ppam-to-Q relationships (Fig. 1). Losartan therapy prevented the increases in Ppam, Ppao, dp/dtmax, Ees, and Ea and reduced the shunt-induced increase in PVR by 51% (Table 2) and partially prevented the shift to higher pressure of Ppam-to-Q relationships (Fig. 1). Losartan reduced the increase in pulmonary arterial MT by an average of 35% (Fig. 2). Losartan was associated with a decrease in plasma ET-1 to 2.0 ± 0.1 pg/ml (P < 0.05 vs. placebo group) without a change in circulating ANG II (losartan, 24.9 ± 0.5 pg/ml).

As illustrated in Fig. 3, systemic-to-pulmonary shunting increased gene expressions for AGT II, the AT1, and AT2 receptors tyrosine kinase with immunoglobulin and EGF homology domains-2 (Tie-2) and partially prevented the shift to higher pressure of Ppam-to-Q relationships (Fig. 1). Losartan reduced the increase in pulmonary arterial MT by an average of 35% (Fig. 2). Losartan was associated with a decrease in plasma ET-1 to 2.0 ± 0.1 pg/ml (P < 0.05 vs. placebo group) without a change in circulating ANG II (losartan, 24.9 ± 0.5 pg/ml).
Table 2. Hemodynamic effects of losartan treatment in overcirculation-induced experimental pulmonary arterial hypertension in piglets

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Placebo</th>
<th>Losartan</th>
</tr>
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<tbody>
<tr>
<td>HR, beats/min(^{-1})</td>
<td>112±8</td>
<td>120±6</td>
<td>110±10</td>
</tr>
<tr>
<td>Q, l/min(^{-1}) · m(^{-2})</td>
<td>3.3±0.1</td>
<td>3.1±0.1</td>
<td>3.3±0.1</td>
</tr>
<tr>
<td>P(_{pam}), mmHg</td>
<td>121±8</td>
<td>130±8</td>
<td>120±4</td>
</tr>
<tr>
<td>P(_{pam}), mmHg</td>
<td>18±1</td>
<td>33±1*</td>
<td>21±1†</td>
</tr>
<tr>
<td>P(_{pam}), mmHg</td>
<td>7±1</td>
<td>11±1*</td>
<td>6±1‡</td>
</tr>
<tr>
<td>PVR, mmHg(^{-1}) · min(^{-2})</td>
<td>2.5±0.2</td>
<td>6.2¡.0.3</td>
<td>4.3¡.0.4†‡</td>
</tr>
<tr>
<td>E(_{es}), mmHg/ml</td>
<td>1.36±0.10</td>
<td>2.05±0.12*</td>
<td>1.24±0.09‡</td>
</tr>
<tr>
<td>E(_{es}), mmHg/ml</td>
<td>0.84±0.07</td>
<td>1.40±0.11*</td>
<td>0.82±0.07‡</td>
</tr>
<tr>
<td>E(<em>{es})/E(</em>{es}), mmHg/ml</td>
<td>1.65±0.10</td>
<td>1.50±0.11</td>
<td>1.54±0.08</td>
</tr>
<tr>
<td>dP/dt(_{max}), mmHg/s</td>
<td>527±34</td>
<td>686±29*</td>
<td>422±44‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. HR, heart rate; Q, cardiac index; P\(_{pam}\), mean systemic arterial pressure; P\(_{pam}\), mean pulmonary arterial pressure; P\(_{pam}\), occluded pulmonary arterial pressure; PVR, pulmonary vascular resistance; E\(_{es}\), end-systolic elastance; E\(_{es}\), pulmonary arterial elastance; and dP/dt\(_{max}\), the rate of change in pressure per unit change in time. *P < 0.05, sham-operated (n = 8 piglets) vs. placebo (n = 10 piglets) group; †P < 0.05, placebo vs. losartan-treated (n = 8 piglets) group; ‡P < 0.05 sham-operated vs. losartan-treated group.

receptors, ET-1, the ET\(_B\) receptor, ECE-1, and angiopoietin-1; decreased gene expressions for BMPR-1A and BMPR-2; and did not change gene expressions for the ETA receptor, angiopoietin-2, and Tie-2. Losartan therapy completely prevented changes in gene expressions for AT\(_1\) and BMPR-2 and reduced the changes in gene expressions for ET-1, ET\(_B\), angiopoietin-1, and BMPR-1A.

As illustrated in Fig. 4, chronic left-to-right shunting increased pulmonary arterial immunostaining for both ANG I and ANG II and ET-1 and ET-1. Losartan reduced the overexpression of ET-1 but did not prevent the increase in ANG I and ANG II proteins.

As shown in Fig. 5, both ANG I and ANG II and ET-1 protein expression in the arteriolar wall were correlated to P\(_{pam}\) and to MT for pulmonary arteries (<75 μm).

**DISCUSSION**

The present results show that the administration of losartan, a selective AT\(_1\) receptor antagonist, partially prevents overcirculation-induced pulmonary hypertension in piglets, with a decrease in pulmonary vascular tone more than in remodeling and a reversal of associated biological derangements, suggesting a significant participation of ANG II and AT\(_1\) signaling in the early stages of PAH.

The pathogenesis of PAH is incompletely understood. A variety of biological abnormalities have been reported in all pulmonary arterial compartments of patients with established PAH (7, 10), but which one initiates the disease remains uncertain. A central role has emerged for abnormal BMPR-2 signaling. BMPR-2 mutations have been reported in 58% of familial PAH and in 26% of idiopathic PAH (5, 11, 14). Pulmonary artery smooth cells from PAH patients exhibit abnormal growth responses to transforming growth factor-β and BMPs (18), and the expression of BMPR-2 is decreased in PAH patients without demonstrable mutation (2). In addition, PAH patients may present with an overexpression of angiopoietin-1, which interacts with an endothelial Tie-2 receptor to decrease the expression of BMPR-1A, a transmembrane protein required for normal BMPR-2 function (6). Naeije’s laboratory (25) previously reported decreased expressions of both BMPR-1A and BMPR-2, together with an overexpression of angiopoietin-1 in overcirculation-induced pulmonary hypertension in piglets, suggesting that abnormal BMPR-2 signaling occurs early in the development of PAH. In the present study, we found no change in the expression of angiopoietin-2 in keeping with a previous report (6) that the involvement of angiopoietin-1 in PAH is not associated with a decreased expression of antagonistic angiopoietin-2. On the other hand, the expression of Tie-2 was normal, suggesting that any effect of angiopoietin-1 in early PAH would be related to phosphorylation of the normally present Tie-2 receptor (6, 29).

The present results confirm that shunt-induced pulmonary hypertension in piglets is associated with increased gene expressions of angiotensinogen and of the AT\(_1\) and AT\(_2\) receptors (25) and show in addition increased ANG I and ANG II protein immunostaining that is correlated to the severity of induced pulmonary hypertension. This finding is consistent with previous observations that suggest a role for the renin-ANG system in the development of PAH. The expression of ACE has been reported to be increased in pulmonary arteries of PAH patients (19, 26) with a functional predominance at the site of arteriolar

**Fig. 1.** Composite plots of mean pulmonary artery pressure (P\(_{pam}\)) versus pulmonary blood flow (Q) in sham-operated, placebo-, and losartan-treated piglets. Losartan partially prevented shunt-induced shift of P\(_{pam}\)-to-Q plots to higher pressures. Values are means ± SE. *P < 0.05, sham-operated versus placebo group; †P < 0.05, placebo versus losartan-treated group; ‡P < 0.05, sham-operated versus losartan-treated group.

**Fig. 2.** Morphometry on pulmonary arterioles of sham-operated, placebo-, and losartan-treated piglets and plot of percent medial thickness versus external diameter. Losartan limited shunt-induced increase in medial thickness to 65%, and this effect was most pronounced in smallest size arterioles. Values are means ± SE. *P < 0.05, sham-operated versus placebo group; †P < 0.05, placebo versus losartan-treated group; ‡P < 0.05, sham-operated versus losartan-treated group.
Because ACE inhibitors and AT1 receptor blockers, but not AT2 receptor blockers, prevent the development of hypoxic pulmonary hypertension (4, 16), it is believed that the contribution of the renin-ANG system to pulmonary hypertension exclusively involves ANG II and AT1 signaling. This is confirmed by the present demonstration of partial but significant prevention of overcirculation-induced PAH by the selective AT1 receptor blocker. One could wonder about the physiological significance of the increased gene expression of the AT2 receptor, previously demonstrated in both overcirculation- and hypoxia-induced pulmonary hypertension (4, 25), which persisted in the present study in losartan-treated animals. ANG II induces AT1-mediated vasoconstriction and remodeling, but several studies suggest that these effects may be limited by AT2 receptor-mediated vasodilatation, growth-antagonizing and apoptosis-enhancing effects (31). The AT2 receptor could also be involved in an ANG II-mediated upregulation of angiopoietin-2, which competes for the Tie-2 receptor with angiopoietin-1 and thereby limits its remodeling effects (8). In the present study, the gene expressions of angiopoietin-2 and Tie-2 were unchanged in shunted piglets and unaffected by losartan therapy. On the other hand, specific AT2 receptor antagonists had no detectable effect in experimental hypoxic pulmonary hypertension (4, 17). Thus, whether an overexpression of the AT2 receptor may contribute to limit the severity of PAH is uncertain.

Losartan therapy was associated with a decreased expression of the AT1 receptor. This unexpected result might be related to losartan-induced decrease in pulmonary artery pressures or to an inhibition of a positive interaction with the ET-1 signaling, because losartan also decreased the lung tissue expression and circulating level of ET-1. These latter effects could be related to decreased pulmonary artery pressures, resulting in decreased shear stress-induced overexpression of ET-1. Losartan could also have inhibited an ANG II-induced inflammatory reaction, part of it being an overexpression of ET-1 (30), or could have prevented ANG II-induced ET-1 gene expression (9, 12). It is of interest that losartan therapy reverted BMPR-2 expression to normal, which could also be related to decreased pulmonary artery pressures or to yet unknown interactions between BMPR-2 expression and ET-1 or ANG II signaling pathways.

In the present study, losartan therapy was associated with a decrease in pulmonary vascular tone more than in remodeling, as assessed by proportionally more important decreases in PVR than in MT. These effects were similar, though less pronounced, to those observed with the administration of the dual ET-1 receptor antagonist bosentan or the PDE-5 inhibitor sildenafil (24, 25). Increased PVR in overcirculation-induced PAH in piglets has previously been shown to be acutely reversible by the inhalation of nitric oxide or the infusion of prostacyclin (32). The present results suggest that increased pulmonary vascular tone in shunt-induced PAH is multifactorial and may be in part ANG II dependent.

In the present study, right ventricular dP/dt max increased with the development of pulmonary hypertension and decreased along with the prevention of increased PVR in losartan-treated animals, suggesting that right ventricular contractility adapted to changes in afterload. This was confirmed by the fact that right ventricular Ees, a load-independent index of contractility (3), changed parallel to changes in Ees, so that ventricularto-arterial coupling estimated by Ees-to-Ea ratio re-

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**Fig. 3. Relative lung tissue mRNA content (2^−ΔΔCt, relative gene expression) for ANG II precursor angiotensigen (AGT II) and ANG II receptor types 1 and 2 (AT1 and AT2, respectively) (A); for endothelin-1 (ET-1), ET-1 types A and B receptors (ETa, ETb, respectively) and ET-converting enzyme-1 (ECE-1) (B); and for angiopoietin-1 (Ang-1), Ang-2, Ang receptor tyrosine kinase with immunoglobulin and EGF homology domains-2 (Tie-2), and bone morphogenetic protein receptor-1A and -2 (BMPR-1A and BMPR-2, respectively) (C) of sham-operated, placebo-, and losartan-treated piglets. Values are means ± SE. *P < 0.05, sham-operated versus placebo group; †P < 0.05, placebo versus losartan-treated group; ‡P < 0.05, sham-operated versus losartan-treated group.**

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remodeling (15). An increased AT1 receptor binding of ANG II has been reported in pulmonary arteries of PAH patients, together with the demonstration of ANG II and AT1 receptor-mediated activation of mitogen-activated protein kinase and increased DNA and protein synthesis in human pulmonary artery smooth muscle cells (17). The present results are also in keeping with previous data suggesting an activation of the renin-ANG system in experimental hypoxic pulmonary hypertension with increased pulmonary arteriolar ACE expression (15) and increased pulmonary binding of ANG II and overexpressions of the AT1 and AT2 receptors (4).

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mained at an optimal value of ~1.5, with and without losartan therapy. This result is in keeping with a previous study (25) in which bosentan prevented overcirculation-induced pulmonary hypertension and confirms that this early PAH model is not associated with right ventricular failure.

It may be of interest that in the present study, the inhibition of ANG II and AT₁ pathway and the associated decrease in PVR was associated with persistently increased expressions of ET-1, the ET<sub>B</sub> receptor, and angiopoietin-1, together with persistently decreased expression of BMPR-1A. This observation argues in favor of dominant roles for ET-1 and angiopoietin-1 pathways in early arteriolar remodeling in shunt-induced PAH.

Attempts to interfere with the renin-ANG system in patients with PAH have been limited until now to the administration of the ACE inhibitor captopril and have produced variable results, with either no effect on PVR, acutely or chronically, at rest and
at exercise (13, 23), or a persistent decrease in PVR (1, 28).

H2324 LOSARTAN IN EXPERIMENTAL PULMONARY HYPERTENSION

Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Stenmark KR. Role of angiotensin-convert-  
ing enzyme and angiotensin II in development of hypoxic pulmonary hyper-  


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