Sildenafil added to sitaxsentan in overcirculation-induced pulmonary arterial hypertension

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Rondelet B, Dewachter L, Kerbaul F, Dewachter C, Hubloue I, Fesler P, Franck S, Remmelink M, Brimioulle S, Naeije R. Sildenafil added to sitaxsentan in overcirculation-induced pulmonary arterial hypertension. Am J Physiol Heart Circ Physiol 299: H1118–H1123, 2010. First published August 6, 2010; doi:10.1152/ajpheart.00418.2010.—Experimental left-to-right shunt-induced pulmonary arterial hypertension (PAH) can be partially prevented by the endothelin-A receptor blocker sitaxsentan or by the phosphodiesterase-5 inhibitor sildenafil. We hypothesized that the combination of these drugs would completely prevent shunt-induced PAH, arguing in favor of a major role of endothelial dysfunction in the initiation of the disease. Twenty-four 3-wk-old piglets were randomized to a sham operation or to placebo, sitaxsentan therapy, or sitaxsentan combined with sildenafil. The coupling of right ventricular end-systolic to arterial elastances (3, 6).

In the present study, we tested the hypothesis that a combination therapy; left-to-right shunt; congenital heart disease; right ventricle

PULMONARY ARTERIAL HYPERTENSION (PAH) is a rare, incurable disease with a poor prognosis (18). The pathobiology of the condition is complex and remains incompletely understood. Mutations of the bone morphogenetic protein receptor (BMPR)-2 are associated with a considerable risk to develop PAH, and there is an imbalance between endothelium-derived constrictor and pro-proliferative mediators such as endothelin-1 and vasodilating and antiproliferative mediators such as prostacyclin and nitric oxide in the pulmonary circulation of PAH patients (20). Progress has been achieved with the chronic administration of targeted therapies with prostacyclins, endothelin receptor antagonists (ERAs), and phosphodiesterase-5 inhibitors (PDE-5i) (18). However, the life expectancy of PAH patients remains limited to 5 to 6 years, and most survivors remain functionally limited with an insufficient quality of life. Therefore, more efficient therapeutic strategies are being sought. Combinations of drugs and earlier administrations are currently under consideration (18). On the other hand, it has recently been better realized that the symptoms and prognosis of PAH patients are largely dependent on right ventricular (RV) function adaptation to afterload, and it has been hypothesized that targeted therapies might improve RV arterial coupling (3, 6).

We previously reported on chronic systemic-to-pulmonary shunting in growing piglets as an experimental model of early congenital heart disease (CHD)-PAH, characterized by increased pulmonary vascular resistance (PVR), medial thickness (MT), and an overexpression of endothelin-1 and angiopoietin-1 and a decreased expression of BMPR-2 (24). In that model, either nonsellective (bosentan) or selective (sitaxsentan) ERAs or the PDE-5i sildenafil partially prevented the increase in PVR, pulmonary vascular remodeling, and associated biological changes (24–26).

In the present study, we tested the hypothesis that a combination of an ERA and a PDE-5i would be a more effective preventive therapy of CHD-PAH than either drug alone. We reasoned that an increased efficacy of the combination would argue in favor of an endothelial imbalance playing a major role in the initiation of the disease. In addition, we used this large animal model of early PAH to explore the possibility that ERA alone or in ERA combined with a PDE-5i might present with intrinsic myocardial effects as evaluated by measurements of RV arterial coupling.

MATERIALS AND METHODS

Twenty-four piglets, 18 ± 1 days old and weighing 5.3 ± 0.2 kg, were included in this study, which was approved by our Institutional Committee on Animal Welfare. The animals were randomized to a

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Hemodynamic evaluation. The animals were anesthetized with remifentanil and midazolam after a premedication with ketamine, midazolam, and atropine; paralyzed with pancuronium; intubated; and ventilated as previously described (24). They were equipped with catheters and an ultrasonic flow probe on the pulmonary artery for measurements of mean pulmonary artery pressure ($P_{pa,m}$), occluded $P_{pa}$, right atrial pressure ($P_{ra}$), mean systemic arterial pressure ($P_{sa,m}$), cardiac output ($Q$), pulmonary arterial flow, and blood gases. PVR was calculated as $(P_{pa,m} - P_{pa,o})/Q$ and systemic vascular resistance (SVR) as $(P_{sa,m} - P_{ra})/Q$. To improve the comparability of PVR at different levels of Q, a total PVR was estimated by $P_{pa,m}$ in an isoflow condition of 3 l·min$^{-1}$·m$^{-2}$ interpolated from $P_{pa,m}$ vs Q relationships obtained by rapid inflation of an inferior vena cava balloon (24). A previously reported single-beat method was used to estimate RV afterload by pulmonary arterial elastance ($E_a$) and RV contractility by end-systolic elastance ($E_{es}$), and the adequacy of RV systolic function adaptation to afterload by the $E_{es}$ vs $E_a$ ratio ($E_{es}/E_a$) (5).

Hemodynamic and blood gas measurements were obtained after ensuring steady-state conditions for 60 min, after shunt closure. After the measurements, the animals were euthanized and pulmonary tissue samples were harvested and snap frozen in liquid nitrogen and stored at $-80^\circ$C or, after overnight fixation, embedded in paraffin.

Morphometry. Pulmonary arterial morphometry was performed as reported previously (24). MT was related to arterial size with the following formula: $\%MT = [(2 \times MT)/(ED \times 100)]$, where ED is external diameter. Only arterioles of $<75 \mu m$ diameter were considered here, since we previously reported the smallest size arterioles to be the major site of remodeling in shunt-induced PAH in piglets (24).

Quantitative real-time polymerase chain reaction. Pulmonary tissue messenger RNA levels were measured by quantitative RT-PCR for endothelin-1, angiotensin-1, BMPR-1A, and BMPR-2 with previously reported primers (24–26). 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 quantitative RT-PCR results was done using the change in threshold cycle ($\Delta Ct$) value (Ct gene of interest – Ct reporter gene). Relative gene expression was obtained by $\Delta \Delta Ct$ methods ($\Delta Ct$ sample – $\Delta Ct$ calibrator) using the sham-operated group as a calibrator. The conversion between $\Delta C_{t}$ and relative gene expression levels is as follows: fold induction $= 2^{-\Delta C_{t}}$ (31).

Radioimmunoassay. Systemic arterial plasma endothelin-1 was measured by radioimmunoassay after extraction as previously described (24, 26), using commercially available antibodies and tracers iodinated and HPLC purified in our laboratory.

Statistical analysis. Values are reported as means ± SE. Five-point $P_{pa,m}$-$Q$ plots were obtained in all animals and always best described by a linear approximation, with correlation coefficients higher than 0.95. A linear regression was calculated on each of them by the least squares method, and $P_{pa,m}$ interpolated at the flow level of 3 l·min$^{-1}$·m$^{-2}$. Effects of the shunt and drugs were analyzed by a repeated-measures analysis of variance. When the $F$ ratio of the analysis of variance reached a $P < 0.05$ critical value, modified $t$-tests were performed to compare specific situations (30).

RESULTS

Weight gain averaged 40 kg and was not different in the four study groups. Arterial blood gases and hematocrits were normal and not different in the four study groups. The ratio of pulmonary-to-systemic flow before closure of the shunt was 1.9 ± 0.1 in the placebo group, 1.8 ± 0.1 in the sitaxsentan group, and 1.9 ± 0.1 in the sitaxsentan plus sildenafil group ($P = not significant$).

Chronic systemic-to-pulmonary shunting increased $P_{pa,m}$ and PVR without a change in heart rate, $Q$, $P_{pa,m}$, SVR, or $P_{pa,o}$ whereas RV systolic function remained adapted to afterload as shown by proportional increases in $E_a$ and $E_{es}$ and no change in $E_{es}/E_a$ (Table 1, and Fig. 1). There were increases in isoflow $P_{pa,m}$ (Fig. 2) and in small arteriolar MT (both, $P < 0.001$; Fig. 3). Plasma endothelin-1 was increased to 4.0 ± 0.5 compared with 2.1 ± 0.1 pg/ml in the sham-operated controls ($P < 0.05$).

Sitaxsentan alone or combined with sildenafil prevented the increase in $P_{pa,m}$, PVR, $E_{es}$, and $E_a$ (Table 1) and did not affect $E_{es}/E_a$ (Fig. 1). Sitaxsentan alone partially prevented the increase in isoflow $P_{pa,m}$ (Fig. 2) and smallest arteriolar MT (Fig. 3). The combination of sitaxsentan and sildenafil normalized isoflow $P_{pa,m}$ (Fig. 2) and almost normalized arteriolar MT ($P = 0.07$ compared with sham-operated controls) (Fig. 3). Plasma endothelin-1 was at 2.4 ± 0.1 pg/ml in the sitaxsentan-treated animals and at 2.2 ± 0.1 pg/ml in the sitaxsentan + sildenafil group ($P = not significant$ between treatments and compared with controls).

Table 1. Hemodynamic effects of preventive sitaxsentan or sitaxsentan + sildenafil treatment in overcirculation-induced experimental pulmonary arterial hypertension in piglets

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Placebo</th>
<th>Sitaxsentan</th>
<th>Sitaxsentan + sildenafil</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>110 ± 7</td>
<td>124 ± 10</td>
<td>115 ± 7</td>
<td>124 ± 6</td>
</tr>
<tr>
<td>$Q$, l·min$^{-1}$·m$^{-2}$</td>
<td>3.1 ± 0.1</td>
<td>3.2 ± 0.2</td>
<td>3.6 ± 0.3†</td>
<td>3.7 ± 0.2†</td>
</tr>
<tr>
<td>$P_{sa,m}$, mmHg</td>
<td>132 ± 6</td>
<td>136 ± 6</td>
<td>144 ± 4</td>
<td>132 ± 5</td>
</tr>
<tr>
<td>SVR, mmHg·l·min$^{-1}$·m$^{-2}$</td>
<td>37.2 ± 3.6</td>
<td>40.7 ± 2.9</td>
<td>38.3 ± 3.2</td>
<td>35.2 ± 2.5</td>
</tr>
<tr>
<td>$P_{pa,m}$, mmHg</td>
<td>19.2</td>
<td>35 ± 1*</td>
<td>22 ± 1†</td>
<td>20 ± 1††</td>
</tr>
<tr>
<td>$P_{pa,o}$, mmHg</td>
<td>8 ± 1</td>
<td>10 ± 1*</td>
<td>9 ± 1*</td>
<td>7 ± 1†</td>
</tr>
<tr>
<td>PVR, mmHg·l·min$^{-1}$·m$^{-2}$</td>
<td>3.7 ± 0.5</td>
<td>8.3 ± 0.4*</td>
<td>4.8 ± 0.3‡</td>
<td>4.4 ± 0.5§</td>
</tr>
<tr>
<td>$E_a$, mmHg/ml</td>
<td>1.32 ± 0.11</td>
<td>2.03 ± 0.21*</td>
<td>1.09 ± 0.16†</td>
<td>1.45 ± 0.17†</td>
</tr>
<tr>
<td>$E_{es}$, mmHg/ml</td>
<td>0.88 ± 0.09</td>
<td>1.33 ± 0.07*</td>
<td>0.68 ± 0.11†</td>
<td>0.99 ± 0.17†</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, number of piglets/group. HR, heart rate; $Q$, cardiac index; $P_{sa,m}$, mean systemic arterial pressure; SVR, systemic vascular resistance; $P_{pa,m}$, mean pulmonary arterial pressure; $P_{pa,o}$, occluded pulmonary arterial pressure; PVR, pulmonary vascular resistance; $E_a$, end-systolic elastance; $E_{es}$, pulmonary arterial elastance. *$P < 0.05$, sham vs. placebo; †$P < 0.05$, placebo vs. sitaxsentan alone or sitaxsentan combined with sildenafil; ‡$P < 0.05$, sitaxsentan alone vs. sitaxsentan combined with sildenafil.

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As illustrated in Figs. 4 and 5, systemic-to-pulmonary shunting increased the expressions of endothelin-1, the endothelin type B (ETβ) receptor, and angiopoietin-1; decreased the expressions of BMPR-1A and BMPR-2; and did not change the expressions of the endothelin type A (ETA) receptor. Sitaxsentan and sitaxsentan combined with sildenafil completely prevented the increased expressions of endothelin-1 and of the ETβ receptor. Both treatment regimens did not prevent the increased angiopoietin-1 expression. Sitaxsentan alone partially restored the expressions of BMPR-1A and BMPR-2. The combination of sildenafil and sitaxsentan further restores the expressions of BMPR-1A and BMPR-2, which remained, however, decreased compared with controls.

**DISCUSSION**

The present results show that the combined administration of sildenafil and sitaxsentan prevents more than sitaxsentan alone vascular remodeling and associated biological changes, together with a trend to a more limited increase in PVR in experimental systemic-to-pulmonary shunt-induced PAH. The results also show that sildenafil combined with sitaxsentan does not affect RV arterial coupling, suggesting the absence of direct myocardial effects in normal conditions or an adequate...
function adaptation to a high flow-induced increase in afterload.

Systemic-to-pulmonary shunting in growing piglets reproduces in a few weeks pulmonary vascular changes that may take years to develop as a complication of congenital cardiac left-to-right shunts such as persistent ductus arteriosus or ventricular septal defects in humans (23). However, as also shown in the present experiments, the histological changes remain limited to medial hypertrophy (23–26). This is a feature of early and still reversible CHD-PAH (13). Therefore, the model is suitable for the investigation of the primum movens of the condition and for the testing of preventive or early therapies.

Sitaxsentan alone prevented part of the increase in PVR and remodeling, together with normalized expressions of endothelin-1 and ETB receptor, and partially normalized expressions BMPRs, confirming previous observations in the same shunt-induced PAH model (25). Since sitaxsentan is a highly specific ETA receptor blocker, it is remarkable that there was no compensatory increase in the expression of the ETB receptor. In vitro studies have suggested that both ETA and ETB receptors are involved in the pro-proliferative effects of endothelin-1 on pulmonary vascular smooth muscle cells (8). Previous studies in an in utero ovine shunt-induced PAH reported a transient increased expression of the ETB receptor that was localized on the pulmonary vascular smooth muscle cells (2). In the present study, sitaxsentan therapy was associated with decreased circulating endothelin-1 and limited pulmonary vascular remodeling to approximately the same extent as observed with the dual ETα/ETβ receptor antagonist bosentan (24). Taken together, these data indicate that the ETβ receptor does not appear to play an important role in the early stages of shunt-induced PAH.

The addition of sildenafil to sitaxsentan allowed for a complete prevention of shunt-induced P\textsubscript{Paw} measured in isoflow conditions to correct for flow-associated changes in PVR, and an almost complete prevention of medial hypertrophy, which remained different from controls at a \( P = 0.07 \) level of significance. This was associated with a further return to normal of the expressions of BMPR-2 and BMPR-1A. All these effects were more marked than previously reported with sildenafil alone in the same PAH model (25).

The expression of angiopoietin-1 was persistently increased in the present experiments, along with downregulated gene expressions of BMPR-2 and BMPR-1A. Angiopoietin-1 has been shown to be overexpressed in pulmonary vascular smooth muscle cells together with a phosphorylation of the endothelial tyrosine kinase receptor Tie-2 in patients with various forms of severe pulmonary hypertension (11). Rats engineered to overexpress angiopoietin-1 present with a marked medial hypertrophy that appears to be related to an endothelial release of serotonin (28). More recent studies on lung tissue and smooth muscle and endothelial cells of idiopathic PAH patients could not confirm an overexpression of angiopoietin-1 but identified an endothelial overexpression of Tie-2 that mediated serotonin and endothelin-induced medial hypertrophy and further activation of angiopoietin signaling (9). Thus angiopoietin-1 appeared involved in a pro-remodeling cross talk between smooth muscle and endothelial cells.

Angiopoietin-1 has also been reported to shut off the expression of BMPR-1A, a receptor required for BMPR-2 signaling, in patients with severe pulmonary hypertension (11). Whether this explains the decreased expressions in both BMPR-1A and BMPR-2 in the present experiments is unclear. Mutations of BMPR-2 have been reported in the majority of patients with familial forms of PAH and in \( \sim 10–20\% \) of patients with sporadic idiopathic PAH and represents the single highest risk factor for the disease (17). The expression of BMPR-2 is markedly decreased in patients with familial PAH but also, although to a lesser extent, in patients with idiopathic PAH and no identified mutations (1). The expression of BMPR-2 has been found to be decreased in experimental hypoxia- or monocrotaline-induced pulmonary hypertension (21, 29). Patients with PAH and BMPR-2 mutations more than those without mutations have a decreased Smad signaling and increased p38MAPK signaling, promoting a proliferation of pulmonary vascular smooth muscle cells (10, 32). What causes the decreased expressions of BMPR-2 and BMPR-1A remains uncertain but may be related to increased angiopoietin-1 or other angiogenic and growth factors. Our results suggest that disturbed angiopoietin-1 and BMPR-2 signaling pathways appear to be involved in the pathogenesis of shunt-induced PAH.

Treatments proved effective until now in PAH: all have aimed at the restoration of the endothelial disequilibrium in release of vasodilator/anti-proliferative and vasoconstrictive/pro-proliferative mediators (1). However, the clinical results of these therapies remain incompletely satisfactory. The combinations of therapies targeting different pathways are currently
considered. There is experimental evidence that this strategy might work. In monocrotaline-induced pulmonary hypertension in rats, bosentan combined with sildenafil showed additional effects by decreasing pulmonary artery pressures, maintaining body weight and improving mortality (7). In the same model, the prostacyclin analog beraprost combined with sildenafil was also more effective than either agent alone (15). However, the effects of combinations have not been reported in other models. In patients with PAH, randomized controlled trials have reported a moderate benefit from the addition of inhaled iloprost to bosentan (19), or sildenafil added to intravenous epoprostenol (27). Further improvement might be expected with an earlier or initial institution of combined therapies. The present results offer additional rationale for randomized controlled strategies to test this more aggressive approach.

In the present study, the RV remained optimally coupled to the pulmonary circulation, as shown by $E_{ac}/E_{es}$ maintained around 1.5 in shunted animals with ERA or PDE-5i therapies. Recent studies have shown the importance of RV systolic adaptation to afterload in PAH patients (15). It has been realized that much of pulmonary hypertension symptomatology and prognosis is explained by RV failure in the presence of afterload, in relation probably with disturbed myocardial hypertrophic or apoptotic pathways, that might possibly be affected by therapies targeting the pulmonary circulation (3, 6). In the conditions of the present experiments, the combination of sitaxsentan to sildenafil had no detectable effect on RV arterial coupling. This confirms a previous report of the absence of direct myocardial effects of either drug alone in experimental shunt-induced PAH (25, 26). However, the present results do not exclude the possible effects in case of failure or more advanced hypertrophy of the overloaded RV in advanced stages of the disease. The inhibition of PDE-5 has been reported to increase contractility in the hypertrophied RV of PAH patients (22), whereas it decreased β-adrenergic-stimulated cardiac contractility in normal subjects (4). Furthermore, sildenafil has been reported to improve RV hypertrophy in relation to an amelioration of nitric oxide synthase coupling, a decrease in reactive oxygen species signaling, and a limitation of RhoA/Rho kinase in a murine model of pulmonary fibrosis associated with intratracchael bleomycin (14).

The limitations of the present study include the absence of an additional arm of sildenafil alone. However, this was thought not to be necessary in view of the previously reported effects of sildenafil alone in the same shunt-induced PAH model, which were actually comparable with those of sitaxsentan alone. Therefore, the repetition of monotherapy observations was limited to sitaxsentan. Another limitation is in the absence of biological studies on the RV myocardium. This was thought to be of secondary interest in the presence of preserved RV arterial coupling in all circumstances. A final limitation is that the prevention of shunt-induced PAH is already marked with either sitaxsentan or sildenafil alone, making it difficult to demonstrate the improved efficacy of combination therapy. However, it was still possible to demonstrate the added value of combination therapy on hemodynamics, remodeling, and BMPR expressions.

In conclusion, chronic systemic-to-pulmonary shunt-induced PAH is more effectively, though still incompletely, prevented by a combined administration of sildenafil and sitaxsentan, compared with sitaxsentan alone. A persistent trend to an increased arteriolar remodeling appears related to the incomplete inhibition of angiotensin-I and BMPR expressions.

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DISCLOSURES

R. Naeije has received research grants from Actelion and Pfizer and has received speaker fees and reimbursements of travel expenses from Pfizer, Actelion, Glaxo, Mondobiotech, United Therapeutics, and LungRX.

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