Signaling Molecules in Overcirculation-Induced Pulmonary Hypertension in Piglets
Effects of Sildenafil Therapy

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Background—The phosphodiesterase type-5 (PDE-5) inhibitor sildenafil has been reported to improve pulmonary arterial hypertension (PAH), but the mechanisms that account for this effect are incompletely understood. Severe pulmonary hypertension has been characterized by defects in a signaling pathway involving angiopoietin-1 and the bone morphogenetic receptor-2 (BMPR-2). We investigated the effects of sildenafil on hemodynamics and signaling molecules in a piglet overcirculation-induced model of early PAH.

Methods and Results—Thirty 3-week-old piglets were randomized to placebo or sildenafil therapy 0.75 mg/kg TID after anastomosis of the left subclavian artery to the pulmonary arterial trunk or after a sham operation. Three months later, the animals underwent a hemodynamic evaluation followed by pulmonary tissue sampling for morphometry, immunohistochemistry or radioimmunoassay, and real-time quantitative-polymerase chain reaction. Chronic systemic-to-pulmonary shunting increased pulmonary mRNA for angiopoietin-1, endothelin-1 (ET-1), angiotensin II, inducible nitric oxide synthase, vascular endothelial growth factor, and PDE-5. Pulmonary messenger RNA for BMPR-1A and BMPR-2 decreased. Pulmonary angiotensin II, ET-1, and vascular endothelial growth factor proteins increased. Pulmonary artery pressure increased from 20/110 to 33/110 mm Hg, and arteriolar medial thickness increased by 91%. The expressions of angiopoietin-1, ET-1, and angiotensin II were tightly correlated to pulmonary hypertension. Sildenafil prevented the increase in pulmonary artery pressure, limited the increase in medial thickness to 41%, and corrected associated biological perturbations except for the angiopoietin-1/BMPR-2 pathway, PDE-5, and angiotensin II.

Conclusions—Sildenafil partially prevents overcirculation-induced PAH and associated changes in signaling molecules. Angiotensin II, PDE-5, and angiopoietin-1/BMPR-2 signaling may play a dominant role in the early stages of the disease. (Circulation. 2004;110:2220-2225.)

Key Words: angiopoietins ■ heart defects ■ nitric oxide ■ remodeling ■ hypertension, pulmonary

Pulmonary arterial hypertension (PAH) is a rare, incurable disease with a poor prognosis.1 The pathobiology of PAH remains incompletely understood. A series of signaling molecules abnormalities have been described in all compartments of the pulmonary arterial wall.2 Mutations in the gene encoding the bone morphogenetic protein receptor-2 (BMPR-2) have been identified in ~60% of patients with familial PAH3,4 and in a minority of patients with sporadic PAH.5 A possible link between acquired and familial pulmonary hypertension has been suggested by a recent study that showed a strong upregulation of angiopoietin-1 in the lungs of patients with various forms of severe pulmonary hypertension.6 Angiopoietin-1, a protein involved in the recruitment of smooth muscle cells around blood vessels, shuts off the expression of BMPR-1A, a transmembrane protein required for BMPR-2 signaling.6 Long-term therapy with the phosphodiesterase type-5 (PDE-5) inhibitor sildenafil may improve patients with PAH7 and patients with inoperable chronic thromboembolic pulmonary hypertension.8 Sildenafil therapy inhibits pulmonary hypertension secondary to long-term hypoxic exposure in rats.9 Sildenafil is believed to act through a PDE-5 inhibition–related increase in pulmonary artery smooth muscle cyclic guanosine monophosphate (cGMP).7-9 How this action relates to the abnormal signaling molecules that are identified in chronically remodeled resistive arterioles in severe pulmonary hypertension remains unclear.

We investigated the effects of long-term sildenafil therapy in overcirculation-induced PAH in growing piglets, which we showed previously to be characterized by small-arteriole medial hypertrophy and increased pulmonary expressions of...
endothelin-1 (ET-1), inducible nitric oxide synthase (iNOS), and vascular endothelial growth factor (VEGF), all of which were prevented by long-term administration of the nonselective endothelin receptor antagonist bosentan. Because lambs with overcirculation-induced pulmonary hypertension present with an increased expression of PDE-5 and angiotensin II might exert angiogenic or antiangiogenic effects, depending on the associated upregulation of its AT-1 or AT-2 receptors, we specifically addressed the questions of whether angioptoein-1/BMPR signaling is abnormal in this early PAH model and how this is related not only to the ET-1, iNOS, and VEGF systems, but also to the expressions of PDE-5 and of angiotensin II.

Methods
Thirty piglets 18 ± 1 days old and weighing 5.8 ± 0.2 kg were included in the present study, which was approved by the institutional committee on animal welfare. The animals were randomized to the operation (n = 10) or to an anastomosis between the left innominate artery and the pulmonary arterial trunk (adapted Blalock-Taussig procedure), followed by random treatment with sildenafil 0.75 mg/kg (n = 10) or placebo (n = 10) administered orally with food 3 times/day for 3 months. The surgical procedure and postoperative care were performed as previously reported. Sildenafil was a gift from Pfizer, Inc. (Cambridge, Mass).

Hemodynamic Evaluation
After 90 ± 1 days, the animals were anesthetized, ventilated, and equipped with catheters and an ultrasonic flow probe on the pulmonary arterial trunk for measurements of heart rate (HR), mean pulmonary artery pressure (Ppa), balloon-occluded Ppa (PpaO), systemic arterial pressure (Psa), thermodilution cardiac output (Q), ultrasonic instantaneous pulmonary arterial flow, and blood gases, as previously described. Pulmonary vascular resistance (PVR) was partitioned into an arterial component (PVRa) and a venous component (PVRv) from the analysis of the Ppa decay curve after inflating the balloon of the pulmonary artery catheter. PVR was defined by multipoint Ppa/Q plots obtained by rapid inflation of the inferior vena cava balloon.

Hemodynamic and blood gases measurements were obtained after ensuring steady-state conditions (stable HR, Psa, and Ppa) for 60 min, after shunt closure in the shunted animals. After the measurements, the animals were killed with an anesthetic overdose and pulmonary tissue samples were immediately harvested and snap frozen in liquid nitrogen and stored at −80°C for radioimmunoassay (RIA) and real-time quantitative-polymerase chain reaction (RTQ-PCR) measurements or, after overnight fixation, embedded in paraffin for morphometric and immunohistochemistry evaluations.

Morphometry
Pulmonary arterial morphometry was performed as reported previously. 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, 76 to 150, 151 to 225, 226 to 300, and 300 to 500 μm. Medial thickness (MT) was related to arterial size with the following formula: %MT = 2 × MT/ED × 100.

Radioimmunoassay
Systematic arterial plasma ET-1 and angiotensin II were measured by RIA after extraction as previously described with commercially available antibodies and standard (ET-1 RAS 6901 and 6901, angiotensin II RAS-7002, both 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.

Pulmonary angiotensin II content also was measured by RIA. Lung tissue samples, 400 mg, were pulverized in liquid nitrogen and transferred at 4°C in 4 mL of 1 mol/L acetic acid–20 mmol/L HCl–9 mmol/L benzamidine. Samples were homogenized at high speed with the IKA ULTRA-TURRAX T-25 dispenser and incubated for 10 min at 4°C, and then centrifuged at 27,000 g at 4°C. The supernatant was processed following the same procedure as that for plasma angiotensin II RIA.

RTQ-PCR
Pulmonary tissue messenger RNA (mRNA) levels were measured by SYBR Green RTQ-PCR as previously described. Primers for the report gene HPRT and ET-1, ETα, and ETβ receptors; ET-1 converting enzyme (ECE-1); neuronal NOS (nNOS); iNOS; endothelial NOS (eNOS); VEGF and its receptor flk-1; and tenasin-C have been used in our laboratory. To develop specific porcine primers adapted to SYBR Green RTQ-PCR conditions, we engaged already reported primers for angioptoein-1 and AT-1 and AT-2 receptors in end point PCR (Perkin Elmer GeneAmp PCR system 2400) to amplify complementary DNA from pig tissue. The PCR product was purified and sequenced using the Big Dye protocol (4303149, Applied Biosystems). These sequences and the already reported sequences for BMPR-1A (GenBank S75359), BMPR-2 (GenBank Z48923), and PDE-5 (GenBank AY266366) were used to design, on Primer Express software (Applied Biosystems), porcine-specific primers adapted to RTQ-PCR conditions. Primers for angiotensin II were already reported. The primers (Table 1) 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. To ensure the quality of the measurements, we systematically included both negative and positive controls in duplicate in each plate. The statistical analysis of the RTQ-PCR results was completed with the ΔCt value (Ct sample − Ct internal standard). Relative gene expression was obtained by ΔΔCt methods (ΔCt sample − ΔCt internal standard), with the sham group as a calibrator, to compare every unknown-sample gene expression level. The conversion between ΔΔCt and relative gene expression levels is fold induction = 2ΔΔCt.

| TABLE 1. Primers Used for RTQ-PCR in Porcine Pulmonary Tissue |
|-------------------|-------------------|
| Genes       | Primer Sequences |
| Angiotensin-1 |                 |
| Sense        | 5′-TGC-TTT-GTG-GCA-ATA-GTA-TGC-3′ |
| Antisense    | 5′-GGA-ATT-CAC-CCA-CAC-AGA-AAC-3′ |
| BMPR-1A      |                 |
| Sense        | 5′-GCA-CCC-GTG-TTA-AGT-TGC-3′ |
| Antisense    | 5′-GGT-GCA-GGC-TTT-CCT-TGA-3′ |
| BMPR-2       |                 |
| Sense        | 5′-GCT-CTG-GCC-GTC-TTG-GTC-CT-3′ |
| Antisense    | 5′-ATC-TGC-AGG-GAA-AAT-TGC-3′ |
| PDE-5        |                 |
| Sense        | 5′-AGA-GGT-TGG-TGG-TGG-GCA-3′ |
| Antisense    | 5′-AAA-ATG-CAC-AAT-AAC-AGC-CAC-3′ |
| Angiotensin II |                |
| Sense        | 5′-CTG-CAG-GTA-TCT-TAT-GAC-3′ |
| Antisense    | 5′-TAC-ACA-GCA-AAC-AGG-AAT-3′ |
| AT-1         |                 |
| Sense        | 5′-CAG-TGC-TAT-GTA-ATA-CCG-CT-3′ |
| Antisense    | 5′-AGG-CAC-GTA-AAG-ATC-GAT-3′ |
| AT-2         |                 |
| Sense        | 5′-GCC-TTT-CCC-ACC-TGA-GAA-3′ |
| Antisense    | 5′-ATC-TTC-AGG-ATC-TGG-TCA-3′ |
TABLE 2. Hemodynamic Effects of Preventive Sildenafil Treatment in Overcirculation-Induced Experimental Pulmonary Arterial Hypertension in Piglets

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=10)</th>
<th>Placebo (n=10)</th>
<th>Sildenafil (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, bpm</td>
<td>117±4</td>
<td>121±6</td>
<td>114±4</td>
</tr>
<tr>
<td>Q, L·min⁻¹·m⁻²</td>
<td>3.4±0.1</td>
<td>3.1±0.1</td>
<td>3.6±0.2</td>
</tr>
<tr>
<td>Psa, mm Hg</td>
<td>131±3</td>
<td>122±6</td>
<td>130±3</td>
</tr>
<tr>
<td>Ppao, mm Hg</td>
<td>21±1*</td>
<td>33±1†</td>
<td>20±2</td>
</tr>
<tr>
<td>PVR, mm Hg·L⁻¹·min⁻¹·m⁻²</td>
<td>4.0±0.3*</td>
<td>7.3±0.5†</td>
<td>4.2±0.6</td>
</tr>
<tr>
<td>PVRa, %</td>
<td>65±2</td>
<td>63±2</td>
<td>68±4</td>
</tr>
</tbody>
</table>

Values expressed as mean±SEM. *P<0.05 sham vs placebo, †P<0.05 placebo vs sildenafil.

Immunohistochemistry

The immunohistochemistry analysis was performed as reported by Aguirre et al.²⁰ with rabbit monoclonal antibody to ET-1 (1/100 dilution) prepared in our laboratory and commercial rabbit polyclonal antibodies against VEGF (A-20 [sc-152], 1/100 dilution, Santa Cruz Biotechnology), nNOS (cat. No. 610310, 1/50 dilution, BD Transduction Laboratories), and iNOS (cat. No. 160862, 1/100 dilution, Cayman Chemical Company), and mouse monoclonal antibody against eNOS (NCL-NOS-3, 1/80 dilution, Novoceastra). Quantitative immunohistochemical assessments were performed as previously reported.²⁰ A mean optical density, which relates to immunohistochemical staining intensity, was calculated for 20 areas previously reported.²⁰ A mean optical density, which relates to immunohistochemical staining by the area of tissue covered by this staining.

Statistical Analysis

Values are reported as mean±SEM. 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⁻².²⁰ The 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, Scheffé post hoc tests were performed to compare specific situations.²¹ Correlations between Ppa and mRNA or protein contents of the investigated signaling molecules were calculated via a linear regression analysis.²¹

Results

Two of the shunted piglets randomized to sildenafil therapy died from acute postoperative heart failure. Weight gain averaged 40 kg and was not different in the 3 study groups. Arterial blood gases and hematocrits were normal and were not different in the 3 study groups. The ratio of pulmonary to systemic flow before closure of the shunt was 1.6±0.1 in the placebo group and 1.7±0.1 in the sildenafil group.

Chronic systemic-to-pulmonary shunting increased Ppa and PVR without change in HR, Q, Psa, and partition of PVR (Table 2). Pulmonary arterial medial thickness increased, and this effect was most pronounced in the smallest arterioles (Figure 1). Ppa/Q relationships were shifted to higher pressures (Figure 1). Plasma ET-1 increased from 1.9±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 angiotensin II (sham 25.2±5.2 pg/mL, placebo 24.4±5.6 pg/mL). Sildenafil therapy prevented the increases in Ppa and PVR (Table 2) and the shift of Ppa/Q plots to higher pressures, but it did not completely prevent the shunt-induced increase in pulmonary arterial medial thickness (Figure 1). Sildenafil was associated with a decrease in plasma ET-1 to 1.7±0.2 pg/mL, P<0.05 versus placebo group, without changes in circulating angiotensin II (sildenafil 22.1±0.9 pg/mL). Medial thickness was correlated to Ppa at all external diameters, but more significantly so for the smallest pulmonary arterioles (Figure 1).

Figure 1. Morphometry (×400) on pulmonary arterioles expressed as % MT vs ED, composite plots of mean Ppa vs Q, and correlations between Ppa and MT of smallest and largest arterioles in sham-operated controls and placebo- or sildenafil-treated shunted piglets. Sildenafil completely prevented shift of Ppa/Q plots to higher pressures and partially prevented medial hypertrophy. MT correlated to Ppa as all vessel diameters. Values expressed as mean±SEM. *P<0.05 sham vs placebo, †P<0.05 placebo vs sildenafil, ‡P<0.05 sham vs sildenafil.
As illustrated in Figure 2, systemic-to-pulmonary shunting increased gene expressions for angiopoietin-1, iNOS, PDE-5, angiotensin II (Ag-II); AT-1 and AT-2; ET-1, ETa, and ETb; ECE-1; VEGF and flk-1; and tenascin-C (TNc) of sham-, placebo-, and sildenafil-treated piglets; and correlation between lung mRNA content of most determinant pathological factors and Ppa. Values expressed as mean±SEM. P values as in Figure 1.

As illustrated in Figure 3, chronic left-to-right shunting increased pulmonary endothelial immunostaining for both ET-1 and VEGF without changes in nNOS, iNOS, and eNOS, and increased the whole lung homogenate protein content for angiotensin II. Sildenafil therapy was associated with a decrease in gene expressions for Ang-1/BMPR-1A and -2, nNOS, iNOS, and eNOS; PDE-5; angiotensin II (Ag-II); AT-1 and AT-2; ET-1, ETa, and ETb; ECE-1; VEGF and flk-1; and tenascin-C (TNc) of sham-, placebo-, and sildenafil-treated piglets; and correlation between lung mRNA content of most determinant pathological factors and Ppa. Values expressed as mean±SEM. P values as in Figure 1.

Discussion

The novel findings of the present study are that overcirculation-induced pulmonary hypertension in piglets, as a model of early PAH, is characterized by abnormal angiopoietin-1/BMPR-2 signaling and overexpressions of PDE-5 and angiotensin II, all of which are only partially prevented by long-term sildenafil therapy.

PAH is a recognized complication of congenital heart disease with right-to-left shunting. This type of PAH can be reproduced experimentally by chronic aorta-pulmonary shunting in growing pigs. In the present study, the model was improved by anastomosing the innominate artery to the pulmonary arterial trunk (adapted Blalock-Taussig procedure), allowing shunt flow to increase with the growth of the animals. This strategy allowed for the induction of moderate to severe pulmonary hypertension, with medial hypertrophy, an increase in PVR, and a mean Ppa between 30 and 40 mm Hg, as seen in early symptomatic PAH. Mean Ppa was closely correlated to arteriolar medial thickness, and more so with the smallest arterioles. This observation, together with...
unchanged partitioning of PVR, is in keeping with the notion that PAH is initiated at the smallest resistive arterioles. Medial hypertrophy is typical of early still-reversible congenital cardiac shunt-associated PAH.\textsuperscript{25}

Shunting-induced PAH was associated with an overexpression of angiopoietin-1, which was correlated to increased PVR, as it was in patients with severe pulmonary hypertension.\textsuperscript{6} Angiopoietin-1 is an angiogenic factor that has been shown to induce severe pulmonary hypertension and medial hypertrophy in rodents.\textsuperscript{26} Angiopoietin-1 shuts off the expression of BMPR-1A, a transmembrane protein required for BMPR-2 signaling.\textsuperscript{6} In our piglets, the expressions of both BMPR-1A and BMPR-2 decreased. Although we have no explanation for decreased BMPR-2 expression, this finding is in keeping with the notion that abnormal angiopoietin-1/BMPR-2 signaling is central to the development of pulmonary hypertension, whether decreased BMPR-2 function is caused by mutations\textsuperscript{3,4} or increased angiopoietin-1.\textsuperscript{6}

The present results confirm that overcirculation-induced PAH is associated with overexpression of ET-1, ECE-1, ET\textsubscript{B}, iNOS, and VEGF, with no change in the expressions of ET\textsubscript{A}, nNOS, eNOS, and flk-1.\textsuperscript{10} Tenascin-C was slightly overexpressed in the present series. Correlations between Ppa and mRNA and protein contents, respectively, are in keeping with ET-1, VEGF, and NOS playing a role in early PAH arteriolar remodeling.\textsuperscript{10} In the present study, overcirculation-induced PAH was associated with an increased expression of PDE-5, which also was correlated to Ppa, though not tightly. This observation is in keeping with the previous report that increased PDE-5 expression accounted for a decrease in vasodilating cGMP in hypoxic pulmonary hypertension\textsuperscript{9} and the impairment of endothelium-dependent pulmonary vasodilation in spite of an increase in soluble guanylate cyclase in lambs with overcirculation-induced pulmonary hypertension.\textsuperscript{11}

Overexpression of angiotensin II and both its AT-1 and AT-2 receptors occurred together with an increase in angiotensin II protein, all correlated to Ppa. This finding agrees with the report of increased endothelial immunoreactivity for the angiotensin-converting enzyme in the lungs of patients with either primary or secondary pulmonary hypertension.\textsuperscript{27}
Angiotensin II is a vasoconstricting and mitogenic mediator, which has been shown to contribute to the medial thickening associated with hypoxic pulmonary hypertension in rats. These effects are attributable to the activation of AT-1, and angiotensin II appears to exert AT-2-mediated angiogenic effects either through an activation of apoptosis or through the upregulation of angiopoietin-2, which may interfere with the effects of angiotensin-I at its soluble endothelial receptor TIE-2. Angiotensin II may also be involved in the increased expression of VEGF. Because in the present experiments expression increased in both AT-1 and AT-2, our data are unclear whether angiotensin II contributed or limited pulmonary hypertension.

Sildenafil in the present study completely prevented the increase in PVR and partially prevented the increase in medial thickness, as previously reported about preventive bosentan therapy. It is of interest that the expression of angiotensin-I/BMPR-2 signaling only partly reverted to normal, whereas the expressions of the endothelin and angiotensin II systems, iNOS, and VEGF normalized. PDE-5 also remained overexpressed, whereas the expressions of eNOS and tenascin-C decreased to lower than normal. In addition, sildenafil decreased iNOS, eNOS, and nNOS and ET-1 and VEGF proteins to below baseline, whereas angiotensin II protein remained increased. These findings contrast with persistent overexpressions of ET-1, ETB, ECE-1, iNOS, and VEGF proteins to below baseline, whereas angiotensin II protein remained increased. These findings contrast with persistent overexpressions of ET-1, ETB, ECE-1, iNOS, and VEGF proteins to below baseline, whereas angiotensin II protein remained increased.

In conclusion, overexpressions of PDE-5 and angiotensin II and abnormal angiotensin-I/BMPR-2 signaling appear to play a dominant role in the early stages of PAH.

Acknowledgments

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References

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