Bosentan for the Prevention of Overcirculation-Induced Experimental Pulmonary Arterial Hypertension

Benoît Rondelet, MD; François Kerbaul, MD; Sophie Motte, DVM; Ronald van Beneden, MSc; Myriam Remmelink, MD, PhD; Serge Brimioulle, MD, PhD; Kathleen McEntee, DVM, PhD; Pierre Wauthy, MD; Isabelle Salmon, MD, PhD; Jean-Marie Ketelslegers, MD, PhD; Robert Naeije, MD, PhD

Background—The dual endothelin-receptor antagonist bosentan has been reported to improve pulmonary arterial hypertension, but the role of endothelins in the pathogenesis of the condition remains uncertain. We investigated the roles of endothelin-1 (ET-1), nitric oxide (NO), vascular endothelial growth factor (VEGF), and tenascin in overcirculation-induced pulmonary hypertension in piglets, as a model of early pulmonary arterial hypertension, with or without bosentan therapy.

Methods and Results—Thirty 3-week-old piglets were randomized to placebo or to bosentan 15 mg/kg BID after the 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 cardiac and pulmonary tissue sampling for morphometry, immunohistochemistry, and real-time quantitative PCR. Chronic systemic-to-pulmonary shunting increased circulating plasma ET-1, pulmonary mRNA for ET-1, ETB receptor, inducible NO synthase, VEGF, and pulmonary ET-1 and VEGF proteins. There were increases in myocardial mRNA for ETA receptor and VEGF and in myocardial VEGF protein. Pulmonary and myocardial tissue mRNA for tenascin did not change. Normalized-flow pulmonary artery pressure increased from 20 (2) to 33 (1) mm Hg [mean (SEM)], arteriolar medial thickness increased on average by 83%, and these changes were completely prevented by bosentan therapy. Right ventricular end-systolic elastance increased in proportion to pulmonary arterial elastance with or without bosentan.

Conclusions—Experimental overcirculation-induced pulmonary arterial hypertension appears to be causally related to an activation of the pulmonary ET-1 system and as such is completely prevented by the dual endothelin receptor antagonist bosentan. (Circulation. 2003;107:1329-1335.)

Key Words: endothelin ■ nitric oxide ■ growth substances ■ hypertension, pulmonary

The pathogenesis of primary pulmonary hypertension (PPH) remains incompletely understood. Biological abnormalities have been identified in all 3 layers of pulmonary arteriolar walls, but which one plays a dominant role in the initiation of the disease is not currently known.1 A possible candidate is the endothelin system. Endothelin (ET)-1 is a potent vasoconstrictor and mitogen.2 Patients with PPH present with increased circulating ET-1 and increased lung tissue expression of the endothelin system3,4 and are improved by the endothelin receptor antagonist bosentan.5

Progress in pathophysiological understanding of PPH, or pulmonary arterial hypertension (PAH) defined as PPH with identifiable associated conditions,6 has been limited until now by the absence of a satisfactory experimental animal model. Rendas et al7 reported on experimental PAH induced by only several weeks of aortopulmonary shunting through an implanted prothetic fistula in growing piglets. However, in this model, the increase in pulmonary artery pressures after shunt closure appeared disappointingly moderate, leaving doubt about its clinical relevance.8 In the present study, we improved the chronic overcirculation-induced PAH model in growing piglets by anastomosing the left subclavian artery to the pulmonary arterial trunk. We surmised that this more natural systemic-to-pulmonary shunting would increase with the animal’s growth, leading to faster and more severe pulmonary hypertension.9 In this improved PAH model, we explored the endothelin system and potentially associated nitric oxide (NO), vascular endothelial growth factor (VEGF), and tenascin pathways by real-time quantitative PCR (RTQ-PCR) and immunohistochemistry and investigated the preventive effects of endothelin receptor blockade. The results are suggestive of activation of the endothelin system as playing a major role in initiating PAH.
Methods
Thirty piglets (Van Gucht, Liedekerke, Belgium) 20±1 days old and weighing 5.9±0.2 kg were included in the present study, which was approved by the institutional Committee on Animal Welfare. The animals were randomized to a sham operation (n=10) or to an anastomosis between the left subclavian artery and the pulmonary arterial trunk followed by random institution of treatment with bosentan 15 mg/kg (n=10) or placebo (n=10) administrated orally with food twice a day for 3 months.

Surgical Procedure
After prophylactic cephaline, premedication with ketamine, midazolam, and atropine, anesthesia with remifentanil and midazolam, paralysis with pancuronium, intubation, and ventilation as previously described except for the replacement of fentanyl by remifentanil 75 μg·kg⁻¹·h⁻¹, a thoracotomy was performed through the left third intercostal space. The left innominate artery was dissected and anastomosed to the main pulmonary trunk according to the classic Blalock-Taussig procedure (Wauthy et al, unpublished observations). Patency of the shunt was checked by the palpation of a thrill. The anastomosis was ligated in the 10 animals that served as sham-operated controls. After careful hemostasis, the chest was tightly closed and pleural air evacuated; anesthesia and paralysis were interrupted, and the animals were weaned from mechanical ventilation. Postoperative analgesia was provided with morphine and paracetamol as required for 72 hours. The day after the procedure, the shunted animals were randomized to bosentan or placebo. In all the shunted animals, daily clinical examination showed a precordial murmur until the end of the 90-day observation period.

Hemodynamic Evaluation
After a 90±1-day observation period, the animals were anesthetized, ventilated, and equipped with catheters and an ultrasonic flow probe on the pulmonary arterial trunk as previously described, with in addition a 5F high-fidelity manometer-tipped catheter (SPC 350, Millar) in the right ventricle. Heart rate, mean pulmonary artery pressure (Ppa), occluded Ppa, systemic arterial pressure, thermodilution cardiac output (Q), ultrasonic instantaneous pulmonary arterial flow, and blood gases were measured as previously reported. An occlusion pressure to partition pulmonary vascular resistance (PVR) into an arterial component and a capillary-venous component was computed by integration of the instantaneous pulmonary arterial occlusion pressure to partition pulmonary vascular resistance (PVR) into an arterial component and a capillary-venous component was computed from a double exponential fitting of the Ppa decay curve after inflation of 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.

The decrease of right ventricular volume during systole was computed by 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 Sunagawa et al., adapted to the right ventricle (Brimioulle et al, unpublished observations), to compute end-systolic elastance (Ees) and arterial elastance (Ea).

Hemodynamic and blood gas measurements were obtained after ensuring steady-state conditions (stable heart rate, systemic arterial pressure, and Ppa) for 60 minutes, after shunt closure in the shunted animals, daily clinical examination showed a precordial murmur until the end of the 90-day observation period. After careful hemostasis, the chest was tightly closed and pleural air evacuated; anesthesia and paralysis were interrupted, and the animals were weaned from mechanical ventilation. Postoperative analgesia was provided with morphine and paracetamol as required for 72 hours. The day after the procedure, the shunted animals were randomized to bosentan or placebo. In all the shunted animals, daily clinical examination showed a precordial murmur until the end of the 90-day observation period.

Radioimmunoassay
Systemic arterial plasma ET-1 was measured by radioimmunoassay after extraction as previously described, using commercially avail-able antibodies and tracers iodinated and HPLC-purified in our laboratory.

Real-Time Quantification PCR
Total RNA was prepared from snap-frozen tissue samples (400 mg) using TRIzol (Gibco Life Technologies). RNA was quantified by absorbance at A=260 nm, and its concentration was adjusted to 0.25 μg/μL. Reverse transcription was performed with the GeneAmp PCR system 2400 (Perkin Elmer) with 1 μg of total RNA in a reaction volume of 20 μL containing 7.5 μmol/L random hexamers, reverse transcription buffer 1×, 9 mmol/L dithiothreitol, 220 μmol/L of each dNTP, 20 U of ribonuclease inhibitor (Applied Biosystems), and 50 U of reverse transcriptase (Superscript, Gibco BRL). Final reverse transcription product was adjusted to 40 μL with RNase-free water. Primers for ET-1 and the report gene, HPRT, were already used in our laboratory. Except for endothelin converting enzyme (ECE)-1 and inductive NO synthase (iNOS), the previously reported end-point PCR primers were unadapted to the Sybr Green RTQ-PCR system. To develop specific porcine primers adapted to Sybr Green RTQ-PCR conditions, we engaged classic primers for ETα, ETβ, neuronal NO synthase (nNOS), endothelial NO synthase (eNOS), and VEGF in end-point PCR (GeneAmp PCR system 2400) to amplify cDNA from pig tissue. PCR product was purified and sequenced using the Big Dye protocol (4303149, Applied Biosystems). These sequences and the already reported sequences for VEGF receptor flk-1 (GenBank AJ245446) and tenascin (GenBank X61599) were used to design, on Primer Express software (Applied Biosystems), porcine specific primers adapted to RTQ-PCR conditions. 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 GeneAmp 5700 (Applied Biosystems). RTQ-PCR was performed using the following cycle parameters: 10 minutes at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 minute at 60°C. For each gene, RTQ-PCR was conducted in duplicate with 25 μL reaction volume of 5 ng of cDNA, 2.5 μL Sybr Green buffer, 250 μmol/L dNTP, 3 mmol/L MgCl₂, 400 nmol/L of each primer, and 0.625 U amplification Taq Gold polymerase (Applied Biosystems). To ensure the quality of the measurements, both negative and positive controls were systematically included in duplicate in each plate. The statistical analysis of the RTQ-PCR results was done using the ΔΔCt method (2−ΔΔCt) using the sham group as a calibrator for comparison of every unknown-sample gene expression level. The conversion between ΔΔCt and relative gene expression levels is Fold induction = 2−ΔΔCt.

Immunohistochemistry
The immunohistochemistry analysis was performed on lung and right ventricular myocardial tissue as reported by Aguie et al., 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 (BD610310, 1/50 dilution; Transduction Laboratories), and iNOS (160862, 1/100 dilution; Cayman Chemical Co) and mouse monoclonal antibody against eNOS (NCL-NOS3, 1/80 dilution; Novacasta). Quantitative immunohistochemical assessments were performed as previously reported. A mean optical density, which relates to immunohistochemical staining intensity, was calculated for 20 areas of 4248 μm² for right ventricular tissue and in the endothelium 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 mean±SEM. Multipoint pressure-flow relations were submitted to linear regression analysis, and standardized pressure values were calculated from individual regressions at Q of 2 and 5 L·min⁻¹·m⁻². Effects of shunt and drugs were analyzed.
by a repeated-measures ANOVA. When the F ratio of the ANOVA
reached a critical value of \( P < 0.05 \), Scheffé post hoc tests were
performed to compare specific situations.\textsuperscript{19}

**Results**

One of the sham-operated piglets died of a mediastinitis, and
one of the shunted piglets randomized to bosentan therapy
died of 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 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 bosentan group.

Chronic systemic-to-pulmonary shunting increased Ppa, occluded Ppa, Ees, and Ea, with no change in heart rate, Q, partition of PVR, or the ratio of Ees to Ea (Table 2). There was an increase in pulmonary arterial medial thickness, and this effect was most pronounced in the smallest arterioles (Figure 1). Ppa/Q relationships were shifted to higher pressures (Figure 2). Plasma ET-1 increased from 2.0±0.1 pg/mL in the sham-operated controls to 2.4±0.1 pg/mL, \( P < 0.05 \).

Bosentan therapy prevented the increases in Ppa, occluded Ppa, and Ea but not the increases in Ees (Table 2), completely prevented the increase in pulmonary arterial medial thickness (Figure 1) and the shift of Ppa/Q plots (Figure 2), and was associated with a further increase in plasma ET-1 to 4.6±0.5 pg/mL, \( P < 0.05 \) versus placebo group.

As illustrated in Figure 3, shunting increased whole-lung homogenate gene expression for ET-1, ET\(_B\), iNOS, and VEGF without changes in gene expression for ET\(_A\), ECE-1, nNOS, eNOS, flk-1, and tenascin and increased pulmonary endothelial immunostaining for both ET-1 and VEGF without changes in nNOS, iNOS, and eNOS. Bosentan therapy was associated with additional increases in ECE-1 and eNOS gene expression.

**Figure 1.** Morphometry (×400) on pulmonary arterioles of sham-, bosentan-, and placebo-treated piglets and plot of % medial thickness (%MT) vs ED. Bosentan completely prevented shunt-induced increase in medial thickness, and this effect was most pronounced in smallest arterioles. Values are expressed as mean±SEM. *\( P < 0.05 \) sham vs placebo. †\( P < 0.05 \) placebo vs bosentan.
As illustrated in Figure 4, shunting increased right ventricular myocardial gene expression for ET₁ and VEGF without changes in gene expression for ET-A, ET-B, ECE-1, nNOS, iNOS, eNOS, flk-1, and tenascin and increased right ventricular myocardial immunostaining for VEGF without changes in ET-1, nNOS, INOS, or eNOS. Bosentan therapy was associated with an additional increase in eNOS expression and immunostaining for iNOS.

Discussion

The present study is the first to show a dominant role of the endothelin system in early overcirculation-induced PAH.

Typical PAH is a classically described complication of congenital heart disease with left-to-right shunts. Previous attempts to reproduce PAH associated with systemic left-to-right shunting often led to disappointingly moderate increases in pulmonary artery pressures related to insufficient duration, pressure, or volume flow of surgically implanted shunting. In the present study, we performed a Blalock-Taussig operation allowing for shunt flow to increase progressively with growth of the animals. This approach resulted in pronounced medial hypertrophy and Ppas between 30 and 40 mm Hg, compatible with changes seen in early PAH. The partitioning of PVR was unaltered, in keeping with the morphometry showing that the site of overcirculation-induced remodeling is at the normal site of resistance, at the periphery of the pulmonary arterial tree.

Overcirculation-induced PAH was associated with increased circulating ET-1, increased pulmonary tissue gene

<table>
<thead>
<tr>
<th>TABLE 1. Primers Used for RTQ-PCR in Porcine Lung and Ventricular Tissue</th>
<th>Shunt</th>
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<tbody>
<tr>
<td>Gene</td>
<td>Primer Sequences</td>
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<tr>
<td>HPRT</td>
<td>Sense: 5'-TCAGGCGATATACTACCAAGATGGT-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-AGTCTGGCTATATACTACCAAGATGGT-3'</td>
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<tr>
<td>ET-1</td>
<td>Sense: 5'-CTCTGCGCTTCCTGATAGGA-3'</td>
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<tr>
<td></td>
<td>Antisense: 5'-GTCTCAGGGTGTGAGACCAA-3'</td>
</tr>
<tr>
<td>ET₁a</td>
<td>Sense: 5'-TTATACCTTGACATCAGAGATG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-GCTCTCGGCTTGTATCAAA-3'</td>
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<tr>
<td>ET₂b</td>
<td>Sense: 5'-CCCTCTCATCTACGAGATG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-GCACACGAGCAATAAAGCATG-3'</td>
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<tr>
<td>ECE-1</td>
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<td>Antisense: 5'-GGGTGTCCTGGAAGTTGTCCTTG-3'</td>
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<tr>
<td>nNOS</td>
<td>Sense: 5'-CTCAACAGTTCTTGGCTACCTGCT-3'</td>
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<td>Antisense: 5'-GATATTACTCGTCAATTAAAAGATTCG-3'</td>
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<td>iNOS</td>
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<td>Tenascin</td>
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</tr>
<tr>
<td></td>
<td>Antisense: 5'-GATGGTGCAGATGTTGCA-3'</td>
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</tbody>
</table>

Figure 2. Composite plots of Ppa vs Q in sham-, placebo-, and bosentan-treated piglets. Bosentan completely prevented shunt-induced shift of Ppa/Q plots to higher pressures. Values are expressed as mean±SEM. *P<0.05 sham vs placebo. †P<0.05 placebo vs bosentan.

As illustrated in Table 1, the present study is the first to show a dominant role of the endothelin system in early overcirculation-induced PAH.
expression for ET-1 and ET₉, and increased pulmonary endothelial ET-1 protein, indicating activation of the pulmonary endothelial endothelin system. Activation of the endothelin system with increased circulating ET-1 has been reported in various experimental models of pulmonary hypertension and in clinical pulmonary hypertension. Endothelin receptor blockade has been shown to prevent experimental hypoxic or monocrotaline-induced pulmonary hypertension. In the present study, the dual endothelin receptor antagonist bosentan completely prevented both medial hypertrophy and increase in PVR, suggesting a causal relationship.

The shunted piglets presented with an overexpressed ET₉ receptor. This has also been observed in rats with hypoxic pulmonary hypertension and in patients with thromboembolic pulmonary hypertension. The ET₉ receptor has been shown to be involved in the clearance of circulating ET-1, the modulation of ET-1 synthesis through a negative feedback, and the release of endothelium-derived vasodilators but may also contribute to ET-1-induced remodeling. Whether selective ET₉ blockade would be more or less effective in preventing overcirculation-induced PAH is unknown at present.

Pulmonary eNOS expression has been reported to be decreased in PAH patients but either increased or unchanged in experimental overcirculation-induced PAH. In the present study, eNOS and nNOS mRNAs were unchanged, iNOS mRNA was increased, but there was no change in the protein levels of either NOS. It may be that in the piglet, and only in the early stages of PAH, increased iNOS could contribute to limit the severity of pulmonary hypertension.

Pulmonary vascular disease has been shown to be associated with induction of tenascin-C, a mitogenic cofactor produced through the action of matrix metalloproteases activated by a smooth muscle cell-derived serine elastase. The present results do not favor a role for this pathway in early overcirculation-induced PAH.

The shunted piglets presented with an increase in pulmonary tissue VEGF mRNA and VEGF protein. An increased VEGF expression has been reported previously in hypoxic rats and in pulmonary hypertensive infants. Overexpres-
expression of VEGF could be considered as counterregulatory to the activation of the endothelin system.\textsuperscript{32}

The myocardial tissue of the shunted piglets showed increased mRNA for \( \text{ET}_A \) receptor and VEGF, together with increased VEGF protein. An increased \( \text{ET}_A \) receptor could contribute increased right ventricular contractility.\textsuperscript{33} Both pathways could have contributed to maintained right ventriculoarterial coupling, as was assessed by the Ees/Ea ratio.

Bosentan therapy was associated with additional expression of pulmonary tissue ECE-1 and eNOS and myocardial tissue eNOS. Increased circulating ET-1 and increased expression of pulmonary tissue ECE-1 have been previously reported with bosentan therapy.\textsuperscript{34} Increased eNOS mRNA could conceivably be secondary to decreased \( \text{ET}_B \)-dependent eNOS activity.\textsuperscript{2}

In summary, overcirculation-induced PAH seems to be causally related to an activation of the pulmonary endothelin system, with counterregulatory NO and VEGF pathways and no role for tenascin. The efficacy of preventive dual endothelin receptor blockade offers a rationale for early therapy with this agent.

\textbf{Acknowledgments}

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\textbf{References}


\begin{figure}
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\caption{Bar graphs showing relative myocardial tissue mRNA content for ET-1, ET\textsubscript{A}, ET\textsubscript{B}, ECE-1, nNOS, iNOS, eNOS, VEGF, VEGF receptor 2 (fK-1), and tenascin (TN) and relative immunostaining in myocardial tissues of ET-1, nNOS, iNOS, eNOS, and VEGF, with illustrative microscopic views (\( \times 400 \)), of sham-, placebo-, and bosentan-treated piglets. Values are expressed as mean \( \pm \) SEM. *\( P < 0.05 \) sham vs placebo. †\( P < 0.05 \) placebo vs bosentan. ‡\( P < 0.05 \) sham vs bosentan.}
\end{figure}
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