Selective Upregulation of Endothelin B Receptor Gene Expression in Severe Pulmonary Hypertension

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Background—The pulmonary circulation is an important site for the production and clearance of endothelin (ET)-1, a potent vasoactive and mitogenic peptide. Increased plasma ET-1 levels are observed in pulmonary arterial hypertension (PHT) and may contribute to the regulation of pulmonary vascular resistance, as well as to proliferative changes in the pulmonary vascular bed.

Methods and Results—We prospectively assessed changes in plasma big ET-1 levels and changes in ET A and ET B receptor gene expression in 14 consecutive patients undergoing pulmonary thromboendarterectomy for thromboembolic PHT. Plasma big ET-1 levels were higher in patients with PHT (median, 2.2 pg/mL; 25th to 75th percentile, 1.5 to 3.0 pg/mL) compared with age-matched controls (median, 1.2 pg/mL; 25th to 75th percentile, 1.0 to 1.4 pg/mL; *P*<0.002). In addition to increased plasma big ET-1 levels, selective upregulation of ET B receptor mRNA transcripts and immunoreactive protein in the pulmonary artery was observed in the patients; however, ET A receptor gene expression was unaffected.

Conclusions—These data suggest that changes in the ET signaling system in PHT caused by thromboembolic disease are not limited to an increased production of ET-1: they also affect ET receptor gene expression. (Circulation. 2002;105:1034-1036.)

Key Words: pulmonary heart disease hypertension, pulmonary receptors

Endothelial mediators, most notably nitric oxide and endothelin (ET)-1, are now widely recognized as exerting potent effects on the cardiovascular system. Impaired release of endothelial-dependent dilators such as prostaglandin I2 has been documented in pulmonary arterial hypertension (PHT), while the release of endothelial-dependent constrictors such as thromboxane A2 is augmented simultaneously. Similarly, circulating levels of ET-1 are increased in various forms of PHT; these levels correlate with disease severity. The ET-1 gene is translated to a 203 amino acid precursor, which is then cleaved to form big ET-1. Big ET-1 is subsequently cleaved by the ET-converting enzyme into functional ET-1.

Two different ET receptors (ET A and ET B) that mediate the effects of ET-1 have been cloned. ET A receptors are located on smooth muscle cells, where they mediate vasoconstriction; ET B receptors are found on both endothelial and smooth muscle cells, where they may mediate vasodilation or vasoconstriction. In addition, pulmonary ET B receptors reflect the predominant site for the clearance of circulating ET-1; these receptors are responsible for the removal of ≈50% of plasma ET-1 during pulmonary transit. Thus, changes in the ratio of ET A to ET B receptor subtypes in the pulmonary vascular bed may significantly affect the functional response to ET-1, which is locally produced in pulmonary hypertension. Consistent with this concept, disruption of the ET B gene in rats was paralleled by higher plasma ET-1 levels and higher resting pulmonary vascular tone.

The objective of the present study was to assess plasma big ET-1 levels in patients undergoing pulmonary thromboendarterectomy (PTE) for thromboembolic PHT, along with changes in ET receptor gene expression in pulmonary artery biopsies obtained during surgery.

Methods

Patient Populations

Fourteen consecutive patients (6 men and 8 women aged a median of 58 years [25th to 75th percentile, 51 to 62 years]) who were undergoing PTE for PHT due to recurrent thromboembolism were enrolled after obtaining consent according to the Declaration of Helsinki. A preoperative mean pulmonary arterial pressure of 45 mm Hg (25th to 75th percentile, 44 to 53 mm Hg) and a resistance of 891 dynes · s−1 · cm−5 (25th to 75th percentile, 773 to 1369 dynes · s−1 · cm−5) were documented in these patients before surgery. Fourteen patients without a history of PHT who were undergoing cardiac surgery served as controls for plasma big ET-1 measurements. Samples were obtained from the radial artery using withdrawal systems containing ethylenediaminetetraacetic acid (Sarstedt). Plasma was stored at −80°C until analysis. Tissue samples obtained from the pulmonary arteries of 5 lung donors in whom

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PHT was excluded and from 14 patients undergoing PTE were used for reverse-transcriptase–polymerase chain reaction (RT-PCR) and immunohistochemistry.

**Measurement of Plasma Big ET-1**
Plasma levels of big ET-1 were measured by means of an immunoassay using polyclonal capture and monoclonal detection antibodies highly specific for human big ET-1, with a detection limit of 0.1 pg/mL (Big ET-1 enzymatic immunoassay, Biomedica).

**RNA Isolation and RT-PCR**
RNA was prepared from 10 mg of pulmonary tissue, as described previously. A total of 1 μg of total RNA was reverse-transcribed after denaturation in a mixture of 1×PCR buffer, 5 mM MgCl₂, 2.5 U/μL reverse transcriptase, 1 U/μL RNase inhibitor, 2.5 μM random hexamer, and 1 mM dNTP each dNTP. After an initial 10-minute incubation step at 20°C, reverse transcription was carried out for 15 minutes at 42°C, followed by 5 minutes of denaturation at 99°C. The following primers (MWG Biotech) were used to amplify a 302-bp ETₐ receptor or a 428-bp ETₐ receptor fragment: 5'-agg cct ttt gat cac aat gac ttg-3' (ETₐ sense); 5'-ttt gat tgt gcc ata cag gtt-3' (ETₐ antisense); 5'-act gcc cat tgt gag ctc aga tgt-3' (ETₐ sense); and 5'-ctg cat gcc act ttg ctc cca-3' (ETₐ antisense). A total of 50 μL of reaction mixture containing 1×PCR buffer, 2 mM MgCl₂, 10 μL of cDNA as a template, 1 μM of each primer, and 2.5 U of Taq Polymerase (Gene Amp RNA PCR Kit, Perkin Elmer) were subjected to the PCR protocol. After an initial denaturation step at 95°C for 2 minutes, denaturation and annealing/elongation were performed at 95°C for 1 minute and 61°C for 1 minute, respectively. The reactions were repeated for 36 cycles. A 175-bp GAPDH fragment was amplified using 5'-cct gag tgt gga tta gtc-3' as the sense primer and 5'-caa ggt tta gca tga tgc-3' as the antisense primer. A total of 50 μL of reaction mixture containing 1×PCR buffer, 2.0 mM MgCl₂, 4 μL of cDNA as a template, 0.15 μM of each primer, 0.5 U of Taq Polymerase, and 1.25 mM dNTP (Gene Amp RNA PCR Kit, Perkin Elmer) were subjected to the PCR protocol. After an initial denaturation step at 94°C for 3 minutes, denaturation, annealing, and elongation were performed at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, respectively. The reactions were repeated for 40 cycles. The PCR products were size-analyzed on a 1.5% agarose gel and stained with ethidium bromide. The reactions were within the linear range for all transcripts studied under these conditions.

**Immunofluorescence Analysis of ETₐ Receptor Gene Expression**
Indirect immunostaining was used to confirm the changes observed for RNA transcripts encoding for the ETₐ receptor subtype on the protein level and to assess the spatial expression pattern of ETₐ immunoreactive protein using a sheep anti-rat ETₐ antibody (Biotrend) that cross-reacts with the human ETₐ protein. After the blockade of unspecific binding sites, sections were incubated overnight with the primary antibody (dilution 1:50) at room temperature. As a secondary antibody, a biotinylated anti-sheep antibody (dilution 1:200) was used; it was detected by fluorescence microscopy after incubation with a streptavidin-Texas red conjugate.

**Statistics**
All data are reported as median and interquartile range. The Mann-Whitney U-test was used to compare control and PHT patient populations. Densitometric values of the RT-PCR gel were used for statistical comparison, although these results are reported as x-fold induction over control. \( P < 0.05 \) was considered significant.

**Results**

**Big ET-1 Concentrations in Patients Suffering From Severe Thromboembolic PHT**
Systemic big ET-1 levels in plasma obtained from patients undergoing PTE (median, 2.2 pg/mL; range, 1.5 to 3.0 pg/mL) were significantly higher than concentrations observed in the plasma of control subjects undergoing coronary bypass grafting or aortic arch repair (median, 1.2 pg/mL; range, 1.0 to 1.4 pg/mL; \( P = 0.002 \)).

**Gene Expression of ETₐ and ETₐ in Severe PHT**

mRNA expression for ETₐ receptors was comparable in the pulmonary arteries obtained from patients undergoing PTE and from donor lungs. In contrast, steady-state transcripts encoding for the ETₐ receptor subtype were selectively increased in samples obtained from patients with PHT over steady-state mRNA levels in tissue from harvested donor lungs (Figure 1). Increased gene expression of the ETₐ receptor subtype observed on the mRNA transcript level was confirmed on the protein level. ETₐ immunoreactive protein was not confined to the endothelium but was primarily observed in the hyperplastic media of pulmonary arterial tissue of the patients undergoing PTE (Figure 2).

**Discussion**
In the present study, we investigated changes in the ET signaling system in patients undergoing PTE for severe thromboembolic pulmonary hypertension. Although increased systemic levels of ET-1 have been documented in PHT of various origins⁴,⁵ and are confirmed in our patient population on the level of the precursor big ET-1, little is known about changes in the expression of ET receptors. While steady-state levels of mRNA encoding for ETₐ receptors were unaffected in pulmonary arterial tissue, a significant increase of transcripts encoding the ETₐ receptor was observed in severe PHT. Although the increase of ET-1, which...
A negative control

B LTX donor

C pulmonary hypertension

Figure 2. Spatial expression of ETB receptors in pulmonary arteries, as assessed by indirect immunofluorescence in specimens from patients suffering from severe PHT (C) or in harvested donor lungs (B; LTX). Samples correspond to biopsies subjected to RT-PCR. Upregulation of ETB immunoreactive protein is observed primarily in the hypertrophied media (arrowheads) in severe thromboembolic PHT. Insets indicate expression in the endothelial layer. Slides in which the primary antibody was omitted served as a negative control (A).

correlates with disease severity in PHT, has been suggested to contribute to the development of PHT and the structural remodeling of the pulmonary circulation.1,2,4,5 The receptors mediating the hemodynamic and fibrogenic effects of ET-1 are unclear.

Accumulating evidence suggests that ETₐ receptors mediate vasoconstriction and proliferation, whereas ETB receptors primarily account for the clearance of ET-1, the release of vasodilators, and the inhibition of ET-converting enzyme.2 In particular, the dichotomous role of the ETₐ receptor subtype has been emphasized lately and has sustained a discussion regarding the use of nonselective as opposed to ETₐ-specific antagonists for clinical use.2,13,14 With respect to the latter, a recent double-blind, placebo-controlled study confirmed the therapeutic potential of the dual ET receptor antagonist bosentan, which improved pulmonary hemodynamics and exercise capacity.14 However, on the basis of an increasing body of experimental evidence, ETₐ-selective antagonists may be superior to nonselective antagonists due to the beneficial effects of ETB stimulation.13,14 Upregulation of ETB gene expression with PHT, as observed in the present study, can be predominantly found in the hyperplastic media of pulmonary arterial biopsies. The relative contribution of ETB receptors expressed in the hyperplastic media for mediating effects other than vasomotion, most notably fibrogenesis or clearance of circulating ET-1 in human cardiovascular disease states, is largely speculative. Nevertheless, recent experimental data with pharmacological blockade of the ETB receptor subtype or targeting the ETB gene in rats suggest that stimulation of the ETB receptor has a net protective effect mediating vasodilation and clearance of ET-1 from the circulation.11

In summary, our data demonstrate that changes in the ET signaling system are not limited to the increased production of the agonist ET-1, but also affect ETB receptor gene expression. These results may have an impact on clinical trials using either nonselective or ETB-selective antagonists such as bosentan or sitaxsentan, respectively.

References
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