Exaggerated hypoxic pulmonary hypertension in endothelin B receptor-deficient rats

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Received 19 July 2001; accepted in final form 30 October 2001

Ivy, D. Dunbar, Masashi Yanagisawa, Cheryl E. Gariepy, Sarah A. Gebb, Kelley L. Colvin, and Ivan F. McMurtry. Exaggerated hypoxic pulmonary hypertension in endothelin B receptor-deficient rats. Am J Physiol Lung Cell Mol Physiol 282: L703–L712, 2002. First published November 9, 2001; 10.1152/ajplung.00272.2001.—Mechanisms by which endothelin (ET)-1 mediates chronic pulmonary hypertension remain incompletely understood. Although activation of the ET type A (ET\textsubscript{A}) receptor causes vasoconstriction, stimulation of ET type B (ET\textsubscript{B}) receptors can elicit vasodilation or vasoconstriction. We hypothesized that the ET\textsubscript{B} receptor attenuates the development of hypoxic pulmonary hypertension and studied a genetic rat model of ET\textsubscript{B} receptor deficiency (transgenic sl/sl). After 3 wk of severe hypoxia, the transgenic sl/sl pulmonary vasculature lacked expression of mRNA for the ET\textsubscript{B} receptor and developed exaggerated pulmonary hypertension that was characterized by elevated pulmonary arterial pressure, diminished cardiac output, and increased total pulmonary resistance. Plasma ET-1 was fivefold higher in transgenic sl/sl rats than in transgenic controls. Although mRNA for prepro-ET-1 was not different, mRNA for ET-converting enzyme-1 was higher in transgenic sl/sl than in transgenic control lungs. Hypertensive lungs of sl/sl rats also produced less nitric oxide metabolites and 6-ketoprostaglandin F\textsubscript{1\alpha}, a metabolite of prostacyclin, than transgenic controls. These findings suggest that the ET\textsubscript{B} receptor plays a protective role in the pulmonary hypertensive response to chronic hypoxia.

MECHANISMS UNDERLYING the development of chronic pulmonary hypertension remain incompletely understood. Recent studies show that the vasoactive peptide endothelin (ET)-1 contributes to the development of hypoxic pulmonary hypertension. Levels of mRNA for prepro-ET-1 and ET-1 peptide and mRNA and protein for ET type A (ET\textsubscript{A}) and type B (ET\textsubscript{B}) receptors are increased in the chronically hypoxic, hypertensive rat lung (9, 38). Similarly, lung ET-1 levels are increased in other animal models of pulmonary hypertension and in patients with pulmonary hypertension (2, 16, 21, 30, 39). A role for the ET\textsubscript{A} receptor has been emphasized, because ET\textsubscript{A} receptor antagonists prevent and may reverse chronic pulmonary hypertension (1, 6, 24). However, the ET\textsubscript{B} receptor can mediate pulmonary vasodilation and vasoconstriction, and its role in chronic hypoxic pulmonary hypertension has not been fully characterized.

We previously showed that the pulmonary vasculature of the transgenic sl/sl rat is deficient in the ET\textsubscript{B} receptor (22). This genetic model was produced by rescue of the spotting lethal rat, which is a naturally occurring rat strain that carries a 301-bp deletion in \(ET\textsubscript{B}\), rendering the gene nonfunctional (12, 14). The transgenic sl/sl rat develops salt-dependent systemic hypertension (13). Although ET\textsubscript{B}-deficient rats lack expression of \(ET\textsubscript{B}\) driven by the endogenous promoter, they express dopamine-\(\beta\)-hydroxylase-driven \(ET\textsubscript{B}\) in adrenergic tissues, such as the adrenal medulla and sympathetic ganglion. Although the pulmonary vasculature of the transgenic sl/sl rat is deficient in expression of mRNA for the ET\textsubscript{B} receptor, ET\textsubscript{B} receptor mRNA is expressed in the lung parenchyma of the transgenic sl/sl rat (22). Furthermore, the pulmonary circulation of the transgenic sl/sl rat lacks ET\textsubscript{B} receptor-mediated vasodilation and exhibits exaggerated pulmonary vasopressor responses to acute hypoxia and exogenous ET-1. When raised in the mild hypoxia of the altitude of Denver, CO, the transgenic sl/sl rat develops mild pulmonary hypertension (22).

The purpose of this study was to examine the role of the ET\textsubscript{B} receptor in the pathogenesis of severe pulmonary hypertension by comparing the development of pulmonary hypertension in response to severe hypoxia in control and transgenic sl/sl rats. After rats were exposed to 3 wk of hypoxia, we localized lung expression of ET\textsubscript{B} receptor mRNA, measured systemic and pulmonary hemodynamics during normoxia and hypoxia, and evaluated lung gene expression of the ET system. Circulating ET-1 protein and lung protein con-

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tent for endothelial nitric oxide (NO) synthase (eNOS) and prostaglandin I$_2$ (PGI$_2$) synthase were also measured. Finally, the basal pulmonary production of NO and PGI$_2$ was evaluated in isolated perfused lungs.

**MATERIALS AND METHODS**

**Animals.** A colony of three groups of rats was studied: transgenic $+/+$ (controls), transgenic sl/sl (heterozygous), and transgenic sl/sl (ET$_B$ deficient). Seventy-three animals were used in these protocols. The colony was established using founder animals provided by Dr. M. Yanagisawa (14). Genotyping of each animal was confirmed by PCR of genomic DNA using standard techniques, as previously described (14). The institution’s animal care and use committee approved all experimental protocols.

Male and female rats at 3 mo of age were exposed to a simulated altitude of 17,000 feet (410 mmHg barometric pressure, $\sim$76 mmHg inspired P$_{O_2}$) for 3 wk in a hypobaric chamber flushed continuously with room air to prevent accumulation of CO$_2$, NH$_3$, and H$_2$O. Hypobaric exposure was 24 h/day, except when the chamber was opened for 10–15 min once a day to remove rats or clean cages and replenish food and water. All rats were exposed to a 12:12-h light-dark cycle and water. All rats were breathing room air and also subsequently while they breathed hypoxic gas (fraction of inspired O$_2$ = 0.10 for 10 min) to simulate the conditions of the exposure to chronic severe hypoxia. Total pulmonary resistance (TPR) was calculated as follows: mean PAP/CO (mmHg·1$^{-1}$·min$^{-1}$). Atrial blood samples were drawn into heparinized syringes through carotid artery catheters. Arterial P$_{O_2}$, arterial P$_{CO_2}$, and pH were measured using a clinical blood-gas analyzer (Radiometer, Copenhagen, Denmark). Hematocrit was measured using a capillary tube and standard techniques.

**RV hypertrophy.** Immediately after the animals were killed with an overdose of pentobarbital sodium, the heart was resected, and the atria were removed to the plane of the atriocentral valves. The free wall of the RV was then dissected free of the left ventricle and septum (LV + S). The RV (LV + S) and the RV-to-(LV + S) ratio was calculated.

**Comparison of PAP and RV hypertrophy in mild and severe hypoxia.** To determine whether the transgenic sl/sl rat developed exaggerated pulmonary hypertension in conditions of severe hypoxia compared with the mild hypoxia of the altitude of Denver, CO, O$_2$ of 5,280 feet (630 mmHg barometric pressure, $\sim$122 mmHg inspired P$_{O_2}$), we compared PAP and RV hypertrophy with previously published data from transgenic $+/+$, sl/+ and sl/sl animals exposed to the altitude of Denver, CO (22).

**Histology and morphometrics.** Rat lungs were fixed for histology by tracheal instillation of 10% buffered formalin under constant pressure (10 cmH$_2$O). The trachea was ligated after sustained inflation, and the lungs were excised and immersed in formalin overnight. Formalin-fixed lung tissue was cut into 4- to 5-mm-thick sections, placed in 10% buffered formalin, and embedded in paraffin. Paraffin sections (5 µm thick) were mounted and stained. A representative hematocoxin-and-eosin-stained section was then coded and evaluated in a blinded manner for measurements of wall thickness of <100-µm pulmonary arteries located at the level of the terminal bronchiole, respiratory bronchiole, or alveolar duct. Ten measurements were obtained from each study animal. External diameter and medial wall thickness were measured with the Zeiss Interactive Digital Analyzer System (Carl Zeiss, Thornwood, NY), as described previously (24). The wall thickness of each artery was expressed as a percentage of the external diameter (ED) by the following formula: (I/2 × medial wall thickness/ED) × 100. Measurements were performed only on vessels that were cut transversely.

**Northern blot analysis.** Total RNA was purified from transgenic $+/+$, sl/+, and sl/sl rat lungs ($n = 4$ for each group) using Tri-Reagent (Molecular Research Center, Cincinnati, OH) and the method of Chomczynski. The RNA was quantified by measuring the absorbance at 260 nm. Twenty micrograms of total RNA per lung were analyzed using standard Northern blot and hybridization techniques using cDNA probes. Rat prepro-ET-1, ET$_A$, ET$_B$, and ET-converting enzyme-1 (ECE-1) cDNA probes were labeled with [z$^{32}$P]dCTP using random primed labeling (RTS Random Primer DNA Labeling System, GibCO BRL, Gaithersburg, MD). A 2.0-kbp rat prepro-ET-1 probe was used. Dr. Kohei Shimada (Sankyo, Tokyo, Japan) kindly provided the 4.0-kbp rat ECE-1 cDNA probe. An 18s rRNA oligonucleotide was labeled using terminal deoxynucleotransferase and [z$^{32}$P]dCTP. After hybridization, blots were washed at room temperature in 1× SSC-0.1% SDS (low stringency) and then at 65°C in 0.4× SSC-0.1% SDS (high stringency). Imaging and quantification of mRNA signals were performed using a Molecular Dynam-
ics Storm 860 PhosphorImager. Normalization to 18S rRNA levels was used in quantification of mRNA signals.

**ELISA for ET-1.** ET-1 peptide was measured in plasma obtained from the aortic catheter of the animals undergoing hemodynamic evaluation during room air breathing. Samples were drawn from EDTA plasma and extracted with acetone. The ET-1 ELISA was performed as described previously (22). An ELISA kit (R & D Systems) was used to determine ET-1 levels by comparison with a standard curve of known ET-1 peptide amounts. The antibody used in this assay cross-reacts with ET-2 (27%) and ET-3 (8%).

**Western blot analysis.** Western blot analysis was performed according to previously published techniques using a monoclonal antibody to eNOS (Transduction Laboratories, Lexington, KY) (26) or a polyclonal antibody to PG I\(_2\) synthase (41). Briefly, lung tissue [transgenic +/+ (n = 5), transgenic sl/sl (n = 5), and transgenic sl/sl (n = 5)] was homogenized in 25 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1% (vol/vol) 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 1 mM pepstatin A. The homogenate was centrifuged at 1,000 g at 4°C for 10 min to remove cell debris. SDS-PAGE was performed on 25-μg aliquots of homogenate protein with a 7.5% (wt/vol) polyacrylamide gel. Proteins were transferred to nitrocellulose paper using an electrophoretic transfer cell. The blot was blocked in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2% (vol/vol) BSA, and 0.1% (vol/vol) Tween 20 overnight at 4°C and then incubated with the primary antibody for 1 h at room temperature. The eNOS antibody was diluted 1:500 in blocking buffer, and the PG I\(_2\) synthase polyclonal antibody was diluted 1:250. The blot was then washed six times, 5 min per wash, with Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) 0.1% (vol/vol) Tween 20 at room temperature. The blot was incubated for 1 h with an anti-mouse IgG antibody coupled to horseradish peroxidase diluted in blocking buffer for eNOS or a goat-anti rabbit IgG antibody coupled to horseradish peroxidase for PG I\(_2\) synthase. The blot was then washed six times, 5 min per wash, with Tris-buffered saline-Tween 20 at room temperature, and protein bands were detected by chemiluminescence and protein bands were detected by chemiluminescence and protein bands were detected by chemiluminescence and protein bands were detected by chemiluminescence. The blot was then washed six times, 5 min per wash, with Tris-buffered saline-Tween 20 at room temperature, and protein bands were detected by chemiluminescence and protein bands were detected by chemiluminescence and protein bands were detected by chemiluminescence.

**Isolated lung studies.** Isolated lung studies were performed in 12 rats [transgenic +/+ (n = 4), transgenic sl/+ (n = 4), and transgenic sl/sl (n = 4)]. After intraperitoneal administration of 30 mg of pentobarbital sodium and intracardiac injection of 200 μl of heparin, the pulmonary artery and LV were cannulated, with the heart-lung block remaining intact (22). The lungs were ventilated with an inspiratory pressure of 9 cmH\(_2\)O and end-expiratory pressure of 2.5 cmH\(_2\)O using a humidified mixture of normoxic gas (21% O\(_2\)-5% CO\(_2\)-74% N\(_2\)) for 60 min. Perfusion was maintained at 0.04 ml·g body wt\(^{-1}\)·min\(^{-1}\) with a peristaltic pump (Gilson). The perfusate was a physiological salt solution containing (in mM) 116.3 NaCl, 5.4 KCl, 0.83 MgSO\(_4\), 19.0 NaHCO\(_3\), 1.04 NaH\(_2\)PO\(_4\), 1.8 CaCl\(_2\)·2H\(_2\)O, and 5.5 d-glucose (Earle’s balanced salt solution; Sigma). Filicol (4 g/100 ml; type 70, Sigma) was included as a colloid. After the lungs were flushed of blood with 30 ml of physiologic salt solution, they were perfused with a circulating volume of 30 ml. Effluent perfusate was drained from the left ventricular cannula into a reservoir and then recirculated. Lung and perfusate temperatures were maintained at 38°C. Perfusion pH was kept at 7.35–7.45. The mean pulmonary arterial catheter was connected to a computer-driven pressure transducer and recorder (Biopac, Santa Barbara, CA). The pressure transducers were calibrated with a mercury column manometer. Samples for measurement of cumulative perfusate NO-containing compounds (NO\(_x\)) and 6-ketoprostaglandin F\(_{1\alpha}\) (6-keto-PGF\(_{1\alpha}\)), a stable metabolite of prostacyclin, were drawn before and after 1 h of normoxic ventilation.

**Measurement of perfusate NO\(_x\).** An NO chemiluminescence analyzer (model NOA 280, Sievers Research) was used to measure the levels of NO\(_x\) (NO\(_2\), NO\(_3\), nitrosothiols, and peroxynitrite) in lung perfusate. Aliquots of perfusate (10 μl) were added to 2 ml of 0.1 M vanadium chloride (type III, Aldrich), dissolved in 1 N HCl, and heated to 90°C in the purge vessel of the NO analyzer to reduce all NO\(_x\), to NO. The liberated NO was driven into the chemiluminescence chamber by bubbling the reaction mixture with argon. Calibration curves for NO\(_x\) levels were generated daily by measuring the amount of NO produced by a range (10–100 pM) of sodium nitrate solutions (Mallicknotrold) (37). Levels were measured in the lung perfusate. Procedures for 6-keto-PGF\(_{1\alpha}\) ELISA were similar to those previously described (42). An ELISA kit (R & D Systems) was used to determine 6-keto-PGF\(_{1\alpha}\) levels by comparison with a standard curve of known 6-keto-PGF\(_{1\alpha}\) amounts.

**Statistical analysis.** Values are means ± SE. Comparisons were made using one- or two-way ANOVA with Fisher’s protected least significant difference post hoc test, with P < 0.05 accepted as significant (Statview, Berkeley, CA). For the Northern and Western analysis, each separate value is divided by the mean value for the transgenic +/+ group and converted to a percentage. Statistical analysis is performed as described above, yielding a mean ± SE for each group.

**RESULTS**

**In situ hybridization.** In the transgenic +/+ lung, we found expression of mRNA for the ET\(_2\) receptor in the endothelium and smooth muscle of pulmonary arteries as well as lung parenchyma (Fig. 1). Minimal staining was noted in the proximal airways. A similar signal was seen in the transgenic sl/+ animals. In contrast, there was no specific ET\(_2\) mRNA signal in the pulmonary arteries of the transgenic sl/sl animals. The signal was localized to the parenchyma of the transgenic sl/sl rat lung.

**Animal and hemodynamic data.** Body weight was not different among the study groups (Table 1). RV weight was 25 and 33% greater in transgenic sl/+ and sl/sl animals, respectively, than in transgenic +/+ animals. (LV + S) weight was not different among the groups. RV hypertrophy, as determined by the ratio of RV to (LV + S) weight, was greater in transgenic sl/+ and sl/sl animals than in transgenic +/+ controls. RV hypertrophy was greater in transgenic sl/sl than in transgenic sl/+ animals. The hematocrit was greater in transgenic sl/sl than in transgenic +/+ or sl/+ animals (Table 1).

The percent wall thickness of small pulmonary arteries was greater in transgenic sl/sl than in transgenic +/+ animals but was not different from transgenic sl/+ animals (Table 1). Wall thickness was increased consistently by exposure to X-ray and protein bands were detected by chemiluminescence.
greater in transgenic sl/+ than in transgenic +/+ lungs.

After 3 wk of chronic hypoxia and 24 h of normoxia (room air in Denver, CO), the mean PAP was higher in transgenic sl/sl than in transgenic +/+ or sl/+ animals (Fig. 2). PAP was not different between transgenic +/+ and sl/+ animals (P = 0.07). CO was not different between the groups (Fig. 3). TPR was greater in transgenic sl/sl than in transgenic sl/+ or +/+ animals (Fig. 4). Aortic pressure was higher in transgenic sl/sl than in transgenic +/+ and sl/+ animals (Table 2). Arterial blood gases were not different among the study groups (Table 2).

When the 3-wk hypoxia-exposed rats were briefly reexposed to 10% O₂ (acute hypoxia) after 24 h of normoxia, all groups showed a significant increase in PAP (P < 0.05). Hypoxic PAP was greater in transgenic sl/sl than in transgenic +/+ or sl/+ animals (Fig. 2). Furthermore, PAP was greater during acute hypoxia in transgenic sl/+ than in transgenic +/+ animals. CO was lower in transgenic sl/sl animals than in the other groups (Fig. 3). TPR was greater in transgenic sl/sl rats than in the other two groups (Fig. 4). Aortic pressure during hypoxia was greater in transgenic sl/sl animals (Table 2). During acute hypoxia, arterial blood gas tensions were similar in the three groups (Table 2).

### Table 1. Body weight, RV hypertrophy, hematocrit, and morphometric changes

<table>
<thead>
<tr>
<th></th>
<th>Trans +/+ (n = 10)</th>
<th>Trans sl/+ (n = 8)</th>
<th>Trans sl/sl (n = 7)</th>
</tr>
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<tr>
<td>Body wt, g</td>
<td>287 ± 14</td>
<td>257 ± 21</td>
<td>295 ± 22</td>
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<td>RV wt, g</td>
<td>0.18 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>(LV + S) wt, g</td>
<td>0.51 ± 0.03</td>
<td>0.51 ± 0.04</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>RV/(LV + S)</td>
<td>0.35 ± 0.01</td>
<td>0.48 ± 0.02</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>57 ± 2</td>
<td>61 ± 3</td>
<td>73 ± 2*</td>
</tr>
<tr>
<td>Wall thickness, %</td>
<td>28 ± 2</td>
<td>33 ± 2*</td>
<td>36 ± 2*</td>
</tr>
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</table>

Values are means ± SE. RV, right ventricle; LV, left ventricle; S, septum; Trans, transgenic. *P < 0.05 vs. Trans +/+; †P < 0.05 vs. Trans sl/+.
Comparison of PAP and RV hypertrophy in mild and severe hypoxia. Mean PAP in 21% O₂ was not different among transgenic +/-, sl/+ sl/sl and sl/sl rats exposed to the mild hypoxia of the altitude in Denver, CO (22). During room air breathing, PAP was 153% higher in transgenic sl/sl rats exposed to severe hypoxia than in those exposed to the mild hypoxia of the altitude of Denver, CO (22) (Table 3). Mean PAP was 42% higher in transgenic +/- animals and 83% higher in transgenic sl/+ animals exposed to severe hypoxia. When the rats were briefly exposed to 10% O₂ (acute hypoxia), mean PAP was 102% higher in transgenic sl/sl animals that had been exposed to 3 wk of severe hypoxia compared with animals raised in Denver, CO.

RV hypertrophy was 58% higher in transgenic sl/sl animals exposed to 3 wk of severe hypoxia than in those exposed only to room air. Furthermore, RV hypertrophy was 25 and 14% greater in transgenic +/- and sl/+ rats, respectively, than in historic controls raised in mild hypoxia (22).

Northern blot analysis. Probing of mRNA for prepro-ET-1 revealed expression of a single 2.3-kb transcript, as previously reported (27). Expression of mRNA for ECE-1 revealed a 4.4- and a 3.4-kb transcript, as previously described (12). Expression of whole lung mRNA for the ET₁ receptor revealed expression of a single 2.3-kb transcript, as previously reported (27). Expression of mRNA for prepro-ET-1, the ETA receptor, and the ETB receptor was not different among transgenic +/-, sl/+ and sl/sl animals (Fig. 5). Northern blots for ET₁ and ETB receptors are not shown. It is likely that there was no difference in expression of the whole lung mRNA for the ETB receptor among the groups, because this mRNA is expressed in the lung parenchyma of all transgenic animals. There was a 77 ± 10% increase in the mRNA for ECE-1 in transgenic sl/sl animals.

Plasma ET-1 protein analysis. Pulmonary artery plasma ET-1 levels were fivefold higher in transgenic sl/sl animals than in the other groups (Fig. 6). ET-1 levels in the transgenic sl/+ animals were not different from those in controls.

eNOS protein content. Lung eNOS protein content was higher in transgenic sl/sl than in transgenic +/- rats (Fig. 7A). In transgenic sl/+ lungs, eNOS protein content tended to be between that in transgenic +/- and sl/sl lungs.

PGI₂ synthase protein content. Lung PGI₂ synthase protein content was lower in lungs of transgenic sl/sl than in transgenic +/- rats (Fig. 7B). Lung PGI₂ synthase protein content was not different between transgenic sl/+ and +/- animals.

Isolated lung studies: perfusate NOx and 6-keto-PGF₁α. To determine whether the hypoxia-induced hypertensive transgenic sl/sl rat lungs produced abnormal amounts of NO and PGI₂, we measured perfusate...
accumulation of NO\textsubscript{x} and 6-keto-PGF\textsubscript{1\alpha} during 1 h of normoxic ventilation of the perfused lung. We found greater initial and final baseline perfusion pressures in transgenic \textit{sl}/H\textsubscript{11001}/H\textsubscript{11001} and \textit{sl}/sl lungs (Table 4).

Perfusate NO\textsubscript{x} increased during 1 h of normoxic ventilation in transgenic \textit{sl}/H\textsubscript{11001}/H\textsubscript{11001} and \textit{sl}/sl lungs (Fig. 8A). In contrast, there was no increase in cumulative NO\textsubscript{x} in transgenic \textit{sl}/sl lungs, suggesting diminished production of NO.

Perfusate 6-keto-PGF\textsubscript{1\alpha} levels increased during 1 h of perfusion of transgenic \textit{sl}/H\textsubscript{11001}/H\textsubscript{11001} and \textit{sl}/sl lungs. In contrast, 6-keto-PGF\textsubscript{1\alpha} levels did not increase in transgenic \textit{sl}/sl lungs, and the levels were greater after 1 h of perfusion in transgenic \textit{sl}/H\textsubscript{11001}/H\textsubscript{11001} than in transgenic \textit{sl}/sl lungs (Fig. 8B).

**DISCUSSION**

The major findings of this study were that deficiency of pulmonary vascular ET\textsubscript{B} receptors predisposed rats to the development of hypoxic pulmonary hypertension. After 3 wk of severe hypoxia, the transgenic \textit{sl}/sl pulmonary vasculature lacked expression of the mRNA for the ET\textsubscript{B} receptor and developed exaggerated pulmonary hypertension that was characterized by ele-

<table>
<thead>
<tr>
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<th>Trans \textit{+/+}</th>
<th>Trans \textit{sl/+}</th>
<th>Trans \textit{sl/sl}</th>
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<tr>
<td>PAP, mmHg</td>
<td></td>
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<tr>
<td>\textit{F\textsubscript{O2}} = 0.21</td>
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<tr>
<td>Mild hypoxia</td>
<td>16.5 ± 0.9*</td>
<td>18.0 ± 1.6*</td>
<td>20.7 ± 2.6*</td>
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<td>Severe hypoxia</td>
<td>23.5 ± 2.8 (42)</td>
<td>33.0 ± 5.2 (83)</td>
<td>52.5 ± 8.5 (153)</td>
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<td>\textit{F\textsubscript{O2}} = 0.10</td>
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<tr>
<td>Mild hypoxia</td>
<td>20.2 ± 1.3*</td>
<td>24.5 ± 3.0*</td>
<td>34.1 ± 4.4*</td>
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<td>Severe hypoxia</td>
<td>33.3 ± 2.9 (65)</td>
<td>48.0 ± 6.0 (96)</td>
<td>69.0 ± 8.3 (102)</td>
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<tr>
<td>RV/(LV + S)</td>
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<tr>
<td>Mild hypoxia</td>
<td>0.28 ± 0.03*</td>
<td>0.42 ± 0.06</td>
<td>0.41 ± 0.04*</td>
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<tr>
<td>Severe hypoxia</td>
<td>0.35 ± 0.01 (25)</td>
<td>0.48 ± 0.02 (14)</td>
<td>0.65 ± 0.04 (58)</td>
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</table>

Values are mean ± SE. Values in parentheses represent percent difference between mild hypoxia (data from Ref. 22) and severe hypoxia. PAP, mean pulmonary arterial pressure; \textit{F\textsubscript{O2}}, fraction of inspired O\textsubscript{2}. *P < 0.05 vs. severe hypoxia.

![Fig. 5. A: after 3 wk of hypoxia, steady-state preproendothelin-1 (ppET-1) mRNA expression was not different among transgenic \textit{+/+}, \textit{sl/+}, and \textit{sl/sl} rat lungs. B: endothelin-converting enzyme-1 (ECE-1) mRNA expression was greater in transgenic \textit{sl/sl} than in transgenic \textit{+/+} or \textit{sl/+} rat lungs.](http://ajplung.physiology.org/).**![Fig. 6. Pulmonary artery plasma ET-1 levels in transgenic \textit{+/+}, \textit{sl/+}, and \textit{sl/sl} rats. ET-1 levels were 5-fold greater in transgenic \textit{sl/sl} than in transgenic \textit{+/+} or \textit{sl/+} animals.](http://ajplung.physiology.org/)
vated PAP and diminished CO. Increased RV hypertrophy and muscularization of small distal pulmonary arteries accompanied the increased pulmonary hypertension in the transgenic \( sl/sl \) rat. Although steady-state mRNA levels of prepro-ET-1 were not greater in transgenic \( sl/sl \) than in transgenic \( /H11001/H11001 \) and \( sl/H11001/H11001 \) lungs, the mRNA for ECE-1 was higher, possibly contributing to the increase in circulating ET-1. The hypertensive lungs of transgenic \( sl/sl \) rats also produced less NO\(_x\) and 6-keto-PGF\(_1\) than transgenic controls. Thus deficiency of the pulmonary vascular ET\(_B\) receptor was associated with elevated ET-1 levels, diminished lung NO and PGI\(_2\) synthesis, and exaggerated pulmonary hypertension. These findings suggest that the ET\(_B\) receptor plays an important physiological role in moderating the pulmonary hypertensive response to chronic hypoxia.

The ETs are a family of isopeptides with potent vasoactive properties. ET-1 regulates pulmonary vascular tone in the normal rat lung (29, 36) as well as the hypertensive rat lung (1, 6, 27). The actions of ET-1 are dependent on activation of at least two receptor subtypes: ETA and ET\(_B\). ETA receptors are located on smooth muscle cells and mediate vasoconstriction and smooth muscle cell proliferation (20, 44). In contrast, ET\(_B\) receptors are present on endothelial and smooth muscle cells in the rat pulmonary circulation (7, 17, 18). Stimulation of endothelial ET\(_B\) receptors causes vasodilation through release of NO and PGI\(_2\). Endothelial ET\(_B\) receptors also function to remove ET-1 from the circulation (11, 19, 36). Stimulation of ET\(_B\) receptors on smooth muscle causes vasoconstriction in the rat lung (36).

### Table 4. Perfusion pressure during isolated lung study

<table>
<thead>
<tr>
<th>PAP, mmHg</th>
<th>Trans +/- (n = 4)</th>
<th>Trans sl/+ (n = 4)</th>
<th>Trans sl/sl (n = 4)</th>
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<tr>
<td>Initial</td>
<td>9.5 ± 0.2</td>
<td>14.5 ± 0.6*</td>
<td>14.1 ± 0.1*</td>
</tr>
<tr>
<td>1 h</td>
<td>10.2 ± 0.2</td>
<td>14.1 ± 1.0*</td>
<td>16.0 ± 0.9*</td>
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Values are means ± SE. *\( P < 0.05 \) vs. Trans +/-.
Blockade of ET<sub>A</sub> receptor activity attenuates PAP and hypertensive structural changes in hypoxia and other models of pulmonary hypertension (1, 6, 24, 30). However, little is known about the role of the ET<sub>B</sub> receptor in the pathogenesis of pulmonary hypertension. Some studies have shown increased lung ET<sub>B</sub> receptor mRNA expression in models of pulmonary hypertension (27, 38), whereas others have shown decreased lung ET<sub>B</sub> receptor mRNA expression (21, 43). The variations may be explained by species differences as well as differences in the hypertensive model used. Although combined ET<sub>AB</sub> receptor blockade prevents and reverses hypoxic pulmonary hypertension in rats (4), the relative contribution of the ET<sub>B</sub> receptor remains uncertain.

Recent studies have shown that selective ET<sub>A</sub> and nonselective ET<sub>AB</sub> receptor antagonists, but not a selective ET<sub>B</sub> receptor antagonist (35), attenuate hypoxic pulmonary vasoconstriction. Thus the ET<sub>A</sub> receptor, but not the ET<sub>B</sub> receptor, contributes to the mechanism of acute hypoxic pulmonary vasoconstriction. In contrast, chronic blockade of the ET<sub>B</sub> receptor causes pulmonary hypertension in fetal lambs (23), and genetic deficiency of the receptor leads to exaggerated pressor responses to ET-1 and acute hypoxia in rats (22). The present study extends these observations and suggests that the ET<sub>B</sub> receptor may provide a protective role in the attenuation of pulmonary hypertension due to chronic severe hypoxia.

Several factors may have contributed to the development of more severe pulmonary hypertension in the ET<sub>B</sub> receptor-deficient rat. First, diminished endogenous vasodilator signals, such as pulmonary vascular NO or PG<sub>L2</sub> production, may exacerbate pulmonary hypertension (10, 15, 40). The chronically hypoxic rat expresses increased ET<sub>B</sub> receptors on the endothelium of pulmonary arteries (27, 38), increased ET<sub>B</sub> receptor-mediated pulmonary vasodilation (31), and an increased capacity for ET<sub>B</sub> receptor-dependent NO production (37) that is associated with increased levels of eNOS (26). Although hypertensive lungs of normal rats exposed to severe hypoxia express increased levels of eNOS, the level of NO production during hypoxic exposure is limited by the decreased Po<sub>2</sub> (37). During normoxic ventilation, the level of NO<sub>x</sub> (NO metabolites) increases, suggesting increased NO production. However, during hypoxic ventilation, NO<sub>x</sub> does not increase. The increase in NO production during normoxic ventilation appears to be mediated by the ET<sub>B</sub> receptor, inasmuch as the ET<sub>B</sub> receptor antagonist BQ-788 prevented the increase in NO<sub>x</sub> during normoxic ventilation (37). Similarly, we found an increase in eNOS protein but limited NO production in the hypertensive sl/sl lung. Because the isolated lung was ventilated with 21% O<sub>2</sub>, the low NO production was apparently due to the absence of ET<sub>B</sub> receptor-mediated stimulation of eNOS (37). The decreased PG<sub>L2</sub> synthesis in the hypertensive sl/sl lung was likely due to the combined effects of the decreased expression of PG<sub>L2</sub> synthase and the lack of ET<sub>B</sub> receptor-mediated stimulation of the pulmonary vascular endothelium. We are unaware of any reported links between ET<sub>B</sub> receptors and PG<sub>L2</sub> synthase expression, and the cause of the decreased expression in the hypertensive sl/sl lung is unknown. Our study suggests that the absence of ET<sub>B</sub> receptor-mediated NO and PG<sub>L2</sub> release may exacerbate pulmonary hypertension. Thus, in chronic hypoxic pulmonary hypertension, enhanced ET<sub>B</sub> receptor activity may be protective because of stimulation of NO and PG<sub>L2</sub> synthesis.

Second, elevated circulating ET-1 levels may have also contributed to the pulmonary hypertension. Although the direct mechanism of the increase in circulating ET-1 in the ET<sub>B</sub> receptor-deficient rat is uncertain, the increased ET-1 levels may have been due to decreased ET<sub>B</sub> receptor-mediated clearance of circulating ET-1 (11). Another possibility was increased ET-1 production. Although there were no differences in steady-state mRNA levels of prepro-ET-1 among transgenic +/+ , sl/+ , and sl/sl lungs, expression of ECE-1 was greater in transgenic sl/sl lungs. Studies have shown that ET<sub>B</sub> receptor stimulation decreases ECE-1 mRNA levels (32); thus deficiency of the ET<sub>B</sub> receptor may lead to increased ECE-1 expression. The transgenic sl/sl rat develops exaggerated ET-1 vasoconstriction, likely through increased ET<sub>A</sub> receptor-mediated vasoconstriction (22). Therefore, the exaggerated pulmonary hypertension to severe hypoxia in the ET<sub>B</sub> receptor-deficient rat may be due to enhanced ET<sub>A</sub> receptor activity.

Mechanisms leading to the increased hematocrit in the transgenic sl/sl rat are unknown. A similar difference in hematocrit was noted in the transgenic sl/sl rats raised under mild hypoxia at the altitude of Denver, CO. It is unlikely that differences in hypoxia may explain the differences in hematocrit, inasmuch as no difference was seen in the arterial Po<sub>2</sub>. Studies have shown that erythropoietin production may be related to proximal renal tubular function and that inhibition of sodium reabsorption reduces tubular O<sub>2</sub> consumption (8). Inasmuch as the ET<sub>B</sub> receptor inhibits sodium reabsorption (14), deficiency of the ET<sub>B</sub> receptor may lead to increased tubular O<sub>2</sub> consumption and increased erythropoietin production. Furthermore, it is unlikely that the elevation in hematocrit alone caused pulmonary hypertension, inasmuch as recent studies have shown that augmented polycythemia does not increase RV hypertrophy and PAP in the normoxic or hypoxic rat (34).

The pulmonary phenotype of the heterozygous transgenic sl/+ rat is intermediate between the transgenic +/+ and sl/sl rats. Under the mildly hypoxic conditions of the altitude of Denver, CO, the transgenic sl/+ rat tends to develop pulmonary hypertension and has a greater acute hypoxic pressor response than transgenic +/+ animals (22). After 3 wk of severe hypoxia, the sl/+ pulmonary vasculature expressed the mRNA for the ET<sub>B</sub> receptor, but there was a trend toward higher mean PAP (P = 0.07), greater RV hypertrophy, and greater muscularization of small pulmonary arteries in transgenic sl/+ than in transgenic +/+ rats. Production of PG<sub>L2</sub> also appeared to be diminished in the
transgenic sl/+ lung after 3 wk of hypoxia. Thus only partial loss of ETB receptor function may also predispose to development of pulmonary hypertension.

The role of the ETB receptor in hypertensive pulmonary vascular remodeling is uncertain. The ability of ET-1 to induce or potentiate vascular smooth muscle proliferation has generally been found to be mediated by the ETA receptor (44), with little or no role for the ETB receptor. Studies have shown that ET-1 stimulation of cAMP production through the ETB receptor suppresses proliferation of human myofibroblastic hepatic stellate cells (28). Although the ETB receptor on rabbit mesenteric smooth muscle may stimulate PG12 synthesis (25), it is unknown whether a similar ETB receptor-dependent effect occurs in pulmonary artery smooth muscle cells and inhibits proliferation. Inasmuch as NO may have antiproliferative effects on vascular smooth muscle (33), the reduced production of NO may enhance vascular smooth muscle proliferation. If the pulmonary artery smooth muscle cell ETB receptor does moderate the mitogenic response to stimulation of the ETA receptor, then the absence of ETB receptors in the sl/sl pulmonary vasculature could have contributed to the greater degree of hypertensive vascular wall thickening.

Our results may be limited by potential genetic alterations in the dopamine-β-hydroxylase-driven ETB in subsequent generations. It is possible that some of the phenotypic differences in the transgenic sl/sl rat lung may be caused by genetic alterations over time. We minimized these limitations by performing studies on animals two to three generations apart.

Elevated immunoreactive ET-1 levels have been found in human disorders of pulmonary hypertension, including primary pulmonary hypertension (16). The combined ETA/ETB receptor antagonist bosentan lowers PAP and improves exercise tolerance in patients with pulmonary hypertension (3). However, the optimal strategy in treatment of pulmonary hypertension with a selective ETA receptor or a combined ETA/ETB receptor antagonist requires further study. The results of our study in ETB receptor-deficient rats raise the possibility that the ETB receptor plays a protective role in pulmonary hypertension.

In summary, our study demonstrated that deficiency of the pulmonary vascular ETB receptor was associated with elevated ET-1 levels, diminished lung NO and PGI2 synthesis, and exaggerated pulmonary hypertension. These findings suggest that the ETB receptor plays an important physiological role in moderating the pulmonary hypertensive response to chronic hypoxia. Further studies are necessary to define the exact mechanisms by which the pulmonary vascular ETB receptor activity attenuates the hypertensive response.

We thank K. Morris and C. Oliver-Pickett for assistance with this study.

This work was supported in part by National Heart, Lung, and Blood Institute Grants HL-03823 (D. D. Ivy) and HL-14985 (J. F. McMurtry), a grant-in-aid from the American Heart Association, Desert/Mountain Affiliate (D. D. Ivy), and grants from the Leibl Pulmonary Hypertension Fund and the Caitlyn Whitley Cardiology Research Fund.

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