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Citation for published version:

Digital Object Identifier (DOI):
10.1159/000231717

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Early version, also known as pre-print

Published In:
Journal of vascular research

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Endothelial ET\textsubscript{B} Limits Vascular Remodelling and Development of Pulmonary Hypertension during Hypoxia

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Key Words
ET\textsubscript{B} receptor · Knockout · Endothelin · Pulmonary hypertension

Abstract
Background: We hypothesised that the potential protective effects of endothelial ET\textsubscript{B} are important in limiting pulmonary vascular muscularisation, vasoconstriction and the development of pulmonary arterial hypertension in response to hypoxia. Methods: EC-specific ET\textsubscript{B} knockout mice (EC ET\textsubscript{B} –/–) and control mice (ET\textsubscript{B} f/f) were subjected to hypobaric hypoxic (10\% FiO\textsubscript{2}) or normoxic conditions for 14 days before assessment of right ventricular pressure and pulmonary vascular morphology and function. Results: During normoxia, no difference in right ventricular pressure was detected between EC ET\textsubscript{B} –/– (23.7 ± 1.7 mm Hg) and ET\textsubscript{B} f/f mice (20.2 ± 1.5 mm Hg). Hypoxia induced an exaggerated increase in right ventricular pressure in EC ET\textsubscript{B} –/– mice (34.4 ± 1.2 mm Hg vs. 24.6 ± 1.4 mm Hg), accompanied by an increase in right ventricular mass. No effect was observed in ET\textsubscript{B} f/f mice. Endothelin-1 constricted pulmonary arteries from both groups, although maximum response was similar irrespective of inspired oxygen or genotype. Hypoxia increased the percentage of muscularised vessels in both groups of mice, but the percentage increase was significantly greater in EC ET\textsubscript{B} –/– mice. Conclusions: The potential protective effects of endothelial ET\textsubscript{B} are important in limiting pulmonary vascular muscularisation and the development of pulmonary arterial hypertension in response to hypoxia.

Introduction

Pulmonary arterial hypertension (PAH) is a progressive condition involving small pulmonary arteries (PAs) characterised by a sustained increase in pulmonary vascular resistance and vascular remodelling leading ultimately to right ventricular failure and premature death [1]. Several lines of evidence implicate endothelin-1 (ET-1) in the aetiology and progression of PAH. First, ET-1 is a potent vasoconstrictor and mitogen [2]. Second, plasma concentration of ET-1 correlates with severity of PAH in animal models [3–5] and patients [6], and third, non-selective and selective endothelin A receptor antagonists improve symptoms and slow the progression of PAH [7, 8]. The lung is an important site of ET-1 production with a concentration of ET-1 five times greater than that seen in other organs [9]. Endothelin B receptor (ET\textsubscript{B}) mRNA is expressed abundantly in the lung [10], particularly in distal segments of the pulmonary vascular tree [11], where
it is found in the endothelium and media of pulmonary blood vessels as well as in bronchioles and alveoli.

In contrast, endothelin A receptors (ET\(_A\)) are localised to the media of large proximal PAs and veins with relatively little expression in distal arterioles [11–13]. Hypoxia increases the expression of ET-1, ET\(_A\) and ET\(_B\) throughout the rat lung [10, 12] with histological evidence to suggest a preferential increase in endothelial cell (EC) ET\(_B\) expression in distal segments [13]. EC ET\(_B\) elicit vasodilatation and anti-mitogenic effects through the release of nitric oxide (NO) and/or prostaglandin I\(_2\) (PGI\(_2\)) [14, 15]. Increased ET-1/EC ET\(_B\)-mediated vasodilatation has thus been hypothesised to protect against hypoxia-induced vasoconstriction [13], and studies in rats suggest that ET\(_B\) deficiency exacerbates monocrotaline-induced PAH [16] and hypoxia-induced PAH [17]. Pulmonary ET\(_B\) also clear ET-1 from the plasma [18], further limiting ET\(_A\)-mediated vasoconstrictor and mitogenic effects.

Vascular smooth muscle cell (VSMC) ET\(_B\) mediate vasoconstriction in humans [19]. In hypoxic rat models [20] and sheep models of embolism-induced PAH and PAH secondary to aortopulmonary shunting, there is evidence of increased VSMC ET\(_B\)-mediated vasoconstriction [21, 22]. Activation of ET\(_A\) and ET\(_B\) on VSMC by ET-1 also promotes cellular hypertrophy [11] and hence may promote the progression of PAH through muscularisation of small pulmonary arterioles. Thus, with respect to the development of PAH, pulmonary ET\(_B\) have the potential to elicit both protective and detrimental effects. Study of the interplay between EC and VSMC ET\(_B\) is likely to increase our mechanistic understanding of the role of the endothelin system in the pathogenesis of PAH. We have previously generated EC-specific ET\(_B\) knockout (KO) mice that exhibit endothelial dysfunction in the absence of systemic hypertension, with evidence of impaired endogenous NO release and increased plasma ET-1 [23]. Otherwise, they have normal feeding and growth rates, exhibit normal behaviour and are healthy. Here we have used this model to determine whether the potential protective effects of EC ET\(_B\) are important in limiting pulmonary vascular muscularisation and the development of PAH during hypoxia.

**Methods**

**Experimental Animals**

Mice featuring selective KO of EC ET\(_B\) were generated using a Cre-LoxP approach as previously described [23]. Briefly, mice with loxP sites flanking exons 3 and 4 of the ET\(_B\) gene (‘floxed’ mice, ET\(_B\)\(^{\text{flox}}\)) were crossed with mice expressing a Cre recombinase transgene in an EC-restricted pattern (WW/Tie2-Cre) [24] to produce EC-specific ET\(_B\) KO mice (EC ET\(_B\)\(^{-/-}\)). Genotyping to identify the flox and recombined alleles was performed by Southern blot and by PCR using primers amplifying a sequence spanning the 3’ and 5’ loxP sites (forward primer: TCA GTT GTA ATG ATG AGA CAC AGA C; reverse primer: AGC CAT AAA GTC ACA GCC ATT C). The Tie2-Cre transgene was detected by PCR as described [24]. Male mice aged 8–12 weeks (weight 25–35 g) were studied and EC ET\(_B\)\(^{-/-}\) mice compared with ET\(_B\)\(^{\text{flox}}\) control mice in all experiments. The genetic background of each group was 129/0la; BKW; C57Bl/6; SJLF. All procedures were carried out with the approval of the University of Glasgow and University of Edinburgh Local Ethical Review Committees, under Home Office Project and Personal Licence authority.

**Hypobaric Chambers**

EC ET\(_B\)\(^{-/-}\) mice and control mice (10 animals/group) were exposed to hypobaric hypoxic conditions for 14 days by housing them in a specially designed hypobaric chamber, as previously described [20]. The chamber was depressurised over the course of 2 days to 550 mbar [55 kPa or 413 mm Hg, equivalent to FiO\(_2\) (percentage oxygen in inspired air) = 10%]. A further 10 age-matched mice of each genotype were maintained in the same room breathing air at atmospheric pressure (FiO\(_2\) = 21%). All mice were allowed free access to standard rodent chow and water throughout the study and kept under 12-hour light/dark cycles.

**Haemodynamic Studies**

Anaesthesia was induced with 2–4% halothane and maintained with 1.5% halothane using a mix (1 part:3 parts) of NO\(_2\) and high-flow O\(_2\). Pressure and heart rate measurements were performed and analysed as previously described [25]. Systemic arterial pressure was measured via a cannula (Portex, 0.75 mm OD) inserted into the right carotid artery. A 25-gauge needle was advanced into the right ventricle (RV) via a transdiaphragmatic approach for measurement of right ventricular pressure (RVP). At the end of the experiment, mice were killed by lethal overdose of halothane. The heart was removed, blotted dry of blood and weighed. The right lung was placed in formal saline (1 part 37% formaldehyde and 9 parts 0.9% saline) for histology and third-order PAs dissected from the left lung for wire myography experiments.

**Assessment of RV Hypertrophy**

Hearts were dissected clean of pericardial tissue, blotted dry and the atria and great vessels removed to the plane of the atrioventricular valves. The RV free wall was dissected free from the left ventricle and septum (LV+S) and weighed. The ratios of RV/ body weight (BW), RV/(LV+S) and RV/total ventricles (TV) were calculated [25].

**Lung Histology**

The right lung was embedded in paraffin and 10-μm sections stained with Miller’s elastin stain [26] and with picrosirius red for collagen [27]. Sections were microscopically assessed for muscularisation of small PAs (25–100 μm external diameter) associated with an airway distal to the respiratory bronchiole. Arteries were considered muscularised if they possessed a distinct double-elastic lamina visible for at least half the diameter of the vessel in cross-section (fig. 2). The percentage of vessels containing double-elastic lamina was calculated as the number of muscularised

\[ \text{ET}_B \text{ in Pulmonary Hypertension} \]
vessels/total number of vessels counted per section × 100. Three sections from each right lung were assessed for every mouse. A total of 6 mice per group were analysed.

**In vitro Wire Myography**

Third-order PAs (first interlobar; approx. 300 μm internal diameter) from the left lung were cut to yield two 2 mm-long segments, which were then mounted onto a wire myograph. Vessels were bathed in Krebs buffer solution (118.4 mM NaCl; 25 mM NaHCO₃; 4.7 mM KCl; 1.2 mM KH₂PO₄; 0.6 mM MgSO₄; 2.5 mM CaCl₂; 11 mM glucose; pH 7.4) at 37°C and constantly bubbled with 16% O₂/5% CO₂. Tension was applied to give transmural pressures equivalent to 12–14 mm Hg for controls and 30–33 mm Hg for hypoxic animals. These pressures are similar to those experienced by pulmonary vessels in vivo in rodents under similar hypobaric conditions [28, 29]. Following equilibration, PA rings were constricted twice with 50 mM KCl solution. Cumulative concentration-response curves were constructed for ET-1 (10⁻¹⁵ to 10⁻⁷ M) following a 30-min incubation with 100 μM N-nitro-L-arginine methylester (L-NAME) or vehicle. All responses were expressed as a percentage of the maximal KCl-induced constriction.

**Drugs**

Halothane, formalin and L-NAME were all purchased from Sigma-Aldrich (Gillingham, UK). ET-1 was purchased from Merck Chemicals Limited (Nottingham, UK).

**Data Analysis and Statistical Procedures**

Data are expressed as means ± SEM. Statistical comparisons were made by two-way ANOVA. When significance was attained (p < 0.05), differences were established using the Bonferroni multiple comparison test. In the myography studies, pEC₅₀ values were calculated from concentration-response curves by graphical interpolation (GraphPad Prism 4.0).

**Results**

**Haemodynamic Studies**

Systemic mean arterial blood pressure was not significantly different between EC ET₄⁻/⁻ mouse and controls under either normoxic (ET₄¹¹⁻⁻ 99 ± 4 mm Hg; EC ET₄⁻/⁻
ETB in Pulmonary Hypertension

ETB in Pulmonary Hypertension

**Table 1.** Indices of right ventricular hypertrophy in ETB<sup>ff</sup> and EC ETB<sup>−/−</sup> mice under normoxic conditions or following 14 days of hypobaric hypoxia

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RV/(LV+S)</th>
<th>RV/TV</th>
<th>RV/BW</th>
<th>RV/(LV+S)</th>
<th>RV/TV</th>
<th>RV/BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxic</td>
<td>0.25 ± 0.009</td>
<td>0.20 ± 0.006</td>
<td>0.80 ± 0.065</td>
<td>0.22 ± 0.009</td>
<td>0.18 ± 0.006</td>
<td>0.75 ± 0.047</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>0.28 ± 0.012</td>
<td>0.22 ± 0.007</td>
<td>1.03 ± 0.083*</td>
<td>0.29 ± 0.017*</td>
<td>0.22 ± 0.010*</td>
<td>0.98 ± 0.045*</td>
</tr>
</tbody>
</table>

Values are means ± SEM (10 animals/group). *p < 0.05 for comparison between normoxic and hypoxic animals within each genotype.

101 ± 4 mm Hg) or hypoxic conditions (ETB<sup>ff</sup> 93 ± 4 mm Hg; EC ETB<sup>−/−</sup> 103 ± 4 mm Hg), as previously reported [23]. No difference in heart rate was observed. Although systolic RVP was similar between genotypes under normoxic conditions, following 2 weeks of hypoxia, systolic RVP in EC ETB<sup>−/−</sup> mice was significantly elevated compared to hypoxic ETB<sup>ff</sup> controls (fig. 1a).

**Right Ventricular Hypertrophy**

Relative right ventricular mass, as measured by RV/(LV+S) and RV/TV ratios, tended to be lower in normoxic EC ETB<sup>−/−</sup> mice compared to normoxic ETB<sup>ff</sup> controls. Body weight fell by approximately 3.4 g in both genotypes following 2 weeks of hypoxia. Both genotypes demonstrated a significant increase in RV/BW ratio when exposed to hypoxia. However, only hypoxic EC ETB<sup>−/−</sup> mice demonstrated a significant increase in RV/(LV+S) ratio and RV/TV, suggesting a preferential increase in RV mass (fig. 1c; table 1).

**Vascular Morphology**

Under normoxic conditions, the percentage of muscularised vessels was similar in EC ETB<sup>−/−</sup> and ETB<sup>ff</sup> mice. Following hypoxia, muscularisation was observed in a significantly greater proportion of vessels from EC ETB<sup>−/−</sup> mice compared to controls (fig. 1b, 2).

**Myography**

Neither genotype nor FiO2 influenced the E<sub>max</sub> or pEC<sub>50</sub> of PAs to ET-1 (table 1). Treatment with L-NAME did not significantly alter the maximum constriction or tissue sensitivity to ET-1 in either genotype (table 2).
Discussion

This study demonstrates that in response to 14 days of hypobaric hypoxia, selective loss of EC ETB results in an exaggerated increase in systolic RVP, an increase in RV mass and an increase in the proportion of muscularised small PAs. However, we did not observe systemic hypertension, implying a selective effect of loss of EC ETB in the pulmonary vasculature.

After 14 days of hypoxia, we observed no increase in systolic RVP in control ETB/−/− mice that were of the same genetic background as EC ETB –/– mice. The development of hypoxia-induced PAH differs between mice of different strains, although even relatively resistant mice have been shown to develop raised systolic RVP after 4 weeks of hypoxia [30]. It is likely that our control ETB/−/− mice would have developed elevated systolic RVP after such a prolonged period of hypoxic exposure. However, we can conclude that loss of EC ETB either accelerated the increase in systolic RVP in this strain or enabled PAH to develop in a strain resistant to the effects of hypoxia.

There are several possible mechanisms that may underlie the exaggerated increase in RVP in EC ETB –/– mice. First, EC ETB vasodilator pathways are likely to be an important protective mechanism that limits the development of PAH during chronic hypoxia. In vitro studies of rat pulmonary microvascular ECs demonstrate that an increase in shear stress increases ETB expression and enhances ET-1-mediated ETB-dependent eNOS activation [31]. Lungs from rescued ETB-deficient rats also demonstrate an exaggerated pressor response to ET-1 [32], due in part to reduced NO and PGI2 production [17]. Studies of eNOS over-expressing [33] and eNOS KO mice [34, 35] have revealed that a reduction in EC-derived NO increases vascular tone and muscularisation of PAs. We have previously reported endothelial dysfunction with decreased NO bioavailability in the absence of systemic hypertension in the aortae of EC ETB –/– mice [23]. Thus, impaired EC ETB-mediated NO/PGI2 release from pulmonary resistance vessels during hypoxia may have contributed to the development of PAH. We also observed an exaggerated increase in the number of muscularised small pulmonary vessels in EC ETB –/– mice following hypoxia. Thus, loss of ETB-mediated NO release may also have had a permissive effect on hypoxia-induced vessel muscularisation.

Second, EC ETB –/– mice have elevated plasma ET-1 concentration [23] that may directly exert pressor and mitogenic actions to further promote the development of PAH. Studies in our laboratory demonstrate that clearance of ET-1 is impaired and plasma ET-1 increased approximately 4-fold in these mice [23]. Increased plasma ET-1 has also been reported in patients with PAH [6, 36, 37], though this may reflect increased production rather than impaired pulmonary clearance [38]. Loss of ETB signalling has also been reported to increase ECE-1 mRNA expression [39], which may further contribute to an increase in ET-1. However, the limited experimental evidence available suggests that an isolated increase in ET-1 is insufficient to cause PAH. Under normoxic conditions, rats chronically infused with ET-1 by subcutaneous pump do not develop raised systolic RVP [40]. Similarly, transgenic mice that over-express preproET-1 have elevated plasma ET-1, exhibit pulmonary inflammation and fibrosis, but do not develop PAH, even when exposed to mild hypoxia (FiO2 16%) [41]. Thus, although an increase in ET-1 may contribute to many of the pathological processes associated with PAH, concomitant loss of NO/PGI2-mediated vasodilator pathways is likely to be necessary for PAH to develop.

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Table 2. Potency and maximum effect of ET-1 in PA rings from EC ETB –/– and ETB/ff mice

<table>
<thead>
<tr>
<th>Condition</th>
<th>ETB/ff  pEC50</th>
<th>Emax (nm)</th>
<th>EC ETB –/–  pEC50</th>
<th>Emax (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxic</td>
<td>9.2 ± 0.1</td>
<td>121.9 ± 4.8</td>
<td>7</td>
<td>8.8 ± 0.1</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>10.1 ± 0.2</td>
<td>116.8 ± 9.0</td>
<td>5</td>
<td>9.6 ± 0.1</td>
</tr>
<tr>
<td>Normoxic + L-NAME</td>
<td>9.7 ± 0.2</td>
<td>126.3 ± 3.4</td>
<td>7</td>
<td>9.1 ± 0.9</td>
</tr>
<tr>
<td>Hypoxic + L-NAME</td>
<td>10.1 ± 0.2</td>
<td>124.3 ± 7.1</td>
<td>6</td>
<td>10.5 ± 0.1</td>
</tr>
</tbody>
</table>

Emax is expressed as a percentage of the maximal contractile response to 50 mM KCl solution.
Although expression of Tie2 was thought to be exclusively restricted to ECs, Tie2-positive monocytes have now been identified [42, 43]. Thus, Cre-Lox-mediated ablation of the ETB gene may have occurred in such inflammatory cells, as well as in ECs, potentially complicating the interpretation of the phenotype of the EC ETB−/− mice. Although no inflammatory infiltrate was seen in the lung sections from the hypoxic EC ETB−/− mice, further studies to clarify the role of such macrophages in the development of PAH are required.

We found no difference in the maximal response or sensitivity of 3rd order PAs to ET-1 in EC ETB−/− mice. The absence of any change in ET-1-mediated constriction in 3rd order PAs to ET-1 in EC ET B−/− mice, further studies to clarify the role of such macrophages in the development of PAH are required.

In conclusion, this study indicates that EC ETB play an important protective role during the prolonged hypoxia that limits vascular remodelling and the development of pulmonary hypertension.

Acknowledgements

N.F.K. was a British Heart Foundation Junior Research Fellow (FS/03/006/15198). A.J.B. was funded by a Wellcome Trust Clinical Research Fellowship (055891/Z/98/Z/DM/H/1). This work was supported by the Wellcome Trust Cardiovascular Research Initiative (Grant No. 065901/Z/01/Z&A). Y.D. and I.M. were funded by the BBSRC and BHF, respectively.

References


Several studies in mice report weight loss following exposure to hypoxia, an effect that is incompletely understood but may involve altered expression of genes regulated by hypoxia-inducible factor α [46]. However, we saw no difference in weight loss (approx. 3.4 g) between controls and EC ETB−/− mice. Although weight loss contributed to the increase in RV/BW ratio in both groups, we only observed an absolute increase in RV mass [as determined by RV/(LV+S) ratio and RV/TV ratio] in EC ETB−/− mice. This increase is, therefore, likely to reflect a true RV hypertrophic response to the increased PAP in EC ETB−/− mice, rather than any disproportionate weight loss in KOs.

In conclusion, this study indicates that EC ETB play an important protective role during the prolonged hypoxia that limits vascular remodelling and the development of pulmonary hypertension.

ETB in Pulmonary Hypertension

J Vasc Res 2010;47:16–22