Deletion of Endothelial Cell Endothelin B Receptors Does Not Affect Blood Pressure or Sensitivity to Salt

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Abstract—Endothelin B receptors in different tissues regulate diverse physiological responses including vasoconstriction, vasodilatation, clearance of endothelin-1, and renal tubular sodium reabsorption. To examine the role of endothelial cell endothelin B receptors in these processes, we generated endothelial cell-specific endothelin B receptor knockout mice using a Cre-loxP approach. We have demonstrated loss of endothelial cell endothelin B receptor expression and function and preservation of nonendothelial endothelin B receptor-mediated responses through binding and functional assays. Ablation of endothelin B receptors exclusively from endothelial cells produces endothelial dysfunction in the absence of hypertension, with evidence of decreased endogenous release of NO and increased plasma endothelin-1. In contrast to models of total endothelin B receptor ablation, the blood pressure response to a high-salt diet is unchanged in endothelial cell–specific endothelin B receptor knockouts compared with control floxed mice. These findings suggest that the endothelial cell endothelin B receptor mediates a tonic vasodilator effect and that nonendothelial cell endothelin B receptors are important for the regulation of blood pressure. (Hypertension. 2006;48:286-293.)

Key Words: endothelin ■ mice ■ endothelium ■ receptors, endothelin ■ vasodilation

Endothelin-1 (ET-1) was initially described as a potent vasoconstrictor and potential mediator of hypertension. More recently, attention has focused on how ET-1 generated within the kidney may promote natriuresis and diuresis and thus contribute to a lowering of blood pressure (BP). ET-1 acts through 2 types of receptors, endothelin receptor type A (ETₐ) and endothelin receptor type B (ET₈). Activation of ETₐ and ET₈ on vascular smooth muscle cells results in vasoconstriction. In contrast, vascular endothelial cells (ECs) exclusively express the ET₈ subtype and mediate vasodilatation. ET₈ has been proposed as the target receptor through which collecting duct (CD)-derived ET-1 regulates natriuresis and diuresis. However, ET₈ may also influence BP through regulation of peripheral vascular tone, renal hemodynamics, and clearance of circulating ET-1. The autocrine/paracrine nature of ET-1 signaling, the close interdependence between renal tubular function and intrarenal blood flow, and the modulation of peripheral vascular tone by ET-1 have made the precise mechanisms by which ET₈ on different cell types contribute to the regulation of BP and natriuresis difficult to define, although evidence of decreased endogenous release of NO and increased plasma ET-1 was observed in models of hypertension. The present study was designed to test the hypothesis that the endothelial cell endothelin B receptor mediates a tonic vasodilator effect and that nonendothelial cell endothelin B receptors are important for the regulation of blood pressure.

Methods

The general approach to producing cell type–specific knockout (KO) of ET₈ through Cre-loxP–mediated recombination has been described previously. A 129/Ola mouse genomic DNA library was screened using an ET₈ cDNA probe, and a 17.7-kb genomic fragment (clone p9261) was cloned, partially sequenced, and mapped. Sequencing of exons 3, 4, and 6 from p9261 showed complete concordance with available published sequences of the ET₈ receptor cDNA.

Construction of Replacement Targeting Vector and Gene Targeting

The replacement vector was based on the LoxP-ROSA26 plasmid. A 956-bp region of pROSA26, spanning exons 3 and 4 of the ET₈ receptor gene, was amplified by PCR (oligo 1: TCCGGATCCAGAGGGTCAGCCG; oligo 2: TCACTAGTGATGGTACCTCACAG) and subcloned into the BamHI site of the vector, such that loxP sites flanked (floxed) the coding regions. A 3.8-kb loxP-flanked MC1: thymidine kinase:phosphoglucokinase:neomycin phosphotransferase (LTNL) selection cassette was then subcloned into the XbaI site 3’ of the PCR product. The 3’ homology arm consisted of the Hpal-SacI fragment of pROSA26 (exon 5, intron 5, and 56 bp of exon 6), subcloned...
between the XbaI and NotI sites 3' of the selection marker, to produce the plasmid p\(\beta H9261\) P2–6LTNL. The 5' homology arm was composed of an 11.9 kb XhoI–NaeI fragment of intron 2. This fragment was subcloned into p\(\beta H9261\) P2–6LTNL upstream of the 5' loxP site. The completed construct, p\(\beta H9261\) PW [Figure 1a (i)], thus featured a deletion of 1.1 kb from intron 2 between the NaeI site and a point 288 bp upstream of exon 3. p\(\beta H9261\) PW was linearized by XhoI/NotI digestion and electroporated into E14Tg2a embryonic stem (ES) cells. G418-resistant clones were selected as described15 and DNA extracted for PCR analysis. Two positive controls were run for each DNA sample: oligo 3 (GCCTCT-GTTCCACATACACTTCATTC) and oligo 4 (GTAGAAACTGAA-CAGCCACCAATC) amplified an 1.4-kb sequence from within the integrated replacement vector; oligo 5 (TTTGGGTGGTCTCTGTGGTTCTGG) and oligo 6 (GAACAGCTGCCACTC) amplified a 2.4-kb sequence from the wild-type (W) allele; oligo 3 and oligo 6 amplified any specific integration event (1.2 kb). Specific recombination at the 3' end was confirmed in HindIII-digested DNA with a 1.2-kb external probe complementary to intron 6/exon 7 (Figure 1a and 1b). Cells from targeted clones were electroporated with a Cre recombinase expression plasmid (pMC-Cre16) to remove the selection marker. Gancyclovir-resistant clones were analyzed by PCR. Oligo 5 and oligo 7 (AGCCATAAAGTCACAGCCATTC) amplified a 420-bp sequence spanning the 3' loxP site; oligo 8 (TCATGTTGAATGAGACAGAC) and oligo 9 (CTTAGAGCACAAAGACTCAGCAC) amplified a 500-bp sequence spanning the 5' loxP site. PCR products were sequenced to confirm loxP sequences. Targeted ES cell clones were injected into C57BL/6 blastocysts and transferred into C57BL/CBA foster mothers. Chimeras were bred to BKW females, and progeny genotyped by Southern blot of EcoRI–digested DNA using an internal probe [Figure 1a (iii)] and by PCR using oligos 7 and 8 (Figure 1a and 1c). These PCR oligos flanked an 1.1-kb sequence spanning both loxP sites in the targeted (flox) allele and a 2.2-kb sequence in the W allele.

**Figure 1.** Targeting of the ET\(_B\) receptor gene and genotyping strategy. a, Structure of the targeting vector p\(\beta PW\) (i), and ET\(_B\) receptor gene (ii): f, exons; \(\mathcal{L}\), loxP sites; MC1, Mario Cappeci-1 promoter; TK, thymidine kinase gene; PGK, phosphogluco kinase promoter; Neo, neomycin resistance gene. The positions of internal and external probes used for Southern analysis and of PCR oligonucleotides used for genotyping are illustrated. After homologous recombination events in ES cells, the selection marker was removed by Cre recombinase-mediated excision. The structures of the targeted (Flox) allele (iii) and of the flox allele after in vivo Cre-mediated recombination in ECs (iv) are illustrated. b, Southern blot hybridization analysis with external probe of HindIII-digested genomic DNA from neomycin-resistant targeted (3B3 and 9D3) and nontargeted (2D1 and E14) ES clones. c, PCR analysis (oligos 7 and 8) of genomic tail DNA from W (Ln 1) heterozygous (Ln 2) and homozygous (Flox/Flox) ET\(_B\) receptor mice (Ln 3). In the presence of the Tie2-Cre transgene, heterozygous (Ln 4) and flox/flox (Ln 5) ET\(_B\) receptor mice demonstrate an additional band corresponding to the recombinated ET\(_B\) receptor gene allele amplified from tail EC. Ln 6, size marker.

**Generation of EC-Specific ET\(_B\) KO Mice**

Homozygous (Flox/Flox) ET\(_B\) mice (background: 50% 129/Ola and 50% BKW) were crossed with Tie2-Cre transgenic mice17 (C57BL/6SJLF1 background). Because of previous reports18,19 of recombination events within germ cells of heterozygous floxed female mice featuring the Tie2-Cre transgene, the transgene was introduced through the male germ line only. Offspring from a single pair in which germ cell recombination was identified were deliberately intercrossed to assess whether a recombination event within the ET\(_B\) gene was sufficient to prevent expression of functional ET\(_B\). Genotyping to identify flox, W, and recombinated alleles was performed by Southern blot and PCR using oligos 7 and 8, as described earlier. The Tie2-Cre transgene was detected by PCR as described.17 All of the mice were given free access to tap water and standard (0.76% NaCl) mouse chow (Special Diet Services, United Kingdom) until implantation of telemeters or in vitro experiments. Mice were housed according to United Kingdom Home
Office recommendations at 22°C with 12-hour diurnal light/dark cycles. All of the procedures were performed under the provisions of the Animals in Scientific Procedures Act (1986) and with the approval of local ethics committees.

**Drugs and Solutions**

ABT627 (ET<sub>A</sub> selective antagonist) and A192621 (ET<sub>B</sub> selective antagonist) were provided by Abbott Pharmaceuticals, United Kingdom. N<sub>ω</sub>-nitro-L-arginine methyl ester hydrochloride (L-NAME), carbachol, acetylcholine (ACh), 2,2-dimethyl-2-aminoethanimine (DETA-NO), and norepinephrine (NE) were supplied by Sigma Aldrich, Germany. Sarafotoxin 6c (S6c; ET<sub>B</sub> selective agonist) was purchased from Calbiochem.

**[<sup>125</sup>I]-ET-1 Binding by Pulmonary EC**

Lungs were removed and collagenase dispersed as described previously. Protein concentration was measured by the Bradford method, and an aliquot of cells equivalent to 50 µg/mL of membrane protein was used for binding reactions. [<sup>125</sup>I]-ET-1 (7.4 × 10<sup>7</sup> TBq/mmol; American Biosciences, United Kingdom) in HEPES-Ringer/0.2% BSA buffer was added and the volume adjusted to 1 mL with additional buffer. ECs were precipitated with biotinylated Griffonia simplicifolia isolecitin B4 (GSL I-B4; Vector Laboratories, United Kingdom) immobilized on streptavidin-coated magnetic beads (CELLLection Biotin Binder kit, Dynal Ltd, United Kingdom; 10<sup>7</sup> beads/mL). Radioactivity was measured in a gamma counter and expressed as counts per minute per 50-µg membrane protein. Receptor-specific binding was calculated by subtracting binding in the presence of 3 × 10<sup>7</sup> M unlabeled ET-1, 3 × 10<sup>-3</sup> M A192621, or 3 × 10<sup>-3</sup> M ABT627 from total [<sup>125</sup>I]-ET-1 binding.

**Plasma ET-1 Measurement**

Under sodium pentobarbitone anesthesia, 1 mL of blood was withdrawn by direct cardiac puncture in 8- to 12-week-old mice fed standard chow (0.76% NaCl) from weaning. Plasma ET-1 concentration was measured by radioimmunoassay according to the manufacturer’s instructions (Peninsula Laboratories Europe Ltd, United Kingdom).

**In Vitro Measurement of Vascular and Tracheal Responses**

Isometric contraction and relaxation was measured in 2-mm aortic and tracheal rings. Eight- to 10-week-old male mice were fed standard chow from weaning and culled by CO<sub>2</sub> asphyxiation. Vessels were mounted in myograph chambers and incubated in physiological saline solution (PSS) at 37°C, constantly bubbled with 95%/5% O<sub>2</sub>/CO<sub>2</sub>. A tension of 1.5 g (7.36 mN) for aortic rings and 0.5 g (2.45 mN) for tracheal rings was applied and vessels equilibrated for 1 hour. Vessels were contracted 3 times with PSS + 125 mmol/L potassium. For aortic rings, a further contraction with 3 × 10<sup>-7</sup> M NaCl was followed by relaxation with 10<sup>-6</sup> M ACh to assess viability of the endothelium. Vessels that failed to relax by >50% were rejected as evidence of endothelial damage during mounting. Concentration response curves (CRCs) were constructed for NE, NE + 10<sup>-5</sup> M L-NAME, ACh, ACh + 10<sup>-5</sup> M A192621, or vehicle (0.1% DMSO) and for DETA-NO. Vasodilatation was measured in aortas contracted with NE to 80% of the maximal NE-induced response (NE<sub>max</sub>) and relaxation expressed as percentage of NE<sub>max</sub>. Aortic relaxation to 10<sup>-7</sup> M S6c was measured after 30 minutes of incubation with either 10<sup>-7</sup> M A192621 or vehicle. For tracheas, CRCs were constructed for carbocoll (<10<sup>-8</sup> M to 3 × 10<sup>-3</sup> M; data not shown) and S6c after incubation with either 10<sup>-7</sup> M A192621 or vehicle. All of the contractile responses were expressed as the percentage of final PSS + 125 mmol/L of potassium-induced contraction.

**Telemetry BP Recording**

Male flox/flox Tie2-Cre and flox/flox litter mate control mice (12 to 16 weeks; 24 to 36g) were housed in single cages for 1 week before surgery and fed from weaning on standard mouse chow prepared as a gel to allow accurate measurement of intake. Under isoflurane anesthesia, a telemetry catheter was inserted into the left carotid artery and the transmitter device (Data Sciences) secured in the left flank as described previously. Mice continued standard gel chow for 7 days after surgery before commencement of high (7.6% NaCl + H<sub>2</sub>O ad libitum), medium (2.5% NaCl + 1% saline ad libitum), or low (0.76% NaCl + H<sub>2</sub>O ad libitum) salt diets. A cohort of mice at the end of the study additionally received A192621 (30 mg/kg per day) in their gel. This dose has been shown previously to inhibit ET<sub>A</sub>-mediated hemodynamic responses in the rat. All of the dietary and drug treatments were maintained for 7 days. Systolic and diastolic BP and heart rate were recorded as described previously and analyzed using the Powerlab data acquisition system. All of the BP results represent the mean of values recorded over the final 24 hours of each dietary/drug intervention.

**Effect of Age on Heart:Body Weight Ratio**

Hearts were dissected from young mice (8 to 12 weeks old) and from older animals (52 to 60 weeks old) fed 0.76% NaCl diet from weaning. The ratio of total ventricular weight (TVW) in milligrams: body weight (BW) in grams was calculated.

**Statistical Analysis**

MAP data were compared by 1-way ANOVA with Newman–Keuls multiple comparison post test analysis. Pulmonary EC [<sup>125</sup>I]-ET-1 binding and TVW:BW ratio were compared using an unpaired Student t test. CRCs were constructed by linear regression analysis and compared by 2-way ANOVA. EC<sub>50</sub> and Emax values, S6c-induced vasodilatation, and plasma ET-1 concentrations were each compared by 1-way ANOVA with Dunnett’s multiple comparison post test analysis. Dose ratios were calculated as EC<sub>50</sub> in the presence of antagonist divided by EC<sub>50</sub> in the absence of antagonist. Values were accepted as statistically significant when <0.05. All of the analyses were performed using Graphpad Prism 3.0 software.

**Results**

**Generation of EC-Specific ET<sub>B</sub> KO Mice**

A fragment of the ET<sub>B</sub> receptor gene was replaced with a targeting vector featuring loxp sites flanking exons 3 and 4 and a loxp-flanked selection cassette (Figure 1a). Homologous recombination was detected in 6 of 97 colonies analyzed (Figure 1b). Of these, 3 clones underwent transfection with pMC-Cre, and 11 of 294 of the resultant gancyclovir-resistant clones were found to retain loxp sites flanking exons 3 and 4 but to lack the selection cassette (floxed ET<sub>B</sub> allele). Sequence analysis confirmed no mutations within loxp sites or coding regions. Germ line transmission was achieved from a single clone and offspring confirmed as heterozygous for the floxed allele by PCR (Figure 1c) and Southern analysis. Flox/flox mice were bred by intercross and backcross of heterozygotes. No abnormality of pigmentation, gut development, litter size, or mortality rate was observed in flox/flox mice, and no deviation from Mendelian distribution of alleles was seen in intercross experiments. Flox/flox mice were crossed with Tie2-Cre transgenic mice. A single PCR reaction (Figure 1c) permitted differentiation between W, flox, and recombinated alleles. In flox/W Tie2-Cre mice, a 186-bp band was detected in addition to the flox and W allele, consistent with recombination in a subgroup of cells. Sequencing confirmed the predicted sequence of the recombinated ET<sub>B</sub> allele. Southern analysis, in contrast to PCR, did not detect the recombinated allele, presumably because of the small proportion of tail DNA derived from EC. Male flox/W Tie2-Cre mice were crossed with flox/flox females to produce flox/flox Tie2-Cre mice (EC-specific ET<sub>B</sub> KOs). Again, the recombinated allele
was detected by PCR but not Southern analysis in homozygous mice (Figure 1c).

**Generation of Complete ET\textsubscript{B} KO (Piebald) Mice**

We exploited the phenomenon of recombination events in germ cells of female flox/W Tie2-Cre mice to produce offspring homozygous for the recombined allele. Consistent with functional ET\textsubscript{B} deficiency, these mice exhibited white coat spotting (piebald appearance) because of the regional absence of neural crest-derived melanocytes and died shortly after weaning from intestinal obstruction.\textsuperscript{25} In contrast to EC-specific KOs, both PCR and Southern analysis detected the recombined allele in piebalds. ET\textsubscript{B}-Mediated ET-1 Binding Is Decreased in ECs of EC-Specific ET\textsubscript{B} KO Mice

ET\textsubscript{B}-mediated binding of [\textsuperscript{125}I]-ET-1 was significantly decreased in EC-enriched pulmonary cells from flox/flox Tie2-Cre mice (P<0.001). Total and ET\textsubscript{A}-mediated binding did not differ between groups (n=3 in each group).

**Figure 2.** EC ET\textsubscript{B}-mediated binding is decreased in flox/flox Tie2-Cre mice. ET\textsubscript{B}-dependent binding of [\textsuperscript{125}I]-ET-1 was significantly decreased in EC-enriched pulmonary cells from flox/flox Tie2-Cre mice (P<0.001). Total and ET\textsubscript{A}-mediated binding did not differ between groups (n=3 in each group).

ET\textsubscript{B}-Mediated Vasodilatation Is Impaired in EC-Specific ET\textsubscript{B} KO Mice

S6c (selective ET\textsubscript{A} agonist) produced vasodilatation of aortic rings in all genotypes (Figure 3a). The vasodilator response was significantly attenuated in flox/flox Tie2-Cre mice compared with W/W and single transgenic litter mates (P<0.05). Previous incubation with A192621 significantly inhibited S6c-induced vasodilatation in W/W and single transgenic mice (P<0.05 versus no antagonist).

**Figure 3.** Selective loss of in vitro EC ET\textsubscript{B}-mediated vasorelaxation in flox/flox Tie2-Cre mice. a, In vitro vasodilatation to 10^{-7} M S6c was significantly impaired in aortic rings from flox/flox Tie2-Cre mice (P<0.05) and in control animals after selective ET\textsubscript{B} antagonism (P<0.05 vs no antagonist; n=10 in all groups). b, In vitro S6c-induced ET\textsubscript{B}-mediated tracheal SMC contraction was normal in flox/flox Tie2-Cre mice (P=0.21 vs W/W; n=16 in each group) but absent in piebald mice (P<0.0001 vs W/W and flox/flox Tie2-Cre; n=10). Previous incubation with A192621 significantly inhibited contractile responses to S6c in flox/flox Tie2-Cre and W/W tracheal rings (E\textsubscript{max} 5.36±1.92 [data not illustrated on graph]; P<0.0001 vs no antagonist; n=16 in all groups).

ET\textsubscript{B}-Mediated Responses in Non-ECs Are Maintained in EC-Specific ET\textsubscript{B} KO Mice

No abnormality of gut development or pigmentation was observed, consistent with functional ET\textsubscript{B} expression on neuroblasts and melanoblasts, respectively. S6c-induced tracheal SMC contraction (Figure 3b) was unaltered in EC-specific ET\textsubscript{B} KO mice. In contrast, tracheas from piebald mice (complete ET\textsubscript{B} KO) demonstrated no contractile response to S6c. Previous incubation with A192621 significantly attenuated maximal S6c-induced contraction in W/W and flox/flox Tie2-Cre mice (P<0.0001 versus no antagonist).

Plasma ET-1 Concentration Is Increased in EC-Specific ET\textsubscript{B} KO Mice

Plasma ET-1 was significantly increased in flox/flox Tie2-Cre mice compared with controls, consistent with the proposed role of EC ET\textsubscript{B} as clearance receptors for ET-1 (Figure 4).

BP and Heart Rate Are Unaffected by EC-Specific ET\textsubscript{B} KO

Increasing dietary salt resulted in an increase in BP in both groups of mice (n=6 to 13). However, at no point during each salt treatment was there a difference in systolic, mean, or diastolic BP between control and EC-specific ET\textsubscript{B} KO mice.
Treatment for 7 days with a selective pharmacological ET$_B$ antagonist during high-salt diet resulted in further increases in BP (flox/flox 159.7±4.0; P<0.05 versus 7.6% NaCl; n=7), although the increase just failed to reach statistical significance in flox/flox Tie2-Cre mice (154±2.9; P=0.06 versus 7.6% NaCl; n=6; Figure 5). Heart rate (=570 bpm) was not influenced by salt intake, genotype, or pharmacological ET$_B$ antagonism (data not shown).

Aged EC-Specific ET$_B$ KO Mice Do Not Develop Cardiac Hypertrophy on Normal Salt

TVW/BW ratio (mg/g) did not differ between control and EC-specific KO mice in either age group, consistent with the lack of BP difference observed during telemetric studies (young mice flox/flox 3.56±0.13, flox/flox Tie2-Cre 3.74±0.22; P=0.48; n=12 to 13; aged mice flox/flox 4.13±0.29, flox/flox Tie2-Cre 4.59±0.32; P=0.31; n=8 to 9).

Impaired ACh-Induced Vasodilatation in Aortas From EC-Specific ET$_B$ KO Mice and W Aortas Pretreated With ET$_B$ Antagonist

EC-specific ET$_B$ KO resulted in significantly impaired ACh-induced vasodilation (Figure 6a). Previous incubation with A192621 impaired ACh-induced vasodilation in W/W (P<0.001) but not flox/flox Tie2-Cre rings. Loss of EC ET$_B$ did not influence vasodilation in response to the endothelium-independent vasodilator DETA-NO (Figure 6b). To examine whether EC-specific ET$_B$ KO mice demonstrated decreased endogenous NO release, we studied vasoconstrictor responses to NE before and after exposure to l-NAME (Figure 6c and 6d).

Figure 4. Plasma ET-1 concentration. Plasma ET-1 concentration was significantly increased in flox/flox Tie2-Cre mice compared with W and single transgenic littermate controls (P<0.001; n=6 in each group).

Figure 5. Effects of EC-specific ET$_B$ KO and salt on BP. MAP was measured in conscious, unrestrained mice fed for 7 days with 0.076%, 0.76%, 2.5%, and 7.6% NaCl diet and after treatment with A192621 for 7 days. Values are MAP during the final 24 hours of dietary or drug treatment (n=6 to 13 per group). There were no differences in BP between genotypes during any of the dietary/drug treatments.

NE-induced contraction was significantly enhanced in both groups of mice (P<0.0001) after NO synthase inhibition. However, the magnitude of the increased contractile response after l-NAME was significantly greater in W than EC-specific KOs (dose ratio before/after l-NAME; W/W, 10.1; flox/flox Tie2-Cre, 2.5).

Discussion

We describe a novel transgenic line in which ET$_B$ expression is regulated by cell type–specific production of Cre recombination. We have demonstrated that the floxed ET$_B$ receptor gene functions normally but that expression of Cre recombinase in flox/flox ET$_B$ mice effectively inactivates ET$_B$ expression from both alleles. EC-specific inactivation of ET$_B$ results in increased plasma ET-1 concentration and impaired endothelium-dependent vasodilatation but not hypertension. In contrast to models of rescued complete ET$_B$ KO,$^8$26 and CD-specific ET-1 KO,$^3$ EC-specific KO mice do not demonstrate increased sensitivity to high-salt diets. This suggests that the likely target(s) for CD-derived ET-1 in the kidney that determine the salt-sensitive phenotype are ET$_B$ located on non-EC, most likely those on inner medullary CD cells.$^27$ Consistent with previous reports,$^26$ the salt-sensitive phenotype seems to be independent of endothelial dysfunction resulting from ET$_B$ deficiency.

We have demonstrated that loss of EC ET$_B$ does not influence BP, regardless of dietary salt intake. After a high-salt diet, rescued ET$_B$-deficient rats$^8$ and mice$^{26}$ and ET$_B$ antagonist–treated rats$^9$ develop grossly elevated BP compared with controls. We observed a clear relationship between salt intake and BP in our mice, with significant increases in BP observed when dietary NaCl intake exceeded =2.5%. Modest hypertension during salt loading has been described in several mouse strains, particularly those featuring 2 copies of the renin gene. The genetic background of our mice features DNA derived from 129/Ola strain (2 renin genes$^{28}$) and C57BL/6, a single renin gene strain that can also develop increased BP after salt loading.$^{24,29}$ Although the influence of salt in our genetic background may have obscured subtle changes in BP secondary to EC ET$_B$ KO, we still found that selective pharmacological antagonism of ET$_B$ during high-salt diet led to further increases in BP, as described previously in the rat.$^9$ Although there was no difference in BP between control and KOs during pharmacological ET$_B$ blockade, the absolute increase in BP just failed to reach statistical significance in EC-specific KOs (P=0.06). The potential effects of EC ET$_B$ KO on a salt-resistant genetic background are, thus, intriguing and merit further study.

We report that loss of endogenous ET$_B$ activity, through either EC-specific ET$_B$ KO or acute exposure to ET$_B$-selective antagonists, results in endothelial dysfunction, as assessed by ACh-induced vasodilatation. The lack of effect on vasodilatation of A192621 in EC-specific ET$_B$ KOs suggests that this is predominantly because of loss of EC ET$_B$ function and is likely related to decreased NO bioavailability, because relaxation to ACh is mainly mediated by NO in the mouse aorta.$^{30,31}$ Diminished NO bioavailability may result from loss of endogenous EC ET$_B$-mediated NO release, as suggested by the acute effect of pharmacological ET$_B$ inhibition in W vessels. In addition, the modest enhancement of NE-induced vasoconstriction after

Figure 6c. Effects of ETB gene deficiency in W and EC-specific ETB KO aortas on vasoconstrictor responses to NE before and after exposure to l-NAME (Figure 6d).
L-NAME indicates that vascular tone in EC-specific ETB KO mice is less dependent on endogenous NO release than in W mice. However, NO bioavailability may also be influenced indirectly by increased plasma ET-1. EC-specific overexpression of a prepro-ET-1 transgene increases reduced nicotinamide-adenine dinucleotide phosphate oxidase activity, superoxide production, and vascular remodeling but does not increase BP.32 In vitro studies33 suggest that the increase in reduced nicotinamide-adenine dinucleotide phosphate oxidase activity is ETA mediated and, hence, may be increased when plasma ET-1 levels are raised. However, whether superoxide contributes significantly to BP in models of exogenous34,35 or endogenous32,36 elevation of ET-1 remains controversial. Our experiments confirm the results of previous transgenic studies demonstrating that an isolated increase in plasma ET-1 does not result in hypertension in young mice.32,37,38 The magnitude of the increase in plasma ET-1 that we observed was similar to that in rescued ETB-deficient rats on a normal salt diet,8 suggesting that EC ETB is likely to be the predominant clearance receptor for ET-1.12 Although we have not analyzed prepro-ET-1 expression in EC-specific ETB KOs, we consider it unlikely that ET-1 production was increased, because EC ETB typically mediates autoinduction of prepro-ET-1 mRNA expression.39

Recent studies40 have identified that primary abnormalities of vascular smooth muscle cell tone in either peripheral resistance vessels or the renal vasculature may directly cause hypertension. This challenges the established hypothesis that primary abnormalities of renal sodium excretion are required for hypertension to develop.41 We have found that deletion of EC ETB from peripheral resistance vessels and from the renal vasculature does not alter BP, despite endothelial dysfunction and an increase in plasma ET-1 concentration. However, prolonged exposure to increased ET-1 may have caused downregulation or desensitization of ETA,42 decreasing both vascular tone and BP in EC ETB KO mice. We analyzed ETA expression by quantitative autoradiography43 and assessed functional responses to infusion of big ET-1 (1 to 10 nmol/kg) during anesthesia. ETA expression was unchanged in EC ETB KO mice, and the increase in BP observed after incremental doses of big ET-1 did not differ from that of control mice (data not shown). Thus, we consider it unlikely that downregulation or desensitization of vascular vasoconstrictor ET\textsubscript{A} activity obscured any hypertensive phenotype in EC ETB KO mice.

Two hypotheses may explain why EC-specific ETB KO mice, in contrast to mice with complete ETB deficiency, do not develop severe salt-sensitive hypertension. First, the ET-1/ET\textsubscript{B} signaling pathway regulating tubular reabsorption of sodium and water...
may be an autocrine process mediated solely by inner medullary CD cells\textsuperscript{27} and, as such, would be unaffected by loss of EC ET\textsubscript{A}. Alternatively, the effect of loss of any paracrine ET-1/ET\textsubscript{A} signaling pathway involving CD cells and medullary vasa recta EC\textsuperscript{13} that might normally regulate natriuresis by alteration of medullary blood flow\textsuperscript{11} may have been masked by changes in the glomerular filtration rate. Both ET\textsubscript{A} and ET\textsubscript{B} regulate afferent arteriolar vasconstriction, whereas EC ET\textsubscript{A} produces vasodilatation of efferent arterioles.\textsuperscript{44} The balance between ET-1-mediated vasconstriction and vasodilatation in the glomerulus may, thus, be important in determining tubular sodium delivery. Key targets for future studies will be to examine the effects of selective inner medullary CD ET\textsubscript{B} KO on sodium and water handling and to determine the influence of vascular ET\textsubscript{B} on glomerular function and medullary blood flow.

**Perspectives**

This article describes a novel murine model of ET\textsubscript{B} deficiency that permits investigation of the physiological influence of ET\textsubscript{B} expressed on a single cell type, without the confounding influence of loss of ET\textsubscript{A} from other cell types. This model will be useful for in vivo studies of ET-1/ET\textsubscript{B} physiology, particularly in organs such as the kidney, where tubular and vascular functions regulated by ET-1 are closely interlinked, making physiological studies using selective antagonists difficult to interpret.

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**Disclosures**

None.

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