Low Blood Pressure in Endothelial Cell–Specific Endothelin 1 Knockout Mice

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Abstract—Endothelin (ET) 1 is a potent vasoconstrictor peptide produced by vascular endothelial cells and implicated in various pathophysiologic states involving abnormal vascular tone. Homozygous ET-1 null mice have craniofacial and cardiac malformations that lead to neonatal death. To study the role of ET-1 in adult vascular physiology, we generated a mouse strain (ET-1<sup>flox/flox</sup>;Tie2-Cre mice) in which ET-1 is disrupted specifically in endothelial cells. ET-1 peptide levels in plasma, heart, lung, kidney, and brain homogenates were reduced by 65% to 80% in these mice. mRNA levels for ET receptors were unaltered except that the ETA receptor mRNA was upregulated in the heart. ET-1<sup>flox/flox</sup>;Tie2-Cre mice had mean blood pressures 10 to 12 mm Hg lower than genetic controls. In contrast, the blood pressure of mice systemically heterozygous for the ET-1 null allele (ET-1<sup>flox/+</sup> mice) was unchanged compared with wild-type littermates. Despite the lower basal blood pressure, acute pharmacological responses to angiotensin II, captopril, phenylephrine, bradykinin, N<sup>ω</sup>-nitro-L-arginine methyl ester, and exogenous ET-1 were normal in ET-1<sup>flox/flox</sup>;Tie2-Cre mice. These results support an essential role of endothelium-derived ET-1 in the maintenance of basal vascular tone and blood pressure. Normal pharmacological responses of ET-1<sup>flox/flox</sup>;Tie2-Cre mice suggest that the renin-angiotensin system, the adrenergic system, and NO are not significantly altered by endothelial ET-1. Taking in conjunction with other lines of genetically altered mice, our results provide evidence for a paracrine vasoregulatory pathway mediated by endothelial cell–derived ET-1 acting on the vascular smooth muscle ETA receptor. (Hypertension. 2010;56:121-128.)

Key Words: hypertension ■ endothelium ■ ET<sub>A</sub> ■ ET<sub>B</sub> ■ cre/loxP ■ blood pressure ■ gene knockout

Endothelin (ET) 1, originally identified as a potent vasoconstrictor derived from vascular endothelial cells, is implicated in the pathogenesis of many cardiovascular disorders. ET-1 is expressed by several tissues during embryogenesis and in the adult under physiological and pathophysiological conditions. However, the most abundant source of ET-1 in the adult animal is vascular endothelial cells. ET-1 acts on 2 distinct G protein–coupled receptors, termed ETA and ETB. Vascular smooth muscle cells express both ETA and ETB, which mediate a direct vasoconstrictor action of ET-1. Vascular endothelial cells express ETB, which exerts vasodilator effects via the release of NO and prostacyclin, and act as “clearance receptors” for circulating ET-1. The ET system also affects blood pressure via activity in renal collecting ducts, the brain, and the adrenal gland. The physiological function of ET-1 in the regulation of blood pressure is the subject of many pharmacological studies. However, the relative contributions of ETA and ETB, as well as the overall physiological roles of endothelial ET-1 in the regulation of the basal vascular tone and blood pressure, remain controversial.

ET-1–deficient mice have craniofacial and cardiac outflow defects and die shortly after birth. ET<sub>A</sub>-deficient mice have the identical developmental phenotype. Curiously, heterozygous ET-1 null (ET-1<sup>+</sup>/–) mice are reported to show an increase in blood pressure. However, heterozygous ET<sub>A</sub> null (ET<sub>A</sub><sup>+/–</sup>) mice do not show a significant change in blood pressure. Although ET-1- and ET<sub>A</sub>-null mice reveal the important role of ET-1/ETA interaction during development,
Materials and Methods

Generation of ET-1<sup>flox/flox</sup>, ET-1<sup>dlox/dlox</sup>, and Tie2-Cre transgenic mice and their tissue genotyping; enzyme immunoassay of plasma and tissue ET-1; quantification of ET-1, ET<sub>A</sub> and ET<sub>B</sub> mRNA by real-time RT-PCR; and ET-1 immunohistochemistry were performed using standard procedures as described in the online Data Supplement. Basal arterial blood pressure was measured in conscious, unrestrained mice via telemetry 5 to 7 days after surgical placement of a transducer in the left carotid artery. Blood pressure response to pharmacological interventions was measured in conscious, unrestrained mice and were apparently healthy into adulthood. This result controls in any tissues tested, including the heart, lung, kidneys, brain, and liver (data not shown). Tie2-Cre transgenic mice carry a silent lacZ transgene from which the β-galactosidase expression is activated only after Cre-mediated recombination.23 LacZ staining was observed specifically in endothelial cells of the organs, including heart, lung, kidney, brain, and intestine of R26R;Tie2-Cre(+) mice but not in any tissues from R26R;Cre(-) mice (Figure 2). Virtually all of the vascular endothelial cells were positive for lacZ staining. We also observed positive lacZ staining in most cells of the atrioventricular valve (Figure 2C), a small number of cardiomyocytes (data not shown), and smooth muscle cells in the intestine, as well as in many of the plasma cells and lymphocytes in lamina propria of the intestine (Figure 2K).

Results

Generation of Endothelial Cell–Specific ET-1 Knockout Mice

We generated mice carrying the flox or dlox ET-1 allele according to the strategy shown in Figure 1A. Mice homozygous for the flox ET-1 allele without the Cre transgene (ET-1<sup>flox/flox</sup>) were born and grew normally into adulthood with no detectable abnormalities (Figure 1B). We also obtained mice homozygous for the dlox ET-1 (null) allele (ET-1<sup>dlox/dlox</sup>). As expected, ET-1<sup>dlox/dlox</sup> mice were born with the identical craniofacial and cardiac defects reported previously in the ET-1 or ET<sub>A</sub> null mice6,11 and died shortly after birth (data not shown).

ET-1<sup>flox/flox</sup> mice were bred with Tie2-Cre transgenic mice to generate ET-1<sup>flox/flox</sup>;Tie2-Cre(+) mice. Cre-mediated recombination in Tie2-Cre transgenic mice is detected as early as embryonic day 7.5, and history of this recombination is traced specifically in vascular endothelial cells, endocardial cells, and their lineages.18 Importantly, ET-1<sup>flox/flox</sup>;Tie2-Cre(+) mice were also born with no developmental defects and were apparently healthy into adulthood. This result indicates that endothelial ET-1 is not essential for the normal development of neural crest-derived tissues.6,11 We harvested various tissues from both ET-1<sup>flox/flox</sup>;Tie2-Cre(+) mice and ET-1<sup>flox/flox</sup>;Cre(-) mice, isolated genomic DNA, and performed Southern blot analysis. Cre-mediated recombination was observed in every tissue examined (including heart, lung, liver, kidney, intestine, and brain) from ET-1<sup>flox/flox</sup>;Tie2-Cre(+) mice but not in any tissues from ET-1<sup>flox/flox</sup>;Cre(-) mice (Figure 1C).

To further assess the functional localization of Cre recombination activity in vivo, we generated double-transgenic mice carrying both R26R and Tie2-Cre transgenes. R26R transgenic mice carry a silent lacZ transgene from which the β-galactosidase expression is activated only after Cre-mediated recombination.23 LacZ staining was observed specifically in endothelial cells of the organs, including heart, lung, kidney, brain, and intestine of R26R;Tie2-Cre(+) mice but not in any tissues from R26R;Cre(-) mice (Figure 2). Virtually all of the vascular endothelial cells were positive for lacZ staining. We also observed positive lacZ staining in most cells of the atrioventricular valve (Figure 2C), a small number of cardiomyocytes (data not shown), and smooth muscle cells in the intestine, as well as in many of the plasma cells and lymphocytes in lamina propria of the intestine (Figure 2K).

Existence of lacZ-positive plasma cells and lymphocytes suggests that these lymphoid cells may be derived from a subset of embryonic “hemangioblasts,” which express Tie2-Cre. The lacZ-positive subpopulation of cardiomyocytes and intestinal smooth muscle cells may also be derived from these hemangioblasts.24

We then immunohistochemically confirmed the localization of ET-1 peptide in these mice. In ET-1<sup>flox/flox</sup>;Cre(-) mice, we detected specific ET-1 signals primarily in the endothelial cells of the lung, heart, and kidney (Figure 3). In contrast, we observed essentially no endothelial staining in these same tissues of ET-1<sup>flox/flox</sup>;Tie2-Cre(+) mice, except that, in their kidneys, a very small number of capillaries surrounding tubules showed positive immunostaining (Figure 3J).

Using an enzyme immunoassay, we determined the level of mature ET-1 peptide in plasma and tissue homogenates from adult ET-1<sup>flox/flox</sup>;Tie2-Cre(+) mice and genetic controls, that is, ET-1<sup>flox/flox</sup>;Cre(-), ET-1<sup>flox/flox</sup>;Tie2-Cre(+), and ET-1<sup>flox/flox</sup>;Cre(-) mice (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). Plasma level of ET-1 was significantly lower in ET-1<sup>flox/flox</sup>;Tie2-Cre(+) mice than in ET-1<sup>flox/flox</sup>;Cre(-) mice. Levels of ET-1 were significantly reduced in tissues from ET-1<sup>flox/flox</sup>;Tie2-Cre(+) mice (20% to <35% of genetic controls). In contrast, the level of ET-1 was not significantly different among the genetic controls in any tissues tested, including the heart, lung, kidneys, brain, and liver (data not shown). This latter observation is consistent with the notion that the ET-1<sup>flox</sup> allele functions normally through adulthood and that integration of Tie2-Cre transgene, per se, does not cause any unexpected effects on the production of ET-1 in vascular endothelial cells.

Real-time PCR showed 2-fold higher levels of ET<sub>A</sub> mRNA in the heart of ET-1<sup>flox/flox</sup>;Tie2-Cre(+) mice compared with ET-1<sup>flox/flox</sup>;Cre(-) mice, whereas the levels of ET<sub>A</sub> mRNA in lung or kidney were not significantly different between the 2 genotypes. The levels of ET<sub>B</sub> mRNA in the heart, lung, and
kidney were not significantly different between ET-1flox/flox;Tie2-Cre(H11001) and ET-1flox/flox;Cre(H11002) mice, although there was a trend of higher ET B mRNA in the ET-1 flox/flox;Tie2-Cre(H11001) heart (Figure S2A). We speculate that these changes reflect a tissue-specific compensatory response to the lack of ET-1 from endocardial and coronary endothelial cells.

Real-time PCR showed 2-fold lower levels of ET-1 mRNA in the renal cortex of ET-1flox/flox;Tie2-Cre(H11001) mice compared with ET-1flox/flox;Cre(H11002) mice. On the other hand, the levels of ET-1 mRNA in the renal medulla were not significantly different between ET-1flox/flox;Tie2-Cre(+) and ET-1flox/flox;Cre(-) mice (Figure S2B). We speculate that decrease in ET-1 expression level in the cortex but not the medulla of kidney in ET-1flox/flox;Tie2-Cre(+) mice is because of an abundance of glomerular/endothelial cell–derived ET-1 mRNA in the cortex.

Reduced Blood Pressure in ET-1flox/flox;Tie2-Cre(+) Mice

Figure 4 shows the results of blood pressure and heart rate measurements from conscious, unrestrained ET-1flox/flox;Tie2-Cre(+) and the genetic controls. We found a consistent decrease (∼10 mm Hg) in blood pressure of ET-1flox/flox;Tie2-Cre(+) mice compared with ET-1flox/flox;Cre(-) mice by tail-cuff measurement (Figure 4A). Basal blood pressures measured via a surgically implanted arterial catheter.
ter confirmed no difference in mean arterial pressures among ET-1flox/Cre(−) mice, ET-1flox/+;Tie2-Cre(+) mice, and ET-1flox/+;Cre(−) mice but showed a reduced mean arterial pressure in ET-1flox/Cre(+) mice (Figure S3). Reduced mean arterial pressure in ET-1flox/Tie2-Cre(+) mice was then further investigated by telemetry measurements. Throughout the light and dark phases, we observed consistent reduced blood pressure in ET-1flox/Tie2-Cre(+) mice compared with ET-1flox/Cre(−) mice (Figure 4B). Mean arterial blood pressure during the light phase of 92.5±0.72 mm Hg in ET-1flox/Tie2-Cre(+) mice compared with 104.7±0.91 mm Hg in ET-1flox/Cre(−) mice. The difference was less pronounced in the dark phase at 112.6±0.90 mm Hg in ET-1flox/Tie2-Cre(+) mice compared with 122.0±0.82 mm Hg in ET-1flox/Cre(−) mice, and systolic pressures were more strongly affected than diastolic pressures (Figure 4D). The lack of difference in blood pressures among ET-1flox/Tie2-Cre(−) mice, ET-1flox/Tie2-Cre(+) mice, and ET-1flox/Cre(−) mice indicates that the reduction of blood pressure in ET-1flox/Tie2-Cre(+) mice was not because of the unexpected effects of overexpression of the Cre protein in endothelial cells or because of unexpected effects of the loxP sites but because of the Cre-mediated recombination of the ET-1flox allele in vascular endothelial cells. We detected no significant difference in the heart rate among the 4 genotypes (Figure 4C and Figure S3).

We measured the weight of hearts and kidneys from both ET-1flox/Tie2-Cre(+) mice and ET-1flox/Cre(−) mice. There is no significant difference in tissue weight (P=0.1959 for heart and P=0.8776 for kidney; n=8 for each group).

**Responses to Acute Administration of Vasoactive Substances**

Acute blood pressure responses to an intra-arterial bolus of captopril, angiotensin II, phenylephrine, or bradykinin measured using this technique were not significantly different between ET-1flox/Tie2-Cre(+) mice and ET-1flox/Cre(−) mice, nor were acute responses to intra-arterial treatment with ET-1 or the NO synthetase inhibitor N^G^-nitro-L-arginine methyl ester (Figure S4). FR139317, a selective ETA antagonist, decreased blood pressure in both ET-1flox/Tie2-Cre(+) mice and ET-1flox/Cre(−) mice, and this decrease was not significantly different between these genotypes.

**Blood Pressure of ET-1dlox/+ Mice**

Because heterozygous ET-1 null (ET-1flox/+ mice were reported previously to show increased blood pressure, we also measured the blood pressure of our ET-1dlox/+ mice via an arterial catheter. Mean arterial blood pressure of ET-1dlox/+ mice (111.9±2.5 mm Hg) was not significantly different from the ET-1flox/+ littermates (Figure S5A). Furthermore, acute responses to ET-1, N^G^-nitro-L-arginine methyl ester, or captopril were not different between ET-1dlox/+ mice and ET-1flox/+ mice (Figure S5B).
Role of Endothelial ET-1 in the Regulation of Basal Blood Pressure

Pharmacological blockade of the ETA reduces blood pressure in a number of animal models. However, this approach is limited by tissue availability, drug half-life, incomplete blockade, and the specificity of drug action. More importantly, pharmacological manipulation at the receptor level does not provide information regarding the location of ET-1 production relevant for the regulation of blood pressure. In this study, we addressed these issues by generating endothelial-specific ET-1 knockout mice.

The observed “residual” amounts of ET-1 peptide in the tissue homogenates from ET-1\(^{\text{flox/flox}}\);Tie2-Cre\(^{-}\) lung (B and D), heart (F), and kidney (H and J), as well as ET-1\(^{\text{flox/flox}}\);Tie2-Cre\(^{+}\) lung (A and C), heart (E), and kidney (G and I), in the lung of control mouse (A and C), robust ET-1 signal is observed in the endothelial cells of arteries, veins, and alveolar walls (arrows) but not in the airway (arrowheads). Very little, if any, ET-1 signal is detected in the lung of ET-1\(^{\text{flox/flox}}\);Tie2-Cre\(^{+}\) mouse (B and D). In the heart of a control mouse (E), endothelial cells show strong ET-1 signals (arrowheads). No ET-1 signals are observed in the endothelial cells of the ET-1\(^{\text{flox/flox}}\);Tie2-Cre\(^{+}\) heart (F). In the kidney of control mouse, ET-1 signal is observed in endothelial cells of glomeruli (arrows in G), peritubular capillaries, and larger blood vessels (arrow in I). In the ET-1\(^{\text{flox/flox}}\);Tie2-Cre\(^{+}\) kidney, no ET-1 signal is detected in glomeruli (H), although a very small fraction of peritubular capillaries shows some ET-1 signals (J).

Responses to Vasodepressor Substances in the Absence of Endothelial ET-1

The interaction of the ET system and the renin-angiotensin system in the blood pressure homeostasis is not fully understood. Different animal models of hypertension demonstrate and opposing affects. Previous studies showed that “rescued” ET\(_B\)-deficient rats\(^{26}\) and ET\(_B\) knockout mice\(^{27}\) exhibit salt-sensitive hypertension. These observations, using animals lacking ET\(_B\) in both endothelial and nonendothelial cells, suggest roles of renal ET\(_B\) in the regulation of salt balance and blood pressure. More recently, using the same Tie2-Cre mice, vascular endothelial-specific ET\(_B\) knockout mice were generated.\(^{28}\) These mice show decreased endothelial release of NO and increased plasma ET-1. Although the data suggest that these mice have impaired endothelial function, they do not develop hypertension. Furthermore, the blood pressure response to a high-salt diet is unchanged in endothelial cell-specific ET\(_B\) knockout mice compared with control mice. These findings suggest that ET\(_B\) in the nonendothelial cells, such as the collecting duct epithelium of the kidney,\(^{16}\) is more important in regulating blood pressure, despite the fact that endothelial ET-1/ET\(_B\) interaction plays a role in vascular NO production and clearance of plasma ET-1. Our present results add further insight as to the overall physiological role of endothelially derived ET-1 in positive control of peripheral vascular tone.
different degrees of dependence on ET-1 in the development of hypertension.29 For example, a subpressor concentration of ET-1 potentiates the effect of a subpressor dose of angiotensin II in normotensive rats, resulting in an increase in blood pressure.30 Pretreatment with bosentan, an ETA/ETB selective antagonist, blunts the elevation of blood pressure caused by infusion of angiotensin II.31,32 These findings suggest that ET-1 is involved in hypertension induced by exogenously administered angiotensin II. In contrast, TGR(mREN-2)27 rats, in which endogenous angiotensin II is elevated by the overexpression of mouse Ren-2, do not respond to bosentan.33 Our present genetic results suggest that the renin-angiotensin system in ET-1 flox/flox;Tie2-Cre(−) mice is not upregulated significantly.29,34,35 Although no change in responsiveness to norepinephrine and decreases the responsiveness to bradykinin,34,35 although no change in responsiveness to norepinephrine was observed in another study. The present genetic evidence for a paracrine vascular regulation that ET-1. Finally, we expected that the lack of endothelial ET-1 would lead to the absence of a depressor response to the selective ETA antagonist FR139317 in ET-1floxflox;Tie2-Cre(+) mice. However, the apparent vascular “ETA tone,” as assessed by hypotensive response to bolus FR139317, was not significantly altered in these mice. ET-1 (or other ETs) produced by nonendothelial cells might also participate in the maintenance of vascular tone through the ETA (see below). Further studies are required to explain this apparent paradox.

**Paracrine Regulation of Vascular Tone by Endothelial ET-1 and Smooth Muscle ETA**

In a separate study, we recently generated smooth muscle–specific ETA knockout mice and found that these mice are also hypotensive compared with genetic controls (R.M.K. and M.Y., unpublished observation). Moreover, the degree of blood pressure reduction was virtually identical (~12 mm Hg) in endothelial ET-1 knockout mice and smooth muscle ETA knockout mice. Like endothelial ET-1 knockout mice described in the present study, smooth muscle ETA knockout mice exhibit no overt sign of alteration of the renin-angiotensin or sympathetic systems. Unlike endothelial ET-1 knockout mice, however, smooth muscle ETA knockout mice showed evidence of compensatory upregulation of vasoconstrictor ETB. Taken together, these results provide genetic evidence for a paracrine vascular regulation that...
involve endothelially derived ET-1 acting on the ET_A expressed by underlying smooth muscle cells. However, with the current data we cannot eliminate the possibility that the observed hypotension in ET-1^{flox/lox};Tie2-Cre(+)/mice is because of reduced stimulation of ET_B on the vascular smooth muscle. Mice and rats lacking endothelial, renal collecting duct, and vascular smooth muscle ET_B have salt-dependent hypertension related to the renal activities of ET_B. If vascular smooth muscle ET_B plays a role in baseline vascular tone, these animals might be predicted to show hypotension on a sodium-restricted diet, which is not the case. On the other hand, Fink et al.\(^{40}\) showed that chronic systemic activation of ET_B produces hypertension. Interpretation of these findings is also complicated by the complex role of ET_B in modulating blood pressure. Mice lacking only vascular smooth muscle ET_B may resolve the issue but are not yet reported.

**Normal Blood Pressure in ET-1^{dlox/+} Mice**

Previous studies reported that the blood pressure of ET-1^{+/−} mice was paradoxically elevated compared with wild-type controls.\(^{13}\) Here we obtained mice heterozygous for systemic disruption of the ET-1 allele (ET-1^{dlox/+}) as a byproduct of generating endothelial cell–specific ET-1 knockout mice. Notably, however, the blood pressure and heart rate of ET-1^{dlox/+} mice were not significantly different from wild-type littersmates. Importantly, the methods for blood pressure measurement in our study and that of Kurihara et al.\(^{11}\) are essentially identical. A possible reason for this discrepancy is the difference in genetic background between our ET-1^{dlox/+} mice (mixed 129SvEv\times C57Bl/6) and the original ET-1^{+/−} mice (mixed ICR\times C57Bl/6). The unexpected hypertension in the original ET-1^{+/−} mice has been speculated to involve ET-1 actions in the central nervous system. Previous studies showed that ET-1 is expressed in specific brain stem neurons and that ET-1^{+/−} mice have blunted respiratory responses against hypercapnia or hypoxia.\(^{35–39}\) If ET-1 deficiency in the brain causes hypertension, we can now test this hypothesis by analyzing neuron-specific ET-1 knockout mice.\(^{40}\) Our present study suggests that a half-normal gene dosage of ET-1 is systemically sufficient to maintain normal basal blood pressure.

**Clinical Perspectives**

The initial finding of a potent pressor action of ET-1 led to the speculation that it might be implicated in hypertension. To date, few studies that have investigated the long-term antihypertensive effects of ET receptor antagonists in humans, which make their place in the antihypertensive armamentarium unclear. Currently, pulmonary arterial hypertension is the only approved indication of ET receptor antagonists in the United States.\(^{41}\) Although unlikely to be considered as first-line treatment, the ET receptor antagonist could be a promising novel drug class to prevent or treat hypertension-induced organ damage, particularly progressive chronic kidney disease.\(^{42}\) Analysis of our genetically altered mice with conditional loss of function should provide valuable information for the novel clinical application of ET receptor antagonists.

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**Disclosures**

None.

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LOW BLOOD PRESSURE IN ENDOTHELIAL CELL-SPECIFIC ENDOThELIN-1 KNOCKOUT MICE
Short Title: Endothelial cell-specific ET-1 knockout mice

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EXPANDED MATERIALS AND METHODS

Generation of Flox ET-1 mice. Using the full-length mouse ET-1 cDNA as a probe, we screened a λFIXII mouse genomic library (Stratagene) in order to clone 129/Sv genomic fragments. The targeting vector contains three loxP sites, which flank exon 2 of ET-1 and neomycin/thymidine kinase cassettes (Fig.1A). Linearized targeting vector was transfections into SM-1 embryonic stem (ES) cells. After selection in 190 mg/ml of G418 (Gibco-BRL), we screened clones with homologous recombination by PCR and subsequently confirmed by Southern blot analysis using an external probe (Probe in Fig.1A). The oligonucleotides for PCR screening were upstream, 5'-TGGGGAATGGTTTATGTTCTGGGGG-3' and downstream, 5'-GAGTGACCCTGTGACAGGTTGAA-3'. The reaction for these primers was cycled 35 times (1 minute at 94°C, 2 minutes at 65°C, and 3 minutes at 72°C), which amplifies an approximately 1.5 Kb fragment. We further confirmed integration of 5' loxP site by Southern blot analysis of Xhol-digested DNA hybridized with the internal neo probe (data not shown). These ES cell clones were further transiently transfected with a circular pBS185 Cre plasmid (Gibco-BRL) to remove the selection marker cassettes through selection against 38.8 µg/ml of FIAU (1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5'-iodouracil). The resultant clones were divided into two groups: cells lacking exon 2 of the ET-1 gene (dlox allele); and cells harboring an allele of the ET-1 gene in which exon 2 is flanked by two loxP sites (flox allele). Both types of alleles were confirmed by Southern blot analysis of EcoRI/EcoRV-digested genomic DNA hybridized with the probe described in Fig.1A. Of 280 FIAU-resistant clones analyzed, we found 95 clones with a dlox allele, and 5 clones with a flox allele. Both types of the ES cell clones were injected into blastocysts obtained from C57BL/6J female mice to produce chimeric mice, and 1 and 2 independent ES cell clones with dlox and flox ET-1 alleles, respectively, gave germline transmission.

Transgenic mice. We used Tie2-Cre transgenic mice to produce endothelial cell-specific ET-1 knockout mice. Tie2-Cre transgenic mice and R26R reporter transgenic mice were previously described.1

Analysis of Cre-mediated recombination. Genomic DNA was prepared from tail biopsies from postnatal day 21 mice or from tissues harvested from mice at the age of 8-12 weeks. For genotyping, this DNA was subjected to PCR and/or Southern blot analysis. Oligonucleotide primers used for the detection of the wild-type ET-1 allele are upstream, 5'-GCTGCCCAAAGATTCTGAATTCTG-3' and downstream, 5'-GATGATGTCAGGTCCAGGTGGCAGAAG-3'. A different upstream primer was used for the detection of the flox ET-1 allele, 5'-CCCAAGATTCTGAATTGATAACTTCG-3'. Cre-mediated recombination with the resulting dlox allele is detected using the latter upstream primer with the following downstream primer, 5'-AACCTCCCAGTCTCCATACTTAC-3'. The reaction for these primers is cycled 35 times (1 minute at 94°C, 2 minutes at 60°C, and 3 minutes at 72°C), which amplifies an approximately 900-bp fragment for both the wild-type and flox ET-1 alleles, and an approximately 300-bp fragment for the dlox allele. Primers for detection of the Tie2-Cre transgene are upstream, 5'-CCCTGTGCTCG-
ACAGAAATGAGA-3' and downstream, 5'-CGCATAACCAGTAAACAGCATTGC-3'. The reaction for these primers is cycled 35 times (94°C at 1 minute, 2 minutes at 60°C, and 3 minutes at 72°C), which amplifies an approximately 550-bp product. For Southern blot analysis, 10 µg of genomic DNA is digested with EcoRI and EcoRV and hybridized with the radiolabeled external probe as described above.

**Determination of tissue ET-1.** Tissues are homogenized using a Polytron (20,000 rpm) in 1 M acetic acid containing 0.01 mM pepstatin A for 1 minute, and immediately placed in boiling water for 10 minutes. After centrifugation at 25,000 g, the supernatant is concentrated with Sep-Pak C18 columns (Waters) as previously described.2 Concentration of mature ET-1 peptide is determined by enzyme immunoassay as previously described.3

**Quantification of ET-1, ET<sub>A</sub> and ET<sub>B</sub> mRNA by real time RT-PCR.** Total RNA is prepared from tissues using Trizol according to manufacturer’s protocol (Gibco BRL). Reverse transcriptase reaction with oligo(dT) priming was carried out using Superscript<sup>™</sup> Choice system (Gibco BRL). Real Time PCR was then performed using CybrGreen buffer in 5700 Thermocycler (Applied Biosystems). Primers used for ET-1 mRNA quantification were 5’-CGCTGCAAGGAAGAACAGGACACGGC-3’ and 5’-CGGAGCTGAGAATGGAGTGC-3’. Primers used for ET<sub>A</sub> mRNA quantitation were 5’-CTGAAACAATTTTTGAATTTCTTGC-3’ and 5’-TACCAAGATGTGAAGGACTGGTG-3’. Primers used for ET<sub>B</sub> mRNA quantitation were 5’-CTGAAACAATTTTTGAATTTCTTGC-3’ and 5’-TACCAAGATGTGAAGGACTGGTG-3’. Primers used for ET<sub>B</sub> mRNA quantitation were 5’-CTGAAACAATTTTTGAATTTCTTGC-3’ and 5’-TACCAAGATGTGAAGGACTGGTG-3’. Primers used for β-actin mRNA quantitation were 5’-ACAGTCCGCTAGAAGCCT-3’ and 5’-TCCGATGCCCTGAGGCTT-3’. Primers used for GAPDH mRNA quantification were 5’-TGACCACAGTCCATGCTGCTCTCTT-3’ and 5’-GACGGACACATTTGCTCTCT-3’. The PCR conditions were 2 min at 50°C, 10 min at 95°C, and followed by 40 cycles of 20 s at 55°C, 30 s at 72°C and 15 s at 94°C. PCR products were 90 bp for ET-1, 390 bp for ET<sub>A</sub>, 250 bp for ET<sub>B</sub>, 150 bp for GAPDH, and 390 bp for β-actin.

**LacZ Staining.** Mice are anesthetized with pentobarbital and transcardially perfused with 30 ml heparinized saline, followed by 30 ml ice cold 4% paraformaldehyde in phosphate-buffered saline (PBS). Tissues (except brains) are harvested and embedded in Frish media for vibratomy.4 Embedded tissues are sectioned at 400 µm by a vibratome. These samples are stained overnight in lacZ staining solution with gentle agitation.1 After staining, samples are placed in an automatic tissue processor for paraffin embedding. To protect β-galactosidase signals, cedar oil is used for the clearing reagent instead of xylene. Samples are sectioned at 5 µm, and counterstained in eosin. Brains are cryoprotected in 30% sucrose for 24 hours at 4°C. Then, they are frozen to a freezing platform (Brain Research Laboratories) using crushed dry ice. Coronal sections are made at 30 microns. LacZ staining is performed as described above. A nuclear fast red is used for counterstain.

**Immunohistochemistry.** For antigen retrieval, the slides are incubated with 0.05% protease (Type XXVII; Sigma, St. Louis, MO, USA) in 50 mM Tris-HCl buffer (pH 7.6) for 10 min, following deparaffinization. Purified mouse anti-mouse ET-1 antibody
(Affinity BioReagents) is used as the primary antibody. The dilution of the primary antibody used in this study was 1/200. Using a Histofine Simple Stain Mouse MAX-PO (M) (Nichirei, Tokyo, Japan), the antigen-antibody complex is visualized with 3, 3’-diaminobenzidine (DAB) solution [1 mM DAB, 50 mM Tris-HCl buffer (pH 7.6), and 0.006% H₂O₂], and counterstained with hematoxylin. For a negative control, normal mouse IgG was used instead of the primary antibody, and no specific immunoreactivity was detected in these slides (data not shown).

**Blood pressure measurement.** All animal experiments were approved by the Institutional Animal Care Research Advisory Committee at The University of Texas Southwestern Medical Center at Dallas and in accordance with the Guidelines for Animal Experiments at Kobe University Graduate School of Medicine, Japan. Mice were maintained on a 12-h light and 12-h dark cycle (lights on at Zeitgeber time/ZT0).

**Tail cuff method:** We measured blood pressure sequentially at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20 using a tail cuff in conscious mice (Softron, Japan). Mean values were determined from at least 5 measurements for each mouse and there were 5 mice of each genotype at every time point.

**Arterial catheter method:** Male mice (15-25 week old) were anesthetized with pentobarbital (50 mg/kg) by intraperitoneal injection. A polyethylene catheter (PE-10, Clay Adams) was placed in the right femoral artery using standard surgical techniques. The catheter was filled with 50 U/ml heparin saline and tunneled under the skin to exit through the scruff of the neck. The free end of the catheter is occluded with a small piece of fishing line and anchored to the skin of the neck. After 24 hr recovery from the catheterization, we connected the catheter to a calibrated blood pressure transducer (BP-100, CB Sciences), amplifier (ETH-400, CB Sciences) and computer-based recorder (PowerLab/8s, CB Sciences). The sampling rate was 200 Hz. Mice with arterial catheters persistently recording narrow pulse pressures (less than 20 mmHg) were not further studied. We recorded pulsatile blood pressure continuously for more than 2 hours under conscious and unrestrained conditions in a quiet environment after at least 30 min of acclimatization. Systolic, mean and diastolic pressure and heart rate were automatically calculated using the PowerLab software. We made pharmacological interventions at the end of the baseline hour, and continued blood pressure measurement for an additional hour. At the end of the experiments, blood samples are withdrawn and the mice are sacrificed.

**Telemetric method:** TA11PA-C10 transmitters (Data Sciences International, St. Paul, MN) were implanted into the left carotid artery under isoflurane anesthesia. They were given 5–7 days to recover from surgery before the measurements were made in conscious, unrestrained mice. Blood pressure and heart rate were recorded every 10 min throughout the study. Data were analyzed with the program Dataquest.

**Pharmacological studies.** After measurement of baseline pressure, we injected vasoactive substances from the catheter in the femoral artery and examine the hemodynamic effects in some mice. Where indicated, mice were treated acutely with angiotensin II (5 µg/kg body weight), captopril (30 mg/kg), phenylephrine (10 µg/kg), bradykinin (1 mg/kg), N⁶-o-nitro-L-arginine methyl ester (L-NAME, 250 µmol/kg), and FR139317 (10 mg/kg). These compounds were dissolved in neutral-pH isotonic
solutions with a dosing volume of 0.5 µl/g. Some mice were given synthetic ET-1 peptide (1 nmol/kg) (American Peptide Company). The injection volume for ET-1 was 100 µl. For all drug treatments, vehicle injection was given as a control.

**Statistics.** We analyzed data using Instat or Prism software (Graph-Pad Software Inc.). Comparisons between two groups were made using the Student’s t-test. Three or more groups were compared using two-way ANOVA. Mean values were reported ±SEM, and *p* values of less than 0.05 were considered significant.

**References**
**Figure S1**

Circulating and tissues ET-1 peptide levels were measured in $ET^{-1}_{flox/flox} \cdot Tie2-Cre(+) \text{ mice (closed circle, n=4)}$ and $ET^{-1}_{flox/flox} \cdot Cre(-) \text{ mice (open circle, n=4).}$ We examined the heart, lung, kidney, and brain. Circles with bars indicate mean ± SEM of the corresponding group.
Fig. S2. Quantification of $ET_A$ and $ET_B$ mRNA in heart, lung and whole kidney (A) and $ET-1$ mRNA in cortex and medulla of kidney (B) from $ET-1^{\text{floxed/floxed};\text{Tie2-Cre}^+}$ mice (closed box, n=4) and $ET-1^{\text{floxed/floxed};\text{Cre}^-}$ mice (open box, n=4). Bars indicate ±SEM. n.s. indicates not significant, * indicates p<0.01.
**Figure S3**

**Arterial blood pressure (mmHg)**

**Heart rate (bpm)**

**Fig S3** Arterial blood pressure and heart rate in endothelial-specific ET-1 knockout mice. Systolic, mean and diastolic blood pressures as well as heart rate are measured by arterial catheter method. *ET-1^flox/flox;Tie2-Cre^+^* mice (closed column), *ET-1^flox/flox;Cre^-^* mice (dark-hatched column), *ET-1^+/+;Tie2-Cre^+^* mice (light-hatched column), and *ET-1^+/+;Cre^-^* mice (open column) are shown. Data are expressed as mean±SEM (* indicates p<0.05 and ** indicates p<0.01 by ANOVA).
Fig. S4. Acute pharmacologic response to exogenous ET-1, L-NAME, FR139317, captopril, angiotensin II, phenylephrine, and bradykinin in endothelial-specific ET-1 knockout mice. Indicated doses of ET-1, the NO synthetase inhibitor L-NAME, the ETA-selective antagonist FR139317, the angiotensin-converting enzyme inhibitor captopril, angiotensin II, the α-adrenergic agonist phenylephrine, or bradykinin were administered in bolus intra-arterially in bolus to ET-1^{flox/flox};Tie2-Cre(+) mice (closed circle) and ET-1^{flox/flox};Cre(-) mice (open circle). Mean blood pressure at baseline and at 30 min after the injection are plotted. ET-1, L-NAME, angiotensin II and phenylephrine significantly increased blood pressure in both ET-1^{flox/flox};Tie2-Cre(+) and ET-1^{flox/flox};Cre(-) mice, and FR139317 and bradykinin significantly decreased blood pressure in ET-1^{flox/flox};Tie2-Cre(+) and ET-1^{flox/flox};Cre(-) mice. However, there was no significant difference (n.s.) in acute response to each of these substances between ET-1^{flox/flox};Tie2-Cre(+) and ET-1^{flox/flox};Cre(-) mice.
**Figure S5**

(A) Arterial blood pressure and heart rate in heterozygous ET-1 null (dlox) mice. Systolic, mean and diastolic blood pressures of ET-1^dlox/+^ mice (closed column, n=19) and ET-1^+/+^ mice (open column, n=18) are shown. Data are expressed as mean±SEM. n.s.: not statistically different. (B) Acute response to ET-1, L-NAME or captopril in ET-1 heterozygous mice. Designated doses of ET-1, L-NAME or captopril are administered intra-arterially in bolus to ET-1^dlox/+^ mice (closed circle) and ET-1^+/+^ mice (open circle). Baseline pressures and the values at 30 min after the injection are plotted. Response to ET-1, L-NAME or captopril was not different between ET-1^dlox/+^ and ET-1^+/+^ mice.