Endothelium

Inducible Human Endothelin-1 Overexpression in Endothelium Raises Blood Pressure via Endothelin Type A Receptors

Yohann Rautureau,* Suellen C. Coelho,* Julio C. Fraulob-Aquino, Ku-Geng Huo, Asia Rehman, Stefan Offermanns, Pierre Paradis, Ernesto L. Schiffrin

See Editorial Commentary, pp 275–277

Abstract—The mechanisms of blood pressure regulation by endothelin-1 produced by endothelial cells are complex and still unclear. Transgenic mice with endothelium-restricted human endothelin-1 (EDN1) overexpression presented vascular damage but no significant change in blood pressure, which could be because of adaptation to life-long exposure to elevated endothelin-1 levels. We now generated a tamoxifen-inducible endothelium-restricted EDN1 overexpressing transgenic mouse (ieET-1) using Cre/loxP technology. Sixteen days after tamoxifen treatment, ieET-1 mice presented ≥10-fold increase in plasma endothelin-1 (P<0.01) and ≥20 mm Hg elevation in systolic blood pressure (P<0.01), which could be reversed by atrasan (P<0.05). Endothelin-1 overexpression did not cause vascular or kidney injury or changes in kidney perfusion or function. However, endothelin type A and B receptor expression was differentially regulated in the mesenteric arteries and the kidney. Our results demonstrate using this ieET-1 mouse model that 21 days of induction of endothelin-1 overexpression caused endothelin-1–dependent elevated blood pressure mediated by endothelin type A receptors. (Hypertension. 2015;66:347-355. DOI: 10.1161/HYPERTENSIONAHA.115.05168.) ● Online Data Supplement

Key Words: blood pressure ■ endothelin-1 ■ endothelin receptors type A ■ endothelium ■ mice, transgenic

Endothelin (ET)-1 is one of the most potent vasoconstrictor peptides.1 ET is produced by endothelial cells (ECs) and by other cell types.2,3 The effects of ET-1 are mediated by 2 G-coupled receptors: ET type A (ET_A) and B (ET_B) receptors. ET-1 secreted by EC acts in paracrine fashion on ET_A and ET_B receptors on underlying vascular smooth muscle cells to induce contraction and growth. It also acts in autocrine fashion on EC ET_A receptors to release vasodilator nitric oxide (NO) and prostacyclin. ET_B receptors also play a role in ET-1 clearance and mediate natriuresis by acting on the kidney.

ET-1 has been implicated in the development of hypertension and vascular damage.3,4 Plasma ET-1 is elevated in patients with essential hypertension,5 particularly in moderate-to-severe hypertensive rats treated with deoxycorticosterone acetate and salt,6 stroke-prone spontaneous hypertensive rats,7 and in Dahl salt-sensitive rats.8 In these rodents, enhanced ET-1 expression is associated with increased vascular oxidative stress, endothelial dysfunction, and hypertrophic vascular remodeling. However, direct evidence that ET-1 may directly raise blood pressure (BP) has been difficult to obtain. Genetic manipulation of ET-1 gene (Edn1) expression levels has yielded contradictory results on the role of ET-1 on BP regulation. Homozygous Edn1 null mice were not viable and presented craniofacial malformation.9 On the contrary, Edn1 haploinsufficiency (Edn1<sup>+</sup>) paradoxically caused hypertension. Endothelial-restricted Edn1 knockout generated by crossing mice with floxed Edn1 exon 2 (Edn1<sup>flx/flx</sup>) with mice expressing Cre recombinase under the transcriptional control of the endothelium-specific angiopoietin-1 receptor (Tek also known as Tie2) promoter, caused
mild hypotension and decrease in both plasma and tissue ET-1 levels. These findings demonstrated that ET-1 participates in the regulation of BP, and that EC are the main source of ET-1 production. Transgenic mice bearing the entire human ET-1 (EDN1) gene presented no change in plasma ET-1, slightly elevated tissue ET-1 concentrations, renal disease with aging (14-month old) and no change in BP. A similar finding was made in mice overexpressing a mouse Edn1 cDNA under the control of the mouse Edn1 promoter. In addition, feeding a high-salt diet (8% NaCl) caused a 20 mmHg BP rise in these mice. We have previously generated mice that overexpressed human EDN1 cDNA in EC under transcriptional control of Tie2 promoter (eET-1). Although eET-1 mice presented increased plasma ET-1, mesenteric artery (MA) endothelial dysfunction, vascular remodeling, oxidative stress, and inflammation, BP was not significantly elevated. All the aforementioned models relied on constitutive loss- or gain-of-function of Edn1, suggesting that there is the possibility that ontogenic adaptation modified the effects of ET-1. Because the Tie2 promoter has been shown to drive gene expression in EC during embryonic development, it is possible that effects of ET-1 overexpression on vascular remodeling and function and absence of BP elevation are, at least in part, because of developmental changes or adaptation to life-long exposure to high ET-1 circulating and tissue concentrations.

In humans, selective ETα receptor or nonselective ET receptor antagonists were effective in reducing BP in resistant hypertensive subjects receiving ≥2 antihypertensive drugs, including a diuretic, at optimized doses, and ameliorated or reversed renal injury in patients with chronic kidney disease. However, the use of ET blockers was limited by adverse side effects, such as liver toxicity, headaches, and fluid retention. Additional studies are required to better understand the complexity of the pathophysiology of ET-1 in hypertension and vascular injury.

To study the role of ET-1 on BP in the absence of developmental effects, we generated a mouse model of inducible endothelium-specific ET-1 overexpression (ieET-1) using a Cre/loxP tamoxifen-inducible system. With this new model, we determined that induction of human ET-1 overexpression (ieET-1) using a developmental effects, we generated a mouse model of inducible vascular injury. Additional studies are required to better understand the complexity of the pathophysiology of ET-1 in hypertension and vascular injury.

To study the role of ET-1 on BP in the absence of developmental effects, we generated a mouse model of inducible endothelium-specific ET-1 overexpression (ieET-1) using a Cre/loxP tamoxifen-inducible system. With this new model, we determined that induction of human ET-1 overexpression in EC of adult ieET-1 mice causes BP rise mediated via ETα receptors.

**Methods**

**Experimental Design**

The study was approved by the Animal Care Committee of the Lady Davis Institute for Medical Research and McGill University, followed recommendations of the Canadian Council for Animal Care and was in agreement with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The bacterial artificial chromosome Tie2-CreER<sup>26</sup> transgenic mice (referred here iCre<sup>26</sup>) were produced by crossing iCre mice with wild-type C57BL/6 mice (Harlan Laboratories, Indianapolis, IN). iCre/ROSA26<sup>EYFP<sup>+</sup></sup> reporter mice were generated by crossing iCre mice with C57BL/6 ROSA26<i>EYFP</i>/iCre reporter mice (Jackson Laboratories, Bar Harbor, ME). The transgenic CAG-cAT-EDN1 and ieET-1 mice were generated in our laboratory as described below and in the online-only Data Supplement, and F4 and F5 C57BL/6 backcrossed generation were used in this study. Mice were treated for 5 days with tamoxifen (1 mg/d SC) or vehicle, as indicated in the text, and studied 16 days later. In a subgroup, BP was determined by telemetry. Mice were anesthetized with isoflurane and surgically instrumented with PA-C10 BP telemetry transmitters (Data Sciences International, St. Paul, MN) as previously done. Mice were allowed to recover for 7 additional days, and BP was determined 2 days before (baseline), during vehicle or tamoxifen treatments, and the 16 days after. One group of tamoxifen-treated ieET-1 mice was also treated with the ET type A receptor blocker atrasentan (10 mg/kg per day PO) from day 10 to the end of the study. In another subgroup of mice, during the 2 last days of the study, 24-hour urine was collected using metabolic cages to determine kidney function and damage by measuring urinary creatinine, sodium, potassium, and protein by the Diagnostic Research Support Services at the Comparative Medicine and Animal Resource Center of McGill University, and urinary nephron (Exocell, Philadelphia, PA) and lipocalin-2 (R&D Systems, Minneapolis, MN) by ELISA. Renal artery flow and resistive index were also assessed by ultrasound. At the end of the protocol, mice were weighed and then anesthetized with isoflurane. Blood was collected by cardiac puncture on EDTA and plasma stored at −80°C until used for ET-1 determination by ELISA (R&D Systems). The MA vascular bed was dissected, and other tissues and tibia harvested in ice-cold phosphate buffered saline (PBS). Tissues were weighed, and tibia length was measured. Kidney sections were dissected under the microscope to separate renal cortex and medulla. Kidney sections and remaining tissues were frozen in liquid nitrogen and stored at −80°C until used. Second-order MA were used for assessment of endothelial function and vessel mechanics function by pressure-scan ultrasound. For assessment of mRNA expression, the MA arcade was dissected away from the attached intestine under RNase-free conditions and stored immediately in RNAlater (Life Technologies, Burlington, Ontario, Canada) until RNA extraction. The expression of ET type A and B receptor (Ednra and Ednrb), renin (Ren1), and ribosomal protein S16 (Rps16) mRNA was determined in MA or renal cortex and medulla by reverse transcription/quantitative polymerase chain reaction (QPCR). For determination of tissue specificity of Cre activation, iCre/ROSA26<sup>EYFP<sup>+</sup></sup> were anesthetized with 300 to 375 mg/kg IP of Avertin. Depth of anesthesia was confirmed by rear foot squeezing. The mice were injected IP with heparin (100 USP units), then perfused through the left ventricle at a constant pressure of 100 mmHg for 5 minutes with PBS to remove the blood, followed by 15-minute perfusion with 4% paraformaldehyde (PFA). Tissues were collected in 4% PFA and incubated in 4% PFA for 24 hours with gentle agitation at 4°C. All tissues with exception of MA were dehydrated by incubation in 30% sucrose in PBS for 24 hours with gentle agitation at 4°C. Tissues were embedded in VWR Clear Frozen Section Compound (VWR international, Edmonton, Alberta, Canada) and stored at −80°C until used. MA were stored in PBS at 4°C until imaged. The numbers of mice used are indicated in the figure and table legends.

**Results**

**Inducible Endothelium-Restricted Human ET-1 Overexpressing Mice**

ieET-1 mice were generated in 2 steps. First, transgenic mice containing a Cre-inducible EDN1 expression were generated as follows (Figure 1A). In brief, a DNA fragment containing the EDN1 cDNA followed by the rabbit β-globin intron and polyadenylation signal (pA) was amplified by quantitative polymerase chain reaction and subcloned into multiple cloning sites of the pCAG-cat-multiple cloning site (pcAT expression vector (generous gift of Dr Thomas N. Sato, Nara Institute of Science and Technology, Ikoma, Nara, Japan). The resulting pcAT-EDN1 expression vector contains a CMV enhancer/chicken β-actin promoter (CAG) that drives the expression of a LoxP-flanked chloramphenicol acetyltransferase (cat) cDNA.
followed by a pA, before Cre-mediated excision, and EDN1 cDNA followed by the rabbit β-globin intron and pA, after excision. A SalI/NotI DNA fragment containing the CAG-cat-EDN1 transgene was used to generate transgenic mice at the Microinjection and Transgenesis core facility of Institut de Recherches Cliniques de Montréal (Montreal, Quebec, Canada). To eliminate the possibility that the effects of the EDN1 transgene expression are because of the integration site, 2 CAG-cat-EDN1 transgenic founders (C134 and C170) that transmitted the transgene contained 1 integration site and expressed cat were selected for the study. Transgenic mice were backcrossed with C57BL/6 mice (Harlan Laboratories, Indianapolis, IN). No difference in plasma ET-1 levels could be observed between CAG-cat-EDN1 C134 and C170 and control mice.

Figure 1. Inducible endothelium-restricted human endothelin (ET)-1 overexpression mice. A, The steps to generate the inducible endothelium-restricted human ET-1 (EDN1) overexpressing (ieET-1) mice are depicted. ►, loxP site; CAG, ubiquitous human cytomegalovirus immediate early enhancer and chicken β-actin promoter; cat, chloramphenicol acetyltransferase; ieCre, mice expressing the tamoxifen-inducible Cre recombinase CreER\(^{\text{T2}}\) under the control of the angioptiotin-1 receptor Tek (also known as Tie2) promoter; MCS, multiple cloning sites; pA, polyadenylation signal; rHbb, rabbit β-globin; right arrow linked to CAG box, initiation of the transcription site; half-arrows pointing to the right and left, primers used for genotyping and relative gene copy number determination (F1 and R1) and quantification of cat expression by reverse transcription/quantitative PCR (F2 and R2). B, Plasma ET-1 was determined in wild-type (WT), CAG-cat-EDN1-C134 and CAG-cat-EDN1-C170, tamoxifen-treated ieCre and vehicle- and tamoxifen-treated ieET-1-C134 and C170 mice. Data are presented as mean±SEM, n=13 for WT and 3 to 5 for the other groups. *P<0.001 vs respective vehicle-treated ieET-1 group, †P<0.001 vs tamoxifen-treated ieCre and ‡P<0.01 vs tamoxifen-treated ieET-1-C134. C and D, Tissue specificity of Cre activation was determined using vehicle- and tamoxifen-treated ieCre/ROSA26\(^{\text{mT-mG}}\)/+ reporter mice by determining the replacement of membrane-targeted tandem dimer tomato (mT) by membrane-targeted enhanced green fluorescent protein (mG) expression using confocal microscopy imaging. Representative mT (red) and mG (green) fluorescence images of mesenteric artery segments and aortic sections (C) and mG, mT and the CD31 endothelium marker (cyan) fluorescence images of renal sections (D) are presented. Blue represents nuclear stain 4',6-diamidino-2-phenylindole (DAPI) fluorescence. n=5 per group.
wild-type mice (Figure 1B), confirming that the expression of 
*EDN1* was blocked by the expression of *cat*. In the second step, 2 transgenic ieET-1 mouse lines were obtained by crossing the CAG-cat-EDN1-C134 and C170 mice with a transgenic mouse having an inducible endothelium-restricted Cre (ieCre), the bacterial artificial chromosome transgenic *Tie2-CreER<sup>2</sup>* mouse. The ieCre mouse expresses a fusion protein of the Cre recombinase with the modified estrogen receptor (ER)-binding domain (CreER<sup>2</sup>) under the control of the endothelium-specific *Tie2* promoter. CreER<sup>2</sup> is activated by tamoxifen but not by natural ER ligands. In the presence of tamoxifen, CreER<sup>2</sup> translocates from the cytoplasm to the nucleus, where it recombines the LoxP sites to remove the *cat*-pa DNA fragment, and consequently, induces the expression of *EDN1* (Figure 1A).

**Tamoxifen-Induced ET-1 Overexpression in ieET-1 Mice**

To evaluate the efficiency of tamoxifen-induced *EDN1* expression, ieET-1-C134 and C170 mice were treated for 5 days with tamoxifen (1 mg/d, SC) or vehicle, and plasma ET-1 levels were determined 16 days later. Tamoxifen-treated ieCre mice were also studied to control for induction of Cre with wild-type mice used as reference. Plasma ET-1 levels in vehicle-treated ieET-1-C134 and ieET-1-C170 were similar to wild-type mice (Figure 1B), demonstrating no leaky Cre activation in the absence of tamoxifen treatment. Similar plasma ET-1 levels were found in tamoxifen-treated ieCre mice, indicating that activation of CreER<sup>2</sup> by tamoxifen or tamoxifen treatment itself did not modify ET-1 expression. However, tamoxifen induced ≥9-fold increase in plasma ET-1 in ieET-1-C134 and ≥15-fold in ieET-1-C170 compared with vehicle-treated ieET-1 and tamoxifen-treated ieCre mice.

**Tamoxifen-Induced Endothelium-Restricted Cre Activation in ieCre Mice**

Tissue specificity of Cre activation was determined using ieCre/ROSA26<sup>mT-mG</sup>/+ reporter mice, which express a loxP-flanked, membrane-targeted tandem dimer tomato (mT), before Cre-mediated excision, and a membrane-targeted enhanced green fluorescent protein (mG) after excision, under the control of the CAG promoter driving the expression in all cell types. The ieCre/ROSA26<sup>mT-mG</sup>/+ mice were treated with tamoxifen or vehicle as above, and the Cre activation was revealed by the replacement of mT by mG expression. Confocal microscopy imaging demonstrated that tamoxifen caused EC-restricted activation of Cre in MA, aorta, and kidneys (Figure 1C and 1D) and in the heart and liver (Figure S2 in the online-only Data Supplement). The EC-restricted Cre activation in kidneys, heart, and liver was confirmed by colocalization of mG fluorescence with an endothelium marker, CD31. Tamoxifen-induced Cre activation in EC was partial in MA and aorta. Cre activation was not observed in the vehicle-treated mice.

**Induction of ET-1 Overexpression in the Endothelium Resulted In ET Type A Receptor-Mediated Systolic BP Rise**

Systolic BP (SBP) was determined by telemetry in ieCre and ieET-1 (C134 and C170) mice 2 days before (baseline), during vehicle or tamoxifen treatments as above, and the 16 days after. Tamoxifen increased SBP in ieET-1-C134 and C170 mice but not in ieCre mice (Figure 2; Figure S3B). SBP rose progressively during the 5 days of tamoxifen treatment in ieET-1-C134 and C170 mice. By the end of the study, nighttime SBP was 25 and 20 mm Hg higher in tamoxifen-treated ieET-1-C134 and C170 mice, respectively, than tamoxifen-treated ieCre mice (Figure 2A; Figure S3A). During the day, when the mice are resting, SBP was 20 mm Hg higher in ieET-1-C134 and C170 mice than in ieCre mice (Figure 2B; Figure S3B). To confirm whether ET-1-mediated SBP rise through ET<sub>R</sub> in ieET-1 mice, another group of ieET-1-C134 mice was treated as above with tamoxifen and then with an ET type A receptor blocker, atrasentan (10 mg/kg per day), from day 10 until the end of the study. Atrasentan treatment abrogated SBP rise during the night and day (Figure 2).

**Induction of ET-1 Overexpression in the Endothelium Did Not Alter Body or Organ Weights**

Induction of endothelial ET-1 overexpression affected neither growth of mice (body weight and tibia length) nor induced blood pressure.

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**Figure 2.** Induction of endothelin (ET)-1 overexpression in the endothelium increased systolic blood pressure (SBP) in an ET type A receptor manner. Nighttime (A) and daytime (B) SBP were assessed by telemetry in ieCre treated with tamoxifen (Tam) and in ieET-1-C134 mice treated with Tam plus or minus the ET type A receptor blocker atrasentan from day 10 to the end of the study. The days of treatment with Tam and atrasentan are indicated by boxes. Data are presented as mean±SEM, n=4–5. †P<0.01 and ††P<0.001 vs ieCre+Tam, and ‡P<0.05 and ‡‡P<0.001 vs ieET-1-C134+Tam. ieCre indicates mice expressing the tamoxifen-inducible Cre recombinase CreER<sup>2</sup> under the control of the angiopoietin-1 receptor Tek (also known as Tie2) promoter; and ieET-1, endothelium-restricted human ET-1 (*EDN1*) overexpressing mice.
changes in heart, kidney, lung, liver, or spleen weight in tamoxifen-treated ieET-1-C134 and C170 mice than in tamoxifen-treated ieCre mice (Table 1).

### Induction of ET-1 Overexpression in the Endothelium Neither Cause Resistance Artery Dysfunction Nor Remodeling but Was Associated With Decrease in ET Receptor Expression

Vascular function and remodeling were assessed in MA of vehicle and tamoxifen-treated ieET-1-C134 mice using pressurized myography. Contractile responses to norepinephrine and endothelium-dependent dilatory response to acetylcholine were unaffected by tamoxifen-induced ET-1 overexpression in ieET-1-C134 mice (Figure 3A and 3B). Likewise, induction of ET-1 overexpression in EC did not cause vascular remodeling within the timeframe of this experimental paradigm (Table 2).

To better understand how induction of endothelial ET-1 overexpression caused a rise in SBP, contractile responses to ET-1 and angiotensin II were examined. The contractile responses to these peptides were identical in tamoxifen and in vehicle-treated ieET-1 mice (Figure 3C and 3D). However, mRNA expression of ET<sub>A</sub> (Ednra) and ET<sub>B</sub> (Ednrb) receptors determined by reverse transcription-QPCR was decreased by ≈60% or tended to decrease, respectively, in MA of tamoxifen-treated ieET-1-C134 mice than that of vehicle-treated mice (Figure 3E and 3F).

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**Table 1. Induction of ET-1 Overexpression in the Endothelium Did Not Alter Body or Tissue Weights**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ieCre+Tamoxifen</th>
<th>ieET-1-C134+Tamoxifen</th>
<th>ieET-1-C170+Tamoxifen</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>BW, g</td>
<td>27.7±0.7</td>
<td>27.7±1.0</td>
<td>26.6±0.5</td>
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<td>TL, mm</td>
<td>18.50±0.14</td>
<td>18.36±0.20</td>
<td>18.37±0.17</td>
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<td>HW/TL, mg/mm</td>
<td>7.44±0.30</td>
<td>7.01±0.21</td>
<td>6.62±0.11</td>
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<td>KW/TL, mg/mm</td>
<td>18.7±0.9</td>
<td>19.2±0.5</td>
<td>19.2±0.6</td>
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<td>LuW/TL, mg/mm</td>
<td>10.2±0.7</td>
<td>10.9±0.6</td>
<td>9.2±0.6</td>
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<td>LiW/TL, mg/mm</td>
<td>77.0±3.9</td>
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<td>72.7±1.4</td>
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<tr>
<td>SW/TL, mg/mm</td>
<td>4.80±0.60</td>
<td>4.33±0.12</td>
<td>4.43±0.26</td>
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</tbody>
</table>

BW, HW, KW, LiW, LuW, SW, TL were determined in tamoxifen-treated ieCre, ieET-1-C134, and ieET-1-C170 mice. Data are mean±SEM. BW indicates body weight; HW, heart weight; ieCre, mice expressing the tamoxifen-inducible Cre recombinase CreER<sup>T2</sup> under the control of the angiopoietin-1 receptor Tek (also known as Tie2) promoter; ieET-1, endothelium-restricted human ET-1 (EDN1) overexpressing mice; KW, kidney weight; LiW, liver weight; LuW, lung weight; n, number; SW, spleen weight; and TL, tibia length.

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**Figure 3.** Induction of endothelin (ET)-1 overexpression in the endothelium did not cause vascular dysfunction in resistance arteries. Contractile responses to norepinephrine (NE, A), relaxation responses to acetylcholine (Ach, B), contractile responses to ET-1 (C) and angiotensin (Ang) II (D) and expression of the ET type A (Ednra, E) and B (Ednrb, F) receptors and ribosomal protein S16 (Rps16) mRNA were determined in mesenteric arteries of ieET-1-C134 mice treated with vehicle (Veh) or tamoxifen (Tam). The decrease in Ang II-induced contraction at concentration ≥10<sup>-9</sup> mol/L could be because of receptor desensitization. Data are presented as mean±SEM, n=5–8 for A, 4–5 for B, 5–7 for C, 5 for D, and 5–7 for E and F. *P<0.05 vs Veh.
Table 2. Induction of Endothelin-1 Overexpression in the Endothelium Did Not Cause Vascular Remodeling in Resistance Arteries

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<th>Parameters</th>
<th>Vehicle</th>
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<tr>
<td>n</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Media/lumen, %</td>
<td>4.30±0.51</td>
<td>4.51±0.27</td>
</tr>
<tr>
<td>Media cross-sectional area, μm²</td>
<td>5956±609</td>
<td>6160±408</td>
</tr>
</tbody>
</table>

Media/lumen and media cross-sectional area were determined in mesenteric arteries of ieET-1-C134 mice treated with vehicle or tamoxifen, pressurized at 45 mmHg. Data are mean±SEM. ET-1 indicates endothelin-1; and n, number.

Kidney Perfusion, Function, and Damage and Renal ET<sub>A</sub> and ET<sub>B</sub> Receptor mRNA Expression

The right renal artery diameter and blood flow were studied by echography using 2-dimensional short-axis view and pulse wave Doppler in tamoxifen-treated ieCre and vehicle- and tamoxifen-treated ieET-1-C134 mice (Figure S4). The renal artery diameter and flow were unaffected by 21 days of endothelial ET-1 overexpression (Table 3). The heart rate and resistive index were also unaltered. Analysis of the urine collected in metabolic cages revealed that kidney function assessed by determining sodium/creatinine, potassium/creatinine, and urea/creatinine, and renal injury by protein/creatinine, nephrin/creatinine, and lipocalin-2/creatinine were unaffected by endothelial ET-1 overexpression in ieET-1.

mRNA expression of Ednra and Ednrb and renin (Ren1) was determined by reverse transcription-QPCR in renal cortex and medulla in the same groups as above. Induction of endothelial ET-1 overexpression was associated with a 3-fold increase in Ednra expression in renal cortex but no change in renal medulla (Figure 4A and 4B). There was a 2- to 3-fold greater Ednrb expression in renal cortex and medulla (Figure 4C and 4D). Ren1 expression was similar in the 3 groups (Figure 4E and 4F).

Discussion

This study demonstrated that generation of a novel mouse model with inducible endothelial ET-1 overexpression, which is devoid of developmental effects, results in ET-1–dependent elevated BP mediated by ET type A receptors. No vascular injury was observed in ieET-1 mice at 21 days after induction of ET-1 overexpression. Furthermore, ET type A and B receptor expression was differentially regulated in MA and kidney.

The ieET-1 mouse was designed to overexpress ET-1 in EC only on tamoxifen treatment. This was realized, in part, by ieCre mice expressing the tamoxifen-inducible CreERT<sup>2</sup> under the control of the EC-specific Tie2 promoter. As previously observed, this study showed that ieCre/Rosa26<sup>Tie2Cre</sup>reporter mice presented Cre activation only in EC of tamoxifen-treated mice. Furthermore, plasma ET-1 levels were increased only in tamoxifen-treated ieET-1 mice, which was independent of the transgene integration site, as this was observed in 2 distinct lines of ieET-1 transgenic mice. Altogether, these results confirmed that the overexpression of ET-1 was inducible and restricted to EC.

Induction of endothelium-restricted ET-1 overexpression in ieET-1 mice with tamoxifen increased BP without affecting at 21-day endothelial or vascular function, or mechanical properties of MA. Moreover, ET-1 overexpression had no effect on body or organ weight. These observations contrast with our previous findings using a constitutive endothelium-specific ET-1 (eET-1) transgenic mouse model. A possible explanation for a different phenotypes observed might be because of developmental changes or adaptation to life-long exposure to elevated ET-1 circulating or tissue levels. In eET-1 mice, ET-1 expression was constitutively driven in EC under the control of the Tie2 promoter. This promoter is known to drive gene expression early in development in all blood vessels, and

Table 3. Induction of ET-1 Overexpression in the Endothelium Did Not Affect Renal Perfusion and Function or Cause Renal Injury

<table>
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<tr>
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<th>ieET-1-C134+Tamoxifen</th>
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<td>Renal artery diameter, mm</td>
<td>0.40±0.00</td>
<td>0.42±0.03</td>
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<td>Renal artery flow, mL/min</td>
<td>1.71±0.16</td>
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<td>Heart rate, bpm</td>
<td>536±8</td>
<td>536±8</td>
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<td>Resistive index, arbitrary units</td>
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<td>Lipocalin-2/creatinine, ng/mg</td>
<td>115±7</td>
<td>123±9</td>
<td>126±7</td>
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</tbody>
</table>

Renal perfusion, function, and damage were determined in tamoxifen-treated ieCre and vehicle- and tamoxifen-treated ieET-1-C134 mice. Renal perfusion was evaluated by measuring renal artery diameter, renal artery flow, heart rate, and resistive index by echography using 2-dimensional short-axis view and pulse wave Doppler of the right renal artery. Renal function was assessed with the sodium/creatinine, potassium/creatinine, and urea/creatinine, and renal injury with the protein/creatinine, nephrin/creatinine, and lipocalin-2/creatinine, all measured in the 24-hour urine collected in metabolic cages. Data are presented as mean±SEM; n=5–6 for renal perfusion; 5–7 for renal function and protein excretion; and 5–6 for nephrin and lipocalin-2. ieCre indicates mice expressing the tamoxifen-inducible Cre recombinase CreER<sup>2</sup> under the control of the angiotensin-1 receptor Tek (also known as Tie2) promoter; and ieET-1, endothelium-restricted human ET-1 (EDN1) overexpressing mice.
thus in utero overexpression of ET-1 could have contributed to the observed vascular damage in the previous constitutive model. ieET-1 mice are devoid of ET-1 overexpression in the absence of induction with tamoxifen. Accordingly, it is likely that the Cre-loxP system was not activated long enough to allow ET-1 to cause vascular injury, whereas vasoconstriction to human ET-1 overexpressed in the endothelium resulted in elevated BP. Longer-term experiments will be necessary to test this hypothesis that chronically the inducible model (ieET-1) will exhibit vascular injury.

The contractile response of MA on exogenous ET-1 was the same between tamoxifen and vehicle-treated ieET-1 mice, suggesting that receptor activity was unaltered by ET-1 overexpression. Interestingly, the MA contractile response to ET-1 was similar to that observed by Yanagisawa et al in porcine coronary arteries. Likewise, the contractile response of MA to Ang II was not modified. Although no vascular dysfunction or remodeling was observed, ET$_A$ and ET$_B$ receptor mRNA expression was downregulated in MA of tamoxifen-treated ieET-1 mice. This suggests that despite the fact that duration of exposure to ET-1 overexpression did not cause vascular damage, it was long enough to induce an adaptive response. Whether the changes in mRNA expression of ET receptors translated into changes in protein is unknown, but they did not result in a reduction of the contractile response to exogenous ET-1. Current ongoing experiments of much longer chronic duration to elevated ET-1 overexpression will elucidate whether indeed in this model adult induction of ET-1 overproduction in the endothelium without exposure in utero will indeed lead to vascular remodeling and modulation of vascular function not found in the present experimental paradigm.

The kidneys play a major role in long-term control of BP and in the development and maintenance of hypertension. Endothelial ET-1 overexpression-induced BP rise could be modulated by the renal ET system that controls multiple aspects of kidney function through regulation of renal blood flow and glomerular filtration rate, control of renin expression, and sodium and water excretion through activation of ET$_A$ and ET$_B$ receptors. This was not the case in tamoxifen-treated ieET-1 mice, as renal artery flow, resistive index, sodium excretion, and renin mRNA level were unaltered. In addition, short-term exposure to endothelial ET-1 overexpression did not cause renal injury, assessed by measuring urinary protein, nephrin, and lipocalin-2 excretion, which may differ in longer-term exposure to endothelial ET-1 overexpression. However, the important conclusion is that BP rose in this inducible model in the short term without renal injury.

The expression of ET$_A$ receptors in the renal cortex and ET$_B$ receptors in renal cortex and medulla was increased. The renal ET system is complex given the fact that both ET receptors present distinct signaling pathways, whose effects depend on which cell type and at what nephron and vascular levels these receptors are present. Changes in ET-1 receptor expression suggest some renal functional adaptation to induction of endothelial ET-1 overexpression or BP rise. A large amount of renal ET receptor mRNA quantified by reverse transcription-QPCR might be coming from the tubular system because the collecting duct (CD) is a major renal site of ET-1 production and in the development and maintenance of hypertension. The kidneys play a major role in long-term control of BP.

In conclusion, we generated a novel inducible EDN1 overexpressing mouse. Twenty-one days of EC-restricted EDN1 overexpression caused ET-1-dependent BP elevation mediated by ET type A receptors, unaccompanied by vascular injury or evident alteration of kidney function or kidney injury.
Limitations

Sex differences have been reported for both endothelin and NO systems. In this study, the effects of the induction of ET-1 were determined only in male mice. Different results might be observed in female mice, which need to be addressed in another study. However, the results obtained in male mice translate to hypertension observed in men and postmenopausal women.

Perspectives

Hypertension is the leading cause for heart disease and stroke, and the first cause of death and disease burden worldwide. This novel mouse model of inducible endothelial restricted human ET-1 overexpression will facilitate understanding the role of ET-1 in the pathophysiology of hypertension and should stimulate development of novel ET-1 antagonists with fewer side effects than those currently available, improving our ability to successfully treat hypertension, particularly difficult to control or resistant hypertension, to improve outcomes of hypertensive subjects.

Sources of Funding

This work was supported by Canadian Institutes of Health Research (CIHR) grants 37917 and 102606, a Canada Research Chair (CRC) on Hypertension and Vascular Research by the CRC Government of Canada/CIHR Program and by the Canada Fund for Innovation, all to E.L. Schiffrin, and by a fellowship to S.C. Coelho and to J.C. Fraulob-Aquino from the program of Science without Borders offered by the National Council for Scientific and Technological Development of Brazil.

Disclosures

None.

References


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Disclosures

None.

Sources of Funding

This work was supported by Canadian Institutes of Health Research (CIHR) grants 37917 and 102606, a Canada Research Chair (CRC) on Hypertension and Vascular Research by the CRC Government of Canada/CIHR Program and by the Canada Fund for Innovation, all to E.L. Schiffrin, and by a fellowship to S.C. Coelho and to J.C. Fraulob-Aquino from the program of Science without Borders offered by the National Council for Scientific and Technological Development of Brazil.


### Novelty and Significance

**What Is New?**
- This is the first study that shows unambiguously that inducible human endothelin-1 overexpression will result in blood pressure elevation in mice and clearly demonstrates in this genetically engineered mouse the hypertensive effects of endothelin-1 mediated by endothelin (ET) type A receptors.

**What Is Relevant?**
- Demonstration of the ET type A–mediated hypertensive effect of human ET-1 should encourage the development of endothelin blockers for treatment of difficult to control or resistant hypertension in which the endothelin system is activated.

### Summary

Hypertension is the number one cause of morbidity and mortality worldwide and is often uncontrolled or resistant to treatment. The ET system is activated particularly in difficult to control or resistant hypertension or hypertension associated with chronic kidney disease or diabetes mellitus. However, the pressor significance in hypertension of endothelin-1, a potent vasoconstrictor, has been difficult to demonstrate. Here, we show unambiguously that when transgenic human ET-1 overexpression is induced in endothelium of mice with a Cre/loxP tamoxifen system, it raises blood pressure via ET type A receptors. These results should encourage the development of ET type A receptor blockers for treatment of difficult to control or resistant hypertension, which has been slowed down by adverse effects of the antagonists developed in the past.
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Inducible human endothelin-1 overexpression in endothelium raises blood pressure via endothelin type A receptors

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Supplemental Methods

**Engineering of the Cre-inducible endothelin-1 expression vector.** The Cre-inducible endothelin (ET)-1 expression vector was constructed using the pCAG-cat-MCS.21 expression vector (generous gift of Dr. Thomas N. Sato, Nara Institute of Science and Technology, Ikoma, Nara, Japan). This vector contain a ubiquitous cytomegalovirus immediate early enhancer/chicken β-actin promoter (CAG) driving the expression in all cell types, followed by a chloramphenicol acetyltransferase (cat) cDNA and a polyadenylation signal, which are flanked by loxP sites, and multiple cloning sites (MCS.21) (Fig. S1A). A DNA fragment containing the human ET-1 (EDN1) cDNA, a rabbit β-globin (rHbb) intron and polyadenylation signal (pA) was amplified from the pTie2-ppET-1 plasmid used to generated our previous transgenic eET-1 mouse,1 by PCR with Platinum® Pfx DNA polymerase (Life Technologies, Burlington, ON, Canada) using forward and reverse oligonucleotides containing SpeI and BclI restriction sites, respectively, and a GATC cap (Table S1). The transgenic DNA fragment was subcloned into the multiple cloning sites of pCAG-cat-MCS expression vector using NheI and BamHI restriction sites that have compatible cohesive ends to SpeI and BclI, respectively, to generate the pCAG-cat-EDN1 expression vector. The subcloned DNA fragment was sequenced completely to ensure that artifactual mutations were not introduced accidentally by the Platinum® Pfx DNA polymerase (see Table S1 for sequencing primers).

**Generation of transgenic CAG-cat-EDN1 founder mouse lines.** The pCAG-cat-EDN1 plasmid was digested with NotI and SalI to free the CAG-cat-EDN1 transgene from the vector backbone. One hundred µg of plasmid was digested with 150 Units of NotI in a final volume of 200 µL overnight at 37°C. After confirmation of complete plasmid digestion by agarose gel electrophoresis, the NotI digesta was heat-inactivated at 65°C for 20 min. The buffer of the digestion was adjusted to perform the SalI digestion with 150 Units in a final volume of 500 µL for 7 h at 37°C. Complete digestion of the plasmid was confirmed by agarose gel electrophoresis. Digested DNA and the picture of the gel with the transgenic fragment circle were sent to the Microinjection and Transgenesis core facility of IRCM (Montreal, QC, Canada) for generation of transgenic mice. In brief, DNA was microinjected into the pronuclei of E0.5 zygotes F1 obtained from C3H crossed with C57BL/6 mice. Injected zygotes were transferred back into the oviducts of CD-1 pseudo-pregnant mice. Pups were weaned, identified and a 3 mm pieceed of tail was cut and stored in a sterile 1.5 mL microtube at -20°C for genotyping. Mice were housed at the Microinjection and Transgenesis core facility until mice positive for the transgene have been identified. Genotyping was performed by PCR (see Genotyping).2-4 Fourteen out of 132 pups were found positive for the transgene. Ten transgenic founder mice were transferred to our mouse facility. Founders were backcrossed with C57BL/6 mice.

**Selection of two CAG-cat-EDN1 transgenic lines for experimentation.** In order to exclude a phenotype dependent on the site of integration of the transgene, two mouse founder transgenic lines were selected for the study as follows.

Seven out of the ten mouse founders transmitted the transgene to the F1 generation and were further studied.
Transgenic mice containing one integration site were determined as follows. The relative copy number of CAG-cat-EDN1 in pups of F1 and F2 generations was determined by quantitative PCR (QPCR) for each transgenic line. No difference in the number CAG-cat-EDN1 copy was observed between F1 and F2 descendent for each of the mouse lines, which suggest a unique site of integration. No mice were eliminated at this point.

Finally, the expression of cat transgene was used as a predictor for the expression of the EDN1 transgene after activation by Cre recombinase to complete the selection of the transgenic mouse lines. The relative mRNA expression level of the cat transgene for all transgenic lines was determined in cardiac ventricles by reverse transcription (RT) followed by QPCR. The ventricles were obtained by removing the base of the heart and the atria. The ventricles were snap frozen in liquid nitrogen and stored at -80°C until used. Six transgenic mouse lines were studied, and the expression levels of cat were in the same range ± 1-fold. The transgenic mouse lines C134 and C170 named after their founders, who presented 2-fold different expression levels were selected. When studied at the F2 generation, the cat relative expression level was $1.23 \pm 0.08$ for the C134 mouse line (n = 6) and $0.45 \pm 0.03$ for the C170 mouse line (n = 6). Selected CAG-cat-EDN1 C134 and C170 transgenic mice were backcrossed with C57BL/6 mice obtained from Harlan Laboratories (Indianapolis, IN). Transgenic mice at F4 and F5 generation were used in this study.

**Generation of inducible endotelial-restricted human ET-1 overexpression (ieET-1) mice.** The ieET-1 mice were generated by crossing the CAG-cat-EDN1 C134 and C170 mice with an inducible endothelial Cre (ieCre) mouse, the bacterial artificial chromosome (BAC) transgenic Tie2-CreERT2 mouse. The ieCre mouse expresses a fusion protein of the Cre recombinase with the modified estrogen receptor (ER) binding domain (CreER$^{T2}$) under the control of the endothelium-restricted angiopoietin-1 receptor (Tek, also known as Tie2) promoter. CreER$^{T2}$ can be induced by tamoxifen but not by natural ER ligands.

**Generation of a Cre activation reporter mouse.** The reporter mouse was generated by crossing the ieCre mice with ROSA26$m^{T\text{-mG/mT-mG}}$ mice (Jackson Laboratories, Bar Harbor, ME). These mice express a loxP-flanked, membrane-targeted tandem dimer tomato (mT) followed by a pA, before Cre-mediated excision, and a membrane-targeted enhanced green fluorescent protein (mG) followed by a pA, after excision, under the control of the CAG promoter driving the expression in all cell types. ROSA26$m^{T\text{-mG/mT-mG}}$ mice were crossed with ieCre mice to generate the reporter ieCre/ROSA26$m^{T\text{-mG/mG}}$ mouse.

**Genotyping.** DNA was extracted using a quick method with a modification. Ninety µL of basic digestion buffer (25 mM NaOH and 0.2 mM EDTA, pH 12) was added to the tubes containing the piece of tail, and the mixture was heated at 95°C for 45 min. Then, the tubes were vortexed to ensure tissue disruption and maximal DNA release. The remaining material and lysis solution was brought down the tube by a quick centrifugation at 12,000 x g, and incubation at 95°C was continued for an additional 15 min. At the end of the digestion, the tubes were vortexed and quickly centrifuged. Samples were stored at -20°C until used. Genotyping was done by PCR using 2 µL of supernatant tail digestion mixture.
Genotyping for CAG-cat-EDN1 transgenic mice was done using TopTaq DNA polymerase kit (Qiagen, Mississauga, ON, Canada) by amplifying a 311 base pairs (bp) cat fragment contained within CAG-cat-EDN1 transgene and a 516 bp fragment of MYB proto-oncogene protein gene (Myb) (Fig. S1A). Myb was used as an internal control to avoid any false-negative result. The oligonucleotides were designed to have a melting temperature (Tm) of 60°C and a 3’ GC clamp using Primer3 (Supplemental Table S1).4 The PCR conditions were 3 min at 94°C, followed by 36 cycles of 1 min at 94°C, 1 min at 66°C and 1 min at 72°C.

Genotyping for Tie2-CreERT2 (ieCre) transgenic mice transgenic mice was done using PCR by amplifying a 350 bp fragment containing a portion of Tie2 gene and CreERT2 transgene, and a 207 bp fragment of Tie2 gene used as an internal control (Fig. S1B). These oligonucleotides sequences have been designed previously (Table S1).5 The PCR conditions were 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C.

Genotyping for ROSA26mT-mG/mT-mG mice transgenic mice was done using PCR by amplifying a 250 bp fragment containing a portion of Rosa26 gene and CAG promoter contained within mT-mG transgene and a 322 bp fragment of the Rosa26 gene used as an internal control (Fig. S1C). These oligonucleotides sequences were obtained from Jackson Laboratories (Table S1). The PCR conditions were 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 1 min at 61°C and 1 min at 72°C.

PCR products were run on a 2% agarose gel in 1x TAE (40 mM tris-acetate, 1 mM EDTA) buffer and picture taken (Fig. S1).

CAG-cat-EDN1 transgene relative copy number. The relative copy number of CAG-cat-EDN1 transgene was estimated by determining the ratio of the transgene over the β-actin (Actb) gene by QPCR. DNA was extracted from a segment of tail for each transgenic line as described in Genotyping section. QPCR was performed with 2 µL of supernatant tail digestion mixture diluted 50-times with water using the SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Mississauga, ON, Canada) with the Mx3005P real-time PCR cycler (Agilent Technologies, Mississauga, ON, Canada). The QPCR conditions used were 2 min at 98°C, followed by 40 cycles of 5 sec at 98°C and 30 sec at 58°C. Oligonucleotides were designed with Primer34 as above to amplify a 311 bp fragment of cat contained within CAG-cat-EDN1 transgene and a 207 bp fragment of Actb gene (Table S1).

Confirmation of absence of CAG-cat-EDN1 transgene leakage. Ten to 11-week old male CAG-cat-EDN1-C134 and CAG-cat-EDN1-C170 mice were treated for 5 days with tamoxifen (1 mg/kg/day, s.c.) and used 16 days later for determination of plasma ET-1 levels. Thirteen to 14-week old male C57BL/6 mice were used as reference for endogenous plasma ET-1 levels. 10

Determination the tissue specificity and extend of Cre activation. Ten to 11-week old male icCre/ROSA26mT-mG/+ mice were treated for 5 days with tamoxifen as above or the vehicle (100 µL of Miglyol 812, S.C., a generous gift of Unipex, Boucherville, QC, Canada) and used 16 days later to determine the tissue specificity and extend of Cre activation by determining the switch in expression of mT to mG.
Effect of induction of ET-1 in endothelial cells on blood pressure regulation. Nine to 10-week old male ieCre and ieET-1-C134 and ieET-1-C170 mice were anesthetized with 3% isoflurane mixed with O₂ at 1 L/min. The depth of anesthesia was confirmed by rear foot squeezing. The non-steroidal anti-inflammatory drug carprofen (20 mg/kg) was administered subcutaneously to minimize the post-operation pain. The mice were then surgically instrumented with PA-C10 telemetry transmitters as recommended by the manufacturer (Data Sciences International, St. Paul, MN). Carprofen was administered as above once a day for the first 3 recovery days. Mice were allowed to recover for 7 additional days. Baseline blood pressure was determined every 5 min for 10 sec 2 days before treatment (Day -2 and, -1), during 5 days of treatment with tamoxifen or vehicle as above, and during the 16 following days. An additional group of ieET-1-C134 mice was treated with tamoxifen as above, followed by the ET type A receptor blocker atrasentan (10 mg/kg/day p.o.) from day 10 to the end of the study.

Kidney function. Ten to 11-week old male ieCre and ieET-1-C134 mice were treated with tamoxifen or vehicle as above, and 24-hour urine was collected using metabolic cages to determine renal function during the two last days of the study. In addition, the renal artery flow and resistive index were determined by ultrasound.

Metabolic cages. Twenty-four-hour urine was collected as follows. In order to reduce stress, mice were first housed in metabolic cages (Tecniplast S.p.A., Buguggiate, VA, Italy) for two consecutive days, one week before the actual experiment to have them acclimatize to the environment and experimental procedure. On the last 2 study days, mice were housed in the metabolic cages, and 24-hour urine collection was initiated early in the morning (between 8 and 9 o’clock). Urine was collected at the end of the first 24-hour period. Food powder and feces attached to the urine collector system were removed by rinsing it extensively with water. The urine collector system was dried with paper towel and fixed to the metabolic cage for the second 24-hour urine collection. At the end of the experiment, 24-hour urine was collected, cleared by centrifugation at 10,000 g for 10 min, and stored at -80°C until used. Mice were anesthetized with isoflurane, and blood and tissues collected as described below. Water and foods were supplied at libitum during the whole procedure.

Renal artery flow and resistive index. Right kidney renal artery flow and resistive index were determined by ultrasound as follows. Briefly, the mice were anesthetized with 3 % isoflurane and 2 L/min O₂. Depth of anesthesia was confirmed by rear foot squeezing. The mice were secured lightly on their chest on a warming pad, and their posterior right back was shaved. Echography was performed using a Visual Sonic VEVO 770 ultrasound machine and a RMV™ 704 high frame rate scanhead with a center frequency of 40 MHz (VisualSonics Inc., Toronto, ON, Canada) as follows. The percentage of isoflurane was adjusted to maintain the heart rate (HR) between 500 and 550 beats/min. A two-dimensional short axis view of the right kidney was obtained and a view of the renal artery emerging from the aorta was positioned at the focus level. The pulse wave (PW) Doppler sample volume was positioned into the renal artery in the focal zone, the scanhead was angled to achieve a beam/flow angle of less than 60 degrees, and the PW Doppler spectrum was recorded. The heart rate (HR), renal artery velocity time integral (VTI), peak systolic velocity (PSV) and end diastolic velocity (EDV) were determined from the PW Doppler waveform on five consecutive beats located between two respirations. The renal artery diameter measured from a two-dimensional guided M-mode image, and HR were used to
calculate the renal artery flow. The resistive index (RI) was calculated as follows. RI = (PSV-EDV)/PSV. Representative images of two-dimensional short axis view of the right kidney and PW Doppler spectrum of ieCre treated with tamoxifen, and in ieET-1-134 mice treated with vehicle or tamoxifen, are presented in Fig. S4.

**Collection of tissues.** At the end of the protocol, mice were weighed and then anesthetized with isoflurane as above, and blood collected by cardiac puncture on EDTA for plasma ET-1 determination. Blood samples were centrifuged at 1,000 g for 15 min at 4°C to remove blood cells, followed by centrifugation at 10,000 g for 10 min at 4°C to remove platelets. Plasma samples were stored at -80°C until tested. The mesenteric artery vascular bed was dissected with the intestine, and aorta, heart, lung, liver, two kidneys, spleen and tibia were harvested in ice-cold phosphate-buffered saline (PBS). Tissues were weighed and tibia length determined. Kidney sections were meticulously dissected under the microscope in order to separate renal cortex and medulla. Kidney sections and remaining tissues were frozen in liquid nitrogen and stored at -80°C until used.

For the study of vascular ET receptor mRNA expression, the mesenteric arcade was dissected away from the attached intestine under RNase-free conditions and stored immediately in RNA later (Life Technologies) until RNA extraction.

For the determination of the tissue specificity and extent of Cre activation, ieCre/ROSA26mT-mG/+ were anesthetized with 300-375 mg/kg IP of Avertin (2.5% solution of 1 mg/mL of 2,2,2-tribromoethanol dissolved in tert-amyl alcohol). Depth of anesthesia was confirmed by rear foot squeezing. The mice were injected intraperitoneally with 100 USP units of sodium heparin (1000 USP units/mL), then perfused through the left ventricle at a constant pressure of 100 mmHg for 5 min with PBS to remove the blood, followed by 15 min perfusion with 4% paraformaldehyde (PFA). The mesenteric vascular bed, aorta, heart, kidneys and liver were collected in 4% PFA. Fat surrounding the mesenteric arteries was removed, and the vessels and other tissues were incubated in 4% PFA for 24 h with gentle agitation at 4°C. All tissues with exception of the mesenteric arteries were dehydrated by incubation in 30% sucrose in PBS for 24 h with gentle agitation at 4°C. Tissues were embedded in VWR Clear Frozen Section Compound (VWR international, Edmonton, AL, Canada) and stored at -80°C until used. The mesenteric arteries were stored in PBS at 4°C until imaged.

**Assessement of mT and mG expression.** The expression of mT and mG was assessed by fluorescence microscopy imaging on 4-μm thick cryosections or intact mesenteric artery segments. Four-μm thick cryosections were stained with 4',6-diamidino-2-phenylindole (DAPI, 6 μM, Life Technologies) for 20 min to label the nuclei. Isolated mesenteric arteries were whole-mount stained in nucleus staining solution overnight. Sections of tissues and isolated mesenteric arteries were mounted in Fluoromount (Sigma-Aldrich, St. Louis, MO) and imaged using a Wave FX Spinning Disc Confocal microscope (Quorum Technologies, ON, Canada).

The localization of endothelial cells within the tissue was determined by immunofluorescence in 4-μm cryostat sections using CD31 staining. Tissue cryosections were permeabilized and blocked for 1 hour with buffer solution containing 50 mM of Tris, 150 mM of NaCl, 1% BSA, 0.4% Triton X-100 and 20% fetal bovine serum. Thereafter, sections were washed once with
PBS. Sections were incubated overnight at 4°C with rat anti-mouse CD31 antibody (1:100, BD Biosciences Pharmingen, Ontario, CA). The sections were then washed 3 times with PBS and incubated for 1 hour at room temperature with Alexa 647-conjugated goat anti-rat IgG antibody (1/200, Life Technologies). Sections were then washed 3 times with PBS and counterstained with DAPI and mounted with Fluoromount. Images were captured using Wave FX Spinning Disc Confocal microscope.

**Assessment of endothelial function and vessel mechanics and structure.** Second-order mesenteric arteries, of average lumen size ~220 µm, were dissected and mounted on a pressurized myograph as previously described. Vessels were equilibrated for 45 min at 45 mmHg intraluminal pressure in Krebs solution (pH 7.4) containing (in mmol/L): 120 NaCl, 25 NaHCO3, 4.7 KCl, 1.18 KH2PO4, 1.18 MgSO4, 2.5 CaCl2, 0.026 EDTA and 5.5 glucose, bubbled continuously with 95% air and 5% CO2. Media and lumen diameter were measured by a computer-based video imaging system (Living Systems Instrumentation, Burlington, Virginia, USA). Contractile responses to cumulative concentrations of norepinephrine (10^-8 to 10^-5 mol/L) were determined. Endothelium-dependent responses to acetylcholine (10^-9 to 10^-4 mol/L) were determined in vessels precontracted with norepinephrine (10^-5 mol/L). Contractile response to cumulative doses of ET-1 (10^-12 to 10^-6 mol/L, Bachem, Torrance, CA) and angiotensin II (10^-12 to 10^-6 mol/L, EMD Chemicals, San Diego, CA) were determined. Thereafter, vessels were perfused with Ca2+-free Krebs solution containing 10 mmol/L EGTA for 30 min to eliminate the tone. Media and lumen diameter were measured at 45 mmHg intraluminal pressure. Media cross-sectional area and media/lumen were calculated as previously described.

**Plasma and urine determinations.** Plasma ET-1 was determined using a human Endothelin-1 QuantiGlo ELISA Kit (R&D Systems, Minneapolis, MN). Urinary sodium, potassium, urea, creatinine and protein were measured using a J&J Vitros 250 chemistry analyzer by Diagnostic Research Support Services at the Comparative Medicine and Animal Resource Centre of McGill University. Urinary nephrin and lipocalin-2 (also known as NGAL) were quantified using the Nephrin ELISA (Exocell, Philadelphia, PA) and Quantikine ELISA Mouse Lipocalin-2/NGAL Immunoassay (R&D Systems, Minneapolis, MN), respectively.

**Quantification of cat, Ednra, Ednrb and Ren1 mRNA expression.** The expression of chloramphenicol acetyltransferase (cat), ET type A and B receptor (Ednra and Ednrb), renin (Ren1) and ribosomal protein S16 (Rps16) was determined in cardiac ventricles or renal cortex and medulla or mesenteric arteries by RT-QPCR. RNA was extracted from frozen tissues using Trizol Reagent (Life Technologies) with a Polytron PT 1600 E homogenizer (Brinkmann Instruments, Mississauga, ON, Canada) and then processed as previously described. The mesenteric arteries were dissected from surrounding tissues, perivascular fat and veins in RNA later, then homogenized for 1 min with a Polytron PT 1600 E homogenizer and processed for total RNA extraction using the mirVana miRNA isolation kit (Life Technologies). RNA concentration was measured using a Nanodrop spectrophotometer ND-100 V3.1.2 (Thermo Fisher Scientific, Wilmington, DE) and RNA quality was assessed by determining the rRNA and mRNA profile by electrophoresis with a RNase free 1% agarose gel with 1X TAE electrophoresis buffer (2 M Tris-acetate and 50 mM EDTA). One and 0.4 µg of total RNA isolated from the frozen tissue and mesenteric arteries, respectively, were reverse-transcribed with the Quantitect RT kit (Qiagen). QPCR was performed using the SsoFast EvaGreen
Supermix with the Mx3005P real-time PCR cycler. Oligonucleotides for cat, Ednra, Ednrb and Ren1 and Rps16 were designed with Primer3 as above (Table S1). The QPCR condition were 2 min at 96 °C, followed by 40 cycles of 5 sec at 96°C and 30 sec at 58°C. Results were normalized with Rps16 and expressed as fold change over control.

**Data Analysis.** Results are presented as means ± SEM. Blood pressure and plasma ET-1 data were compared by two-way analysis of variance (ANOVA) for repeated measures and one-way ANOVA respectively, all followed by a Student-Newman-Keuls post-hoc test. All other results were compared using a Student t-test or one-way ANOVA followed by a contrast post-hoc test, as appropriate. P<0.05 was considered statistically significant.
References


Table S1. Oligonucleotides, product sizes and applications.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Product size (bp)</th>
<th>Application</th>
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<tbody>
<tr>
<td><strong>EDN1-rHbb</strong></td>
<td>F: 5’-GATC ACTAGTAGATGGATTAATTGCTCATGATTATTT-3’&lt;br&gt;R: 5’-GATC TGATCACTGTCCTCAGGAGA-3’</td>
<td>1525</td>
<td>PCR subcloning of the EDN1-rHbb fragment</td>
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<tr>
<td></td>
<td>Seq: 5’-GGGACCCTTGATTGTTCTTTC-3’&lt;br&gt;Seq: 5’-AAAATTCGTGAAATCGACGAC-3’&lt;br&gt;Seq: 5’-CCCTGAGTTCTTTTCCTGC-3’</td>
<td>N/A</td>
<td>Sequencing of the subcloned EDN1-rHbb fragment</td>
</tr>
<tr>
<td><strong>cat</strong></td>
<td>F: 5’-TTTCTGCTCAAGGCAATCCC-3’&lt;br&gt;R: 5’-TTTCTGCTCAAGGCAATCCC-3’</td>
<td>311</td>
<td>PCR genotyping and QPCR copy number determination of CAG-cat-Edn1</td>
</tr>
<tr>
<td><strong>Actb</strong></td>
<td>F: 5’-TCCTCCTCCCGACTCCAGC-3’&lt;br&gt;R: 5’-CCATCCGCTATTATATATCC-3’</td>
<td>316</td>
<td>Control for CAG-cat-Edn1 PCR genotyping</td>
</tr>
<tr>
<td><strong>Tie2-CreER&lt;sup&gt;T2&lt;/sup&gt;</strong></td>
<td>F: 5’-GAAGTCGCAAGGAGTTGAGTG3’&lt;br&gt;R: 5’-TGGCTTGGCAGGAGAGGA-3’&lt;br&gt;(Tie2)</td>
<td>350</td>
<td>PCR genotyping of Tie2-CreER&lt;sup&gt;T2&lt;/sup&gt;. Product confirming CreER&lt;sup&gt;T2&lt;/sup&gt; transgene.</td>
</tr>
<tr>
<td><strong>Tie2</strong></td>
<td>F: 5’-ATCCTAATTGGAAGCTACAG3’&lt;br&gt;R: 5’-ATCCTAATTGGAAGCTACAG3’</td>
<td>207</td>
<td>Control for Tie2-CreER&lt;sup&gt;T2&lt;/sup&gt; PCR genotyping.</td>
</tr>
<tr>
<td><strong>cat</strong></td>
<td>F: 5’-ATCCTAATTGGAAGCTACAG3’&lt;br&gt;R: 5’-ATCCTAATTGGAAGCTACAG3’</td>
<td>314</td>
<td>RT-QPCR mRNA expression levels of cat</td>
</tr>
<tr>
<td><strong>Ednra</strong></td>
<td>F: 5’-TTGCTCTTGAGACCTATAC3’&lt;br&gt;R: 5’-TGGCTCTTGAGACCTATAC3’</td>
<td>339</td>
<td>RT-QPCR mRNA expression levels of Ednra</td>
</tr>
<tr>
<td><strong>Ednrb</strong></td>
<td>F: 5’-GCTCCCATCCGGTATTAC3’&lt;br&gt;R: 5’-GCTCCCATCCGGTATTAC3’</td>
<td>327</td>
<td>RT-QPCR mRNA expression levels of Ednrb</td>
</tr>
<tr>
<td><strong>Ren1</strong></td>
<td>F: 5’-ATGATGCACACATGACGAC-3’&lt;br&gt;R: 5’-AGAATGACGACGACCACTGC-3’</td>
<td>278</td>
<td>RT-QPCR mRNA expression levels of Ren1</td>
</tr>
<tr>
<td><strong>Rps16</strong></td>
<td>F: 5’-ATCTCAAAAGGCCCTCGTACG3’&lt;br&gt;R: 5’-ATCTCAAAAGGCCCTCGTACG3’</td>
<td>211</td>
<td>Housekeeping gene for relative quantification of mRNA by QPCR</td>
</tr>
</tbody>
</table>

Forward (F) and reverse (R) oligonucleotides used to subclone by PCR the human endothelin-1/rabbit beta-globin transgene (EDN1-rHbb) contain respectively Spe I and Bcl I restriction sites (bold italic) and both a cap GATC sequence (underline). Actb, β-actin; cat, chloramphenicol acetyltransferase; Ednra and Ednrb, endothelium type A and B receptor; Myb, mouse transcriptional activator myeloblastosis; Ren1, renin; Rps16, 40S ribosomal protein S16; Tie2-CreER<sup>T2</sup>, a fusion protein of Cre recombinase with the modified estrogen receptor binding domain (CreER<sup>T2</sup>) under the control of the endothelium-specific angiopoietin-1 receptor (Tie2 and alias of Tek) promoter.
Figure S1. Genotyping of mice for CAG-cat-EDN1 (A), Tie2-CreERT2 (B) and ROSA26<sup>mT-mG/+</sup> (c). Representative images of agarose gel for genotyping of wild-type (WT, A-C) and heterozygote (HT) cat-EDN1 (A), Tie2CreERT2 (B) and ROSA26<sup>mT-mG/+</sup> (c) mice are shown. 

a. CAG-cat-EDN1 genotyping: PCR product of 516 base pairs (bp) is used for detection of Myb control gene (WT) and 311 bp for detection of CAG-cat-EDN1 (cat) transgene. 

b. Tie2-CreERT2 genotyping: PCR product of 207 bp is used for detection of Tie2 gene (WT) and PCR product of 350 bp for detection of Tie2-CreERT2 transgene (Cre). 

c. ROSA26<sup>mT-mG/+</sup>: PCR product of 322 bp is used for detection of Rosa26 gene and 250 bp for detection of CAG promoter contained within mT-mG transgene. Sizes in bp on the left side of each panel are for the DNA ladder.
**Figure S2.** Tissue specificity of Cre activation was determined using vehicle- and tamoxifen-treated ieCre/ROSA26mT-mG/+ reporter mice by determining the replacement of membrane-targeted tandem dimer tomato (mT) by membrane-targeted enhanced green fluorescent protein (mG) expression using confocal microscopy imaging. Representative mG, mT and the CD31 endothelium marker (cyan) fluorescence images of heart and liver sections are presented. Blue represents nuclear stain DAPI fluorescence. n = 5 per group.
Figure S3. Induction of endothelin-1 overexpression in the endothelium increased systolic blood pressure (SBP) in ieET-1-C170 mice. Nighttime (A) and daytime (B) SBP was assessed by telemetry in ieCre and in ieET-1-C170 mice treated with tamoxifen (Tam). Days of Tam treatment are indicated by a box. Data are presented as means ± SEM, n = 5. †P<0.05 vs. IeCre + TAM.
Figure S4. Renal artery blood flow was determined by echography using two-dimensional short axis view and pulse wave (PW) Doppler as described in the Online Methods section. Representative images of two-dimensional short axis view of the right kidney (upper panels) and PW Doppler spectrum (lower panels) of ieCre treated with tamoxifen (A) and in ieET-1-134 mice treated with vehicle (B) or tamoxifen (C). The PW Doppler sample volume indicated by a pair of yellow lines was positioned into the renal artery (RA) at the focal zone indicated by an arrow head. The PW Doppler angle line (yellow dashed line) was positioned parallel to the blood flow. The PW angle was measured between the PW Doppler line of acquisition (red line) and PW Doppler angle line. Aorta, A, PSV, peak systolic velocity and EDV, end diastolic velocity.