Lack of Endothelial Nitric Oxide Synthase Promotes Endothelin-Induced Hypertension: Lessons from Endothelin-1 Transgenic/Endothelial Nitric Oxide Synthase Knockout Mice

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Endothelin-1 (ET-1) is one of the most potent biologic vasoconstrictors. Nevertheless, transgenic mice that overexpress ET-1 exhibit normal BP. It was hypothesized that vascular effects of ET-1 may be antagonized by an increase of the endothelial counterpart of ET-1, nitric oxide (NO), which is produced by the endothelial NO synthase (eNOS). Therefore, cross-bred animals of ET transgenic mice (ET+/+) and eNOS knockout (eNOS−/−) mice and were generated, and BP and endothelial function were evaluated in these animals. Endothelium-dependent and -independent vascular function was assessed as relaxation/contraction of isolated preconstricted aortic rings. The tissue ET and NO system was determined in aortic rings by quantitative real-time PCR and Western blotting. Systolic BP was similar in ET+/+ and wild-type (WT) mice but was significantly elevated in eNOS−/− mice (117 ± 4 mmHg versus 94 ± 6 mmHg in WT mice; P < 0.001) and even more elevated in ET+/+ eNOS−/− cross-bred mice (130 ± 4 mmHg; P < 0.05 versus eNOS−/−). Maximum endothelium-dependent relaxation was enhanced in ET+/+ mice (103 ± 6 versus 87 ± 4% of preconstriction in WT littermates; P < 0.05) and was completely blunted in eNOS−/− (−3 ± 4%) and ET+/+ eNOS−/− mice (−4 ± 4%), respectively. Endothelium-independent relaxation was comparable among all groups. Quantitative real-time PCR as well as Western blotting revealed an upregulation of the aortic ETₐ and ETₐ receptors in ET+/+ eNOS−/−, whereas eNOS was absent in aortic rings of eNOS−/− and ET+/+ eNOS−/− mice. ET-1 aortic tissue concentrations were similar in WT mice and ET+/+ eNOS−/− mice most probably as a result of an enhanced clearance of ET-1 by the upregulated ETₐ receptor. These data show for the first time that in transgenic mice that overexpress human ET-1, additional knockout of eNOS results in a further enhancement of BP as compared with eNOS−/− mice. The human ET+/+ eNOS−/− mice therefore represent a novel model of hypertension as a result of an imbalance between the vascular ET-1 and NO systems.


The intact endothelium produces a variety of vasoactive substances, such as nitric oxide (NO), thromboxane, and the 21-amino acid peptide endothelin-1 (ET-1). ET-1 is a potent vasoconstrictor (1) and mitogen in vitro and in vivo and exerts its biologic effects via activation of specific receptors: Whereas on vascular smooth muscle cells the predominant ET receptor is the ETₐ receptor (ETₐR) subtype (2,3), contributing to long-lasting vasoconstriction, endothelial cells express the ETₐ receptor (ETₐR), mediating the formation of NO (4) and prostacyclin (5) and featuring the pulmonary clearance of circulating ET-1 (6) as well as the reuptake of ET-1 by endothelial cells (7).

Correspondingly, intravenous administration of ET-1 causes a rapid and transient vasodilation followed by a sustained increase in BP (8). In addition to the negative feedback loop via ETₐR, ET-1 production in the vascular wall is inhibited by NO (9) and prostacyclin (10) via cGMP-dependent mechanisms. Therefore, the NO system may be considered as the functional counterpart of the ET system (11–16), thus warranting the sub-
tile balance of vascular tone in a wide range of conditions and emphasizing the pivotal role of the endothelium as a regulator of vascular tone. ET-1 has been considered to play an important role in several animal models of hypertension (12,14,15).

The normalization of BP by ET receptor agonists in various forms of experimental hypertension (14,17) as well as in human essential hypertension (18) demonstrates its impact on the regulation of vascular tone. However, two independent ET-1 transgenic mouse models do not support the concept that the ET system might play an important role in the pathogenesis of hypertension, because these ET-1-overproducing mice are not hypertensive (19,20). In line with these data is the finding of normal BP in ET-2 transgenic rats (21,22).

Counterregulatory interactions between the NO and ET systems are well established (9,23). Therefore, we suspected that an activation of the NO system accompanied the activation of the ET system. Indeed, through enhanced vasodilation, NO could antagonize vascular ET effects. Consistent with this hypothesis, we were able to demonstrate an increased NO-dependent vasodilation in ET+/+ mice before (24,25). Considering the short half-life of NO, an increase in NO availability most likely will be due to an increase in NO production. Recently, we were able to demonstrate that the inducible NO synthase (iNOS) is upregulated in ET-1 transgenic mice and contributes to BP regulation (26). However, because endothelial nitric oxide synthase (eNOS) is the quantitatively most important source of NO in blood vessels, the eNOS might likewise contribute to the maintenance of normal BP in ET-1 transgenic mice.

To verify this hypothesis, we generated cross-bred mice of ET+/+ and eNOS knockout (eNOS−/−) mice. These ET+/+ eNOS−/− mice combined the attributes of ET-1 overexpression and the lack of relevant eNOS-derived NO production. The aim of this study was to elucidate the impact of reduced NO synthesis on BP and endothelial function in states of an activated ET system.

**Materials and Methods**

**Mice**

Male heterozygous human ET-1 transgenic mice (line 856) were generated by microinjection using the human prepro-ET-1 expression cassette as described previously (19). Genotype was confirmed by PCR of genomic DNA using standard techniques (19). Because of the small number of resulting homozygous ET-1 transgenic mice, heterozygous mice, which were shown to exhibit a similar phenotype as compared with homozygous mice (19), were used for all experiments. ET-1 transgenic mice exhibited elevation of ET tissue levels in kidney, lung, and brain (19). They furthermore presented with an increase in development of interstitial fibrosis and glomerulosclerosis (19,27).

eNOS−/− mice were a gift from Prof. David Rodman and were generated as described previously (28). Female eNOS−/− mice and male homozygous ET-1 transgenic mice (19) were used for breeding.

Transgenic/knockout mice and their wild-type (WT) littermates were kept under controlled environmental conditions with respect to temperature (20°C), humidity (64%), and a 12-h night-day light cycle. Mice were fed on a standard breeding rodent diet and water *ad libitum*. Study groups were composed as follows: WT (n = 17), ET transgenic mice (ET+/+; n = 18), eNOS−/− mice (eNOS−/−; n = 25), and cross-bred mice (ET+/+eNOS−/−; n = 22). At the age of at least 4 mo, all mice were killed, organs were weighed, and aortas were harvested for histologic studies. In a subset of each group, aortic segments were used for isometric tension recording in an organ chamber under physiologic conditions (see the Organ Chamber Experiments section). Study design and experimental protocols were conducted according to the local institutional guidelines for the care and use of laboratory animals and are in accordance with the American Heart Association guidelines for research animal use.

**Blood Pressure**

BP was measured by the tail-cuff method (Blood Pressure Monitor BMN-1756; Führ Medical Instruments, Seeheim, Germany) in unanesthetized mice that underwent 4 d of extensive training to get used to this procedure (24). For every data point, five measurements were performed and mean values of five subsequent measurements were calculated.

**Tissue Harvesting**

Mice were anesthetized with pentobarbital (40 mg/kg body wt, intraperitoneally) and were killed by cervical dislocation. The aorta was isolated in no-touch technique as described previously (29), removed, and placed immediately into cold (4°C) modified Krebs-Ringer bicarbonate solution (in mmol/L): 118.6 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.1 NaHCO₃, 0.026 EDTA, and 10.1 glucose. Under a microscope, vessels were rinsed with a cannula to remove residual blood cells, cleaned of adherent tissue, and cut into rings 3 mm long.

**Organ Chamber Experiments**

Aortic rings were suspended by fine tungsten stir-ups (diameter 50 μM), placed in an organ bath filled with 10 ml of Krebs solution, and connected to force transducers (Führ Medical Instruments) for isometric tension recording as described previously (30). After an equilibration period of 60 min, aortic rings were progressively stretched to their optimal passive tension (2.0 ± 0.2 g) as assessed by the response to 100 μmol/L KCl in modified Krebs solution (31). Rings were precontracted with norepinephrine (approximately 70% of KCl 100 μmol/L) and relaxations to acetylcholine (10−10 to 10−7 mol/L) or sodium nitroprusside (10−11 to 10−5 mol/L) were acquired. To rule out the impact of cyclooxygenase products, we performed experiments with and without preincubation with 10−5 mol/L diclofenac for 30 min. In additional experiments, cumulative concentration–response curves to ET-1 (10−10 to 10−7 mol/L) were obtained in quiescent preparations and after preincubation with the selective ET₄R antagonist BQ-788 (10−5 mol/L) or the selective ET₄R antagonist BQ-123 (10−5 mol/L), respectively. All chemical substances and drugs used in this study were purchased from Sigma Aldrich Chemical Co. (Munich, Germany), apart from ET-1, which was purchased from Calbiochem AG (La Jolla, CA), and BQ-123 and BQ-788, which were obtained from A.G. Scientific (San Diego, CA).

**Histologic Evaluation**

Computer-aided image analysis was performed as recently described (32). Briefly, for histologic evaluation, aortas were embedded in paraffin, cut in 3-μm sections, and submitted to Elastica–van Gieson staining. Quantitative stereology (i.e., intima/media and lumen area of the aortas) was analyzed using a computer-aided image analysis system. In brief, microscopic pictures of aortas in Elastica–van Gieson staining were transferred from the microscope to a PowerMAC via a Hitachi CCD camera. Then the inner and outer diameters of aortas were measured using ImageJ, an image-processing software (shareware from the National Institutes of Health); afterward area contents of media and
lumen were calculated using standard formulas. Data were expressed as media-lumen ratio serving as marker for aortic wall thickening.

**Quantitative Real-Time PCR**

Total RNA was extracted from snap-frozen aortic tissue using Trizol reagent (Invitrogen 15996-026, Carlsbad, CA). A DNase treatment was performed using the Nuclease Pip II extraction kit from Merckery & Nagel (cat. no. 746955). The RNA concentration was determined by absorbance at 260 nm. RNA was reverse-transcribed to cDNA using the Superscript III reverse transcriptase from Invitrogen (18080-044) according to the manufacturer’s protocol. The specific primers for mouse and human ET-1 and the primers for hypoxanthine guanine phosphoribosyl transferase (HPRT) were designed with the online available computer program Primer 3 (33) using as template the sequence from the Ensembl Database (human ET-1 Ensembl Transcript ID ENST00000075232; murine ET-1 Ensembl Transcript ID ENSMUST0000021796; murine HPRT Ensembl Transcript ID ENSMUST0000026723). The specificity of the primers were verified by sequence analysis using the BLAST program. The other primers used were derived from the literature (Table 1). A total of 5 µl of cDNA 10 µg/ml (50 ng) was used as template for the amplification. A total of 0.5 µl of each specific primer (5 pmol), 6.5 µl of water, and 12.5 µl of SYBR Green PCR Master Mix (4309155; Applied Biosystems, Foster City, CA) were added. All spots were performed in triplicate. The PCR was performed on an Mx3000P thermal cycler (Stratagene, La Jolla, CA). Reaction conditions were 95°C for 10 min followed by 40 cycles of the amplification steps (95°C for 15 s, 60°C for 30 s, and 72°C for 45 s). We normalized quantity (nQ) was then normalized to the expression in WT mice using the formula sQ = nQtest/mQWT, where Ct(gene of interest) and Ct(HPRT) represent the threshold cycle for the gene of interest and for HPRT, respectively; minCt(gene of interest) and minCt(HPRT) are the lower Ct values for the gene of interest and HPRT, respectively; nQtest is the normalized quantity in a given genotype, and mQWT is the mean of the nQ in WT mice.

eNOS/iNOS and ET<sub>A</sub>R/ET<sub>B</sub>R Western Blot Analysis

**Protein Extraction.** Snap-frozen aorta were pulverized with mortar and pestle under liquid nitrogen. The powder was dissolved in 1 ml of RIPA Buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium deoxycholic acid, 0.1% SDS, and 1% Triton X100) supplemented with a protease inhibitor cocktail (Complete; Roche Diagnostic 1697498). The samples were vortexed and sonicated for 5 s for five cycles. The samples were incubated for 10 min at room temperature and then centrifuged for 12 min at 13,000 rpm at 4°C. The supernatants were collected and stored at −20°C. The protein concentration was measured using the BCA assay from Pierce (Rockford, IL; cat. no. 23225).

**Western Blotting.** Proteins (25 µg) were loaded on an SDS-PAGE gel 10% acrylamide and separated for 2.5 h at 100 V at 4°C. The proteins were transferred onto a nitrocellulose membrane for 25 min (intensity 2 mA/cm²) using semidry transferring equipment.

The unspecific sites were blocked overnight at 4°C with TBS Tween/5% milk. The anti-ET<sub>A</sub>R (M60 sc33536; Santa Cruz Biotechnol-ogy, Santa Cruz, CA), anti-ET<sub>B</sub>R (M74 sc33538; Santa Cruz Biotechnology), and anti-β-actin (C11 sc1615; Santa Cruz Biotechnology) antibodies were diluted 1:500 in TBS Tween/4% BSA and incubated for 1 h at room temperature. The anti-eNOS antibody (Alexis ALX-210-509) and the anti-iNOS antibody (gift from J. Pfeilschifter, Institute of Pharmacology, University of Frankfurt, Frankfurt, Germany) were diluted 1:500 in TBS Tween/5% milk and incubated overnight at 4°C. The horseradish peroxidase–coupled anti-rabbit Ig secondary antibody (NA934V; Amersham Biosciences, Buckinghamshire, UK; for ET<sub>A</sub>R, ET<sub>B</sub>R, eNOS, and iNOS detection) and the horseradish peroxidase–coupled anti-goat Ig secondary antibody (P0449; DakoCytomation, Amersham Biosciences) were used for secondary antibody detection.

**Table 1.** PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence 5’ to 3’</th>
<th>Amplicon Length (bp)</th>
<th>Spanned Intron</th>
<th>Reference</th>
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<tr>
<td>ET&lt;sub&gt;A&lt;/sub&gt;R</td>
<td>+GCTGGTCCCTCCTCACATTAAGC -TCATGTTGCCAGGTAAATGC</td>
<td>129</td>
<td>6</td>
<td>(50)</td>
</tr>
<tr>
<td>ET&lt;sub&gt;B&lt;/sub&gt;R</td>
<td>+GCTGCCTAAGTGACAGATCGAG -GCCTGTCTTGAACTGATGAA</td>
<td>240</td>
<td>2, 3</td>
<td>(50)</td>
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<tr>
<td>mET-1</td>
<td>+GGAACCTCGAGGTTGAGGCC -CTTGAAGCAGCCACAGATGGTCT</td>
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<td>4</td>
<td></td>
</tr>
<tr>
<td>hET-1</td>
<td>+AGGTCAGAGGGACACTACAAACACCAAC -GAGGCTATGGCTTACACAGG</td>
<td>170</td>
<td>4, 5</td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>+CAGCTGGCGTCTACAACCTTT -CTTGAAGTGGAGCTTGTTTG</td>
<td>95</td>
<td>17, 18</td>
<td>(51)</td>
</tr>
<tr>
<td>eNOS</td>
<td>+CCTCCCGCTTACCAGCCCAAGA -CAGAGATCCCTACTGCGATGCTA</td>
<td>105</td>
<td>10,11,12</td>
<td>(52)</td>
</tr>
<tr>
<td>ECE</td>
<td>+ATGACGGCGCCTATGGTGAAC -TGCGCTGAGAAAGACATGAC</td>
<td>110</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>HPRT</td>
<td>+CAGGCCAGACTTTGTGGAT -TTGGCTCTCATCATTGCTTT</td>
<td>147</td>
<td>7, 8, 9</td>
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</table>

*+, forward; −, reverse; ECE, endothelin-converting enzyme; eNOS, endothelial nitric oxide synthase; ET<sub>A</sub>R, endothelin subtype A receptor; ET<sub>B</sub>R, endothelin subtype B receptor; hET-1, human endothelin-1; HPRT, hypoxanthine guanine phosphoribosyl transferase; iNOS, inducible nitric oxide synthase; mET-1, mouse endothelin-1.
Glostrup, Denmark; for β-actin detection) were diluted 1:4000 and 1:2000 in TBST Tween/4% BSA, respectively, and incubated for 1 h at room temperature. Between each detection, the membrane was stripped by incubation with glycine 25 mM/SDS 1% (pH 2) for 30 min at 50°C. Every incubation was preceded by three washes with TBS Tween for 10 min.

Immunoreactive sites were visualized using a chemiluminescence detection system (ECL Western Blotting Detection Reagents; RPN2109; Amersham Bioscience). Membranes were exposed to autoradiography film (Kodak BioMax Light Film, Rochester, NY; 8194540), and the films were scanned on a Canon scanner (Canoscan 5000F; Canon, Tokyo, Japan). The bands were analyzed using the AlphaEaseFC software (Alpha Innotech, San Leandro, CA) for densitometry.

**ET-1 Tissue Levels**

**Tissue Preparation.** The probes were stored in liquid nitrogen until further analysis. The frozen tissue was powdered in the presence of liquid nitrogen. The powdered samples were suspended and subsequently homogenized using a motor-driven pestle homogenizer in 2 ml of buffer solution at 4°C (0.14 mol/L NaCl, 2.6 mmol/L KCl, 8 mmol/L Na₂HPO₄, 1.4 mmol/L KH₂PO₄, and 1% Triton-X 100 [pH 7.4]). The homogenates were centrifuged at 4°C for 60 min at 100,000 × g. The supernatants were retained for ET-1 ELISA. The recovery rate for ET-1 after preparation of tissue ET-1 as described previously was always between 97.5 and 99%.

**ELISA.** The commercially available enzyme immunoassays for ET-1 that were suitable for direct measurement of ET-1 in plasma and after tissue preparation were obtained from Biomedic (Vienna, Austria) and were performed as described previously (15,27).

**Statistical Analyses**

Relaxations to agonists in isolated vessels are given as percentage of precontraction in rings that were precontracted with norepinephrine to approximately 70% of contraction induced by KCl (100 mmol/L). The contractions were expressed as a percentage of 100 mmol/L KCl-induced contractions, which were obtained at the beginning of every experiment. Results are presented as means ± SEM. In all experiments, n equals the number of mice per experiment. For statistical analysis, the sensitivity of the vessels to the drugs was expressed as the negative logarithm of the concentration that caused half-maximal relaxation or contraction (pD₂). Maximal relaxation (expressed as a percentage of precontraction) or contraction was determined for each individual concentration–response curve by nonlinear regression analysis with the use of MatLab software (Math Works, Natick, MA). For comparison between two values, the unpaired t test or the nonparametric Mann-Whitney test was used when appropriate. For multiple comparisons, results were analyzed by ANOVA followed by Bonferroni correction (34). Pearson correlation coefficients were calculated by linear regression. For quantitative real-time PCR, values were reported as means ± SEM, and differences between groups were determined using the unpaired t test. P < 0.05 was considered significant.

**Results**

**Systolic BP**

Systolic BP was different between the study groups (Figure 1): Compared with WT mice both in eNOS−/− mice and in cross-bred ET+/+ eNOS−/−, a significantly elevated systolic BP was observed (117 ± 4 mmHg for eNOS−/− mice versus 94 ± 6 mmHg in WT mice; P < 0.001), whereas in ET+/+ mice, BP was not different from that in WT mice. Furthermore, BP in cross-bred animals with both attributes was significantly enhanced compared with eNOS−/− alone (130 ± 4 mmHg; P < 0.05 versus eNOS−/−).

**Body Weight and Organ Weight**

Body weight was comparable in all groups (ET+/+ mice 34 ± 1 g; ET+/+ eNOS−/− mice 34 ± 1 g; eNOS−/− mice 37 ± 1 g; WT controls 37 ± 2 g). In addition, weight of right and left kidney, spleen, lungs, and heart did not differ significantly among the four groups.

**Endothelium-Dependent Relaxation**

In ET+/+ mice, maximum endothelium-dependent relaxation of preconstricted aortic rings to acetylcholine was significantly elevated in comparison with WT littermates (103 ± 6 versus 87 ± 4% of preconstriction with norepinephrine; P < 0.05; Figure 2). Absence of eNOS completely blunted endothelium-dependent relaxation in both ET+/+ eNOS−/− (−4 ± 4% of preconstriction) and eNOS−/− only mice (−3 ± 4% of preconstriction; each P < 0.01 versus WT controls; Figure 2). Preincubation with diclofenac (10⁻⁵ mol/L for 30 min) did not significantly influence endothelium-dependent relaxation (data not shown).

**Endothelium-Independent Relaxation**

In contrast to endothelium-dependent relaxation, maximal endothelium-independent relaxation to the NO donor sodium nitroprusside was comparable in all groups (Figure 3), indicating that the NO-dependent intracellular signal transduction pathway was not affected in this model. Preincubation with diclofenac (10⁻⁵ mol/L for 30 min) did not significantly alter endothelium-independent relaxation (data not shown).
Concentration-Dependent Contraction to ET-1

Maximum contraction to ET-1 was reduced in ET+/+ mice as compared with their WT littermates (30 ± 4 versus 87 ± 8% of 100 mmol/L KCl; P < 0.05; Figure 4). Reduction of ET-1-mediated contraction in ET+/+ eNOS−/− mice was similar (38 ± 5% of 100 mmol/L KCl; P < 0.05 versus WT littermates; Figure 4). Contraction to ET-1 in eNOS−/− mice (90 ± 8% of 100 mmol/L KCl; Figure 4) did not differ from WT controls.

In vitro preincubation of vessel rings with the selective ETAR antagonist BQ-123 (10−5 mol/L) completely blunted contractions to ET-1 in all groups (Figure 5A). In contrast, preincubation of vessel rings with the selective ETBR antagonist BQ-788 (10−5 mol/L) did not significantly affect contractions to ET-1 (Figure 5B).

Histology Results

Media-lumen ratio of aorta was assessed using computer-aided histomorphometry devices: Compared with WT controls, a significantly decreased media-lumen ratio was observed in cross-bred mice, whereas eNOS−/− mice did not differ from controls, and in ET+/+ mice, a trend (P = 0.07) toward higher values was observed. Within the transgenic groups, cross-bred mice exhibited significantly lower media-lumen ratio when compared with both ET+/+ and eNOS−/− mice (Figure 6). When absolute lumen area was compared between the groups, ET+/+ mice (18.8 ± 1.8) exhibited a significantly smaller lumen area than ET+/+eNOS−/− mice (31.9 ± 4.0) or eNOS−/− mice (23.0 ± 1.3) and a trend (P = 0.07) toward lower lumen area compared with WT mice (25.5 ± 3.2).

mRNA Expression

mRNA expression of endogenous murine ET as well as transgenic human ET, ETARB, ECE, iNOS, and eNOS was assessed in aortic tissue using quantitative real-time PCR. Results are illustrated in Table 2. Whereas no significant differences regarding all of these parameters between ET+/+ and WT mice were detected, both groups that carried an eNOS knockout (eNOS−/− and ET+/+eNOS−/−) exhibited a significantly enhanced iNOS mRNA expression when compared with WT mice. In cross-bred mice, expression of all parameters related to the endothelin system was markedly upregulated when compared with WT controls: Endogenous murine ET as well as transgenic human ET (compared with ET+/+ mice), ETAR, ETBR, and ECE (Table 2).

\( \text{ET}_A, \text{ET}_B, \text{iNOS}, \text{and eNOS Western Blot Analysis} \)

In ET transgenic mice, ETARB protein was not significantly altered as compared with WT mice (0.79 ± 0.17-fold versus WT; NS). In eNOS−/− mice, ETAR expression was reduced (0.27 ± 0.7-fold versus WT; P < 0.005), whereas in combined ET+/+...
eNOS−/− mice, ETAR protein expression was significantly increased (1.61 ± 0.18-fold versus WT; P < 0.05; Figure 7A). In addition, ETAR was markedly overexpressed at the protein level in ET+/+ eNOS−/− mice as compared with WT mice (3.05 ± 0.75-fold; P < 0.05; Figure 7B).

In ET-1 transgenic mice, iNOS protein level was significantly increased (4.68 ± 0.82-fold versus WT; P < 0.05; Figure 7C). Knockout of eNOS did not significantly alter iNOS protein expression (1.32 ± 0.29-fold in eNOS−/− mice versus WT [NS]; 2.58 ± 0.88-fold in ET+/+ eNOS−/− mice versus WT [NS]; Figure 7C). eNOS Western blotting confirmed absence of eNOS protein in eNOS−/− mice, whereas eNOS protein expression in ET+/+ mice was not significantly altered (1.67 ± 0.22; NS; Figure 7D).

Figure 5. (A) Contraction to ET-1 in aortic segments of 12-wk-old ET+/+ mice, eNOS−/− mice, ET+/+ eNOS−/− mice, and their WT littermates. Preincubation with the selective ETA receptor antagonist BQ123 10^{-5} mol/L. Results are means ± SEM (n = 6 per group). (B) Contraction to ET-1 in aortic segments of 12-wk-old ET+/+ mice, eNOS−/− mice, ET+/+ eNOS−/− mice, and their WT littermates. Preincubation with the selective ETβ receptor (ETBR) antagonist BQ788 10^{-5} mol/L. Results are means ± SEM (n = 6 per group). *P < 0.05 versus WT mice.

Figure 6. (A) Aortic media-lumen ratio of ET+/+ mice (n = 15), eNOS−/− mice (n = 23), ET+/+ eNOS−/− mice (n = 18), and their WT littermates (n = 14). *P < 0.05 versus WT mice; **P < 0.05 versus eNOS−/− mice; †P < 0.05 versus ET+/+ mice; §P < 0.001 versus ET+/+ mice. (B) Typical aortic sections in Elastica–Van Gieson staining. (A) WT mice. (B) ET+/+ mice. (C) eNOS−/− mice. (D) ET+/+ eNOS−/− mice.

ET-1 Tissue Levels

ET-1 concentration in aortic tissue as measured by ELISA was not significantly different among the four groups, independent of whether it referred to wet weight or to milligrams of protein in aortic tissue (Table 3).

Discussion

In this study, we evaluated the relevance of eNOS activity in states of overproduction of ET-1. We generated cross-bred mice of ET+/+ and eNOS−/− mice. Systolic BP was similar in ET+/+ and WT mice but was significantly elevated in eNOS−/− mice and even more elevated in ET+/+ eNOS−/− cross-bred mice. Endothelium-dependent relaxation was en-
enhanced in ET+/+ mice and was completely blunted in eNOS−/− and ET+/+ eNOS−/− mice, respectively. Therefore, in transgenic mice that overexpressed ET-1, additional knockout of eNOS results in a further enhancement of BP and represents a novel model of hypertension as a result of an imbalance between the vascular ET-1 and NO systems.

Table 2. mRNA levels in aortic tissue

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT</th>
<th>ET+/+</th>
<th>eNOS−/−</th>
<th>ET+/+ eNOS−/−</th>
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<tr>
<td></td>
<td>1.00 ± 0.25</td>
<td>0.54 ± 0.11</td>
<td>0.95 ± 0.24</td>
<td>5.36 ± 0.91b</td>
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<tr>
<td>ETAR</td>
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<td>2.65 ± 0.73b</td>
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<tr>
<td>ETBR</td>
<td>1.00 ± 0.19</td>
<td>0.58 ± 0.07</td>
<td>2.45 ± 0.35b</td>
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<tr>
<td>iNOS</td>
<td>1.00 ± 0.14</td>
<td>0.75 ± 0.04</td>
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<td>mET</td>
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<tr>
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<td>4.26 ± 1.1d</td>
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<tr>
<td>ECE</td>
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<td>0.82 ± 0.11</td>
<td>1.33 ± 0.12b</td>
<td>1.45 ± 0.15b</td>
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</table>

* mRNA levels are relative expression normalized to wild-type (WT) expression levels. Data are means ± SEM. ET+/+, endothelin transgenic mice; eNOS−/−, eNOS knockout; ET+/+ eNOS−/−, cross-bred with both attributes.

**P < 0.05 versus WT mice.

**P < 0.01 versus WT mice.

***P < 0.05 versus ET+/+ mice.

Figure 7. Western Blot analysis of ETA (A), ETB (B), iNOS (C), and eNOS (D) in aortic tissue from WT, ET+/+, eNOS−/−, and ET+/+ eNOS−/− mice. Densitometric analysis is shown normalized to the average expression in WT aortas and presented as means ± SEM (n = 4 to 6). *P < 0.05, **P < 0.005 versus WT.
An increase in endothelium-dependent relaxation in the aorta of ET+/+ mice has been described for this model before (24), whereas Amiri et al. (35) demonstrated reduced endothelium-dependent relaxation in the resistance vessels in ET-1 overproduction. It is of note that ET-1 concentrations in aortic tissue were not elevated. This is in accordance with a previous description of the model (19), which demonstrated an increase of ET levels in several organ tissues but not in the aorta or in resistance vessels. In our model, ET-1 is mainly expressed in the lung, brain, and kidney (19), whereas Amiri et al. (35) induced ET-1 overproduction selectively in endothelial cells. In any case, our model represents a new model of an imbalance between the aortic ET and NO systems. ET-1 remains constant; the ET$_A$R and ET$_B$R are upregulated, whereas eNOS is not expressed.

Aortic rings from ET+/+ eNOS$-/-$ mice exhibit a similar response to exogenously added ET-1 (10$^{-10}$ to 10$^{-7}$ mol/L) alone or in the presence of 10$^{-5}$ mol/L BQ123 or 10$^{-5}$ mol/L BQ788, respectively, as compared with pure ET+/+ mice. The response to acetylcholine (10$^{-10}$ to 10$^{-4}$ mol/L), however, was completely different.

The direct vascular effects, at least in aortic vessel segments, seem to be mainly mediated by the ET$_A$R (Figure 5). These novel findings represent the proof of concept of our earlier work that suggested that the interaction between the vascular ET and NO systems is critical for BP regulation under the condition of an activated ET system (22,28). In this study, we demonstrate for the first time that a lack of eNOS in the aorta of ET transgenic mice leads to an increase in both vascular ETAR and ETBR, while eNOS is not completely downregulated in the presence of a ETA antagonist. Second, the aortic contraction response to exogenously added ET-1 (10$^{-10}$ to 10$^{-7}$ mol/L) was markedly elevated. It is of note that the latter is in part mediated by protein kinase C (PKC) (38) and can be abolished by experimental downregulation of PKC (39).

Because media-lumen ratio was reduced rather than elevated in hypertensive ET+/+ eNOS$-/-$ mice, there is obviously no linear relationship between hypertension and vascular remodeling in this model. This may at least in part be due to the type of vessel that was used for histomorphometry: Hypertrophy as a result of hypertension is mainly described for small-resistance vessels rather than for the aorta. It is of note that on the other side, vascular hypertrophy in the absence of hypertension has been described in states of ET-1 overexpression (35).

Our results on the mRNA level indicating a stimulation of the ET system in ET+/+ eNOS$-/-$ mice are confirmed to a large extent by investigation on the protein level: We demonstrated a significant increase of ET$_A$R and ET$_B$R protein levels in aortic tissue of ET+/+ eNOS$-/-$ mice by Western blot analysis, and likewise we confirmed the absence of eNOS in both eNOS knockout groups. However, because ET-1 protein levels in aortic tissue as measured by ELISA were not different between the groups, the increase of ET-1 mRNA expression in ET+/+ eNOS$-/-$ mice has not been detected on the protein level.

This discrepancy between the mRNA and the protein level for ET-1 is in line with literature describing a differential regulation of ET-1 mRNA and protein levels (40–42) as a result of regulation of ET-1 biosynthesis by ECE and other factors apart from gene transcription. Furthermore, one has to bear in mind that the ET$_B$R was upregulated in ET+/+ eNOS$-/-$ mice. It is widely known that the ET$_B$R acts as a clearance receptor for ET-1; therefore, regulation or pharmacologic intervention at the ET$_B$R was shown to have an impact on ET-1 levels (6,7,43,44). Furthermore ET-1 is known to exhibit high affinity to its receptors. This may result on one hand in a reduced contractile response to exogenously added ET-1 (as demonstrated in Figures 4 and 5B) because many ET$_A$R still may be occupied. On the other hand, high receptor affinity may at least in part account for the absence of enhanced ET-1 levels in the supernatant of aortic tissue (Table 3).

Regarding iNOS mRNA and protein levels, we also observed a discrepancy between mRNA and protein levels: iNOS mRNA was upregulated in both eNOS knockout groups, whereas on

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>ET+/+</th>
<th>eNOS$-/-$</th>
<th>ET+/+ eNOS$-/-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1 in aortic tissue (pg/g wt wt)</td>
<td>14.1 ± 1.9</td>
<td>11.5 ± 1.5</td>
<td>14.0 ± 2.9</td>
<td>10.3 ± 2.1</td>
</tr>
<tr>
<td>ET-1 in aortic tissue (ng/g protein)</td>
<td>2.5 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>1.7 ± 0.5</td>
</tr>
</tbody>
</table>

*ET-1 levels were determined by ELISA. Data are means ± SEM.*
the protein level, the ET+/+ group displayed enhanced iNOS expression only. These findings may also be attributed to the fact that proteins are subjected to regulations apart from gene transcription; for example, iNOS protein levels depend on degradation by the ubiquitin-proteasome pathway (45), which is known to be altered in cardiovascular diseases such as cardiac hypertrophy (46) and atherosclerosis (47). Furthermore, our findings of an upregulation of iNOS on the protein level in ET+/+ mice are supported by previous studies of our group indicating a vital role for iNOS in counterregulating the effects of ET-1 overexpression in ET+/+ mice (26).

Apart from the direct interplay between NO and ET-1, other adaptive mechanisms may be involved in our model. By preincubation of aortic rings with diclofenac, we were able to exclude significant effects of prostaglandins on vasoreactivity. Furthermore, it is known that the endothelium-derived hyperpolarizing factor plays an important role in the relaxation of resistance vessels and may contribute significantly, particularly in the absence of eNOS, to endothelium-dependent vasodilation (48,49). In contrast to resistance vessels, endothelium-derived hyperpolarizing factor does not contribute to relevant vasodilation in the aorta of mice (48). Nevertheless, other mediators may also be involved in adaptive mechanisms; their potential contribution was not evaluated in this study.

Conclusion

This study demonstrates for the first time that in the presence of an activated ET system, additional knockout of eNOS results in further activation of the endothelin production and elevation of BP. The human ET+/+ eNOS−/− mice therefore represent a novel model of hypertension as a result of an imbalance between the vascular ET-1 and NO systems.

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Disclosures

None.

References


