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 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 ET₆ 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 ET₆ 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.


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tle 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+/+ /eNOS− /− mice 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 pro-pre-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 with homozygous mice (19), were used for all experiments. ET-1 trans-

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.

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 Elastic–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 RNA extraction kit from Merck & Nagel (cat. no. 740955). 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 ENST00000079232; murine ET-1 Ensembl Transcript ID ENSMUST00000021796; murine HPRT Ensembl Transcript ID ENSMUST00000026723). 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.

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>
</tr>
</thead>
<tbody>
<tr>
<td>ETAR</td>
<td>+GCTGTTCTCCCTTCATTAAGGC</td>
<td>129</td>
<td>6</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td>−TCATGTTGCGGAGTTAATGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETBR</td>
<td>+TGTCCTCAAGTAGCATGCAGAG</td>
<td>240</td>
<td>2, 3</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td>−GGCGTCTTCTAAATGCATGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mET-1</td>
<td>+GAAACTCAAGGGTGGAGGCC</td>
<td>235</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−CTGTAAGAGCCACACAGATGGCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hET-1</td>
<td>+AAGTTCCAGGAGGACACTAAGAACAC</td>
<td>170</td>
<td>4, 5</td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>+CAGCTGGGCTGTCAAAACCTTT</td>
<td>95</td>
<td>17, 18</td>
<td>(51)</td>
</tr>
<tr>
<td></td>
<td>−CATTTGAAGTAAAGGCTTTCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>+CCCTCCGCTACACGAGCCAGA</td>
<td>105</td>
<td>10,11,12</td>
<td>(52)</td>
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<tr>
<td></td>
<td>−CAGAGATCTTCTACTGCTTGGTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECE</td>
<td>+ATGACGCCCAGCATTGTGAAAC</td>
<td>147</td>
<td>7, 8, 9</td>
<td>(53)</td>
</tr>
<tr>
<td></td>
<td>−TGTTGGCTTAAGACATAAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPRT</td>
<td>+CAGGCCGACAGCTTTGTTGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>−TGGGCTCCTACTTTAGGCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+, forward; −, reverse; ECE, endothelin-converting enzyme; eNOS, endothelial nitric oxide synthase; ETAR, endothelin subtype A receptor; ETBR, endothelin subtype B receptor; hET-1, human endothelin-1; HPRT, hypoxanthine guanine phosphoribosyl transferase; iNOS, inducible nitric oxide synthase; mET-1, mouse endothelin-1.
The homogenates were centrifuged at 4°C for 60 min at 50°C. Every incubation was preceded by three washes with TBS Tween for 10 min.

**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 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 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 contraction (pD2). Maximal relaxation (expressed as a percentage of 100 mmol/L KCl–induced contractions) was 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 preconstriction) 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).

mRNA Expression
mRNA expression of endogenous murine ET as well as transgenic human ET, ETαR, ETβR, 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−/−) 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−/−) was significantly enhanced in 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).

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).

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

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).

**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>
</tr>
</thead>
<tbody>
<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>
</tr>
<tr>
<td>ETaR</td>
<td>1.00 ± 0.18</td>
<td>0.56 ± 0.10</td>
<td>0.74 ± 0.21</td>
<td>2.65 ± 0.73b</td>
</tr>
<tr>
<td>ETR</td>
<td>1.00 ± 0.19</td>
<td>0.58 ± 0.07</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>eNOS</td>
<td>1.00 ± 0.14</td>
<td>0.75 ± 0.04</td>
<td>2.45 ± 0.35b</td>
<td>3.93 ± 0.76b</td>
</tr>
<tr>
<td>iNOS</td>
<td>1.00 ± 0.46</td>
<td>0.86 ± 0.22</td>
<td>1.76 ± 0.66</td>
<td>6.59 ± 1.1c</td>
</tr>
<tr>
<td>mET</td>
<td>–</td>
<td>1.00 ± 0.35</td>
<td>–</td>
<td>4.26 ± 1.1d</td>
</tr>
<tr>
<td>hET</td>
<td>1.00 ± 0.07</td>
<td>0.82 ± 0.11</td>
<td>1.33 ± 0.12b</td>
<td>1.45 ± 0.15b</td>
</tr>
<tr>
<td>ECE</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</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 ETAR and ETBR 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 ETAR (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. 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 expression, resulting in an impaired vascular relationship between the ET and NO systems. As a consequence, BP in these ET+/- eNOS-/- mice was markedly elevated.

Our experiments concerning aortic contraction by exogenously administered ET-1 can be summarized in two qualitative results: First, contraction in all of our mouse models is ETA dependent, and ETB does not play a contractile role in our setting as indicated by absence of contraction in all mouse models in the presence of an ETA antagonist. Second, the aortic contraction response to exogenously administered ET-1 of both groups that carry the ET transgene seems to be decreased, which is possibly due to occupation of the receptors by endogenous ET-1 in those groups. This finding corresponds well with the absence of enhanced ET-1 levels in the supernatant of aortic tissue in those two groups, because the amount of receptor-bound ET-1 possibly escapes detection by this method. However, we suggest not extending interpretation of contraction experiments from a qualitative to a quantitative level (i.e., anticipating that different levels of ETAR between our groups as indicated by Western blot analysis are automatically translated into corresponding differences in contraction responses). It is widely known that corresponding to differences between mRNA and protein levels, differences between receptor expression and receptor-related response also exist (36,37). To illustrate the complex pathway between ET-1 and its contractile response, 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 ETAR and ETBR 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 ETBR was upregulated in 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.

Table 3. ET-1 in aortic tissue

<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 wet 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 vasodilatation (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


