Vasomotor control in mice overexpressing human endothelial nitric oxide synthase

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Submitted 19 July 2006; accepted in final form 25 April 2007

van Deel ED, Merkus D, van Haperen R, de Waard MC, de Crom R, Duncker DJ. Vasomotor control in mice overexpressing human endothelial nitric oxide synthase. Am J Physiol Heart Circ Physiol 293: H1144–H1153, 2007. First published May 11, 2007; doi:10.1152/ajpheart.00773.2006.—Nitric oxide (NO) plays a key role in regulating vascular tone. Mice overexpressing endothelial NO synthase [eNOS-transgenic (Tg)] have a 20% lower systemic vascular resistance (SVR) than wild-type (WT) mice. However, because eNOS enzyme activity is 10 times higher in tissue homogenates from eNOS-Tg mice, this in vivo effect is relatively small. We hypothesized that the effect of eNOS overexpression is attenuated by alterations in NO signaling and/or altered contribution of other vasoregulatory pathways. In isoflurane-anesthetized open-chest mice, eNOS inhibition produced a significantly greater increase in SVR in eNOS-Tg mice compared with WT mice, consistent with increased NO synthesis. Vasodilation to sodium nitroprusside (SNP) was reduced, whereas the vasodilator responses to phosphodiesterase-5 (PDE-5) blockade and 8-bromo-cGMP (8-Br-cGMP) were maintained in eNOS-Tg compared with WT mice, indicating blunted responsiveness of guanylyl cyclase to NO, which was supported by reduced guanylyl cyclase activity. There was no evidence of eNOS uncoupling, because scavenging of reactive oxygen species (ROS) produced even less vasodilation in eNOS-Tg mice, whereas after eNOS inhibition the vasodilator response to ROS scavenging was similar in WT and eNOS-Tg mice. Interestingly, inhibition of other modulators of vascular tone [including cyclooxygenase, cytochrome P-450 2C9, endothelin, adenosine, and Ca-activated K+ channels] did not significantly affect SVR in either eNOS-Tg or WT mice, whereas the marked vasoconstrictor responses to ATP-sensitive K+ and voltage-dependent K+ channel blockade were similar in WT and eNOS-Tg mice. In conclusion, the vasodilator effects of eNOS overexpression are attenuated by a blunted NO responsiveness, likely at the level of guanylyl cyclase, without evidence of eNOS uncoupling or adaptations in other vasoregulatory pathways.

nitric oxide signaling; vasodilation; reactive oxygen species; redundancy

ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS)-derived nitric oxide (NO) plays a key role in cardiovascular homeostasis (23). Loss of NO bioavailability is implicated in the etiology and pathogenesis of ischemic heart disease (14, 48), whereas an increase in NO bioavailability has been shown to ameliorate ischemic heart disease at its various stages, including atherosclerosis (47), ischemia-reperfusion injury (26), and heart failure (27).

To study the beneficial effects of increased eNOS activity, we have previously developed and described transgenic (Tg) mice that overexpress the human eNOS gene (46, 47). In these eNOS-Tg mice, eNOS protein level is significantly increased as demonstrated by Western blot analysis and immunohistochemistry (27, 46, 47). Phosphorylated eNOS levels were highly increased in eNOS-Tg mice indicating that the overexpressed eNOS is active (46). Furthermore, the overexpressed eNOS is localized exclusively in the endothelial cells of smaller and larger blood vessels of several organs such as the aorta, heart, and kidney, but not in myocardial cells or parenchymal cells of these organs (46, 47). Within the endothelial cells, eNOS is expressed in the Golgi complex and at the plasma membrane, which exactly matches the sites where eNOS is known to be primarily located (46). As a consequence of the eNOS overexpression, eNOS enzyme activity was increased 10-fold in eNOS-Tg mice (27, 46, 47). Accordingly, eNOS-Tg mice have a significantly lower aortic blood pressure compared with wild-type (WT) mice as a result of a lower systemic vascular resistance (SVR). After administration of the NOS inhibitor Nω-nitro-arginine methyl ester (l-NNAME), SVR increases more in Tg animals, indicating that in the eNOS-Tg mice an elevated eNOS activity is indeed responsible for the lower SVR (47). However, from the 10-fold increase in vascular eNOS protein level and in vitro enzyme activity (47), the 20% lower SVR appears rather modest when compared with the 150% increase in SVR observed in WT mice produced by inhibition of the endogenous murine eNOS with l-NNAME (47). These observations lead us to hypothesize that the vasodilator influence of a 10-fold overexpression of eNOS is blunted through alterations in vasomotor control of the resistance vessels in the systemic bed of eNOS-Tg mice.

A variety of vasomotor control mechanisms could have contributed to the blunted vasodilator influence of eNOS overexpression. For example, a chronic increase in NO production has been shown to blunt the responsiveness of soluble guanylyl cyclase (sGC) and protein kinase G (PKG) in aortic rings obtained from mice overexpressing bovine eNOS (51). Alternatively, under conditions of oxidative stress and eNOS substrate and cofactor depletion, eNOS can become uncoupled and produce superoxide instead of NO (2, 50). It is possible that high levels of eNOS expression result in simultaneous NO and superoxide production, thereby reducing the bioavailability of NO. Finally, control of vascular tone is characterized by cross-talk between redundant vasoregulatory pathways. Thus loss of eNOS activity can result in a compensatory increase in the vasodilator influence of cytochrome P-450 2C9 (CYP2C9)
(13, 36), cyclooxygenase (COX) (33, 38), adenosine (28), or ATP-sensitive K⁺ (Kₘₚ) channels (24), and increase endothelin (21). Consequently, overexpression of eNOS may attenuate the vasodilator influence of other vasodilator pathways, thereby counteracting the NO-induced decrease in SVR.

From these considerations, the aim of the present study was to investigate in vivo the alterations in vasomotor control of SVR in mice overexpressing human eNOS. Specifically, we investigated whether alterations in vascular NO signaling and/or alterations in the vasomotor influence of other pathways blunted the vasodilator influence of eNOS overexpression.

METHODS

All experiments were performed in accordance with the “Guiding Principles in the Care and Use of Animals” as approved by the Council of the American Physiological Society and with prior approval of the Animal Care Committee of the Erasmus MC.

A total of 178 eNOS-Tg mice and 179 WT littermates in C57BL/6 background of both sexes entered the study (19–35 g). The generation of eNOS-Tg mice has been previously described (46, 47). Briefly, a DNA fragment, containing the human eNOS gene and ~6 kb of 5'-natural flanking sequence, including the native eNOS promoter, and ~3 kb of 3'-sequence to the gene, was used for microinjection of fertilized oocytes. Transgenic offspring was backcrossed to C57BL/6 for >10 generations.

Western Blot Analysis and Enzyme Activity Measurement

To determine the expression level and activity of guanylyl cyclase (GC), the aorta, kidney, and brain of WT and eNOS-Tg mice were homogenized in 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.25 M sucrose, and 20 mM CHAPS. Western blot analysis was performed as previously described (12). Anti-GC was obtained from Sigma-Aldrich. GC activity was determined with an immunoassay for the quantification of total cGMP (Sigma-Aldrich).

Surgical Instrumentation

Mice were weighed, sedated with 4% isoflurane, intubated, and connected to a pressure-controlled ventilator (SAR-830/P; CWE). Respiration rate was set at 90 breaths/min with a peak inspiratory pressure of 18 cmH₂O and a positive end-expiratory pressure of 4 cmH₂O. All animals were ventilated with a gas mixture of O₂-N₂O (1:2 vol/vol) containing 2.5% isoflurane to maintain anesthesia while temperature at 37°C. A polyethylene catheter (PE-10) was inserted into the right femoral artery to measure aortic pressure and heart rate, mean aortic pressure, and mean aortic blood flow were measured, followed by intravenous infusion of various drugs or intravenous infusion of an equal volume of vehicle (0.5 ml saline). All drugs were purchased from Sigma-Aldrich (except the PDE5 inhibitor EMD-360527 and the ET₄/ET₉ receptor antagonist teimazolazide, which were generous gifts from Prof. Schelling, Merck kGaA, and Dr. M. Clozel, Actelion, respectively) and were dissolved in saline and administered intravenously. Unless otherwise stated, each mouse was subjected to single drug treatment. Hemodynamics were recorded continuously throughout the 10-min infusion period and subsequent 10-min washout period. Fifteen WT and 15 eNOS-Tg mice were infused with saline to determine the effect of the vehicle on SVR.

Alterations in systemic vascular NO signaling. To confirm previous observations (46, 47) that eNOS contributes to the lower vascular tone in the systemic bed of eNOS-Tg mice, we first studied the effect of the eNOS inhibitor L-NAME (100 mg/kg) in 10 WT and 10 eNOS-Tg mice. Subsequently, to study alterations in the NO signal transduction pathway, we determined the systemic vasodilator responses to the eNOS-dependent vasodilator acetylcholine (ACH, 200 μg/kg) (10 WT and 10 eNOS-Tg mice), the NO donor sodium nitroprusside (SNP, 300 μg/kg) (15 WT and 15 eNOS-Tg mice), the PDE5 inhibitor EMD-360527 (EMD, 30 mg/kg) (10 WT and 10 eNOS-Tg mice), or the PKG activator 8-bromo-cGMP (8-Br-cGMP, 10 mg/kg) (10 WT and 10 eNOS-Tg mice).

Higher NO background concentrations in eNOS-Tg mice might blunt the effect of exogenous NO (via SNP infusion), because the relative amount of extra NO would be lower. To circumvent this issue, we additionally studied the effects of SNP (300 μg/kg) after pretreatment with L-NAME (100 mg/kg) (15 WT and 15 eNOS-Tg mice). Furthermore, to determine whether the difference in vasodilator response to SNP in WT and eNOS-Tg mice was nonspecific to the NO pathway and not a general blunting of the vascular smooth muscle responsiveness, we also studied the vasodilator response to the NO independent K⁺ channel opener bimakalim (0.5 mg/kg), both in the presence (6 WT and 6 eNOS-Tg mice) and the absence (10 WT and 10 eNOS-Tg mice) of L-NAME (100 mg/kg).

To investigate whether eNOS uncoupling [due to either substrate or cofactor tetrahydrobipterin (BH₄) deficiency] resulted in reduced NO bioavailability and increased superoxide formation, thereby reducing the effects of eNOS overexpression, we determined the systemic vascular effects of the ROS scavenger N-acetyl cysteine (NAC, 500 mg/kg) and the superoxide dismutase (SOD) mimetic 4-hydroxy-2,2,6,6-tetramethyl-piperidin-1-oxyl (TEMPOL, 100 mg/kg) in 10 WT and 10 eNOS-Tg mice. To determine the NO independent effect of ROS scavenging on SVR, the effects of NAC were also studied in the presence of L-NAME (100 mg/kg) (10 WT and 10 eNOS-Tg mice). Additionally, superoxide potentially increases endothelin levels either through a direct effect (44) or by upregulation of gene expression (10). In this way eNOS uncoupling could increase the vasconstrictor effect of endothelin and counterbalance eNOS overexpression. To establish whether the vasomotor influence of endothelin was indeed higher in eNOS-Tg mice, 6 WT and 6 eNOS-Tg mice received the mixed ETA/ETB receptor antagonist tezoazolazide (50 mg/kg) in both the absence and presence of L-NAME (100 mg/kg).

Alterations in vasomotor control by other mediators of vasodilation and their end effectors. To determine alterations in vasomotor influence of other endothelial vasodilators system in eNOS-Tg mice, 6 WT and 6 eNOS-Tg mice received the COX inhibitor indomethacin (100 mg/kg), or CYP2C9 inhibitor sulfaphenazole (10 mg/kg). To investigate alterations in adenosine-mediated vasomotor control, we infused the adenosine receptor antagonist 8-3-phenylephthaline (8SPT, 50 mg/kg) to 6 WT and 6 eNOS-Tg mice. To study alterations in the vasomotor influence of ATP-sensitive K⁺ (K₅), Ca-activated K⁺ (KᵥCa), and voltage-dependent K⁺ (Kᵥ) channels, 15 WT and 15 eNOS-Tg mice were given the K₅ channel blocker glibenclamide (10 mg/kg). The KᵥCa channel blocker tetraethylammonium chloride (TEA, 100 mg/kg) and the Kᵥ channel blocker 4-aminoypyrindin (4-AP, 16 mg/kg) were administered to 6 WT and 6 eNOS-Tg mice.

Data Analysis

All hemodynamic data were recorded and digitized (400 Hz/channel) on-line using an eight-channel data-acquisition program (ATCODAS, Datasq Instruments, Akron, OH) and stored on a computer for off-line analysis. Data were analyzed at baseline and at various time points during drug infusion. A minimum of 10 consecutive beats was selected for analysis of each time point, from which heart rate, mean aortic pressure, and mean aortic blood flow were...
SVR was subsequently computed as the ratio of mean aortic pressure and mean aortic blood flow at each time point. From these values, absolute changes from baseline were determined for heart rate, aortic blood flow, aortic blood pressure, and SVR.

Statistics

All hemodynamic data were tested using two-way (genotype × effect of drug) analysis of variance followed by post hoc testing with Student-Newman-Keuls test. A value of $P \leq 0.05$ was considered statistically significant. All data are presented as means ± SE.

RESULTS

eNOS overexpression had no effect on heart rate but resulted in a lower aorta pressure, despite a higher aorta flow (Figs. 1 and 2 and Table 1), implying that the lower aorta pressure was caused by a reduction in SVR. The difference in SVR between WT and eNOS-Tg mice was abolished after eNOS inhibition with L-NAME, indicating that increased eNOS activity indeed underlies the lower SVR in eNOS-Tg mice (Fig. 2). Consequently, eNOS inhibition induced a significantly larger increase in SVR in eNOS-Tg than that in WT animals (Fig. 3).

Infusion of the vehicle saline (0.5 ml) had no effect on SVR in either WT or eNOS-Tg mice (Fig. 3).

Alterations in Systemic Vascular NO Signaling

Infusion of the eNOS-dependent vasodilator ACh produced similar degrees of vasodilation in WT and eNOS-Tg animals, whereas the systemic vasodilation by the endothelium-independent NO-donor SNP was blunted in eNOS-Tg animals (Fig. 4). Inhibition of PDE5 with EMD-360527 and stimulation of PKG with 8-Br-cGMP resulted in similar vasodilator responses in both genotypes (Fig. 4). Western blot analyses of aorta, kidney, and brain homogenates of WT and eNOS-Tg mice, showing a protein band of the expected molecular size (70 kDa), demonstrated a trend toward a reduced expression of the GC protein, although this difference was not statistically significant after densitometric analysis (Fig. 5). However, subsequent enzymatic activity measurements clearly showed a decreased GC activity in eNOS-Tg mice compared with WT mice in organs that are of importance in controlling SVR, but not in the aorta (Fig. 5).
The blunted vasodilator response to SNP in eNOS-Tg mice was not merely due to a dilution of the effect of a dose of exogenous NO in eNOS-Tg mice (caused by higher NO background levels), because the vasodilator response to SNP in the presence of l-NAME was similarly blunted in eNOS-Tg compared with that of WT mice (Fig. 6). Furthermore, the blunted vasodilation to SNP could not be ascribed to a nonspecific decrease in vascular smooth muscle responsiveness, because the vasodilation produced by the K<sub>ATP</sub> channel opener bimakalim was identical in eNOS-Tg and WT mice both in the absence and in the presence of l-NAME (Fig. 6).

The ROS scavenger NAC elicited modest vasodilation in both WT and eNOS-Tg mice. However, the vasodilation was significantly less in eNOS-Tg mice (Fig. 6). Similar observations were made with the SOD mimic TEMPO (Table 1), which indicates that superoxide levels are initially lower in eNOS-Tg mice. After infusion of l-NAME, the vasodilation by NAC was the same in WT and eNOS-Tg mice. ET<sub>A</sub>/ET<sub>B</sub> blockade with tezosentan had minimal effects on SVR in WT mice.

Table 1. Hemodynamic changes in WT and eNOS-Tg mice produced by 10 min infusion of various vasoactive drugs

<table>
<thead>
<tr>
<th>Number</th>
<th>AoP, mmHg</th>
<th>AoF, ml/min</th>
<th>SVR, mmHg·min·ml&lt;sup&gt;−1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>eNOS-Tg</td>
<td>WT</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (vehicle)</td>
<td>15</td>
<td>15</td>
<td>1±2</td>
</tr>
<tr>
<td>l-NAME</td>
<td>10</td>
<td>10</td>
<td>24±3*</td>
</tr>
<tr>
<td>ACH</td>
<td>10</td>
<td>10</td>
<td>−16±2*</td>
</tr>
<tr>
<td>Nitroprusside</td>
<td>15</td>
<td>15</td>
<td>−25±3*</td>
</tr>
<tr>
<td>EMD</td>
<td>10</td>
<td>10</td>
<td>−11±2*</td>
</tr>
<tr>
<td>8-Br-cGMP</td>
<td>10</td>
<td>10</td>
<td>−18±2*</td>
</tr>
<tr>
<td>Bimakalim</td>
<td>10</td>
<td>10</td>
<td>−29±2*</td>
</tr>
<tr>
<td>NAC</td>
<td>10</td>
<td>10</td>
<td>−9±2*</td>
</tr>
<tr>
<td>TEMPOL</td>
<td>10</td>
<td>10</td>
<td>−12±1*</td>
</tr>
<tr>
<td>Tezosentan</td>
<td>6</td>
<td>6</td>
<td>−8±1*</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>6</td>
<td>6</td>
<td>−13±3*</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>6</td>
<td>6</td>
<td>−3±2</td>
</tr>
<tr>
<td>8-SPT</td>
<td>6</td>
<td>6</td>
<td>2±2</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>15</td>
<td>15</td>
<td>17±2*</td>
</tr>
<tr>
<td>TEA</td>
<td>6</td>
<td>6</td>
<td>−6±3</td>
</tr>
<tr>
<td>4-AP</td>
<td>6</td>
<td>6</td>
<td>16±6*</td>
</tr>
<tr>
<td>Post-l-NAME</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitroprusside</td>
<td>15</td>
<td>15</td>
<td>−50±3*</td>
</tr>
<tr>
<td>Bimakalim</td>
<td>6</td>
<td>6</td>
<td>−43±3*</td>
</tr>
<tr>
<td>NAC</td>
<td>10</td>
<td>11</td>
<td>2±2</td>
</tr>
<tr>
<td>Tezosentan</td>
<td>6</td>
<td>6</td>
<td>−4±4</td>
</tr>
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</table>

Values are means ± SE. AoP, mean arterial blood pressure; AoF, mean aortic blood flow; SVR, mean systemic vascular resistance; WT, wild-type; eNOS-Tg, endothelial nitric oxide synthase transgenic; l-NAME, N<sup>ω</sup>-nitro-l-arginine methyl ester. Data are presented as absolute change from corresponding baseline either under Control conditions (Control) or in the presence of l-NAME (Post-l-NAME); 8-SPT, 8-S-phenylthioephylline; Glib, Glibenclamide; TEA, tetraethylammonium chloride; ACh, acetylcholine; EMD, PDE5 inhibitor EMD-360527; 8-Br-cGMP, 8-bromo-cGMP; NAC, N-acetyl cysteine. *P ≤ 0.05 vs. corresponding saline; †P ≤ 0.05 eNOS-Tg vs. WT.

Fig. 3. Changes in SVR (ΔSVR) from baseline produced by infusion of saline (Vehicle) and l-NAME in WT (open bars) and eNOS-Tg mice (solid bars). *P ≤ 0.05 vs. corresponding saline; †P ≤ 0.05 vs. change in corresponding WT.
and eNOS-Tg mice, either in the absence or in the presence of l-NAME (Fig. 6).

Alterations in vasomotor control by other mediators of vasodilation and their end effectors. Blockade of COX with indomethacin or of CYP2C9 with sulfaphenazole had no significant effect on SVR in either WT or eNOS-Tg mice. Adenosine receptor blockade with 8-SPT had no significant effect on SVR (Fig. 7). K<sub>ATP</sub> channel blockade with glibenclamide and K<sub>V</sub> channel blockade with 4-AP resulted in marked, but identical, vasoconstriction in WT and eNOS-Tg mice (Fig. 7 and 8). In contrast, K<sub>Ca</sub> channel blockade with TEA had no effect in either WT or eNOS-Tg mice (Fig. 7).

DISCUSSION

The present study is the first to investigate in detail the regulation of systemic vasomotor control in mice in vivo and was designed to investigate alterations in vasomotor control of systemic resistance vessels in mice overexpressing human eNOS (47). In these eNOS-Tg mice, eNOS protein expression as well as in vitro eNOS activity, were markedly elevated, resulting in an eNOS-dependent ~20% decrease in SVR in eNOS-Tg mice. The main findings are that 1) the vasodilator response to exogenous NO was blunted in eNOS-Tg mice, which appeared to occur at the level of GC, and which was corroborated by reduced activity of GC and a trend toward lower GC protein expression; 2) there was no evidence for increased ROS production [e.g., due to eNOS uncoupling (2)]; and 3) finally, there were no apparent adaptations in other vasomotor control pathways. The implications of these findings will be discussed below.

Methodological Limitations

The present study was performed in open-chest, isoflurane-anesthetized mice. Isoflurane anesthesia results in heart rate values that approximate values observed in the awake state, suggesting that sympathetic activity under isoflurane anesthesia is similar to that in the awake state (6, 25, 29, 52). Conversely, blood pressures in isoflurane-anesthetized, open-chest mice are lower than values typically observed in awake mice (6, 25, 29, 52). The latter may have resulted in activation of the renin-angiotensin system (32), thereby influencing systemic vasomotor control and potentially confounding interpretation of the results. However, we previously reported that blood pressure differences between WT and eNOS-Tg mice were present independently of the employed anesthetic regimen (15), suggesting that any alteration in renin-angiotensin system due to the lower blood pressure affected WT and
eNOS-Tg mice in a similar fashion. Therefore, it is unlikely that the differences in vasomotor control between WT and eNOS-Tg mice, as observed in the present study, are due to the lower blood pressures associated with isoflurane anesthesia and open-chest conditions.

In the present study we observed an exaggerated increase of SVR in response to L-NAME in eNOS-Tg compared with WT mice. Although this finding [which confirms previous observations in our laboratory (27, 46, 47)] is consistent with the overexpression of eNOS, we cannot entirely exclude that part of the effect of L-NAME is mediated by inhibition of other NOS isoforms. For example, L-NAME could have inhibited central neuronal NOS (nNOS). However, since we have previously shown that the eNOS transgene is selectively overexpressed in endothelial cells, (46, 47), it is unlikely that central nNOS would be different between WT and eNOS-Tg mice. Moreover, blockade of nNOS by L-NAME will result in sympathetic activation (41), thereby increasing heart rate. In contrast, heart rate did not increase after L-NAME infusion in the present study. Based on these observations, it is unlikely that the different effect of L-NAME on SVR in eNOS-Tg versus WT mice is due to inhibition of nNOS by L-NAME.

Alterations in Systemic Vascular NO Signaling

Infusion of the same dose of exogenous NO by administration of the NO donor SNP resulted in a vasodilator response that was smaller in eNOS-Tg mice indicating a reduced vascular sensitivity to NO. Because the effects of inhibiting cGMP breakdown and administering 8-Br-cGMP were similar in WT and eNOS-Tg mice, the signal transduction pathway downstream of cGMP was apparently unaffected by eNOS overexpression. A critical role for sGC in response to alterations in NO bioavailability is also suggested by studies in which pharmacological inhibition of eNOS or knocking out eNOS resulted in enhanced sensitivity to SNP but not to 8-Br-cGMP (8, 16, 22, 34).

The reduced effect of SNP in eNOS-Tg mice did not result from the higher amount of NO already present in these mice as the same effect was observed after inhibition of eNOS. There-
fore, the reduced responsiveness to NO within the NO pathway could be attributed exclusively to sGC. Indeed, the enzyme activity of GC was lower in eNOS-Tg mice compared with that of WT animals, and also the expression level of GC tended to be smaller in eNOS-Tg mice, which is evidently one of the mechanisms by which eNOS-Tg mice partly compensate for the eNOS overexpression. The observation that the vasodilator response to SNP and enzyme activity of GC was reduced are in partial agreement with the report by Yamashita et al. (51), who described not only a reduced activity of sGC but also of PKG as well as decreased levels of PKG in eNOS-Tg mice. The difference between the outcome of their study and our observations could be explained by differences in experimental design. For example, in our eNOS-Tg mice, we used a cosmid with the human eNOS gene and the native eNOS promoter that contains all the regulatory sequences of eNOS. Yamashita et al.
used a murine prepro-endothelin-1 promoter-bovine eNOS cDNA fusion construct that does not include any of the natural regulatory noncoding sequences of eNOS and has a heterologous promotor. Furthermore, some stimuli affect these promoters differently (7, 31), and NO has been shown to inhibit the expression of the prepro-endothelin-1 gene (19). Consequently, the different promoters used in the mice of Yamashita et al. and our mice may result in different expression levels of eNOS. Indeed, NOS activity measured in vitro in the aorta demonstrated a 1.8-fold increase in enzyme activity in the mice of Yamashita et al. (37) and a 10-fold increase in our eNOS-Tg mice (42). Perhaps more important is the observation that the experiments by Yamashita et al. were performed in vivo on mouse aortic rings. Because the aorta does not contribute to the regulation of SVR, the vasodilator properties of the aorta may well be different from those of the integrated systemic resistance vessels (17).

Interestingly, stimulation of endogenous NO production with ACh produced similar vasodilator responses in WT and eNOS-Tg animals. From the blunted vasodilator response in eNOS-Tg mice to exogenous NO via SNP infusion, it follows that the maintained ACh-induced vasodilatation is likely the result of increased ACh-mediated NO production that is counteracted by a reduced sGC responsiveness to NO.

NO signaling is also influenced by the presence of ROS. eNOS overexpression could result in substrate and cofactor depletion, leading to uncoupling of eNOS and production of superoxide instead of NO (2, 49, 50). Superoxide rapidly reacts with NO to form peroxynitrite (40) thereby reducing NO bioavailability. Moreover, peroxynitrite oxidizes eNOS cofactor BH4, resulting in further eNOS uncoupling (9, 30). In addition, superoxide can also increase endothelin levels through either a direct effect (44) or by upregulation of gene expression (10), which could blunt the vasodilator effects of eNOS overexpression. Enhanced production of superoxide caused by uncoupling of eNOS has been described in nitrate tolerance (35) and could also occur in eNOS-Tg mice in which eNOS expression is increased 10-fold. However, we have previously shown that supplementation of L-arginine to eNOS-Tg mice did not affect aortic blood pressure and SVR (47), suggesting that substrate availability is not limited. Furthermore, scavenging of ROS by NAC resulted in a smaller decrease in SVR in eNOS-Tg than in WT mice, suggesting that eNOS-Tg mice have lower rather than higher ROS levels, possibly as a result of increased superoxide scavenging by NO. Although NAC is known to scavenge ROS other than superoxide (3), the effects of NAC on SVR are likely mediated through superoxide scavenging, because similar observations were made with the SOD mimetic TEMPOL. Finally, our hypothesis that superoxide could exert a vasoconstrictor influence via an increased endothelin vasoconstrictor influence was...
not supported by our data as the endothelin blocker tezosentan produced similar effects on SVR in eNOS-Tg or WT mice, in both the absence or presence of L-NAME. Taken together these findings fail to provide evidence for eNOS uncoupling and increased superoxide production.

There are several other potential mechanisms that could have counteracted the effects of eNOS overexpression. For example, in the endothelial cell, eNOS is targeted into the Golgi complex and the plasma membrane but the individual role of each pool of eNOS is incompletely understood. However, it has been shown that eNOS localized in the Golgi complex or in the plasma membrane is activated by different stimuli (18). It is therefore possible that the vasodilator effect of eNOS can be influenced by alterations in the subcellular targeting. NO itself can also inhibit NOS activity by forming a ferrous-nitrosyl complex with the heme iron in the NOS enzyme (1, 20). It is likely that this negative feedback mechanism is increased in eNOS-Tg mice, which produce more NO. Therefore, the markedly elevated eNOS activity, as measured under optimal in vitro conditions, does not necessarily imply that all overexpressed eNOS is functionally active in vivo. Isolated aortas of eNOS transgenic mice showed only a 30% increase in NO release (46). Measuring NO directly in the blood of mice in vivo is difficult with the currently available techniques. Alternatively, an indication of NO concentrations could be obtained by measuring the NO metabolite (nitrite and nitrate) levels (5, 39). Such measurements should be the subject of future studies.

In conclusion, eNOS-overexpressing mice demonstrate a blunted responsiveness to NO, which appears to be, at least in part, due to a reduced responsiveness of sGC. Despite significant redundancy of pathways involved in the regulation of vascular tone, other vasodilator and constrictor pathways were not affected by eNOS overexpression. This feature makes the eNOS-Tg mouse model very reliable for studying the effect of eNOS on the physiology and pathophysiology of the cardiovascular system, without the confounding influence of alterations in other vasomotor control pathways.

GRANTS

This study was supported by grants from The Netherlands Heart Foundation [2000T038 (to D. J. Duncker) and 2000T042 (to D. Markus)].

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AJP-Heart Circ Physiol • VOL 293 • AUGUST 2007 • www.ajpheart.org