Mechanisms of Reduced Nitric Oxide/cGMP–Mediated Vasorelaxation in Transgenic Mice Overexpressing Endothelial Nitric Oxide Synthase

Tomoya Yamashita, Seinosuke Kawashima, Yoshitaka Ohashi, Masanori Ozaki, Yoshiyuki Rikitake, Nobutaka Inoue, Ken-ichi Hirata, Hozuka Akita, Mitsuhiro Yokoyama

Abstract—NO, constitutively produced by endothelial NO synthase (eNOS), plays a key regulatory role in vascular wall homeostasis. We generated transgenic (Tg) mice overexpressing eNOS in the endothelium and reported the presence of reduced NO-elicited relaxation. The purpose of this study was to clarify mechanisms of the reduced response to NO-mediated vasodilators in eNOS-Tg mice. Thoracic aortas of Tg and control mice were surgically isolated for vasomotor studies. Relaxations to acetylcholine and sodium nitroprusside were significantly reduced in Tg vessels compared with control vessels. Relaxations to atrial natriuretic peptide and 8-bromo-cGMP were also significantly reduced in Tg vessels. Reduced relaxations to these agents were restored by chronic N^G-nitro-L-arginine methyl ester treatment. Basal cGMP levels of aortas were higher in Tg mice than in control mice, whereas soluble guanylate cyclase (sGC) activity in Tg vessels was ≈50% of the activity in control vessels. Moreover, cGMP-dependent protein kinase (PKG) protein levels and PKG enzyme activity were decreased in Tg vessels. These observations indicate that chronic overexpression of eNOS in the endothelium resulted in resistance to the NO/cGMP-mediated vasodilators and that at least 2 distinct mechanisms might be involved: one is reduced sGC activity, and the other is a decrease in PKG protein levels. We reported for the first time that increased NO release from the endothelium reduces sGC and PKG activity in mice. These data may provide a new insight into the mechanisms of nitrate tolerance and cross tolerance to nitrovasodilators. (Hypertension. 2000;36:97-102.)

Key Words: nitric oxide synthase ■ mice, transgenic ■ guanylyl cyclase ■ protein kinases

Nitric oxide plays critical roles in vascular biology, including its action on vascular tone and regulation of vascular structure.1 In physiological conditions, NO is mainly produced by endothelial NO synthase (eNOS) in vessels. eNOS produces small amounts of NO continuously by physiological stimuli, such as shear stress and endogenous vasoactive substances.2 Released NO diffuses to overlying vascular smooth muscle and binds to the ferrous heme moiety of soluble guanylate cyclase (sGC), thereby activating the enzyme. Activated sGC converts guanosine triphosphate to the intracellular second messenger cGMP, which relaxes vascular smooth muscle cells.3 Most of the effects of cGMP are mediated by stimulation of the cGMP-dependent protein kinase (PKG).4 Recently, we generated transgenic (Tg) mice overexpressing the bovine eNOS gene in endothelial cells. The Tg mice exhibited increased basal NO production and basal cGMP levels in the vascular wall. Furthermore, in previous reports, we found that the overproduction of NO caused reduced endothelium-dependent and NO-mediated relaxations without changes in cAMP-mediated relaxation.5,6 Those were the first reports indicating that increased intrinsic NO induced resistance to NO-mediated vasodilators. This alteration in vascular reactivity resembles “nitrate tolerance.” Organic nitrates induce vasodilatation by releasing NO and are in widespread use as therapeutic agents for the treatment of myocardial ischemia and heart failure. However, chronic treatment of organic nitrates or NO donors leads to the development of nitrate tolerance, which sometimes yields a therapeutic limitation. The mechanism of nitrate tolerance in humans has been the subject of intense debate but remains poorly defined. The present study was undertaken to clarify the mechanisms of reduced relaxation to NO/cGMP-mediated vasodilators in eNOS-Tg mice. The results might help to elucidate one of the mechanisms of nitrate tolerance.

Methods

Materials

Prostaglandin F_2alpha (PGF_2alpha), acetylcholine (ACh), sodium nitroprusside (SNP), 8-bromoguanosine-cGMP (8Br-cGMP), N^G-nitro-L-arginine methyl ester (L-NAME), and other drugs were purchased.

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Hypertension is available at http://www.hypertensionaha.org
from Sigma Chemical Co. Atrial natriuretic peptide (ANP) was purchased from the Peptide Institute.

Animals
We have generated Tg mice overexpressing the bovine eNOS gene in the endothelium by using the murine preproendothelin-1 promoter. Heterozygous Tg mice and their littermate control mice, at 12 to 16 weeks of age, were used in the present study. To inhibit NO synthase chronically, mice were provided water containing 1 mg/mL L-NAME for 3 weeks. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University School of Medicine.

Measurement of NO Release
Plasma nitrite and nitrate (NOx) levels were measured as nitrite by using the Griess reaction after enzymatic conversion by nitrate reductase as previously described. NO release from the ex vivo aorta was measured by examining the production of nitrite with the use of a NOx analyzer (ozone chemiluminescence) as previously described. The mouse aorta was incubated in O2-saturated Krebs’ solution containing 30 μmol/L ACh at 37°C for 60 minutes. After these preparations, the buffer was removed and used for the assay.

Studies of Vascular Reactivity Ex Vivo
Isometric tension was recorded as previously described. In the experiments with L-NAME pretreatment, the drug was given in an organ bath 30 minutes before precontraction. The EC value for each experiment was obtained from a sigmoid logistic curve.

Detection of Superoxide Anion
Aortic rings were incubated with a Cu-Zn superoxide dismutase inhibitor (diethyldithiocarbamate) for 30 minutes at 37°C, and vascular superoxide levels were measured by use of lucigenin chemiluminescence according to the method modified from that of Münzel et al.

Measurement of cGMP Levels in Aorta
Aortas from control and Tg mice were incubated in O2-saturated Krebs’ solution at 37°C for 60 minutes. After the preparation, the buffer was replaced with Krebs’ solution containing either SNP or ANP, and 1 minute later, the aorta was rapidly frozen in liquid nitrogen. Samples were prepared, and cGMP was measured as previously described.

Measurements of sGC Protein Levels and Activity in Aorta
A crude soluble extract of the aorta from control and from Tg mice was obtained and analyzed by Western blot analysis with the use of 8% SDS polyacrylamide gels. After electrophoresis, protein was transferred to a nitrocellulose membrane. Blots were then incubated in a blocking buffer consisting of 5% nonfat dry milk for 2 hours at room temperature and incubated overnight at 4°C with rabbit polyclonal sGC IgG (dilution 1:1000, Calbiochem). Immunoreactive bands were visualized by use of an ECL detection kit (Amersham) and quantified by densitometry. The sGC assay was performed according to the method described previously.

Immunological Quantification and Assay of PKG in Aorta
Western blot analysis for PKG was performed with the use of 12% SDS polyacrylamide gels. After electrophoresis, protein was transferred to a nitrocellulose membrane. After incubation with blocking buffer, blots were incubated overnight at 4°C with rabbit polyclonal PKG IgG (dilution 1:300, Calbiochem), which recognizes PKG-α and -β. Immunoreactive bands were visualized and quantified by densitometry as described above. PKG enzyme activity was measured according to the method of Diwan et al with some modifications.

Statistics
Data are presented as mean±SEM. An unpaired Student t test was used to detect significant differences when 2 groups were compared. Statistical analysis for multiple comparisons was performed by ANOVA with the Bonferroni correction. A value of P<0.05 was considered to be statistically significant.

Results

NO Production Is Increased in eNOS-Tg Mice
We have already reported that basal NO release from the aorta was increased in Tg mice. Moreover, the plasma NOx level was higher in Tg mice than in control mice (38.9±4.4 versus 17.0±2.5 μmol/L, respectively; P<0.01; Figure 1a). In the present study, we also demonstrated that ACh-induced NO production from the aorta was increased in Tg mice compared with control mice (42.8±7.5 versus 13.9±1.9 pmol·min⁻¹·mg protein⁻¹, respectively; P<0.01; Figure 1b).

Comparison of Contractions to KCl and PGI2
Contractions to 40 mmol/L KCl were significantly reduced in vessels from Tg mice compared with control mice (1.52±0.04 versus 1.28±0.05 g, respectively; P<0.05). Similarly, the receptor-mediated contractions to PGI2 were also attenuated in vessels from Tg mice compared with vessels from control mice (data not shown).

Reduced Relaxations to NO/cGMP-Mediated Vasodilators in eNOS-Tg Mice
In accordance with our previous report, we found that ACh- and SNP-induced relaxations were significantly reduced in vessels from Tg mice compared with vessels from control mice (Figure 2a and 2b). We also confirmed that ATP-γ-S–induced relaxation was reduced in Tg mice (data not shown).
Indomethacin had no effects on those vasorelaxations (data not shown). To clarify the mechanisms of the reduced NO-mediated relaxations, we examined responses to other cGMP-dependent vasodilators (ANP and 8Br-cGMP). Relaxations to ANP were significantly reduced in vessels from Tg mice compared with vessels from control mice, as measured by shifts in EC (0.29 ± 0.07 versus 0.67 ± 0.10 μmol/L, respectively) and by maximal relaxations (97.9 ± 31.7% versus 91.8 ± 2.0%, respectively; P < 0.01; Figure 2c). Likewise, relaxations to a cGMP analogue, 8Br-cGMP, were also significantly reduced in vessels from Tg mice compared with control mice, as measured by shifts in EC (Table 1) and by maximal relaxations (74.3 ± 3.7% versus 96.8 ± 1.2%, respectively; P < 0.01; Figure 2d). On the other hand, as we reported already, cAMP-mediated vasorelaxations were not different between control and Tg mice.5

Effects of L-NAME Pretreatment on Vascular Reactivities
For the L-NAME study, control and Tg mice were provided with water containing 1 mg/mL L-NAME for 3 weeks to inhibit NOS chronically, and then ex vivo experiments were performed with 100 μmol/L L-NAME in the organ baths. L-NAME treatment enhanced the sensitivities to both SNP and 8Br-cGMP in both genotypes. Chronic L-NAME treatment reversed the reduced responses in vessels from Tg mice to levels similar to those in vessels from L-NAME-treated control mice (Figure 3, Table 1).

Vascular Superoxide Levels
Increased superoxide production from the endothelium was shown in a rabbit model of nitrate tolerance.8 To examine the contribution of increased superoxide production to the vascular hyporeactivity in our mouse model, steady-state vascular superoxide levels were measured by use of lucigenin chemiluminescence. Superoxide production from the aorta with endothelium was not significantly different between the 2 genotypes (for control mice, 46.4 ± 6.4 arbitrary units, n = 12; for Tg mice, 42.1 ± 5.8 arbitrary units, n = 10). Moreover, Mn-tetrakis-4-benzoic acid porphyrin chloride (Calbiochem), a cell-permeable superoxide dismutase mimetic and

### Table 1. NO Synthase Inhibition Reversed the Reduced NO/cGMP-Mediated Vasorelaxation in eNOS-Tg Mice

<table>
<thead>
<tr>
<th></th>
<th>Control Mice</th>
<th>Tg Mice</th>
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<tbody>
<tr>
<td></td>
<td>SNP, nmol/L</td>
<td>3.8 ± 0.8 (6)</td>
</tr>
<tr>
<td></td>
<td>8Br-cGMP, μmol/L</td>
<td>12.7 ± 2.7 (6)</td>
</tr>
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Values are mean ± SEM and were obtained from individual aortas; the values in parentheses indicate the number of aortas. For precise comparison of the sensitivity of the mouse aorta, EC (concentration that produces 50% of the maximal relaxation to each agent) was calculated from the dose-response curves obtained. Values for L-NAME–treated mice are measurements of isometric tension of aortas from 3-wk L-NAME–treated mice in the presence of 100 μmol/L L-NAME in organ baths. *P < 0.01 vs control mice at the same conditions; †P < 0.01 and ‡P < 0.05 relative to nontreated mice.
peroxynitrite scavenger, could not reverse the reduced relaxation in Tg mice (data not shown).

**NO Donor–Induced cGMP Production Is Reduced in eNOS-Tg Mice**

As we reported previously, basal cGMP levels increased in the aortas from Tg mice compared with those from control mice.\(^5,6\) On the contrary, cGMP levels stimulated by SNP were significantly reduced in the aortas from Tg mice compared with those from control mice (Table 2). However, cGMP levels stimulated by ANP were not different in the 2 groups (Table 2). These results suggest that cGMP production by sGC was reduced but that cGMP production by particulate guanylate cyclase was not changed in Tg mice.

**Reduced sGC Enzyme Activity in eNOS-Tg Aorta**

We examined sGC protein levels with the use of anti-sGC antibody, which mainly recognizes \(\beta_1\) subunits. There were no significant differences in aortic sGC protein levels between the 2 groups (Figure 4a). However, in vitro sGC activity was markedly reduced in the Tg aorta compared with the control aorta (Figure 4b).

**Reduced PKG Levels and Enzyme Activity in eNOS-Tg Aorta**

Protein levels of aortic PKG detected by Western blotting were decreased ≈20% in Tg mice compared with control mice (Figure 5a and 5b). Moreover, PKG enzyme activity in the aorta detected by an assay that used protein kinase–specific substrates was also reduced in Tg mice compared with control mice (70.5±7.4 versus 106.6±9.1 pmol·mg\(^{-1}\)·min\(^{-1}\), respectively; \(P<0.05\); Figure 5c).

**Discussion**

We reported for the first time that chronic overproduction of endothelium-derived NO induced resistance to NO/cGMP/PKG-mediated vasodilators. The reduced vascular response in eNOS-Tg mice resembles “nitrate tolerance,” which results from chronic treatment of nitrovasodilators (exogenous NO donors). Although a large number of studies have tried to clarify the underlying mechanisms of nitrate tolerance, the precise mechanisms remain to be elucidated.\(^{11}\) So far, several different mechanisms for nitrate tolerance have been proposed by experimental investigation, including an increased

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**TABLE 2. Increase in Aortic cGMP Levels in Response to Vasodilators**

<table>
<thead>
<tr>
<th>cGMP, pmol/mg</th>
<th>Control Mice</th>
<th>Tg Mice</th>
</tr>
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<tbody>
<tr>
<td>No stimulation</td>
<td>0.68±0.11 (6)</td>
<td>1.42±0.52 (6)</td>
</tr>
<tr>
<td>SNP (300 nmol/L)</td>
<td>13.63±1.92 (5)</td>
<td>6.56±1.89* (5)</td>
</tr>
<tr>
<td>ANP (100 nmol/L)</td>
<td>2.26±0.14 (5)</td>
<td>2.80±0.24 (5)</td>
</tr>
</tbody>
</table>

Values are mean±SEM and were obtained from individual aortas; the values in parentheses indicate the number of aortas. Response of cGMP elevation was evaluated as absolute cGMP level determined by enzyme immunoassay in ex vivo aortas stimulated by agents at indicated concentrations for 1 minute. No stimulation means stimulation with vehicle after preincubation with Kreb’s solution for 60 minutes. *\(P<0.05\) vs control mice at the same conditions.
superoxide production from the endothelium, \textsuperscript{8} impaired biotransformation of nitrovasodilators to NO, \textsuperscript{12} desensitization of sGC (which is the target enzyme of NO), \textsuperscript{9,13} and an increased phosphodiesterase activity leading to enhanced cGMP breakdown. \textsuperscript{9,14}

Münzel et al \textsuperscript{10} reported that increased superoxide production from endothelial cells induced inactivation of NO and caused nitrate tolerance in the rabbit model. An augmentation of vasoconstriction due to increased endothelin-1 production was shown in that model. \textsuperscript{15} On the other hand, vascular superoxide levels in eNOS-Tg mice were not increased compared with those levels in control mice. Moreover, in contrast to the rabbit nitrate tolerance model, contractions to KCl and PGF\textsubscript{2}\alpha were reduced in vessels from Tg mice. Therefore, an increase in superoxide production does not relate to the resistance to NO in eNOS-Tg mice. Regarding the impaired biotransformation of nitrovasodilators to NO\textsuperscript{12} and an increased phosphodiesterase activity, \textsuperscript{14} both are unlikely related to the reduced relaxation in our Tg mice, because endothelium-dependent relaxation was reduced and cGMP-selective phosphodiesterase inhibitors (zaprinast)\textsuperscript{14} could not reverse the reduced response (data not shown).

In the present study, we demonstrated that at least 2 distinct mechanisms were associated with the resistance to NO. One is reduced cGMP elevations in response to the vasodilators, and the other is decreased PKG protein levels. Several reports have shown that the desensitization of sGC is associated with nitrate tolerance, \textsuperscript{9,13} and our finding is consistent with them. Moncada et al \textsuperscript{16} reported that vascular reactivity to nitrovasodilators was increased by inhibiting basal NO release from the endothelium. They showed that SNP-induced increases in cGMP were significantly potentiated by removal of the endothelium or by eNOS inhibition. They further reported that the specific supersensitiveness to nitrovasodilators, which follows removal of the basal NO release, occurs at the level of its receptor sGC. Recently, Faraci et al \textsuperscript{17} reported that relaxation of the carotid artery to NO was enhanced in eNOS knockout mice compared with wild-type mice. In the present study, in vitro sGC activity was attenuated in aortas from Tg mice compared with those from control mice, whereas sGC protein levels were not changed (Figure 4). Moreover, we demonstrated that the inhibition of basal NO release with chronic L-NAME treatment could reverse the reduced relaxations (Figure 3, Table 1). These results are consistent with the concept of altered vascular reactivity at the level of sGC after changes in the basal NO release. \textsuperscript{16}

The reduced vascular reactivity cannot be explained by the reduced sGC activity alone. Indeed, despite the reduced relaxation to ANP, there was no difference in cGMP elevations by ANP administration (Figure 2c, Table 2). Moreover, 8Br-cGMP–mediated relaxation was also reduced in Tg mice (Figure 2d). Thus, we further examined the downstream pathway and found the reduced expression and activity of PKG. We demonstrated that protein levels and enzyme activity of PKG were significantly reduced in Tg mice (Figure 5). Until recently, PKG and its downstream pathway were not thought to be associated with nitrate tolerance. However, Matsumoto et al \textsuperscript{18} reported that treatment of isolated canine coronary arteries with nitroglycerin induced hyporesponsiveness not only to nitrovasodilators but also to ANP and 8Br-cGMP. Furthermore, Soff et al \textsuperscript{19} demonstrated that continuous exposure to nitrovasodilators suppressed PKG transcription and resulted in reduced PKG protein levels in rat vascular smooth muscle cells in vivo and in vitro. They suggested that the reduced PKG might contribute to the mechanisms of nitrate tolerance. Our finding is in accordance with their data; thus, it is likely that the reduced PKG expression is at least partly responsible for the resistance to NO/cGMP-mediated vasodilators in eNOS-Tg mice. In addition, we cannot deny the possibility that the downstream pathway of PKG may also be altered and involved in the mechanisms of the reduced response to NO in Tg mice.

Other mechanisms extraneous to the vessel wall were thought to be responsible for nitrate tolerance, and the phenomenon induced by these mechanisms was called pseudotolerance. These mechanisms include neurohumoral counterregulation and intravascular volume expansion. \textsuperscript{20} However, there was no difference in plasma concentrations of catecholamines, renin, and endothelin-1 between control and Tg mice. \textsuperscript{5}

Recently, it has been reported by several investigators that transient eNOS gene transfer to the vascular wall changes the vascular reactivity. \textsuperscript{21–23} In contrast with our findings, adenovirus-mediated eNOS gene transfer to rabbit carotid arteries has been reported to enhance endothelium-dependent relaxations. \textsuperscript{21,22} The period of overexpressing the eNOS gene might be responsible for the difference in vascular reactivity between their studies and ours. In their gene transfer studies, the period of overexpressing the eNOS gene was only 1 to
several days before vascular tonus experiments. In our Tg mice, eNOS was overexpressed chronically and throughout the lives of the mice. Regarding contractile responses, both receptor-independent (KCl) and receptor-mediated (PGF2α) contractions were reduced in vessels from eNOS-Tg mice. The same observation applies to the studies of virus-mediated eNOS gene transfer. Hemagglutinating virus of Japan liposome–mediated eNOS gene transfer in injured rat carotid arteries resulted in reduced contractions to KCl. Moreover, contractions to phenylephrine were also reduced in adenovirus-mediated eNOS gene–transferred arteries. However, eNOS gene transfer did not change phenylephrine-induced contraction in other reports. The discrepancy may be due to the difference in the extent of eNOS overexpression in these studies. The diminished sensitivity to contractile agonists is commonly seen in the condition with increased basal NO release from the vascular wall.

In conclusion, the eNOS-overexpressing mice displayed reduced relaxant responses in the NO/cGMP pathway but not in the cAMP pathway. At least 2 distinct mechanisms are involved: one is reduced sGC activity, and the other is reduced PKG protein levels. Furthermore, the reduced reactivity could be reversed by chronic NOS inhibition. These findings indicate that not only exogenous NO but also intrinsic NO from the endothelium could induce reduced vascular responses to nitroglycerin and other nitrovasodilators, like nitrate tolerance. The implications of these findings will not be confined to the Tg mice, and our mouse model will be a novel useful tool to explore the real mechanisms of tolerance to nitrovasodilators. Further studies are warranted to reveal relevant roles of NO in physiological and pathologic situations.

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References

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