Increased Nitrovasodilator Sensitivity in Endothelial Nitric Oxide Synthase Knockout Mice

Role of Soluble Guanylyl Cyclase

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Abstract—Endogenously produced nitric oxide (NO) modulates nitrovasodilator-induced relaxation. We investigated the underlying mechanism in wild-type (WT) mice and endothelial NO synthase knockout (eNOS−/−) mice to determine whether a chronic lack of endothelial NO alters the soluble guanylyl cyclase (sGC) pathway. In aortic segments from eNOS−/− mice, the vasodilator sensitivity to sodium nitroprusside (SNP) was significantly greater than that in WT mice. There was no difference in sensitivity to the G-kinase I activator 8-para-chlorophenylthio-cGMP or to cromakalim. Nω-Nitro-L-arginine had no effect on the SNP-induced relaxation in eNOS−/− but increased the sensitivity in WT mice so it was no longer different than that of eNOS−/−. Basal cGMP levels in aortic rings were significantly lower in eNOS−/− mice than in WT mice. SNP (300 nmol/L) induced a significantly greater cGMP accumulation in eNOS−/− mice than in WT mice. The maximal SNP-induced (10 μmol/L) increase in cGMP was similar in both strains. SNP-stimulated sGC activity was significantly greater in eNOS−/− mice than in WT mice. Incubation of aortic segments from WT mice with Nω-nitro-L-arginine increased sGC activity, an effect prevented by coincubation with SNP (10 μmol/L). The aortic vasorelaxations of the sGC α1 and β1 subunits in WT and eNOS−/− mice were identical as determined with Western blot analysis. These data suggest that chronic exposure to endothelium-derived NO, as well as acute exposure to nitrovasodilator-derived NO, desensitizes sGC to activation by NO but does not alter sGC expression. Both the acute cessation of endothelial NO formation in WT mice and the chronic deficiency of NO in eNOS−/− mice restore the NO sensitivity of sGC and enhance vascular smooth muscle relaxation in response to nitrovasodilator agents. (Hypertension. 2000;35[part 2]:231-236.)

Key Words: nitric oxide ■ mice ■ genes ■ vasodilator agents

Vascular relaxation responses elicited by nitrovasodilator agents and by endothelium-derived nitric oxide (NO) are mainly mediated via the activation of soluble guanylyl cyclase (sGC) and a subsequent increase in intracellular cGMP levels.1 Interactions between endogenous and exogenous NO have been reported to modulate vasodilatory responsiveness.2–5 Different mechanisms may underlie this phenomenon, such as the downregulation of sGC, sGC desensitization, or inhibition of the cGMP-dependent signal transduction cascade. In the present study, we investigated the mechanism by which endothelium-derived NO affects the nitrovasodilator-induced relaxation in wild-type (WT) mice and in endothelial NO synthase knockout mice (eNOS−/−).

Methods

Animals and Tissue Preparations

WT c57 black b6 mice were purchased from Charles River, and eNOS−/− mice and age-matched control animals were obtained from the Department of Physiology at Heinrich Heine Universität Düsseldorf.6 Mice were housed under conditions that conformed with the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health publication No. 85-23).

Mice were killed by cervical dislocation. The aorta was excised rapidly and freed of surrounding fat and connective tissue. The aortic arch was used for Western blot analysis, whereas the thoracic aorta was cut into rings for cGMP determination and organ chamber studies. For assessment of sGC activity, as many as 3 mouse aortas were pooled.

Organ Chamber Experiments

Aortic rings were mounted on stainless steel wires connected to force transducers and placed in individual organ chambers containing Krebs’ buffer of the following composition (in mmol/L): NaCl 119, KCl 4.7, CaCl2 1.6, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, EDTA 0.026, and glucose 12, gassed with 95% O2/5% CO2, pH 7.4, at 37°C. Diclofenac (10 μmol/L) was present in all experiments to inhibit prostaglandin synthesis. Passive tension was gradually increased to 1g. Each ring was challenged twice with K+–rich Krebs’ buffer. Precontraction was elicited with phenylephrine (0.01 to 1 μmol/L).

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Phenylephrine concentrations were adjusted to obtain a similar level of precontraction in each ring (~80% of initial KCl-induced contraction). When a stable contraction plateau was obtained, concentration-relaxation curves were performed in response to cumulatively increasing concentrations of various vasodilators in the presence or absence of N\textsuperscript{-}nitro-L-arginine (L-NA; 300 \mu M/L, applied 30 minutes before the experiments).\textsuperscript{7}

**Determination of Intracellular cGMP**

Aortic rings were incubated in HEPES-modified Tyrode's solution containing the phosphodiesterase inhibitor isobutylmethylxanthine (100 \mu M/L). After 27 minutes, rings were stimulated with either solvent or sodium nitroprusside (SNP; 300 \mu M/L or 10 \mu M/L) for 3 minutes. Thereafter, rings were frozen in liquid nitrogen and homogenized in ice-cold 10% trichloroacetic acid. cGMP was extracted with water-saturated diethylether, acetylated, and quantified via radioimmunoassay as described previously.\textsuperscript{8}

**Western Blot Analysis**

Aortic segments were boiled for 10 minutes in 40 \mu L agitated Laemmli's buffer,\textsuperscript{2} and the supernatants (35 \mu g protein) were subjected to SDS-PAGE and blotted onto nitrocellulose membranes as described previously.\textsuperscript{9} Proteins were detected with their respective antibodies linked with the appropriate horseradish peroxidase-coupled secondary antibody (Calbiochem).

**Assessment of GC Activity in Aortic Protein Extracts**

After preparation of the aorta, samples (at least 1 mouse aorta per data point) were incubated in Krebs' buffer (37°C in 5% CO\textsubscript{2}) in the presence or absence of L-NA (300 \mu M/L) and SNP (10 \mu M/L). After 2 hours, the tissue was shocked frozen and homogenized in liquid nitrogen, dissolved in 200 \mu L ice-cold lysis buffer (20 \mu M/L Tris-HCl, pH 7.4, 0.25 \mu M/L succrose, 200 \mu M/L EDTA, 10 \mu M/L dithiothreitol, 2 \mu M/L benzamidine, and 10 \mu g/mL leupeptin), and cleared through centrifugation (13 000 \times g protein) were incubated at 37°C for 10 minutes in a Tris-HCl--buffered solution (30 \mu M/L, pH 7.4, 100 \mu L containing 50 \mu M/L [\textsuperscript{32}P]GTP (0.2 \mu Ci), 100 \mu M unlabeled cGMP, 3 \mu M/L MgCl\textsubscript{2}, 100 \mu g/mL bovine \textgamma-globulin, 5 \mu M/L creatine phosphate, 100 \mu g/mL creatine phosphokinase (1 U), 3 \mu M/L glutathione, 0.5 \mu M/L isobutylmethylxanthine, and 0.5 \mu M/L DTPA in the presence or absence of 100 \mu M/L SNP. Reactions were stopped by the addition of 0.4 mL zinc acetate (120 \mu M/L) and 0.5 mL sodium carbonate (120 \mu M/L). After centrifugation (10 000 \times g for 10 minutes) 0.95 mL supernatant was loaded onto acid-activated alumina, and [\textsuperscript{32}P]GMP was isolated and determined as described previously.\textsuperscript{10}

**Results**

**Organ Chamber Studies**

The endothelium-dependent vasodilator acetylcholine (10\textsuperscript{-9} to 10\textsuperscript{-5} mol/L) relaxed aortas from WT mice but not from eNOS\textsuperscript{−/−} mice (E\textsubscript{max} 86±4% versus 2±5%, P<0.0001). SNP (10\textsuperscript{-11} to 10\textsuperscript{-5} mol/L) induced complete relaxation of aortic rings from WT mice and eNOS\textsuperscript{−/−} mice. The sensitivity to SNP was significantly greater in eNOS\textsuperscript{−/−} mice than in WT mice. In rings from WT mice, the NOS inhibitor L-NA shifted the concentration-response curve to SNP to the left, whereas relaxations in eNOS\textsuperscript{−/−} mice were unaffected. SNP-induced relaxations in aortic rings from WT mice and eNOS\textsuperscript{−/−} mice in the presence of L-NA did not differ (Figure 1 and Table). Relaxations in response to the G-kinase I-activator 8p-cpt-cGMP were similar in aortas from WT mice and eNOS\textsuperscript{−/−} mice. L-NA had no effect on 8p-cpt-cGMP–induced relaxation in either strain (Figure 2 and Table). Relaxations in response to the K\textsubscript{ATP} channel opener cromakalin (10\textsuperscript{-9} to 10\textsuperscript{-3} mol/L) were slightly greater in preparations from WT mice than in preparations from eNOS\textsuperscript{−/−} mice (n=14, P<0.02), but E\textsubscript{max} and EC\textsubscript{50} values were not significantly different (Figure 3 and Table).

**Western Blot Analysis**

eNOS protein was detected in aortic tissue from WT mice but not from eNOS\textsuperscript{−/−} mice. The expression of the sGC subunits \textalpha and \beta1 was not significantly different in aortas from WT mice and eNOS\textsuperscript{−/−} mice (P=0.8; Figure 4).
cGMP Measurements
Basal cGMP levels were \( \approx 50 \) times greater in aortic rings from WT mice than in rings from eNOS \( ^{-/-} \) mice (10.28 \( \pm \) 2.07 versus 0.20 \( \pm \) 0.06 pmol/mg cGMP; \( n=9 \)). L-NA markedly reduced the cGMP levels in aortic rings from WT mice but had no effect in rings from eNOS \( ^{-/-} \) mice (0.47 \( \pm \) 0.12 versus 0.20 \( \pm \) 0.06 pmol/mg cGMP; \( n=9 \), \( P<0.05 \); data not shown). SNP induced a concentration-dependent increase in cGMP levels in aortas from both strains. cGMP accumulation in response to SNP (300 nmol/L) was significantly greater in rings from eNOS \( ^{-/-} \) mice than in rings from WT mice. The maximal SNP-induced (10 \( \mu \)mol/L) increase in cGMP was comparable in both strains (Figure 5).

sGC Activity
The SNP-stimulated (100 \( \mu \)mol/L) sGC activity in aortic homogenates was significantly greater in samples from eNOS \( ^{-/-} \) mice than in samples from WT mice (\( n=6 \)). Incubation of intact aortas with L-NA (300 \( \mu \)mol/L) for 2 hours significantly increased the SNP-stimulated sGC activity (\( n=6 \); Figure 6A). This increase was prevented when SNP (10 \( \mu \)mol/L) was present during incubation with L-NA (Figure 6B).

Discussion
In the present study, we demonstrate that aortic rings from mice lacking eNOS exhibit an increased sensitivity to nitrovasodilator agents but not to downstream targets of the cGMP pathway (G-kinase) and cGMP-unrelated vasodilator mechanisms, such as opening of \( K_{ATP} \) channels by cromakalim. A comparable effect could be observed after the application of
an NOS inhibitor to aortas from WT mice. Although sGC protein expression was identical in aortas of WT and eNOS\(^{-/-}\) mice, the latter exhibited an increased sensitivity of sGC to SNP in both intact aortas and aortic homogenates. Inhibition of NOS in aortas from WT mice increased the SNP-stimulated sGC activity in protein extracts, and this effect was prevented by concomitant incubation with SNP.

Numerous studies have demonstrated that the acute inhibition of NOS increases the sensitivity of arterial segments to NO donors,\(^2\)\(^{-}\)\(^5\) whereas the exposure to higher doses of exogenous NO has the opposite effect.\(^1\)\(^2\) Moreover, it has been reported that increasing the vascular generation of NO by inducing the inducible NOS\(^1\)\(^3\) or by overexpressing eNOS\(^2\)\(^{/-}\) mice, the sensitivity to this group of vasodilators is increased.\(^1\)\(^5\),\(^1\)\(^6\)

In the present study, knockout of the eNOS gene had no effect on the expression of the sGC\(^\alpha_1\) and \(\beta_1\) subunit proteins, which suggests that the amount of NO generated by eNOS is not sufficient to alter sGC expression and in this way affect nitrovasodilator sensitivity. eNOS\(^2\)\(^{/-}\) mice do not appear to have a higher sensitivity to relaxants in general, because contraction did not differ between the 2 strains (data not shown) and relaxations to the K\(_{ATP}\) channel opener cromakalim were even slightly greater in aortas from WT mice than in those from eNOS\(^2\)\(^{/-}\) mice.

We initially reported that NO has an inhibitory effect on relaxations induced by the G-kinase I activator 8-bromo-cGMP and that 8-bromo-cGMP inhibits NO-induced vasodilation by shifting the concentration-responses curve, probably as the result of the fact that the 2 vasodilators activate the same downstream target (G-kinase I).\(^2\)\(^4\) In the present study, endogenously formed NO had no effect on relaxations induced by 8p-cpt-cGMP, which was apparent by the lack of effect of L-NA on 8p-cpt-cGMP relaxations and the similarity of the concentration-response curves to this substance in aortas from WT and eNOS\(^2\)\(^{/-}\) mice. It is not known whether species differences or different properties of the 2 G-kinase I activators (8p-cpt-cGMP lacks the inhibitory effect on phosphodiesterases and the cAMP-like activity of 8-bromo-cGMP\(^2\)\(^5\)) are responsible for this marked difference, but certainly alterations of the cGMP effector pathway are

![Figure 4](image-url). eNOS and sGC protein expression. Aortic extracts from WT and eNOS\(^2\)\(^{/-}\) mice were subjected to SDS-PAGE. eNOS and sGC\(^\alpha_1\) and \(\beta_1\) subunit expressions were detected with use of respective antibody. Numbers below blot indicate results of relative densitometry (n=9 in each group).

![Figure 5](image-url). Levels of cGMP in basal and SNP-stimulated aortic segments. cGMP concentrations in aortic rings (WT or eNOS\(^2\)\(^{/-}\) mice) treated with phosphodiesterase inhibitor isomethylbutylxanthine (100 \(\mu\)mol/L, 30 minutes) under basal conditions or after stimulation with SNP (300 nmol/L and 10 \(\mu\)mol/L) for 3 minutes as determined with radioimmunoassay (n=10 each group).

![Figure 6](image-url). sGC activity in aortic homogenates from WT and eNOS\(^2\)\(^{/-}\) mice (A) as well as from WT mice control preparation (CTL) compared with WT mice aorta pretreated with L-NA (300 \(\mu\)mol/L) or L-NA and SNP (10 \(\mu\)mol/L) for 2 hours (B) (n=6 in each group).

**Figure 6.** sGC activity in aortic homogenates from WT and eNOS\(^{2\/-}\) mice (A) as well as from WT mice control preparation (CTL) compared with WT mice aorta pretreated with L-NA (300 \(\mu\)mol/L) or L-NA and SNP (10 \(\mu\)mol/L) for 2 hours (B) (n=6 in each group).
excluded as a cause for the increased nitrovasodilator sensitivity in eNOS−/−.

Because neither sGC expression nor the pathways downstream of sGC appear to be altered in eNOS−/− mice, an increased activity or sensitivity of sGC in eNOS−/− mice is likely to be the underlying mechanism. Indeed, SNP-stimulated sGC activity was significantly higher in eNOS−/− mice than in WT mice, and the accumulation of cGMP in aortic rings from eNOS−/− mice stimulated with SNP (300 nmol/L) was also higher than that in aortas from WT mice. This effect could be simulated in aortas from WT mice, because the inclusion of L-NA in the organ chamber experiments not only increased the relaxant sensitivity to SNP to a similar level as in eNOS−/− mice but also increased the SNP-stimulated sGC activity in aortic homogenates. SNP (10 μmol/L) prevented this effect of L-NA on the SNP-stimulated sGC activity and masked the differences in cGMP accumulation in intact aortic segments from both strains. It has been reported that the S-nitroso-N-acetyl-DL-penicillamine (another NO donor)-stimulated sGC activities in lungs from eNOS−/− and WT mice are identical.15 One possible explanation for this conflicting result is that the amount of NO produced from lung endothelial cells is likely to be very different from that from aortic endothelial cells because of the markedly different hemodynamic forces exerted on the cells. The fact that basal cGMP levels were 50-fold higher in WT mice than in eNOS−/− mice emphasizes that basal NO release from endothelial cells is substantial in the aorta of WT mice. It is perhaps important to note that lung homogenates usually contain blood contaminants that may interfere with the determination of SNP-stimulated sGC activity due to the scavenging of NO by the hemoglobin.

It was previously reported that acute inhibition of endothelial NO release results in a supersensitivity of sGC to NO in rat aorta, but the underlying molecular mechanism at the enzyme level has not been clarified.3 In another study, oxidation of the heme iron of sGC in rat aortic smooth muscle cells was suggested as a possible mechanism to explain the loss of NO responsiveness after short-term treatment with higher doses of exogenous NO.23 This hypothesis is consistent with our present observation that a relatively short incubation of mouse aorta with SNP prevented the L-NA-induced increase in SNP-stimulated sGC activity in aortic protein extracts. Although the oxidation of sGC heme iron (from ferrous to ferric) by the sGC inhibitors ODQ26 or NS202827 renders sGC insensitive to activation by NO, oxidation of the heme iron of sGC by NO has not yet been demonstrated. Therefore, it remains to be shown whether endothelium-derived NO desensitizes vascular sGC by partially oxidizing its heme iron and whether the absence of the continuous basal oxidation of sGC by NO account for the higher nitrovasodilator sensitivity of eNOS−/− mice. If this mechanism is operative in endothelium-intact aorta of WT mice (and other species), it is expected to be readily reversible, because exposure of the intact aorta to L-NA for 120 minutes was sufficient to desensitize sGC to NO. The presence of an endogenous mechanism that desensitizes oxidized sGC to NO is supported by the observation that NS2028-induced inhibition of nitrovasodilator-induced relaxation is reversible, although this compound oxidizes the heme moiety of sGC.27 Recently, indirect evidence for such a mechanism was provided in endothelium-denuded bovine coronary arteries, in which a flavin-dependent enzymatic process appears to sensitize oxidized sGC to NO.28

In summary, we demonstrated that the aorta of eNOS−/− mice exhibits a higher sensitivity to SNP than the aorta of WT mice. This effect can be attributed to an enhanced sensitivity, but not expression, of sGC and is due to the lack of endothelium-derived NO in these animals.

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References

18. Brandes RP, Gräper S, Busse R, Mügge A, Mülsch A. Lipopolysaccharide treatment induces nitrate tolerance and down-regulates the soluble...


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