Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial cell nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control

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Abstract

Objective: Both disruption of the endothelial nitric oxide synthase (eNOS) gene and pharmacological inhibition of the NOS produce modest hypertension. It is unclear if and to what extent NOS isoforms other than eNOS contribute to this effect and how loss of one copy of the eNOS gene might impact on vascular reactivity or eNOS protein expression. Methods: We examined protein expression, vascular reactivity, activity of soluble guanylate cyclase, blood pressure and heart rate in mice completely lacking the eNOS gene (eNOS\textsuperscript{-/-}), wild-type mice (eNOS\textsuperscript{+/+}), and mice heterozygotic for the eNOS gene (eNOS\textsuperscript{+/-}). Results: While eNOS\textsuperscript{-/-} mice had mild hypertension and bradycardia, eNOS\textsuperscript{+/-} mice were normotensive. In control mice, oral administration of l-NAME (approximately 100 mg/kg/day \times 21 days) increased blood pressure to levels observed in eNOS\textsuperscript{-/-} mice. In eNOS\textsuperscript{+/-} mice, chronic oral administration of l-NAME had no effect on blood pressure, suggesting that inhibition of other NOS isoforms unlikely contribute to hypertension. l-NAME treatment induced bradycardia in both control and eNOS\textsuperscript{+/-} mice, suggesting that both eNOS and other isoforms of NOS might be involved in heart rate control. Studies of aortic rings from eNOS\textsuperscript{-/-} mice revealed a complete lack of endothelium-dependent vascular relaxation in response to acetylcholine and the calcium ionophore A23187 and an increase in sensitivity to phenylephrine, serotonin and nitroglycerin. Aortic rings from eNOS\textsuperscript{+/-} mice demonstrated only minor alterations of responses to nitroglycerin and a normal relaxation to either acetylcholine or A23187 compared to vessels from eNOS\textsuperscript{+/-}. Western analysis demonstrated that eNOS expression was virtually identical between eNOS\textsuperscript{+/-} and eNOS\textsuperscript{-/-} mice and was absent in eNOS\textsuperscript{+/-} mice. The activity of lung-isolated soluble guanylate cyclase was identical in the three strains of mice. Conclusions: We conclude that loss of one copy of the eNOS gene, as observed in heterozygotic animals, has no effect on vascular reactivity, blood pressure or eNOS protein expression. Isoforms of NOS, other than eNOS are unlikely involved in blood pressure regulation but may participate in heart rate control. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The endothelial cell nitric oxide synthase (eNOS) is one of three known isoforms of NO-synthase and plays a major role in modulation of vascular tone [1–3]. This enzyme is most abundantly expressed in endothelial cells, and catalyzes a complex oxidation/reduction reaction in which L-arginine is converted to citrulline and nitric oxide (nitrogen monoxide). Activation of eNOS by acetylcholine in vitro and in vivo contributes to endothelium-dependent

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vasorelaxation. While the enzyme is activated by a variety of agonists and physiological shear stress [4–6], it is constitutively present in vivo. Inhibition of the enzyme using arginine analogs results in various degrees of hypertension. This observation has led to the concept that vessels in vivo are under a constant state of vasodilation produced by endogenously released nitric oxide. A confounding factor in studies using NO synthase inhibitors in vivo is that these antagonists are, for the most part, not specific to any of the various NOS isoforms. This lack of specificity may be important, because inhibition of the neuronal isoform has been shown to increase sympathetic nerve firing and circulating levels of norepinephrine [7–9]. Given this, it is unclear as to what degree inhibition of the neuronal vs the endothelial isoform might contribute to the hypertension observed following administration of non-specific NOS inhibitors.

Recently, it has been shown that mice lacking the eNOS gene have modest hypertension [10,11]. To date, the effect of loss of one eNOS gene on either eNOS protein expression or vascular reactivity has not been examined. It is interesting to speculate that a partial loss of the eNOS gene might likewise result in hypertension or might alter vascular reactivity to an extent intermediate between that observed in normal animals and eNOS-deficient animals. Thus, the purpose of the present study was two-fold. First, we wished to examine how loss of one vs both eNOS genes might affect eNOS protein expression or vascular reactivity. Secondly, we sought to determine if inhibition of forms of NOS other than eNOS could contribute to hypertension.

2. Methods

2.1. Animals studied

Mice deficient in the endothelial nitric oxide synthase (eNOS<sup>−/−</sup>) mice, wild-type mice (eNOS<sup>+/+</sup>) and mice heterozygotic for the presence of the eNOS gene (eNOS<sup>+/−</sup>) were obtained by mating eNOS<sup>−/−</sup> mice as described previously [11]. The eNOS<sup>+/−</sup> mice were hybrids of 129/Ola and C57BL/6J. Genotypes of the offspring were identified from tail clippings using polymerase chain reactions. Primers complementary to Exon 12 of the eNOS gene were used to identify wild-type mice (sense 5′GCAATCCAAGGAAAGACCTC′, and antisense 5′GAGCCATAAGATGGTTGCC′), and primers complementary to the neomycin-resistance cassette were used to identify the presence of the disrupting genetic insert (sense 5′CTCGACCTTGTCACGTAGC′, and antisense 5′TCAAGAAGGCGATAAGGC′). Results were confirmed using Southern Blot analysis in selected instances as described previously [11]. In addition to these animals, eight C57/BL6 mice were included (see below). Studies were performed when the mice were 12–14 weeks or age. Mice were sacrificed by CO₂ inhalation.

Permission for these studies was provided and the experiments were performed according to the guidelines for the use of experimental animals as given by the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health.

2.2. Determination of eNOS protein levels

After removal, the aortas were immersed in iced tris-buffer (5 mM, pH 7.4) containing the protease inhibitors leupeptin, benzamidine, aproitin, phenylmethylsulfonyl fluoride and antipain (10 μg/ml). The tissues were homogenized for 30 s using a polytron-homogenizer. The homogenates were then centrifuged for 10 min at 100000 × g to remove particulate matter and unbroken cells. Total protein levels were determined using the Bradford method [12]. Western blot analysis was performed as described previously [13] using a commercially available monoclonal antibody (Transduction Laboratories) and the ECL–detection system (Amersham).

2.3. Determination of guanylate cyclase activity

Studies of soluble guanylate cyclase isolated from the lung were performed on ten each of the eNOS<sup>+/+</sup>, eNOS<sup>+/−</sup>, and eNOS<sup>−/−</sup> mice. The animals were sacrificed and the lungs were flash-frozen in liquid nitrogen. A cytosolic fraction was obtained from each lung by homogenisation in 1 ml of hypotonic Tris buffer (5 mM, pH 7.6) containing leupeptin (0.05%), phenylmethylsulfonyl fluoride (PMSF 0.2 mM) and dithiothreitol (DTT 1 mM). The supernatant from the following centrifugation (10 0000 × g for 10 min) was collected and centrifuged again with 105 000 × g for 1 h. The specific activity of soluble guanylate cyclase was measured in aliquots of the resulting supernatant (20–40 μg protein) by the formation of [³²P]-cGMP from [³²P]-GTP as described previously [14]. Briefly, aliquots were incubated for 12 min in a total volume of 100 μl of a triethanolamine–HCl buffer (50 mM, pH 7.4, 37°C) containing [³²P]-GTP (5 nM, 0.4 μCi), GTP (100 μM), cGMP (1 mM), 3-isobutyl-1-methylxanthine (IBMX, 1 mM), MgCl₂ (1 mM) and DTT (1 mM) in the presence of 500 μM S-nitroso-N-acetyl-DL-penicillamine (SNAP) or vehicle (0.25% dimethylsulfoxide). To determine dose-dependent effects of SNAP, the assay volume contained concentrations of these drugs or vehicle as indicated in Section 3.

2.4. Isolated vessel studies

Studies of isolated vessels were performed on six each of the eNOS<sup>+/+</sup>, eNOS<sup>+/−</sup>, and eNOS<sup>−/−</sup> mice. Four ring segments (3 mm width) of thoracic aorta from each mouse were mounted between stainless steel triangles in a water-
jacketed organ bath (37°C) for measurement of tension-development as previously described [15]. Preliminary experiments demonstrated that the optimal resting tension for development of active contraction was 1.0 g. The vessels were gradually stretched over a one-hour period to this tension. Responses to vasocostrictrors were examined at this resting tension and related to maximal vasocostriction elicited by depolarization with 80 mM KCl. Responses to vasodilator substances were examined after achieving a preconstricted tone with 0.2 μM phenylephrine.

2.5. Measurement of blood pressure and heart rate

Blood pressure and heart rate were measured in eNOS+/+ (n=7) eNOS−/− (n=4) and eNOS−/+ (n=7) for fourteen consecutive days using an automated tail cuff system (Visitech Systems, Apex, NC, USA) specifically made for studies of mice [16]. Mice of both sexes were used. On each day, three repeated cycles of ten pressure determinations each were made. The first seven days of blood pressure measurements were used only to accustom the mice with the procedure and data were only used from the final seven days of pressure measurement.

To examine the potential role of isoforms of NO synthase other than eNOS in the control of blood pressure and heart rate, eight eNOS−/− mice and eight C57/Bl6 were treated for 21 days with l-NAME. To accomplish this, l-NAME was added to the drinking water of the mice to achieve a final concentration of 1.5 mg/ml. Based on preliminary experiments which demonstrated that the mice drank approximately 2 ml per day, this concentration resulted in the mice receiving approximately 100 mg/kg of body weight. Following 17 days of l-NAME treatment, a second 6-day series of blood pressures were recorded and the last four used for data analysis.

2.6. Statistical analysis

Data in the manuscript are presented as mean±standard error of the mean. Relaxations of the isolated vessels to vasodilator stimuli are expressed as a percentage of the preconstriction caused by phenylephrine achieved prior to addition of the respective vasodilator agents. Vasocostrictrions to KCl are expressed in grams and constrictions to other agents are expressed as a percent of the peak KCl response. Dose–response curves were compared between groups of animals using analysis of variance (ANOVA for repeated measures, Graph Pad Prism®, 2.01). Comparisons of blood pressure between the eNOS−/+ and eNOS−/− mice before and after l-NAME treatment were made using ANOVA. P<0.05 was considered significant.

The concentrations of the half-maximal vasodilator effect of nitroglycerin (pD2-values) were calculated from the individual concentration–effect–curves as proposed by Hafner et al. [17].

3. Results

3.1. Western blot analysis of eNOS protein expression

Western analysis revealed a 133-kd band in homogenates of both eNOS−/+ and eNOS−/− mouse aortas and bovine aortic endothelial cells. The intensity of this band appeared virtually identical between the eNOS−/+ and eNOS−/− mice and in both cases increased as increasing amounts of total protein were loaded in each lane (n=3, Fig. 1). No band was detected in the homogenates of eNOS−/− mice aortas (Fig. 1).

3.2. Studies of isolated aortic vessels

In aortas of eNOS−/− mice, acetylcholine and A23187 produced only vasoconstriction (Fig. 2). Thus, disruption of the eNOS gene resulted in complete loss of endothelium-dependent relaxation. Endothelium-dependent relaxations evoked by acetylcholine and the calcium ionophore A23187 were identical between aortic rings of eNOS−/+ and eNOS−/− mice.

Endothelium-independent vasorelaxations induced by nitroglycerin are illustrated in Fig. 3. In vessels from all
mice, nitroglycerin produced near complete relaxations from the preconstricted tensions, however this response occurred at substantially lower concentrations of nitroglycerin in the eNOS mice as compared to the eNOS mice, resulting in a shift of the EC50 by approximately seven-fold. The pD2 values (−log EC50) were significantly shifted from 7.5 ± 0.1 (eNOS mice) to 7.8 ± 0.1 (eNOS mice) and 8.2 ± 0.1 (eNOS, eNOS, and eNOS mice, Fig. 3).

Constrictions caused by 80 mM of KCl were similar in all groups of mice, averaging 1.7 ± 0.2, 2.2 ± 0.2, and 1.9 ± 0.3 grams in the eNOS mice, eNOS mice, and eNOS mice respectively. Phenylephrine and serotonin produced vasoconstrictions in vessels from all groups of mice (Fig. 4). There was no difference in the concentration dependency or the magnitude of vasoconstriction between aortic rings of eNOS mice and eNOS mice. In contrast, aortic rings of the eNOS mice constricted to a markedly greater extent to serotonin and phenylephrine. In other experiments, it was found that acute incubation of aortic rings of normal mice (C57BL/6J) with N-nitro-l-arginine (100 μM × 30 min) resulted in a similar hypersensitivity to phenylephrine (data not shown).

3.3 Blood pressure and heart rate

There was no difference in either blood pressure or heart rate between eNOS and eNOS mice. As previously reported, blood pressure was substantially increased in the eNOS−/− mice [11]. This was associated with a significant decrease in heart rate (Fig. 5).

Treatment of normal C57BL/6J-mice with L-NAME for 21 days resulted in a significant increase of blood pressure (Fig. 6) that equaled the increase of blood pressure caused by disruption of the eNOS gene (Fig. 6, left panel). Treatment of eNOS mice with L-NAME had no effect on blood pressure. By contrast, L-NAME treatment induced a significant decrease of heart rate in both C57BL/6J and eNOS mice (Fig. 6, right panel).

3.4 Studies on isolated soluble guanylate cyclase

The activity of soluble guanylate cyclase isolated from the lung showed no differences between eNOS mice, eNOS mice, and eNOS mice. The concentration response curves of SNAP were identical in each of the mouse strains (Fig. 7) suggesting that disruption of the eNOS gene is not associated with changes of the sensitivity of vascular soluble guanylate cyclase to nitric oxide.

4. Discussion

In this study, we sought to further characterize the consequences of disruption of the eNOS gene on both vascular reactivity and systemic hemodynamics. As previously reported, eNOS mice have modest hypertension and an absence of endothelium-dependent vascular relaxation to acetylcholine [10,11]. In isolated vessels, we also found that relaxations to the calcium ionophore A23187 were absent while relaxations to the endothelium-independent vasodilator nitroglycerin were enhanced. In keeping with the role of nitric oxide in modulating vascular tone, contractions to phenylephrine, and to a lesser extent serotonin, were potentiated.

In rats treated with NOS inhibitors such as L-NAME,
Fig. 5. Blood pressure and heart rate in eNOS<sup>+/-</sup>, eNOS<sup>-/-</sup>, and eNOS<sup>-/-</sup> mice. Measurements were obtained in resting awake animals using a tail-cuff method. Plotted are average values of 210 single measurements performed at seven consecutive days in each animal. Significant changes (* = P < 0.05) were only observed in mice homozygotic for the disruption of the eNOS gene.

Fig. 6. Blood pressure (left panel) and heart rate (right panel) in normal C57/BL6<sup>+/-</sup> and eNOS<sup>-/-</sup> mice before and after 21 days of oral treatment with l-NAME (approximately 100 mg/kg). Measurements were obtained in resting awake animals using a tail-cuff method. Significant changes are indicated (* = P < 0.05).

plasma norepinephrine levels increase substantially [9]. Acute administration of NO synthase inhibitors increase sympathetic nerve activity, even when administered intracysternally into the third ventricle [7,8]. These observations have raised the suspicion that a portion of the hypertension observed following administration of NOS-antagonists may be due to inhibition of the neuronal isoform of NOS. The present studies would suggest that this is not the case. Administration of l-NAME to eNOS<sup>-/-</sup> mice did not increase blood pressure. Further, chronic l-NAME treatment increased blood pressure in control mice to a level similar to that observed in the eNOS<sup>-/-</sup> mice. These findings strongly suggest that the major effect of NOS inhibition on blood pressure in mice is mediated by blockade of the eNOS enzyme.

The lack of a change in blood pressure during prolonged inhibition of NOS is in contrast to the acute effect of NOS inhibition in eNOS<sup>-/-</sup> mice reported by Huang et al. [10]. While different NOS antagonists were used in these two
studies, it is unlikely that this accounts for the differing results. L-NAME is rapidly converted to L-nitroarginine in vivo, and is not active until this de-esterification occurs [18]. It is more likely that differences in modes of administration and eventual effective concentrations of the inhibitors might account for these varying results. It is also possible that anaesthesia, not used during blood pressure measurement in our study, could have affected the response to NOS inhibition in the previous study. The decrease in heart rate observed in our study might be related to a similar underlying mechanism as the decrease in blood pressure reported in Huang’s previous study. Of note, in a recent preliminary study, chronic oral administration of L-NAME paradoxically lowered blood pressure by about 9%, in a fashion similar to that observed by Huang et al. [10,19]. Notwithstanding the issue of whether or not NOS antagonists might lower blood pressure, it is clear that neither acute nor chronic administration of these agents increase blood pressure in eNOS<sub>−/−</sub> mice. This finding suggests that other isoforms of NOS unlikely contribute, either directly or indirectly, to vasodilation in vivo.

As previously described, the hypertension in the eNOS<sub>−/−</sub> mice was found to be associated with a relative bradycardia compared to mice with intact eNOS gene [11]. Of interest, we also found that treatment of control mice with L-NAME produced a marked decrease in heart rate to values below that observed in the untreated eNOS<sub>−/−</sub> mice identical to those observed in the eNOS<sub>−/−</sub> mice treated with L-NAME. Taken together, these results show that endogenous nitric oxide production contributes substantially to heart rate control and that both eNOS and likely other isoforms of NOS are involved. The mechanism whereby nitric oxide could control heart rate has recently been studied in detail [20]. The results of this investigation showed that nitric oxide can increase heart rate by stimulating the hyperpolarization-activated inward current. By contrast, it is unlikely that the bradycardic effect of L-NAME shown here is solely due to a baroreflex slowing of heart rate due to the increase in blood pressure. L-NAME treatment produced a mild decrease in heart rate in the eNOS<sub>−/−</sub> mice, even in the absence of a change in blood pressure. One might have expected that a baroreflex effect would be lost over time due to baroreflex resetting, which is commonly observed in other hypertensive models. There is some evidence to suggest that endothelium-derived mediators in the carotid sinus may be involved in heart rate control and that the production of these may be affected by loss of nitric oxide [21].

As previously reported, acetylcholine-induced endothelium-dependent vascular relaxation were absent in aortas from eNOS<sub>−/−</sub> mice [10]. In our study, both acetylcholine and the calcium ionophore A23187 produced marked constrictions in the eNOS<sub>−/−</sub> mice, while producing potent vasodilation in the eNOS<sub>−/−</sub> mice. The contractions caused by these agents are likely due to their direct effect on the vascular smooth muscle to activate muscarinic receptors and increase intracellular calcium levels respectively.

In previous studies, it has been reported that either removal of the endothelium or pharmacological inhibition of NO synthase enhances the vasodilation produced by exogenously administered nitrovasodilators [22,23]. In accordance with these prior studies, we found that relaxations to nitroglycerin were markedly enhanced in aortic segments from eNOS<sub>−/−</sub> mice. These results demonstrate that the enhanced sensitivity to nitrovasodilators caused by endothelial-denudation or pharmacologic NO synthase inhibitors is independent of non-specific effects. It is unlikely that the chronic loss of an effect of endogenous nitric oxide on the vascular smooth muscle guanylate cyclase sensitizes this enzyme to the effects of exogenously administered nitric oxide. The sensitivity of isolated vascular soluble guanylate cyclase to the nitric oxide donor SNAP was not changed by disruption of the eNOS gene (Fig. 7). In contrast, it is conceivable that the enhanced aortic relaxations to nitroglycerin are related to an increased vascular bioactivation of this organic nitrate caused by the lack of endogenous production of nitric oxide. Previous investigations have shown that nitric oxide is capable of inhibiting the bioconversion of nitroglycerin [24]. Interestingly, the response to nitroglycerin in vessels from eNOS<sub>−/−</sub> mice was also modestly enhanced. The mechanism for this remains unclear. This finding could be due to an alteration of basal production of nitric oxide in vessels from the eNOS<sub>−/−</sub> mice, however we believe this is unlikely based on our other physiological findings and the Western analysis. It is conceivable that the bioactivation process is also altered in mice with only one copy of the eNOS gene.

In addition to alterations of vasodilation in aortic segments from eNOS<sub>−/−</sub> mice, we also found that these vessels demonstrated a markedly enhanced vasoconstriction to phenylephrine and a modestly enhanced constriction to serotonin. This effect was likely not due to a long-term adaptation to loss of endogenous nitric oxide, as it could be mimicked by acute administration of L-nitroarginine in the organ chamber. Nevertheless, the marked increase in sensitivity to these vasoconstrictor compounds could contribute to the hypertension observed in the eNOS<sub>−/−</sub> mice and in other models in which NOS is pharmacologically inhibited. Recent data suggests that cGMP-dependent protein kinase participates in phosphorylation of the cytoplasmic domain(s) of G-protein dependent receptors, leading to uncoupling to downstream activation pathways [25]. The loss of NO might therefore increase receptor mediated vasoconstrictor mechanisms, such as those mediated by alpha-adrenergic and serotoninergic receptors.

The mice used in this study were offspring of F2 hybrids of C57BL/6J and 129/Ola mice. The use of F3 hybrids raises the possibility that abnormalities of blood pressure,
heart rate and vascular reactivity could be due to differences between the strains not related to the disrupted eNOS gene. The likelihood of this possibility is reduced by the use of wild-type siblings as controls in most experiments. Further, prior analysis of F2 hybrid mice with similar strain backgrounds and intact eNOS genes revealed no differences of heart rate or blood pressure associated with an origin of the eNOS gene [11].

In several genetically transmitted diseases, the loss of one gene has no effect on the phenotype of the carrier. Examples of these include Tay–Sachs disease, Alkaptonuria, Wilson’s disease and some rare sex-linked hereditary diseases. In contrast, loss of one gene in other conditions such as sickle cell trait, LDL-receptor deficiency, and phenylketonuria, produce disease states of intermediate severity. Because loss of one eNOS gene might have implications for several important cardiovascular diseases, we sought to determine if the eNOS+/- mice exhibited any distinguishing characteristics. In the present experiments we found that eNOS protein expression, as detected by Western analysis, was not different between eNOS+/- and eNOS+/- mice, suggesting that the loss of one gene has no effect on ultimate protein levels. In keeping with this finding, there was no alteration in blood pressure, heart rate, endothelium-dependent vascular relaxation to acetylcholine and A23187 or vasoconstrictions to phenylephrine or serotonin in the eNOS+/- as compared to eNOS+/- mice.

In summary, our findings demonstrate that in eNOS+/- mice, aortic contractions to phenylephrine and serotonin and relaxations to glyceryl trinitrate are potentiated, while endothelium-dependent relaxations and eNOS protein are completely absent. The activity of isolated guanylate cyclase is unchanged. Inhibition of eNOS by treatment with L-NAME did not aggragate the mild hypertension but did effect bradycardia present in eNOS+/- mice. None of these changes were observed in eNOS+/- mice. We suggest that isoforms other than eNOS are unlikely involved in blood pressure regulation but may participate in heart rate control.

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References
