Role of nNOS in Blood Pressure Regulation in eNOS Null Mutant Mice

Nobutaka Kurihara, Marcos E. Alfie, David H. Sigmon, Nour-Eddine Rhaleb, Edward G. Shesely, Oscar A. Carretero

Abstract—The role of neural nitric oxide synthase (nNOS) in regulating blood pressure (BP) remains uncertain. Recently it was reported that in mice lacking functional endothelial NOS (eNOS) genes (\(+/−\)), acute administration of a nonselective NOS inhibitor, \(N^\text{o}\)-nitro-L-arginine, decreased mean BP, suggesting that NO released by non-eNOS isoforms increases BP. Because the inducible NOS isoform is not constitutively expressed and when induced causes hypotension, we hypothesize that it is NO produced by nNOS that increases BP in the absence of eNOS activity. To test this hypothesis, we studied the acute effect of selective and nonselective nNOS inhibitors on BP and cerebellar NOS activity in eNOS (−/−), wild-type (+/+), and heterozygous (+/−) mice as well as in +/+ mice with renovascular hypertension. Because it is not known whether the decrease in BP caused by acute NO inhibition in −/− mice can occur chronically, we also studied the effect of chronic NOS inhibition on both BP and cerebellar NOS activity. eNOS (−/−) mice had higher BP than +/+ or +/− mice, and acute administration of the selective nNOS inhibitor 7-nitroindazole (7-NI) decreased their mean BP from 137±13 to 124±12 mm Hg (\(P<0.01\)). In +/+, +/−, or renovascular hypertensive +/+ mice, 7-NI caused a small but insignificant rise from 105±5 to 110±6 mm Hg, from 115±9 to 119±13 mm Hg, and from 146±6 to 150±6 mm Hg, respectively. Fifteen minutes after administration of 7-NI, cerebellar NOS activity decreased by 70%; however, this inhibitory effect was brief, since 2 hours after 7-NI administration NOS returned toward control values. Chronic oral or intraperitoneal administration of 7-NI did not inhibit cerebellar NOS activity, whereas the nonselective NOS inhibitor \(N^\text{o}\)-nitro-L-arginine methyl ester (L-NAME) decreased this activity by 50%. Therefore, we studied the effect of chronic L-NAME administration (4 weeks) on BP. In −/− mice, chronic L-NAME administration decreased BP from 135±4 to 120±3 mm Hg (\(P<0.05\)), whereas in +/+ and +/− mice, as expected, it increased BP from 109±2 to 125±3 mm Hg (\(P<0.001\)) and from 107±6 to 119±5 mm Hg (\(P<0.02\)), respectively. After L-NAME administration was stopped, BP returned to baseline. These results suggest that in eNOS −/− mice, NO derived from nNOS increases BP both acutely and chronically. (Hypertension. 1998;32:856-861.)

Key Words: nitric oxide ▪ blood pressure ▪ nitric oxide synthase, neural ▪ nitric oxide synthase, endothelial

It is well known that nitric oxide (NO) plays an important role in regulating blood pressure (BP).\(^1\)\(^-\)\(^4\) Three isoforms of the enzyme responsible for NO formation, nitric oxide synthase (NOS), have been identified: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS).\(^5\)\(^,\)\(^6\) eNOS is expressed in endothelial cells and produces NO, which dilates blood vessels. It is stimulated by a variety of receptor agonists and by shear stress produced by flowing blood.\(^3\)\(^,\)\(^4\) Recently, 2 groups acting independently developed mice lacking functional eNOS genes (eNOS −/− mice).\(^6\)\(^,\)\(^7\) These mutant mice showed elevated BP in both conscious and anesthetized states,\(^6\)\(^,\)\(^7\) confirming that NO derived from eNOS plays an important role in regulating BP as a vasodilator.

The role of nNOS in regulating BP is unclear. Most investigators have shown no changes in BP after acute inhibition of nNOS.\(^8\)\(^-\)\(^10\) However, it was recently reported that chronic administration of the selective nNOS inhibitor 7-nitroindazole (7-NI) in drinking water significantly increased BP in rats.\(^11\) Therefore, it has been believed alternatively that NO derived from nNOS has no effect or that it decreases BP. Complicating matters, there is evidence that 7-NI may inhibit eNOS in vivo,\(^12\) suggesting that 7-NI increases BP through inhibition of both nNOS and eNOS. On the other hand, in mice lacking functional eNOS (−/−), acute injection of a nonselective NOS inhibitor, \(N^\text{o}\)-nitro-L-arginine (L-NNA), decreased mean BP,\(^7\) suggesting that NO derived from isoforms other than eNOS increases BP in the absence of eNOS activity. Because the iNOS isoform is not expressed constitutively and when induced causes hypotension,\(^2\) we hypothesize that it is NO derived from nNOS that increases...
BP in eNOS (−/−) mice. This effect may occur at the level of the central nervous system (CNS) or baroreceptors, since circulating NO would cause vasodilatation independent of the enzyme that produces it. To test the hypothesis that NO derived from nNOS increases BP, we studied the effect of selective and nonselective nNOS inhibitors on BP and cerebellar NOS activity (because this organ is rich in nNOS) in eNOS (−/−), wild-type (+/+), and heterozygous (+/−) mice as well as in +/+ mice with renovascular hypertension. The latter group was included to rule out the possibility that the acute hypotensive effect of 7-NI was due to the high BP. Because it is not known whether the decrease in BP caused by acute NO inhibition in −/− mice can occur chronically, we also studied the effect of chronic NOS inhibition on both BP and cerebellar NOS activity. However, since preliminary studies showed that chronic administration of 7-NI did not inhibit cerebellar NOS whereas L-NAME did, the chronic studies were performed using L-NAME.

Methods

Animals

Experimental eNOS mice were obtained by breeding F1 hybrid mice composed of strains 129 and C57BL/6J that were heterozygous (+/−) for an eNOS null mutant generated by gene targeting. The result was F2 offspring of all 3 eNOS genotypes: wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−). The genotype of each experimental mouse was determined by Southern blot analysis as described previously. Those littermate F2 mice are the experimental eNOS mice used in this study. All mice were housed in a quiet room at 25°C with a 12-hour light/dark cycle and had free access to food and water. This study was approved by the Henry Ford Hospital Care of Experimental Animals Committee.

Renovascular Hypertension

Because eNOS −/− mice are hypertensive, we performed an additional control in which the effect of acute 7-NI injection (Alexis) was studied in mice with 2-kidney, 1-clip renovascular hypertension (+/+/2K1C). For this, 129/SvEv mice weighing 25 to 30 g (Taconic Farms), and the left renal artery was carefully dissected from the renal vein under antiseptic conditions. A silver clip with an ID of 0.102 mm was placed around the left renal artery. Five mice were sham-clipped. Tail-cuff systolic blood pressures (SBPs) before and 3 weeks after clipping were 114 ± 6 for 7-NI and 114 ± 6 mm Hg, compared with 114 ± 3 and 114 ± 4 mm Hg in the sham group. Only mice with a mean BP of ≥120 mm Hg were used as controls for the −/− group.

Direct Mean BP, SBP, and Heart Rate

To measure mean BP, mice were anesthetized with thiobutabarbital sodium salt (Inactin, RBI; 125 mg/kg body wt IP). They were placed on a heating pad to maintain constant body temperature, and a catheter constructed of PE-10 tubing connected to PE-50 was inserted into the right carotid artery. Mean BP was monitored with a pressure transducer (Ohmeda) and recorded with a chart recorder (Gould). After a 20-minute stabilization period, mean BP was recorded for 5 minutes (basal mean BP), followed by drug injection. After a 15-minute stabilization period, mean BP was recorded for another 5 minutes.

Systolic blood pressure (SBP) and heart rate (HR) were measured in conscious mice using a noninvasive computerized tail-cuff system (BP-2000, Visitech Systems) as described previously. 13,14

Cerebellar NOS Activity

To see whether nNOS activity was inhibited, cerebellar NOS activity was assayed by measuring the ability of the homogenized tissue to convert [3H]-L-arginine (Amersham) to [3H]-L-citrulline as described previously. 15 Mice were decapitated, and the cerebella were stored at −70°C until the assay was performed. Each value for nNOS activity from a given sample was subtracted from a negative control obtained from a “blank” sample (without tissue). The value of the negative control was <5% compared with samples from control mice. All values were corrected for the protein concentration in the homogenate and are expressed in cpm citrulline per minute per milligram of protein. Protein concentration was measured with Coomassie assay reagent (Pierce).

Experimental Protocols

Acute Effect of 7-NI on Cerebellar NOS Activity

Because the solubility of 7-NI in water is very low, it was necessary to dissolve it in peanut oil (20 mg/mL). Thirty-three 129/SvEv mice were anesthetized with Inactin. Six mice were not injected but were decapitated (controls). Eighteen were injected with 7-NI (50 mg/kg body wt IP) and 9 with vehicle (peanut oil, 2.5 mL/kg body wt). All animals were placed on a heating pad and killed at 15, 60, or 120 minutes for each period. n = 6 for 7-NI and n = 3 for vehicle. After the mice were killed, the cerebellum was removed and assayed for NOS activity.

Acute Effect of 7-NI on Mean BP in +/+, +/−, and −/− eNOS and +/+ 2K1C Mice

Mean BP was measured before and after acute injection of 7-NI (50 mg/kg body wt IP) in +/+ , +/− , −/− eNOS mice (n = 6 per group) and in +/+ 2K1C mice (n = 9). As controls, +/+ , +/−, and −/− eNOS mice (n = 4 per group) and +/+ 2K1C mice (n = 8) were injected with vehicle (peanut oil, 2.5 mL/kg body wt).

Acute Effect of L-NAME on Mean BP and Cerebellar NOS Activity in +/+ , +/−, and −/− eNOS Mice

Mean BP was measured before and after acute injection of L-NAME (100 mg/kg body wt IP) in +/+ , +/−, −/− eNOS mice. Mice were decapitated, and the cerebellum was assayed for NOS activity (n = 6 per group). NOS activity was also measured in vehicle-treated +/+ , +/−, and −/− eNOS groups (n = 3 per group).

Chronic Effect of 7-NI on Cerebellar NOS Activity

129/SvEv mice received either tap water (n = 3) or 7-NI in tap water (50 mg/L; n = 3) for 4 weeks. Another group received injections of peanut oil (10 mL/kg body wt IP; n = 3) or 7-NI (20 mg/kg body wt; n = 3) twice a day for 7 days. Mice were then decapitated, and the cerebellum was removed and assayed for NOS activity.

Effect of Chronic Administration of L-NAME on Cerebellar NOS Activity

129/SvEv mice received either tap water (n = 6) or L-NAME in tap water (0.2 g/L; n = 12) for 4 weeks. Half of each group was killed at the fourth week, and the cerebellum was removed and assayed for NOS activity; the other half received tap water for another 3 weeks after discontinuation of L-NAME, and the cerebellum was then removed and assayed.

Effect of Chronic Administration of L-NAME on SBP

First, SBP and HR were measured in conscious mice (+/+ , +/−, and −/− eNOS). Then each group was divided into 2 subgroups at random and given either vehicle (n = 5, n = 9, and n = 6, respectively) or L-NAME (0.2 g/L; n = 9, n = 5, and n = 5) ad libitum. All mice had free access to food. Four weeks later, SBP and HR were measured as described. After that, L-NAME was stopped, and all mice were given tap water. Three or 8 weeks later, SBP and HR were measured again.

Statistical Analysis

Data are expressed as mean ± SEM. All data were subjected to ANOVA. Individual comparisons were performed using a paired/
Acute Effect of 7-NI on Cerebellar NOS Activity

Figure 1 shows the effect of 7-NI on cerebellar NOS activity. Acute administration of 7-NI significantly inhibited cerebellar NOS activity at 15 and 60 minutes after injection (P<0.001 versus vehicle). Activity tended to return to baseline after 2 hours, although it remained lower than in the vehicle-treated group. Mice injected with vehicle had cerebellar NOS activity similar to that in the control group (not injected with vehicle).

Unpaired t test or Wilcoxon rank sum test with Bonferroni’s correction.

TABLE 1. Mean BP (mm Hg) Before and 15 Minutes After L-NAME Administration in Wild-Type (+/+), Heterozygous (+/−), and Homozygous (−/−) eNOS Mutant Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Before L-NAME</th>
<th>15 Min L-NAME</th>
<th>P</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>101±3</td>
<td>139±8</td>
<td>&lt;0.001</td>
<td>6</td>
</tr>
<tr>
<td>+/−</td>
<td>120±8</td>
<td>152±9</td>
<td>&lt;0.005</td>
<td>6</td>
</tr>
<tr>
<td>−/−</td>
<td>156±9</td>
<td>138±6</td>
<td>0.038</td>
<td>6</td>
</tr>
</tbody>
</table>

Acute Effect of 7-NI on Mean BP in +/+, +/−, and −/− eNOS and +/+ 2K1C Mice

Figure 2 shows the effect of 7-NI or vehicle on mean BP in +/+, +/−, and −/− eNOS and +/+ 2K1C mice. Before the injection, mean BP was higher in −/− than in +/+ and +/− mice (P<0.01 and P<0.03, respectively). After acute 7-NI injection, mean BP increased slightly in +/+, +/−, and +/+ 2K1C mice by 5, 4, and 4 mm Hg, respectively, but the changes were not statistically significant; conversely, in −/− mice, mean BP decreased by 14 mm Hg (P<0.01 versus control period). Injection of vehicle did not change mean BP in any of the groups.

Acute Effect of L-NAME on Mean BP and Cerebellar NOS Activity in +/+, +/−, and −/− eNOS Mice

Table 1 shows mean BP before and after acute injection of L-NAME. Before the injection, −/− mice were hypertensive compared with +/+ and +/− mice (P<0.001 and P<0.02, respectively). In +/+ and +/− mice, L-NAME increased mean BP by 37 and 32 mm Hg, respectively (P<0.001 and P<0.005 versus control period). In −/− mice, L-NAME functioned similarly to 7-NI, decreasing mean BP by 18 mm Hg (P<0.05).

Table 2 shows cerebellar NOS activity in +/+, +/−, and −/− eNOS mice given vehicle and L-NAME acutely. The L-NAME group was composed of the same mice used for mean BP measurement (Table 1); after mean BP was measured, the cerebellum was immediately removed. Cerebellar NOS activity was 95% less in all 3 genotype groups given L-NAME than in the same genotype groups given vehicle (P<0.025 for each group). There were no significant differences in cerebellar NOS activity among genotypes in either vehicle or L-NAME groups.

Chronic Effect of 7-NI on Cerebellar NOS Activity

Cerebellar NOS activity in mice receiving 7-NI in drinking water for 4 weeks was 1560±70 compared with 1510±140 cpm citrulline·min⁻¹·mg protein⁻¹ in mice receiving tap water (NS). In mice treated with 7-NI IP for 7 days, cerebellar NOS activity was 1790±130 compared with 1760±60 cpm citrulline·min⁻¹·mg protein⁻¹ in the vehicle group (NS).

Effect of Chronic Administration of L-NAME on Cerebellar NOS Activity

In 129/SvEv mice receiving L-NAME in drinking water for 4 weeks, cerebellar NOS activity was 780±110 cpm citrulline·min⁻¹·mg protein⁻¹, compared with 1600±120 in mice.
receiving tap water ($P=0.0054$). Three weeks after L-NAME was stopped, cerebellar NOS was $1500\pm 100$ versus $1650\pm 60$ cpm citrulline $\cdot$ min$^{-1}$ $\cdot$ mg protein$^{-1}$ in the controls ($P=\text{NS}$).

**Effect of Chronic Administration of L-NAME on SBP**

Because chronic L-NAME inhibited cerebellar NOS activity whereas oral or intraperitoneal administration of 7-NI did not, we studied only the effect of chronic L-NAME administration on BP in the 3 genotypes. Figure 3 shows the effect of chronic oral administration of L-NAME on SBP and HR in $+/+$, $+/-$, and $-/-$ eNOS mutant mice. L-NAME increased SBP by 16 mm Hg in $+/+$ mice ($P<0.001$) and 13 mm Hg in $+/-$ mice ($P<0.02$), while in $-/-$ mice SBP decreased by 14 mm Hg ($P<0.05$). SBP did not change in the vehicle-treated groups. After L-NAME administration was stopped, SBP returned to baseline in all 3 groups. HR tended to decrease in the L-NAME groups, but the change was not significant by paired $t$ test ($P=0.09$, $P=0.09$, and $P=0.17$ for $+/+$, $+/-$, and $-/-$ eNOS mice, respectively). However, ANOVA suggested that HR decreased with chronic L-NAME throughout the 3 groups independent of group. This tendency was reversible after L-NAME was discontinued. HR did not change in the vehicle-treated groups.

**Basal BP and HR in Wild-Type, Heterozygous, and Homozygous eNOS Mutant Mice**

Basal mean BP of anesthetized mice, obtained by combining the data from the protocols on the acute effect of 7-NI and L-NAME, was $104\pm 3$, $115\pm 4$, and $145\pm 7$ mm Hg in $+/+$, $+/-$, and $-/-$ mice, respectively ($n=11$ per group). Mean BP was significantly higher in $-/-$ mice than in $+/+$ ($P<0.0001$) or $+/-$ ($P<0.05$) mice. There were no significant differences in mean BP between $+/+$ and $+/-$ mice.

In conscious mice, SBP measured by the computerized tail-cuff system was $109\pm 1$ mm Hg in $+/+$ ($n=14$), $110\pm 3$ mm Hg in $+/-$ ($n=14$), and $131\pm 2$ mm Hg in $-/-$ ($n=11$) mice. It was significantly higher in $-/-$ mice than in $+/+$ ($P<0.0001$) or $+/-$ ($P<0.0001$) mice. HR was $617\pm 15$ bpm in $+/+$, $636\pm 14$ bpm in $+/-$, and $573\pm 17$ bpm in $-/-$ mice. It was significantly lower in $-/-$ mice than in $+/+$ ($P<0.01$) and tended to be lower than in $+/+$ mice ($P=0.058$). There was no significant difference in HR between $+/+$ and $+/-$ mice.

**Discussion**

A previous study has shown that acute administration of the nonselective NOS inhibitor L-NNA decreased mean BP in eNOS $-/-$ mice.\textsuperscript{7} Here we expanded this observation using the selective nNOS inhibitor 7-NI. In eNOS $-/-$ mice, a bolus injection of 7-NI decreased mean BP, whereas in $+/+$ and $+/-$ mice it caused a small but statistically insignificant increase (Figure 2). Also, in a separate group of 129/SvEv mice, we showed that 7-NI decreased cerebellar NOS activity, with the maximum effect occurring between 15 and 60 minutes after injection. This decrease was relatively brief, since in $-/-$ mice treated with vehicle cerebellar NOS activity was similar to that in $+/+$ and $+/-$ mice (Table 2). Furthermore, although 7-NI may not be completely specific for nNOS and could have an inhibitory effect on eNOS,\textsuperscript{12} the fact that it caused an impressive decrease in cerebellar NOS while producing a very small increase in BP in eNOS $+/+$ and $+/-$ mice further supports the conclusion that its effect was mediated by inhibition of nNOS. In contrast, when we used the nonselective NOS inhibitor L-NAME acutely in eNOS $+/+$ and $+/-$ mice, it decreased cerebellar NOS activity but also produced a significant increase in BP. This increase can be explained by inhibition of eNOS. In eNOS $-/-$ mice, L-NAME, like 7-NI, decreased both mean BP and cerebellar NOS activity (Tables 1 and 2).

Independent of the NOS isoform, if NO is abundant in the circulation it will decrease both vascular resistance and BP;

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**Table 2. Cerebellar NOS Activity in Wild-Type (+/+), Heterozygous (+/−), and Homozygous (−/−) eNOS Mutant Mice Injected With Either Vehicle or L-NAME**

<table>
<thead>
<tr>
<th>Vehicle, cpm</th>
<th>L-NAME, cpm</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrulline $\cdot$ min$^{-1} \cdot$ mg protein$^{-1}$</td>
<td>Citrulline $\cdot$ min$^{-1} \cdot$ mg protein$^{-1}$</td>
<td>n</td>
</tr>
<tr>
<td>$+/+$</td>
<td>1530±70</td>
<td>3</td>
</tr>
<tr>
<td>$+/-$</td>
<td>1600±110</td>
<td>3</td>
</tr>
<tr>
<td>$-/-$</td>
<td>1630±110</td>
<td>3</td>
</tr>
</tbody>
</table>

*The L-NAME-treated group comprises the same mice as in Table 1.

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![Figure 3. Systolic BP (SBP) and HR in wild-type (+/+), heterozygous (+/−), and homozygous eNOS mutant mice (−/−) before chronic treatment with vehicle (●) or L-NAME (○). After 4 weeks of treatment, and 3 or more weeks after treatment. $*P<0.05$, $**P<0.001$ vs SBP before L-NAME.](http://hyper.ahajournals.org/)
thus, inhibition of its release should increase rather than decrease BP. The hypotensive effect of chronic L-NAME in −/− mice is unlikely to be related to iNOS inhibition for the following reasons: (1) iNOS usually is not expressed constitutively except in some tissues such as the lung and kidney; (2) iNOS is present in renal tubules, and NO in tubules inhibits sodium and water reabsorption, so that chronic inhibition of iNOS in the kidney would increase rather than decrease BP by sodium and water retention; (3) when iNOS is induced, it stimulates the release of very high amounts of NO, which in turn lower BP, while inhibition of iNOS increases BP; and (4) chronic administration of a selective iNOS inhibitor, aminoguanidine, reportedly had no effect on BP or renal hemodynamics in rats. Thus, it seems unlikely that blocking the release of NO by iNOS could explain the decreased BP observed in eNOS −/− mice after L-NAME treatment. One possibility is that NO released by nNOS in the CNS and/or baroreceptor pathways may increase sympathetic nerve activity (SNA) and cause BP to increase, whereas inhibition of NO may have the opposite effect. The immediate question, then, is why in eNOS +/+ or +/− mice 7-NI did not decrease BP but rather caused it to increase slightly. Perhaps 7-NI has a slight inhibitory effect on eNOS; therefore, in normal animals receiving 7-NI, the hypotensive effect of nNOS inhibition could be masked or even reversed by concomitant inhibition of eNOS. Alternatively, since eNOS −/− mice are hypertensive due to increased vascular resistance, it is possible that the hypotensive effect of 7-NI is more evident in hypertensive than normotensive mice. For this reason, we also set up a control group in which hypertension was produced by constriction of the renal artery; this reason, we also set up a control group in which hyper- tensive response to both L-NAME and 7-NI implies that the hypotension was not due to a direct effect of 7-NI on vascular smooth muscle cells.

In summary, acute and chronic L-NAME and acute 7-NI administration decreased BP in eNOS −/− mice and also inhibited cerebellar NOS activity. These findings suggest that NO derived from iNOS increases BP in both acute and chronic states, at least in eNOS −/− mice. Because NO causes vasodilatation independently of its origin, this effect of nNOS-derived NO may occur at the level of the CNS or baroreceptor pathways.

Acknowledgments

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