Maternal Endothelial Nitric Oxide Synthase Genotype Influences Offspring Blood Pressure and Activity in Mice

Bruce N. Van Vliet, Linda L. Chafe

Abstract—Deficiencies in maternal endothelial NO synthase (eNOS) have been associated with pregnancy complications, intrauterine growth retardation, and altered vascular function in offspring. In the present study, we investigated the influence of the maternal eNOS genotype on offspring’s blood pressure, heart rate, and locomotor activity. The effect of maternal eNOS genotype was made by comparing telemetered blood pressure and activity between 2 groups of 13- to 16-week-old male heterozygous eNOS knockout mice, 1 produced by a cross of eNOS knockout (eNOS+/−) mothers and wild-type (eNOS+/+) fathers (eNOS+/−-MAT offspring, N=11), the other by a cross of eNOS+/− mothers and eNOS−/− fathers (eNOS−/−-PAT offspring, N=10). Data were also collected for homozygous eNOS−/− and eNOS+/+ mice (N=15 each). Heterozygous eNOS knockout mice exhibited blood pressures that were intermediate to the eNOS+/− and eNOS−/− groups. Relative to eNOS+/−-PAT mice, eNOS+/−-MAT mice exhibited significant increases in nocturnal diastolic arterial pressure and diurnal variations (dark–light difference) in systolic, mean, and diastolic arterial pressure. In addition, indices of spontaneous nocturnal locomotor activity, including both the proportion of time spent active and the intensity of activity when active, were also significantly increased. Heart rate did not differ between the groups. Our results suggest that the maternal eNOS genotype influences both blood pressure and behavior of offspring, possibly as a consequence of developmental programming associated with intrauterine growth retardation. (Hypertension. 2007; 49:556-562.)

Key Words: experimental hypertension ■ pregnancy ■ developmental programming ■ maternal environment ■ hyperactivity ■ knockout mice ■ telemetry ■ nitric oxide

The characteristics of many physiological systems are influenced by the conditions prevailing during development.1 Perturbations of the maternal environment that lead to intrauterine growth retardation (IUGR) can have a marked impact on the grown offspring, including increased cardiovascular risk factors (eg, blood pressure and insulin resistance2,3) and reduced adult life span.3 A wide range of experimental interventions have been used to provoke developmental programming, including disturbances in maternal nutrition (eg, undernutrition and nutritional imbalances4), restriction of uterine blood flow,5 and the administration of drugs (eg, glucocorticoids5 and NO synthase inhibitors6,7).

A relatively unexplored possibility is that developmental programming could also occur as a consequence of maternal genotype. Maternal endothelial NO synthase (eNOS) is a potential candidate in this regard, because eNOS has been shown to play an important role in the regulation of uterine blood flow,8,9 and IUGR has been observed in rats treated with inhibitors of NO synthase6,7 and in mice with targeted deletion of the eNOS gene (eNOS−/−)8,9,10. In humans, polymorphisms of the eNOS gene are common and are associated with endothelial dysfunction,11–13 increased uterine artery resistance,14 and poor pregnancy outcomes.14–16 A recent study has provided direct evidence of developmental programming induced by a maternal eNOS genotype.17 In that study, mice produced by a cross of eNOS−/− mothers and wild-type fathers (eNOS−/−-MAT offspring) exhibited functional and structural changes in the carotid and mesenteric arteries that were significantly greater than those of genetically identical mice produced by a cross of wild-type mothers and eNOS−/− fathers (eNOS−/−-PAT offspring). Acetylcholine-induced relaxation and calcium-induced contractions were severely affected in the offspring of eNOS−/− mothers (eNOS−/−-MAT and eNOS−/− mice), whereas the offspring of wild-type mothers (eNOS−/−-PAT and wild-type mice) were largely unaffected.17 The effects of maternal eNOS genotype on other aspects of cardiovascular function, such as blood pressure, have not been reported.

The main objective of the present study was to test the hypothesis that the maternal eNOS genotype influences the blood pressure of grown offspring. Our approach was to use telemetry to compare the blood pressure phenotype of undisturbed eNOS+/−-MAT and eNOS+/−-PAT mice, genetically identical mice that differ only with respect to the eNOS genotype of their mother and father. According to our hypothesis, an effect of the maternal eNOS genotype would be manifest as...
a higher level of blood pressure in eNOS\textsuperscript{+/-}\textsubscript{MAT} mice relative to that of eNOS\textsuperscript{+/-}\textsubscript{PAT} mice. To put our results into perspective, we also report results for homozygous wild-type (eNOS\textsuperscript{+/-}) and eNOS\textsuperscript{-/-} mice.

Methods

General
Mice were maintained on a 12-hour light cycle and fed a Prolab RMH 3000 rodent chow (0.26% Na\textsuperscript{+}, 0.91% K\textsuperscript{+}, 22.5% protein, and 6.4% fat). Experiments were approved by the Memorial University Animal Care Committee.

Breeding
Mice were bred using colony founders obtained from Jackson Laboratories, where the eNOS\textsuperscript{+/-} strain\textsuperscript{19} had been backcrossed to the C57BL/6j background. Females were bred starting at 8 weeks of age, and offspring produced in their first 2 litters were used. eNOS\textsuperscript{+/-} mice were generated by crossing eNOS\textsuperscript{+/-} dams and eNOS\textsuperscript{+/-} sires (eNOS\textsuperscript{+/-}\textsubscript{MAT} offspring) or eNOS\textsuperscript{-/-} dams and eNOS\textsuperscript{-/-} sires (eNOS\textsuperscript{-/-}\textsubscript{PAT} offspring). eNOS\textsuperscript{+/-} and eNOS\textsuperscript{-/-} strains were propagated by mating homozygotes.

Experimental Protocol
Mice were weaned at 4 weeks, moved to the recording room (21–22°C) at 6 weeks, housed individually at 9 to 12 weeks, and underwent implantation of a telemeter (D.S.I. model TA11PA-C20)\textsuperscript{13} under ketamine–xylazine anesthesia (90 and 10 mg/kg) at 11 to 14 weeks (day 0). Mice were returned to the recording room within 48 hours of telemeter implantation. The 24-hour data set to be used for analysis was recorded starting on the morning of day 11 after implantation surgery. To assess the contribution of eNOS to the resting blood pressure in the 4 groups, on day 12, blood pressure was recorded while mice were treated with 5% dextrose (2 ml/kg IP) followed 2 hours later by Na\textsubscript{+}-nitro-l-arginine methyl ester hydrochloride (l-NNAME; 50 mg/kg IP, in 5% dextrose, 2 ml/kg volume). The response 15 minutes after vehicle administration was used as an index of the blood pressure response to handling and disturbance. The effects of l-NNAME and vehicle were assessed 90 minutes after administration, because the increase in activity and blood pressure caused by drug administration requires 60 to 90 minutes to dissipate.\textsuperscript{19} On day 14, the telemeter was removed under deep anesthesia and recalibrated.

Analysis of Telemetry Data
Telemetry data were sampled at 500 Hz in 3-s bursts at 30-s intervals using a DSI DataQuest ART Silver 2.1 acquisition system (Data Sciences). In-house routines (http://www.med.mun.ca/basic/pages/faculty/vanvliet.htm) were used to analyze 24-hour data sets as described previously\textsuperscript{19–22} with the following exception. Because raw activity values from this telemetry system are skewed toward high values, logarithmic transformation of activity data is required.\textsuperscript{19}

Therefore, in addition to raw activity reported by the telemetry system, we also report a total activity index calculated as the mean log(activity+1). The addition of 1 to activity values before applying the logarithm allows the calculation to be applied to all of the activity values (ie, including activity=0). To discern the intensity of activity when active, we also report an activity intensity index calculated as the mean log(activity+1) for activity values >0. Finally, we also report active time, the proportion of samples with raw activity counts >0.

Statistics and Analysis
Data are expressed as the mean±SE. The principal hypothesis of the study (the influence of maternal eNOS genotype) was evaluated by comparison of data from eNOS\textsuperscript{+/-}\textsubscript{MAT} and eNOS\textsuperscript{-/-}\textsubscript{PAT} mice using an unpaired 2 tailed Student’s t test. To put our results into broader perspective, we also report data for eNOS\textsuperscript{+/-} and eNOS\textsuperscript{-/-} mice and include the results of a 1-way ANOVA followed by Tukey’s multiple comparison test (family error rate=5%) in Table I of the data supplement (available online at http://hyper.ahajournals.org). REGRESSIONS were performed using the least-squares method. 

Results

General Characteristics
Litter size and body weight data are provided in the Table. The number of pups weaned from each litter was significantly reduced in eNOS\textsuperscript{+/-}\textsubscript{MAT} relative to eNOS\textsuperscript{-/-}\textsubscript{PAT} mice. Small litter size was also characteristic of eNOS\textsuperscript{-/-} mice. Although the body weights of eNOS\textsuperscript{+/-}\textsubscript{MAT} and eNOS\textsuperscript{-/-}\textsubscript{PAT} mice did not differ, body weight was significantly reduced in eNOS\textsuperscript{-/-} mice.

Hemodynamics
The blood pressure level of eNOS\textsuperscript{+/-} mice was intermediate to that of the control and eNOS\textsuperscript{-/-} groups (Figure 1 and Table I). Relative to eNOS\textsuperscript{-/-}\textsubscript{PAT} mice, the blood pressure level of eNOS\textsuperscript{+/-}\textsubscript{MAT} tended to be elevated, an effect that reached statistical significance in the case of the nocturnal diastolic arterial pressure level (+4.6 mm Hg). The day–night difference of systolic arterial pressure, mean arterial pressure, and diastolic arterial pressure was also significantly elevated in the eNOS\textsuperscript{-/-}\textsubscript{MAT} group relative to the eNOS\textsuperscript{-/-}\textsubscript{PAT} group (Figure 1 and Table I). The increased day–night difference appeared to be predominantly because of the elevation of nocturnal pressure levels (Figure 2). Although heart rate was similar among all 4 of the groups, a tendency for the day–night difference in heart rate to be slightly elevated in eNOS\textsuperscript{-/-}\textsubscript{MAT} mice reached borderline statistical significance (P=0.05).

Locomotor Activity Signal
eNOS\textsuperscript{-/-}\textsubscript{MAT} exhibited increased spontaneous nocturnal locomotor activity relative to eNOS\textsuperscript{-/-}\textsubscript{PAT} mice. This difference was evident for the overall average level of activity (raw

<table>
<thead>
<tr>
<th>Characteristic of the 4 Mouse Groups Used in the Study</th>
<th>eNOS\textsuperscript{+/-} (n=15) (a)</th>
<th>eNOS\textsuperscript{-/-}\textsubscript{PAT} (n=10) (b)</th>
<th>eNOS\textsuperscript{-/-}\textsubscript{MAT} (n=11) (c)</th>
<th>eNOS\textsuperscript{-/-} (n=15) (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal genotype</td>
<td>eNOS\textsuperscript{+/-}</td>
<td>eNOS\textsuperscript{-/-}</td>
<td>eNOS\textsuperscript{-/-}</td>
<td>eNOS\textsuperscript{-/-}</td>
</tr>
<tr>
<td>Paternal genotype</td>
<td>eNOS\textsuperscript{+/-}</td>
<td>eNOS\textsuperscript{-/-}</td>
<td>eNOS\textsuperscript{-/-}</td>
<td>eNOS\textsuperscript{-/-}</td>
</tr>
<tr>
<td>No. of pups weaned from litter</td>
<td>7.9±0.5 (c d)</td>
<td>8.4±0.6 (c d)</td>
<td>5.5±0.7* (a b)</td>
<td>4.9±0.3 (a b)</td>
</tr>
<tr>
<td>Weight at weaning (3 wk of age, g)</td>
<td>19.8±0.4 (d)</td>
<td>19.5±0.5 (d)</td>
<td>18.2±0.6</td>
<td>16.8±0.5 (a b)</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>26.1±0.3 (d)</td>
<td>24.9±0.5</td>
<td>25.8±0.3 (d)</td>
<td>23.4±0.5 (a c)</td>
</tr>
</tbody>
</table>

[a–d] indicates a significant difference from groups a to d.

*P<0.05 for unpaired t test between eNOS\textsuperscript{-/-}\textsubscript{MAT} and eNOS\textsuperscript{-/-}\textsubscript{PAT} groups.
activity and total activity index), the proportion of time spent active (active time), and the average level of activity when active (activity intensity index; Figure 3 and Table I). The increased activity appeared to be broadly distributed throughout the dark period (Figure 4). Although nocturnal locomotor activity also tended to be elevated in eNOS<sup>−/−</sup> mice, this tendency did not reach statistical significance (Table I).

**Responses to l-NAME and Vehicle**

Injection of vehicle (1 mL/kg<sup>−1</sup> 5% dextrose, IP) in mice resting in their home cages elicited a prompt increase in mean arterial pressure, pulse pressure, heart rate, and activity that dissipated over 60 to 90 minutes (as described previously in detail<sup>19</sup>). We assessed the change in mean arterial pressure 15 minutes after injection of the vehicle as an index of the response to handling and disturbance. However, there were no significant differences between the responses of the eNOS<sup>−/−</sup>/MAT and eNOS<sup>−/−</sup>/PAT groups or in multiple comparisons among the 4 groups (eNOS<sup>−/−</sup>/: +18.6±2.4 mm Hg; eNOS<sup>−/−</sup>/PAT: +24.2±4.4 mm Hg; eNOS<sup>−/−</sup>/MAT: 20.8±4.2 mm Hg; and eNOS<sup>−/−</sup>/: 28.0±9.8 mm Hg).

Administration of the NOS inhibitor l-NAME elicited an increase in mean arterial pressure and pulse pressure and decreases in heart rate that were well sustained after 90 minutes in eNOS<sup>−/−</sup>, eNOS<sup>−/−</sup>/MAT, and eNOS<sup>−/−</sup>/PAT mice but not eNOS<sup>−/−</sup> mice (Figure 5). The responses of eNOS<sup>−/−</sup>/MAT and eNOS<sup>−/−</sup>/PAT groups were not statistically different.
Discussion

In the present study, we tested the hypothesis that the maternal eNOS genotype would have a significant influence on the blood pressure of grown offspring. To address this question, we compared eNOS$^{-/-,Pat}$ and eNOS$^{-/-,Mat}$ mice, mice that are genetically identical but nevertheless differ with respect to the eNOS genotype of their parents. To facilitate detection of even small differences between the 2 groups, we used telemetry to obtain a detailed assessment of 24-hour blood pressure data in undisturbed mice$^{19–22}$ and used a sample size of $\geq10$ animals per group. Our data revealed a significant elevation of the nocturnal diastolic arterial pressure level of the eNOS$^{-/-,Mat}$ group relative to the eNOS$^{-/-,Pat}$ group. Because the pressor response to L-NAME did not differ between the eNOS$^{-/-,Mat}$ and eNOS$^{-/-,Pat}$ groups, our results are consistent with the differences in their blood pressures being secondary to the IUGR that has been described in the offspring of eNOS$^{-/-}$ mothers$^{8,10}$ and not a reduced activity of eNOS within the vascular system of eNOS$^{-/-,Mat}$ mice as a consequence of genetic imprinting or other factors (Figure 5). These results provide a novel illustration of the influence that maternal genotype can have on offspring blood pressure independent of the offspring’s genotype. Although our data are based on mothers in which the eNOS gene was completely nonfunctional, they may be relevant to humans in which polymorphisms of the human eNOS gene are known to be common and have been associated with endothelial dysfunction, increased uterine artery resistance, and poor pregnancy outcomes. Thus, our results raise the possibility that a similar influence of maternal genotype could occur among offspring born to mothers with polymorphisms of the human eNOS gene.

The influence of maternal eNOS genotype amounted to a 4.5-mm Hg elevation in the nocturnal diastolic arterial pressure level in eNOS$^{-/-,Mat}$ mice, relative to eNOS$^{-/-,Pat}$ mice. Although a larger effect might occur under other circumstances, we were unable to find any studies of the effect of developmental programming on blood pressure in mice. In rat models of IUGR, increases in offspring blood pressure have frequently been reported including, in some cases, large increases in tail cuff blood pressure in rats of either gender at ages as young as 4 weeks in the absence of any additional experimental treatments.$^{23,24}$ More modest and less consistent effects of IUGR on blood pressure have been reported in studies in which blood pressure was assessed by long-term recordings in undisturbed rats by telemetry (eg, no effect in a model of unilateral uterine artery ligation$^{25}$; $+4$ mm Hg systolic pressure in offspring of mothers undergoing protein

![Figure 3. Effects of eNOS genotype on overall activity (total activity index), intensity of activity when active (activity intensity index), and the proportion of time spent active (active time) during the 12-hour dark period (left side) and 12-hour light period (right side). Open symbols represent the data of individual mice. Group means and SEs are represented by filled symbols and error bars. Mean values for control (++) and homozygous knockout (−−) groups are indicated by an interrupted line except in cases in which the values are similar or it would obscure data. *$P<0.05$ (t test) between eNOS$^{-/-,Pat}$ and eNOS$^{-/-,Mat}$ groups. Statistical outcomes for multiple between-group comparisons are given in Table I. N = 10 to 15 per group.](http://hyper.ahajournals.org/issue/559/11/VanVlietAndChafeMaternaleNOSGenotypeAffectsOffspringBP)
restriction during pregnancy; an 8-, 6-, and 4-mm Hg increase in nocturnal systolic, mean, and pulse pressures, respectively, in a model of bilateral uterine artery ligation; and a 10- and 14-mm Hg increase in diastolic and systolic pressures in offspring of mothers fed a high-fat diet during pregnancy). The available data suggest that, when telemetry methods are used to minimize potential stress-induced artifacts, IUGR is associated with a statistically significant though modest impact on offspring blood pressure.

Tonkiss et al observed an increased blood pressure response to stress in IUGR rats and suggested that the large and frequently positive findings obtained with tail cuff measurements could, to some extent, reflect an effect of programming to increase the blood pressure response to stress. A tendency toward an increased blood pressure response to stress was also apparent in 1 other study of IUGR rats but absent in another. Although we did not observe any effect of maternal eNOS genotype on the blood pressure response to handling and disturbance, we did observe an effect of maternal eNOS genotype on other aspects of behavior. During the dark phase, the proportion of time spent active and the intensity of activity were clearly increased in eNOS+/−MAT mice relative to eNOS++/−MAT (Figures 3 and 4). This effect on nocturnal activity may have contributed to the increased night–day difference in blood pressures that we also observed in eNOS+/−MAT mice. A tendency toward increased activity was also evident in eNOS−/− mice of the present (Table I) and a previous study but did not reach statistical significance in either case. An effect of developmental programming on spontaneous locomotor activity was also described in the rat model of IUGR induced by low maternal protein intake by Tonkiss et al. Although reductions in the spontaneous locomotor activity have also been reported in a rat model of developmental programming (induced by a maternal high-fat diet), this was not a consistent observation, being present in only 1 of 6 groups investigated. Other reports of reduced activity associated with IUGR have been based on the use of a specialized apparatus in which subjects are placed for short periods of testing. Such tests are likely to evaluate a different aspect of behavior than that provided by long-term telemetric monitoring of spontaneous activity from undisturbed animals in their usual cage and room.

We also observed a significant effect of the maternal eNOS genotype on reproductive productivity. The litter size at weaning was significantly reduced in eNOS+/−MAT mice relative to eNOS++/−MAT mice. A similar effect was evident in
eNOS\textsuperscript{+/−} mice and has been described by others.\textsuperscript{8,17,18} The litter size data reported in the Table actually overestimate the productivity of eNOS\textsuperscript{+/−} mothers, because only successful pregnancies producing the pups used in the present study are included. In our broader experience breeding these animals, eNOS\textsuperscript{+/−} mothers frequently had pregnancies in which all of the pups were lost or destroyed (46% of litters [23 of 50] of pregnancy) amounted to 2.1 loss\textsuperscript{15} and abrupto placentae\textsuperscript{16} that have been associated with correspond with the increased risk of recurrent pregnancy loss\textsuperscript{11006} for wild-type pups). These pregnancy losses may include. In our broader experience breeding these animals, those born of eNOS-deficient mothers (pups weaned per pregnancy) amounted to 2.1±0.3 for eNOS\textsuperscript{+/−} pups and 3.1±0.5 for eNOS\textsuperscript{+/+\textsuperscript{MAT}} pups. Results for wild-type mothers were considerably higher (5.3±0.8 for eNOS\textsuperscript{+/−\textsuperscript{PAT}} pups and 5.6±0.5 for wild-type pups). These pregnancy losses may correspond with the increased risk of recurrent pregnancy loss\textsuperscript{15} and abrupto placenta\textsuperscript{16} that have been associated with polymorphisms of the human eNOS gene.

**Perspectives**

Disturbances of the maternal environment that result in UGR can alter the programming of cardiovascular structures and function in grown offspring. Because eNOS plays an important role in uterine blood flow regulation and eNOS deficiency has been associated with UGR, we investigated the potential impact of the maternal eNOS genotype on blood pressure of offspring. Our results demonstrate that, among genetically identical mice, those born of eNOS-deficient mothers exhibited increased blood pressure and spontaneous locomotor activity. These findings are a novel illustration of the influence that maternal genotype can have on blood pressure and behavior of grown offspring independent of the offspring’s genotype. Although our results are based on the use of mothers in which the eNOS gene was completely nonfunctional, polymorphisms of the human eNOS gene are common and have been associated with endothelial dysfunction, increased uterine arterial resistance, and poor pregnancy outcomes. Our findings, therefore, raise the possibility that maternal eNOS genotype could also influence the blood pressure and/or behavior of grown offspring in humans.

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**Disclosures**

None.

**References**


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