Hypertensive disorders in pre-term pregnancy: management and long-term consequences
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Enhanced growth and improved vascular function in offspring from successive pregnancies in endothelial nitric oxide synthase knockout mice

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Gary D.V. Hankins
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Abstract

Objective: Transgenic mice that lack endothelial nitric oxide synthase have offspring with growth deficiency and abnormal vascular reactivity in later life. Our objective was to evaluate the role of parity in the modulation of the fetal programming of growth and vascular responses in these transgenic mice.

Study design: Oligoparous (0-2 previous pregnancies) and multiparous (5-9 previous pregnancies) nitric oxide synthase knockout (−/−KO) female mice were bred with nitric oxide synthase−/−KO and wild type (+/+WT) male mice to produce nitric oxide synthase−/−KO and maternally derived heterozygous (+/−Mat) litters. The pups were weighed weekly. Carotid arteries of the adult females from these litters were used for in vitro vascular reactivity studies.

Results: Nitric oxide synthase knockout and nitric oxide synthase maternal litters that were born to oligoparous mothers had significant growth lag compared with corresponding multiparous litters. Length-tension characteristics were not different between the groups. However, optimal diameter, which is a measure of vascular tensile properties and resistance, was decreased in oligoparous compared with multiparous female offspring. Acetylcholine-mediated vasorelaxation was abolished, and contraction by phenylephrine and Ca++ was increased in oligoparous, but not multiparous, female offspring (P < .05).

Conclusion: These data support the role of abnormal uterine environment in the fetal programming of postnatal growth and vascular function in later life. Successive pregnancies may lead to maternal uterine adaptations that bypass the lack of a functional nitric oxide synthase, which leads to improvement in postnatal growth and vascular function in the offspring. Given the reported effect of parity on the risk of preeclampsia, similar mechanisms may be operative in human pregnancy.
**Introduction**

As hypothesized by Barker and Osmund,\(^1\) stimuli or insults to the fetus during the critical period of intrauterine development lead to “fetal programming” and produce adaptive changes in fetal anatomy, physiology, and metabolism that have long-term consequences.

Maternal hemodynamic changes during pregnancy influence fetal growth through their impact on uteroplacental perfusion.\(^3\) These changes, with onset in early pregnancy, contribute to the increase in the uteroplacental blood flow.\(^4\) There is growth of new vessels and remodeling of existing vessels; vascular refractoriness to vasoconstrictor agents and increased production of vasodilators include nitric oxide.\(^5,6\)

Defects in the nitric oxide pathway have been implicated in the causation of various vascular pathologic states that include hypertension and intrauterine growth restriction.\(^7\) Three nitric oxide synthase (NOS) isoforms (ie, neuronal NOS [NOS1], inducible NOS [NOS2], and endothelial NOS [NOS3]) have been described.\(^8\) In the vasculature, NOS3 is the main isoform that is responsible for the production of nitric oxide.\(^8\) Infusion of NOS inhibitors during pregnancy causes hypertension and fetal growth restriction.\(^9,10\) Nitric oxide plays a significant role in maintaining adequate uteroplacental perfusion.\(^7\) NOS3-knockout mice are hypertensive and lack endothelium-dependent vasodilation.\(^11,12\)

We have shown previously that female offspring of NOS3-knockout female mice are growth restricted and have abnormal vascular function during their adult life, as compared with the genetically identical offspring of wild-type female mice.\(^13,14\)

The results in this animal model of genetically induced abnormal uteroplacental perfusion indicate a fetal programming effect of the abnormal uterine environment, with impact on postnatal growth and future vascular reactivity in the offspring.\(^13,14\) Compromised uteroplacental blood flow is a common denominator in hypertensive disorders during pregnancy, especially preeclampsia.\(^6,15\) Indeed, there is a strong relationship between preeclampsia and fetal growth restriction.\(^16\) In addition, preeclampsia occurs more frequently in nulliparous women (3%-7%) than in multiparous women (0.8%-5%).\(^17\) Thus, we hypothesized that, with sequential pregnancies in the same animal, adaptive changes occur in the maternal/uterine environment that result in amelioration of the fetal programming effects in offspring of successive gestations.
Material and methods

Animals
Female mice that are homozygous for the disruption of the endothelial NOS gene (NOS3<sup>−/−</sup> KO strain: C57BL/6J-NOS3<sup>−/−</sup>) and their age matched wild-type controls (NOS3<sup>+/+</sup> WT strain: C57BL/6J-NOS3<sup>+/+</sup>) were obtained from Jackson Laboratory (Bar Harbor, Me). The animals were housed separately in temperature- and humidity-controlled quarters with constant light/dark cycles of 12 hours/12 hours and were provided with food and water ad libitum.

Oligoparous (0-2 previous pregnancies) and multiparous (5-9 previous pregnancies) NOS3<sup>−/−</sup> KO female mice were mated with NOS3<sup>−/−</sup> KO and NOS3<sup>+/+</sup> WT male mice to produce 4 types of litters: oligoparous-NOS3<sup>−/−</sup> KO, oligoparous-NOS3<sup>+/−</sup> Mat, multiparous-NOS3<sup>−/−</sup> KO, and multiparous-NOS3<sup>+/−</sup> Mat (superscript Mat indicates maternal source of the nonfunctional NOS3 allele). In the oligoparous-NOS3<sup>+/−</sup> Mat and oligoparous-NOS3<sup>−/−</sup> KO litters, the pups matured in an oligoparous mother; the multiparous-NOS3<sup>+/−</sup> Mat and multiparous-NOS3<sup>−/−</sup> KO pups matured in a multiparous mother.

After delivery, female pups from the 4 groups were weighed weekly until early adulthood (week 6). Mature cycling female mice (7-8 weeks old) from the 4 litters were used for in vitro vascular reactivity experiments. The animals were killed by carbon dioxide inhalation. All procedures were approved by the Animal Care and Use Committee of the University of Texas Medical Branch.

In vitro experiments
Two-millimeter segments of carotid artery from the mice were dissected and mounted over 25-μm tungsten wires in a myograph (model 410A; J.P. Trading I/S, Aarhus, Denmark). The preparations were bathed in physiologic salt solution that was maintained at 37°C (pH approximately 7.4), and a mixture of 95% oxygen/5% carbon dioxide was bubbled continuously through the solution. Force was recorded continuously by an isometric force transducer and analyzed with Windaq software (Dataq Instruments, Akron, Ohio). With Myosight software (J.P. Trading I/S), length-tension relationship between internal circumference and wall tension (length-tension curve) was determined to characterize the viscoelastic properties of vascular rings. The slope that was obtained from the length-tension curve is a measure of elasticity of the vessel wall; the greater the slope, the more rigid the vessel wall. Optimal diameter of the vessels was calculated with the Laplace equation. In addition to being dependent on vessel elasticity, the optimal diameter of the vessels is a measure of the vascular diameter at a specific transmural pressure and therefore an estimate of vascular resistance. The rings
were contracted twice with 60 mmol/L KCl to stabilize vascular responsiveness. The second KCl response was used as the reference contraction for data analysis. Contractile responses to α-adrenergic agonist phenylephrine (10⁻⁹–10⁻⁵ mol/L) were assessed. In addition, relaxant responses to endothelium-dependent relaxant acetylcholine (10⁻⁹–10⁻⁵ mol/L) were examined in vessels that were precontracted with phenylephrine (10⁻⁷–10⁻⁶ mol/L to match amplitude of contractions in the different groups). Finally, and after equilibration of the vessels in high-K⁺ Ca⁺⁺-free solution, contractile responses to cumulative concentrations of Ca⁺⁺ (0.05-5 mmol/L) were studied to evaluate responsiveness of the vascular smooth muscle.

**Data analysis**

Data are expressed as mean ± SEM. For the experiments that examined vascular elastic properties, data are expressed as the length-tension slope and optimal diameter of the vessels. For the vascular reactivity studies, the concentration-response curves to the agents that were tested were constructed. In addition, the logarithm of the concentration that produced 50% of the maximal effect (log IC₅₀) and the maximal effect were also compared. The Kolmogorov-Smirnov test was used to check for normality of data; 1-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparisons test were used for statistical analysis. A probability value of <.05 was considered significant.

**Drugs and solutions**

The drugs that were used in the in vitro experiments were acetylcholine hydrochloride and phenylephrine hydrochloride (Sigma Chemical Company, St Louis, Mo). Stock solutions of the drugs (10⁻² mol/L) were prepared in deionized water and stored at −20°C. The following composition of physiologic salt solution was used: NaCl, 119 mmol/L; KCl, 4.7 mmol/L; NaH₂PO₄, 1.2 mmol/L; NaHCO₃, 25 mmol/L; MgCl₂, 1.2 mmol/L; CaCl₂, 2.5 mmol/L; ethylenediaminetetraacetic acid, 0.026 mmol/L; and glucose, 11.5 mmol/L. In experiments that used high-K⁺ Ca⁺⁺-free physiologic salt solution (80 mmol/L K⁺), Ca⁺⁺ was omitted from the physiologic salt solution, and K⁺ replaced NaCl to maintain the solution iso-osmotic.
Results

Postnatal weight gain

The weight of female pups from the oligoparous female mice (ie, oligoparous-NOS3\(^{-/-}\)KO and oligoparous-NOS3\(^{+/−}\)Mat mice) was significantly less than female pups of the multiparous female mice (ie, multiparous-NOS3\(^{-/-}\)KO and multiparous-NOS3\(^{+/−}\)Mat mice) in the first and last 2 weeks (Figure 1).

Passive characteristics of carotid artery

The length-tension curves were not significantly different among the 4 groups (Figure 2). However, the optimal diameter of the vessels was significantly smaller in the oligoparous-NOS3\(^{-/-}\)KO and oligoparous-NOS3\(^{+/−}\)Mat mice, compared...
with multiparous-NOS3\(^{-/-}\)KO and multiparous-NOS3\(^{+/−}\)Mat mice (Figure 3), which indicates a possible increase in the vascular smooth muscle that could lead to the increased vascular resistance in the oligoparous offspring compared with the multiparous offspring.

**In vitro reactivity of carotid artery**

Phenylephrine contraction was increased significantly in oligoparous-NOS3\(^{-/-}\)KO and oligoparous-NOS3\(^{+/−}\)Mat mice, compared with multiparous-NOS3\(^{-/-}\)KO and multiparous-NOS3\(^{+/−}\)Mat mice (\(P < .05\); Figure 4, Table 1). However, the IC\(_{50}\) was significantly different only in the oligoparous-NOS3\(^{-/-}\)KO mice, compared with oligoparous-NOS3\(^{+/−}\)Mat, multiparous-NOS3\(^{-/-}\)KO, and multiparous-NOS3\(^{+/−}\)Mat mice.

Acetylcholine produced vasorelaxation in a dose-dependent manner in multiparous-NOS3\(^{-/-}\)KO and multiparous-NOS3\(^{+/−}\)Mat mice. In contrast,
acetylcholine produced a small contractile effect in the oligoparous-NOS3$^{-/-}$KO and oligoparous-NOS3$^{+/+}$Mat mice (Figure 5, Table II)

The responses to the cumulative concentration of Ca$^{++}$ were significantly higher in oligoparous-NOS3$^{-/-}$KO and oligoparous-NOS3$^{+/+}$Mat mice, compared with

Table I. Area under the phenylephrine concentration-response curves (AUC; arbitrary units), logarithm of the molar concentration that produces log IC$_{50}$ and maximal effect (expressed as percentage of reference contraction to 60 mmol/L KCl) in the carotid arteries of female homozygous NOS3$^{-/-}$KO and NOS3$^{+/+}$Mat offspring of oligoparous and multiparous NOS3$^{-/-}$KO female mice (n=8-10 mice in each group)

<table>
<thead>
<tr>
<th></th>
<th>AUC</th>
<th>Log IC$_{50}$</th>
<th>Maximal effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligoparous-NOS3$^{-/-}$KO</td>
<td>436.93 ± 33.64*</td>
<td>-8.1 ± 0.4‡</td>
<td>178.39 ± 8.6*</td>
</tr>
<tr>
<td>Oligoparous-NOS3$^{+/-}$Mat</td>
<td>521.61 ± 51.60*</td>
<td>-6.9 ± 1.3</td>
<td>210.78 ± 21.3*</td>
</tr>
<tr>
<td>Multiparous-NOS3$^{-/-}$KO</td>
<td>173.18 ± 16.65</td>
<td>-6.7 ± 0.07</td>
<td>92.0 ± 8.8</td>
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<tr>
<td>Multiparous-NOS3$^{+/-}$Mat</td>
<td>216.57 ± 19.93</td>
<td>-6.8 ± 0.07</td>
<td>111.0 ± 6.6</td>
</tr>
</tbody>
</table>

*P < .05 for oligoparous-NOS3$^{-/-}$KO and oligoparous-NOS3$^{+/-}$Mat mice versus multiparous-NOS3$^{-/-}$KO and multiparous-NOS3$^{+/-}$Mat mice by 1-way ANOVA and Newman-Keuls test.

‡P < .05 oligoparous-NOS3$^{-/-}$KO versus oligoparous-NOS3$^{+/-}$Mat mice, multiparous-NOS3$^{-/-}$KO mice, and multiparous-NOS3$^{+/-}$Mat mice by 1-way ANOVA and Newman-Keuls test.

Table II. Area under the acetylcholine concentration-response curves (AUC; arbitrary units) and maximal effect (expressed as percentage relaxation of phenylephrine contraction) in the carotid arteries of female NOS3$^{-/-}$KO and NOS3$^{+/-}$Mat offspring of oligoparous and multiparous NOS3$^{-/-}$KO female mice (n = 8-10 mice in each group)

<table>
<thead>
<tr>
<th></th>
<th>AUC</th>
<th>Log IC$_{50}$</th>
<th>Maximal effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligoparous-NOS3$^{-/-}$KO</td>
<td>64.39 ± 11.36*</td>
<td>NA</td>
<td>-12.5 ± 6.39*</td>
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<tr>
<td>Oligoparous-NOS3$^{+/-}$Mat</td>
<td>90.30 ± 17.46*</td>
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<td>-16.3 ± 9.9*</td>
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<tr>
<td>Multiparous-NOS3$^{-/-}$KO</td>
<td>239.32 ± 27.33</td>
<td>NA</td>
<td>68.7 ± 7.0</td>
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<tr>
<td>Multiparous-NOS3$^{+/-}$Mat</td>
<td>309.0 ± 27.33</td>
<td>NA</td>
<td>83.2 ± 3.3</td>
</tr>
</tbody>
</table>

NA, Not applicable. *P < .05 for oligoparous-NOS3$^{-/-}$KO and oligoparous-NOS3$^{+/-}$Mat mice versus multiparous-NOS3$^{-/-}$KO and multiparous-NOS3$^{+/-}$Mat mice by 1-way ANOVA and Newman-Keuls test.
These results show greater sensitivity to Ca ++ and/or greater contractile potential in oligoparous offspring, compared with multiparous offspring.

Comment

Our results support the hypothesis that cumulative adaptive changes occur in the uterine environment during successive gestations that may serve to counterbalance an inherent vascular perfusion defect, which improves fetal growth and future vascular function in the offspring. In humans, this is seen as a decrease in the incidence of preeclampsia and as a trend for greater birth weights in successive pregnancies. In this animal model with a genetic defect in vascular function, the fetal programming effect that is evident in the offspring of oligoparous NOS3−/−KO mice and that manifests as a delay in postnatal growth
and abnormalities in vascular function during adulthood was not seen in the genetically similar offspring of multiparous NOS3−/−KO mice.

Programming is defined as a permanent response to an insult or stimulus that is experienced at a critical period of fetal development, during which organogenesis and differentiation take place. During these periods of rapid growth and maturation, the fetus is vulnerable to variations in nutrient or oxygen supply and other perturbations that occur in the uterine environment. The immediate response of the fetus is to survive using its own substrate, which leads to a slowing of fetal growth. As such, when the fetal programming occurs during the defined critical periods of development, it may result in long-term or permanent changes to organ morphology and physiology that can lead to permanent consequences later in life. Low birth weight is known to be associated with an increased risk of the development of cardiovascular disease, coronary artery disease, and diabetes mellitus in adulthood.

The results from this study confirm our previous findings that, in the absence of maternal NOS3 expression, the abnormal uterine environment results in fetal programming, with delay in postnatal growth. Interestingly, this occurred in the offspring of the oligoparous, but not the multiparous, female mice, which suggests that the fetal programming effects of the abnormal uterine environment in mice that lacks functional NOS3 diminish with successive pregnancies. Similarly, we have demonstrated an increased response to contractile agents and the absence of endothelium-dependent vasodilation that are associated with decreased vascular distensibility in offspring of oligoparous mothers compared with offspring of multiparous mothers. It is unlikely that advancing maternal age may be responsible for the noted effects, because age has a negative rather than a positive effect on reproduction. These observations support a cumulative adaptive change in the uterine environment, with the deleterious effects diminishing with successive pregnancies, which lead to an improvement in the vascular function of the successive offspring in their later life. In the absence of NOS3 expression in the maternal/uterine environment, alternate pathways that may include the other NOS isoforms and other vasodilators, such as prostacyclin or endothelium-derived hyperpolarizing factor, may serve to compensate for the decreased nitric oxide production in the maternal vasculature. These changes may lead to an improvement in uteroplacental perfusion, which prevents the abnormalities in postnatal growth and future vascular function that are seen in the first pregnancies. Pregnancy-induced hypertensive disorders (which include preeclampsia) are known to occur primarily in first pregnancies, with a risk of recurrence of 47% in the second pregnancy and 21% overall. The beneficial effects of a previous pregnancy also have been explained by the vascular remodeling in the spiral arteries that occur early in pregnancy and can lead to increased uteroplacental
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perfusion with increasing parity. In addition, pregnancy-induced increase in cardiac output and a decrease in peripheral vascular resistance is greater in multiparous women. The adaptations that occur during the first pregnancy may persist in the following pregnancies and may lower the risk of cardiovascular disease, which includes pregnancy-induced hypertension or preeclampsia in the mother. Further studies are needed to examine maternal cardiovascular and uteroplacental function during successive gestations in this animal model. Despite several epidemiologic studies that have pointed to an association between fetal programming and the onset of cardiovascular disease in later life, few studies have been conducted into the long-term consequences of hypertensive disorders during pregnancy because of the intrinsic limitations of long-term retrospective and prospective studies. Our data raise the possibility that, in women with poor obstetric history, multiparity may reduce the associated risk of fetal programming, thereby improving fetal and postnatal growth and future vascular function of the offspring.

In conclusion, multiparity appears to circumvent the adverse effects of abnormal maternal vascular function on fetal programming, as evidenced by improved postnatal growth and future vascular function in the offspring of mice that lack a functional endothelial NOS. Further comparative studies are needed in offspring from human pregnancies that are associated with preeclampsia or intrauterine growth restriction and subsequent pregnancies to confirm the clinical relevance of our findings.
References


