Enhanced Vascular Reactivity During Inhibition of Nitric Oxide Synthesis in Pregnant Rats

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Abstract—Pregnancy-induced hypertension has been suggested to be mediated by several mechanisms, including reduced nitric oxide (NO) synthesis. In this study, the effects of chronic treatment with the NO synthase inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) on blood pressure and the underlying changes in vascular reactivity were investigated in virgin and late-pregnancy Sprague-Dawley rats. The systolic blood pressure was 120±2, 124±5, 116±4, and 171±5 mm Hg in untreated virgin, virgin treated with L-NAME, untreated pregnant, and pregnant treated with L-NAME rats, respectively. The rats were killed, and the thoracic aorta was cut into strips for measurement of active stress in response to α\textsubscript{1}-adrenergic stimulation with phenylephrine and membrane depolarization by high KCl. In pregnant rats, the maximal active stress to phenylephrine (0.31±0.03×10\textsuperscript{4} N/m\textsuperscript{2}) and the high-KCl–induced active stress (0.55±0.09×10\textsuperscript{4} N/m\textsuperscript{2}) were smaller than those in virgin rats. By contrast, in the L-NAME–treated pregnant rats, the maximal phenylephrine-induced active stress (0.76±0.1×10\textsuperscript{4} N/m\textsuperscript{2}) was greater than that in virgin rats (0.52±0.1×10\textsuperscript{4} N/m\textsuperscript{2}), whereas the high-KCl–induced active stress (1.08±0.14×10\textsuperscript{4} N/m\textsuperscript{2}) was indistinguishable from that in virgin rats (1.03±0.14×10\textsuperscript{4} N/m\textsuperscript{2}). Treatment with L-NAME did not affect the phenylephrine-releasable Ca\textsuperscript{2+} stores in pregnant rats and had minimal effect on active stress in virgin rats. Thus, reduction of NO synthesis during late pregnancy is associated with a significant increase in blood pressure and vascular responsiveness to α-adrenergic stimulation, which can possibly be explained in part by enhanced Ca\textsuperscript{2+} entry from extracellular space. However, other mechanisms such as increased myofilament force sensitivity to Ca\textsuperscript{2+} and/or activation of a completely Ca\textsuperscript{2+}-independent mechanism cannot be excluded. (Hypertension. 1998;31:1065-1069.)

Key Words: blood pressure ■ calcium ■ muscle, smooth ■ contraction

Normal pregnancy is associated with many hemodynamic changes such as increased heart rate and cardiac output,\textsuperscript{1} increased plasma volume, and an increase in uterine\textsuperscript{2-4} and renal blood flow.\textsuperscript{5} Despite the increase in heart rate, blood volume, and cardiac output, normal pregnancy is usually associated with a significant decrease in arterial blood pressure and total peripheral resistance.\textsuperscript{6} This normal pregnancy-associated decrease in peripheral resistance has been explained by several mechanisms, including an increase in the metabolic requirements of both maternal and fetoplacental tissues and/or a decrease in vascular reactivity.\textsuperscript{7} Several explanations have been proposed for the decrease in vascular reactivity during normal pregnancy, such as decreased presor response to vasoconstrictors.\textsuperscript{8-10} Specific alterations within the vascular wall,\textsuperscript{11} and an increase in nitric oxide (NO) synthesis.\textsuperscript{12,13} Also, Conrad and Vernier\textsuperscript{14} have found that the plasma level, metabolic production, and urinary excretion of cGMP, a second messenger of NO and a cellular mediator of vascular smooth muscle relaxation, are increased during pregnancy.

In 5% to 7% of pregnancies, women develop a condition called pregnancy-induced hypertension.\textsuperscript{15} In contrast to normal pregnancy, pregnancy-induced hypertension is characterized by increased arterial blood pressure, generalized vasoconstriction, increased systemic vascular resistance, increased capillary permeability, decreased plasma volume, severe edema, increased intravascular coagulation, reduced tissue perfusion, decreased glomerular filtration rate, proteinuria, and widespread vascular endothelial damage.\textsuperscript{15,16}

Although pregnancy-induced hypertension is a leading cause of maternal and fetal mortality, the exact mechanism of this disorder has not yet been clearly identified. Several mechanisms have been suggested, including reduction of NO synthesis\textsuperscript{17,18} and/or changes in the vasoconstrictor receptor affinity or density.\textsuperscript{9} Consistent with these suggested mechanisms are reports that chronic NO synthase blockade during mid to late gestation in rats results in many pathological changes similar to those found in women with pregnancy-induced hypertension, such as increased blood pressure, proteinuria, thrombocytopenia, and intrauterine growth retardation.\textsuperscript{19-22} These observations have led investigators to suggest the use of pregnant rats chronically treated with NO synthase blockers as a model to study pregnancy-induced hypertension.
hypertension. Although chronic NO synthase blockade in pregnant rats has been shown to enhance the pressor response to vasoconstrictor substances, it is not clear whether these pressor responses reflect changes in the vascular reactivity. The present study was designed to investigate whether chronic NO synthase blockade in pregnant rats is associated with changes in vascular reactivity. Pregnant rats were treated chronically with N\textsuperscript{-}nitro-L-arginine methyl ester (L-NAME) during mid to late gestation. L-NAME is a structural analogue of L-arginine and is known to inhibit the synthesis of NO. The L-NAME–induced changes in blood pressure were measured, and the underlying changes in vascular reactivity were investigated for possible alterations in the Ca\textsuperscript{2+}-handling mechanisms.

Methods

Animals
Female Sprague-Dawley rats (10 to 12 weeks of age) were purchased from Harlan Sprague-Dawley, Inc (Indianapolis, Ind). Rats were divided into four groups: (1) virgin, (2) virgin treated with L-NAME, (3) pregnant, and (4) pregnant treated with L-NAME. The first day of pregnancy was verified by the presence of sperm in vaginal smears (full term is 21 to 22 days). All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and the American Physiological Society.

Measurement of Blood Pressure
Systolic blood pressure was measured in the four groups of rats with an automated sphygmomanometer with a tail-cuff device (IITC Life Science Inc). On the day of the experiment (typically 18 to 20 days of gestation in pregnant rats or the equivalent period in virgin rats), pretrained rats were placed in a warm chamber maintained at 30°C for 1 hour, then each rat was transferred to an individual Plexiglas restrainer. The pneumatic tail-cuff device was placed around the rat’s tail; at least three pressure measurements were recorded for each rat, and the average systolic blood pressure was calculated.

Tissue Preparation
Rats were terminally anesthetized by inhalation of chloroform. The thoracic aorta was rapidly removed, placed in oxygenated Krebs’ solution, and cleaned of connective tissue. The aorta was cut transversely into 3-mm-wide rings. The endothelium was removed by rubbing of the vessel interior with forceps. Rings were cut open into strips. One end of the strip was attached to a glass hook using a thread loop, and the other end was connected to a Grass force transducer (FT03, Astro-Med Inc).

Isometric Tension
Aortic strips were stretched to 2 g of tension and allowed to equilibrate for 1 hour in an organ bath filled with 50 mL Krebs’ solution continuously bubbled with 95% O\textsubscript{2}/5% CO\textsubscript{2} at 37°C. The changes in isometric tension were recorded on a Grass polygraph (model 7D, Astro-Med Inc). Removal of the endothelium was routinely verified by the absence of acetylcholine (10\textsuperscript{-5} mol/L)–induced vasorelaxation in aortic strips precontracted with phenylephrine (3×10\textsuperscript{-7} mol/L).

Three different protocols were followed in this study. In the first protocol, after the rat aortic strip was allowed to equilibrate for 1 hour, a control contraction was elicited by applying 10\textsuperscript{-5} mol/L phenylephrine to the organ bath solution. Once the phenylephrine contraction reached a plateau, the tissue was rinsed with Krebs’ solution three times for a duration of 10 minutes each. The whole procedure of contraction and washing was repeated two times. Phenylephrine was then added cumulatively to the organ bath at concentrations ranging from 10\textsuperscript{-5} mol/L to 10\textsuperscript{-4} mol/L, and the changes in isometric tension were recorded. For each phenylephrine concentration, the contraction was allowed to reach a plateau before the addition of the next concentration.

In the second protocol, control phenylephrine (10\textsuperscript{-5} mol/L) contractions were elicited; then the tissue was incubated in Ca\textsuperscript{2+}-free (2 mmol/L EGTA) Krebs’ solution for 10 minutes. Phenylephrine (10\textsuperscript{-5} mol/L) was added, and the transient phenylephrine contraction in Ca\textsuperscript{2+}-free solution was recorded.

In the third protocol, control contractions were elicited using 96 mmol/L KCl solution, and the tissue was rinsed with Krebs’ solution three times for a duration of 10 minutes each. This procedure was repeated two times. Increasing concentrations of KCl solution (16, 24, 36, 51, 66, 78, 86, and 96 mmol/L) were added to the organ bath separately. Contractions were allowed to reach a steady plateau before the solution was drained and the next concentration of KCl solution was added.

Solutions
The Krebs’ solution contained (in mmol/L) NaCl 120, KCl 5.9, NaHCO\textsubscript{3} 25, NaH\textsubscript{2}PO\textsubscript{4} 1.2, dextrose 11.5, MgCl\textsubscript{2} 1.2, and CaCl\textsubscript{2} 2.5. The solution was bubbled with 95% O\textsubscript{2}/5% CO\textsubscript{2} to adjust the pH to 7.4. For the Ca\textsuperscript{2+}-free Krebs’ solution, CaCl\textsubscript{2} was omitted and replaced with 2 mmol/L EGTA. The high-KCl depolarizing solution was prepared as Krebs’ solution but with equimolar substitution of NaCl with KCl.

Drugs and Chemicals
The stock solution of phenylephrine (L-phenylephrine hydrochloride; Sigma Chemical Co) was prepared as 10\textsuperscript{-1} mol/L in distilled water. Diluted phenylephrine solutions were also made in distilled water. The L-NAME (Sigma) solution was prepared by adding 50 mg to 1 L of the rat’s drinking water. This L-NAME solution concentration was selected after studies of Molnar and coworkers\textsuperscript{17} that showed that a dose of approximately 1 mg/d resulted in significant elevation of blood pressure in pregnant rats while having minimal effect in virgin rats. The L-NAME–treated rats were allowed to drink the water containing L-NAME for 4 to 6 days before blood pressure measurements were taken or excision of the aorta. All other chemicals were of reagent grade or better.

Statistical Analysis
The developed force was corrected for the cross-sectional area of each individual strip and expressed as active stress (N/m\textsuperscript{2}) using the following equation: stress = force/cross-sectional area, where cross-sectional area equals wet weight/tissue density\texttimes length of the strip, and tissue density equals 1.055 g/cm\textsuperscript{3}. Data were analyzed and expressed as mean±SEM. Data were compared using one-way ANOVA with Scheffé’s test and unpaired Student’s t test. Differences <.05 were considered statistically significant.

Results
Fig 1 shows the systolic blood pressure recorded in the four groups of rats. In the virgin rats, the systolic blood pressure was
The systolic blood pressure of the pregnant rats was slightly lower (116 ± 4 mm Hg, n=17) when compared with the virgin rats. In contrast, the systolic blood pressure of the pregnant rats treated with L-NAME (171 ± 5 mm Hg, n=14) was significantly higher than that in the virgin rats. On the other hand, the systolic blood pressure of the virgin rats treated with L-NAME (124 ± 5 mm Hg, n=12) was not significantly different from that in the virgin rats.

Fig 2 shows the contractile response to phenylephrine in rat aortic strips isolated from the four groups of rats. All groups showed a concentration-dependent increase in active stress to phenylephrine. In the virgin rats, phenylephrine (10⁻² mol/L) produced an active stress of 0.52 ± 0.10 × 10⁴ N/m² (n=7). The active stress in the pregnant rats was markedly reduced. In the pregnant rats, phenylephrine (10⁻² mol/L) produced an active stress of 0.31 ± 0.03 × 10⁴ N/m² (n=8). In contrast, the active stress in the pregnant rats treated with L-NAME was markedly increased. In the pregnant rats treated with L-NAME, phenylephrine (10⁻³ mol/L) produced an active stress of 0.76 ± 0.10 × 10⁴ N/m² (n=8). On the other hand, active stress in the virgin rats and the virgin rats treated with L-NAME showed no significant difference. In the virgin rats treated with L-NAME, phenylephrine (10⁻³ mol/L) produced an active stress of 0.57 ± 0.11 × 10⁴ N/m² (n=8).

Fig 3 shows that when the contractile response to increasing concentrations of phenylephrine was presented as a percentage of the maximal phenylephrine contraction, no significant difference among any of the four groups was observed.

Phenylephrine-induced contraction in Ca²⁺-free solution is often used as a measure of the phenylephrine-releasable intracellular Ca²⁺ stores. Fig 4 shows the phenylephrine contraction of rat aortic strips in Ca²⁺-free (2 mmol/L EGTA) Krebs’ solution. In all groups of rats, phenylephrine showed a transient increase in active stress in Ca²⁺-free solution. However, there was no significant difference in the active stress among any of the four groups.

Membrane depolarization by high-KCl solution is known to activate Ca²⁺ entry through voltage-gated Ca²⁺ channels. Fig 5 shows the effect of increasing concentrations of extracellular KCl on contraction. In the virgin rats, 96 mmol/L KCl produced an active stress of 1.03 ± 0.14 × 10⁴ N/m² (n=6). The 96 mmol/L KCl active stress in the pregnant rats was reduced to 0.55 ± 0.09 × 10⁴ N/m² (n=8). On the other hand, the 96 mmol/L KCl-induced active stress in the virgin rats treated with
L-NAME (0.94±0.12×10⁶ N/m² n=17) and pregnant rats treated with L-NAME (1.08±0.14×10⁶ N/m² n=9) was not significantly different from that in the virgin rats.

Discussion
The major findings of this study are that (1) the systolic blood pressure of pregnant rats treated with L-NAME was significantly greater than that of the pregnant rats, the virgin rats, and the virgin rats treated with L-NAME; (2) the vascular reactivity to phenylephrine was decreased in the pregnant rats and increased in the pregnant rats treated with L-NAME when compared with the virgin rats and the virgin rats treated with L-NAME; (3) the transient phenylephrine contraction in Ca²⁺-free solution, a measure of the phenylephrine-releasable Ca²⁺ stores, showed no difference among any of the four groups; and (4) an observed reduced vascular reactivity to membrane depolarization by KCl in pregnant rats was corrected only to the virgin levels when the pregnant rats were treated with L-NAME.

Our results show that systolic blood pressure is slightly lower in pregnant rats than in virgin rats. These results are consistent with the findings of other laboratories.7–9,11,12,23 This normal pregnancy-induced drop in blood pressure could be explained by changes in the function of the kidneys, metabolic changes, and/or changes in vascular reactivity. On the other hand, chronic NO synthase blockade with L-NAME caused severe hypertension in pregnant rats. The same dose of L-NAME had minimal effect on blood pressure in virgin rats. These data are consistent with the findings of Molnar and colleagues24 and further support a role for NO synthase blockade in pregnancy-induced hypertension.

The present study showed that the active stress in response to phenylephrine was greater in the pregnant rats treated with L-NAME than in the pregnant rats. However, since the pregnant rats had a lower blood pressure than the virgin control animals, and the pregnant rats treated with L-NAME showed a significant increase in blood pressure, the possible role of vascular wall remodeling should be considered. Several studies have shown that the increase in blood pressure is often associated with an increase in the thickness of the vascular wall.24,25 If this is the case, then the vascular wall thickness is predicted to be greater in the pregnant rats treated with L-NAME than in the untreated pregnant rats. Because the wall thickness was part of the denominator in the “active stress” calculation (N/m²), the observed increase in active stress in the pregnant rats treated with L-NAME is probably underestimated.

It has been hypothesized that an increase in the production of NO during pregnancy leads to a decrease in peripheral resistance and a decrease in vascular reactivity.1–14 If this is the case, one would expect that blocking the formation of NO during pregnancy would bring the vascular reactivity back to the level observed in virgin rats. However, our data show that the vascular reactivity to phenylephrine in pregnant rats treated with L-NAME is greater than that in virgin rats. These results suggest that treatment of pregnant rats with L-NAME not only blocks the synthesis of NO by endothelial cells but may also cause an increase in the synthesis of or sensitivity to other vasoactive compounds that would increase vascular reactivity. It has been suggested that the reduction in the placental blood flow during pregnancy may be associated with placental release of cytotoxic factors that alter the endothelial cell function, leading to reduction in the synthesis of vasodilator such as NO or prostacyclin or, more importantly, increased production of vasoconstrictor factors such as endothelin.19–22 This is consistent with a recent study showing that long-term inhibition of NO synthesis during mid to late gestation in rats is associated with increased blood pressure and elevated plasma levels of endothelin-1.20

The observed increase in vascular reactivity to phenylephrine in pregnant rats treated with L-NAME could be explained by an increase in the sensitivity to phenylephrine at the α-adrenergic receptor level. However, our results showed no significant difference between the four groups of rats in the phenylephrine concentration-response curve when the contraction was presented as a percentage of the maximum. These results suggest that the change in the α-adrenergic receptor sensitivity to phenylephrine may not be responsible for the observed increase in vascular reactivity in pregnant rats treated with L-NAME and that the increased vascular reactivity could be due to activation of a signaling mechanism downstream from receptor activation.

It is generally accepted that α-adrenergic agonists, such as phenylephrine, react with α-adrenergic receptors, causing activation of phospholipase C and increased hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol.27 IP₃ stimulates Ca²⁺ release from intracellular stores,28 and diacylglycerol stimulates protein kinase C.29 In addition, α-adrenergic agonists cause plasma membrane Ca²⁺ channels to open.30

We tested whether the amount of Ca²⁺ released from intracellular stores in response to phenylephrine is different in the four groups of rats. We found that the transient phenylephrine contraction in Ca²⁺-free solution, which is often used as a measure of phenylephrine-releasable intracellular Ca²⁺ stores, is not different in the four groups of rats. This suggests that the enhanced vascular reactivity observed in pregnant rats treated with L-NAME is not due to changes in Ca²⁺ uptake to or Ca²⁺ release from intracellular stores.

To test the possible role of plasma membrane Ca²⁺ channels in the observed enhanced vascular reactivity to phenylephrine in pregnant rats treated with L-NAME, we tested the effect of KCl, which exclusively stimulates Ca²⁺ entry through voltage-gated Ca²⁺ channels. We found that the KCl-induced contraction in the pregnant rats was significantly reduced when compared with that in the virgin rats. These results suggest that normal pregnancy is associated with decreased Ca²⁺ entry through voltage-gated Ca²⁺ channels. On the other hand, in the pregnant rats treated with L-NAME, we found that the pregnancy-induced decrease in vascular reactivity to KCl was corrected but only to the level observed in the virgin rats. These results can possibly be explained in part by an increase in the permeability of voltage-gated Ca²⁺ channels in blood vessels of pregnant rats treated with L-NAME. However, because direct measurements of Ca²⁺ or myosin light chain phosphorylation were not performed in the present study, we cannot make a definite conclusion that L-NAME treatment increases Ca²⁺ entry into
smooth muscle cells, and other possible mechanisms cannot be excluded. These mechanisms may involve an increase in the myofilament force sensitivity to Ca\(^{2+}\) entry or possibly activation of a completely Ca\(^{2+}\)-independent mechanism.

The rebound increase in vascular reactivity to phenylephrine in the pregnant rats treated with L-NAME above that in the virgin rats can then be explained by the following: (1) Phenylephrine may activate an additional group of Ca\(^{2+}\) channels that have a different sensitivity to activation by phenylephrine when compared with the voltage-gated Ca\(^{2+}\) channels. These Ca\(^{2+}\) channels have been found in several smooth muscle preparations and have been designated “receptor-operated Ca\(^{2+}\) channels.”\(^{3,12}\) (2) Phenylephrine may further activate different pathways that increase the myofilament force sensitivity to Ca\(^{2+}\) or possibly activate a completely Ca\(^{2+}\)-independent pathway. For example, phenylephrine may activate protein kinase C through increased formation of diacylglycerol.\(^\text{!!}\)

In conclusion, the increased blood pressure in late-pregnancy rats treated with L-NAME is associated with an increased vascular reactivity to sympathetic amines. This increased vascular reactivity can possibly be explained in part by an enhancement of Ca\(^{2+}\) entry through Ca\(^{2+}\) channels. However, other mechanisms such as an increase in the myofilament force sensitivity to Ca\(^{2+}\) entry or activation of a completely Ca\(^{2+}\)-independent pathway may also be involved. Further studies are needed to investigate these possible mechanisms.

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