Stimulated mechanisms of Ca\textsuperscript{2+} entry into vascular smooth muscle during NO synthesis inhibition in pregnant rats

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Crews, Janice K., Jacqueline Novak, Joey P. Granger, and Raouf A. Khalil. Stimulated mechanisms of Ca\textsuperscript{2+} entry into vascular smooth muscle during NO synthesis inhibition in pregnant rats. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R530–R538, 1999.—We have previously found that the vascular responsiveness to \(\alpha\)-adrenergic agonists is reduced in pregnant rats and enhanced in a rat model of pregnancy-induced hypertension produced by chronic treatment of pregnant rats with the nitric oxide (NO) synthase inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME). The purpose of this study was to investigate whether the observed changes in vascular reactivity during normal pregnancy and during pregnancy-induced hypertension reflect changes in the mechanisms of Ca\textsuperscript{2+} entry into vascular smooth muscle. \(^{45}\text{Ca}^2\) influx and active stress during \(\alpha\)-adrenergic stimulation by phenylephrine and membrane depolarization by 96 mM KCl were measured in denudated aortic strips isolated from virgin and pregnant Sprague-Dawley rats untreated or treated with 1 mg/day L-NAME for 4–6 days and incubated in Krebs solution containing increasing concentrations of extracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{o}). In all groups of rats, both phenylephrine and 96 mM KCl caused [Ca\textsuperscript{2+}]\textsubscript{i}-dependent increases in active stress and \(^{45}\text{Ca}^2\) influx. The phenylephrine- and 96 mM KCl-induced active stress and Ca\textsuperscript{2+} influx were significantly reduced in pregnant rats but significantly enhanced in pregnant rats treated with L-NAME. The phenylephrine-induced Ca\textsuperscript{2+} influx-stress relationship was significantly greater than that induced by 96 mM KCl in pregnant rats treated with L-NAME. The phenylephrine-induced Ca\textsuperscript{2+} influx-stress relationship was reduced in pregnant rats but enhanced in pregnant rats treated with L-NAME. Chronic treatment with L-NAME had minimal effect on active stress, Ca\textsuperscript{2+} influx, and the Ca\textsuperscript{2+} influx-stress relationship in virgin rats. These results provide evidence that the mechanisms of Ca\textsuperscript{2+} entry into vascular smooth muscle are inhibited during pregnancy but enhanced during inhibition of NO synthesis in late pregnancy. The enhancement of the phenylephrine-induced Ca\textsuperscript{2+} influx-stress relationship in pregnant rats treated with L-NAME suggests activation of other contractile mechanisms in addition to stimulation of Ca\textsuperscript{2+} entry. These mechanisms appear to be inhibited during normal pregnancy.

arterial pressure; peripheral vascular resistance; calcium; contraction; nitric oxide

NORMAL PREGNANCY is usually associated with a significant decrease in arterial blood pressure and total peripheral resistance (9). Several mechanisms have been suggested for the observed decrease in peripheral resistance during normal pregnancy, including decreased vascular reactivity to vasoconstrictors (12, 16, 19, 24, 29). The decrease in vascular reactivity during pregnancy has been attributed to specific alterations within the vascular wall (22) and/or increased nitric oxide (NO) synthesis (1, 7, 23). However, the signaling mechanisms involved in the reduced vascular reactivity during pregnancy are not clearly understood.

In 5–7% of pregnancies, women develop a condition called pregnancy-induced hypertension, characterized by increased arterial blood pressure, generalized vasoconstriction, increased systemic vascular resistance, increased intravascular coagulation, proteinuria, and widespread vascular endothelial damage (6). Although pregnancy-induced hypertension is a major 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 (11, 18). This is supported by 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 (3, 4, 17, 21). 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 (3, 4, 12, 17, 21).

In our search for the possible signaling mechanisms underlying the pregnancy-associated changes in arterial blood pressure and total peripheral vascular resistance, we have recently found that the vascular reactivity to the \(\alpha\)-adrenergic agonist phenylephrine is reduced in pregnant rats (12). Also, we and others have found that chronic NO synthase blockade in late pregnant rats increases the pressor response to vasoconstrictor substances (16) and enhances the vascular reactivity to \(\alpha\)-adrenergic agonists (12). We have also reported that the changes in vascular reactivity during pregnancy are not due to changes in the \(\alpha\)-adrenergic receptor sensitivity to phenylephrine or to changes in the releasable intracellular Ca\textsuperscript{2+} stores (12). We have also found that the vascular responsiveness to membrane depolarization by high-KCl solution, an activator of Ca\textsuperscript{2+} entry through voltage-gated Ca\textsuperscript{2+} channels, is reduced in pregnant rats but enhanced in pregnant rats treated with L-NAME, suggesting possible changes in the Ca\textsuperscript{2+} entry mechanisms during pregnancy (12).

The purpose of the present study was as follows. First, we wanted to determine whether the observed pregnancy-associated changes in vascular reactivity reflect changes in the mechanisms of Ca\textsuperscript{2+} entry into...
vascular smooth muscle. Therefore, Ca\(^{2+}\) influx was measured in parallel with active stress in aortic strips isolated from pregnant rats untreated or treated chronically with N\(^\text{\textsuperscript{3}}\)-nitro-L-arginine methyl ester (L-NAME) during mid- to late gestation. L-NAME is a structural analog of L-arginine and is known to inhibit the synthesis of NO. Second, we wanted to investigate the possible Ca\(^{2+}\) entry pathway(s) involved, if any. Therefore, the phenylephrine- and 96 mM KCl-induced changes in active stress were measured at increasing concentrations of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_e\)) and the underlying changes in Ca\(^{2+}\) entry were compared. Third, we wanted to investigate whether the observed pregnancy-associated changes in vascular reactivity involve other contractile mechanisms in addition to changes in Ca\(^{2+}\) entry. Therefore, the Ca\(^{2+}\) entry-active stress relationships were constructed and compared in pregnant and virgin rats untreated or treated with L-NAME.

**METHODS AND MATERIALS**

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

Protocol for L-NAME treatment. Pregnant and virgin rats in the untreated groups received drinking water. Pregnant and virgin rats in the treated groups received L-NAME (Sigma) at a dose of \(-1\) mg/day. This dose of L-NAME was chosen on the basis of recent studies from our laboratory and others, which showed that a dose of \(-1\) mg/day resulted in significant elevation of blood pressure in pregnant rats but had minimal effect in virgin rats (12, 17). L-NAME treatment of the pregnant rats began at day 15 of gestation. The L-NAME-treated rats were allowed to drink the water containing L-NAME for 4–6 days before the rats were killed and the tissues harvested at day 20 of gestation. Some of the L-NAME-treated pregnant rats simultaneously received L-arginine (Sigma) in the drinking water at a dose of \(-20\) mg/day for the same period of time (4–6 days). Tissues from untreated pregnant rats were also obtained after 19–20 days of gestation. After this protocol we recorded systolic blood pressure on the day of the experiment using an automated sphygmonanometer with a tail-cuff device; results were (in mmHg) 118 \pm 3 (n = 8) in virgin rats, 125 \pm 6 (n = 10) in virgin rats treated with L-NAME, 113 \pm 5 (n = 8) in pregnant rats, and 172 \pm 6 (n = 10) in pregnant rats treated with L-NAME as previously described (12). The systolic blood pressure in pregnant rats simultaneously treated with L-NAME and L-arginine was significantly reduced to 124 \pm 3 mmHg (n = 12) compared with that in L-NAME-treated pregnant rats but was not significantly different from that in untreated pregnant rats.

Tissue preparation. On the day of the experiment (typically 19–20 days of gestation in pregnant rats or the equivalent period in virgin rats), the 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 the vessel interior with forceps. Aortic rings were cut open into strips.

Isometric tension. One end of the aortic 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, West Warwick, RI). Aortic strips were stretched to \(2\) g of tension and allowed to equilibrate for \(1\) h in a water-jacketed, temperature-controlled tissue bath filled with 50 ml Krebs solution continuously bubbled with \(95\%\) O\(_2\)-5% CO\(_2\) at 37°C. The changes in isometric tension were recorded on a Grass polygraph (model 7D, Astro-Med). Removal of the endothelium and/or dysfunction of the endothelium-dependent NO-releasing pathway (in rats chronically treated with L-NAME) was routinely verified by the absence of Ach (10\(^{-6}\)M)-induced vasorelaxation in aortic strips precontracted with phenylephrine (3 \times 10\(^{-7}\) M).

After the rat aortic strips were allowed to equilibrate for \(1\) h, a control contraction was elicited by adding \(10\) \(^{-5}\) M phenylephrine to the tissue bath solution. Once the phenylephrine contraction reached a plateau, the tissue was rinsed with Krebs solution three times for a duration of 10 min each. The whole procedure of contraction and washing was repeated two times. The bath solution was changed to nominally 0 Ca\(^{2+}\) Krebs solution for 10 min, and the tissues were stimulated with phenylephrine (10\(^{-5}\) M). Increasing [Ca\(^{2+}\)]\(_e\), (0.1, 0.3, 0.6, 1.0, and 2.5 mM) was achieved by adding Ca\(^{2+}\) cumulatively to the tissue bath, and the changes in isometric tension were recorded. For each [Ca\(^{2+}\)]\(_e\), the contraction was allowed to reach a plateau before the next [Ca\(^{2+}\)]\(_e\).

In other experiments, control contractions were elicited using 96 mM KCl solution, and the tissue was rinsed with Krebs solution three times for a duration of 10 min each. This procedure was repeated two times. The bath solution was changed to nominally 0 Ca\(^{2+}\) 96 mM KCl solution for 10 min. Increasing [Ca\(^{2+}\)]\(_e\), (0.1, 0.3, 0.6, 1.0, and 2.5 mM) was achieved by adding Ca\(^{2+}\) cumulatively to the tissue bath, and the changes in isometric tension were recorded. For each [Ca\(^{2+}\)]\(_e\), the contraction was allowed to reach a steady plateau before the next [Ca\(^{2+}\)]\(_e\).

Ca\(^{2+}\) influx. The changes in Ca\(^{2+}\) influx were measured in the aortic strips as previously described (14). Briefly, the aortic strips were incubated in normal Krebs solution for \(1\) h. The strips were transferred to Krebs solution containing specific [Ca\(^{2+}\)]\(_e\), for 10 min, then stimulated with phenylephrine (10\(^{-5}\) M) or 96 mM KCl for 30 min because at that time point the contractile response consistently reached steady state. Because the unidirectional Ca\(^{2+}\) influx, and not the net Ca\(^{2+}\) uptake, was measured in the present study, at steady state the rate of Ca\(^{2+}\) influx is not dependent on the time of incubation with phenylephrine or KCl. The tissues were transferred to the respective radioactive Ca\(^{2+}\)-labeled solution (specific activity 2 µCi/ml; ICN Radiochemical, Irvine, CA) for 90 s. Preliminary experiments showed that the relationship between Ca\(^{2+}\) uptake vs. time is linear during 15-, 30-, 60-, and 90-s exposures to the Ca\(^{2+}\)-labeled solution. The tissues were transferred to ice-cold Ca\(^{2+}\)-free (2 mM EGTA) Krebs for 45 min to quench extracellular Ca\(^{2+}\) label. The ice-cold Ca\(^{2+}\)-free (2 mM EGTA) Krebs displaced the extracellular Ca\(^{2+}\) label with minimal effect on the intracellular Ca\(^{2+}\) content, because the cellular Ca\(^{2+}\) extrusion mechanisms such as the plasmaemal Ca\(^{2+}\) extrusion pump require energy and therefore are significantly inhibited in the ice-cold conditions. Also, all tissues from the different groups of rats were treated identically using the same experimental protocol. The tissue samples were weighed and placed in 2 ml of hypotonic (5 mM) EDTA for 24 h at 4°C to disrupt the cell membranes and release the intracellular content of Ca\(^{2+}\).
The next day, 4 ml of Ecolite scintillation cocktail was added, and the samples were counted in a scintillation counter (Beckman LS 6500, Beckman Instruments, Houston, TX).

Solutions, drugs, and chemicals. Normal Krebs solution contained (in mM) 120 NaCl, 5.9 KCl, 25 NaHCO3, 1.2 NaH2PO4, 11.5 dextrose, 1.2 MgCl2, and 2.5 CaCl2. The solution was bubbled with 95% O2-5% CO2 to adjust the pH to 7.4. For the nominally 0 Ca2+-Krebs solution, CaCl2 was omitted. The high-KCl depolarizing solution was prepared as Krebs solution but with equimolar substitution of NaCl with KCl. Stock solution of phenylephrine (L-phenylephrine HCl, Sigma) was prepared as 10−1 M in distilled water. 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/m2) using the equation: stress = force/cross-sectional area, where cross-sectional area = wet wt/(tissue density × length of the strip), and tissue density = 1.055 g/cm3. Data were analyzed and expressed as means ± SE. Data were compared using one-way ANOVA with Scheffé's test and unpaired Student's t-test. Differences at P < 0.05 were considered statistically significant.

RESULTS

The effect of phenylephrine on active stress in rat aortic strips isolated from the different groups of rats and incubated at increasing [Ca2+]e was first investigated. All groups of rats showed increases in active stress to phenylephrine with increasing [Ca2+]e (Fig. 1A, Table 1). In virgin rats, phenylephrine (10−5 M) increased active stress to 0.9 ± 0.1 × 104 N/m2 (n = 7) at 2.5 mM [Ca2+]e. In virgin rats treated with L-NAME, the phenylephrine-induced active stress was not significantly different from that in virgin rats. The phenylephrine-induced active stress in pregnant rats was significantly reduced. In pregnant rats, phenylephrine (10−5 M) increased active stress to only 0.5 ± 0.1 × 104 N/m2 (n = 10) at 2.5 mM [Ca2+]e. In contrast, the active stress in pregnant rats treated with L-NAME was significantly increased. In pregnant rats treated with L-NAME, phenylephrine (10−5 M) increased active stress to 1.4 ± 0.1 × 104 N/m2 (n = 8) at 2.5 mM [Ca2+]e. At 2.5 mM [Ca2+]e, the phenylephrine-induced active stress in pregnant rats simultaneously treated with L-NAME and L-arginine was significantly reduced to 0.6 ± 0.1 × 104 N/m2 (n = 12) compared with that in L-NAME-treated pregnant rats but was not significantly different from that observed in untreated pregnant rats.

The effect of membrane depolarization with 96 mM KCl on active stress was also investigated in rat aortic strips isolated from the different groups of rats and incubated at increasing [Ca2+]e. All groups of rats showed increases in the magnitude of 96 mM KCl-induced active stress with increasing [Ca2+]e (Fig. 1B, Table 1). In virgin rats, 96 mM KCl increased active stress to 0.8 ± 0.2 × 104 N/m2 (n = 8) at 2.5 mM [Ca2+]e. The 96 mM KCl-induced active stress in virgin rats treated with L-NAME showed no significant difference from that in virgin rats. In virgin rats treated with L-NAME, 96 mM KCl increased active stress to 0.8 ± 0.1 × 104 N/m2 (n = 8) at 2.5 mM [Ca2+]e. The 96 mM KCl-induced active stress in pregnant rats was significantly reduced. In pregnant rats, 96 mM KCl increased active stress to 0.6 ± 0.1 × 104 N/m2 (n = 8) at 2.5 mM [Ca2+]e. The 96 mM KCl-induced active stress in pregnant rats treated with L-NAME was increased to levels not distinguishable from those in virgin rats. In pregnant rats treated with L-NAME, 96 mM KCl-induced active stress to only 0.4 ± 0.1 × 104 N/m2 (n = 7) at 2.5 mM [Ca2+]e. In contrast, the 96 mM KCl-induced active stress in pregnant rats treated with L-NAME was increased to levels not distinguishable from those in virgin rats. In pregnant rats treated with L-NAME, 96 mM KCl-induced active stress to only 0.4 ± 0.1 × 104 N/m2 (n = 7) at 2.5 mM [Ca2+]e. In contrast, the 96 mM KCl-induced active stress in pregnant rats treated with L-NAME was increased to levels not distinguishable from those in virgin rats. In pregnant rats treated with L-NAME, 96 mM KCl-induced active stress to only 0.4 ± 0.1 × 104 N/m2 (n = 7) at 2.5 mM [Ca2+]e. In contrast, the 96 mM KCl-induced active stress in pregnant rats treated with L-NAME was increased to levels not distinguishable from those in virgin rats. In pregnant rats treated with L-NAME, 96 mM KCl-induced active stress to only 0.4 ± 0.1 × 104 N/m2 (n = 7) at 2.5 mM [Ca2+]e. In contrast, the 96 mM KCl-induced active stress in pregnant rats treated with L-NAME was increased to levels not distinguishable from those in virgin rats. In pregnant rats treated with L-NAME, [Ca2+]e producing equivalent phenylephrine-or KCl-induced active stress in rats

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Values are concentrations of extracellular Ca2+ ([Ca2+]e) required to produce half-maximal phenylephrine (10−5 M) or 96 mM KCl-induced active stress in virgin rats or equivalent stress in virgin rats treated with Nω-nitro-arginine methyl ester (L-NAME), pregnant rats, and pregnant rats treated with L-NAME.
mM KCl increased active stress to 1.0 ± 0.2 × 10^4 N/m^2 (n = 6) at 2.5 mM [Ca^{2+}]_e. At 2.5 mM [Ca^{2+}]_e, the 96 mM KCl-induced active stress in pregnant rats simultaneously treated with L-NAME and L-arginine was significantly reduced to 0.5 ± 0.1 × 10^4 N/m^2 (n = 12) compared with that in L-NAME-treated pregnant rats but was not significantly different from that observed in untreated pregnant rats.

To determine whether the observed changes in active stress reflect changes in Ca^{2+} entry, we measured the phenylephrine-induced 45Ca^{2+} influx in rat aortic strips isolated from the different groups of rats and incubated at increasing [Ca^{2+}]_e. All groups of rats showed increases in phenylephrine-induced 45Ca^{2+} influx with increasing [Ca^{2+}]_e (Fig. 2A). In virgin rats, phenylephrine (10^{-5} M) increased Ca^{2+} influx from 5.0 ± 0.4 µmol·kg^{-1}·min^{-1} (n = 5) at 100 µM [Ca^{2+}]_e to 25.0 ± 2.2 µmol·kg^{-1}·min^{-1} (n = 10) at 2.5 mM [Ca^{2+}]_e. The phenylephrine-induced Ca^{2+} influx in virgin rats treated with L-NAME was not significantly different from that in virgin rats. In virgin rats treated with L-NAME, phenylephrine (10^{-5} M) increased Ca^{2+} influx from 4.8 ± 0.9 µmol·kg^{-1}·min^{-1} (n = 5) at 100 µM [Ca^{2+}]_e to 26.5 ± 1.6 µmol·kg^{-1}·min^{-1} (n = 10) at 2.5 mM [Ca^{2+}]_e. The phenylephrine-induced Ca^{2+} influx in pregnant rats was significantly reduced compared with that in virgin rats. In pregnant rats, phenylephrine increased Ca^{2+} influx from 3.6 ± 0.1 µmol·kg^{-1}·min^{-1} (n = 5) at 100 µM [Ca^{2+}]_e to 17.4 ± 2.2 µmol·kg^{-1}·min^{-1} (n = 5) at 2.5 mM [Ca^{2+}]_e. In contrast, the phenylephrine-induced Ca^{2+} influx in pregnant rats treated with L-NAME was significantly increased compared with virgin rats. In pregnant rats treated with L-NAME, phenylephrine increased Ca^{2+} influx from 3.9 ± 0.3 µmol·kg^{-1}·min^{-1} (n = 5) at 100 µM [Ca^{2+}]_e to 40.1 ± 4.2 µmol·kg^{-1}·min^{-1} (n = 10) at 2.5 mM [Ca^{2+}]_e. At 2.5 mM [Ca^{2+}]_e, the phenylephrine-induced Ca^{2+} influx in pregnant rats simultaneously treated with L-NAME and L-arginine was significantly reduced to 18.8 ± 1.2 µmol·kg^{-1}·min^{-1} (n = 12) compared with that in L-NAME-treated pregnant rats but was not significantly different from that observed in untreated pregnant rats.

To determine whether the observed changes in active stress reflect changes in Ca^{2+} entry through voltage-gated Ca^{2+} channels, we also measured the effect of membrane depolarization by 96 mM KCl solution on 45Ca^{2+} influx in rat aortic strips isolated from the different groups of rats and incubated at different [Ca^{2+}]_e. All groups of rats showed increases in 96 mM KCl-induced 45Ca^{2+} influx with increasing [Ca^{2+}]_e (Fig. 2B). In virgin rats, 96 mM KCl increased Ca^{2+} influx from 6.7 ± 1.0 µmol·kg^{-1}·min^{-1} (n = 5) at 100 µM [Ca^{2+}]_e to 31.9 ± 2.6 µmol·kg^{-1}·min^{-1} (n = 10) at 2.5 mM [Ca^{2+}]_e. The 96 mM KCl-induced Ca^{2+} influx in virgin rats treated with L-NAME was not significantly different from that in virgin rats. In virgin rats treated with L-NAME, 96 mM KCl increased Ca^{2+} influx from 7.0 ± 0.8 µmol·kg^{-1}·min^{-1} (n = 10) at 100 µM [Ca^{2+}]_e to 33.2 ± 4.2 µmol·kg^{-1}·min^{-1} (n = 10) at 2.5 mM [Ca^{2+}]_e. The 96 mM KCl-induced Ca^{2+} influx in pregnant rats was significantly reduced when compared with that in virgin rats. In pregnant rats, 96 mM KCl increased Ca^{2+} influx from 3.6 ± 0.2 µmol·kg^{-1}·min^{-1} (n = 5) at 100 µM [Ca^{2+}]_e to only 23.1 ± 2.5 µmol·kg^{-1}·min^{-1} (n = 10) at 2.5 mM [Ca^{2+}]_e. In contrast, the 96 mM KCl-induced Ca^{2+} influx in pregnant rats treated with L-NAME was significantly increased. In pregnant rats treated with L-NAME, 96 mM KCl increased Ca^{2+} influx from 5.4 ± 0.2 µmol·kg^{-1}·min^{-1} (n = 5) at 100 µM [Ca^{2+}]_e to 46.8 ± 5.2 µmol·kg^{-1}·min^{-1} (n = 5) at 2.5 mM [Ca^{2+}]_e. At 2.5 mM [Ca^{2+}]_e, the 96 mM KCl-induced Ca^{2+} influx in pregnant rats simultaneously treated with L-NAME and L-arginine was significantly reduced to 24.2 ± 2.5 µmol·kg^{-1}·min^{-1} (n = 12) compared with that in L-NAME-treated pregnant rats but was not significantly different from that observed in untreated pregnant rats.

**Fig. 2.** Phenylephrine (Phe)- and 96 mM KCl-induced 45Ca^{2+} influx at increasing [Ca^{2+}]_e in aortic strips isolated from virgin and pregnant rats untreated or treated with L-NAME. After 1-h incubation in normal Krebs solution, rat aortic strips were incubated in Krebs solution containing specific [Ca^{2+}]_e (0.1, 0.3, 0.6, 1.0, or 2.5 mM) for 10 min, then either activated by 10^{-5} M phenylephrine (A) or rapidly switched to 96 mM KCl solution containing specific [Ca^{2+}]_e (B). Tissues were transferred to corresponding 45Ca^{2+}-radiolabeled solution containing 0.1, 0.3, 0.6, 1.0, or 2.5 mM Ca^{2+} for 90 s, and 45Ca^{2+} influx was measured as described in Methods and Materials. Data points represent means ± SE of measurements in aortic strips from 5–15 rats.
To further investigate the possible Ca$^{2+}$ entry pathways that might be involved in the observed changes in active stress, the phenylephrine- and 96 mM KCl-induced [Ca$^{2+}$]$_e$-active stress relationships were compared in each group of rats. As shown in Fig. 3, the phenylephrine-induced [Ca$^{2+}$]$_e$-active stress relationship was not significantly different from that induced by 96 mM KCl in the virgin (Fig. 3A) and pregnant rats (Fig. 3C). In contrast, in pregnant rats treated with L-NAME the phenylephrine-induced [Ca$^{2+}$]$_e$-active stress relationship was significantly enhanced when compared with that induced by 96 mM KCl (Fig. 3D). On the other hand, treating the virgin rats with L-NAME did not show any significant difference between the phenylephrine- and the 96 mM KCl-induced [Ca$^{2+}$]$_e$-active stress relationships (Fig. 3B).

The phenylephrine- and the 96 mM KCl-induced [Ca$^{2+}$]$_e$-$^{45}$Ca$^{2+}$ influx relationships were also compared in each group of rats. At all [Ca$^{2+}$]$_e$ tested, the 96 mM KCl-induced $^{45}$Ca$^{2+}$ influx was greater than that induced by phenylephrine in virgin rats (Fig. 4A), virgin rats treated with L-NAME (Fig. 4B), pregnant rats (Fig. 4C), and pregnant rats treated with L-NAME (Fig. 4D). In other words, at the same [Ca$^{2+}$]$_e$, 96 mM KCl caused greater Ca$^{2+}$ influx than that induced by phenylephrine in all groups of rats.

The phenylephrine- and 96 mM KCl-induced Ca$^{2+}$ influx-active stress relationships were constructed using the data from Figs. 3 and 4. If the pregnancy-associated changes in vascular reactivity to phenylephrine involve changes only in Ca$^{2+}$ entry through voltage-gated Ca$^{2+}$ channels, then the pregnancy-associated changes in the phenylephrine-induced Ca$^{2+}$ influx-stress relationship would be similar to the changes in the 96 mM KCl-induced Ca$^{2+}$ influx-stress relationship. In all groups of rats, the phenylephrine-induced Ca$^{2+}$ influx-stress relationship was located to the left of that induced by 96 mM KCl (Fig. 5). In other words, for the same level of Ca$^{2+}$ influx, phenylephrine caused greater active stress than that induced by 96 mM KCl. In pregnant rats treated with L-NAME, the phenylephrine-induced Ca$^{2+}$ influx-active stress relationship was significantly shifted to the left of that induced by 96 mM KCl compared with the Ca$^{2+}$ influx-stress relationships in the other groups of rats.

To further investigate whether other contractile mechanisms in addition to Ca$^{2+}$ entry may be involved in the observed changes in active stress, we compared the phenylephrine-induced Ca$^{2+}$ influx-stress relationship in the different groups of rats (Fig. 6). If the observed pregnancy-associated changes in active stress involve changes only in the Ca$^{2+}$ entry mechanisms, then the Ca$^{2+}$ influx-stress relationship in pregnant rats would not be different from that in virgin rats. As shown in Fig. 6A, the phenylephrine-induced Ca$^{2+}$ influx-stress relationship in pregnant rats was smaller when compared with that in virgin rats. In other words, for the same level of Ca$^{2+}$ influx, phenylephrine caused less active stress in pregnant rats than in virgin rats. In contrast, the phenylephrine-induced Ca$^{2+}$ influx-stress relationship in pregnant rats treated with L-NAME was enhanced compared with that in virgin rats (Fig. 6A). On the other hand, the phenylephrine-induced Ca$^{2+}$ influx-stress relationship in virgin rats treated with L-NAME was not significantly different from that in virgin rats.

Fig. 3. Phenylephrine- and 96 mM KCl-induced steady-state active stress at increasing [Ca$^{2+}$]$_e$ in virgin (A), virgin + L-NAME (B), pregnant (C), and pregnant + L-NAME (D) rats. Data points represent means ± SE of measurements in aortic strips from 6–10 rats.
The 96 mM KCl-induced Ca\textsuperscript{2+} influx-stress relationship was also compared in the different groups of rats (Fig. 6). As shown in Fig. 6B, the 96 mM KCl-induced Ca\textsuperscript{2+} influx-stress relationship in pregnant rats was smaller than that in virgin rats. Treating the pregnant rats with L-NAME increased the 96 mM KCl-induced Ca\textsuperscript{2+} influx-stress relationship to levels not distinguishable from those in virgin rats (Fig. 6B). On the other hand, the treatment did not affect the relationship in virgin rats.

Fig. 4. Phenylephrine- and 96 mM KCl-induced \textsuperscript{45}Ca\textsuperscript{2+} influx at increasing [Ca\textsuperscript{2+}]e in virgin (A), virgin + L-NAME (B), pregnant (C), and pregnant + L-NAME (D) rats. Data points represent means ± SE of measurements in aortic strips from 5–15 rats.

Fig. 5. \textsuperscript{45}Ca\textsuperscript{2+} influx-stress relationship during activation of rat aortic strips with 10^{-5} M phenylephrine or 96 mM KCl in virgin (A), virgin + L-NAME (B), pregnant (C), and pregnant + L-NAME (D) rats. Data points represent means ± SE of measurements in aortic strips from 5–15 rats.
DISCUSSION

The main findings of the present study are that 1) the vascular reactivity to phenylephrine and 96 mM KCl at increasing [Ca\(^{2+}\)]\(_e\) as well as Ca\(^{2+}\) entry from the extracellular space are reduced in pregnant rats but enhanced in pregnant rats treated with L-NAME, 2) the phenylephrine-induced Ca\(^{2+}\) influx-stress relationship is slightly greater than that induced by 96 mM KCl in pregnant rats but significantly greater in pregnant rats treated with L-NAME, and 3) the Ca\(^{2+}\) influx-stress relationship is reduced in pregnant rats but significantly enhanced in pregnant rats treated with L-NAME compared with virgin rats.

The present study showed that the contractile response to phenylephrine at increasing [Ca\(^{2+}\)]\(_e\) was reduced in pregnant rats compared with virgin rats. These results are consistent with our previous finding that the vascular reactivity to increasing concentrations of phenylephrine is reduced in pregnant rats (12). Also, we previously found no significant difference between the different groups of rats in the phenylephrine concentration-response curve when the contraction was presented as percentage of the maximum, suggesting that the observed reduced vascular reactivity in pregnant rats may not be due to a change in the \(\alpha\)-adrenergic receptor sensitivity to phenylephrine but rather due to inhibition of a signaling mechanism downstream from receptor activation.

It is generally accepted that activation of \(\alpha\)-adrenergic receptors by agonists such as phenylephrine causes activation of phospholipase C and increases the hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (5). IP\(_3\) stimulates Ca\(^{2+}\) release from intracellular stores (25), and diacylglycerol stimulates protein kinase C (20). In addition, \(\alpha\)-adrenergic agonists enhance Ca\(^{2+}\) entry through the plasma membrane Ca\(^{2+}\) channels (15).

We have previously found that the transient phenylephrine contraction in Ca\(^{2+}\)-free solution is not significantly changed in pregnant rats compared with virgin rats, suggesting that the reduced vascular reactivity observed in pregnant rats is not due to changes in Ca\(^{2+}\) uptake or Ca\(^{2+}\) release from intracellular stores (12). On the other hand, the present results showed that the phenylephrine-induced Ca\(^{2+}\) entry into vascular smooth muscle was reduced in pregnant rats. To investigate the possible Ca\(^{2+}\) entry pathways that might be involved, we compared the phenylephrine-induced response with that induced by high KCl. Membrane depolarization by high-KCl solution is known to stimulate Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels. The present results showed that the 96 mM KCl response at all [Ca\(^{2+}\)]\(_e\) tested was reduced in pregnant rats, providing evidence that Ca\(^{2+}\) entry from the extracellular space into vascular smooth muscle may be reduced. We also found that the 96 mM KCl-induced Ca\(^{2+}\) influx in aortic strips from pregnant rats was reduced, suggesting that Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels may be reduced during pregnancy. However, the present results cannot exclude the possibility that other types of Ca\(^{2+}\) channels may also be involved. These Ca\(^{2+}\) channels have been described in several types of smooth muscle and have been termed receptor-operated Ca\(^{2+}\) channels (27).

The present study showed that phenylephrine-induced contraction at all [Ca\(^{2+}\)]\(_e\) tested was significantly enhanced in pregnant rats treated with L-NAME compared with that in pregnant and virgin rats. It has been hypothesized that an increase in the production of NO during pregnancy (1) leads to a decrease in peripheral resistance and vascular reactivity (23, 24). 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, suggesting that treatment of pregnant rats with L-NAME not only...
blocks the synthesis of NO by endothelial cells but may also increase the synthesis of or sensitivity to other vasoactive contractile compounds. 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 vasodilators such as NO or prostacyclin or, more importantly, increased production of vasoconstrictor factors such as endothelin (3, 4, 17, 21). 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 (10).

The signaling mechanisms of the observed increase in vascular reactivity to phenylephrine in pregnant rats treated with L-NAME can possibly involve increased Ca\(^{2+}\) release from intracellular stores and/or enhanced Ca\(^{2+}\) entry from the extracellular space. We have recently reported that the enhanced vascular reactivity observed in pregnant rats treated with L-NAME is not due to changes in Ca\(^{2+}\) uptake or Ca\(^{2+}\) release from intracellular stores (12). To test the possible role of Ca\(^{2+}\) entry, we compared the phenylephrine response with that induced by high KCl and found that the apparent reduction in the high-KCl-induced contraction was corrected to levels not distinguishable from those in the virgin rats when the pregnant rats were chronically treated with L-NAME. We also measured Ca\(^{2+}\) influx in aortic strips from pregnant rats treated with L-NAME and found that both phenylephrine and 96 mM KCl caused significant stimulation of Ca\(^{2+}\) influx, suggesting that Ca\(^{2+}\) entry through excitable Ca\(^{2+}\) channels is enhanced.

The present study showed that in pregnant rats simultaneously treated with L-NAME and L-arginine the systolic blood pressure and the phenylephrine- and 96 mM KCl-induced vascular reactivity and Ca\(^{2+}\) entry were significantly reduced compared with the L-NAME-treated pregnant rats, and that these measurements were at levels not significantly different from those observed in the untreated pregnant rats. These data provide evidence that the increased systolic blood pressure and the enhanced vascular reactivity and Ca\(^{2+}\) entry in the L-NAME-treated pregnant rats are reversible and thus lend support to the contention that the enhanced responses may be due to L-NAME treatment.

Although the present results suggest that the enhanced Ca\(^{2+}\) entry in L-NAME-treated pregnant rats may be related to chronic inhibition of NO synthesis, these results should be interpreted with caution because the enhanced Ca\(^{2+}\) entry may also be secondary to blood pressure elevation. Several studies have shown that Ca\(^{2+}\) entry into vascular smooth muscle is enhanced in nonpregnant hypertensive rat models (15). These studies have not provided definite answers on whether the increase in Ca\(^{2+}\) entry is the cause or the consequence of increased blood pressure. However, studies in cultured aortic smooth muscle cells from the spontaneously hypertensive rat model, in which the effects of high blood pressure on intracellular Ca\(^{2+}\) in vivo are expected to disappear during cell culture, have shown that the intracellular Ca\(^{2+}\) is still elevated in these cells (26). These studies suggested that the elevated intracellular Ca\(^{2+}\) in the spontaneously hypertensive rat model is genetically regulated and may constitute one of the mechanisms rather than the consequence of blood pressure elevation.

The present results suggest that the enhanced vascular reactivity in pregnant rats treated with L-NAME may involve stimulation of Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels because 1) the contractile response to high KCl, a known activator of voltage-gated Ca\(^{2+}\) channels, was enhanced, and 2) the high-KCl-induced Ca\(^{2+}\) entry was increased. However, the present results cannot exclude the possibility that other types of Ca\(^{2+}\) channels, such as the receptor-operated Ca\(^{2+}\) channels (27), may also be involved. The present results also showed that in all groups of rats the phenylephrine-induced Ca\(^{2+}\) influx-stress relationship is located to the left of that induced by 96 mM KCl. Assuming that the depolarization-induced contraction is mainly due to stimulation of Ca\(^{2+}\) entry, the enhanced phenylephrine response could be due to activation of other contractile mechanisms in addition to Ca\(^{2+}\) entry (8). These possible mechanisms may include the following: 1) phenylephrine may inhibit Ca\(^{2+}\) extrusion mechanisms such as the plasmalemmal Ca\(^{2+}\) pump and the Na\(^+\)/Ca\(^{2+}\) exchanger or Ca\(^{2+}\) uptake by the sarcoplasmic reticulum Ca\(^{2+}\) pump, 2) phenylephrine may disrupt superficially located Ca\(^{2+}\) buffering systems and thus allow more Ca\(^{2+}\) to be available for the myofilaments to cause contraction (28), or 3) phenylephrine may increase the myofilament force sensitivity to Ca\(^{2+}\) or perhaps stimulate a completely Ca\(^{2+}\)-independent pathway. For example, phenylephrine may activate protein kinase C through increased formation of diacylglycerol (2, 13). Because one or more of these mechanisms could contribute to the observed shift in the phenylephrine Ca\(^{2+}\) influx-stress relationship compared with that of 96 mM KCl, we suggest that one or more of these additional contractile mechanisms may be significantly stimulated in pregnant rats treated with L-NAME compared with other groups of rats.

To further investigate the possible contribution of mechanisms other than Ca\(^{2+}\) entry to the pregnancy-associated changes in vascular reactivity, we compared the relationship between Ca\(^{2+}\) entry and active stress in pregnant and virgin rats. If the pregnancy-associated changes in active stress are merely due to changes in Ca\(^{2+}\) entry, then one would not expect the Ca\(^{2+}\) entry-active stress relationship in pregnant rats treated with L-NAME to be different from that in virgin rats. The present study showed that, regardless of the type of stimulant, the Ca\(^{2+}\) influx-stress relationship was enhanced in pregnant rats treated with L-NAME compared with pregnant rats. These data lend further support to the contention that other contractile mechanisms in addition to stimulation of Ca\(^{2+}\) entry are enhanced in pregnant rats treated with L-NAME. The rebound increase in vascular reactivity to phenylephrine in the pregnant rats treated with L-NAME above...
that in the virgin rats can be explained by the possibility that phenylephrine may further activate these additional contractile mechanisms. It is also important to note that the Ca\(^{2+}\) entry-stress relationship appeared to be reduced in pregnant rats regardless of the type of stimulant, suggesting that these additional contractile mechanisms may be inhibited during pregnancy.

In conclusion, Ca\(^{2+}\) entry into vascular smooth muscle appears to be reduced during pregnancy and significantly enhanced during inhibition of NO production in late pregnancy. The results suggest that the reduced vascular reactivity during normal pregnancy is in part due to reduction of Ca\(^{2+}\) entry. Likewise, the enhanced vascular reactivity observed in the pregnant rats during chronic inhibition of NO synthesis can possibly be related to the stimulated Ca\(^{2+}\) entry from extracellular space observed under the same conditions. Also, the enhanced Ca\(^{2+}\) influx-stress relationships with inhibition of NO synthesis suggest activation of other contractile mechanisms in addition to stimulation of Ca\(^{2+}\) entry. These mechanisms appear to be inhibited during normal pregnancy. Further studies are needed to investigate the changes in these additional contractile mechanisms during pregnancy.

This work was supported by National Heart, Lung, and Blood Institute (HL61971 and HL-33849 (J. P. Granger) and grants from the University of Mississippi Medical Center, the American Health Assistance Foundation, the American Heart Association (Grant-in-Aid, Mississippi Affiliate), and the NHLBI (HL-52686, R. A. Khalil).

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Received 3 JUne 1998; accepted in final form 21 October 1998.

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