Ca$^{2+}$-Insensitive Vascular Protein Kinase C During Pregnancy and NOS Inhibition

Celia A. Kanashiro, Barbara T. Alexander, Joey P. Granger, Raouf A. Khalil

**Abstract**—Pregnancy-induced hypertension is associated with increased vascular resistance; however, the cellular mechanisms involved are unclear. We have previously found that the relation between Ca$^{2+}$ entry and the developed force in vascular smooth muscle is altered during normal pregnancy and in a rat model of pregnancy-induced hypertension produced by long-term treatment with the nitric oxide synthase inhibitor $N^\omega$-nitro-$L$-arginine methyl ester (L-NAME). The purpose of this study was to investigate whether the pregnancy-associated changes in the vascular Ca$^{2+}$ entry-force relation reflect changes in the amount and/or activity of Ca$^{2+}$-insensitive protein kinase C (PKC) isoforms. Active stress and the amount and activity of PKC were measured in deendothelialized aortic strips from nonpregnant and pregnant rats untreated or treated with L-NAME and incubated in Ca$^{2+}$-free (2 mmol/L EGTA) Krebs solution. In nonpregnant rats, the PKC activator phorbol 12,13-dibutyrate (PDBu, $10^{-6}$ mol/L) and the $\alpha$-adrenergic agonist phenylephrine (Phe, $10^{-5}$ mol/L) caused significant, maintained increases in active stress and PKC activity that were inhibited by the PKC inhibitors staurosporine and calphostin C. Western blots in aortic strips of nonpregnant rats revealed the Ca$^{2+}$-insensitive $\delta$-PKC and $\xi$-PKC isoforms. Both PDBu and Phe caused translocation of $\delta$-PKC from the cytosolic to the particulate fraction. Compared with nonpregnant rats, the amount of $\delta$-PKC and $\xi$-PKC and the PDBu-stimulated and Phe-stimulated stress, PKC activity and translocation of $\delta$-PKC were significantly reduced in late pregnant rats but significantly enhanced in pregnant rats treated with L-NAME. The PDBu-induced and Phe-induced responses in nonpregnant rats treated with L-NAME were not significantly different from nonpregnant rats, whereas the responses in pregnant rats treated with L-NAME and pregnant rats treated with L-NAME and L-arginine were not significantly different from pregnant rats. These results provide evidence that a signaling pathway in vascular smooth muscle possibly involving the Ca$^{2+}$-insensitive $\delta$-PKC and $\xi$-PKC isoforms is reduced in late pregnancy and enhanced during long-term inhibition of nitric oxide synthesis. The changes in the amount and activity of vascular PKC isoforms may, in part, explain the changes in vascular resistance during normal pregnancy and pregnancy-induced hypertension. (Hypertension. 1999;34[part 2]:924-930.)

**Key Words:** nitric oxide ■ muscle, smooth, vascular ■ hypertension, pregnancy ■ preeclampsia

Normal pregnancy is characterized by several hemodynamic changes including increased renal, uterine, and feto-placental blood flow,\textsuperscript{1,2} decreased vascular resistance, and reduced pressor responses to circulating vasoconstrictor agents.\textsuperscript{3,4} These hemodynamic changes have been attributed in part to increased nitric oxide (NO) synthesis by many cell types including vascular endothelial cells.\textsuperscript{5} This is supported by a recent report that the metabolic production and plasma level of cGMP, a second messenger of NO and a cellular mediator of vascular smooth muscle relaxation, are increased during pregnancy.\textsuperscript{6}

In ≈5% to 10% of all pregnancies, women have a condition called preeclampsia, characterized by severe edema, proteinuria, vascular endothelial damage, generalized vasoconstriction, increased vascular resistance, and pregnancy-induced hypertension.\textsuperscript{7,8} The exact mechanism of pregnancy-induced hypertension is unclear, and several mechanisms have been suggested including reduction of NO synthesis by endothelial cells.\textsuperscript{9} We and others have shown that long-term inhibition of NO synthase (NOS) in rats during mid to late pregnancy is associated with many pathological changes similar to those observed in women with pregnancy-induced hypertension.\textsuperscript{10-12} We have also shown that the vascular reactivity and Ca$^{2+}$ entry in response to the $\alpha$-adrenergic agonist phenylephrine (Phe) are decreased in pregnant rats and increased in pregnant rats treated with the NOS inhibitor $N^\omega$-nitro-$L$-arginine methyl ester (L-NAME) when compared with nonpregnant rats.\textsuperscript{13,14} Furthermore, we have reported that the relation between Ca$^{2+}$ entry and force in vascular smooth muscle is decreased in pregnant rats and increased in pregnant rats treated with L-NAME and suggested the involvement of other signaling pathways in addition to Ca$^{2+}$ entry such as an increase in the myofilament force sensitivity to Ca$^{2+}$ or perhaps activation of a Ca$^{2+}$-independent pathway.\textsuperscript{13,14}
KAlishiro et al Vascular Protein Kinase C in Pregnancy 925

In numerous cell types including vascular smooth muscle, the interaction of the agonist with its receptor is coupled to increased breakdown of the plasma membrane phospholipids and increased production of diacylglycerol (DAG)\textsuperscript{15,16}. DAG binds to and activates the enzyme protein kinase C (PKC). PKC is mainly cytosolic under resting conditions and undergoes translocation from the cytosolic to the particulate fraction when the cells are activated by DAG or phorbol esters\textsuperscript{15,16}. Also, direct activation of PKC by phorbol esters causes sustained contraction of vascular smooth muscle\textsuperscript{17} with no significant change in [Ca\textsuperscript{2+}]\textsubscript{i}.\textsuperscript{18} These reports have suggested a role for PKC in regulating the contractile responses of vascular smooth muscle by increasing the myofilament force sensitivity to Ca\textsuperscript{2+} or perhaps activating a completely Ca\textsuperscript{2+}-independent contractile pathway.

PKC is now known to be a family of several isoforms that have different enzyme properties, substrates, and functions and exhibit different subcellular distributions in the same blood vessel from different species and in different vessels from the same species\textsuperscript{15,16,19,20}. Although the changes in PKC activity have been well characterized in blood vessels of normal male rats and ferrets\textsuperscript{19,20}, it is not clear whether the changes in vascular reactivity observed during pregnancy or pregnancy-induced hypertension are associated with changes in the amount and/or activity of specific Ca\textsuperscript{2+}-dependent PKC isoforms in vascular smooth muscle. Active stress as well as the amount, distribution, and activity of Ca\textsuperscript{2+}-insensitive PKC isoforms were measured in rat aortic strips isolated from nonpregnant and pregnant rats untreated or treated with L-NAME. The effects of the \alpha-adrenergic agonist Phe were compared with direct activation of PKC by phorbol esters.

Methods

Animals

Female Sprague-Dawley rats (10 to 12 weeks, Harlan) were housed individually. Nonpregnant rats were either untreated (n=24) or treated with L-NAME (n=24). Pregnant rats were studied at day 6 or early pregnancy (n=8), day 13 or mid pregnancy (n=8), day 19 to 21 or late pregnancy (n=32), and 3 days postpartum (n=8). Other late pregnant rats were treated with L-NAME (n=32) or with L-NAME and l-arginine (n=16). The first day of pregnancy was verified by the presence of sperm in vaginal smears (full term is 21 days). All procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee and the American Physiological Society.

Protocol for L-NAME Treatment

Pregnant and nonpregnant rats in the untreated groups received drinking water. Pregnant and nonpregnant rats in the treated groups received 4 mg/kg per day L-NAME (Sigma). This dose resulted in significant elevation of blood pressure in pregnant rats while having minimal effect in nonpregnant rats.\textsuperscript{11,13,14,22} L-NAME treatment in pregnant rats started at day 15 of gestation and continued for 4 to 6 days before the rats were killed and tissues harvested at day 19 to 21 of gestation. Some L-NAME–treated pregnant rats simultaneously received 80 mg/kg per day l-arginine (Sigma) in drinking water for the same period of time. Because water intake in pregnant rats was 2 times that in nonpregnant rats, the amount of L-NAME in the drinking water was adjusted to maintain a daily dose of 4 mg/kg per day in both the pregnant and nonpregnant rats. L-Arginine did not significantly affect the amount of drinking water in pregnant rats. Therefore the amount of L-NAME the animals ingested was similar between pregnant rats treated with L-NAME and pregnant rats treated with L-NAME plus l-arginine.

Isometric Tension

Rats were anesthetized by inhalation of isoflurane. The thoracic aorta was removed, placed in oxygenated Krebs solution, cleaned of connective tissue, and cut into 3-mm-wide strips; the endothelium was removed by rubbing with forceps. One end of the strip was attached to a glass hook and the other end was connected to a Grass force transducer (FT03, Astro-Med). Aortic strips were stretched to L\textsubscript{max} (1.5 times their initial unloaded length) and allowed to equilibrate for 1 hour in oxygenated Krebs solution at 37°C. The changes in isometric tension were recorded on a Grass 7D polygraph. Removal and/or dysfunction of the endothelium-dependent NO-releasing pathway (in rats given long-term treatment with L-NAME) was verified by the absence of acetylcholine-induced (10\textsuperscript{-7} mol/L) vasorelaxation in aortic strips precontracted with Phe (3\texttimes 10\textsuperscript{-7} mol/L).

Tissue Fractions

Deendothelialized aortic strips were homogenized, then centrifuged at 10 000g for 2 minutes; the supernatant was used as whole tissue fraction. Other tissues were stimulated with phorbol 12,13-dibutyrate (PDBu, 10\textsuperscript{-6} mol/L) or Phe (10\textsuperscript{-7} mol/L) in Ca\textsuperscript{2+}-free Krebs solution for 30 minutes, transferred to ice-cold equilibrating buffer A, homogenized in homogenizing buffer B, and centrifuged at 100 000 rpm for 20 minutes; the supernatant was used as the cytosolic fraction. The pellet was resuspended in homogenizing buffer containing 1% Triton X-100 and centrifuged at 100 000 rpm for 20 minutes; the supernatant was used as the particulate fraction.\textsuperscript{22} Protein concentrations were determined with the use of a protein assay kit (Bio-Rad).

PKC Activity

The cytosolic and particulate fractions were applied to diethylaminooethyl cellulose columns (0.8 × 4.0 cm; Bio-Rad) and the protein was eluted with 0.1 mol/L NaCl. PKC activity in the aliquots was determined by measuring the incorporation of \textsuperscript{32}P from [\gamma\textsuperscript{32}P]ATP (ICN) into histone II\textsubscript{S}.\textsuperscript{22} The assay mixture contained 25 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl\textsubscript{2}, 200 \mu g/mL histone II\textsubscript{S}, 80 \mu g/mL PS, 30 \mu g/mL diolein, [\gamma\textsuperscript{32}P]ATP (1 to 3 \times 10\textsuperscript{4} cpm/nmol), and 0.5 to 3 \mu g protein. After 5 minutes of incubation at 30°C, the reaction was stopped by spotting 25 \mu L of the assay mixture onto phosphocellulose disks. The disks were washed 3 × 5 minutes with 5% trichloroacetic acid and placed in 4 mL of Ecolite scintillation cocktail; the radioactivity was measured in a liquid scintillation counter.

Immunoblotting

Protein-matched samples of the whole tissue and cytosolic and particulate fractions were subjected to electrophoresis on 8% SDS-polyacrylamide gels, then transferred electrophoretically to nitrocellulose membranes. The membranes were incubated in 5% dried milk in PBS-Tween for 1 hour, then incubated in the primary anti-PKC antibody solution at 4°C overnight. Polyclonal antibodies to the Ca\textsuperscript{2+}-dependent \(\delta\)-PKC, \(\epsilon\)-PKC, \(\eta\)-PKC, and \(\zeta\)-PKC (Gibco) were used. We used the same titer of the anti-PKC antibodies (1:500) and the same protein concentration (10 \mu g). The nitrocellulose membranes were washed 5 × 15 minutes, then incubated in horseradish peroxidase–conjugated anti-rabbit secondary antibody for 1.5 hours.\textsuperscript{22} The blots were washed 5 × 15 minutes and visualized with an ECL detection system (Amersham). The reactive bands corresponding to PKC isoforms were analyzed with the use of an optical densitometer (GS-700, Bio-Rad).
eliminate the contribution of Ca2+ at pH 7.4. For Ca2+-free Krebs solution, CaCl2 was omitted and replaced with 2 mmol/L EGTA. Equilibrating buffer A contained (in mmol/L) Tris-HCl 25 (pH 7.5), EGTA 5, leupeptin 0.02, phenylmethylsulfonyl fluoride 0.2, and dithiothreitol 1. Homogenizing buffer B had the same composition as buffer A plus sucrose 250 mmol/L. Phe (Sigma) was prepared in distilled water. PDBu and 4-α-PDBu (Alexis Laboratory), staurosporine, and calphostin C (Kamiya) were dissolved in DMSO to form a stock solution of 10−3 mol/L. The final concentration of DMSO in solution was 0.1%. All other chemicals were of reagent grade or better.

Statistical Analysis
The developed force was normalized for the cross-sectional area of the aortic strip and expressed as active stress (N/m²) by use of 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 are presented as mean ± SEM. Data were analyzed and compared by use of the Student’s t test for unpaired data, with a value of P < 0.05 considered significant.

Results
We first investigated whether vascular PKC activity changes during different stages of pregnancy. In aortic strips of nonpregnant rats, the basal PKC activity was significantly greater in the cytosolic fraction than in the particulate fraction (Figure 1A). Also, in nonpregnant rats, PDBu (10−6 mol/L) and Phe (10−5 mol/L) caused significant increases in PKC activity in the particulate fraction and a concomitant decrease in the cytosolic fraction (Figure 1B and C). During all stages of pregnancy, the basal PKC activity in the cytosolic fraction was significantly greater than that in the particulate fraction (Figure 1A). However, during late pregnancy, the basal PKC activity in both the cytosolic and particulate fractions was significantly reduced when compared with that during early and mid pregnancy (Figure 1A). At different stages of pregnancy, treatment with PDBu (Figure 1B) or Phe (Figure 1C) caused a significant increase in PKC activity in the particulate fraction and a concomitant decrease in the cytosolic fraction. However, during late pregnancy, the PDBu-induced and Phe-induced PKC activity was significantly reduced when compared with that during early and mid pregnancy (Figure 1, B and C). In the postpartum rats, the total PKC activity and the relative PKC activity in the particulate fraction compared with the cytosolic fraction were not significantly different from that in nonpregnant rats (Figure 1).

We tested the effect of long-term L-NAME treatment in nonpregnant and late pregnant rats on vascular reactivity and PKC activity (Figure 2). In aortic strips of nonpregnant rats incubated in Ca2+-free (2 mmol/L EGTA) Krebs solution to eliminate the contribution of Ca2+ entry from the extracellular space to tissue contraction, PDBu (10−3 mol/L) caused a slow increase in active stress that reached a maximum of 5.1 ± 0.65 × 103 N/m² (n = 8) in ∼30 minutes. Also, in Ca2+-free Krebs solution, Phe (10−5 mol/L) caused a transient increase in active stress to 3.23 ± 0.4 (n = 8) followed by a smaller but maintained increase in stress to 2.22 ± 0.3 × 103 N/m² (n = 8) for at least 30 minutes. The transient Phe-induced stress was not significantly different among the different groups of rats.

On the other hand, the PDBu-induced and Phe-induced maintained increases in stress were significantly reduced in late pregnant rats but significantly enhanced in pregnant rats treated with L-NAME compared with nonpregnant rats (Figure 2, A and B). The PDBu-induced and Phe-induced maintained stress in nonpregnant rats treated with L-NAME was not significantly different from that in nonpregnant rats, whereas the stress in pregnant rats simultaneously treated with L-NAME and L-arginine was not significantly different from that in pregnant rats. No significant changes in active stress were observed in tissues treated with the inactive 4-α-PDBu.

We tested the effect of 2 chemically unrelated PKC inhibitors with 2 different sites of action in the PKC molecule. Staurosporine is known to interact with the catalytic domain of PKC at the ATP binding site, whereas calphostin C is known to interact with the regulatory domain of PKC at the DAG/phorbol ester binding site. In all groups of rats, pretreatment of the aortic strips with 10−7 mol/L staurosporine or calphostin C for 10 minutes completely abolished the
contractile responses induced by PDBu in Ca²⁺-free Krebs solution (Figure 2A). The PKC inhibitors did not affect the transient Phe contraction but completely inhibited the maintained Phe contraction in Ca²⁺-free Krebs solution (Figure 2B).

We measured the effects of long-term treatment with L-NAME on the PKC activity of aortic smooth muscle by measuring the changes in the particulate/cytosolic (P/C) PKC activity ratio. In nonpregnant rats, the basal P/C PKC activity ratio was 0.51 ± 0.05 (n=8). PDBu (10⁻⁶ mol/L) and Phe (10⁻⁵ mol/L) caused significant increases in the P/C PKC activity ratio (Figure 2C). Compared with nonpregnant rats, the basal and PDBu-stimulated and Phe-stimulated P/C PKC activity were significantly reduced in late pregnant rats but significantly increased in pregnant rats treated with L-NAME. The basal and PDBu-induced and Phe-induced changes in PKC activity in nonpregnant rats treated with L-NAME were not significantly different from that in nonpregnant rats, whereas the PKC activity in pregnant rats simultaneously treated with L-NAME and L-arginine was not significantly different from that in pregnant rats (Figure 2C).

Immunoblots were performed in the tissue samples with the use of primary antibodies specific to the Ca²⁺-independent δ-PKC, ε-PKC, η-PKC, and ζ-PKC isoenzymes, respectively (Figure 3). The specificity of the δ-PKC and ζ-PKC reactive bands was confirmed by the loss of immunoreactive signal in the presence of specific synthetic peptide to which the antibody was raised. No significant immunoreactive bands were detected with antibodies to ε-PKC or η-PKC isoforms. In nonpregnant rats, the optical density (OD)/µg protein for δ-PKC and ζ-PKC was 0.11±0.01 (n=8) and 0.16±0.01 (n=8), respectively. The OD/µg protein for δ-PKC and ζ-PKC was significantly reduced in late pregnant rats but significantly increased in pregnant rats treated with L-NAME. On the other hand, the OD/µg protein for δ-PKC and ζ-PKC in pregnant rats simultaneously treated with L-NAME and L-arginine was not significantly different from that in pregnant rats (Figure 3).
Immunoblots for the internal control protein β-actin did not show any significant difference in tissue samples from the different groups of rats.

In unstimulated tissues from late pregnant rats, the OD/μg protein for δ-PKC was greater in the cytosolic than in the particulate fraction (Figure 4A). In contrast, in unstimulated tissues from pregnant rats treated with L-NAME, the amount of δ-PKC in both fractions was significantly increased and the distribution of δ-PKC in the particulate fraction was significantly greater than that in the cytosolic fraction (Figure 4A). In unstimulated tissues from pregnant rats treated with L-NAME plus L-arginine, the amount and distribution of δ-PKC in both fractions was not significantly different from that in pregnant rats. In pregnant rats, PDBu (Figure 4B) and Phe (Figure 4C) caused a <2-fold increase in the distribution of δ-PKC in the particulate fraction compared with the cytosolic fraction. In contrast, PDBu and Phe caused a >3-fold increase in δ-PKC in the particulate fraction compared with the cytosolic fraction in pregnant rats treated with L-NAME. The PDBu-induced and Phe-induced redistribution of δ-PKC in pregnant rats treated with L-NAME plus L-arginine was not significantly different from that in pregnant rats. Compared with δ-PKC, ζ-PKC was equally distributed between the cytosolic and particulate fraction in pregnant rats, pregnant rats treated with L-NAME, and pregnant rats treated with L-NAME plus L-arginine both under basal conditions (Figure 4A) and during stimulation with PDBu (Figure 4B) or Phe (Figure 4C).

**Figure 4.** Distribution of δ-PKC and ζ-PKC isoforms in aortic strips of pregnant rats under basal conditions (A) and during stimulation with 10^−6 mol/L PDBu (B) or 10^−5 mol/L Phe (C). Cytosolic (Cyt) and particulate (Part) fractions of aortic strips were prepared for Western blot analysis with anti-δ-PKC and anti-ζ-PKC antibodies (Gibco 1:500). Data bars represent mean±SEM of measurements in aortic strips from 8 rats of each group.

**Discussion**

The current study showed that in aortic smooth muscle of nonpregnant rats incubated in Ca^2+^-free solution, PDBu caused significant contraction. Phorbol esters are known to bind to and increase PKC activity; therefore the observed PDBu-evoked contraction suggests activation of PKC. This is supported by the finding that 2 chemically unrelated PKC inhibitors caused complete inhibition of the phorbol ester–induced contraction and that PDBu caused a significant increase in PKC activity. Also, in aortic smooth muscle of nonpregnant rats, the α-adrenergic agonist Phe caused a significant, maintained contraction in Ca^2+^-free solution that was associated with a significant increase in PKC activity; these responses were significantly inhibited by the PKC inhibitors staurosporine and calphostin C at the same concentrations that completely inhibited the phorbol ester–induced responses. These results are consistent with other reports and suggest that PKC is involved in the maintained Phe-induced contraction of rat aortic smooth muscle in Ca^2+^-free solution.

We found that the PDBu-induced and Phe-induced contractions were smaller in aortic strips from late pregnant rats compared with nonpregnant rats. We also observed that the basal and PDBu-induced and Phe-induced PKC activity were significantly reduced during late pregnancy as compared with nonpregnant rats. These results suggest that the decrease in the rat aortic vascular reactivity to PDBu and Phe during late pregnancy is associated with a decrease in the amount and/or activity of PKC.

The observed changes in vascular reactivity and PKC activity during pregnancy could be, in part, due to changes in eNOS activity and NO production by the endothelium in vivo. This is supported by reports that NO production by many cell types including endothelial cells is increased during late pregnancy, leading to a decrease in vascular reactivity, perhaps through increased formation of cGMP in vascular smooth muscle. It has also been reported that NO causes reversible inactivation of PKC either directly through the formation of disulfide bridges with the PKC molecule or indirectly through the inhibition of membrane phospholipid breakdown and DAG production. Other studies have also shown that NO, by increasing cGMP formation, inhibits the activation of PKC and the PKC-mediated contractions by endothelin in rat aorta by mechanisms involving inhibition of phospholipid metabolism and DAG production. Thus the pregnancy-associated changes in vascular reactivity and PKC activity could be related, at least in part, to the increased NO production and cGMP formation that we and others reported to occur during late pregnancy.

On the basis of these premises, one
would predict that the blockade of NO production during late pregnancy would bring the vascular reactivity and PKC activity back to the levels observed in nonpregnant rats. However, we observed that the PDBu-induced and Phe-induced contractions as well as the basal and PDBu-induced and Phe-induced PKC activity in pregnant rats treated with L-NAME were significantly greater than that in nonpregnant rats or nonpregnant rats treated with L-NAME. These results suggest that treatment of pregnant rats with L-NAME not only inhibits NO synthesis but may alter the synthesis of or sensitivity to other vasoactive compounds that would increase the amount and/or activity of PKC. It has been suggested that the reduction in the placental blood flow during pregnancy is associated with placental release of cytotoxic factors that alter the function of many cell types including endothelial cells, leading to reduction in the synthesis of vasodilators such as NO or prostacyclin or, more importantly, increased production of vasoconstrictor factors such as thromboxane or endothelin.11–13 This is consistent with the report that long-term inhibition of NO synthesis during mid to late gestation in rats is associated with elevated plasma levels of endothelin-1.31 Because the current study was performed on deendothelialized aortic strips, the observed pregnancy-associated changes in vascular reactivity and PKC activity should represent additional pregnancy-induced changes in the cellular mechanisms of vascular smooth muscle contraction independent of eNOS activity and NO production by the endothelium.

The immunoblot analysis showed that significant amounts of δ-PKC and both phorbol esters and Phe caused significant translocation of δ-PKC in aortic smooth muscle of nonpregnant rats. These results are consistent with other reports that have shown significant amounts of δ-PKC in the aorta of male rats19 and suggest that this PKC isoform may be involved in the phorbol ester–induced and Phe–induced contraction. Interestingly, the amount of δ-PKC was reduced in late pregnant rats but significantly increased in pregnant rats treated with L-NAME. Also, under resting conditions, δ-PKC was equally distributed between the cytosolic and the particulate fraction in late pregnant rats but mainly distributed in the particulate fraction in pregnant rats treated with L-NAME. In addition, the phorbol ester–induced and Phe–induced translocations of δ-PKC were reduced in pregnant rats but significantly enhanced in pregnant rats treated with L-NAME. These results suggest that the reduction in vascular reactivity in pregnant rats and its enhancement during inhibition of NO synthesis is related, in part, to underlying changes in the amount and activity of the δ-PKC isoform in vascular smooth muscle. The causes of the pregnancy-associated changes in the amount and activity of δ-PKC are not clear but could be related to changes in the rate of phospholipid turnover and DAG production in vascular smooth muscle and should represent important areas for future investigations.

The current study showed that compared with the L-NAME–treated pregnant rats, in pregnant rats simultaneously treated with L-NAME and L-arginine, the PDBu-induced and Phe-induced contraction and the activation and translocation of δ-PKC were significantly reduced to levels not significantly different from those observed in the untreated pregnant rats. These data provide evidence that the PDBu-induced and Phe-induced contraction as well as the activation and translocation of δ-PKC in the L-NAME–treated pregnant rats are reversible and thus lend support to the contention that the enhanced responses may be due to inhibition of the L-arginine–NO pathway.

We also found that the amount of ζ-PKC was reduced in pregnant rats and increased in pregnant rats treated with L-NAME. The observation that ζ-PKC did not show significant redistribution with phorbol ester or Phe is consistent with the reports that ζ-PKC lacks the phorbol ester/DAG binding site15,16 and thus provided a control experiment and increased the level of confidence in the immunoblot analysis. Although the absence of ζ-PKC translocation during PDBu and Phe contraction suggests that ζ-PKC might not be involved in rat aortic contraction, the significant decrease in the amount of ζ-PKC in pregnant rats and significant increase in pregnant rats treated with L-NAME suggest that ζ-PKC may still play a role in the vascular changes during pregnancy, for example, vascular smooth muscle growth. This is supported by reports that vascular ζ-PKC is localized in the vicinity of the nucleus and may be involved in cell growth and proliferation.20

It is important to note that although the observed changes in the amount and distribution of Ca2+-insensitive PKC isoforms suggest that the PKC-associated contraction is through a Ca2+-independent pathway, these results should be interpreted with caution because (1) incubation of the tissues in Ca2+-free (2 mmol/L EGTA) Krebs solution is known to completely abolish Ca2+ entry from the extracellular space; however, the possibility of slow release of finite amounts of Ca2+ from the intracellular Ca2+ stores cannot be ruled out under these experimental conditions. Further experiments are needed to investigate whether similar results can be obtained in tissues loaded with intracellular Ca2+ chelators such as BAPTA. (2) We have previously found that Ca2+ mobilization into vascular smooth muscle through the Ca2+ entry pathway is reduced during pregnancy and increased during inhibition of NO synthesis in late pregnant rats. Whether the pregnancy-associated changes in intracellular Ca2+ mobilization in vascular smooth muscle affect the content and activity of Ca2+-dependent PKC isoforms is currently unknown and should represent an important area for future investigation.

Last, because the current study was performed on strips of thoracic aorta, we cannot make a definite conclusion on whether the observed changes in vascular reactivity and PKC activity also occur in resistance vessels, which should represent an important area for future investigation.

In conclusion, a PKC-mediated contractile pathway in vascular smooth muscle is reduced during pregnancy and significantly enhanced in pregnant rats pretreated with the NOS inhibitor L-NAME. The results suggest that the pregnancy-associated changes in vascular reactivity may reflect changes in the amount and activity of the Ca2+-independent δ-PKC and ζ-PKC isoforms. The changes in the amount and activity of vascular PKC isoforms may, in part, explain the changes in vascular resistance during normal pregnancy and pregnancy-induced hypertension.
Acknowledgments

This work was supported by grants from the American Heart Association (grant-in-aid) and the National Heart, Lung, and Blood Institute (HL-52696, HL-33849, and HL-51971).

References
Ca²⁺-Insensitive Vascular Protein Kinase C During Pregnancy and NOS Inhibition
Celia A. Kanashiro, Barbara T. Alexander, Joey P. Granger and Raouf A. Khalil

_Hypertension_. 1999;34:924-930
doi: 10.1161/01.HYP.34.4.924

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/34/4/924

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/