Pregnancy Downregulates Actin Polymerization and Pressure-Dependent Myogenic Tone in Ovine Uterine Arteries

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Abstract—Pregnancy is associated with significantly decreased uterine vascular tone and increased uterine blood flow. The present study tested the hypothesis that the downregulation of actin polymerization plays a key role in reduced vascular tone of uterine arteries in the pregnant state. Uterine arteries were isolated from nonpregnant and near-term pregnant sheep. Activation of protein kinase C significantly increased the filamentous:globular actin ratio and contractions in the uterine arteries, which were inhibited by an actin polymerization inhibitor cytochalasin B. The basal levels of filamentous:globular actin were significantly higher in nonpregnant uterine arteries than those in near-term pregnant sheep. Prolonged treatment (48 hours) of nonpregnant sheep with 17β-estradiol (0.3 nmol/L) and progesterone (100.0 nmol/L) caused a significant decrease in the filamentous:globular actin. In accordance, the treatment of near-term pregnant sheep for 48 hours with an estrogen antagonist ICI 182 780 (10.0 μmol/L) and progesterone antagonist RU 486 (1.0 μmol/L) significantly increased the levels of filamentous:globular actin. Increased intraluminal pressure from 20 to 100 mm Hg resulted in an initial increase in uterine arterial diameter and vascular wall Ca2+ concentrations, followed by a decrease in the diameter at a constant steady-state level of Ca2+. Cytochalasin B blocked pressure-induced myogenic constrictions without effect on vascular wall Ca2+ levels and eliminated the differences in pressure-dependent myogenic tone between nonpregnant sheep and near-term pregnant sheep. The results indicate a key role of actin polymerization in protein kinase C–induced myogenic contractions and suggest a novel mechanism of sex steroid hormone–mediated downregulation of actin polymerization underlying the decreased myogenic tone of uterine arteries in pregnancy. (Hypertension. 2010;56:1009-1015.)

Key Words: pregnancy ▪ uterine artery ▪ steroids ▪ protein kinase C ▪ actin polymerization ▪ myogenic tone

The striking increase of uterine blood flow during pregnancy is critical both for the growth and survival of the fetus and for cardiovascular well being of the mother. The mechanisms underlying adaptation of the uteroplacental circulation to pregnancy are complex and poorly understood. Among others, during pregnancy the vascular myogenic reactivity plays an important physiological role in the regulation of uterine blood flow.1–7 Our recent studies in sheep have demonstrated that pressure-induced myogenic tone is decreased significantly in the pregnant uterine artery.7–9 In addition, we have demonstrated that protein kinase C (PKC) plays a key role in regulating uterine arterial myogenic tone, and the reduced myogenic tone in uterine arteries of pregnant sheep is primarily mediated by a decreased PKC signaling pathway.7–10

The mechanisms underlying the PKC-mediated regulation of myogenic tone remain largely unknown. Previous studies have demonstrated that PKC mediates pressure-induced myogenic tone without increasing intracellular Ca2+ concentrations or myosin light chain phosphorylation.7,11–13 This suggests a thin filament regulatory pathway. Indeed, it has been demonstrated that PKC is an upstream signal and induces actin polymerization in vascular smooth muscle cells.14–21 The polymerization of actin filaments from monomeric globular actin (G actin) to filamentous actin (F actin), occurring independent of changes in intracellular Ca2+ and myosin light chain phosphorylation, is an essential cellular event during smooth muscle contraction.22–29 Both pressure- and stretch-induced actin polymerization in vascular smooth muscle are important mechanisms underlying myogenic contractions.30–37

Little is known, however, about the effect of pregnancy on PKC-mediated actin polymerization in regulating pressure-dependent myogenic tone of uterine arteries. The present study investigated the role of actin polymerization in PKC-induced contractions of uterine arteries in nonpregnant and pregnant sheep and tested the hypothesis that actin polymerization plays a key role in pressure-induced myogenic tone in the uterine artery. In addition, we tested the hypothesis that
decreased actin polymerization contributes to the reduced myogenic tone of uterine arteries in pregnancy. Given our recent findings that sex steroid hormones play an important role in downregulating pressure-dependent myogenic tone of the uterine artery in pregnancy,8 we further tested the hypothesis that sex steroid hormones downregulate uterine artery actin polymerization.

Materials and Methods

Tissue Preparation and Treatment

As described previously,8 nonpregnant and near-term pregnant (~140 days’ gestation) sheep were anesthetized with thiobutyl and administered via the external left jugular vein. The ewes were then intubated, and anesthesia was maintained on 1.5% to 2.0% halothane in oxygen throughout surgery. An incision in the abdomen was made and the uterus exposed. The uterine arteries were isolated and removed without stretching and placed into a modified Krebs solution. For steroid hormone treatments, arterial preparations were incubated in phenol red-free DMEM with 1% charcoal-stripped FBS for 48 hours at 37°C in a humidified incubator with 5% CO2/95% air in the presence or absence of 17β-estradiol (E2; 0.3 nmol/L, Sigma), progesterone (P4; 100.0 nmol/L, Sigma), ICI 182 780 (10.0 μmol/L, Torcis Bioscience), and RU 486 (1.0 μmol/L, Sigma), as reported previously.8 The concentrations of E2, P4, ICI 182 780, and RU 486 were chosen based on the previous study showing their direct chronic effects on PKC-mediated contractions and pressure-dependent myogenic tone in the uterine artery. All of the procedures and protocols were approved by the Institutional Animal Care and Use Committee and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Measurement of Myogenic Tone

Resistance-sized uterine artery segments (~150 μm in diameter) were dissected. The arterial segments were mounted and pressurized in an organ chamber (Living Systems). Arterial diameter and vascular intracellular Ca2+ concentrations ([Ca2+]i) were measured in the same tissues loaded with the Ca2+-indicator Fura 2-acetoxyethyl ester and recorded using the SoftEdge Acquisition Subsystem (IonOptix), as described previously.28 The passive pressure–diameter relationship was conducted in Ca2+-free physiologic saline solution containing 3 mmol/L of EGTA to determine the maximum passive diameter. The following formula was used to calculate percentage of myogenic tone at each pressure step: % myogenic tone = (D1-D2)/D1 × 100, where D2 is the passive diameter in Ca2+-free physiologic saline solution (0 Ca2+ with 3.0 mmol/L of EGTA) and D1 is the active diameter with normal physiologic saline solution in the presence of extracellular Ca2+.

Contraction Studies

The fourth-generation branches of the main uterine arteries from both pregnant and nonpregnant sheep were isolated and cut into 2-mm ring segments. The PKC agonist phorbol 12,13-dibutyrate (PDBu; Sigma)–induced isometric tensions in the absence or presence of cytochalasin B (Cyt B; 10.0 μmol/L) for 20 minutes. Data are mean ± SEM of tissues from 6 animals. *P < 0.05, PDBu + Cyt B vs PDBu; †P < 0.05, pregnant vs nonpregnant animals.

Figure 1. Effect of cytochalasin B on PDBu-induced contractions. PDBu (10.0 μmol/L)-induced contractions were determined in both nonpregnant and pregnant uterine arteries in the absence or presence of cytochalasin B (Cyt B; 10.0 μmol/L for 20 minutes). Data are mean ± SEM of tissues from 6 animals. *P < 0.05, PDBu + Cyt B vs PDBu; †P < 0.05, pregnant vs nonpregnant animals.

Measurement of Actin Polymerization

F actin and G actin were separated by differential sedimentation, as described previously.27 Briefly, tissues were homogenized in 200 μL of F-action stabilization buffer and centrifuged at 100 000 g for 60 minutes at 30°C. The supernatants were collected and the pellets were resuspended in ice-cold distilled H2O with 1.0 μmol/L of cytochalasin D followed by incubation on ice for 1 hour to dissociate F actin. The resuspended pellets were then centrifuged at 2300g for 2 minutes at 4°C, and the supernatants were collected. The proteins from the first supernatant (G actin) and second supernatant (F actin) were subjected to analysis by Western immunoblotting using antiactin antibody (Sigma) to determine the filamentous-globular (F/G) actin ratio, an index of actin polymerization. In separate studies, F-actin fluorescence stained with rhodamine-phalloidin was determined in collagenase-elastase digestion mixture with gentle shaking, as described previously.25 After enzyme incubation, the dissociated cells were washed onto siliconized coverslips. After treatments, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) and permeabilized with 0.1% Triton X-100. The F actin was labeled with rhodamine-phalloidin (1:400, Molecular Probe), and the cell nuclei were stained with rhodamine-phalloidin. The F actin fluorescence index of actin polymerization was determined in freshly isolated smooth muscle cells, as described previously.25 Briefly, smooth muscle cells were freshly isolated from uterine arteries using a collagenase-elastase digestion mixture with gentle shaking, as described previously.25 After enzyme incubation, the dissociated cells were washed onto siliconized coverslips. After treatments, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) and permeabilized with 0.1% Triton X-100. The F actin was labeled with rhodamine-phalloidin (1:400, Molecular Probe), and the cell nuclei were stained with DNA-binding fluorescence dye Hoechst 33258 at room temperature. After labeling, cells were washed to remove excess label and examined using a fluorescent microscope with the SPOT digital camera.

Data Analysis

Results were expressed as mean ± SEM obtained from the number of experimental animals given. Differences were evaluated for statistical significance (P < 0.05) by ANOVA or t-test, where appropriate.

Results

Role of Actin Polymerization in PKC-Mediated Contractions

As shown in Figure 1, the PKC activator PDBu-induced contraction was significantly greater in the uterine artery of nonpregnant sheep than that in pregnant animals (67.5 ± 7.9% versus 37.0 ± 4.6% KCl maximum; P < 0.05). The actin polymerization inhibitor cytochalasin B significantly decreased PDBu-induced contractions in uterine arteries of both nonpregnant (67.5 ± 7.9% versus 26.7 ± 3.0% KCl maximum; P < 0.05) and pregnant (37.0 ± 4.6% versus 16.8 ± 3.1% KCl maximum; P < 0.05) animals and eliminated the difference in PKC-mediated contractions of uterine arteries between nonpregnant and pregnant sheep (26.7 ± 3.0% versus 16.8 ± 3.1% KCl maximum; P > 0.05). Similarly, PDBu-induced contractions were inhibited by a different inhibitor of actin polymerization, latrunculin B (67.5 ± 7.9% versus 23.6 ± 0.1% KCl maximum; P < 0.05). Consistent with the contractile results, studies with the rhodamine-phalloidin–labeled F actin demonstrated that PDBu significantly increased F-actin fluorescence density in freshly isolated uterine artery smooth muscle cells (Figure 2A). The PKC-induced actin polymerization...
Effect of Pregnancy on Actin Polymerization

As shown in Figure 3, the basal levels of the F/G actin ratio were significantly higher in uterine arteries of nonpregnant sheep than those in pregnant animals (39.2 ± 5.3 versus 5.3 ± 0.9; P < 0.05). PDBu significantly increased the levels of F/G actin in uterine arteries of both nonpregnant (75.2 ± 6.2 versus 39.2 ± 5.3; P < 0.05) and pregnant (22.9 ± 1.4 versus 5.3 ± 0.9; P < 0.05) animals. To test whether the pregnancy-mediated decrease in actin polymerization was regulated through sex steroid hormones, uterine arteries of nonpregnant sheep were treated in the absence or presence of E2β (0.3 nmol/L) and P4 (100.0 nmol/L) for 48 hours. As shown in Figure 4, the treatment with E2β and P4 significantly decreased the F/G actin ratio in uterine arteries of nonpregnant sheep (9.4 ± 0.6 versus 23.2 ± 3.5; P < 0.05). Consistently, the treatment of uterine arteries of pregnant animals with ICI 182 780 (10.0 μmol/L) plus RU 486 (1.0 μmol/L) in the presence of E2β and P4 for 48 hours significantly increased the F/G actin ratio (7.3 ± 0.4 versus 3.0 ± 0.7; P < 0.05).

Role of Actin Polymerization in Pressure-Induced Myogenic Tone

As shown in Figure 5A and 5B, increased pressure from 20 to 100 mm Hg caused an increase in vessel wall Ca2+ concentrations and an initial increase in uterine artery diameter, followed by a decrease in diameter, that is, myogenic contractions. The pretreatment of the tissue with cytochalasin B blocked the myogenic constriction without significantly changing the vessel wall Ca2+ concentrations in the uterine artery (Figure 5C and 5D). To determine the extent to which...
actin polymerization regulates pregnancy-mediated changes of myogenic tone, we measured pressure-induced myogenic tone and vessel wall Ca\(^{2+}\) concentrations in uterine arteries of both nonpregnant and pregnant animals. Figure 6 shows pressure-dependent increases in myogenic tone and vessel wall Ca\(^{2+}\) concentrations in the uterine arteries. The uterine arterial myogenic tone of nonpregnant sheep was significantly greater than that in pregnant animals. However, the pressure-induced changes of vessel wall Ca\(^{2+}\) concentrations were decreased in uterine arteries of nonpregnant as compared with pregnant animals. In both nonpregnant and pregnant sheep, pretreatment of the tissues with cytochalasin B significantly inhibited uterine arterial pressure–induced myogenic tone without affecting Ca\(^{2+}\) concentrations and eliminated the difference of pressure-dependent myogenic tone between nonpregnant and pregnant animals (Figure 6).

### Discussion

#### PKC Activation and Actin Polymerization

The present study demonstrates for the first time that activation of PKC causes actin polymerization and contractions of the uterine artery and that inhibition of actin polymerization blocks PKC-mediated contractions. Previous studies have reported that PDBu increases PKC activity and induces slow and sustained arterial contractions.\(^{39-41}\) In addition, we have demonstrated that PDBu-induced contractions of uterine arteries are independent of changes in [Ca\(^{2+}\)], or myosin light chain phosphorylation levels.\(^{38,41}\) This is consistent with other studies that demonstrated a dissociation between myosin light chain phosphorylation and development of arterial tension in response to phorbol esters.\(^{12,42,43}\) These observations suggest that, in the uterine artery, PKC-induced contractions are mediated predominantly through thin filament regulatory pathways, that is, tension development independent of changes in myosin light chain phosphorylation. The present study also demonstrates that actin filament dynamics are sensitive to PKC activation in the uterine artery. PDBu caused a decrease in the content of G actin but an increase in F actin and the F/G actin ratio, an index of actin polymerization. Although a possible involvement of the endothelium to measured F actin and G actin cannot be excluded, its contribution is likely to be minimal given the much lower quantity of the endothelium as compared with smooth muscle cells. Both cytochalasin B and latrunculin B inhibited PDBu-induced actin polymerization and uterine artery contractions. Cytochalasins and latrunculins are structurally unrelated compounds, which inhibit actin polymerization by different mechanisms.\(^{25,27,31,32,34-37}\) Cytochalasins bind to the rapidly growing end of actin filaments and inhibit actin polymerization. In contrast, latrunculins bind and induce highly specific sequestration of G actin, resulting in inhibition of actin polymerization and increases in actin depolymerization. In
agreement, previous studies have shown that inhibition of actin polymerization by cytochalasins blocks smooth muscle contractions.25,35,44 Taken together, these findings suggest that actin polymerization is a key mechanism in PKC-mediated contractions in the uterine artery.

**Actin Polymerization and Myogenic Tone**

Given that PKC regulates pressure-dependent myogenic tone in the uterine artery,7–10 we further investigated the role of actin polymerization in PKC-mediated myogenic contractions. The present study shows that an increase in intraluminal pressure from 20 to 100 mm Hg causes an initial increase in the Ca$^{2+}$ signal. This suggests that, in addition to the initial elevation of [Ca$^{2+}$], pressure-induced myogenic contractions are mediated by the increased myofilamental Ca$^{2+}$ sensitivity. This is consistent with growing evidence suggesting that mechanisms that regulate the Ca$^{2+}$ sensitivity of the contractile apparatus in vascular smooth muscle form the backbone of pressure-induced myogenic vasoconstriction.7,45 The finding that cytochalasin B inhibited the pressure-induced myogenic tone, with no effect on vascular wall [Ca$^{2+}$], suggests that actin polymerization has a modulatory but not a sensory role in the development of uterine artery myogenic tone. Similarly, previous studies have demonstrated that cytochalasins inhibit smooth muscle contractions without affecting [Ca$^{2+}$], myosin light chain phosphorylation, or myosin ATPase activity.32,44–47 Along with the findings that pressure-induced activation of PKC induces myogenic tone with no changes in intracellular Ca$^{2+}$ concentrations or myosin light chain phosphorylation,7,11,12,13 and that cytochalasins inhibit PKC-mediated contractions in the uterine artery, the present study demonstrates a key role of actin polymerization in PKC-mediated myogenic tone and provides strong evidence that actin polymerization regulates vascular tone development by a cellular process that is distinct from and independent of cross-bridge cycling in the uterine arteries.

**Actin Polymerization and Pregnancy**

Pregnancy is associated with attenuated PKC signaling pathway and decreased myogenic tone in the uterine artery.7–10 The present findings that cytochalasin B inhibited PDBu-induced actin polymerization and contractions and eliminated the difference in PKC-mediated contractions of uterine arteries between nonpregnant and pregnant sheep suggest that decreased PKC-induced myogenic contractions of uterine arteries in pregnant animals are mediated by the downregulation of actin polymerization. This is further supported by the observation of a significantly decreased F/G actin ratio in uterine arteries of pregnant sheep, as compared with that of nonpregnant animals. Additional evidence derives from the results that inhibition of actin polymerization by cytochalasin B eliminated the differences in pressure-dependent myogenic tone in uterine arteries between nonpregnant and pregnant ewes. The finding that estrogen and P4 significantly reduced the F/G actin ratio in the uterine artery is intriguing and suggests a direct genomic effect of the steroid hormones in the downregulation of actin polymerization in pregnancy. This is further supported by the finding that hormonal receptor antagonists significantly increased the F/G actin ratio in the uterine artery of pregnant animals. Nonetheless, the F/G actin ratios in pregnant uterine arteries in the presence of the antagonists were lower than the values in the nonpregnant control vessels, suggesting that sex steroids may not be the only mechanism, and some other mechanisms are also involved in the decrease in the F/G actin in pregnant vessels. Consistent with the present findings, our recent study with the same treatment of the steroid hormones and the antagonists demonstrated a direct genomic effect of the hormones in downregulating the PKC signaling pathway resulting in attenuated myogenic tone of the uterine artery in pregnancy.8 In addition, the study demonstrated that the hormonal effect was the same between the endothelium-intact and -denuded arteries.8 The notion that the steroids act primarily on vascular smooth muscle to regulate myogenic tone is also supported by other studies.48–52 Nonetheless, the potential regulatory role of the endothelium in hormonal-mediated myogenic response of the uterine artery remains to be further investigated. Although the mechanisms underlying the hormonal-mediated downregulation of actin polymerization in the uterine artery are not clear at present, recent studies demonstrated that E2β downregulated the gene expression...
of the Arp2/3 complex and cortactin, both of which are known to be involved in the polymerization and reorganization of actin.53,54

Perspectives
The present study has demonstrated a novel mechanism of actin polymerization in the regulation of myogenic tone in the uterine artery and its adaptation to pregnancy. Pressure-dependent myogenic contraction is an important physiological mechanism in regulating basal vascular tone and organ blood flow, and decreased pressure-induced myogenic response of the uterine arteries contributes significantly to the adaptation of uteroplacental circulation in pregnancy. Thus, dysregulation of myogenic tone is likely to contribute to the maladaptation of uterine vascular hemodynamics in pregnancy and may result in an increased risk of preeclampsia. Indeed, a reduction of uteroplacental perfusion leads to the characteristics of preeclampsia found in pregnant women. In addition to the improved understanding of mechanisms in regulating the uteroplacental circulation, the novel finding of estrogen-mediated downregulation of actin polymerization is likely to have broader implications in understanding the hormonal regulation of myogenic tone of resistance arteries in general and the lower risk of developing hypertension and coronary heart disease in premenopausal women.

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Disclosures
None.

References


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