Direct Chronic Effect of Steroid Hormones in Attenuating Uterine Arterial Myogenic Tone

Role of Protein Kinase C/Extracellular Signal–Regulated Kinase 1/2

Daliao Xiao, Xiaohui Huang, Shumei Yang, Lubo Zhang

Abstract—Pregnancy is associated with a significant decrease in uterine vascular tone and an increase in uterine blood flow. The present study tested the hypothesis that estrogen and progesterone differentially regulate the extracellular signal–regulated kinase (ERK)1/2 and protein kinase C (PKC) signaling pathways in vascular smooth muscle, resulting in a decrease in uterine vascular myogenic tone in pregnancy. Uterine arteries were isolated from nonpregnant and near-term pregnant sheep. Chronic treatment (48 hours) of nonpregnant uterine arteries with 17β-estradiol and progesterone caused a significant decrease in PKC-mediated contractions and pressure-induced myogenic tone. In accordance, treatment of near-term pregnant uterine arteries for 48 hours with ICI 182780 and RU 486 significantly increased PKC-induced contractions and myogenic tone. In contrast, acute treatment for 30 minutes had no effect on uterine artery contractility. An ERK1/2 inhibitor, PD098059, restored the chronic effect of steroids on PKC-mediated contractions in nonpregnant sheep. ERK1/2 protein and mRNA levels were greater in near-term pregnant as compared with nonpregnant uterine arteries. 17β-Estradiol and progesterone increased ERK1/2 protein in nonpregnant sheep. In agreement, ICI 182780 and RU 486 caused significant decreases in ERK1/2 protein in near-term pregnant sheep. Western blot showed 6 PKC isozymes, α, β1, βII, δ, ε, and ζ, in the uterine arteries. 17β-Estradiol and progesterone decreased the particulate:cytosolic ratios of PKCα, ε, and ζ, respectively, in nonpregnant sheep. ICI 182780 and RU 486 increased the ratios in near-term pregnant sheep. The results indicate a direct chronic effect of the steroid hormones in the upregulation of ERK1/2 expression and downregulation of the PKC signaling pathway, resulting in attenuated myogenic tone of the uterine artery in pregnancy. (Hypertension. 2009;54:352-358.)

Key Words: pregnancy ■ uterine artery ■ steroids ■ protein kinase C ■ ERK ■ myogenic tone

Pregnancy is associated with a significant decrease in uterine vascular tone and an increase in uterine blood flow, which are essential for the growth of the fetus and the cardiovascular well being of the mother. Recent studies have demonstrated that pressure-induced myogenic response is significantly decreased in the uterine artery in pregnancy.1–3 The physiological importance of myogenic response in the regulation of uterine blood flow in human pregnancy has been demonstrated in myometrial arteries in term pregnant women.4,5 Given that pressure-dependent myogenic contraction is an important physiological mechanism that regulates basal vascular tone and is a major contributor to the modulation of organ blood flow, the decreased myogenic tone of the uterine artery is likely to contribute significantly to the adaptation of uterine vascular hemodynamics in pregnancy.

Protein kinase C (PKC) plays an important role in regulating arterial myogenic response.6,7 We have demonstrated that the reduced myogenic tone of resistance-sized uterine arteries in pregnant sheep is mediated primarily by a decreased PKC signaling pathway.1,8,9 In addition, we have demonstrated that extracellular signal–regulated kinase (ERK)1/2 functions as an upstream signal in suppressing the PKC activity in pregnant uterine arteries.1,8,9 The inhibition of ERK1/2 increased PKC-mediated contractions and myogenic tone in pregnant uterine arteries,1,8 suggesting a physiological mechanism of ERK1/2 in the increased uterine blood flow by suppressing the basal vascular tone during pregnancy.

The mechanisms in the regulation of the PKC/ERK1/2 signaling pathway and the myogenic tone of the uterine artery during pregnancy remain undetermined. Previous in vivo studies have suggested an important role for the steroid hormones in the regulation of uterine blood flow during pregnancy. Both estrogen and progesterone receptors have been identified in uterine artery vascular smooth muscle.10,11 Studies in ovariectomized and pregnant ewes have demonstrated a key role of 17β-estradiol (E2β) in the regulation of uterine blood flow.12–14 The effect of progesterone in regulating uterine blood flow during pregnancy is less clear and

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appears controversial, possibly because of a relative difficulty of executing in vivo studies with prolonged treatment of a progestosterone receptor antagonist in pregnant animals. Nonetheless, most in vivo studies to date have focused primarily on acute and nongenomic effects of the steroid hormones on relaxation of the uterine artery, observed at the concentrations substantially higher than physiological concentrations. The chronic action of physiologically relevant concentrations of the steroid hormones on uterine artery contractility and myogenic tone and their adaptation to pregnancy remain poorly understood. The differences between pharmacological and physiological responses of uterine blood flow to estrogen have been recognized.

The present study investigated the effects of estrogen and progesterone on pressure-dependent myogenic tone of the resistance-sized uterine arteries obtained from nonpregnant and near-term pregnant ewes in an ex vivo tissue culture model system. We hypothesized that the steroid hormones have direct and chronic effects in the upregulation of ERK1/2 expression and downregulation of PKC signaling pathway in vascular smooth muscle, resulting in attenuated myogenic tone of the uterine artery in pregnancy.

**Materials and Methods**

An expanded Materials and Methods section is available in the online data supplement at http://hyper.ahajournals.org.

**Tissue Preparation and Treatment**

Uterine arteries were isolated from nonpregnant and near-term pregnant (≈140 days’ gestation) sheep, and arterial preparations were incubated in phenol red-free DMEM (Mediatech Cellgro) with 1% charcoal-stripped FBS for 48 hours at 37°C in a humidified incubator with 5% CO2/95% air in the absence or presence of E2β, progesterone, ICI 182780, and RU 486, respectively. All of the procedures and protocols were approved by the Institutional Animal Care and Use Committee guidelines.

**Measurement of Myogenic Tone**

Pressure-dependent myogenic tone of resistance-sized uterine arteries was measured as described previously.

**Contraction Studies**

Isometric tensions were measured in tissue baths at 37°C, as described previously.

**Measurement of ERK1/2 mRNA Levels**

ERK1/2 mRNA was quantified by coupled RT-PCR amplification in a single tube assay, as described previously.

**Western Immunoblotting Analysis**

ERK1/2 protein abundance was measured in freshly isolated uterine arteries and after the hormonal and/or antagonist treatments by Western blot analysis.

**Measurement of PKC Isozyme Translocation**

After the treatments, tissues were homogenized in an ice-cold lysis buffer. The cytosolic and particulate fractions were separated as described previously. Proteins from cytosolic and particulate fractions were subjected to electrophoresis on 10% SDS-PAGE. PKCα, βI, βII, δ, e, and ζ were detected and analyzed as described previously.

**Results**

**Effect of Steroid Hormones on Pressure-Dependent Myogenic Tone**

As shown in Figure 1, pressure-dependent myogenic tone was significantly less in the uterine artery of pregnant sheep as compared with that of nonpregnant animals. E2β (0.3 nmol/L) and progesterone (100 nmol/L) treatment for 48 hours resulted in a significant decrease in myogenic responses in nonpregnant uterine arteries (Figure 1A) and abolished the difference in pressure-induced myogenic tone between nonpregnant and pregnant uterine arteries (Figure 1). In accordance, the chronic treatment with the estrogen receptor (ER) antagonist ICI 182780 (10 μmol/L) and the progesterone receptor antagonist RU 486 (1 μmol/L) caused significant increases in myogenic responses in pregnant uterine arteries (Figure 1B).
Effect of Steroid Hormones on PKC-Mediated Contractions

Phorbol 12,13-dibutyrate (PDBu)-induced contractions were significantly attenuated in uterine arteries from pregnant (−logEC50 [pD2]: 5.52±0.05; Emax: 66.7±7.8% KCl maximum) as compared with nonpregnant (pD2: 6.61±0.12, P<0.05; Emax: 136.8±6.9% KCl maximum, P<0.05) ewes. Neither E2β (0.3 nmol/L) and progesterone (30 nmol/L) nor ICI 182780 (10 μmol/L) and RU 486 (1 μmol/L) had acute (30 minutes of pretreatment) effects on PDBu-induced contractions in uterine arteries from nonpregnant or pregnant sheep (data not shown).

In nonpregnant uterine arteries, the chronic treatment with E2β produced a concentration-dependent attenuation of PDBu-induced contractions (Figure S1A, available in the online data supplement). Treatment with 0.3 nmol/L of E2β significantly decreased the pD2 value (6.6±0.1 versus 5.9±0.1; P<0.05) but not the Emax. However, 10.0 nmol/L of E2β significantly decreased the Emax (105.5±5.3% versus 71.2±7.2% control response; P<0.05) but not the pD2 value. Similarly, progesterone also produced a concentration-dependent decrease in PDBu-mediated contractions (Figure S1B). Chronic treatment with 30.0 nmol/L of progesterone significantly reduced the Emax (105.5±5.3% versus 76.7±7.4% control response; P<0.05) but not the pD2 value. The higher concentration of 100.0 nmol/L of progesterone decreased the pD2 value (6.6±0.1 versus 5.8±0.1, P<0.05) but not the Emax. In addition, the combined treatment of E2β plus progesterone significantly inhibited PDBu-induced contractions (Figure 2A). A combination of 0.3 nmol/L of E2β and 30.0 nmol/L of progesterone significantly attenuated the Emax (105.5±5.3% versus 63.6±9.0% control response; P<0.05) but not the pD2 value. The combination of 0.3 nmol/L of E2β and 100.0 nmol/L of progesterone significantly decreased the pD2 value (6.6±0.1 versus 6.0±0.1; P<0.05) but not the Emax. Unlike uterine arteries, the same concentrations of the steroid hormones had no significant effects on PDBu-induced contractions in mesenteric arteries (Figure S2). In contrast to PDBu-induced contractions, neither acute (30-minute) nor chronic (48-hour) pretreatments with E2β (0.3 and 10.0 nmol/L), progesterone (30.0 nmol/L and 100.0 nmol/L), or their combinations had effects on KCl-induced contractions (data not shown).

In pregnant uterine arteries, the chronic treatment with ICI 182780 significantly increased the pD2 value of PDBu-induced contractions from 5.6±0.2 to 6.5±0.2 (P<0.05; Figure 2B). On the other hand, RU 486 alone had no significant effect on PDBu-induced contractions. However, the combined treatment with RU 486 and ICI 182780 significantly increased the pD2 value (5.6±0.2 versus 6.7±0.2; P<0.05; Figure 2B). To determine whether the endothelium plays a role in the steroid hormone–mediated effect, the studies were repeated in endothelium-denuded uterine arteries. As shown in Figure S3, the effects of the steroid hormone receptor antagonists on PDBu-mediated contractions were similar to those determined in the endothelium-intact preparations, with the combined treatment of ICI 182780 plus RU 486 increasing the pD2 values from 5.3±0.1 to 6.4±0.1 (P<0.05).

Given that RU 486 blocks glucocorticoid receptors as well as progesterone receptors, we determined a possible involvement of glucocorticoid receptors in progesterone-mediated effects in uterine arteries using a potent and selective glucocorticoid receptor antagonist, 21-hydroxy-6,19-epoxyprogesterone.21 As shown in Figure S4, 10 μmol/L of 21-hydroxy-6,19-epoxyprogesterone had no effect on progesterone-mediated inhibition of PDBu-induced contractions in the uterine arteries. We further tested its effect on glucocorticoid receptor–mediated responses in the uterine arteries. Consistent with our previous studies,18 chronic treatment with cortisol significantly enhanced norepinephrine-induced contractions in nonpregnant uterine arteries, which were blocked by 10 μmol/L of 21-hydroxy-6,19-epoxyprogesterone (Figure S5).

Effect of ERK1/2 in Steroid Hormone–Suppressed PKC-Mediated Contractions

As shown in Figure 3, an ERK inhibitor, PD098059, reversed the effect of E2β plus progesterone on PDBu-induced contractions in nonpregnant uterine arteries. ERK1/2 protein and mRNA abundance were determined in uterine artery vascular smooth muscles. Western blot analysis showed a significant increase in ERK1/2 protein abundance in pregnant, as compared with nonpregnant, uterine arteries (Figure 4A). In
accordance, there were significantly higher levels of phospho-ERK1/2 in pregnant uterine arteries (Figure 4A). However, the ratio of phospho-ERK1/2:total-ERK1/2 was not significantly different in the uterine arteries between nonpregnant and pregnant ewes. In agreement with the increased protein abundance, ERK1/2 mRNA levels were also significantly greater in pregnant, as compared with nonpregnant, uterine arteries (Figure S6).

As shown in Figure 4B, the chronic treatment with 0.3 nmol/L of E2β produced a significant increase in ERK2 but not ERK1 in nonpregnant uterine arteries. The treatments with progesterone (30 nmol/L) or combined E2β plus progesterone resulted in significant increases in both ERK1 and ERK2 (Figure 4B). In accordance, the chronic treatment with 10 μmol/L of ICI 182780 decreased ERK2 but not ERK1 in pregnant uterine arteries (Figure S7). RU 486 (1 μmol/L) or combined ICI 182780 and RU 486 produced significant decreases in both ERK1 and ERK2 (Figure S7).

**Effect of Steroid Hormones on PKC Isozyme Translocation**

The subcellular distribution of PKC isozymes in cytosolic and particulate fractions in the uterine artery vascular smooth muscle after chronic treatments with steroid hormones or their receptor antagonists is shown in Figure 5. Six isozymes of PKC, α, βI, βII, δ, ε, and ζ, were determined in the uterine arteries. In nonpregnant uterine arteries, the combined treatment of E2β plus progesterone produced significant decreases in the particulate:cytosolic ratios of PKCα, ε, and ζ (Figure 5). The subcellular distributions of PKCβI, βII, and δ were not significantly affected. In accordance, in pregnant uterine arteries, the combined ICI 182780 plus RU 486 significantly increased the particulate:cytosolic ratio of PKCα, ε, and ζ (Figure 5).

**Discussion**

The present study demonstrated for the first time that physiologically relevant concentrations of E2β and progesterone, as observed in ovine pregnancy,22 had direct chronic effects on downregulating pressure-dependent myogenic tone in the uterine artery. The finding that the steroids eliminated the difference in myogenic contractions between nonpregnant and pregnant uterine arteries suggests a major role for the hormones in the downregulation of basal vascular tone of the uterine artery in pregnancy. This is further supported by the finding that hormonal receptor antagonists significantly increased pressure-induced myogenic tone in the uterine artery of pregnant animals. The concentration of ICI 182780 (10 μmol/L) used in the present study is consistent with the previous studies showing that 10 μmol/L of ICI 182780 selectively blocked estrogen-mediated responses.23–25 In agreement with the present study, the similar temporal response of the rise in uterine blood flow has been demonstrated in ovary-intact ewes, showing the maximal increase of uterine blood flow at ≈45 to 55 hours in animals treated physiologically with the steroid hormones.17
Myogenic tone is an intrinsic property of the smooth muscle and is independent of neural, metabolic, and endothelial influences. In the present study, the hormonal effects on the myogenic reactivity were determined in the presence of an endothelial NO synthase inhibitor, suggesting a smooth muscle effect of the hormones. Although a possible role for prostacyclin may not be excluded in the present study, it has been shown that the inhibition of prostaglandin synthesis does not alter basal uterine blood flow.26,27 Consistent with the present finding, a recent study in resistance arteries of ovariectomized female rats demonstrated that although E2 alone improved flow-mediated dilation, pressure-dependent myogenic tone was significantly reduced only in patients receiving combined E2 plus progesterone.28 Together with the findings in human subcutaneous arteries,1,6,7 and a decrease in the PKC signaling pathway accounts for the attenuated myogenic tone of the uterine artery in pregnancy.1,8,9,31,32 These findings suggest that the chronic action of the steroids in attenuating myogenic tone of the uterine artery is mediated by downregulating the PKC signaling pathway in the vascular smooth muscle. This is supported by the finding that a blockade of the hormonal receptors significantly increased PKC-mediated contractions in the uterine artery of pregnant animals. Given that RU 486 blocks glucocorticoid receptors, as well as progesterone receptors, and that there is no highly selective progesterone receptor antagonist available, the present finding that a potent and selective glucocorticoid receptor antagonist, 21-hydroxy-6,19-epoxyprogestosterone,21 blocked cortisol-induced effects but had no significant effect on the progesterone-mediated response in the uterine arteries, suggests a minimum role for glucocorticoid receptors in the action of progesterone. The finding that the effects of hormonal receptor antagonists were the same in the endothelium-denuded arteries is consistent with the results of hormonal effects on myogenic tone and, thus, further supports a smooth muscle action of the steroids in the uterine artery. In agreement, previous studies in ovariectomized monkeys have demonstrated that physiological concentrations of estrogen and progesterone have inherent effects in vascular smooth muscle cells and suppress the PKC activity and contractility of the coronary artery.33,34 The present finding of the effects of steroids on vascular smooth muscle in the regulation of uterine artery myogenic tone provides a mechanism in addition to estrogen-mediated endothelium-dependent vasodilatation in the uterine artery that accounts only in part for increased uterine blood flow during pregnancy in sheep.13,17

Consistent with the present finding, it has been shown that PDBu-mediated contractions and PKC activity are significantly greater in ovariectomized female rats than in intact female animals.35 In addition, treatment of ovariectomized females with E2β, but not 17α-estradiol (E2α), caused a significant reduction in PDBu-induced contractions and PKC activity in endothelium-denuded aortic strips, which was blocked by ICI 182780.35 Together with the findings in human subcutaneous arteries28,29 and monkey coronary arteries,33,34 these studies suggest a systemic effect of estrogen on vascular tone. In the present study, the same concentrations of the steroids had no significant effects on PDBu-induced contractions in mesenteric arteries. This is probably because of the differences in the tissue sensitivity and ER density between uterine and mesenteric arteries. The chronic action of estrogen in suppressing PKC activity and vascular tone is consistent with its role in the regulation of uterine blood flow demonstrated in ovariectomized and pregnant ewes. The effect of progesterone in regulating uterine blood flow is less clear and appears controversial in animal studies between the ovarian cycle and the pregnancy. Studies in ewes have shown that, during the estrous cycle and when hormones are given, changes in uterine blood flow directly relate to the ratio of concentrations of estrogen to progesterone and indirectly with the concentrations of progesterone alone.36 However, in the second half of pregnancy and at term, changes in uterine blood flow directly related with progesterone concentrations and even more prominently with the sum of progesterone and estrogen.37,38 The present study provided evidence of a direct chronic effect of progesterone in attenuating PKC-mediated
vascular tone in the uterine artery. Similar findings were obtained in primate coronary vascular smooth muscle. In contrast, other studies demonstrated an acute effect of progesterone in sensitizing α-adrenoceptor-mediated contractions of the uterine arteries. Taken together, these findings suggest that progesterone may have a dual role in the regulation of uterine artery contractility, ie, sensitization of the phasic contraction but downregulation of basal vascular tone, which may be of physiological importance during pregnancy in maintaining low myogenic tone in response to increased uterine blood flow, as well as sustaining tissue reactivity and allowing a redistribution of blood by contracting the uterine artery in response to circulating catecholamine under stress.

The question arises as to how the steroids might affect PKC activity in uterine artery vascular smooth muscle. Our previous studies have demonstrated that pregnancy-increased ERK1/2 acts as an upstream signal in suppressing PKC-mediated contractions and pressure-dependent myogenic tone in the uterine arteries, suggesting a physiological mechanism of ERK1/2 in the increased uterine blood flow by suppressing the basal vascular tone during pregnancy. In ovine uterine arteries, an ERK1/2 inhibitor, PD098059, inhibited phosphorylation and activation of ERK1/2. In the present study, we demonstrated that the inhibition of ERK1/2 by PD098059 restored the steroid-mediated attenuation of PDBu-induced contractions in the uterine arteries. This indicates a key role for ERK1/2 activation in the hormone-mediated suppression of PKC activity. Consistent with this finding, protein levels of total and phosphorylated ERK1/2 were significantly greater in pregnant, as compared with nonpregnant, uterine arteries. The finding that the ratio of phospho-ERK1/2:total-ERK1/2 was not significantly different between pregnant and nonpregnant uterine arteries suggests that pregnancy increases the expression of ERK1/2, resulting in elevated phospho-ERK1/2, rather than stimulating artery activities, per se. The increased expression of ERK1/2 in pregnant uterine arteries is likely mediated through a direct action of the steroids, because it has been demonstrated in the present study that E2β plus progesterone significantly increases ERK1/2 protein abundance in nonpregnant uterine arteries. This is further supported by the finding that the ICI 182780 and RU 486 decreased ERK1/2 in pregnant uterine arteries. Unlike progesterone that increased both ERK1 and ERK2, the finding that E2β upregulated only ERK2 is intriguing and suggests that ERK2 may be involved in the steroid-mediated attenuation of PKC activity and myogenic tone in the uterine artery. Although many previous studies showed that both E2β and progesterone increased ERK1/2 activity acutely, few examined the effect of the steroids on the transcriptional control of ERK1/2. Future studies of the transcriptional mechanisms are needed.

The present study further demonstrated that the steroids differentially regulated the subcellular distribution of PKC isozymes in uterine artery vascular smooth muscle. Six PKC isozymes, α, β1, βII, δ, ε, and ζ, have been detected in the smooth muscle of the uterine artery. E2β plus progesterone significantly reduced PKCα, ε, and ζ in the particulate fraction in nonpregnant uterine arteries, suggesting an attenuation of basal activities of these isozymes. This is supported by the finding that ICI 182780 plus RU 486 significantly increased the particulate fractions of PKCα, ε, and ζ in pregnant uterine arteries. Both PKCα and PKCe have been implicated in contractions of the vascular smooth muscle by increasing the Ca2+ sensitivity. Our recent studies demonstrated that PDBu-induced activation of PKCα was similar in the uterine arteries between nonpregnant and pregnant ewes, in the absence or presence of PD098059. In contrast, PDBu-induced PKCe activation was significantly attenuated in pregnant uterine arteries, which was restored by PD098059. Taken together, these studies suggest that the steroid-mediated decrease in the activity of PKCe, but not PKCα, may be involved in the attenuation of PKC-mediated contractions in pregnancy. Although PKCζ is an atypical PKC isozyme that is not activated by Ca2+, diacylglycerol, or phorbol esters, and it may be less likely to be involved in the PDBu-induced contractions, its role in pressure-dependent myogenic contractions of the uterine artery may not be excluded. In agreement with the present study, it has been shown that a sex-related decrease in PKC-mediated vascular smooth muscle contractions in female rats is associated with reductions in the activity of PKCα, δ, and ζ.

Perspectives

The present study has demonstrated a direct chronic effect of the steroid hormones in the upregulation of ERK1/2 expression and downregulation of the PKC signaling pathway, resulting in a reduced myogenic tone of the uterine artery in pregnancy. Given that pressure-dependent myogenic contraction is an important physiological mechanism that regulates basal vascular resistance and is a major contributor to the modulation of organ blood flow, dysregulation of myogenic tone is likely to contribute significantly to the maladaptation of uterine vascular hemodynamics in pregnancy and an increased risk of preeclampsia. Not only do the present findings provide an understanding of the mechanisms of the steroid hormone-mediated adaptation of uterine artery contractility to pregnancy, but they also offer insights into the mechanisms in the hormonal regulation of myogenic tone of resistance arteries in general and improve our understanding of vascular benefits of hormone replacement therapy in postmenopausal women, given the well-established finding that premenopausal women are at lower risk of developing hypertension and coronary heart disease than men of the same age and that the cardiovascular risk increases only after the cessation of ovarian function.

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Disclosures

None.
References


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DIRECT CHRONIC EFFECT OF STEROID HORMONES IN ATTENUATING UTERINE ARTERIAL MYOGENIC TONE: ROLE OF PKC/ERK1/2

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Materials and Methods

Tissue preparation and treatment
Nonpregnant and near-term pregnant (~140 days gestation) sheep were anesthetized with thiamylal (10 mg/kg) 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 as described previously. Briefly, the middle uterine artery from each uterine horn was dissected and 4th generation branches from the middle uterine artery were isolated in the Krebs solution. These vessels have been extensively examined in our previous studies, and we have demonstrated that the characteristics of PKC-mediated effects are not altered among branches of small uterine arteries. The Krebs solution was oxygenated with a mixture of oxygen-carbon dioxide (95:5%). After removal of the tissues, animals were killed with T-61 (euthanasia solution, Hoechst-Rousel, Somervile, NJ). Arterial preparations were incubated in a complete medium of phenol red-free Dulbecco’s Modified Eagle’s Medium (DMEM, Mediatech Cellgro Inc., VA) with 1% charcoal-stripped fetal bovine serum for 48 h at 37 °C in a humidified incubator with 5% CO2/95% air, as described previously, in the absence or presence of E2β (Sigma, St. Louis, MO), progesterone (Sigma), ICI 182,780 (Tocris Bioscience, Ellisville, MO), and RU 486 (Sigma), respectively. All procedures and protocols used in the present study were approved by the Animal Research Committee of Loma Linda University 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. After the hormonal or antagonist treatments for 48 h, arteries were cannulated in an organ chamber (Living Systems, Burlington, VT), followed by placement on the stage of an inverted microscope. The proximal cannula was connected to a pressure transducer and reservoir of physiological salt solution (PSS), and the intraluminal pressure was controlled by a servo-system to set transmural pressures. The distal cannula was connected to a luer-lock valve that was open to flush the lumen during the initial cannulation. Following cannulation, the valve was closed, and all measurements were conducted under no-flow conditions. Arterial diameter was recorded using the SoftEdge Acquisition Subsystem (IonOptix Milton, MA), as described previously. After being mounted, the vessels were equilibrated in PSS for 10 min at an intraluminal pressure of 20 mmHg, followed by increasing the pressure from 20 to 70 mmHg, and returning to 20 mmHg immediately. The vessels were then allowed to equilibrate at 20 mmHg in the presence of a nitric oxide synthase inhibitor \( \text{N}^\text{G}-\text{nitro-L-arginine (L-NNA, 100 } \mu\text{M)} \) for 30 min. After the equilibration period, the pressure was increased in a stepwise manner from 10 to 100 mmHg in 10-mmHg increments, and each pressure was maintained for 3 min to allow vessel diameter to stabilize before measurement. The passive pressure-diameter relationship was conducted in Ca\(^{2+}\)-free PSS containing 3 mM EGTA to determine the maximum passive diameter. The following formula was used to calculate percent myogenic tone at each pressure step: \( \%\text{myogenic tone} = \frac{(D_1 - D_2)}{D_1} \times 100 \), where \( D_1 \) is the passive diameter in Ca\(^{2+}\)-free PSS (zero Ca\(^{2+}\) with 3 mM EGTA), and \( D_2 \) is the active diameter with normal PSS in the presence of extracellular Ca\(^{2+}\).
Contraction studies

The fourth branches of middle uterine arteries were separated from the surrounding tissue, and cut into 2-mm ring segments. In some rings the endothelium was removed, as described previously. After the hormonal and/or antagonist treatments for 48 h, isometric tensions were measured in tissue baths at 37 °C, as described previously. After 60 min of equilibration, each ring was stretched to the optimal resting tension as determined by the tension developed in response to 120 mM KCl added at each stretch level. Concentration-response curves of phorbol 12,13-dibutyrate (PDBu, Sigma) were obtained by a cumulative addition of the agonist in approximate one-half log increments. Some arteries were treated with the steroid hormones in the presence of an ERK1/2 inhibitor PD098059 (30 μM, Sigma) for 48 h. To determine the acute effect of steroid hormones, some arteries were treated in the tissue bath with the steroids for 30 min, and then stimulated with increasing concentrations of PDBu. EC50 values for the agonist in each experiment were taken as the molar concentration at which the contraction-response curve intersected 50% of the maximum response, and were expressed as pD2 (-logEC50) values.

Measurement of ERK1/2 mRNA levels

Extracellular signal-regulated kinase (ERK1/2) mRNA was quantified by coupled RT-PCR amplification in a single tube assay as described previously. Total cellular RNA was isolated from the uterine arteries using TRizol reagent (Gibco BRL, Life Technologies, Rockville, MD, USA). RNA (0.1 μg) was used for first-strand cDNA synthesis with AMV reverse transcriptase (RT) and oligo (dT) as primer. Polymerase chain reaction (PCR) amplifications were carried out on a portion of the cDNA produced using specific oligonucleotide primers for ERK1 and 2. The primer sequences are as follows: ERK1 forward: 5’gtctggacggagttaac3’, reverse: 5’gaatgcaccaaacagcagagatgtc3’; ERK2 forward: 5’gcagccacatggeggegg3’, reverse: 5’tctggatctgcaacacgggc3’. PCR products were resolved on a 1% agarose gel containing ethidium bromide. The band intensities at the size of 661 (ERK1) and 539 (ERK2) bases were measured and analyzed by using densitometry, as described previously.

Western immunoblotting analysis

ERK1/2 protein abundance was measured in freshly isolated uterine arteries and after the hormonal and/or antagonist treatments. Tissues were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris HCl, 10 mM EDTA, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin, PH 7.4. Homogenates were then centrifuged at 4 °C for 10 min at 10,000g and the supernatants were collected. Proteins were quantified in the supernatant with a protein assay kit (Bio-Rad). Samples with equal proteins were loaded onto 7.5% polyacrylamide gel with 0.1% sodium dodecyl sulfate (SDS), and were separated by electrophoresis at 100 V for 2 h. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites in the membranes were blocked by an overnight incubation at 4 °C in Tris-buffered saline solution (TBS) containing 5% dry milk. The membranes were incubated with primary antibodies against ERK1/2 and phospho-ERK1/2 (Tyr202/Tyr204) antibodies (New England Biolabs, Beverly, MA). After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software.
Measurement of PKC isozyme translocation

After the hormonal or antagonist treatments for 48 h, tissues were snap-frozen in liquid N₂ and homogenized in ice-cold homogenization buffer containing Tris-HCl 20 mM, sucrose 250 mM, EDTA 5 mM, EGTA 5 mM, PMSF 1 mM, β-mercaptoethanol 10 mM, and benzamide 1 mM. The cytosolic and particulate fractions were separated as previously described.\(^\text{10}\) Briefly, the homogenate was centrifuged at 100,000g for 60 min at 4 °C, and the supernatant was used as the cytosolic fraction. The pellet corresponding to the membrane particulate fraction was solubilized in buffer containing Triton X-100 at a final concentration of 0.1% by stirring on ice for 45 min at 4 °C, followed by centrifugation at 100,000g for 60 min at 4 °C to remove insoluble membrane particles. The supernatant was collected and was referred to as the membrane particulate fraction. Protein concentrations were determined with a protein assay kit (Bio-Rad). Proteins from cytosolic and particulate fractions were subjected to electrophoresis on 10% SDS-PAGE as described above. The membranes were incubated with primary antibodies against PKCα, βI, βII, δ, ε, and ζ (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were detected and analyzed as described above.

Data analysis

Concentration-response curves were analyzed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad software, San Diego, CA). Results were expressed as means ± SEM obtained from the number (n) of experimental animals given. Differences were evaluated for statistical significance (P < 0.05) by ANOVA or t-test, where appropriate.

References


Figures and Legends

A

B

Figure S1. Effect of steroid hormones on PDBu-induced contractions of nonpregnant uterine arteries. Uterine arteries from nonpregnant ewes were treated with 17β-estradiol (E2β) and progesterone (P4) or vehicle control for 48 h and then submitted to the cumulative additions of PDBu in the tissue bath. Data are means ± SEM of 5-11 animals. pD₂ and E_max values are presented in Results.
Figure S2. Effect of steroid hormones on PDBu-induced contractions of mesenteric arteries. Mesenteric arteries from nonpregnant ewes were treated with 17β-estradiol (E2β) and progesterone (P₄) or vehicle control for 48 h and then submitted to the cumulative additions of PDBu in the tissue bath. Data are means ± SEM of 7 animals.
Figure S3. Effect of steroid hormone receptor antagonists on PDBu-induced contractions of endothelium-denuded pregnant uterine arteries. Uterine arteries were isolated from pregnant ewes and the luminal endothelium was denuded. The arteries were then treated with ICI 182,780 (10 μM) and RU 486 (1 μM) or vehicle control for 48 h in the presence of E_2β (0.3 nM) and P_4 (100 nM) and then submitted to the cumulative additions of PDBu in the tissue bath. Data are means ± SEM of 4 animals. pD_2 and E_{max} values are presented in Results.
Figure S4. Effect of glucocorticoid receptor antagonist on the progesterone-mediated response in uterine arteries. Nonpregnant uterine arteries were treated with progesterone ($P_4$) in the absence or presence of a potent and selective glucocorticoid receptor antagonist 21-hydroxy-6,19-epoxyprogesterone (5b) for 48 h, and then submitted to the cumulative additions of PDBu in the tissue bath. Data are means ± SEM of 5 animals.
Figure S5. Effect of glucocorticoid receptor antagonist on the cortisol-mediated response in uterine arteries. Nonpregnant uterine arteries were treated with cortisol in the absence or presence of a potent and selective glucocorticoid receptor antagonist 21-hydroxy-6,19-epoxyprogesterone (5b) for 24 h, and then submitted to the cumulative additions of norepinephrine in the tissue bath. Data are means ± SEM of 6 animals.
Figure S6. Effect of pregnancy on ERK1/2 mRNA abundance in the uterine arteries. Uterine arteries were isolated from pregnant (PUA) and nonpregnant (NPUA) sheep. mRNA levels of ERK1/2 were determined by RT-PCR. Data are means ± SEM of 3 animals. * P < 0.05, vs. NPUA.
Figure S7. Effect of steroid hormone antagonists on ERK1/2 protein abundance in pregnant uterine arteries. Uterine arteries from pregnant ewes were treated with ICI 182,780 (ICI) or/and RU 486 (RU) or vehicle control for 48 h in the presence of E₂β (0.3 nM) plus P₄ (100 nM). ERK1/2 protein abundance was determined by Western blotting. Data are means ± SEM of 4 animals. * P < 0.05, vs. control.