Chronic hypoxia increases pressure-dependent myogenic tone of the uterine artery in pregnant sheep: role of ERK/PKC pathway

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Chang K, Xiao D, Huang X, Longo LD, Zhang L. Chronic hypoxia increases pressure-dependent myogenic tone of the uterine artery in pregnant sheep: role of ERK/PKC pathway. Am J Physiol Heart Circ Physiol 296: H1840–H1849, 2009. First published April 3, 2009; doi:10.1152/ajpheart.00090.2009.—Chronic hypoxia during pregnancy has profound effects on uterine artery (UA) contractility and attenuates uterine blood flow. The present study tested the hypothesis that chronic hypoxia inhibits the pregnancy-induced reduction in pressure-dependent myogenic tone of resistance-sized UAs. UAs were isolated from nonpregnant ewes (NPUAs) and near-term pregnant ewes (PUAs) that had been maintained at sea level (~300 m) or at high altitude (3,801 m) for 110 days. In normoxic animals, the pressure-dependent myogenic response was significantly attenuated in PUAs compared with NPUAs. Hypoxia significantly increased myogenic tone in PUAs and abolished its difference between PUAs and NPUAs. Consistently, there was a significant increase in PKC-mediated baseline Ca2+ sensitivity of UAs in hypoxic animals. Hypoxia significantly increased phosphor 12,13-dibutyrate (PDBu)-induced contractions in PUAs but not in NPUAs. Whereas the inhibition of ERK1/2 by PD-98059 potentiated PDBu-mediated contractions of UAs in normoxic animals, it failed to do so in hypoxic animals. Hypoxia decreased ERK1/2 expression in PUAs. PDBu-induced membrane translocation of PKC-α and PKC-ε. Whereas there were no significant differences in PKC-ε translocation among all groups, the translocation of PKC-ε was significantly enhanced in PUAs compared with NPUAs in normoxic animals, and hypoxia significantly increased PKC-ε translocation in PUAs. In the presence of PD-98059, there were no significant differences in PDBu-induced PKC-ε translocation among all groups. Treatment of UAs isolated from normoxic animals with 10.5% O2 for 48 h ex vivo significantly increased PDBu-induced contractions and eliminated its difference between PUAs and NPUAs. The results suggest that hypoxia upregulates pressure-dependent myogenic tone through its direct effect in suppressing ERK1/2 activity and increasing the PKC signal pathway, leading to an increase in the Ca2+ sensitivity of the myogenic mechanism in the UA during pregnancy.

hypothesis; protein kinase C

DURING PREGNANCY, the development of uteroplacental circulation with decreased vascular tone accommodates a >30-fold increase in uterine blood flow in term pregnant sheep and humans, which helps to ensure normal fetal development. Chronic hypoxia during pregnancy has profound effects on uterine artery (UA) contractility and attenuates the pregnancy-induced increase in uterine blood flow, which is associated with an increased risk of preeclampsia and fetal intrauterine growth restriction (16, 19, 33, 46, 54, 55). The adaptation of UA contraction and relaxation mechanisms to pregnancy is complex and poorly understood. In addition to growth and remodeling of the uterus vasculature, the decreased UA resistance is accomplished by both increased endothelial nitric oxide synthesis/release and decreased vascular contractility and myogenic response. Our recent study (47) in sheep has demonstrated that the pressure-induced myogenic response is significantly less and the distensibility of the UA is greater in pregnant ewes. Similar findings have been demonstrated in pregnant mice and rats (10, 28, 29, 41). The physiological importance of the myogenic response in the regulation of uterine blood flow in human pregnancy has been demonstrated in myometrial arteries in term pregnant women (20, 21). 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 UA is likely to contribute significantly to the adaptation of uterine vascular hemodynamics in pregnancy.

Myogenic activity is an intrinsic property of vascular smooth muscle in response to pressure or stretch. It is modulated by paracrine or endocrine substances but does not require the nerves or endothelium to occur. Among other mechanisms, numerous studies (5, 22, 30, 32) have demonstrated an important role of PKC in the regulation of the arterial myogenic response. We (47, 48, 51, 53) have recently demonstrated that PKC plays a key role in the regulation of myogenic tone of resistance-sized UAs, and the reduced myogenic tone in the pregnant UA is primarily mediated by a decrease in the PKC signaling pathway. Consistent with our findings, it has been shown that pregnancy is associated with attenuated arterial PKC activity (6, 7, 17, 18, 25). In addition, we (47, 49, 50, 51, 53) have demonstrated that ERK1/2 functions as an upstream signal in the suppression of PKC activity in pregnant UAs. The activation of ERK1/2 depends on the dual phosphorylation on Tyr185 and Thr187 by MEK (1). In ovine UAs, the ERK1/2 inhibitor PD-98059 inhibited the phosphorylation and activation of ERK1/2 (50, 51, 53). The inhibition of ERK1/2 increased PKC-mediated contractions and myogenic tone in pregnant UAs (47, 53), suggesting a physiological mechanism of ERK1/2 in the increased uterine blood flow by suppressing the basal vascular tone during pregnancy.

It is unknown whether and to what extent chronic hypoxia modulates the adaptation of myogenic mechanisms of the UA during pregnancy. In the present study, we investigated the effects of chronic hypoxia on pressure-dependent myogenic tone of resistance-sized UAs obtained from nonpregnant and near-term pregnant ewes with normoxic control or high-altitude (3,801 m) hypoxic (arterial PO2: 60 mmHg) treatment for 110 days. We hypothesized that chronic hypoxia inhibited the pregnancy-mediated attenuation of the myogenic response in pregnancy.
UAs, which was due to a suppression in ERK1/2 activity and an increase in the PKC signal pathway, leading to an increase in the Ca\textsuperscript{2+} sensitivity of the myogenic mechanism. To address whether chronic hypoxia has direct effects on the ERK/PKC pathway in the regulation of the UA myogenic response and its adaptation to pregnancy, experiments were also performed in isolated UAs treated ex vivo with prolonged hypoxia.

**METHODS**

**Tissue preparation.** As previously described (52), nonpregnant and time-dated pregnant sheep were obtained from the Nebecker Ranch in Lancaster, CA (altitude: ~300 m, arterial PO\textsubscript{2}: 102 ± 2 mmHg). UAs were obtained from nonpregnant and near-term (~140 days of gestation) pregnant sheep. For chronic hypoxic treatment, nonpregnant and pregnant (30 days of gestation) animals were transported to the Barcroft Laboratory, White Mountain Research Station, in Bishop, CA (altitude, 3,801 m, maternal arterial PO\textsubscript{2}: 60 ± 2 mmHg) and maintained there for ~110 days. Animals were transported to the laboratory immediately before the experiments. Ewes were anesthetized with thiampyl (10 mg/kg) administered via the external jugular vein. Animals were then intubated, and anesthesia was maintained on 1.5–2.0% halothane in oxygen throughout surgery. An incision in the abdomen was made, and the uterus was exposed. The UAs were isolated, removed without being stretched, and placed into solutions containing intermediate free Ca\textsuperscript{2+} (pH 7.4). After removal of the tissues, animals were killed with T-61 (euthanasia solution, Hoechst-Roussel, Somerville, NJ). All procedures and protocols used in the present study were approved by the Animal Research Committee of Loma Linda University and followed guidelines put forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Measurement of myogenic tone.** Resistance-sized UA segments (~150 \(\mu\)m in diameter) were dissected and cannulated in an organ chamber (Living Systems, Burlington, VT) by placement on the stage of an inverted microscope. The proximal cannula was connected to a pressure transducer and reservoir of PSS, and the intraluminal pressure was controlled by a servo system to set transmural pressures. The distal cannula was connected to a user-lock valve that was opened to flush the lumen during the initial cannulation. After 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 previously described (47). After being mounted, vessels were equilibrated in PSS for 10 min at an intraluminal pressure of 20 mmHg followed by a pressure increase from 20 to 70 mmHg and a return to 20 mmHg immediately. Vessels were then allowed to equilibrate at 20 mmHg in the presence of the nitric oxide synthase inhibitor L-NAME (100 \(\mu\)M) for 30 min. After the equilibration period, the pressure was increased in a stepwise manner from 20 to 100 mmHg in 20-mmHg increments, and each pressure was maintained for 3 min to allow the vessel diameter to stabilize before measurements. The passive pressure-diameter relationship was conducted in Ca\textsuperscript{2+}-free PSS containing 3 mM EGTA to determine the maximum passive diameter. The following formula was used to calculate the percent myogenic tone at each pressure step: percent myogenic tone = \((D_1 - D_2)/D_1 \times 100\), where \(D_1\) is the passive diameter in Ca\textsuperscript{2+}-free PSS (0 mM Ca\textsuperscript{2+} with 3 mM EGTA) and \(D_2\) is the active diameter with normal PSS in the presence of extracellular Ca\textsuperscript{2+}.

**Contraction experiments.** The fourth branches of main UAs were separated from the surrounding tissue and cut into 2-mm ring segments. Isometric tension was measured in PSS in a tissue bath at 37°C, as previously described (53). 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. Tissues were pretreated with either the ERK1/2 inhibitor PD98059 (30 \(\mu\)M, Sigma, St. Louis, MO) or vehicle (DMSO) for 30 min followed by stimulations with the PKC activator phorbol 12,13-dibutyrate (PDBu; Sigma) in the presence of 1-NA. Concentration-response curves of PDBu were obtained by the cumulative addition of the agonist in approximate one-half log increments. To investigate the direct effect of hypoxia, some arterial rings obtained from normoxic nonpregnant and pregnant ewes were incubated in a given culture dish with 5 ml of complete DMEM (Mediatech Cellgro) containing 1% FBS, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Tissues were incubated at 37°C in a humidified incubator with either 21% or 10.5% O\textsubscript{2} for 48 h before they were subjected to contraction experiments. EC\textsubscript{50} 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 pD\textsubscript{2} (=log EC\textsubscript{50}) values. Responses were normalized to the maximal KCl contraction.

**Measurement of baseline Ca\textsuperscript{2+} sensitivity.** Two main Ca\textsuperscript{2+} buffer solutions were used in the present study, as previously described (48). One solution was zero Ca\textsuperscript{2+} relaxing solution, which contained (in mM) 110 K-acetate, 5 EGTA, 5 ATP, 6 Mg-acetate, 1 DTT, 0.01 leupeptin, 20 imidazole, and 2 HEPES at pH 6.8 (titrated with KOH). The other solution contained 1 mM Ca\textsuperscript{2+} in addition to the same components in the zero Ca\textsuperscript{2+} buffer. As described in a previous study (48), Ca\textsuperscript{2+} buffer solutions were prepared by solving the multi-equilibrium equations for interactions among the different ions, and solutions containing intermediate free Ca\textsuperscript{2+} concentrations were prepared by mixing the appropriate amounts of zero Ca\textsuperscript{2+} relaxing solution and maximum Ca\textsuperscript{2+} buffer solution and then titrated to pH 7.0 with 1 M KOH. Arterial rings were attached to isometric force transducers and bathed in PSS at 37°C. After 60 min of equilibration in the tissue bath, 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. Arterial rings were then placed into the zero Ca\textsuperscript{2+} relaxing solution and permeabilized as previously described (48). Briefly, chemical permeabilization was achieved by adding 40 \(\mu\)M β-escin to the relaxing solution and allowing the arteries to incubate for 20 min at 25°C. The permeabilized solution was then replaced with the relaxing solution containing 1 mM A-23187 to deplete Ca\textsuperscript{2+} from the sarcoplasmic reticulum. Concentration-response curves to Ca\textsuperscript{2+} were obtained by cumulative increases of Ca\textsuperscript{2+} concentrations in approximate one-half log increments in β-escin-permeabilized arterial rings in the presence or absence of the PKC activator PDBu.

**Immunoblot analysis.** The protein abundance of ERK1/2 and phospho-ERK1/2 was determined as previously described (53). Briefly, tissues were homogenized in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.1 mM PMSF, 5 \(\mu\)g/ml leupeptin, and 5 \(\mu\)g/ml aprotinin (pH 7.4). Sample homogenates were then centrifuged at 4°C for 5 min at 6,000 g, and the supernatants were collected. Samples with equal protein were loaded onto a 10% polyacrylamide gel with 0.1% SDS and separated by electrophoresis. Proteins were then transferred onto Immobilon P membranes and incubated with ERK1/2 and phospho-ERK1/2 antibodies (New England Biolabs, Beverly, MA). After an incubation with horseradish peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, IL), immunoreactive bands and separated by electrophoresis. Proteins were then transferred onto Immobilon P membranes and incubated with ERK1/2 and phospho-ERK1/2 antibodies (New England Biolabs, Beverly, MA). After an incubation with horseradish peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, IL), immunoreactive bands were visualized by enhanced chemiluminescence. Blots were exposed to Hyperfilm and analyzed with Kodak 1D image analysis software. Data were normalized by an internal standard loaded in the same membrane within each group.

**Measurement of PKC isozyme translocation.** To determine PDBu-induced translocation of PKC isozymes from the cytosolic and particular fractions in UAs, arterial rings were equilibrated in the tissue bath, and the optimal tension was obtained as described above.
Tissues were then incubated for 20 min with PD-98059 (30 μM) or vehicle alone in the organ bath at 37°C. After incubation, they were stimulated with 3 μM PDBu for 10 min. At the end of the treatment, tissues were snap frozen in liquid N2, and the cytosolic and particulate fractions were prepared as previously described (56). Briefly, tissues were homogenized in ice-cold homogenization buffer A containing 20 mM Tris-Cl, 250 mM sucrose, 5 mM EDTA, 5 mM EGTA, 10 mM β-mercaptoethanol, 1 mM benzamidine, 1 mM PMSF, 50 μM leupeptin, 1 mM DTT, and 2 μg/ml aprotinin (pH 7.5). Homogenates were centrifuged at 100,000 g for 20 min at 4°C, and supernatants were collected and used as the cytosolic fraction. Pellets were then resuspended in homogenization buffer A containing 1% Triton X-100 by stirring overnight at 4°C, diluted with buffer A to a final concentration of 0.2% Triton X-100, and then centrifuged at 100,000 g for 20 min at 4°C. Supernatants were then collected and referred to as the particulate fraction. Protein concentrations were determined with a protein assay kit (Bio-Rad). Protein samples (5 μg) of particulate fractions were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel and then transferred electrophoretically to nitrocellulose membranes. Membranes were incubated at room temperature for 1 h in Tris-buffered saline solution containing 5% dried milk and 0.5% Tween 20 followed by an incubation with primary antibodies for PKC-α and PKC-ε (Santa Cruz Biotechnology, Santa Cruz, CA), respectively, overnight at 4°C and secondary antibody for 1 h at room temperature. To confirm the phosphorylation of PKC-α and PKC-ε in PDBu-induced membrane translocation, some membranes were incubated with phospho-PKC-α and phospho-PKC-ε antibodies (Upstate, Lake Placid, NY). Bands were detected with enhanced chemiluminescence, visualized on Hyperfilm, and analyzed with Kodak 1D image analysis software. To normalize the loading variation of each sample, the corresponding actin level presented in each sample was determined using monoclonal anti-actin as the primary antibody (Santa Cruz Biotechnology).

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 are expressed as mean ± SE obtained from the number of experimental animals given (n). Differences were evaluated for statistical significance (P < 0.05) by two-way ANOVA followed by the Newman-Keuls post hoc test.

RESULTS

Effect of long-term high-altitude hypoxia on myogenic tone in pressurized UAs. In the presence of Ca2+-containing PSS, UAs from all four groups of sheep (normoxic nonpregnant UAs, normoxic pregnant UAs, hypoxic nonpregnant UAs, and hypoxic pregnant UAs) developed myogenic tone in response to stepwise increases of intraluminal pressure (Fig. 1). In normoxic animals, pressure-dependent myogenic tone was significantly less in pregnant compared with nonpregnant UAs. Long-term high-altitude hypoxia resulted in a significant increase in the myogenic response in the UA of pregnant ewes but not in the UA of nonpregnant ewes (Fig. 1). Unlike normoxic animals, there were no significant differences in the myogenic response of UAs between nonpregnant and pregnant ewes in long-term high-altitude hypoxic animals.

Effect of long-term high-altitude hypoxia on PKC-mediated contractions in UAs. Given that PKC plays a key role in the regulation of the pressure-dependent myogenic response in UAs, and that reduced myogenic tone in pregnant UAs is primarily mediated by a decrease in the PKC signaling pathway (47), we determined the effect of hypoxia on PDBu-induced contractions. Consistent with our previous study (53), PDBu-induced contractions of UAs were significantly decreased in pregnant compared with nonpregnant ewes in normoxic animals (Figs. 2 and 3). Long-term high-altitude hypoxia did not alter pD2 values in either nonpregnant or pregnant UAs but significantly increased the maximal response of PDBu-induced contractions of UAs in pregnant but not nonpregnant ewes (Figs. 2 and 3). As we reported in a previous study (53) in normoxic animals, PD-98059 had no effect on PDBu-induced contractions of UAs in either pregnant or nonpregnant ewes (Figs. 2 and 3). Furthermore, in hypoxic animals, there were no significant differences in PDBu-induced maximal responses in UAs between pregnant and nonpregnant ewes in the absence or presence of PD-98059 (Fig. 3). Consistent with these findings, chronic hypoxia caused a significant downregulation of ERK1/2 expression in UAs of pregnant but not nonpregnant ewes (Fig. 4, top). The decreased ERK1/2 expression in UAs of hypoxic pregnant animals was associated with a significant reduction in ERK1/2 activity and reduced phospho-ERK1/2 levels (Fig. 4, bottom).

Effect of long-term high-altitude hypoxia on PKC-mediated baseline Ca2+ sensitivity. Our previous study (48) in normoxic animals demonstrated that pregnancy attenuated PKC-medi-
ated baseline Ca$^{2+}$/H_11001 sensitivity in the UA. Figure 5 shows that cumulative increases of Ca$^{2+}$/H_11001 produced concentration-dependent contractions of permeabilized UAs from both nonpregnant and pregnant ewes in long-term high-altitude hypoxic animals. In the absence of PDBu, both pD$_2$ values (4.7 ± 0.1 vs. 5.2 ± 0.1, P < 0.05) and maximal responses (78.2 ± 3.4 vs. 133.8 ± 3.2% of the KCl maximum, P < 0.05) of Ca$^{2+}$/H_11001-induced contractions were significantly increased in UAs from pregnant compared with nonpregnant ewes. In the presence of PDBu, there were significant increases in pD$_2$ values (4.7 ± 0.1 vs. 5.0 ± 0.1, P < 0.05) and maximal responses (78.2 ± 3.4 vs. 137.3 ± 5.3% of the KCl maximum, P < 0.05) of Ca$^{2+}$/H_11001-induced contractions of UAs in pregnant ewes but not in pregnant ewes (pD$_2$: 5.2 ± 0.1 vs. 5.1 ± 0.1, P > 0.05; maximal response: 133.8 ± 3.2 vs. 147.3 ± 6.4% of the KCl maximum, P > 0.05). PDBu abolished the difference in Ca$^{2+}$/H_11001-induced contractions of UAs between nonpregnant and pregnant ewes in long-term high-altitude hypoxic animals.

Effect of long-term high-altitude hypoxia on PDBu-induced translocation of PKC isoforms. PDBu significantly increased the levels of PKC-α in membrane particulate fractions, suggesting that PDBu induced translocation and activation of PKC-α in UAs (Fig. 6). However, these PDBu-induced translocations of PKC-α in UAs were not significantly different among all four groups and were not significantly affected by PD-98059 (Fig. 6). Unlike PKC-α, in normoxic animals, PDBu-induced translocations of PKC-ε were significantly decreased in UAs of pregnant ewes compared with nonpregnant animals (Fig. 6). PD-98059 significantly increased PDBu-induced translocations of PKC-ε in pregnant but not nonpregnant UAs of normoxic ewes. In the presence of PD-98059, there were no significant differences in PDBu-induced translocations of PKC-ε between nonpregnant and pregnant UAs (Fig. 6). Long-term high-altitude hypoxia significantly increased PDBu-induced translocations of PKC-ε in UAs of pregnant ewes (Fig. 6). PD-98059 had no significant effect on PDBu-induced translocations of PKC-ε in either pregnant or nonpregnant UAs in hypoxic animals (Fig. 6). Whereas PKC translocation from cytosolic to membrane particulate fractions generally has been considered the hallmark of activation and frequently has been used as a surrogate measure of PKC isoform activation in cells, phosphorylation of PKC-α and PKC-ε was confirmed in the PDBu-induced translocation in UAs (Fig. 7).

![Non-pregnant Uterine Artery](image1)

![Pregnant Uterine Artery](image2)

![Fig. 2. Effect of high-altitude hypoxia on phorbol 12,13-dibutyrate (PDBu)-induced contractions in UAs. Cumulative concentration-response curves to PDBu were determined in UAs obtained from nonpregnant and pregnant ewes with normoxia and long-term high-altitude hypoxia treatment in the absence or presence of PD-98059 (PD; 30 μM, pretreatment for 20 min). Data are expressed as percentages of contraction generated by 120 mM KCl, and each point represents the mean ± SE of tissues from 4–10 animals.](image3)

![Fig. 3. Effect of high-altitude hypoxia on the maximal response ($E_{max}$) and potency (pD$_2$) of PDBu-induced contractions in UAs. $E_{max}$ and pD$_2$ values were determined from the PDBu concentration-response curves shown in Fig. 2 by computer-assisted nonlinear regression to fit the data using GraphPad Prism. Data are expressed as means ± SE of tissues from 4–10 animals. *P < 0.05, pregnant vs. nonpregnant animals; **P < 0.05, hypoxia vs. normoxia.](image4)
Direct effect of chronic hypoxia on PKC-mediated contractions in UAs. To determine the direct effect of hypoxia on contractility, UAs isolated from nonpregnant and pregnant ewes of normoxic animals were treated ex vivo with either 21% or 10.5% O2 for 48 h before they were subjected to contraction experiments. The hypoxic treatment of 10.5% O2 for 48 h had no significant effect on KCl-induced contractions in either nonpregnant (10.4 ± 0.8 g/mm², P > 0.05) or pregnant (10.7 ± 0.7 vs. 11.4 ± 0.6 g/mm², P > 0.05) UAs. In contrast, PDBu-induced contractions were significantly increased by the hypoxic treatment in UAs of pregnant ewes (Figs. 8 and 9). PD-98059 significantly increased PDBu-induced contractions of pregnant UAs (Fig. 9). In the presence of PD-98059, hypoxia had no further effect on the PDBu-mediated contraction of pregnant UAs, which was not significantly different from that of the hypoxic treatment in the absence of PD-98059 (Fig. 9). Whereas PDBu-mediated contractions were significantly increased in UAs of nonpregnant ewes compared with those of pregnant animals, hypoxia did not affect PDBu-mediated contractions in nonpregnant UAs in the absence or presence of PD-98059 (Figs. 8 and 9).

DISCUSSION

The following several key observations were made in the present study: 1) long-term high-altitude hypoxia during pregnancy increases the pressure-dependent myogenic tone of resistance-sized UAs and eliminates the difference in the myogenic response between nonpregnant and pregnant UAs, 2) high-altitude hypoxia potentiates PKC-mediated contractions of pregnant UAs and abolishes the difference in PKC-mediated contractions between nonpregnant and pregnant UAs in the absence or presence of ERK1/2 inhibition with PD-98059, 3) chronic hypoxia caused a decrease in ERK1/2 and phospho-ERK1/2 protein abundance in pregnant UAs, 4) high-altitude hypoxia results in an increase in PKC-mediated baseline Ca²⁺ sensitivity in UAs of pregnant ewes, 5) high-altitude hypoxia inhibits ERK1/2-mediated down-regulation of PKC-ε in UAs of pregnant ewes, and 6) chronic hypoxia has a direct effect in the upregulation of PKC-induced contractions in pregnant UAs by removing the ERK1/2-mediated inhibition.
Our previous study (47) in sheep has demonstrated that pregnancy is associated with a significant decrease in pressure-induced myogenic tone in the UA. Similar findings of a pregnancy-mediated decrease in the vascular myogenic response have been obtained in mice and rats (10, 28, 29, 41). In the present study, we found that long-term high-altitude hypoxia during pregnancy increased the pressure-dependent myogenic response in UAs of pregnant ewes and abolished the differences in myogenic tone in UAs between pregnant and nonpregnant animals. To our knowledge, this is the first study showing that chronic hypoxia increases myogenic contractions.

Fig. 6. Effect of high-altitude hypoxia on PDBu-induced membrane translocation of PKC isozymes in UAs. PDBu-induced membrane translocations of PKC-α and PKC-ε were determined in NNUAs, NPUAs, HNUAs, and HPUAs in the absence or presence of PD-98059 (30 μM, pretreatment for 20 min). PDBu-induced increases in the ratio of the particulate to cytosolic distribution of PKC-α and PKC-ε are expressed as fold changes of basal levels of each isozyme blotted in the same membrane. Lane 1, basal; lane 2, PD-98059; lane 3, PDBu; lane 4, PDBu + PD-98059. Data are means ± SE of tissues from 5–6 animals. *P < 0.05, PDBu vs. basal; **P < 0.05, PDBu + PD98059 vs. PDBu alone; ***P < 0.05, all groups vs. NPUAs.

Fig. 7. PDBu-induced membrane translocation of p-PKC-α and p-PKC-ε in UAs. PDBu-induced membrane translocations of p-PKC-α and p-PKC-ε were determined in UAs obtained from four pregnant ewes.

Fig. 8. Effect of ex vivo hypoxia on PDBu-induced contractions in UAs. UAs isolated from normoxic nonpregnant and pregnant ewes were incubated at 37°C in a humidified incubator with either 21% or 10.5% O₂ for 48 h before they were subjected to contractions induced by increased concentrations of PDBu. Data are expressed as percentages of contraction generated by 120 mM KCl, and each point represents the mean ± SE of tissues from 7–12 animals.
increased concentrations of PDBu in the absence or presence of PD-98059 (30 or chronic hypoxia. The lack of a pressure-dependent myogenic response in the UA, which was not affected by either pregnancy or chronic hypoxia showed an absence of the pressure-dependent myogenic response of arteries, including the UA (5, 11, 22, 30, 32, 47). We have demonstrated that the reduced myogenic tone of the UA in pregnancy is primarily mediated by a decrease in the PKC signaling pathway. Consistent with our findings, it has been shown that pregnancy is associated with attenuated arterial PKC activity (6, 7, 17, 18, 25). Taken together, these findings indicate that long-term high-altitude hypoxia inhibits the pregnancy-induced downregulation of the myogenic response in the UA by upregulating the PKC signaling pathway. This is most likely due to the direct effect of chronic hypoxia because of the finding in the present study that ex vivo prolonged hypoxic treatment of isolated UAs from normoxic ewes produced similar effects on PKC-induced contractions as those found in UAs from high-altitude hypoxic animals. In agreement with the present finding, several previous studies (3, 24, 31, 34, 39, 44) have demonstrated that the activation of PKC played a central role in modulating hypoxic pulmonary vasoconstriction and hypertension.

The lack of a direct effect of prolonged hypoxia on KCl-induced contractions of the UA is consistent with the previous finding in long-term high-altitude hypoxic animals (57) and reinforces the notion that it is unlikely that the hypoxia-mediated increase in UA contractility is attributed to increased L-type Ca\(^{2+}\) channels and influx of extracellular Ca\(^{2+}\). It has been demonstrated that the PKC-dependent pathway modulates primarily the Ca\(^{2+}\) sensitivity of myogenic mechanisms (2, 8, 23, 26, 32). In the UA, the activation of PKC by PDBu decreased the diameter of pressurized vessels without increasing intracellular Ca\(^{2+}\) concentrations, indicating a key role of Ca\(^{2+}\) sensitization in PKC-mediated changes in vascular tone in the UA (47). In the present study, we determined the effect of chronic hypoxia on baseline myofilament Ca\(^{2+}\) sensitivity in pregnancy is primarily mediated by a decrease in the PKC signaling pathway. Consistent with our findings, it has been shown that pregnancy is associated with attenuated arterial PKC activity (6, 7, 17, 18, 25). Taken together, these findings indicate that long-term high-altitude hypoxia inhibits the pregnancy-induced downregulation of the myogenic response in the UA by upregulating the PKC signaling pathway. This is most likely due to the direct effect of chronic hypoxia because of the finding in the present study that ex vivo prolonged hypoxic treatment of isolated UAs from normoxic ewes produced similar effects on PKC-induced contractions as those found in UAs from high-altitude hypoxic animals. In agreement with the present finding, several previous studies (3, 24, 31, 34, 39, 44) have demonstrated that the activation of PKC played a central role in modulating hypoxic pulmonary vasoconstriction and hypertension.

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signaling in pregnant UAs, resulting in increased baseline Ca\(^{2+}\) sensitivity and the myogenic response. Unlike UAs, hypoxia-mediated augmentation of Ca\(^{2+}\) sensitivity in pulmonary arteries appeared through the activation of RhoA/Rho kinase but not PKC signaling (15), suggesting heterogeneity in the vascular response to chronic hypoxia.

Both PKC-\(\alpha\) and PKC-\(\varepsilon\) have been implicated in contractions of vascular smooth muscle through increasing Ca\(^{2+}\) sensitivity (12, 42, 43). The present finding that PDBu-induced activation of PKC-\(\varepsilon\) was similar in UAs from all four groups of animals in the absence or presence of PD-98059 suggests that PKC-\(\alpha\) is not involved in pregnancy- and/or hypoxia-mediated changes in PKC-induced contractions of UAs. In contrast, the finding of a significant decrease in PDBu-induced PKC-\(\varepsilon\) activation in UAs of pregnant ewes indicates a key role of PKC-\(\varepsilon\) in the pregnancy-mediated decrease in PKC-mediated contractions of UAs. Chronic hypoxia significantly increased the activation of PKC-\(\varepsilon\) in pregnant UAs and abolished the difference in PKC-\(\varepsilon\) activity between nonpregnant and pregnant UAs. This suggests that chronic hypoxia inhibits the pregnancy-mediated downregulation of PKC-\(\varepsilon\) activity, resulting in increased PKC-mediated contractions in UAs. Consistent with the present finding, a previous study (24) in a PKC-\(\varepsilon\) knockout mouse in which PKC-\(\varepsilon\) was absent, and expression of all other isozymes was unaffected, demonstrated a direct involvement of PKC-\(\varepsilon\) in pulmonary pressor responses to hypoxia. The effect of PKC-\(\varepsilon\) ablation appeared to be specific to hypoxia-mediated vasoconstriction in perfused lungs, because no differences in the pressure response to infused angiotensin II or cumulative KCl were detected in PKC-\(\varepsilon^{-/-}\) mice compared with PKC\(^{\alpha\gamma\delta\theta^{++}}\) mice (24).

Additionally, the present study suggests that hypoxia-mediated upregulation of PKC activation is likely due to an inhibition of ERK1/2 activity. Our previous studies (47, 50, 53) have demonstrated that pregnancy-increased ERK1/2 acted as an upstream signal in suppressing PKC-mediated contractions and pressure-dependent myogenic tone in UAs. In the present study, we demonstrated that inhibition of ERK1/2 by PD-98059 increased PDBu-induced PKC-\(\varepsilon\) activation in pregnant UAs, and, in the presence of PD-98059, there were no significant differences in PKC-\(\varepsilon\) activity between nonpregnant and pregnant UAs. This supports the previous findings and indicates a selective cross talk between PKC-\(\varepsilon\) and ERK1/2 pathways in the adaptation of the UA myogenic response to pregnancy. The finding of the lack of effect of PD-98059 on PDBu-induced PKC-\(\varepsilon\) activation in UAs of pregnant ewes of high-altitude animals suggests that chronic hypoxia during pregnancy inhibits ERK1/2 activity, resulting in increased PKC activity in UAs. This is further supported by the findings that chronic hypoxia abolished the effect of PD-98059 and increased PDBu-induced contractions in pregnant UAs in the absence or presence of PD-98059. Additionally, we demonstrated in the present study that long-term high-altitude hypoxia resulted in a significant decrease in ERK1/2 protein expression in UAs of pregnant but not nonpregnant ewes, which was associated with a reduction in ERK1/2 activity. Although many previous studies have suggested that hypoxia increased ERK activity, most if not all of these studies were conducted in cell cultures with short-term hypoxia treatment within 24 h. Few studies have examined the effect of long-term hypoxia in animals on ERK1/2 expression and activity in tissue. A study (45) of male rats treated with hypoxia for 14 days showed an increase in ERK activity in pulmonary but not in aortic fibroblasts. This tissue-specific response of ERK to long-term hypoxia was also demonstrated in the heart, in which 25-day hypoxia treatment of rats resulted in a partial upregulation of ERK2 in the right ventricle, but expression of ERK1/2 in the left ventricle as well as their activities in both ventricles were not affected by chronic hypoxia (38). In the present study, given that in normoxic animals there was a significant increase in ERK1/2 in pregnant compared with nonpregnant UAs and this difference was abolished in hypoxic animals, the results suggest that chronic hypoxia during pregnancy inhibits the upregulation of ERK1/2 in the UA that normally occurs in pregnancy. This is supported by previous studies (35, 36) demonstrating that long-term high-altitude hypoxia diminished the proliferative response of UA vascular smooth muscle cells in pregnant guinea pigs, although ERK1/2 was not measured. The similar findings obtained in UAs of long-term high-altitude hypoxic animals and those of prolonged ex vivo hypoxic treatment strongly suggest a direct effect of chronic hypoxia on the cross talk between ERK1/2 and PKC pathways in the adaptation of the UA myogenic response to pregnancy.

In summary, we have shown in the ovine UA that pregnancy-induced downregulation of pressure-dependent myogenic tone is inhibited by chronic hypoxia in pregnancy. The increased myogenic response by hypoxia is mediated in part by a decrease in the inhibitory effect of ERK1/2, resulting in an increase in the PKC signal pathway, which leads to an increase in baseline Ca\(^{2+}\) sensitivity of the myogenic mechanism in the UA during pregnancy. It should be noted that it is unlikely that ERK1/2 is the only possible mechanism in hypoxia-induced changes in UA contractility because hypoxia is known to activate many contractile signaling pathways in vascular smooth muscle. For instance, inhibition of K\(^{+}\) channels may also be important in the hypoxic effect. 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 increased myogenic response of the UA in pregnancy with chronic hypoxia is likely to contribute significantly to the dysregulation of uterine vascular hemodynamics and results in reduced uterine blood flow, which may have deleterious consequences on fetal development during pregnancy. Indeed, it has been shown that long-term high-altitude hypoxia attenuates the pregnancy-induced increase in uterine blood flow, which is associated with an increased risk of preeclampsia and fetal intrauterine growth restriction (46, 54, 55). The potential mechanisms of sex steroid hormones in the regulation of the PKC/ERK1/2 pathway and the myogenic response of UAs in the adaptation to pregnancy and chronic hypoxia present an intriguing area for future investigation.

**REFERENCES**


