Chronic Hypoxia Inhibits Sex Steroid Hormone-Mediated Attenuation of Ovine Uterine Arterial Myogenic Tone in Pregnancy

Katherine Chang, DaLiao Xiao, Xiaohui Huang, Zhice Xue, Shumei Yang, Lawrence D. Longo, Lubo Zhang

Abstract—Previous studies in ovine uterine arteries have demonstrated that sex steroid hormones upregulate extracellular signal–regulated kinase 1/2 expression and downregulate the protein kinase C signaling pathway, resulting in the attenuated myogenic tone in pregnancy. The present study tested the hypothesis that chronic hypoxia during gestation inhibits the sex steroid-mediated adaptation of extracellular signal–regulated kinase 1/2 and protein kinase C signaling pathways and increases the myogenic tone of uterine arteries. Uterine arteries were isolated from nonpregnant and near-term pregnant sheep that had been maintained at sea level (≈300 m) or exposed to high-altitude (3801 m) hypoxia for 110 days. In contrast to the previous findings in normoxic animals, 17β-estradiol and progesterone failed to suppress protein kinase C–induced contractions and the pressure-induced myogenic tone in uterine arteries from hypoxic animals. Western analyses showed that the sex steroids lost their effects on extracellular signal–regulated kinase 1/2 expression and phospho-extracellular signal–regulated kinase 1/2 levels, as well as the activation of protein kinase C isozymes in uterine arteries of hypoxic ewes. In normoxic animals, pregnancy and the sex steroid treatments significantly increased uterine artery estrogen receptor-α and progesterone receptor B expression. Chronic hypoxia selectively downregulated estrogen receptor-α expression in uterine arteries of pregnant animals and eliminated the upregulation of estrogen receptor-α in pregnancy or by the steroid treatments observed in normoxic animals. The results demonstrate that, in the ovine uterine artery, chronic hypoxia in pregnancy inhibits the sex steroid hormone–mediated adaptation of decreased myogenic tone by downregulating estrogen receptor-α expression, providing a mechanism linking hypoxia and maladaptation of uteroplacental circulation and an increased risk of preeclampsia in pregnancy. (Hypertension. 2010;56:750-757.)

Key Words: hypoxia • pregnancy • steroids • uterine artery • myogenic tone

Pregnancy is associated with a significant decrease in uterine vascular tone and an increase in uterine blood flow to meet the increasing metabolic demands of the mother and developing fetus. Pressure-dependent myogenic contraction is an important physiological mechanism in regulating basal vascular tone and organ blood flow, and decreased pressure-induced myogenic responses of the uterine arteries contribute significantly to the adaptation of uterine vascular hemodynamics in pregnancy.1–5 Recently, we have demonstrated a direct genomic effect of the sex steroid hormones, estrogen and progesterone, in upregulating the extracellular signal–regulated kinase (ERK) 1/2 expression and downregulating protein kinase C (PKC) signaling pathway, resulting in the attenuated myogenic tone of uterine arteries in pregnancy.6

Several studies in animal models suggest that a chronic reduction of uteroplacental perfusion leads to the characteristics of preeclampsia found in pregnant women, including hypertension, reduced glomerular filtration rate, proteinuria, intrauterine growth restriction, and endothelial dysfunction.7–9 Chronic hypoxia during gestation is a common stress to maternal cardiovascular homeostasis and has profound adverse effects on uterine artery contractility, leading to the attenuation of pregnancy-induced increase in uterine blood flow and an increased risk of preeclampsia and fetal intrauterine growth restriction.10–15 Recently, we have demonstrated in sheep that long-term high-altitude hypoxia during pregnancy significantly increases the pressure-dependent myogenic tone of resistance-sized uterine arteries by suppressing the ERK1/2 activity and increasing the PKC signaling pathway, leading to the increased Ca^{2+} sensitivity of myogenic mechanisms.16 The finding that chronic hypoxia eliminated the difference in the pressure-induced myogenic response of the uterine arteries between pregnant and nonpregnant animals16 is intriguing and suggests that hypoxia...
inhibits the normal adaptation of uterine vascular myogenic responses during pregnancy. Herein, we present evidence that chronic hypoxia in pregnancy inhibits the sex steroid hormone–mediated adaptation of decreased myogenic tone in the uterine artery by downregulating estrogen receptor (ER)-α expression, providing a mechanism linking hypoxia and maladaptation of uteroplacental circulation and an increased risk of preeclampsia 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 of gestation) sheep maintained at high altitude (altitude: ≈300 m; PaO₂: 102 ± 2 mm Hg) or at high altitude (altitude: 3801 m; PaO₂: 60 ± 2 mm Hg) for ≈110 days. As described previously, 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 absence or presence of 17β-estradiol (E₂β), progesterone, ICI 182 780, RU 486, propyl pyrazole triol, and diarylpropionitrile, respectively. To investigate the direct effect of hypoxia, some arterial rings obtained from normoxic animals were treated in a humidified incubator with either 21.0% or 10.5% O₂ for 48 hours, as described previously. All of the procedures and protocols were approved by the Institutional Animal Care and Use Committee guidelines.

Measurement of Plasma E₂β
Blood samples were collected from sheep, and plasma E₂β concentrations were measured by a radioimmunoassay, as reported previously.

Measurement of Myogenic Tone
Pressure-dependent myogenic tones of resistance-sized uterine arteries were measured as described previously.

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

Real-Time RT-PCR
mRNA abundance of ER-α and ER-β and progesterone receptor (PR) was determined by real-time RT-PCR, as described previously.

Western Blot Analysis
Protein abundance of E-Rα, ER-β, PR, ERK1/2, and phospho-ERK1/2 was determined with Western blot analysis, as described previously.

Measurement of PKC Isozyme Translocation
Protein abundance of PKC-α, -βI, -βII, -δ, -ε, and -ζ in the cytosolic and particulate fractions was determined with Western blot analysis, as described previously.

Data Analysis
Data were expressed as mean ± 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.

Results
Chronic Hypoxia Abolishes the Steroid Hormone–Mediated Attenuation of Pressure-Dependent Myogenic Tone
In contrast to the previous finding in normoxic animals, in high-altitude hypoxic animals there were no significant differences in the pressure-dependent myogenic tone of uterine arteries between pregnant and nonpregnant sheep (Figure 1A). The E₂β (0.3 nmol/L) and progesterone (100.0 nmol/L) treatments for 48 hours did not alter uterine arterial myogenic responses in high-altitude nonpregnant sheep (Figure 1B). In accordance, the treatment with the ER antagonist ICI 182 780 (10.0 nmol/L) plus RU 486 (1.0 μmol/L) had no significant effects on myogenic responses of uterine arteries from high-altitude pregnant animals (Figure 1C).

Chronic Hypoxia Inhibits Steroid Hormone Regulation of PKC-Mediated Contractions
In normoxic animals, E₂β and progesterone caused a significant decrease in PKC-mediated contractions of the uterine arteries. In hypoxic animals, E₂β and progesterone treatments had no significant effects on phorbol 12,13-dibutyrate (PDBu)-induced contractions of the uterine arteries in nonpregnant sheep (Figure 2A). In accordance, ICI 182 780 and RU 486 did not alter the PDBu-induced contractions in pregnant animals (Figure 2B). To determine the direct effect of hypoxia on the inhibition of steroid hormone regulation of PKC-induced contractions, uterine arteries isolated from low-altitude normoxic nonpregnant and pregnant ewes were...
treated ex vivo with the steroid hormones and their receptor antagonists under either 21.0% O2 or 10.5% O2 for 48 hours before they were subjected to contraction studies. Similar to the findings in the arteries from high-altitude hypoxic animals, the uterine arteries treated with prolonged hypoxia ex vivo lost the responses mediated by the sex steroid hormones (Figure 2C and 2D).

**Chronic Hypoxia Diminishes Steroid Hormone-Mediated PKC Isozymes Translocation**

The subcellular distributions of PKC isozymes α, βI, βII, δ, ε, and ζ in cytosolic and particulate fractions in the uterine arteries after chronic treatments with E2β and progesterone or their receptor antagonists are shown in Figure 3. In contrast to the previous finding in normoxic animals that E2β and progesterone stimulated the membrane translocation of PKC-α, -ε, and -ζ in the uterine arteries,6 E2β and progesterone failed to cause the membrane translocation in any of the 6 PKC isozymes in the uterine arteries of high-altitude hypoxic pregnant sheep (Figure 3). Similarly, the ICI 183 780 and RU 486 treatment had no significant effects on the PKC isozyme translocation in the uterine arteries of hypoxic pregnant animals (Figure 3).

**Chronic Hypoxia Eliminates Steroid Hormone Upregulation of ERK1/2 Expression**

As shown in Figure 4, ERK1/2 and phospho-ERK1/2 protein abundance was determined by Western blot analyses in the uterine arteries of high-altitude hypoxic ewes. In contrast to the finding in normoxic animals,6 E2β and progesterone treatments had no significant effects on the protein abundance of ERK1/2 and phospho-ERK1/2 in the uterine arteries of hypoxic nonpregnant animals. In accordance, ICI 182 780 and RU 486 did not significantly alter ERK1/2 and phospho-

---

**Figure 2.** Effect of chronic hypoxia on the steroid hormone-mediated regulation of PDBu-induced contractions. Uterine arteries were isolated from high-altitude hypoxic nonpregnant (A) and pregnant (B) ewes and from low-altitude normoxic nonpregnant (C) and pregnant (D) animals and incubated under 10.5% O2. Nonpregnant arteries were treated with E2β, progesterone (P4), and E2β plus P4 or vehicle control for 48 hours and pregnant vessels with ICI 182 780 plus RU 486 for 48 hours in the presence of E2β and P4. After the treatments, PDBu-induced contractions were determined. Data are mean±SEM of 7 to 19 high-altitude animals and 8 to 11 low-altitude animals.

**Figure 3.** Effect of steroid hormones on PKC activities of uterine arteries in high-altitude hypoxic sheep. Nonpregnant uterine arteries (NPUAs) were treated with E2β (0.3 nmol/L) plus progesterone (P4; 100.0 nmol/L) or vehicle control for 48 hours. Pregnant uterine arteries (PUAs) were treated with ICI 182 780 (ICI; 10.0 μmol/L) plus RU 486 (RU; 1.0 μmol/L) or vehicle control for 48 hours in the presence of E2β and P4. PKC isozymes in the cytosolic and particulate fractions were determined by Western blotting and expressed as a ratio of particulate:cytosolic fractions. Data are mean±SEM of 4 to 6 animals. *P<0.05 vs control.
ERK1/2 protein abundance in the uterine arteries of hypoxic pregnant animals.

**Chronic Hypoxia Downregulates ER-α Expression in Uterine Arteries**

The expression and transcription levels of ER-α, ER-β, and PRs in the vascular smooth muscle of uterine arteries were determined by Western analyses and real-time RT-PCR (Figure 5). In uterine arteries of pregnant animals, the mRNA and protein abundance of combined cytosolic and nuclear ER-α, but not ER-β, were significantly decreased in high-altitude hypoxic sheep, as compared with low-altitude control animals (Figure 5A). In contrast, there were no significant differences in plasma E2β concentrations between low-altitude control (81.9 ± 3.5 pg/mL) and high-altitude hypoxic (82.3 ± 4.2 pg/mL) pregnant sheep. Interestingly, although PR (A/B) mRNA levels were significantly decreased in the hypoxic animals, the protein abundances of PR A and B were not significantly different in the uterine arteries between hypoxic and control animals (Figure 5A). To determine the direct effect of hypoxia on the expression of sex hormone receptors, uterine arteries isolated from low-altitude normoxic pregnant ewes were treated ex vivo with 21.0% O2 or 10.5% O2 for 48 hours. Figure 5B shows that the ex vivo hypoxic treatment of uterine arteries produced the results similar to those found in high-altitude hypoxic animals (Figure 5A).

To determine the extent to which chronic hypoxia during gestation inhibits the normal adaptation of sex hormone receptor expression in the uterine artery, protein and mRNA abundances of estrogen and PRs were measured in uterine arteries of nonpregnant and pregnant sheep in both low-altitude and high-altitude groups. Western analyses showed that the expression of combined cytosolic and nuclear ER-α, as well as PRs A and B, were significantly greater in the uterine arteries of pregnant ewes as compared with that in the nonpregnant state in low-altitude animals (Figure 6A and 6B). In agreement, the mRNA levels for ER-α and ER-β, PR A (PRa), and B (PRb) protein and mRNA abundance were determined by Western blotting and real-time RT-PCR, respectively. A, Uterine arteries were isolated from low-altitude normoxic (control) and high-altitude hypoxic (hypoxia) pregnant ewes. B, Uterine arteries from low-altitude normoxic pregnant ewes were treated under 21.0% O2 (control) or 10.5% O2 (hypoxia) for 48 hours. Data are mean ± SEM of 5 animals. *P < 0.05 vs control.

**Figure 5.** Effect of chronic hypoxia on estrogen and PR expression in uterine arteries of pregnant ewes. ER-α and ER-β, PR A (PRa), and B (PRb) protein and mRNA abundance were determined by Western blotting and real-time RT-PCR, respectively. A, Uterine arteries were isolated from low-altitude normoxic (control) and high-altitude hypoxic (hypoxia) pregnant ewes. B, Uterine arteries from low-altitude normoxic pregnant ewes were treated under 21.0% O2 (control) or 10.5% O2 (hypoxia) for 48 hours. Data are mean ± SEM of 5 animals. *P < 0.05 vs control.

**Figure 4.** Effect of steroid hormones on ERK1/2 protein abundance in uterine arteries in high-altitude hypoxic sheep. Non-pregnant uterine arteries (NPUAs) were treated with E2β (0.3 nmol/L) plus progesterone (P4; 100.0 nmol/L) or vehicle control for 48 hours. Pregnant uterine arteries (PUsAs) were treated with ICI 182 780 (ICI; 10.0 μmol/L) plus RU 486 (RU; 1.0 μmol/L) or vehicle control for 48 hours in the presence of E2β plus P4. Protein abundance of total and phosphorylated ERK1/2 was determined by Western blotting. Data are mean ± SEM of 5 animals. *P < 0.05 vs control.
Effect of pregnancy on estrogen and PR expression in uterine arteries in low- and high-altitude ewes. Uterine arteries were isolated from nonpregnant ewes in low-altitude (normoxia) and high-altitude (hypoxia) animals and were treated with E2β (0.3 nmol/L), progesterone (P4; 100.0 nmol/L), and E2β plus P4 or vehicle control for 48 hours. ERα and ERβ, PR A (PRa), and B (PRb) protein and mRNA abundance were determined by Western blotting and real-time RT-PCR, respectively. Data are mean±SEM of 5 animals. *P<0.05 vs control.

**Discussion**

The present study demonstrates for the first time that chronic hypoxia diminishes the inhibitory effect of sex steroid hormones on the pressure-dependent myogenic tone of uterine arteries in pregnancy. Estrogen plays a key role in regulating uterine blood flow.19–24 The finding that maternal plasma E2β others, as compared with their respective nonpregnant groups (Figure 6B).

To further examine the role of sex hormones in the regulation of their receptors, uterine arteries isolated from nonpregnant ewes of low-altitude and high-altitude animals were treated ex vivo with sex steroid hormones under either 21.0% O2 or 10.5% O2 for 48 hours. In low-altitude normoxic animals, the treatment of nonpregnant vessels with E2β, progesterone, and E2β plus progesterone, respectively, demonstrated a greater expression of ERα and PR B and a reduced expression of PR A, as compared with the untreated groups (Figure 7). In the uterine arteries of high-altitude hypoxic animals, the steroid hormone treatments had no significant effects on the abundance of ERα and PR A (Figure 7). Although E2β decreased ERβ expression, progesterone increased the expression of both ERβ and PR B in the uterine arteries in hypoxic animals (Figure 7).

**Figure 6.** Effect of pregnancy on estrogen and PR expression in uterine arteries in low- and high-altitude ewes. ERα and ERβ, PR A (PRa), and B (PRb) protein and mRNA abundance were determined by Western blotting and real-time RT-PCR, respectively. A, Uterine arteries were isolated from pregnant and nonpregnant ewes in low altitude. B, Uterine arteries were isolated from pregnant and nonpregnant ewes in high altitude (hypoxia). C, Uterine arteries were isolated from pregnant and nonpregnant ewes in low altitude. Data are mean±SEM of 4 to 5 animals. *P<0.05 vs nonpregnant.

**Figure 7.** Effect of E2β and progesterone on estrogen and PR expression in uterine arteries in low- and high-altitude ewes. Uterine arteries were isolated from nonpregnant ewes in low-altitude (normoxia) and high-altitude (hypoxia) animals and were treated with E2β (0.3 nmol/L), progesterone (P4; 100.0 nmol/L), and E2β plus P4 or vehicle control for 48 hours. ERα and ERβ, PR A (PRa), and B (PRb) protein and mRNA abundance were determined by Western blotting and real-time RT-PCR, respectively. Data are mean±SEM of 5 animals. *P<0.05 vs control.

**Figure 8.** Effect of selective ER agonists on PDBu-induced contractions in uterine arteries. Uterine arteries were isolated from low-altitude nonpregnant ewes and were treated with propyl pyrazole triol (PPT; 10.0 μmol/L), or vehicle control for 48 hours. Data are mean±SEM of 5 animals.
levels were similar in low- and high-altitude pregnant ewes is consistent with the previous observations in guinea pigs in which plasma estrogen levels were unaltered by exposure to high altitude, although estrogen-specific responses were diminished in the uterine artery. These findings suggest that the hypoxia-mediated effects are imposed on the functional responsiveness of the uterine artery to sex steroid hormones. This is further supported by the present finding that exogenous E$_2$/$\beta$ and progesterone treatments failed to regulate the pressure-dependent myogenic tone of uterine arteries in high-altitude ewes.

Previous studies have demonstrated that E$_2$/$\beta$ and progesterone suppress arterial myogenic contractions in women and downregulate myogenic tone of uterine arteries in pregnant sheep. The lack of hormone-mediated myogenic responses in hypoxic animals is likely attributed to the loss of their inhibitory effects on PKC-induced contractions. This is consistent with our previous studies demonstrating that high-altitude hypoxia significantly increased PKC-induced contractions in pregnant ewes and abolished the differences in PKC-mediated uterine arterial contractions between nonpregnant and pregnant animals. PKC plays an important role in regulating pressure-dependent myogenic responses of resistance arteries, and a decrease in the PKC signaling pathway accounts for attenuated myogenic tone of the uterine artery in pregnancy. In low-altitude normoxic sheep, the genomic action of the sex hormones in attenuating the myogenic tone of uterine arteries is mediated by the downregulation of the PKC signaling pathway in the vascular smooth muscle. Similar findings have been obtained in monkeys, showing that physiological concentrations of estrogen and progesterone have inherent effects in vascular smooth muscle cells and that they suppress PKC activity and myogenic contractions of the coronary artery. In contrast to the findings in the normoxic animals that E$_2$/$\beta$ and progesterone significantly reduced the basal activity of PKC, the hormones had no significant effects on the activities of PKC isozymes determined in the hypoxic animals. In a previous study, we demonstrated that chronic hypoxia significantly increased the activity of PKC- in pregnant uterine arteries and abolished the difference in the PKC- activity in the uterine arteries between nonpregnant and pregnant sheep. These findings suggest that chronic hypoxia during pregnancy inhibits the sex hormone–mediated downregulation of PKC- activity, resulting in increased PKC-mediated contractions and myogenic responses in uterine arteries.

The finding that E$_2$/$\beta$ and progesterone had no effect on protein levels of total and phosphorylated ERK1/2 in the uterine arteries from high-altitude hypoxic animals is intriguing and suggests a possible upstream mechanism for the loss of PKC-induced contractions and myogenic tone to the sex hormones in the hypoxic animals. The previous studies have demonstrated that hormone-mediated increases in ERK1/2 gene expression act as upstream signals in suppressing PKC-mediated contractions and myogenic tone of uterine arteries in pregnant animals. In addition to the genomic function in the nucleus, estrogens activate multiple signal transduction cascades, including ERK, in the extranuclear compartment, possibly through protein methylation of ER-$\alpha$. Unlike breast cancer cells in which increased ERK activity represses ER-$\alpha$, both ER-$\alpha$ and ERK are upregulated and activated in the uterine arteries of pregnant animals. The potential effect of ERK on ER-$\alpha$ expression in the uterine artery remains to be determined. The finding that inhibition of ERK1/2 restored the sex hormone–mediated attenuation of PKC-induced contractions in uterine arteries indicates a key role for ERK1/2 activation in the hormone-induced suppression of PKC activity and myogenic responses. Consistent with the present finding, the previous study demonstrated that high-altitude hypoxia resulted in a significant decrease in ERK1/2 protein expression in uterine arteries of pregnant sheep and abolished the difference in ERK1/2 levels between pregnant and nonpregnant ewes, as demonstrated in low-altitude animals. This lack of the hormonal upregulation of ERK1/2 in uterine arteries of pregnant ewes in high-altitude hypoxic animals is likely to result in the increased PKC- activity and PDBu-induced contractions in uterine arteries in the hypoxic animals, as demonstrated previously.

The question arises as to the mechanism by which chronic hypoxia during gestation might alter the sex hormone–mediated responses in uterine arteries. Consistent with the previous study, the present study demonstrated that ER-$\alpha$ was the dominant receptor in the uterine artery. In contrast to the previous finding that ER-$\beta$ but not ER-$\alpha$ was increased in the vascular smooth muscle of uterine arteries in pregnant sheep, the present study demonstrated a greater abundance of ER-$\alpha$ and ER-$\beta$ mRNA and ER-$\alpha$ protein in the uterine artery in pregnant animals. This may be because the cytosolic and nuclear hormonal receptors were mainly determined in the previous study, whereas in the present study the combined cytosolic and nuclear hormonal receptors were measured. Although the conditions of the ovarian cycle of luteal or follicular phases were not determined in nonpregnant animals in the present study, pregnancy is a stage of high steroid hormone concentrations. The potential effect of the ovarian cycle on the steroid hormone receptors needs to be further investigated.

The physiological importance of a greater ER-$\alpha$ expression in pregnancy has been demonstrated in human uterine arteries. This leads to a lower collagen concentration resulting in reduced tissue stiffness and increased distensibility of the uterine artery to accommodate the marked elevation in uterine blood flow. Similar findings were observed in sheep showing a correlation of higher ER-$\alpha$ levels (the present study) and the increased distensibility of the uterine artery in pregnant animals.

To our knowledge, the effect of pregnancy on PRs in the uterine artery has not been studied previously. The increased expression of PR A and B in the uterine artery smooth muscle in pregnant animals is consistent with the role of progesterone in downregulating myogenic contractions of the uterine arteries in pregnancy. In several other tissues, including the endometrium, progesterone may antagonize the actions of estrogen, but this does not occur in uterine arteries. Although 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, it has been demonstrated that, in the second half of pregnancy and at term, increases in
uterine blood flow directly relate to progesterone concentrations and even more prominently with the sum of progesterone and estrogen.\textsuperscript{40,41}

Of interest, the present study demonstrates that high-altitude hypoxic exposure during pregnancy suppresses ER-\(\alpha\) expression in the uterine artery, providing a mechanism for the hypoxia-induced inhibition of sex hormone–mediated responses in uterine arteries. Consistent with this finding, the diameter of uterine arteries and uterine blood flow were found to be significantly reduced in pregnant women residing at high altitude.\textsuperscript{14} This is most likely because of the direct effect of chronic hypoxia, given that the ex vivo prolonged hypoxic treatment of isolated uterine arteries from the normoxic ewes produced the similar effects on ER-\(\alpha\) expression as those found in uterine arteries in high-altitude hypoxic animals. This is consistent with the finding that chronic hypoxia had a direct effect on increased PKC-induced contractions and myogenic responses of the uterine artery in pregnant ewes.\textsuperscript{16} The direct effects of steroid hormones in regulating the hormonal receptors in the vascular smooth muscle of uterine arteries demonstrated in the present study are consistent with the premise that ovarian steroids maintain and regulate expression of their own receptors and are largely in agreement with the previous findings of the effect of in vivo hormonal treatments on steroid receptor expression in uterine arteries in sheeph.\textsuperscript{38} Of interest, in high-altitude hypoxic animals the hormonal-mediated upregulation of ER-\(\alpha\) expression was abolished, leading to no significant difference in the ER-\(\alpha\) density in uterine arteries between nonpregnant and pregnant animals. The mechanism(s) for the hypoxia-mediated loss of hormonal effects on the upregulation of ER-\(\alpha\) expression in uterine arteries is not clear at present. Given the findings that chronic hypoxia increases global DNA methylation levels\textsuperscript{42} and that ER-\(\alpha\) gene promoter contains CpG islands,\textsuperscript{43–45} it is plausible to speculate that increased promoter methylation may contribute to ER-\(\alpha\) gene repression in the uterine artery in response to chronic hypoxia. Indeed, it has been shown that hypermethylation of promoter CpG islands is associated with the loss of ER-\(\alpha\) expression and function.\textsuperscript{46–49} The finding that hypoxia decreased PR mRNA but not protein abundance is intriguing and suggests a compensatory upregulation of translation, supporting an essential role for PRs in maintaining the pregnancy.

It has been demonstrated that E2\(\beta\) confers its vascular protective effect primarily via ER-\(\alpha\) rather than ER-\(\beta\).\textsuperscript{50,51} However, it is unknown whether ER-\(\alpha\), ER-\(\beta\), or both mediate the estrogen-induced downregulation of uterine artery myogenic responses and rise in uterine blood flow. The present study provides the first evidence that activation of either ER-\(\alpha\) or ER-\(\beta\) suppresses PKC-induced contractions and, hence, myogenic responses of uterine arteries. The 2 selective agonists, propyl priol triol and dipropionitrile, have been widely used in studying the effects of ER-\(\alpha\) and ER-\(\beta\). The present finding is somewhat different from the other studies showing that ER-\(\alpha\), but not ER-\(\beta\), is implicated in the regulation of myogenic responses in subcutaneous and mesenteric arteries.\textsuperscript{52,53} However, the differences in the expression and function of ERs between uterine arteries and other systemic vessels have been well demonstrated.\textsuperscript{38} Given that both ER-\(\alpha\) and ER-\(\beta\) agonists suppress PKC-mediated contractions, whereas only ER-\(\alpha\) but not ER-\(\beta\) is downregulated by chronic hypoxia, it is possible for ER-\(\beta\) to play a parallel or compensatory role to suppress the myogenic tone.

**Perspectives**

The present study demonstrates that chronic hypoxia inhibits the steroid hormone–mediated adaptation of ERK1/2 and PKC signaling pathways to cause an increase in the myogenic tone of uterine arteries in pregnancy. Although it may be difficult to translate the present findings directly to humans, the possibility that the hypoxia-mediated dysregulation of myogenic tone contributes to the maladaptation of uteroplacental circulation with a consequence of increased pregnancy complications provides a mechanistic understanding worthy of investigation in humans. This is because hypoxia during gestation is a common stress to maternal cardiovascular homeostasis, causing a reduction of uteroplacental perfusion and an increased risk of preeclampsia. Given that ER-\(\alpha\) is the predominant receptor in the estrogen-mediated protective effect of vascular remodeling, the novel finding of the hypoxia-induced downregulation of ER-\(\alpha\) expression in the vascular smooth muscle is likely to have broader implications in addition to the regulation of vascular contractility. In addition, the potential epigenetic mechanism of DNA methylation in the hypoxia-mediated ER-\(\alpha\) gene repression in the vascular smooth muscle presents an intriguing area for the future investigation.

**Sources of Funding**

This work was supported by National Institutes of Health grants HD31226 (to L.D.L. and L.Z.), HL89012 (to L.Z.), HL83966 (to L.Z.), and DA025319 (to S.Y.).

**Disclosures**

None.

**References**


9. Walsh SK, English FA, Johns EJ, Kenny LC. Plasma-mediated vascular dysfunction in the reduced uterine perfusion pressure model of pre-


Chronic Hypoxia Inhibits Sex Steroid Hormone-Mediated Attenuation of Ovine Uterine Arterial Myogenic Tone in Pregnancy

Katherine Chang, DaLiao Xiao, Xiaohui Huang, Zhice Xue, Shumei Yang, Lawrence D. Longo and Lubo Zhang

*Hypertension*. 2010;56:750-757; originally published online July 26, 2010; doi: 10.1161/HYPERTENSIONAHA.110.155812

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/56/4/750

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2010/07/23/HYPERTENSIONAHA.110.155812.DC1

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

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

Subscriptions: Information about subscribing to *Hypertension* is online at:
http://hyper.ahajournals.org//subscriptions/
Chronic Hypoxia Inhibits Sex Steroid Hormones-Mediated Attenuation of Ovine Uterine Arterial Myogenic Tone in Pregnancy

By

Katherine Chang,* DaLiao Xiao,* Xiaohui Huang, Zhice Xue, Shumei Yang, Lawrence D. Longo and Lubo Zhang

The Center for Perinatal Biology (K.C., D.X., X.H., Z.X., L.D.L., L.Z.), Department of Physiology and Pharmacology, Loma Linda University School of Medicine, Loma Linda; and the Department of Chemistry and Biochemistry (S.Y.), California State University, San Bernardino, California

Running title: Chronic hypoxia and myogenic tone

Corresponding author:

Lubo Zhang, PhD
Center for Perinatal Biology
Department of Physiology and Pharmacology
Loma Linda University School of Medicine
Loma Linda, CA 92350
Tel: 909-558-4325
Fax: 909-558-4029
E-mail: lzhang@llu.edu

* These two authors contribute equally to the work.
Materials and Methods

Tissue preparation and treatment
Uterine arteries were isolated from nonpregnant and near-term pregnant (~140 days’ gestation) sheep from the Nebeker Ranch in Lancaster, CA (altitude: ~300 m, arterial PO2: 102 ± 2 mmHg). For the chronic hypoxic treatment, nonpregnant and pregnant (30 days of gestation) animals were transported to the Barcroft Laboratory, White Mountain Research Station, Bishop, CA (altitude, 3,801 m; maternal PaO2, 60 ± 2 mmHg) and maintained there for ~110 days, as previously described.1 Animals were transported to the laboratory immediately before the experiments. To isolate uterine arteries, animals 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. Uterine arteries were isolated and removed without stretching and placed into a modified Krebs solution as described previously.1 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.2-8 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, Somerville, NJ). For the ex vivo treatments, 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 hours at 37 °C in a humidified incubator with 5% CO2/95% air, as described previously,9 in the absence or presence of 17β-estradiol (E2β) (Sigma, St. Louis, MO), progesterone (Sigma), ICI 182,780 (Tocris Bioscience, Ellisville, MO), RU 486 (Sigma), propyl pyrazole triol (PPT) and diarylpropionitrile (DPN), respectively. To investigate the direct effect of hypoxia, some arterial rings obtained from normoxic nonpregnant and pregnant ewes were maintained in a humidified incubator with either 21% or 10.5% O2 for 48 hours, as described previously.1 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 plasma 17β-estradiol
Blood samples were collected from maternal jugular vein into ice-cold tubes containing lithium heparin, centrifuged and stored at –80 °C before assays. Plasma E2β concentrations were measured by a radioimmunoassay (RIA) in a blind manner, as reported previously.10 The sensitivity of each assay was <5 pg/ml. The interassay variation was 3.4%. Cross reaction with estriol, progesterone, and testosterone was <0.09%, <0.01%, and <0.01%, respectively.

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 N\textsuperscript{G}-nitro-L-arginine (L-NNA, 100 µmol/L) for 30 minutes. 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\textsuperscript{2+}-free PSS containing 3 mmol/L EGTA to determine the maximum passive diameter. The following formula was used to calculate percent myogenic tone at each pressure step: \( \% \text{myogenic tone} = (D_1-D_2)/D_1 \times 100 \), where \( D_1 \) is the passive diameter in Ca\textsuperscript{2+}-free PSS (zero Ca\textsuperscript{2+} with 3 mmol/L EGTA), and \( D_2 \) is the active diameter with normal PSS in the presence of extracellular Ca\textsuperscript{2+}.

**Contraction studies**

The fourth branches of middle uterine arteries were separated from the surrounding tissue, and cut into 2-mm ring segments. After the hormonal and/or antagonist treatments for 48 hours, isometric tensions were measured in tissue baths at 37 °C, as described previously. After 60 minutes of equilibration, each ring was stretched to the optimal resting tension as determined by the tension developed in response to 120 mmol/L 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. 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} (-logEC\textsubscript{50}) values.

**Real-time RT-PCR**

RNA was extracted from uterine arteries using TRIzol protocol (Invitrogen, Carlsbad, USA). mRNA abundance of estrogen receptor \( \alpha \) (ER\( \alpha \)) and \( \beta \) (ER\( \beta \)) and progesterone receptor (PR) was determined by real-time RT-PCR using Icycler Thermal cycler (Bio-Rad, Hercules, CA), as described previously. \( \text{ER} \alpha \) primers were 5’-TCTGGAAGAGAAGGACCAC-3’ (forward) and 5’-AAGTGAGAGGAGGAGGAG-3’ (reverse). \( \text{ER} \beta \) primers were 5’-CCCAATAACTCAGAAGATGG-3’ (forward) and 5’-CTTTTCAATGTCTCCCTGTT-3’ (reverse). PR primers were 5’-TGTCGCCTTAGAAAGTGC-3’ (forward) and 5’-GCTTGGTGAAAAAGTGATT-3’ (reverse). Real-time RT-PCR was performed in a final volume of 25 µl. Each PCR reaction mixture consisted of 600 nmol/L of primers, 33 units of M-MLV reverse transcriptase (Promega, Madison, WI), and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) containing 0.625 unit Taq polymerase, 400 µmol/L each of dATP, dCTP, dGTP, and dTTP, 100 mmol/L KCl, 16.6 mmol/L ammonium sulfate, 40 mmol/L Tris-HCl, 6 mmol/L MgSO\textsubscript{4}, SYBR Green I, 20 nmol/L fluorescing and stabilizers. We used the following RT-PCR protocol: 50 ºC for 10 minutes, 95 ºC for 5 minutes, followed by 45 cycle of 95 ºC for 10 seconds, 56 ºC for 30 seconds, 72 ºC for 10 seconds. GAPDH was used as an internal reference and serial dilutions of the positive control was performed on each plate to create a standard curve. PCR was performed in triplicate, and threshold cycle numbers were averaged.
Western blot analysis
Uterine arteries were isolated and the endothelium was removed. To determine the combined cytosolic and nuclear protein abundance, tissues were homogenized in a lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris.HCl, 10 mmol/L EDTA, 0.1% Tween-20, 0.1% β-mercaptoethanol, 0.1% Triton X-100, 0.1 mmol/L phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, and 5 µg/ml aprotinin, pH 7.4 and allowed to incubate for 1 hour on ice. Homogenates were then centrifuged at 4 °C for 10 minutes at 10,000 g, and supernatants collected. Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA). Samples with equal amounts of protein were loaded onto 7.5% polyacrylamide gel with 0.1% sodium dodecyl sulfate (SDS) and were separated by electrophoresis at 100 V for 90 minutes. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites were blocked for 1 hour at room temperature in a Tris-buffered saline solution containing 5% dry-milk. The membranes were then probed with primary antibodies against extracellular signal-regulated kinase (ERK1/2) and phospho-ERK1/2 (Tyr202/Tyr204) (New England Biolabs, Beverly, MA), estrogen α (ERα), β (ERβ) receptors, and progesterone receptor (PR) (Santa Cruz Biotechnology; Santa Cruz, CA). Beta actin antibody (Santa Cruz Biotechnology) was used to normalize loading. After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. The results were analyzed with the Kodak ID image analysis software.

Measurement of PKC isozyme translocation
After the hormonal or antagonist treatments for 48 hours, tissues were snap-frozen in liquid N2 and homogenized in ice-cold homogenization buffer containing Tris-HCl 20 mmol/L, sucrose 250 mmol/L, EDTA 5 mmol/L, EGTA 5 mmol/L, PMSF 1 mmol/L, β-mercaptoethanol 10 mmol/L, and benzamide 1 mmol/L. The cytosolic and particulate fractions were separated as previously described. Briefly, the homogenate was centrifuged at 100,000g for 60 minutes 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 minutes at 4 °C, followed by centrifugation at 100,000g for 60 minutes 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


