Chronic Hypoxia During Gestation Causes Epigenetic Repression of the Estrogen Receptor-α Gene in Ovine Uterine Arteries via Heightened Promoter Methylation

Chiranjib Dasgupta, Man Chen, Haitao Zhang, Shumei Yang, Lubo Zhang

Abstract—Estrogen receptor-α (ERα) plays a key role in the adaptation of increased uterine blood flow in pregnancy. Chronic hypoxia is a common stress to maternal cardiovascular homeostasis and causes increased risk of preeclampsia. Studies in pregnant sheep demonstrated that hypoxia during gestation downregulated ERα gene expression in uterine arteries. The present study tested the hypothesis that hypoxia causes epigenetic repression of the ERα gene in uterine arteries via heightened promoter methylation. Ovine ERα promoter of 2035 bp spanning from −2000 to +35 of the transcription start site was cloned. No estrogen or hypoxia-inducible factor response elements were found at the promoter. Two transcription factor binding sites, USF−15 and Sp1−20, containing CpG dinucleotides were identified, which had significant effects on the promoter activity. The USF element binds transcription factors USF1 and USF2, and the Sp1 element binds Sp1, as well as ERα through Sp1. Deletion of the Sp1 site abrogated 17β-estradiol–induced increase in the promoter activity. In normoxic control sheep, CpG methylation at the Sp1 but not the USF site was significantly decreased in uterine arteries of pregnant as compared with nonpregnant animals. In pregnant sheep exposed to long-term high-altitude hypoxia, CpG methylation at both Sp1 and USF sites in uterine arteries was significantly increased. Methylation inhibited transcription factor binding and the promoter activity. The results provide evidence of hypoxia causing heightened promoter methylation and resultant ERα gene repression in uterine arteries and suggest new insights of molecular mechanisms linking gestational hypoxia to aberrant uteroplacental circulation and increased risk of preeclampsia. (Hypertension. 2012;60:00.) ● Online Data Supplement

Key Words: hypoxia ■ estrogen receptor ■ methylation ■ epigenetic modulation ■ uterine artery

The striking increase in uterine blood flow during pregnancy is a key adaptation to maintain normal fetal development, as well as maternal cardiovascular well being. Animal studies have demonstrated that a chronic reduction of uteroplacental perfusion leads to a hypertension state that closely resembles preeclampsia in women.1,2 Chronic hypoxia during gestation is a common stress to maternal cardiovascular homeostasis and has profound adverse effects on uteroplacental circulation, leading to attenuated uterine blood flow and increased risk of preeclampsia and fetal intrauterine growth restriction.3–6 It has been shown that both endogenous and exogenous estrogen play a pivotal role in regulating uterine blood flow during pregnancy.9–14 We have demonstrated recently in sheep a direct genomic effect of exogenous estrogen in downregulating the protein kinase C signaling pathway and upregulating the large-conductance Ca2+-activated K channel activity, resulting in a reduced myogenic tone of the uterine artery in pregnancy.15,16 Further studies demonstrated that pregnancy or exogenous estrogen-induced upregulation of Ca2+-activated K channel activity and a reduction of pressure-induced myogenic tone of the uterine artery were abrogated in pregnant sheep acclimatized to long-term high-altitude hypoxia.17,18 Chronic hypoxia during gestation did not alter maternal plasma estrogen levels but significantly suppressed estrogen receptor-α (ERα) expression in the uterine artery.18

The molecular mechanisms underlying gestational hypoxia-mediated ERα gene repression in the uterine artery remain undetermined. Epigenetic mechanisms are essential for the homeostasis in response to the environment through changes in gene expression patterns.19–21 DNA methylation is a chief mechanism in epigenetic modification of gene expression patterns and occurs at cytosines of the dinucleotide sequence CpG.21–23 Although cytosine is methylated in 70% of CpGs of mammalian DNA, CpGs in the promoter/enhancer regions of many mammalian genes are not methylated. Increased methylation in promoter regions is generally associated with transcription repression of the associated genes.
Although methylation of the ERα promoter has been reported to occur as a direct function of physiological regulation in several tissue types and as part of a pathological progression of numerous types of cancerous tissues, little is known about the epigenetic regulation of ERα gene expression patterns in vascular smooth muscle and its functional consequences. Herein, we present novel evidence that chronic hypoxia during gestation causes repression of the ERα gene in the uterine artery via heightened promoter methylation, providing a molecular mechanism linking hypoxia and maladaptation of uteroplacental circulation and increased risk of preeclampsia in pregnancy.

Materials and Methods

An expanded Materials and Methods section is available in the online-only Data Supplement.

Tissue Preparation and Treatment

Uterine arteries were harvested from nonpregnant sheep regardless of stages of the estrous cycle, and near-term pregnant (≈140 days’ gestation) ewes were maintained at sea level (≈300 m, arterial PaO₂ ≈102 mm Hg) or exposed to high-altitude (3801 m, arterial PaO₂ ≈60 mm Hg) hypoxia for 110 days. To investigate the direct effect of hypoxia, some arteries obtained from normoxic control nonpregnant and pregnant 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 and followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Reporter Gene Assay

Genomic DNA isolated in uterine arteries from control nonpregnant animals was used as a PCR template. Using primers designed based on the bovine ERα gene promoter sequence (Gene ID, 407238), a 2035-bp ovine genomic fragment spanning –2000 bp to +35 bp relative to the transcription start site was cloned. The activities of wild-type or site-specific deletion of USF and Sp1 promoters in pregnant animals was used as a reporter gene assay. Site-directed CpG methylation and methylation at the Sp1 promoter were subjected to gel shift assays using the Biotin 3’ end-labeling kit and Light-Shift Chemiluminescent electrophoretic mobility shift assay kit (Pierce Biotechnology, Rockford, IL), as described previously.

Electrophoretic Mobility Shift Assay

Nuclear extracts were collected in uterine arteries of control nonpregnant animals. The oligonucleotide probes with CpG and CmG in the 3 putative transcription factor binding sites, USF₁₋₁₅, Sp1₋₁₂₀, and PRA/B₋₅₆₃, respectively, were labeled and subjected to gel shift assays using the Biotin 3’ end-labeling kit and Light-Shift Chemiluminescent electrophoretic mobility shift assay kit.

Chromatin Immunoprecipitation

Chromatin extracts were prepared from uterine arteries of all 4 group animals. Chromatin immunoprecipitation assays were performed using the ChIP-IT kit (Active Motif, Carlsbad, CA), as described previously. Primers flanking USF₁₋₁₅ and Sp1₋₁₂₀ binding sites are shown in Table S2.

Site-Directed CpG Methylation Mutagenesis and Reporter Gene Assay

The effect of site-directed CpG methylation on the ERα promoter activity was determined in uterine arterial smooth muscle cells of control nonpregnant sheep, as described previously. Table S3 lists the oligonucleotide probes used in site-directed CpG methylation mutagenesis and reporter gene assay.

Data Analysis

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

Results

Sequencing of Ovine ERα Gene Proximal Regulatory Region

The sequence of the regulatory region consisting of a 2035-bp ovine genomic fragment spanning –2000 bp to +35 bp relative to the transcription start site of ERα gene is shown in Figure S1, which has 96% homology to the bovine ERα proximal promoter obtained from GenBank (Gene ID, 407238). Similar to the bovine ERα promoter, the ovine ERα promoter lacks a TATA box-like element. In silico analysis of the ovine ERα promoter sequence identified 3 putative transcription factor binding sites that contained CpG dinucleotides in their core binding sequences, USF₁₋₁₅, Sp1₋₁₂₀, and PRA/B₋₅₆₃ (Figure S1).

Deletion of USF₁₋₁₅, Sp1₋₁₂₀, and PRA/B₋₅₆₃ Inhibits ERα Promoter Activity

The 2035-bp ovine ERα promoter was first cloned into a pCR4-TOPO vector and subsequently cloned in Xhol-HindIII orientation into pGL3 basic vector to drive the transcription of the luciferase reporter gene transfected in smooth muscle cells of uterine arteries from control nonpregnant sheep. Site-specific deletion of USF₁₋₁₅, Sp1₋₁₂₀, or PRA/B₋₅₆₃, respectively, caused a significant inhibition of the promoter activity (Figure 1), indicating a strong stimulatory role of these promoter elements in driving the transcription of the ovine ERα promoter.

Chronic Hypoxia Increases CpG Methylation at ERα Promoter

Our previous studies in sheep demonstrated that pregnancy upregulated ERα gene expression and significantly increased ERα mRNA and protein abundance in the uterine artery, which was abrogated in animals acclimatized to long-term high-altitude hypoxia. The effects of pregnancy and hypoxia on methylation patterns of CpG dinucleotides in the 3 putative binding sites at the ovine ERα promoter were determined in uterine arteries by quantitative methylation-specific PCR. In normoxic control animals, there were no significant changes in CpG methylation status at the USF₁₋₁₅ and PRA/B₋₅₆₃ sites but a significant decrease in CpG methylation at the Sp1₋₁₂₀ site in uterine arteries of pregnant as compared with nonpregnant sheep (Figure 2A). In animals acclimatized to long-term high-altitude hypoxia, there were significant increases in CpG methylation at USF₁₋₁₅ and Sp1₋₁₂₀ but not at PRA/B₋₅₆₃ sites in uterine arteries of pregnant sheep (Figure 2A). Although it appeared to be a
trend of increased methylation at the Sp1-520 site, chronic hypoxia had no significant effect on ERα promoter methylation in uterine arteries of nonpregnant animals (Figure 2A), suggesting that it is the decrease in methylation in pregnancy that is abrogated by hypoxia. In the previous study, we demonstrated that ex vivo hypoxic treatment of uterine arteries under 10.5% O2 for 48 hours produced results similar to those found in high-altitude hypoxic animals and caused a significant decrease in ERα mRNA and protein abundance. To determine the direct effect of hypoxia on Cpg methylation patterns at the ERα promoter, uterine arteries from normoxic control nonpregnant and pregnant sheep maintained at sea level (normoxic control, C) or exposed to high-altitude hypoxia (H) for 110 days (A) and in uterine arteries of normoxic animals treated ex vivo with 21.0% O2 (normoxic control, C) or 10.5% O2 (H) for 48 hours (B). Data are mean±SEM. *P<0.05 vs normoxic control. n=6 to 10.

CpG Methylation Inhibits Transcription Factor Binding to the Promoter

Given the finding that pregnancy and hypoxia had no significant effects on methylation status at the putative PRA/B-563 site, our further investigation was focused on USF-15 and Sp1-520 binding sites. To evaluate the nuclear protein binding to the putative USF-15 and Sp1-520 elements at the ERα promoter, electrophoretic mobility shift assays were performed. Incubation of nuclear extracts from uterine arteries of control nonpregnant animals with double-stranded oligonucleotide probes encompassing the putative Sp1-520 binding site resulted in the appearance of a major DNA-protein complex (Figure 3A). Supershift analyses showed that Sp1 antibody caused supershifting of the DNA-protein complex (Figure 3A). Unlike the Sp1 binding site, incubation of nuclear extracts with double-stranded oligonucleotide probes encompassing the putative USF1-520 site are blocked by the antibodies. To determine whether methylation of the Sp1-520 and USF-15 sites inhibits the transcription factor binding to the ERα promoter, electrophoretic mobility shift assay was performed with nuclear extracts isolated from uterine arteries with methylated and unmethylated Cpg of oligonucleotide probes containing the Sp1-520 and USF-15 sites. As shown in Figure 3A and 3B, nuclear extracts from uterine arteries bound and shifted the double-stranded unmethylated Sp1 and USF oligonucleotide probes, respectively, but failed to cause a shift of methylated Sp1 and USF oligonucleotides. To further determine whether a hypoxia-mediated increase in Cpg methylation of the Sp1-520 and USF-15 binding sites inhibits Sp1 or USF1/USF2 binding to the ERα promoter in
significant effects on the transcription factor binding to the arteries of pregnant sheep (Figure 4). Chronic hypoxia had no cells of uterine arteries from control nonpregnant sheep. To binding at the Sp1

The functional significance of hypoxia-mediated increase in Promoter Activity the promoter activity compared with nonpregnant sheep (Figure 4). In animals acclimatized to long-term high-altitude hypoxia, there were significant decreases in Sp1 and USF1/USF2 binding to Sp1$_{-520}$ or USF$_{-15}$ binding sites, respectively, in uterine arteries of pregnant sheep (Figure 4). Chronic hypoxia had no significant effects on the transcription factor binding to the ER$\alpha$ promoter in uterine arteries of nonpregnant animals (Figure 4).

Methylation of Sp1 and USF Sites Reduces ER$\alpha$ Promoter Activity

The functional significance of hypoxia-mediated increase in CpG methylation rendering the inhibition of Sp1 and USF binding to the promoter was further investigated by determining the effect of site-specific CpG methylation of Sp1$_{-520}$ and USF$_{-15}$ sites on ER$\alpha$ promoter activity in smooth muscle cells of uterine arteries from control nonpregnant sheep. To create a constitutive site-directed CmG at the Sp1 site, a SacII site was engineered at 3’ downstream of the Sp1$_{-520}$ site in ER$\alpha$ promoter-luciferase reporter gene constructs. As shown in Figure 5, the insertion of SacII site had no significant effect on the promoter activity compared with wild-type ER$\alpha$ promoter. The mutation of CmG at the Sp1$_{-520}$ site significantly reduced the promoter activity (Figure 5). Similarly, to create a constitutive site-directed CmG at the USF site, SacII and an EcoRI sites were engineered at both 3’ downstream and 5’ upstream of the USF$_{-15}$ site in ER$\alpha$ promoter-luciferase reporter gene constructs. As shown in Figure 5, the insertion of SacII and EcoRI sites had no significant effect on the promoter activity, but the mutation of CmG at the USF$_{-15}$ site significantly reduced the promoter activity.

Deletion of the Sp1 Site Inhibits Estrogen-Mediated ER$\alpha$ Promoter Activity

Our previous studies in sheep demonstrated that ex vivo treatment of uterine arteries with 17$\beta$-estradiol (E$_2$) significantly increased ER$\alpha$ expression, which was abrogated by chronic hypoxia.$^{18}$ These findings suggest that estrogen regulates its own receptor expression in uterine arteries, and this regulation is inhibited by chronic hypoxia. Nonetheless, the analysis of the ovine ER$\alpha$ promoter sequence revealed no complete estrogen response element. Given that estrogen is known to stimulate transcription of many genes through its interaction with Sp1 promoter elements,$^{37,38}$ we reasoned that E$_2$-induced ER$\alpha$ expression in uterine arteries was mediated via the Sp1 element at the ER$\alpha$ promoter. As shown in Figure 6A, chromatin immunoprecipitation assay demonstrated the PCR products of the Sp1$_{-520}$ binding site in DNA sequences pulled down by both ER$\alpha$ and estrogen receptor-$\beta$ (ER$\beta$) antibodies in ovine uterine arteries, suggesting an interaction between estrogen receptors and the Sp1$_{-520}$ binding site at the ER$\alpha$ promoter. We then determined the effect of E$_2$-E$_2$ on ER$\alpha$ promoter activity. Smooth muscle cells were isolated from uterine arteries of nonpregnant sheep and were transiently transfected with the ER$\alpha$ promoter-luciferase reporter gene constructs. As shown in Figure 6B, the treatment of cells with 10 nmol/L of E$_2$ for 24 hours significantly increased

![Figure 3](https://hyper.ahajournals.org/)

**Figure 3.** SP1 and USF binding to unmethylated and methylated binding elements at estrogen-receptor (ER)-$\alpha$ promoter. Nuclear extracts (NE) from uterine arteries were incubated with double-stranded oligonucleotide probes containing unmethylated (UM) or methylated (M) Sp1$_{-520}$ binding site (A) or USF$_{-15}$ binding site (B) in the absence or presence of antibodies (Ab) against Sp1, USF1, or USF2. Cold competition (CC) was performed with unlabeled competitor oligonucleotide at a 200-fold molar excess. Free oligo indicates that no nuclear extracts were added; S, shift; SS, supershift.

![Figure 4](https://hyper.ahajournals.org/)

**Figure 4.** Hypoxia decreases SP1 and USF binding to estrogen receptor (ER)-$\alpha$ promoter. Sp1, USF1, and USF2 binding to ER$\alpha$ promoter in vivo in the context of intact chromatin was determined with chromatin immunoprecipitation (ChIP) assays in uterine arteries from nonpregnant (CNPUA) or pregnant (CPUA) sheep of normoxic control and from nonpregnant (HNPUA) and pregnant (HPUA) sheep of long-term high-altitude hypoxia. Data are mean±SEM. $^\ast$$P<0.05$ vs nonpregnant; $^+$$P<0.05$ vs normoxic control. n=5.
the promoter activity, which was blocked by a site-specific deletion of the Sp1 element at the ERα promoter.

**Discussion**

Here for the first time we report cloning of a 2035-bp ovine ERα promoter sequence, relate the promoter activity to specific transcription factors that transcribe this region, and demonstrate that the transcription in uterine arteries is affected by pregnancy and chronic hypoxia. The DNA sequence of the 471-bp ovine ERα promoter region identified previously (GenBank No. AF159145.1) is in complete agreement with the proximal DNA sequence of the ovine ERα promoter that we cloned in the present study (from −471 to −1). The ovine ERα promoter has 96% homology to the bovine ERα promoter sequence obtained from GenBank. The USF, Sp1, and PRAB response elements are present in both bovine and ovine promoters and are located almost in the same region. Although the PRAB element in the ovine promoter is identical in sequence to the PRAB site in the bovine promoter, unlike the bovine USF site (CAGCATG) that lacks CpG dinucleotide, the ovine USF site (CA CG TG) is similar to the consensus USF binding sequence (CAGCGT) and contains CpG in its core binding sequence. Similarly, unlike the bovine Sp1 site, the Sp1 element in the ovine ERα promoter (AGGGCGGGCT) contains CpG in its core binding sequence.

In the 5′ region of human ERα gene, other than protein coding exons, 9 additional exons have been described. The 2 proximal exons A and B are functional in the majority of tissue types studied, including breast, uterus, brain, heart, and vascular smooth muscle cells. Many of these alternative exons do not code for proteins but produce tissue-specific splice variant mRNA. The structural resemblance at the 5′ region of bovine and human ERα genes has been described. Like the human ERα gene, the bovine ERα promoter also contains 2 similar proximal exons A and B. In both human and bovine ERα genes, the alternative exons are spliced to a single acceptor site. Following the transcription initiation site (+1), translation starts at +245 nucleotides for the human sequence and +153 nucleotides for the bovine sequence. In the sequence of ovine ERα promoter cloned in the present study, we have indicated the putative transcription initiation site using our sequencing data based on the bovine ERα gene.
This is in agreement with the ovine ERα mRNA reported previously (GenBank No. Z49257.1). The ovine ERα transcription initiation site has almost the same nucleotide sequence as it is in the bovine ERα mRNA (GenBank No. NM001001443.1). Comparison of the ovine ERα mRNA with that of the bovine ERα gene sequence indicates that, like bovine, the ovine gene also contains the 2 proximal exons. Furthermore, the short sequence of open reading frame that could potentially code a short peptide of 18 amino acid is also present in the bovine gene. The ovine ERα protein translation for a long open reading frame begins at +149 nucleotide downstream from the transcription initiation site. Taken together, thus, there is a high degree of conservation of the ERα gene between bovine and ovine species. Figure 2 presents the comparison of human, bovine, and ovine ERα promoter, transcription, and translation initiation structures.

The functional significance of USF, Sp1, and PRα/β response elements in the regulation of the ovine ERα gene activity was demonstrated by the finding that deletion of these sites significantly decreased the ERα promoter activity. The finding that pregnancy and chronic hypoxia differentially regulated Cpg methylation at the Sp1 and USF sites is intriguing and suggests an important epigenetic mechanism in regulating ERα gene expression patterns in uterine arteries. The present study demonstrated clearly that an antiserum to Sp1 caused supershifting of the DNA-protein complex resulting from binding of nuclear extracts from uterine arteries with the double-stranded oligonucleotide probes containing the putative Sp1-520 element, indicating a true Sp1 binding site in the ovine ERα promoter. The finding of >1 band in the gel-retarded complex formed between the USF oligonucleotide probes and nuclear extracts is in agreement with the previous studies showing a highly heterogeneous group of USF proteins that bind to the USF site, with USF1 and USF2 being the major USF species. In the present study, USF1 and USF2 antibodies failed to produce the supershifting of nuclear extracts binding to the putative USF-15 site but significantly inhibited the binding. This is possibly because the binding domains of USF1/2 to the USF-15 site are blocked by the antibodies. Similar findings were reported previously in several cell types.

Our previous studies in sheep demonstrated that pregnancy caused a significant increase in ERα mRNA and protein abundance in uterine arteries, which was abolished in animals acclimatized to long-term high-altitude hypoxia. Consistent with this finding, the present study found that Cpg methylation at the Sp1 binding site was significantly decreased in uterine arteries of pregnant sheep, which was abolished by chronic hypoxia. Unlike the Sp1 site, Cpg methylation at the USF binding site was not significantly changed in uterine arteries of pregnant animals, suggesting its minimal role at the USF site in pregnancy-induced upregulation of ERα expression. However, Cpg methylation at the USF site was significantly increased in uterine arteries of pregnant sheep exposed to long-term high-altitude hypoxia, suggesting its role in suppressing ERα gene expression in uterine arteries caused by chronic hypoxia during gestation. These effects observed in hypoxic animals are most likely attributed to the direct effect of chronic hypoxia, given the finding that the ex vivo prolonged hypoxic treatment of isolated uterine arteries from the normoxic ewes produced similar effects on Cpg methylation patterns as those found in uterine arteries from high-altitude hypoxic animals. This finding is consistent with the previous studies showing that chronic hypoxia had a direct effect in suppressing ERα expression in uterine arteries. Taken together, these findings provide evidence of a novel epigenetic mechanism of Cpg methylation at sequence-specific transcription factor binding sites in regulating ERα gene expression patterns in the uterine artery.

Although the transcriptional regulation by DNA methylation is often observed in Cpg islands located around the promoter region via the sequence-nonspecific and methylation-specific binding of inhibiting methylated Cpg-binding proteins. DNA methylation of sequence-specific transcription factor binding sites can alter gene expression through changes in the binding affinity of transcription factors by altering the major groove structure of DNA to which the DNA-binding proteins bind. The finding that both Sp1 and USF probes with methylated Cpg dinucleotides at the core of the consensus elements at the ERα promoter inhibited the binding of Sp1 and USF1/2 in the present study indicates that Cpg methylation in sequence-specific binding sites may directly inhibit the transcription factor binding, resulting in downregulation of ERα gene expression in uterine arteries. Similar findings were demonstrated in the heart showing that site-specific Cpg methylation at Sp1 and Egr1 binding sites suppressed protein kinase C-e gene expression. The results of chromatin immunoprecipitation assays in the present study demonstrate further that the hypoxia-induced increase in methylation of the Sp1 and USF binding sites inhibits the transcription factor binding to the ERα promoter in vivo in uterine arteries in the context of intact chromatin. The functional significance of Cpg methylation at sequence-specific transcription factor binding sites in regulating ERα gene expression was further addressed by site-directed methylation of ERα promoter selectively at Sp1-520 or USF-15 binding sites, showing that the mutation of Cpg at either Sp1-520 or USF-15 sites significantly reduced the promoter activity.

In the previous study, we demonstrated the direct ex vivo effect of exogenous E2β in upregulating ERα expression in uterine arteries of sheep. This is consistent with the premise that ovarian steroid estrogen maintains and regulates the expression of their own receptors, ERα and ERβ, and is largely in agreement with the finding of exogenous E2β treatments in vivo on steroid receptor expressions in uterine arteries of sheep. Of interest, in sheep acclimatized to long-term high-altitude hypoxia, pregnancy or exogenous E2β-mediated upregulation of ERα was abrogated, leading to no significant difference in the ERα density in uterine arteries between nonpregnant and pregnant animals. Nonetheless, the ovine ERα promoter sequence contains no estrogen response element. Several studies have indicated that hormone-activated ERα often uses an alternative mode of activation, known as “tethering,” in which ERα does not directly bind to DNA but interacts with another DNA-binding transcription factor. Sp1 is a transcription factor through which activated ERα drives transcription despite the lack of...
Estrogen response element in the genome. In the present study, we demonstrated that both ERα and ERβ interacted with the Sp1 binding site at the ovine ERα promoter in intact chromatin, indicating a mechanism of the Sp1 binding site in E2β-mediated regulation of ERα transcription in uterine arteries. This was further demonstrated by the finding that the E2β-induced increase in ovine ERα promoter activity in uterine arterial smooth muscle cells was abrogated by the deletion of the Sp1–520 binding site.

**Perspectives**

Hypoxia during gestation is a common stress to maternal cardiovascular homeostasis, causing a reduction of uteroplacental perfusion and an increased risk of preeclampsia. Estrogen via the activation of ERα and ERβ plays a pivotal role in regulating uterine blood flow during pregnancy. The present investigation provides evidence of a novel mechanism of promoter methylation at sequence-specific transcription factor binding sites in epigenetic modifications of ERα gene expression patterns in uterine arteries, linking heightened promoter methylation and ERα gene repression in hypoxia-mediated maladaptation of uteroplacental circulation. These findings will not only significantly advance our knowledge of the molecular mechanisms underlying aberrant uteroplacental circulation in response to hypoxia in pregnancy and, hence, improve our understanding of the pathophysiology of preeclampsia and intrauterine growth restriction, but they also provide important original insights into epigenetic mechanisms in regulating ERα gene expression patterns in vascular smooth muscle in general and, hence, a comprehensive understanding of the role of ERα in the estrogen-mediated protective effect of vascular function in physiology and pathophysiology.

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**Disclosures**

None.

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Gestation hypoxia and reduced uteroplacental perfusion are major risk factors of preeclampsia. Increased methylation of the estrogen receptor Fc gene in uterine arteries via its interaction with Sp1.

What Is Relevant?
- Gestation hypoxia and reduced uteroplacental perfusion are major risk factors of preeclampsia.
- Estrogen via activation of ERα and ERβ plays a pivotal role in regulating uterine blood flow in pregnancy, and suppression of ERα leads to aberrant uteroplacental circulation.

Novelty and Significance

What Is New?
- Gestation hypoxia increases promoter methylation of the ERα gene in uterine arteries.
- Heightened methylation inhibits transcription factor binding and ERα gene promoter activity.
- Estrogen plays a key role in regulating ERα gene promoter activity in uterine arteries via its interaction with Sp1.

What Is Relevant?
- Gestation hypoxia and reduced uteroplacental perfusion are major risk factors of preeclampsia.
- Estrogen via activation of ERα and ERβ plays a pivotal role in regulating uterine blood flow in pregnancy, and suppression of ERα leads to aberrant uteroplacental circulation.

Epigenetic regulation of ERα gene expression patterns provides a molecular mechanism linking gestation hypoxia, impaired uteroplacental perfusion, and increased risk of preeclampsia, as well as estrogen-mediated protective effect of vascular function, in physiology and pathophysiology in general.

Summary
The present study demonstrates a novel mechanism of promoter methylation in epigenetic regulation of ERα gene expression patterns in uterine arteries linking gestation hypoxia, impaired uteroplacental perfusion, and increased risk of preeclampsia.
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By

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Running title: Hypoxia represses ERα gene in uterine arteries

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Materials and Methods

**Tissue Preparation and Treatment.** Uterine arteries were harvested from nonpregnant sheep regardless of stages of the estrous cycle and near-term pregnant (~140 days’ gestation) ewes maintained at sea level (~300m, arterial PaO₂ ~102 mmHg) or exposed to high-altitude (3801m, arterial PaO₂ ~60 mmHg) hypoxia for 110 days. Animals were anesthetized with thiamylal (10 mg/kg, i.v.) followed by inhalation of 1.5% to 2.0% halothane. An incision was made in the abdomen and the uterus exposed. The resistance-sized uterine arteries (~150 μm in diameter) were isolated and removed without stretching and placed into a modified Krebs solution. To investigate the direct effect of hypoxia, some arteries obtained from normoxic control nonpregnant and pregnant animals were treated in a humidified incubator with either 21.0% or 10.5% O₂ for 48 hours, as described previously. All procedures and protocols were approved by the Institutional Animal Care and Use Committee and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Reporter Gene Assay.** Genomic DNA isolated in uterine arteries from control nonpregnant animals was used as a PCR template. Using primers designed based on the bovine ERα gene promoter sequence (Gene ID: 407238), a 2035 bp ovine genomic fragment spanning -2000 bp to +35 bp relative to the transcription start site was first cloned into pCR4-TOPO vector and subsequently cloned in Xho I-Hind III orientation in pGL3 basic vector (Promega) to drive the transcription of the luciferase reporter gene. Site-specific deletion mutations were constructed at three putative transcription factor binding sites, USF-15, Sp1-520, and PRA/B-563, as described previously. All promoter constructs sequences were confirmed with DNA sequencing analyses. Smooth muscle cells isolated from uterine arteries of normoxic control nonpregnant sheep were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in 95% air/5% CO₂. Cells were grown and sub-cultured in 24-well plates with experiments performed at 70-80% confluent. Cells were transiently co-transfected with 1 μg of promoter/reporter vector along with 0.1 μg of internal control pRL-SV40 vector using endotoxin free plasmid DNA plus X-tremeGENE HP DNA Transfection Reagent (Roche) following manufacturer’s instructions. After 48 hours, firefly and Renilla reniformis luciferase activities in cell extracts were measured in a luminometer using a dual-luciferase reporter assay system (Promega), as described previously. The activities of the wild-type or site specific deletion constructs were then calculated by normalizing the firefly luciferase activities to R. reniformis luciferase activity, and expressed as relative to wild-type reporter activity (% WT).

**Quantitative Methylation-Specific PCR.** Genomic DNA was isolated from uterine arteries of all four group animals using a GenElute Mammalian Genomic DNA Mini-Prep kit (Sigma), denatured with 2 N NaOH at 42°C for 15 minutes, treated with sodium bisulfite at 55°C for 16 hours, and purified by EZ DNA Methylation-Gold Kit™ (Zymo Research), as previously described. Bisulfite-treated DNA was used as a template for real-time fluorogenic methylation-specific PCR (MSP) using specific primers designed to amplify the regions of interest with unmethylated CpG dinucleotides or methylated CpG dinucleotides (CmG), respectively (Table S1, available in the online data supplement). GAPDH was used as an internal reference gene. Real-time MSP was performed using the iQ SYBR Green Supermix with iCycler real-time PCR system (Bio-Rad). Data are presented as the percent of methylation at the region of interest (methylated CpG/methylated CpG + unmethylated CpG x100), as described before.
Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were collected in uterine arteries from control nonpregnant animals using NXTRACT CelLytic Nuclear Extraction Kit (Sigma, St. Louis, MO). The oligonucleotide probes with CpG and CmG in the three putative transcription factor binding sites, USF-15, Sp1-520, and PRA/B-563 at ovine ERα promoter region were labeled and subjected to gel shift assays using the Biotin 3’ end labeling kit and Light-Shift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL), as previously described.3,4 Briefly, single stranded oligos were incubated with Terminal Deoxynucleotidyl Transferase (TdT) and Biotin-11-dUTP in binding mixture for 30 minutes at 37°C. The TdT adds a biotin labeled dUTP to the 3’-end of the oligonucleotides. The oligos were extracted using chloroform and isoamyl alcohol to remove the enzyme and unincorporated biotin-11-dUTP. Dot blots were performed to ensure the oligos were labeled equally. Combining sense and antisense oligos and exposing to 95°C for 2 minutes was done to anneal complementary oligos. The labeled oligonucleotides were then incubated with or without nuclear extracts in the binding buffer (from Light-Shift kit). Binding reactions were performed in 20 μl containing 50 fmol oligonucleotide probes, 1x binding buffer, 1 μg of poly (dIdC), and 10 μg of nuclear extracts. For competitions studies, 200-fold molar excess of non-labeled oligonucleotides were added to binding reactions. For super-shift assays, 2 μl of affinity purified Sp1 antibody (Active Motif) or USF1 or USF2 antibodies (Santa Cruz) were added to the binding reaction. The samples were then run on a native 5% polyacrylamide gel. The contents of the gel were then transferred to a nylon membrane (Pierce Biotechnology, Rockford, IL) and crosslinked to the membrane using a UV crosslinker (125 mJoules/cm²). Membranes were blocked and then visualized using the reagents provided in the LightShift kit.

Chromatin Immunoprecipitation (ChIP). Chromatin extracts were prepared from uterine arteries of all four group animals. ChIP assays were performed using the ChIP-IT kit (Active Motif, Carlsbad, CA), as previously described.3,4 Briefly, tissues were exposed to 1% formaldehyde for 10 minutes to crosslink and maintain DNA/protein interactions. After the reactions were stopped with glycine, tissues were washed, chromatin isolated and DNA sheared into medium fragments (100 - 1000 bp) using a sonicator. ChIP reactions were performed using Sp1, USF1 or USF2 antibodies to precipitate the transcription factor/DNA complex. Crosslinking was then reversed using a salt solution and proteins digested with proteinase K. Primers flanking USF-15 and Sp1-520 binding sites are shown in Table S2 (available in the online data supplement). PCR amplification products were visualized on 2% agarose gel stained with ethidium bromide. To quantify PCR amplification, 45 cycles of real-time PCR were carried out with 3 minutes initial denaturation followed by 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds, using the iQ SYBR Green Supermix with iCycler real-time PCR system (Bio-Rad, Hercules, CA).

Site-Directed CpG Methylation Mutagenesis and Reporter Gene Assay. The effect of site-directed CpG methylation on ERα promoter activity was determined as described previously.3 Briefly, to determine the effect of methylation at Sp1-520 on ERα promoter activity, a unique SacII cutting site was engineered at 3’ downstream of the Sp1 site. A customized 90-bp PflMI/SacII oligonucleotide fragment with methylation of CpG-516 at the Sp1 site was synthesized by IDT, and was then ligated back to the ERα promoter-reporter plasmid with pGL3. An identical 90-bp PflMI/SacII fragment with unmethylated CpG-516 at the Sp1 site was served as a control. To determine the effect of methylation at USF-15 on ERα promoter activity, two unique cutting sites of SacII and EcoRI were engineered at 3’ downstream and 5’ upstream, respectively, of the USF site. A customized 30-bp EcoRI/SacII oligonucleotide fragment with
methylation of CpG_{13} at the USF site was synthesized by IDT, and was then ligated back to the ERα promoter-reporter plasmid with pGL3. Amount of formed ligation product was quantified by real-time PCR and equal amount of plasmid was used in a transfection assay. Activities of each promoter-reporter constructs were determined in smooth muscle cells of uterine arteries from control nonpregnant sheep, as described above. Table S3 (available in the online data supplement) lists the oligonucleotide probes used in site-directed CpG methylation mutagenesis and reporter gene assay.

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

**References**


### Supplemental Data

**Table S1. Primers used in quantitative methylation-specific PCR**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’→3’)</th>
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<tr>
<td><strong>PRA/B_563</strong></td>
<td></td>
</tr>
<tr>
<td>F (M or UM):</td>
<td>gaggtggaggaatatattatatttag</td>
</tr>
<tr>
<td>RM:</td>
<td>acctctaaacacacacactattacccg</td>
</tr>
<tr>
<td>RUM:</td>
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<tr>
<td><strong>Sp1_520</strong></td>
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</tr>
<tr>
<td>RUM:</td>
<td>taccttaacctacccaacccca</td>
</tr>
<tr>
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</tr>
<tr>
<td>RUM:</td>
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F, forward  
R, reverse  
M, methylated  
UM, unmethylated
Supplemental Data

Table S2. Primers used in ChIP assay

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<tr>
<th>Primer</th>
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<tr>
<td>Sp1-520</td>
<td>5’-gttcagaggcaggggatg-3’</td>
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<td>5’-cateccacatgcaacacat-3’</td>
<td>5’-gaagtcagggctgagaca-3’</td>
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### Supplemental Data

#### Table S3. Oligonucleotide probes used in site-directed CpG methylation mutagenesis and reporter gene assay

<table>
<thead>
<tr>
<th>Probe</th>
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<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp1-520</td>
<td>M-sense</td>
<td>5’-P-ctggtttccgccgtcaatgggacggcaacagctgtgctgtccagggaagggatgggtgaggagggacagggcggctgggcg-OH-3’</td>
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<tr>
<td></td>
<td>M-antisense</td>
<td>5’-P-ggccccagcgcgccggtcctcctcaccatcccctgccctctgaacagacacacttgtgcctgcctccattgcacccggaaaccagact-OH-3’</td>
</tr>
<tr>
<td>Sp1-520</td>
<td>UM-sense</td>
<td>5’-P-ctggtttccgccgtcaatgggacggcaacagctgtgctgtccagggaagggatgggtgaggagggacagggcggctgggcg-OH-3’</td>
</tr>
<tr>
<td></td>
<td>UM-antisense</td>
<td>5’-P-ggccccagcgcgccggtcctcctcaccatcccctgccctctgaacagacacacttgtgcctgcctccattgcacccggaaaccagact-OH-3’</td>
</tr>
<tr>
<td>USF-15</td>
<td>M-sense</td>
<td>5’-P-gggagccacagcagcacagggagacattg-OH-3’</td>
</tr>
<tr>
<td>USF-15</td>
<td>M-antisense</td>
<td>5’-P-aattcgaatgtctccctgcccgtgctctgcttgetccccg-OH-3’</td>
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<tr>
<td>USF-15</td>
<td>UM-sense</td>
<td>5’-P-gggagccacagcagcacagggagacattg-OH-3’</td>
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<tr>
<td>USF-15</td>
<td>UM-antisense</td>
<td>5’-P-aattcgaatgtctccctgcccgtgctctgcttgetccccg-OH-3’</td>
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</table>

M, methylated  
UM, unmethylated
**Supplemental Data**

Figure S1. Ovine ERα gene promoter sequence. The PR/A/B, Sp1, and USF binding sites are indicated in red. The CpG units in these sites are denoted in blue.
**Figure S2. Comparison of human, bovine and ovine ERα promoter and transcription and translation initiation structure.** Two proximal exons A and B are shown. The alternative exons are spliced to a single acceptor site (shown by arrow). Following the transcription initiation site (+1), translation starts at +254 nucleotides for human, +153 nucleotides for bovine, and +149 nucleotides for ovine sequence.