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Induction of Uterine Prostaglandin H Synthase 2 by Estradiol Following Fetal Adrenalectomy

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In sheep, fetal cortisol stimulates the conversion of progesterone to estradiol in late gestation initiating labor. It is unclear whether an intact fetal hypothalamic–pituitary–adrenal (HPA) axis is required to induce the estradiol-triggered subsequent endocrine changes including enhanced intrauterine prostaglandin (PG) synthesis associated with the onset of labor. We have shown that maternal estradiol administration stimulates PG H synthase (PGHS)-2 expressions in pregnant ovine intrauterine tissues. The current study was undertaken to determine whether the fetal adrenal mediates estradiol's stimulation of the intrauterine PGHS-2 in pregnant sheep. Placenta, myometrium, and endometrium were collected from two groups of ewes at 123–127 d of gestational age (dGA) after fetal adrenalectomy and vehicle treatment (ADX; *n* = 5); or fetal ADX and maternal estradiol administration (5 mg twice a day for 2 d, ADX+E2, *n* = 5). PGHS-2 mRNA and protein were analyzed by Northern and Western Blot analyses in both groups and presented as the ratios to beta actin mRNA for Northern and G protein β subunit for Western blot analysis. Fetal plasma cortisol was measured by radioimmunoassay. Data were analyzed by Student's *t* test. Fetal plasma cortisol levels were low in ADX and ADX+E2 groups (<6 ng/mL). The cervix of all ADX+E2 treated ewes was dilated at necropsy. Three out of five ADX+E2–treated ewes delivered within 48 h. The cervix was closed in all fetal ADX ewes at necropsy. PGHS-2 mRNA and protein increased ($p < 0.05$) in myometrium and endometrium, but not placenta in ADX+E2–treated ewes compared with ADX group. These data provide the first in vivo evidence for estradiol upregulation of intrauterine PGHS-2 in late gestation in the absence of an intact fetal HPA axis. Thus, the fetal adrenal is not required to mediate estradiol's stimulation of uterine PGHS-2 expression associated with the onset of labor.

Key Words: Estradiol; PGHS-2; fetus; adrenalectomy; sheep.

Introduction

Fetal glucocorticoid can trigger the onset of premature labor [after 88 d of gestational age (dGA), term 148 dGA] in pregnant sheep *(1)*. The endocrine cascade associated with the onset of labor induced by fetal glucocorticoid administration in pregnant sheep has been studied extensively. Increasing fetal plasma glucocorticoid concentrations result in an increase in estrogen and a decrease in progesterone in maternal plasma through induction of the $P450_{c17\alpha}$ hydroxylase enzyme *(2)*. Estrogen then recruits many positive systems, including enhanced prostaglandin production in the intrauterine tissues. These enzymatic steps constitute the estrogen-dependent stimulation pathway of prostaglandin synthesis. Indeed, we have recently shown that maternal estradiol administration stimulates the PG system in intrauterine tissues and induces fetal delivery in pregnant sheep with an intact fetal hypothalamic–pituitary–adrenal (HPA) axis at 121 dGA *(3)*.

It is unclear whether an intact fetal HPA axis is required to mediate the estradiol-triggered subsequent endocrine changes including enhanced intrauterine PG synthesis associated with the onset of labor. In particular, recent studies have suggested that estrogen may not be the sole regulator of the intrauterine PG system in late gestation, and pathways such as the direct fetal cortisol stimulation of PGHS-2 may exist *(4–6)*. For example, in vitro studies have demonstrated that glucocorticoid stimulates prostaglandin production directly in cultured human fetal membranes *(7)*. Furthermore, evidence has also been presented for the existence of a cortisol-dependent/estradiol-independent mechanism on PGHS-2 induction within trophoblast tissue leading to the elevation of fetal plasma PGE2 *(5)* in pregnant sheep using an in vivo approach. Recently, we have shown that cortisol administration to adrenalectomized fetuses to clamp fetal cortisol at levels present early in the late gestation rise, which are inadequate to produce labor, stimulates intrauterine PGHS-2 mRNA and protein expression in the absence

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of increased estradiol action in maternal plasma *(6)*. Taken together, these findings support an action of cortisol on prostaglandin production in intrauterine tissues by a direct pathway. However, the physiological interplay between cortisol and estrogen in the regulation of intrauterine PG production during pregnancy and at labor is not clear.

Increased intrauterine PG production is observed in glucocorticoid-induced premature labor and spontaneous term labor. In these situations there are increases in both fetal plasma cortisol and maternal plasma estradiol and a fall in maternal plasma progesterone associated with the onset of labor. The use of the adrenalectomized fetus allows us to remove the contribution of the fetal adrenal to the endogenous fetal glucocorticoid milieu and to limit the fall of progesterone induced by increased fetal cortisol associated with the onset of labor. The purpose of the present study was to characterize the regulation of intrauterine PGHS-2 by estrogen in the absence of an intact fetal HPA axis and to determine whether the presence of the fetal adrenal is required to mediate estradiol's stimulation of the intrauterine PGHS-2 in pregnant sheep. This approach addresses the interplay between fetal cortisol and maternal estrogen. We administered estradiol at a dose that produced estradiol concentrations observed in maternal plasma during labor and was not associated with altered progesterone concentrations in maternal plasma *(3)*. In addition, we used both in vivo and in vitro techniques to determine the role of estrogen at both the systemic and molecular levels in the absence of fetal cortisol in the stimulation of the intrauterine PG system associated with onset of labor.

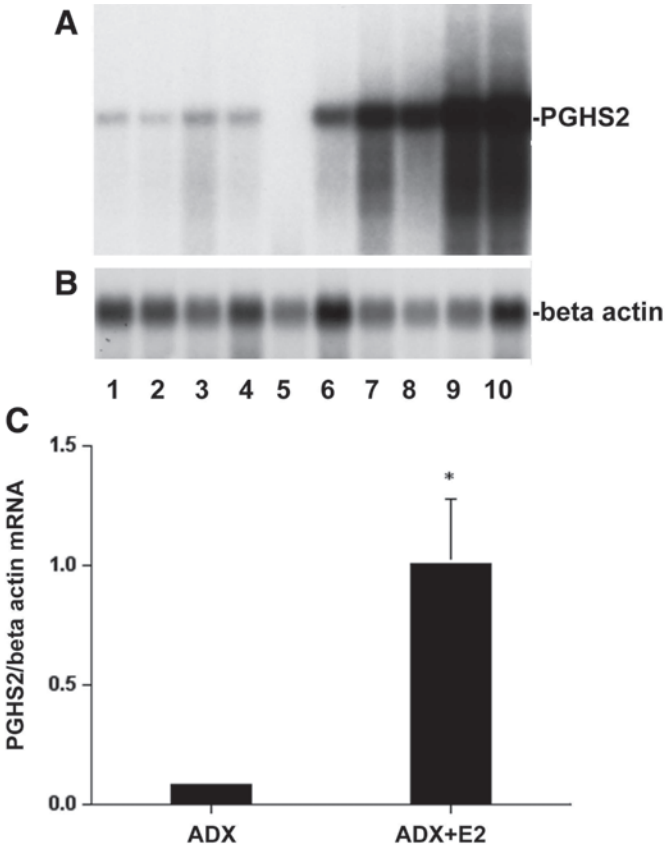

Fig. 1. Northern blot analysis of PGHS-2 mRNA in the endometrium (**A**) from controls (ADX, lanes 1–5) and estradiol-treated animals after fetal adrenalectomy (ADX+E2, lanes 6–10). (**B**) Beta actin in each corresponding lane. (**C**) Densitometry analysis of the ratios of endometrial PGHS-2 and beta actin mRNAs. There was a significant increase of PGHS-2 mRNA in endometrium in ADX+E2 group (*$p < 0.05$) compared with ADX group. Values are presented as mean ± SEM.

Results

Effect of Fetal Adrenalectomy and Estradiol Treatment on Fetal Blood Gases and pH

Representative values for fetal arterial blood gases and pH during the experimental period were obtained in each treatment group by averaging averaged daily values on d 5 or 6 after surgery in each fetus. Values for arterial pH in ADX and ADX+E2 fetuses were 7.32 ± 0.01 and 7.30 ± 0.02, respectively. Values for PaCO2 in ADX and ADX+E2 fetuses were 55.9 ± 1.5 and 55.7 ± 2.0 mmHg, respectively. Values for PaO2 in ADX and ADX+E2 fetuses were 18.4 ± 0.8 and 17.9 ± 0.5 mmHg, respectively. There was no significant difference between two groups in pH, $PaCO_2$, or PaO_2.

Induction of Labor by Estradiol

The cervix of every ADX+E2–treated ewe was dilated at necropsy. Three out of five ADX+E2 treated ewes delivered within 48 h. Five milligrams estradiol twice a day intramuscularly administered to the mother produced concentrations of estradiol in maternal plasma similar to those observed at labor without an altered progesterone concentration in maternal plasma as we demonstrated in our previous study *(3)*. Control ewes carried their pregnancies until they were necropsied, and the cervix was closed at necropsy in all ewes in the control group.

Fetal Cortisol Level in Fetal Plasma in Control and Estradiol-Treated Animals

Fetal plasma cortisol levels were similar in baselines in the two groups (5.36 ± 0.52 and 5.5 ± 1.29 ng/mL for controls and estradiol-treated animals) and did not change after vehicle and estradiol-treatment (5.14 ± 1.52 and 4.7 ± 1.04 ng/mL, respectively).

Induction of PGHS-2 mRNA in Myometrium, Endometrium, but not Placenta

There were significant increases of PGHS-2 mRNA analyzed by Northern blot analysis (Figs. 1–3) in the endometrium (Fig. 1) and the myometrium (Fig. 2), but not in the placenta (Fig. 3) in all five estradiol-treated ewes carrying adrenalectomized fetuses. We validated our cloned PGHS-2 riboprobes by Northern blot analysis in our previ-

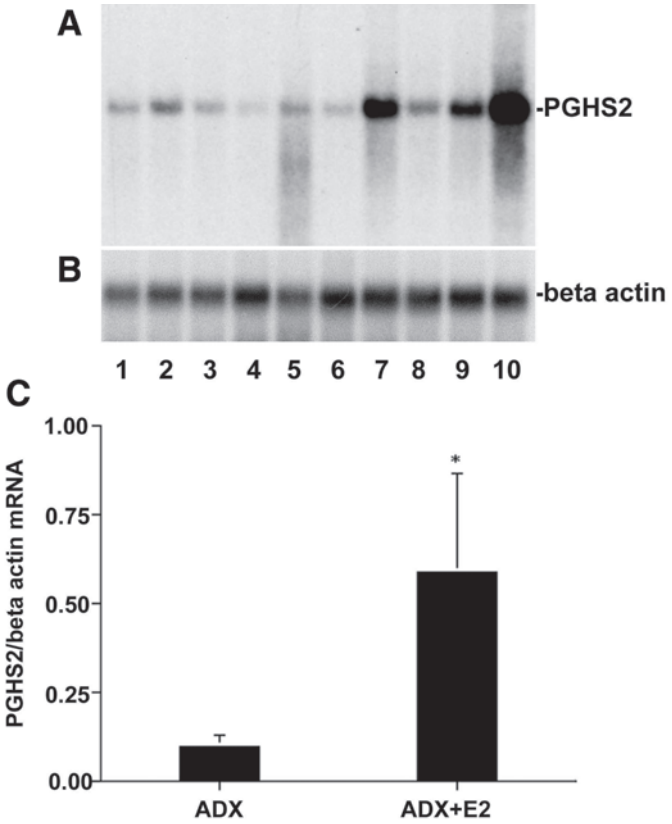

Fig. 2. Northern blot analysis of PGHS-2 mRNA in the myometrium (**A**) from controls (ADX, lanes 1–5) and estradiol-treated animals after fetal adrenalectomy (ADX+E2, lanes 6–10). (**B**) Beta actin in each corresponding lane. (**C**) Densitometry analysis of the ratios of myometrial PGHS-2 and beta actin mRNAs. There was a significant increase of PGHS-2 mRNA in myometrium in ADX+E2 group (*$p < 0.05$) compared with ADX group. Values are presented as mean ± SEM.

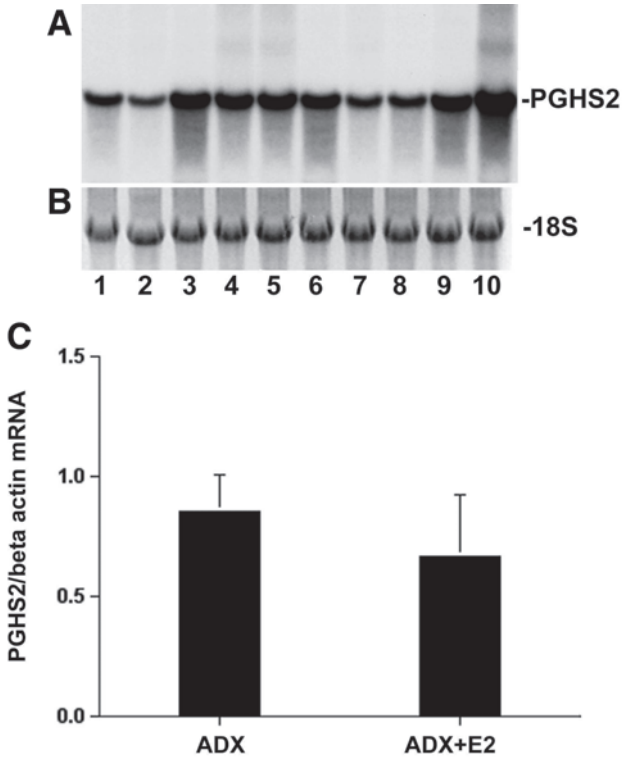

Fig. 3. Northern blot analysis of PGHS-2 mRNA in the placenta (**A**) from controls (ADX, lanes 1–5) and estradiol-treated animals after fetal adrenalectomy (ADX+E2, lanes 6–10). (**B**) Beta actin in each corresponding lane. (**C**) Densitometry analysis of the ratios of placenta PGHS-2 and beta actin mRNAs. There was no change of PGHS-2 mRNA in placenta in ADX+E2 compared with ADX group. Values are presented as mean±SEM.

ous study *(3)*. Only the PGHS-2 antisense probe generated specific signals at the expected molecular weight (about 4.5 kb) *(3)*.

Induction of PGHS-2 Protein in Myometrium, Endometrium, but not Placenta

PGHS-2 protein was analyzed by Western blot analysis in all three intrauterine tissues (Figs. 4–6). After estradiol treatment, there were significant increases of PGHS-2 protein in the endometrium (Fig. 4) and myometrium (Fig. 5), but not in the placenta (Fig. 6), which is consistent with the change at mRNA level (Figs. 1–3).

Discussion

There is very little information on the relative degree of participation and the interaction between fetal cortisol and maternal estrogen in regulating the intrauterine PG system during labor. In our previous studies we have consistently demonstrated induction of PGHS-2 associated with both glucocorticoid-induced premature labor *(8)* and spontaneous term labor *(9)* in pregnant sheep. In both situations there are three major endocrine changes in fetal and maternal plasma associated with onset of spontaneous or induced premature labor: (1) increased fetal plasma cortisol concentration; (2) decreased maternal progesterone concentration; and (3) increased maternal estradiol concentration. In these spontaneous and induced labor models it is impossible to dissect out effects of each individual hormone on induction of the intrauterine PG system from the sum effect of the combined hormonal changes. Although we have demonstrated estrogen's stimulating effect on intrauterine PGHS-2 in pregnant sheep *(3)*, basal levels of glucocorticoids and the products of the adrenal are consistently produced by fetal adrenal throughout the estrogen challenge. Therefore, the changes in the intrauterine PG system observed might result from the combined effect of hormones that continue to be produced by fetal HPA activity in addition to the effects of the exogenously infused estrogen. By conducting fetal adrenalectomy in all animals investigated in the present studies,

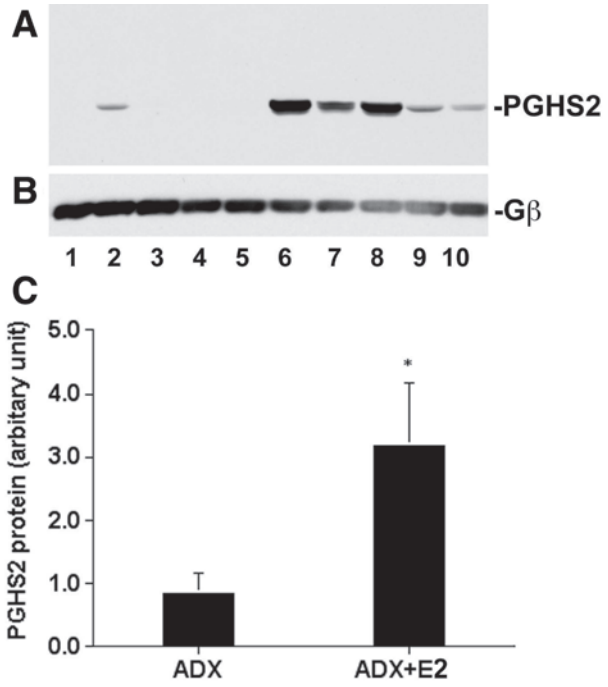

Fig. 4. Western blot analysis of PGHS-2 protein in endometrium (**A**) from controls (ADX, lanes 1–5) and estradiol-treated animals after fetal adrenalectomy (ADX+E2, lanes 6–10). (**B**) Gβ in each corresponding lane. (**C**) Densitometry analysis of PGHS-2 protein in endometrium from ADX- and ADX+E2–treated animals. There was a significant increase of PGHS-2 protein in endometrium (*$p < 0.05$) in ADX+E2 group compared with ADX group. Values are presented as mean ± SEM.

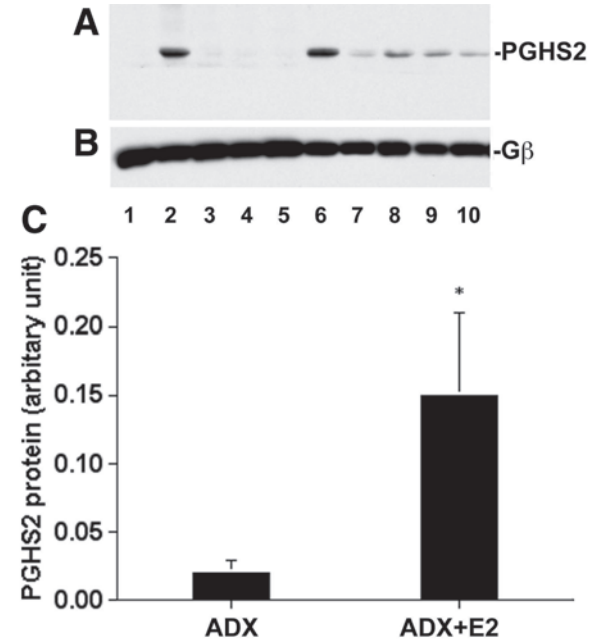

Fig. 5. Western blot analysis of PGHS-2 protein in myometrium (**A**) from controls (ADX, lanes 1–5) and estradiol-treated animals after fetal adrenalectomy (ADX+E2, lanes 6–10). (**B**) Gβ in each corresponding lane. (**C**) Densitometry analysis of PGHS-2 protein in myometrium from ADX- and ADX+E2–treated animals. There was a significant increase of PGHS-2 protein in myometrium (*$p < 0.05$) in ADX+E2 group compared with ADX group. Values are presented as mean±SEM.

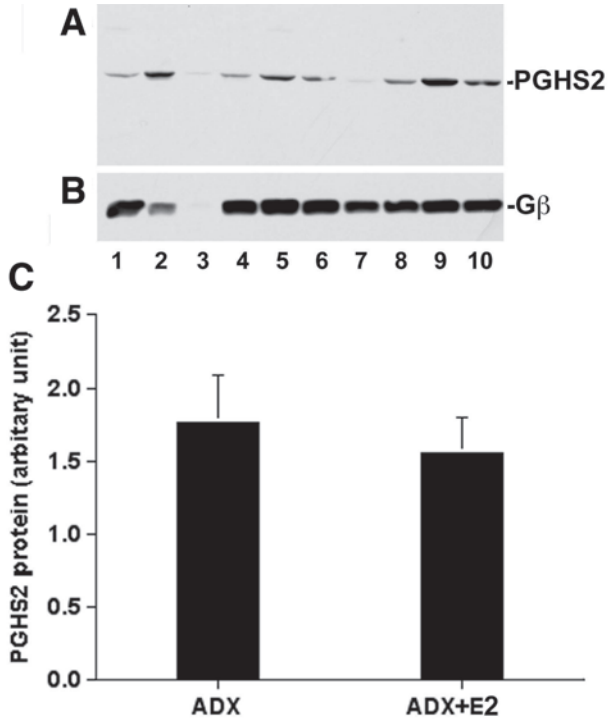

Fig. 6. Western blot analysis of PGHS-2 protein in placenta (**A**) from controls (ADX, lanes 1–5) and estradiol-treated animals after fetal adrenalectomy (ADX+E2, lanes 6–10). (**B**) Gβ in each corresponding lane. (**C**) Densitometry analysis of PGHS-2 protein in placenta from ADX- and ADX+E2–treated animals. There was no change of PGHS-2 protein in placenta in ADX+E2 group compared with ADX group. Values are presented as mean ± SEM.

we removed endogenous fetal cortisol as well as other products of fetal adrenal origin and at the same time limited the maternal plasma progesterone alteration, which is usually induced by increased fetal cortisol in conjunction with onset of labor in pregnant sheep. Therefore, our approach provided the ability to identify the specific function of estradiol on induction of intrauterine PGHS-2 in the pregnant ovine uterus. We present the first in vivo evidence that maternal estradiol administration, independent of any other steroids or products of fetal adrenal origin, resulted in increased PGHS-2 expression in the endometrium and myometrium and induced cervical dilation in pregnant sheep.

Removal of endogenous fetal cortisol production by fetal adrenalectomy is of particular physiological importance in light of recent evidence produced by several investigators including ourselves *(6)* that direct fetal cortisol stimulation of the intrauterine PGHS-2 may occur (cortisol-dependent/estrogen-independent pathway) *(4–6)*, which is an addition to our classic views of estradiol-dependent pathway on regulation of intrauterine PGHS-2. Therefore, it is important to differentiate the relative roles of both pathways in experimental models that allow simultaneous study of the various tissues that are critical to normal labor.

The pregnant sheep has been a central model for study of the process of parturition by research groups throughout the world *(1,3,5,10,11)*. There are differences in the biology of onset of labor between sheep and primates; for example, fall in progesterone is induced by increased fetal cortisol in pregnant sheep *(1)*, but not in primates *(12–14)*. However, increased activity of the pituitary and adrenal systems before onset of labor has been demonstrated in many different species including monkeys and humans *(14–16)*. Adrenal weights dramatically increase over the last 30% of gestation in both fetal sheep and monkeys *(14)*. Infusion of ACTH into the fetus can precipitate premature labor in pregnant rhesus monkey *(17)* and increased cortisol matures fetal organs such as the lung in sheep and humans *(1)*. Thus, there are parallels in biology of fetal adrenal development and onset of labor between the primates and sheep that make the fetal adrenal an important organ to study in parturition research.

The current study confirms our previous demonstration *(3)* of positive stimulation by estradiol on PGHS-2 in maternal uterine tissues of pregnant sheep, and extends it by demonstrating that estradiol's stimulating effect on maternal uterine PGHS-2 is independent of the fetal HPA axis. These two studies support each other by providing similar conclusions from different approaches that address both the interactive and independent functions of fetal cortisol and maternal estrogen in the regulation of intrauterine PGHS-2 during late gestation and labor in pregnant sheep. Our data strongly suggest that estradiol plays a central role in the labor-type myometrial contraction and cervical dilation and mediates increased PG production from maternal uterine tissues.

Consistent with our previous findings *(3,6)*, we were unable to demonstrate an increase of PGHS-2 in the sheep placenta, providing further evidence for a tissue-specific action of estrogen in regulating the intrauterine PGHS-2 in pregnant sheep *(9)*. In light of the observation that fetal placental PGHS-2 increases in late gestation *(10,11,18)* well before the rise in maternal plasma estradiol at the end of gestation *(19)*, we and others have proposed that in late gestation fetal adrenal cortisol is responsible for the increased fetal placental PGHS-2 expression and PG production *(5,6)*. Indeed we have reported recently that fetal cortisol at concentrations seen in late gestation, independent of any other steroids of fetal origin, is able to stimulate fetal placental PGHS-2 expression *(6)*. In addition, maternal administration of glucocorticoid at a dose inadequate to induce labor resulted in increased PGHS-2 expression in the fetal placenta *(20)*. Therefore, it is likely that the increase in placental PGHS-2 in late gestation is under the control of the late gestation rise of fetal cortisol, while estradiol may control PGHS-2 expression in maternal intrauterine tissues associated with the onset of labor in pregnant sheep. Taken together, our past and present observations have consistently shown the tissue-specific regulation of PGHS-2 in pregnant sheep suggesting that specific PG production from different intrauterine tissues may play differential functions in preparation, activation, and completion of labor. Induction of the PG synthetic pathway in *maternal* uterine tissues is strongly dependent on stimulation by estrogen, whereas fetal cortisol may be responsible for regulation of the placental PG system.

This is the first study to examine the effect of exogenously administered estradiol on the physical and molecular changes in the uterus of pregnant sheep after fetal adrenalectomy. Estradiol administration induced dilation of cervix in all five treated animals and delivery of the fetus in three out of five animals. We have demonstrated in our previous study that the dosage of estradiol used in the present study was able to produce maternal plasma estradiol concentrations at the level observed in dexamethasone induced premature labor *(21)* and spontaneous term labor *(19)*, while leaving maternal plasma progesterone concentrations unchanged *(3)*. Therefore, our data once again suggest that estradiol alone is able to induce two out of three major uterine physical changes associated with onset of labor, labor-type myometrial contraction, and cervical dilation.

The retention of the placenta in all estradiol-induced premature fetal deliveries at the stage of gestation studied suggests that estradiol alone is not able to complete the process of labor. Other mechanisms have to interact with estradiol to carry labor to completion. Prostaglandins produced by fetal membranes and placenta might be of importance in regulating placental separation.

Conclusions

These data provide the first in vivo evidence for estradiol upregulation of the uterine PGHS-2 in late gestation in the absence of an intact fetal HPA axis. Thus the fetal adrenal is not required to mediate estradiol's stimulation of maternal uterine PGHS-2 associated with the onset of labor.

Materials and Methods

Animals and Tissue Collection

Pregnant ewes bred on a single occasion and carrying fetuses of known gestational age were studied. Experimental procedures were approved by Oklahoma University Institutional Animal Care and Use Committees. The Oklahoma facilities are approved by the American Association for the Accreditation of Laboratory Animal Care.

Placement of fetal carotid arterial and jugular venous catheters and bilateral fetal adrenalectomy at 117–121 dGA were performed under halothane general anesthesia in 10 pregnant ewes. Four days after surgery (at 121–125 dGA) ewes were treated with vehicle (sesame oil, $n = 5$), or 17 beta estradiol ($n = 5$, 5 mg twice a day), administered intramuscularly for 2 days to produce concentrations of estradiol in maternal plasma similar to those observed at labor as we demonstrated in our previous study *(3)*. At 123–127 dGA after 2 d of vehicle or estradiol treatment, tissues were obtained under halothane anesthesia, after which the ani-

mals were euthanized. Indication of labor was confirmed at necropsy with the observation of cervical dilation. Endometrium, myometrium, and placental cotyledons separated from the uterine muscle and fetal membranes were collected for later RNA and protein analysis. Fetal arterial blood samples were collected on the day before vehicle or estradiol treatment and on the day before necropsy. Plasma was separated from blood cells by centrifugation at 1500*g* for 10 min at 4°C. Plasma samples were frozen at –20°C for subsequent assay.

RIA

The RIA for fetal plasma cortisol was performed as described previously *(3)* using commercially available ^{125}I RIA kits (Diagnostic Products Co., Los Angeles, CA). The sensitivity of the assays was 2 ng/mL. The specificity was provided by the manufacturer. The intraassay coefficient of variation was 5%.

Northern Analysis

Total RNA was extracted from placenta. Polyadenylated RNA was extracted from myometrium and endometrium by oligo dT cellulose affinity chromatography using a commercial kit (Invitrogen, San Diego, CA). Samples of total RNA from placenta (30 μg) or polyadenylated RNA from myometrium and endometrium (2 μg) were separated by electrophoresis on a 1.4% (wt/vol) agarose–0.66 *M* formaldehyde gel and transferred onto a nylon membrane (NEN Life Science, DE, USA) and then subjected to Northern blot analysis for PGHS-2 mRNA as described previously *(3,6,22)*. Beta-actin mRNA was used to control RNA loading.

Synthesis of Probes

Our cloned ovine PGHS-2 cDNA *(3)* in pCR II vector (Invitrogen), which includes promoters for phage polymerases SP-6 to produce antisense probe and T-7 to produce sense probe, was linearized by an appropriate restriction enzyme. The antisense and sense riboprobes were synthesized using a commercial kit (MAXIscript, Ambion, TX) labeled with [α-^{32}P]UTP for Northern (NEN Life Science).

Solubilized Cell Membrane Extraction and Western Blot Analysis

To prepare solubilized cell extracts, approx 1 g of tissue was homogenized for 1 min (Polytorn, KINEMATICA. AG, Switzerland) on ice in TE buffer (50 m*M* Tris and 10 m*M* EDTA) containing 2 m*M* octyl glucoside and 0.2 m*M* phenylmethylsulfony fluoride and centrifuged at 30,000*g* for 1 h at 4°C. The crude pellets (membrane, nuclei, and mitochondria) were sonicated in 1 mL TE sonication buffer (20 m*M* Tris and 50 m*M* EDTA) containing 45 m*M* octyl glucoside and 0.2 m*M* phenylmethylsulfony fluoride. The supernatants were centrifuged at 13,000*g* for 25 min at 4°C. The recovered supernatants (solubilized cell extract) were stored at –70°C until electrophoresis analysis. The protein concentration was determined by the method of Bradford (Bio-Rad Laboratories, Hercules, CA) as we described previously *(22)*. The solubilized proteins (50 μg/lane) separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were electrophoretically transferred to a Nylon membrane (Imobilon, Millipore Corp., Bedford, MA), using a Bio-Rad transfer blot cell. The protein bands were visualized using an enhanced chemiluminescence Western blotting detection kit (ECL, Amersham Life Sciences, Arlington Heights, IL). The molecular sizes of the proteins were determined by running standard molecular-weight-marker proteins (Bio-Rad) in an adjacent lane. G protein β subunit was used to normalize the loading for each lane. Chemiluminescence signals were analyzed and quantified with the scanner and data were analyzed with a densitometry program Scan Analysis and quantified against an arbitrary scale in the plot.

Statistical Analysis

Comparison of two means was made with the Student's *t* test. Statistical significance was assumed at the 5% level. Data are presented throughout as mean ± SEM.

Acknowledgment

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