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The Effects of Estradiol-17β Infusion into Fetal Sheep in Late Gestation

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Activation of the hypothalamic–pituitary–adrenal (HPA) axis of fetal sheep during late gestation is associated with increases in plasma concentrations of adrenocorticotropic hormone (ACTH) and cortisol, and ultimately results in parturition. However, the mechanisms contributing to the concurrent increases in ACTH and cortisol are unclear. Plasma estradiol-17β (E_2) concentrations increase progressively in the prepartum ovine fetus, and we hypothesized that E_2 may influence HPA activity by affecting either basal and/or hypoxemia-stimulated ACTH release. We examined potential mechanisms, including altered expression of pro-opiomelanocortin (POMC) in fetal pituitary corticotrophs, and changes in corticosteroid binding globulin (CBG) and/or the enzymes 11β hydroxy steroid dehydrogenase (11β HSD)-1 or 11β HSD-2 in liver and placenta, that could alter negative feedback control. We infused fetal sheep at 127 d of gestation with either E_2 (100 μg/24 h) or saline for 100 h. Fetal arterial blood samples were collected at 8 h intervals during the infusion of E_2 or saline ($n = 4$), for measurement of basal plasma ACTH and cortisol concentrations, as well as plasma corticosteroid binding capacity (CBC). Placenta and fetal liver samples were collected at 100 h for measurement of placental 11β HSD-1 and 11β HSD-2 mRNA and hepatic CBG and 11β HSD-1 mRNA, by Northern blotting. Fetal pituitary samples were collected for measurement of POMC mRNA by *in situ* hybridization. In a separate experiment, fetuses were exposed to 2 h of hypoxemia at 75 h of E_2 or saline infusion ($n = 4$), and fetal arterial blood samples were collected during the period of hypoxemia for measurement of plasma ACTH and cortisol concentrations. E_2 infusion had no effect on basal plasma concentrations of ACTH or total cortisol, or on the stimulated levels of ACTH or total cortisol achieved in response to hypoxemia. Basal fetal pituitary POMC mRNA also did not change significantly with E_2 infusion. No significant increases were observed in plasma CBC during E_2 administration. However, hepatic CBG and 11β HSD-1 mRNA were significantly elevated in the livers of E_2-treated fetuses. Placental 11β HSD-1 mRNA; but not 11β HSD-2 mRNA was increased by E_2 treatment. These data do not support a direct effect of exogenous E_2 at the level of basal or hypoxemia-stimulated ACTH output, but suggest that elevated E_2 concentrations may alter the expression of genes encoding proteins implicated in tonic regulation of fetal HPA function.

Key Words: Estradiol; sheep fetus; adrenocorticotropin; cortisol; corticosteroid binding globulin; 11β hydroxysteroid dehydrogenase.

Introduction

Maturation and activation of the fetal hypothalamic–pituitary–adrenal (HPA) axis is central to the initiation of parturition in sheep. HPA activity increases during gestation such that highest concentrations of adrenocorticotropic hormone (ACTH) and cortisol are achieved in fetal plasma at the time of birth *(1,2)*. However, the mechanisms contributing to the concurrent increases in fetal plasma ACTH and cortisol toward term is not clearly understood. Experiments involving adult rats suggest that estrogens increase the activity of the HPA axis *(3)*. Female rats have higher basal and stimulated plasma ACTH *(4)* and corticosterone *(5)* levels than male rats. Adult male rats treated with estradiol also have higher basal plasma ACTH concentrations than untreated rats *(6)*. In response

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to stress, ovariectomized female rats given estrogen have a more prolonged ACTH and corticosterone response to stress than untreated rats *(7)*. The site of action of estrogen has been postulated to be the hypothalamus and/or the pituitary.

It is possible that estradiol may promote HPA activity in fetal sheep. In this species, maternal and fetal plasma concentrations of estradiol increase prepartum *(8,9)*, at the time of late HPA activation. Saoud and colleagues *(10)* have reported that treatment of fetal sheep with estradiol increased basal and hypotension-stimulated plasma ACTH concentrations. We hypothesized that intrafetal estradiol infusion would increase fetal HPA activity, through either an effect on pituitary corticotroph activity, or as a result of diminished negative feedback. Feedback may be decreased by either an increase in plasma corticosteroid binding globulin (CBG) or altered metabolism of cortisol in the placenta or fetal liver as a result of changes in expression of the enzyme 11β hydroxysteroid dehydrogenase (11β HSD). There are two isoforms of this enzyme, 11β HSD-1 and 11β HSD-2. 11β HSD-1 is bidirectional, and interconverts cortisol and cortisone *(11)*. 11β HSD-2 functions preferentially as a unidirectional dehydrogenase, converting cortisol to cortisone *(11)*.

Therefore, we examined the effect of estradiol infusion on basal fetal pituitary pro-opiomelanocortin (POMC) mRNA levels, and basal and hypoxemia-stimulated fetal plasma ACTH and cortisol concentrations. We also determined the effect of estradiol infusion on fetal hepatic CBG mRNA levels, plasma corticosteroid binding capacity (CBC), and on levels of mRNA encoding 11β HSD-1 and 11β HSD-2 in the placenta and 11β HSD-1 in the liver.

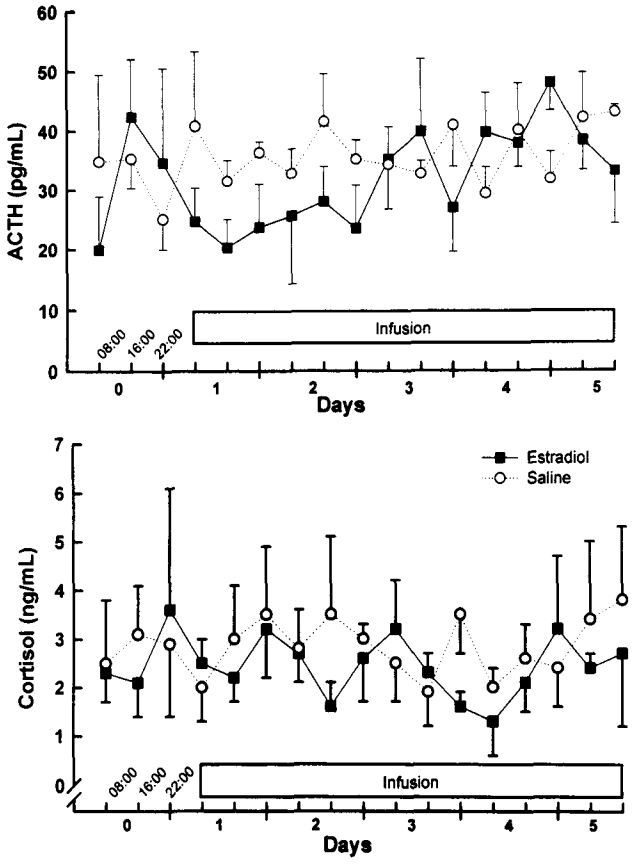

Fig. 1. Effect of estradiol treatment to the fetus on basal plasma immunoreactive ACTH and cortisol concentrations. Fetal plasma concentrations of immunoreactive ACTH (top panel) and cortisol (bottom panel) for 100 h of infusion either with estradiol-17β (■; $n = 4$) or saline (○; $n = 4$), mean ± S. E. M.

Results

A gestational age of d 127 was chosen for the start of the infusion because the prepartum activation of the HPA axis is demonstrable at this gestational age *(2)*. Also, the infusion rate of estradiol used in this study was calculated based on maternal metabolic clearance rates for the ewe *(12)* corrected for an estimated fetal weight at a gestational age of 130 d, and was expected to elevate fetal plasma concentrations to 200–500 pg/mL. This estradiol concentration is comparable to or greater than estradiol concentrations in sheep fetal plasma at term *(8)*.

Basal Fetal Plasma ACTH and Cortisol Concentrations

The effects of estradiol and saline infusion on basal fetal plasma immunoreactive (ir)-ACTH and cortisol concentrations are shown in Fig. 1. The mean plasma ir-ACTH concentrations were similar between the two groups during both the control period (d 0, 08:00 h; estradiol, $n = 4$, 19.9 ± 9.0 pg/mL; saline, $n = 4$, 34.9 ± 14.6 pg/mL) and after 100 h of infusion (estradiol, 33.0 ± 8.9 pg/mL; saline, 42.8 ± 1.2 pg/mL). Overall there was no significant change in the mean concentration of ir-ACTH in the plasma of the fetuses with estradiol or saline treatment.

The mean plasma cortisol concentrations before the start of the infusion (d 0, 08:00 h) were 2.3 ± 0.6 ng/mL ($n = 4$) and 2.5 ± 1.3 ng/mL ($n = 4$), and after 100 h of infusion 2.7 ± 1.5 and 3.8 ± 1.5 ng/mL, in the estradiol- and saline-treated fetuses, respectively. Again, there was no significant change in the mean concentration of cortisol in the plasma of the fetuses.

Hypoxemia-Stimulated Fetal Plasma ACTH and Cortisol Concentrations

The fetal partial pressures of oxygen, plasma ir-ACTH, and cortisol responses to hypoxemia are shown in Fig. 2. The mean fetal partial pressures of oxygen prior to hypoxemia were similar (22.3 ± 0.7 and 18.6 ± 1.3 mmHg, estradiol- and saline-infused fetuses, respectively), but were reduced significantly by 30 min and maintained at reduced levels for an additional 90 min. The fetal partial pressures of oxygen returned to similar prehypoxemia values 60 min following the 2 h period of hypoxemia. The mean fetal plasma ir-ACTH concentrations prior to hypoxemia were 53.4 ± 5.7 and 27.9 ± 4.6 pg/mL for estradiol- and saline-infused fetuses, respectively. There were no significant differences in plasma ir-ACTH concentrations

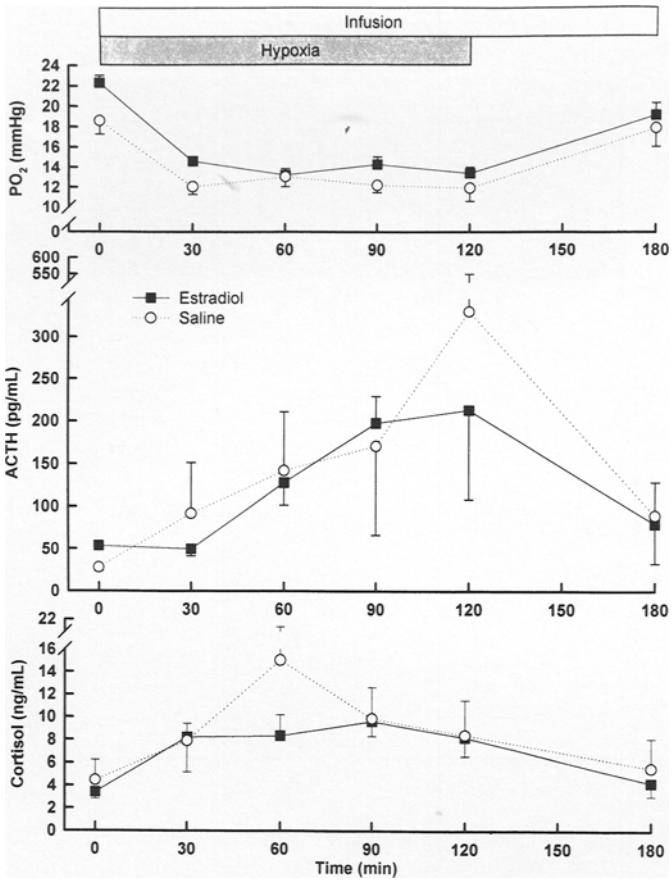

Fig. 2. Effect of fetal hypoxemia on plasma immunoreactive ACTH and cortisol in fetuses infused with estradiol or saline. Fetal arterial partial pressures of oxygen (top panel), hypoxemia-stimulated immunoreactive ACTH concentrations (middle panel), and plasma cortisol concentrations (bottom panel), of fetuses infused with either estradiol-17β (■; $n = 4$) or saline (○; $n = 4$). Values are mean ± S. E. M.

between estradiol- and saline-infused fetuses, but both groups of fetuses responded to the hypoxemia. The ir-ACTH concentrations were increased after 2 h of hypoxemia (212.7 ± 104.8 pg/mL for estradiol- and 330.2 ± 219.6 pg/mL for saline-treated fetuses). The mean cortisol concentrations prior to hypoxemia were 3.4 ± 0.6 and 4.5 ± 1.8 ng/mL for estradiol- and saline-infused fetuses, respectively. In both estradiol- and saline-infused fetuses, fetal plasma cortisol concentrations increased in response to hypoxemia. However, estradiol treatment did not augment the plasma cortisol responses. Both ir-ACTH and cortisol concentrations returned to prehypoxic values by 60 min after the end of the hypoxemic episode.

Basal Pituitary POMC mRNA

The pituitaries were analyzed in two regions: the *pars distalis* (Fig. 3, left panel) and the *pars intermedia* (Fig. 3, right panel). There was no significant difference in the level of POMC mRNA in either region of the pituitary from estradiol-infused fetuses compared to saline-infused fetuses.

Hepatic CBG mRNA and Plasma CBC Concentrations

A 1.8 kb CBG mRNA was detected by Northern blotting of liver RNA from both estradiol- ($n = 4$) and saline- ($n = 4$) infused fetuses. The relative level of CBG mRNA:18S rRNA, was increased significantly in estradiol-infused fetuses (Fig. 4, $p < 0.05$).

The plasma CBC was similar in the two groups of fetuses prior to the start of infusion (d 0, 08:00 h; estradiol, 26.6 ± 5.1 ng/mL, $n = 4$; saline, 29.8 ± 9.2 ng/mL; Fig. 5). Despite the difference in hepatic CBG mRNA, there was no significant effect of estradiol infusion on fetal plasma CBC.

Hepatic 11β HSD-1 mRNA

A single 11β HSD-1 mRNA of 1.8 kb was detected in total RNA extracts of the fetal liver. There was a significant increase in the relative amount of hepatic 11β HSD-1 mRNA in estradiol-infused fetuses ($p < 0.05$, Fig. 6).

Placental 11β HSD-1 and 11β HSD-2 mRNA

The mean relative amount of 11β HSD-1 mRNA was almost threefold higher in the placentae of fetuses that had been treated with estradiol compared with those of saline-infused fetuses ($p < 0.05$, Fig. 7). In contrast, there was no significant difference in the amount of 11β HSD-2 mRNA between the placentae of fetuses that had been treated with estradiol compared with those of saline-infused fetuses (Fig. 8). A single 11β HSD-2 transcript of 2.0 kb was identified in total RNA extracts of placentae.

Discussion

We have demonstrated that infusion of estradiol to the fetus does not directly affect basal and/or hypoxemia-stimulated ACTH release. The lack of effect of estradiol on basal plasma ACTH concentrations is consistent with the lack of change in levels of mRNA encoding POMC in the pituitary. Therefore, basal synthesis of POMC from the anterior lobe of the fetal pituitary and plasma ACTH concentrations were not affected by estradiol in this study. Although we have demonstrated that estradiol increases fetal hepatic CBG mRNA, this increase was not reflected in plasma CBC. We have also determined that the synthesis of hepatic, as well as placental, 11β HSD-1 was augmented with estradiol treatment, suggesting that this gene may be positively regulated by estradiol. Estradiol treatment had no effect on placental 11β HSD-2 mRNA, indicating that it has differential, but specific effects on different genes.

This report is in contrast to the results of Saoud et al., *(10)*, who demonstrated an increase in basal and nitroprusside-stimulated, fetal plasma ACTH concentrations in fetuses treated with estradiol. In that study *(10)*, silastic capsules containing estradiol were implanted into fetuses; however, the length of treatment and the gestational ages of the fetuses were not reported. It is possible that if the fetuses were older than those in this study, HPA maturation

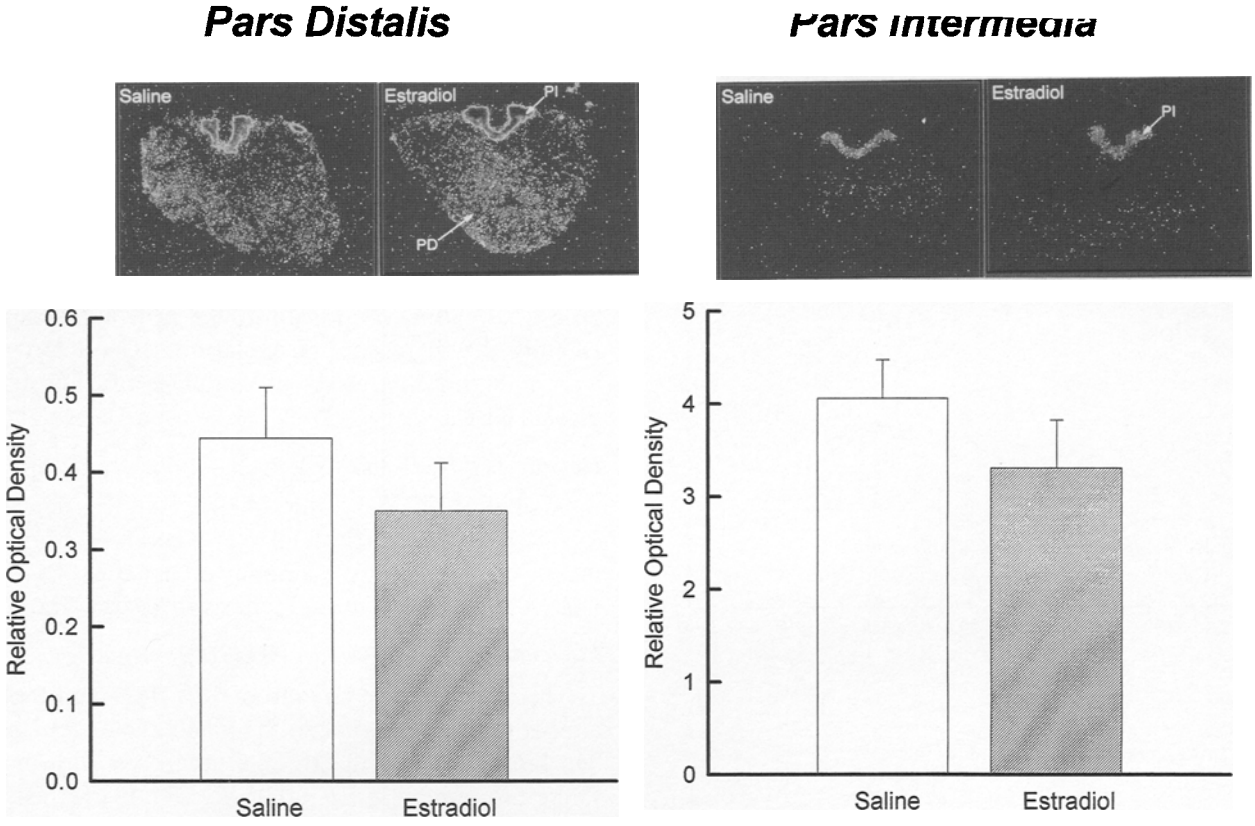

Fig. 3. Effect of estradiol or saline infusion on POMC mRNA levels in the ovine fetal pituitary gland. The top panels show representative computerized autoradiograms of POMC expression as determined by *in situ* hybridization, and the bottom panels show the results from computerized image analysis of the autoradiograms, in the *pars distalis* (PD; left panels) and *pars intermedia* (PI; right panels) of fetuses treated with saline or estradiol-17β. Values are mean ± S.E.M., $n = 4$.

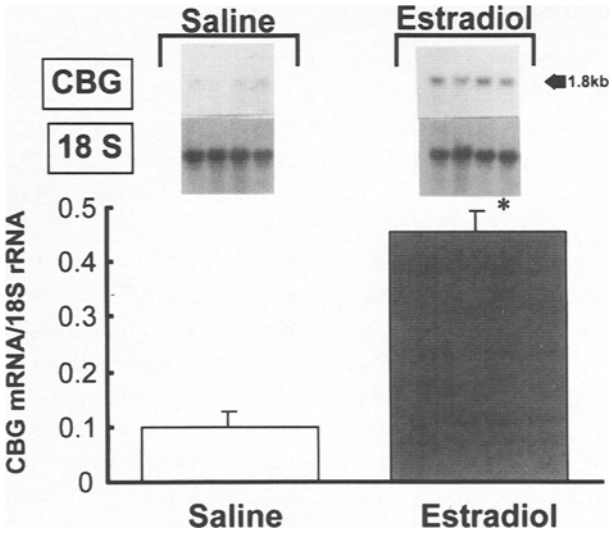

Fig. 4. Hepatic CBG mRNA levels in estradiol- and saline-infused fetuses. Photograph of autoradiographic film showing hepatic CBG mRNA and 18S rRNA (top panel) and ratio of CBG mRNA:18S rRNA relative optical densities (ROD; bottom panel) in saline- ($n = 4$, open bars) and estradiol-17β- ($n = 4$, shaded bars) treated fetuses, mean ± S.E.M., $*p < 0.05$.

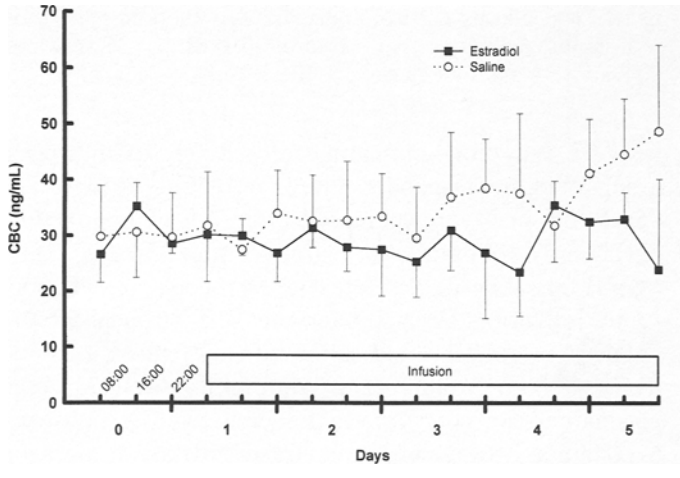

Fig. 5. Effect of estradiol and saline infusion on plasma CBC. Plasma CBC of fetuses infused with estradiol-17β (■; $n = 4$) or saline (○; $n = 4$), mean ± S.E.M., are shown.

may have already been activated. Subsequently, Wood and Saoud *(13)* demonstrated that androstenedione, but not estradiol, changed the length of gestation, consistent with the results of the present study that exogenous estradiol has no effect on fetal ACTH secretion and HPA axis activation in fetal sheep.

In rats, there are increases in basal corticosterone and stress-stimulated ACTH and corticosterone responses with estrogen administration to males or females *(3,14)*

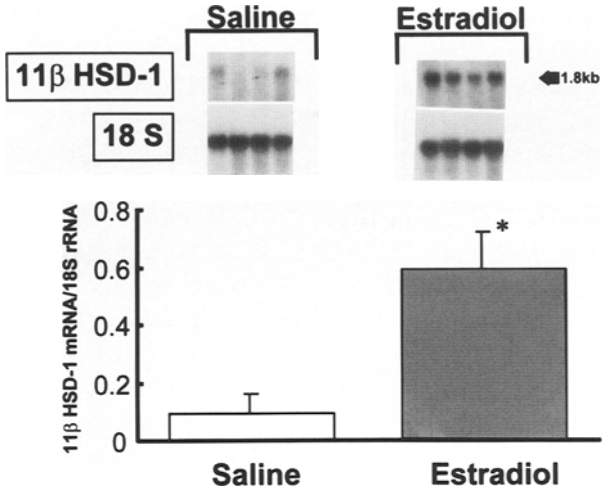

Fig. 6. Hepatic 11β HSD-1 mRNA levels in fetuses infused with estradiol or saline. Photograph of autoradiographic film showing hepatic 11β HSD-1 mRNA and 18S rRNA (top panel) and ratio of 11β HSD-1 mRNA:18S rRNA relative optical densities (ROD; bottom panel) in saline- (n = 4, open bars) and estradiol-17β- (n = 4, shaded bars) treated fetuses, mean ± S. E. M., *$p < 0.05$.

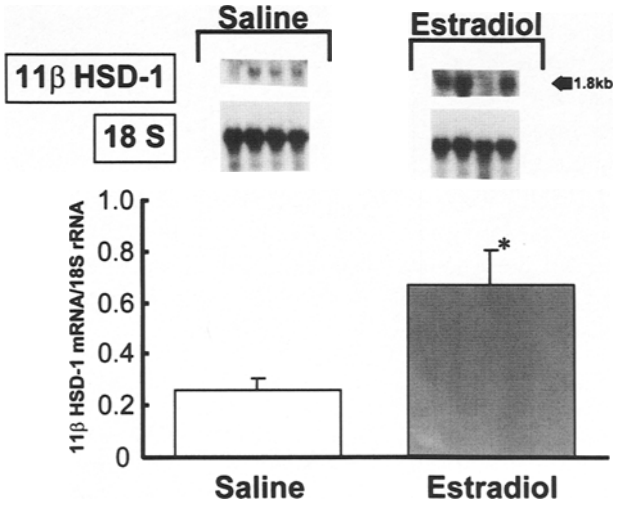

Fig. 7. Effect of estradiol or saline infusion on placental 11β HSD-1 mRNA levels. Autoradiographic film showing placental 11β HSD-1 mRNA and 18S rRNA (top panel) and ratio of 11β HSD-1 mRNA:18S rRNA relative optical densities (ROD; bottom panel) in saline- (n = 4, open bars) and estradiol-17β- (n = 4, shaded bars) treated fetuses, mean ± S.E.M., *$p < 0.05$.

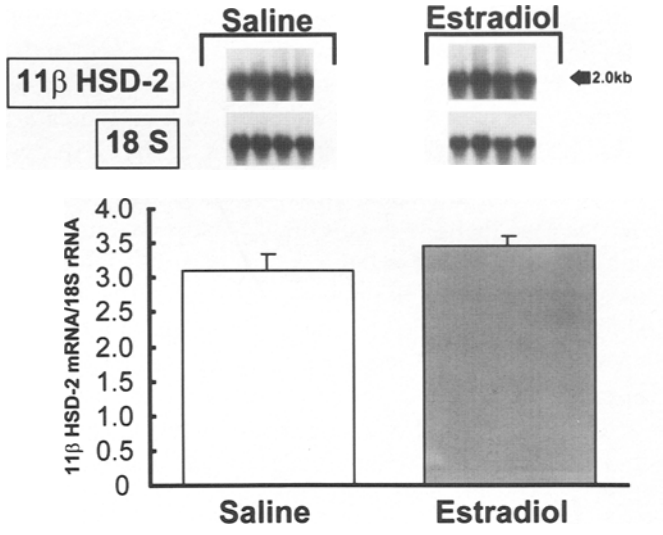

Fig. 8. Placental 11β HSD-2 mRNA levels after 100 h of estradiol or saline infusion. Photograph of autoradiographic film showing placental 11β HSD-2 mRNA and 18S rRNA (top panel) and ratio of 11(HSD-2 mRNA:18S rRNA relative optical densities (ROD; bottom panel) in saline- (n = 4, open bars) and estradiol-17β- (n = 4, shaded bars) treated fetuses, mean ± S.E.M.

and during the estrous cycle in female rats *(3,7)*. However, POMC mRNA levels in ovariectomized rats treated with estradiol were significantly decreased in the anterior pituitary *(15)*, as well as in the medial basal hypothalamus *(16)* and the arcuate nucleus *(17)*. Exogenous estrogen increased POMC mRNA levels in the arcuate nucleus of nonpregnant ovariectomized ewes *(18)*. These results suggest that there may be species differences in the HPA axis response to estrogen treatment. In the present study, there was no effect of estradiol infusion to the sheep fetus on either levels of POMC mRNA in the fetal pituitary, or on basal or hypoxemia-stimulated concentrations of ACTH and cortisol in plasma. Athough there was some individual variation in hormone concentrations, the mean and the pattern of change in individual animals were not significantly affected with estradiol infusion. It is possible that the fetuses have not yet developed, or that the fetuses do not have the same HPA axis regulatory mechanisms as in the adult and, therefore, do not respond to exogenous estradiol treatment in a similar manner.

The sheep fetal HPA axis is influenced by negative feedback of cortisol at the level of the hypothalamus and the pituitary *(2)*. Cortisol negative feedback can be modulated in the fetus by CBG, the specific, high-affinity binding protein of cortisol *(19)*. Augmentation of CBG concentrations in the sheep fetus may indirectly affect ACTH synthesis and secretion by the pituitary, since it has been proposed that CBG may diminish the inhibitory effects of cortisol on the hypothalamus and the pituitary *(20,28)*. Studies in humans have demonstrated that exogenous estrogens stimulate an increase in CBG in men *(21)*, and in women taking oral contraceptives *(22,23)*. The elevated concentrations of systemic estrogen during pregnancy are believed to contribute to the raised levels of CBG, synthesized in the maternal liver *(23,24)*. However, in fetal sheep, Ballard et al. *(25)*, were unable to influence fetal plasma CBC with exogenous estradiol. Consistent with these findings, Challis et al. *(26)*, demonstrated that increases in fetal plasma CBC occur prior to changes in maternal estrogen, which are believed to reflect temporal changes in fetal estrogen *(8)*. No consensus sequence resem-

not reflec-ted in plasma CBC, but there may be a lag between the increase in CBG mRNA accumulation and a subsequent increase in the secretion of CBG. We did not measure pitui-tary CBG mRNA, although CBG has been shown to be produced by the fetal pituitary and may be an additional, novel way of modifying cortisol feedback effects *(28)*.

The enzyme 11β HSD is an important regulator of local, and also potentially of circulating, cortisol concentrations. 11β HSD-1 is found predominately in the liver. This isoform interconverts cortisol and cortisone, although it appears to favor reductase activity. We did not find differences in plasma total cortisol concentrations with estradiol treatment in this study, even though there were changes in the levels of 11β HSD-1 mRNA in the liver and placenta. In this study, we did not determine 11β HSD activity, although previously we have reported good correlation between changes in 11β HSD-1 mRNA levels and enzyme activity, in vitro in the ovine pituitary *(11)*, and placental tissue *(29)*. In the tissues investigated from those studies *(11,29)*, the dehydrogenase activity exceeded the reductase activity, but this does not preclude reductase activity from predominating in the liver. Although we did not observe differences in systemic fetal plasma cortisol, it is possible that local concentrations of cortisol may be elevated in specific tissues as a result of changes in 11β HSD expression. For example, alterations in the local concentration of cortisol in the liver may affect the synthesis of CBG, which is also produced in the liver. In the sheep fetus, the synthesis of CBG is stimulated by cortisol *(30)*. In the present study, therefore, the increase in levels of mRNA encoding CBG in the sheep fetal liver could result from a direct action of estradiol, or indirectly through altered cortisol bioformation in fetal hepatocytes.

The placenta is also a source of 11β HSD-1 and of 11β HSD-2. Both of these enzymes may be important regulators of the transplacental transfer of maternal cortisol, which can affect fetal cortisol concentrations and fetal HPA feedback. Changes in the synthesis and/or activity of these enzymes by estradiol may have indirect effects on fetal pituitary ACTH synthesis and secretion. In the baboon placenta, it has been demonstrated in vivo that estrogen regulates corticosteroid metabolism *(31)* via regulation of 11β HSD activity *(32)*, where the predominant activity at term was the oxidation of cortisol to cortisone *(29,32)*. The authors hypothesized that this increase in corticosteroid metabolism results in a decline in the maternal contribution to fetal cortisol levels and allows maturation of the fetal HPA axis. This is consistent with results demonstrating increased fetal pituitary POMC mRNA expression *(33)*

study. We infused estradiol directly into the fetus. treatment affected the level of placental 11β HSD-1, but not placental 11β HSD-2 mRNA. However, there was no effect on total cortisol or ACTH concentrations in fetal plasma, or fetal pituitary POMC mRNA levels. The potential physiological significance of 11β HSD-1 and 11βHSD-2 in the ovine placenta has not been elucidated, and it remains possible that these enzymes regulate local cortisol concentrations within the placenta and not the transplacental transfer of maternal cortisol to the fetal circulation. Furthermore, it remains to be shown whether substrate concentrations achieved in the placenta or the liver are sufficient for optimal activity of 11β HSD-1, which has a K_m in the μM range.

In summary, we have shown that exogenous estradiol given to the sheep fetus has no significant effect on basal fetal pituitary POMC mRNA, basal or hypoxemia-stimulated plasma ACTH, or total cortisol concentrations. Although estradiol infusion did not change pituitary-adrenal function, an increase in mRNA levels of hepatic CBG was observed, though no change in plasma CBC was measured. Infusion of estradiol also increased both hepatic and placental 11β HSD-1 mRNA, but not placental 11β HSD-2 mRNA. We suggest that estradiol does not affect circulating hormones of the fetal pituitary–adrenal axis, but does have a stimulatory effect on the levels of mRNA encoding proteins involved with modulating free cortisol concentrations in specific tissues.

Methods

Animals and Surgical Procedures

Experiments were performed on 16 fetuses of mixed-breed ewes of known gestational age. The studies were performed according to protocols approved by the Animal Care Committees of St. Joseph's Health Centre and the University of Western Ontario, in accordance with the guidelines of the Canadian Council for Animal Care.

Surgery was performed on ewes using similar techniques to those described previously *(35)* on d 121 ± 1 (mean ± S. E. M.) of gestation. Polyvinyl catheters (V4, Bolab, Lake Havasu City, AZ) were inserted into fetuses as described for each protocol. A polyvinyl catheter (V11, Bolab) was inserted into the amniotic fluid and secured for support to the fetal vascular catheters at the exit point from the uterus. At the time of surgery, a prophylactic antibiotic treatment was given, and this was repeated daily for at least 3 d post-operatively. After surgery, the ewes were housed in individual metabolic cages, with access to food and water ad libitum. To monitor fetal health, fetal arterial blood

samples (0.8 mL) were collected each day for blood gas determinations using an ABL-5 blood gas analyzer (Radiometer, Copenhagen, Denmark). These measurements were corrected to a fetal temperature of 39.5°C. The animals were allowed at least 4 d to recover from surgery before the start of the experiment.

Experimental Protocols

To examine the effects of estradiol-17β on basal HPA function, in eight fetuses, polyvinyl catheters were inserted into a carotid artery and a jugular vein, with the catheter tips advanced 7 cm toward the heart. The fetuses received a continuous infusion of either estradiol-17β (100 μg in 10 mL heparinized saline/24 h; $n = 4$), or heparinized saline (10 mL/24 h; $n = 4$) for 100 h, beginning on day 127 ± 1 of gestation. Fetal blood (3 mL) was collected from the carotid artery three times daily, beginning 24 h before the start of the infusion (control day) and continuing for 100 h. Blood samples were collected into heparinized syringes before being transferred to chilled plastic tubes and centrifuged immediately at 1500*g* for 10 min. Plasma was stored at –20°C until analysis.

After 100 h of infusion the ewe and fetus(es) were euthanized by administration of an overdose of sodium pentobarbital (Euthanyl, MTC Laboratories, Cambridge, Ontario, Canada). At the end of the study, samples of placenta and liver from the fetuses were collected quickly, frozen in liquid nitrogen, and stored at –80°C. Pituitary glands were also collected from the fetuses, slow-frozen on solid carbon dioxide, and stored at –80°C until tissue sectioning.

In another eight fetuses, to examine the effect of estradiol-17β on hypoxemia stimulated HPA axis responses, catheters were inserted into a femoral artery and femoral vein. The fetuses also received a continuous infusion of either estradiol-17β (100 μg in 10 mL heparinized saline/24 h; $n = 4$), or heparinized saline (10 mL/24 h; $n = 4$). On the fourth day, 75 h after the start of the infusion, these fetuses were exposed to an acute episode of hypoxemia. At least 12 h before the induction of fetal hypoxemia, the ewe's head was placed in a large Perspex chamber placed inside the ewe's own metabolic cage, which allowed the ewe access to food and water ad libitum *(36)*. A moderate level of fetal hypoxemia was induced by reducing the percentage of oxygen in the maternal inspired air *(35)* to obtain a predetermined fetal PO_2 decrease of approx 8 mmHg, and was maintained in this way for 2 h. Blood samples were collected prior to the start of the hypoxemia (0 min) and every 30 min thereafter for a total of 120 min. At the end of the hypoxemia, the Perspex box was removed, the ewe was left to breathe room air, and a final blood sample was collected (+180 min). Fetal blood (3 mL) was collected from the femoral artery into heparinized syringes before being transferred to chilled plastic tubes and centrifuged immediately at 1500*g* for 10 min. Plasma was stored at –20°C until analysis.

At the end of the study, the ewe and fetus(es) were euthanized by administration of an overdose of sodium pentobarbital (Euthanyl, MTC Laboratories, Cambridge, Ontario, Canada).

Measurements of Plasma Hormone Concentrations

Fetal plasma immunoreactive ACTH and cortisol concentrations were determined as described by Norman et al. *(37)*. CBC was measured in fetal plasma using a saturation assay procedure also described and validated previously *(26)*.

RNA Extraction and Northern Blot Analysis

Total RNA from placenta and fetal liver was extracted, electrophoresed, and transferred to a Zeta-Probe blotting membrane (Bio-Rad Canada, Ltd., Mississauga, Ontario, Canada) as described previously *(30)*. The membranes were probed using a ^{32}P-labeled ovine CBG, 11β HSD-1 or 11β HSD-2 cDNA probe, and then exposed to autoradiographic film (XAR-5, Eastman Kodak Co., Rochester, NY) using a Cronex Hi-Plus intensifying screen (DuPont, Wilmington, DE). To correct for differences in RNA loading and transfer, the blots were stripped and probed with a ^{32}P-labeled cDNA for mouse 18S ribosomal RNA (rRNA). The data were analyzed using computerized image analysis (Imaging Research Inc., St. Catherines, Ontario, Canada) using autoradiographic signals that were all within the linear range of the autoradiographic film. Results were expressed as the optical densities of signals obtained using specific cDNAs relative to the signals for 18S rRNA.

In situ *Hybridization*

Frozen fetal pituitary glands were cut in coronal sections (12 μm) on a cryostat (Jung CM3000, Leica Instruments GmbH, Nussloch, Germany), freeze-thaw mounted onto poly-L-lysine- (Sigma Chemical, St. Louis, MO) coated slides, and air-dried. Slides were then postfixed in 4% paraformaldehyde (pH 7.4, 4°C; 5 min), rinsed twice in phosphate-buffered saline (pH 7.4, 1 min), dehydrated in an ascending ethanol series, and stored in 95% ethanol at 4°C until analysis by *in situ* hybridization.

The *in situ* hybridization technique used has been described in detail previously *(38,39)*. Briefly the sections were hybridized overnight in a moist chamber at 42°C with a radiolabeled synthetic 45-mer oligonucleotide complementary to bases 711–756 of the porcine POMC gene *(40)* that has previously been characterized *(18)*. The oligonucleotide was labeled using terminal deoxynucleotidyl transferase and [α-^{35}S]dATP. After hybridization, the sections were washed and exposed to autoradiographic film (Biomax, Eastman Kodak Co., Rochester, NY). The autoradiographic films were developed using standard procedures.

Data Analysis

Results are presented as the mean ± S.E.M. for the number of observations indicated. The effects of treatment on plasma hormone concentrations were determined by two-way analysis of variance (ANOVA) corrected for repeated measures, and Student-Newman-Keuls multiple-range tests were used to assess the effects of individual times of treatment. Relative optical density determinations were analyzed by the Wilcoxon Rank Sum Test. Statistical significance was set at $p < 0.05$. Calculations were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA).

Acknowledgment

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