Long-term hypoxia represses the expression of key genes regulating cortisol biosynthesis in the near-term ovine fetus

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Myers, Dean A., Kimberly Hyatt, Malgorzata Mlynarczyk, Ian M. Bird, and Charles A. Ducsay. Long-term hypoxia represses the expression of key genes regulating cortisol biosynthesis in the near-term ovine fetus. Am J Physiol Regul Integr Comp Physiol 289: R1707–R1714, 2005.—Basal plasma ACTH1–39 concentrations are elevated in long-term hypoxic (LTH) fetal sheep. This study was designed to determine whether the expression of genes regulating cortisol biosynthesis was altered after LTH. Pregnant ewes were maintained at high altitude (3,820 m) from day 30 of gestation to near term, when the animals were transported to the laboratory. Reduced PO2 was maintained by nitrogen infusion through a maternal tracheal catheter. On days 137–141, fetal adrenal glands were collected from LTH and normoxic control fetuses. Real-time PCR was used to quantify mRNA for steroidogenic acute regulatory protein, 17α-hydroxylase (CYP17), 21-hydroxylase (CYP21), cholesterol side-chain cleavage (CYP11A1), 3β-hydroxysteroid dehydrogenase type II (HSD3B2), and the ACTH receptor. We analyzed mRNA by slot-blot hybridization and also quantified mRNA for transcription factors necessary for adrenocortical development by quantitative real-time PCR: steroidogenic factor 1 and dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X chromosome (DAX-1). Protein was quantified by Western blot analysis. Adrenal mRNAs for CYP17, CYP11A1, and the ACTH receptor were significantly reduced in LTH fetal sheep compared with levels shown in controls. Similarly, CYP11A1 protein and CYP17 protein were reduced in the LTH group. CYP21, steroidogenic acute regulatory protein, HSD3B2, steroidogenic factor 1, and DAX-1 expressions were not altered in response to LTH. We conclude that expression of two key steroidogenic enzymes (CYP17, CYP11A1) regulating cortisol biosynthesis and the ACTH receptor is lower in response to LTH. This likely represents an adaptive response to LTH, to prevent excessive cortisol production that would restrict fetal growth and potentially induce preterm delivery.

CYP17; CYP11A1; steroidogenic acute regulatory protein

Fetal plasma cortisol concentrations rise exponentially during the final 3 wk of gestation in sheep (term pregnancy is ~146 days in sheep). This increase in fetal plasma cortisol induces organ maturation essential for neonatal survival and, in sheep, has an additional role of triggering the onset of labor and delivery (18, 19). Thus regulation of adrenocortical glucocorticoid production is critical for both the timing of birth and neonatal survival in this species. Two key steroidogenic enzymes regulating cortisol biosynthesis [17α-hydroxylase (CYP17) and cholesterol side chain cleavage (CYP11A1)] undergo a hypothalamic-pituitary-dependent increase in expression in the adrenal cortex of fetal sheep approximately paralleling plasma cortisol as term gestation approaches (26, 27, 34). During late gestation, in addition to the increase in basal plasma cortisol, there is also increased cortisol production in response to stress in fetal sheep, reflecting the maturation of the adrenal cortex, as well as the hypothalamic and anterior pituitary components of the fetal hypothalamic-pituitary-adrenocortical (HPA) stress axis (31). Like that shown in the adult, the capacity to produce cortisol in response to stress is paramount in the ability of the late-gestation fetus to survive physiological stressors.

Hypoxia is a potent fetal stressor that increases both fetal plasma ACTH and cortisol (2, 6). Various methods have been used to induce experimental hypoxia in the sheep fetus, with the duration of hypoxia ranging from acute [minutes to hours (2)] to prolonged [several hours to several days (11)] to chronic [a few weeks (23)]. In addition to the duration, the severity of the experimentally induced hypoxia has varied greatly among experimental paradigms. Our laboratory has developed a model of long-term hypoxia (LTH) in which pregnant ewes are maintained at high altitude (3,820 m), resulting in a sustained, moderate hypoxic state from day 30 of gestation to term (1, 14, 15).

ACTH has been demonstrated as the major regulator of acute cortisol production; it also regulates the expression of key genes essential for cortisol biosynthesis in both adult and fetal adrenals (8, 10, 34, 37, 38). Our group recently reported that basal plasma ACTH1–39 concentrations are elevated in the LTH fetus (24) and that LTH fetal sheep exhibit enhanced cortisol production in response to a secondary stressor (1, 15). These findings indicated that the adrenal cortex of LTH fetal sheep responds to the increased circulating ACTH1–39 with enhanced expression of genes regulating cortisol biosynthesis. The purpose of the present study was to determine the effect of LTH on expression of genes involved in the production of cortisol in the adrenal gland. Specifically, we examined the expression of the steroidogenic enzymes CYP17, CYP11A1, CYP21, and 3β-hydroxysteroid dehydrogenase (HSD3B2), known to be regulated by ACTH in the late-gestation sheep fetus. In addition, we examined the expression of steroidogenic acute regulatory protein (STAR), which mediates cholesterol transport to the mitochondrial inner membrane, and expression of the ACTH receptor, which measures the adrenal capacity to respond to this important regulatory peptide. We also determined the expression of two transcription factors fundamental to the regulation of steroidogenic gene expression in the adrenals. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
for the development and differentiation of the adrenal cortex: steroidogenic factor 1 (SF-1) and the dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X chromosome (DAX-1) gene.

**METHODS**

**Animals.** All studies were conducted with the approval of the Institutional Animal Care and Use Committees at either Loma Linda University School of Medicine or the University of Oklahoma Health Sciences Center. Fetal sheep were obtained from pregnant ewes maintained at sea level (~300 m, arterial Po2 = 102 ± 2 Torr) or at high altitude (Barcroft Laboratory, White Mountain Research Station, Bishop, CA, altitude 3,820 m) for ~110 days beginning on day 30 of gestation (term = 146 days). Animals from the latter group were then transported to Loma Linda Medical Center at which time animals received a nonocclusive maternal tracheal catheter. Reduced Po2 was maintained at a level comparable to that observed at altitude (50 Torr) by nitrogen infusion. On days 137–141 of gestation, the ewes were sedated with pentobarbital sodium, intubated, and maintained under general anesthesia with 1.5–2% halothane in oxygen while the fetuses were delivered through a midline laparotomy. Adrenal glands were rapidly collected, frozen in liquid nitrogen, and stored at −80°C until analysis. Adrenal glands were also obtained from additional normoxic control fetuses (n = 20) between 100 and 145 days of gestation for determination of the normal ontogeny of adrenal steroidogenic enzyme expression.

**Quantitative real-time PCR analysis.** Quantitative real-time (qRT) PCR was used to quantify the mRNA for CYP17, CYP11A1, CYP21, HSD3B2, ACTH receptor, SF-1, DAX-1, and cyclophilin. Real-time PCR was performed using cDNA generated from the first-strand synthesis reaction. All PCRs were performed in triplicate. Initial qRT-PCRs were performed with serial dilutions ranging from 250 to 15.625 ng (250, 125, 62.5, 31.25, and 15.625 ng) to determine the linear range of amplification for each primer set. For each mRNA, a starting amount of the cDNA reaction (based on quantity of the input RNA) was chosen within the linear amplification range. For CYP11A1, CYP17, CYP21, ACTH receptor, SF-1, DAX-1, and cyclophilin, 100 ng/PCR reaction was used. For each primer set, the amplicon was subcloned into the TA cloning vector (Invitrogen) and subjected to Sanger dideoxysequencing (Oklahoma Medical Research Foundation Sequencing Core, Oklahoma City, OK) to confirm the identity of the amplicon as the correct product. For the qRT-PCR, Sybr green (1× Sybr green master mix; Bio-Rad, Hercules, CA) was utilized as the fluorophore, and real-time PCR was performed utilizing a Bio-Rad iCycler equipped with the real-time optical fluorescent detection system. The primer sequences used are listed in Table 1; the primers were derived from cDNA sequences available at the National Center for Biotechnology Information (ovine CYP17: AF251388; bovine CYP21: M11267; ovine CYP11A1: D50057; bovine HSD3B2: NM_174343; ovine StAR: AF290202; ovine ACTH receptor: NM_001009442; ovine SF-1: AF299081; bovine DAX-1: AF421373; bovine GAPDH: U85042; bovine cyclophilin B: BT020966). A three-step PCR was used: an initial denaturation step of 95°C for 10 min to activate the hot-start Taq DNA polymerase, followed by sequential cycles consisting of denaturation at 95°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. A total of 35 PCR cycles were performed. A melt-curve analysis was conducted on each sample after the final cycle to ensure that a single product was attained, and agarose gel electrophoresis confirmed that a single PCR product was of the predicted length. qRT-PCR was performed for each sample (in triplicate) for cyclophilin as a control mRNA using the identical first-strand cDNA used for quantification of mRNA for the gene of interest and in the same PCR run as for the gene of interest to circumvent any between-run variation. Cyclophilin was used as a “housekeeping” mRNA, since we previously found that cyclophilin mRNA is not glucocorticoid responsive and does not change in expression in adrenal cells in vitro in response to a variety of stimuli, including ACTH. We also analyzed GAPDH as a control mRNA for each fetal adrenal. For quantification purposes, a synthetic, double-stranded

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Fw, forward; Rv, reverse; CYCLO, cyclophilin; CYP17, 17α-hydroxylase; CYP21, 21-hydroxylase; CYP11A1, cholesterol side-chain cleavage; SF-1, steroidogenic factor 1; StAR, steroidogenic acute regulatory protein; NCBI, National Center for Biotechnology Information.
DNA standard was used to generate a standard curve for extrapolation of starting cDNA concentrations per reaction using the Ct (threshold at which the fluorescence of each PCR reaction increased above baseline) values for standards to create a linear standard curve (100, 10, 1, 0.1, 0.01, and 0.001 pg of standard cDNA). The standards were run in duplicate along with the unknowns in the same PCR block. Extrapolation of unknowns from the standard curve was performed using Prism 3 (GraphPad Software, San Diego, CA), predicting unknowns from the standard curve Ct values.

Slot-blot analysis of mRNA. Adrenals were homogenized using a Polytron (Powergen 700, Fisher Scientific) at 4°C into RNazol B solution (Cinna Biotec, Houston, TX) before transfer in 1-ml volumes to microcentrifuge tubes. Phase separation was achieved by mixing with 0.15 ml CHCl₃, incubation at 4°C for 5 min, and centrifugation (12,000 g; 20 min, 4°C). The recovered upper phase (0.6 ml) was extracted twice with phenol-chloroform-isooamyl alcohol in the presence of heavy-grade phase lock gel (5 Prime, Bolder, CO). Total RNA was then precipitated by the addition of isopropanol (1 h, −20°C), recovered by centrifugation (30 min, 12,000 g, 4°C) and washed in 75% ethanol (1.0 ml) before it was dissolved in molecular biology-grade water (0.1 ml). Recovery and purity were determined by absorbance at 260 and 280 nm, and samples were stored at −70°C before analyses.

Samples of total RNA (10 μg) were diluted to 100-μl volumes before precipitation with 10 μl of 3 M sodium acetate (pH 5.2) and 500 μl of ethanol. After recovery by centrifugation, RNA was dissolved in 100 μl of TE buffer (pH 7.5), denatured in the presence of 40 μl of formaldehyde and 70 μl of 20× sodium chloride-sodium citrate (SSC) for 15 min at 65°C, and finally applied directly to Magna NT membrane under 1 psi vacuum using a slot blower (Bio-Rad). Sample wells were rinsed with 500 μl of 20× SSC to ensure efficient loading. Membranes were then removed, cross-linked, and hybridized as follows. Prehybridization was carried out at 42°C overnight in a final buffer composition of 50% formamide, 5× SSC, 1× PE, and 50 mg/ml transfer RNA [20× SSC contains 3.0 M NaCl and 0.3 M trisodium citrate, pH 7.0. 5× PE contains 500 mM Tris·HCl (pH 7.5), 0.5% sodium pyrophosphate, 5% SDS, 1% polyvinylpyrrolidone, 1% Ficoll, 25 mM EDTA, and 1% BSA]. Hybridizations were performed sequentially in the same buffer at 42°C for 16–24 h using antisense probes of at least 500 base lengths to protein coding sequences of human ACTH receptor, mouse StAR, and human CYP11A1, CYP17, CYP21, and HSD3B2 exactly as described previously (4, 5, 21, 22). Each antisense probe was labeled with 32P by asymmetric PCR in the presence of [32P]dCTP (Amersham, Arlington Heights, IL). The blots were then washed in 2× SSC containing 0.1% SDS at room temperature for 30 min and in 0.1× SSC containing 0.1% SDS at room temperature for 2 × 60 min before direct radioimaging quantification of bound probe (Bio-Rad model GS-250 Phosphorimager, BI Screen; 4–24 h) and subsequent exposure to film (Hyperfilm, Amersham; 1–4 days). Blots were subsequently stripped by repeated washing in 0.1× SSC-0.5% SDS (65°C, 1 h) and checked for lack of radioactivity before reprobing. Finally, all blots were probed for GAPDH mRNA using an antisense probe generated by asymmetric PCR against bases 39–900 of the human cDNA, and bound probe was quantified as above. Binding of GAPDH probe per lane was then used to normalize data against minor variations in lane loading.

Western blot analysis. Western blot analysis was performed similarly to what has been described for our laboratory (24). Adrenal glands were homogenized (4°C, 1 ml, 0.1 M acetic acid, 100 mM

Fig. 1. Concentrations of CYP17 and CYP11A1 (A), HSD3B2 and CYP21 (B), and GAPDH and cyclophilin (C) mRNA expressed as pg mRNA/100 ng cDNA (RNA) from the first-strand synthesis reaction. Quantitative real-time PCR was used to determine the amount of mRNA for each gene of interest as described in METHODS. All values represent means ± SE for control (CONT; n = 6) and LTH (n = 5) animals.
sodium chloride, pH 5.0, containing 1 mM peptatin, 0.4 mM pefablock, 1 µg/ml leupeptin) and centrifuged at 12,000 g for 2 min, and the supernatant was collected. Protein concentrations were determined by the Bio-Rad method. Samples (50 µg of protein per adrenal gland) were electrophoresed by SDS-PAGE (4–10%; Invitrogen), and the proteins were subsequently electrophoretically transferred to nitrocellulose membranes and subjected to Western blot analysis. The membranes were blocked for 1 h with TBS (10 mM Tris·HCl, pH 7.2, 100 mM sodium chloride, pH 5.0, containing 0.1% Tween 20 (TTBS) and 10% nonfat dry milk. Membranes were then washed twice in TTBS and incubated with primary antibodies (anti-ovine HSD3B2 from Dr. Mason; anti-bovine CYP17 generously supplied by Dr. Alan Conley, University of California, Davis, CA; anti-bovine CYP11A1 from Chemicon, Temecula, CA; anti-human DAX-1 from Santa Cruz; anti-human SF-1 generously supplied by Dr. Ken-ichiro Morohoshi, National Institute for Basic Biology, Okazaki, Japan; and affinity-purified anti-rat StAR from Abcam, Cambridge, MA) for varying lengths of time. For controls, we omitted of the primary antiserum and used a nonexpressing tissue, such as liver or muscle. Films were quantified by densitometry. To ensure that equal protein amounts were loaded for each sample, identical SDS-PAGE gels were performed with the same protein samples from control and LTH adrenal glands and were subsequently stained with Coomassie blue.

Statistical analysis. Differences between normoxic control and LTH fetuses in mRNA and protein for each gene of interest were compared using Student’s t-test. All results were expressed as means ± SE.

RESULTS

qRT-PCR analysis. As determined by qRT-PCR, concentrations of mRNA for CYP17 (P < 0.001) and CYP11A1 (P < 0.01) were significantly lower in LTH fetal adrenal glands compared with normoxic controls (Fig. 1A). The concentrations of mRNA for CYP21 and HSD3B2 (Fig. 1B), as well as cyclophilin and GAPDH (Fig. 1C) were not different between LTH and control fetal adrenal glands. Figure 2 illustrates the normal ontogeny of CYP11A1, CYP17, and HSD3B2 mRNA in the ovine fetal adrenal gland, as determined by qRT-PCR. Messenger RNA levels for all three enzymes in the adrenal glands from the normoxic control fetal sheep were at the expected gestational age on the curves. In marked contrast, mean values for both CYP17 and CYP11A1 from the LTH adrenals were delayed by ~10 days based on curve fitting. HSD3B2 mRNA values were within the normal developmental range for both LTH and control fetal adrenal glands. Cyclophilin values did not change across gestation. Similar to CYP17 and CYP11A1, concentrations of mRNA for the ACTH receptor were lower (P < 0.05) in the adrenal glands of LTH fetal sheep (Fig. 3A). However, concentrations of StAR (Fig. 3B) and SF1 and DAX-1 (Fig. 3C) mRNA were not different in the adrenal glands of LTH vs. normoxic controls.

Slot-blot analysis of mRNA. All individual hybridization signals were corrected for GAPDH as described in Methods. As determined by slot-blot analysis, HSD3B2 expression did not differ between the groups. Levels of other mRNAs of interest are expressed as the ratio of the gene of interest to HSD3B2 (Table 2). Using this methodology, CYP17 and ACTH receptor mRNA were significantly lower in the adrenal glands of LTH compared with control fetal sheep. There was also a trend (P = 0.06) for CYP11A1 being decreased compared with control. CYP21 and StAR mRNA were not different between LTH and control fetal adrenal glands. With the exception of CYP11A1, the results obtained via slot-blot analysis confirmed those obtained using qRT-PCR and Western blot analysis (see below). The reason for the discrepancy between the techniques used for mRNA quantification for CYP11A1 is unclear but may reflect variability introduced using ratio of CYP11A1 to HSD3B2.

Western blot analysis. CYP11A1 and CYP17 protein levels were significantly lower in the adrenal glands of LTH compared with normoxic control fetal sheep (Fig. 4A), whereas
values for HSD3B2 did not differ (Fig. 4B). For StAR, levels of the 36-kDa precursor and the 32-kDa mature form were not different between LTH and control fetuses, whereas the 30-kDa fully processed and inactive form was greater (P < 0.05) in the adrenal cortex of LTH fetal sheep (Fig. 5A). SF-1 and DAX-1 (Fig. 5B) were not different between LTH and normoxic control fetal sheep. These results are in agreement with the mRNA levels obtained via qRT-PCR.

**DISCUSSION**

In the present study, we report that expression of CYP11A1 and CYP17 were significantly lower at 137–141 days of gestation in the adrenal cortex of fetal sheep that developed under conditions of LTH. Similarly, mRNA for the ACTH receptor was also lower. These findings suggest a diminished capacity of the LTH fetal adrenal to synthesize cortisol (lower CYP11A1 and CYP17) coupled with a decreased responsiveness to ACTH (decreased ACTH receptor expression). The decreased expressions of CYP11A1, CYP17, and the ACTH receptor were somewhat unexpected on the basis of our previous demonstration that basal plasma ACTH_{1-39} concentrations are nearly threefold higher in LTH fetus compared with control during late gestation (24), whereas basal plasma cortisol concentrations are not different (1, 15). The maintenance of basal plasma cortisol concentrations in the LTH fetus appears to reflect a balance between changes observed in expression of the key steroidogenic genes in the adrenal and the higher concentrations of fetal plasma ACTH_{1-39} (24). The diminished expression of the ACTH receptor would provide an effective counter to the elevated plasma ACTH_{1-39} and as such, a possible insight into the decreased expression of CYP11A1 and CYP17 in these fetuses. Albeit lower, the level of expression of these enzymes must be sufficient for maintaining basal cortisol production in the LTH fetus. The increase observed in the mature, inactive (30 kDa) form of StAR in the LTH fetal adrenal is indicative of enhanced translocation of cholesterol to the inner mitochondrial membrane. An increased availability of cholesterol may compensate for the decreased expression of CYP11A1, the first essential limiting step in cortisol synthesis. However, StAR transport of cholesterol is also regulated by phosphorylation, and at present, we do not know whether the phosphorylation state of StAR is altered in the adrenal glands of LTH fetal sheep. Of interest, it has been reported that elevation of ACTH in fetal sheep is accompanied by an increase in the 30-kDa form of StAR and a decrease in mRNA for this gene (40).

The present findings of the decreased expression of ACTH receptor, coupled with the lower expression of CYP11A1 and CYP17, are also consistent with the diminished capacity of LTH fetal sheep to produce cortisol in response to exogenous ACTH (14). Paradoxically, LTH fetal sheep demonstrate higher plasma concentrations of cortisol compared with normoxic controls in response to a secondary stressor (1, 15). Considering the changes observed in the present study in expression of key genes regulating cortisol synthesis, the higher levels of cortisol in response to the secondary stress is seemingly difficult to reconcile. However, there are other experimental situations where ACTH and adrenal cortisol production are not well correlated (16). In near-term fetal sheep, sinoaortic denervation (9) or splanchnic nerve section (20, 25) altered the cortisol response to acute hypoxia or hypotension without affecting the increase in ACTH. Thus, in the LTH sheep fetus, in addition to increased release of ACTH_{1-39}, other mechanisms may compensate for the decreased responsiveness.
of the adrenal gland to ACTH1–39, resulting in an enhanced cortisol production in response to stress.

In vivo paradigms designed to disrupt basal release of ACTH have also suggested a less clear role for ACTH in the regulation of adrenocortical glucocorticoid synthesis in the ovine fetus. For instance, hypophysectomy, which removes the entire anterior pituitary, reduces adrenocortical expression of CYP11A1, CYP17, and HSD3B2 in fetal sheep but has no effect on the expression of the ACTH receptor (36). Likewise, hypothalamo-pituitary disconnection, which severs all hypothalamic connections in both the anterior and neurointermedi-

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HSD3B2 mRNA was not different between control and long-term hypoxic (LTH) fetal adrenal glands [control = 24,330 ± 1,720 integrated densitometric units (IDU) vs. LTH: 32,690 ± 5,369 IDU] as determined from Northern analysis.

Fig. 4. Western blot analysis for CYP11A1 and CYP17 (A) and HSD3B2 (B) as described in METHODS. Top: representative lanes from control and LTH fetuses. Bottom: densitometric analyses of the Western blots. Data are expressed in densitometric arbitrary units (DU); n = 5 animals for each group. All values represent means ± SE.

Fig. 5. Western blot analysis for StAR (A) and SF-1 and DAX-1 (B) as described in METHODS. Top: representative lanes from control and LTH fetuses. Bottom: densitometric analysis of the Western blots. Data are expressed in DU; n = 5 animals for each group. All values represent means ± SE.
ate lobes of the pituitary, decreases the expression of CYP11A1, CYP17, and HSD3B2 (29).

We have also previously shown that a lesion of the fetal hypothalamic paraventricular nucleus (PVN), the source of the major neuropeptides corticotropin-releasing hormone (CRH) and AVP, regulation of anterior pituitary ACTH release, or stereotaxic placement of dexamethasone at the PVN, which selectively suppresses CRH and AVP expression and release, prevents the late-gestation emergence of expression of CYP11A1 and CYP17 (26, 27) but not HSD3B2 (26). PVN lesion effectively reduces circulating ACTH1–39 to below the limit of detection (3, 26). The differences observed between these studies on adrenocortical expression of these key genes involved in glucocorticoid production point toward factors in addition to ACTH that regulate cortisol biosynthesis in the late-gestation ovine fetus. The results of the present study reinforce this concept.

An intriguing question that remains to be answered is the mechanism by which expression of this battery of genes essential for cortisol biosynthesis is suppressed in the face of elevated ACTH1–39 in the LTH fetus. In addition to plasma ACTH1–39 concentrations, we also previously reported that LTH fetal sheep have significantly elevated ACTH precursors, proopiomelanocortin, and 22-kDa proACTH (24). These precursors, which circulate in the fetus at a 5- to 10-fold excess compared with ACTH, have been previously reported in fetal sheep (32), rhesus monkey (30), and rats (31) to attenuate the actions of ACTH on cortisol output in vitro. Thus the increase in ACTH precursors may negate the effect of elevated ACTH1–39 at the level of the fetal adrenal. The finding that inhibitory actions of these precursors are observed in ovine fetal but not adult adrenocortical cells (32) implies that the capacity to respond to the ACTH precursors is regulated, and thus it is possible that the LTH fetus may be more sensitive to the inhibitory actions of these precursors.

Another possibility, based on the lower level of expression of CYP11A1 and CYP17, is that LTH may have delayed the maturation of the adrenal cortex. Interestingly, when the values for the LTH fetuses were fit to the developmental gestational age curve for these genes, they were placed −7–10 days behind the normoxic controls, suggestive of delayed maturation. However, expression of SF-1 and DAX-1, orphan nuclear receptors essential in the differentiation of the adrenal cortex (34, 39) and integral in regulating expression of CYP11A1, CYP17, StAR, and the ACTH receptor, was not different between the LTH and normoxic control fetuses. Although SF-1 is fundamental for tissue-specific expression and ACTH-stimulated expression of these genes, DAX-1 has been shown to inhibit certain steroidogenic genes (17). Indeed, despite maintenance of expression of SF-1, expression levels of genes known to be specifically regulated by SF-1, including CYP11A1, CYP17, and the ACTH receptor, were lower. It is noteworthy that expression of HSD3B2, a steroidogenic gene not known to be regulated by SF-1, was maintained. SF-1 has been observed to decrease in response to hypophysectomy in the late-gestation ovine fetus (36). However, ACTH replacement to the hypophysectomized fetal sheep did not restore adrenal expression of SF-1 mRNA to control. Thus adrenocortical SF-1 may not be subject to ACTH regulation in the fetal sheep, a finding backed by the present study.

Because DAX-1 has been shown to inhibit SF-1-mediated expression of certain steroidogenic genes such as CYP17 (13), a possible explanation was that DAX-1 expression may have been elevated in response to LTH. However, like SF-1, there were no differences between LTH and control fetal sheep in expression of DAX-1. Recent studies have, however, indicated that SF-1 regulation of steroidogenesis may be mediated by the phosphorylation state of this orphan nuclear receptor (12, 33, 35). Indeed, it has been suggested that ACTH-stimulated dephosphorylation of SF-1 is needed for ACTH-mediated expression of CYP17 in adrenocortical cells (33, 35). This implies that, in the LTH fetal adrenals, factors other than ACTH play a major role in regulating the ACTH signal via modulation of SF-1 activity.

Together with our previous studies, data from the present study demonstrate that the HPA axis of fetal sheep undergoes significant adaptations in response to development under conditions of LTH. These changes include increased circulating basal ACTH1–39 and a heightened ACTH1–39 release in response to secondary stressors. Contrary to this apparent activation of the hypothalamic-anterior-pituitary arm of the HPA axis, the present study demonstrates that the adrenal cortex of the LTH fetus has adapted quite differently, with a reduced capacity to respond to ACTH at both the plasma membrane level (ACTH receptor) and at the level of steroidogenesis (decreases in key steroidogenic genes). However, this divergent adaptation may serve to limit cortisol production in the basal state yet allow the increased production of cortisol when needed during situations of acute, secondary stress. Clearly, future studies are needed to focus on the mechanisms invoked by the LTH fetus leading to this adaptive state.

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