Expression of StAR and Key Genes Regulating Cortisol Biosynthesis in Near Term Ovine Fetal Adrenocortical Cells: Effects of Long-Term Hypoxia

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Reproductive Sciences
2018, Vol. 25(2) 230-238
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sagepub.com/journalsPermissions.nav
DOI: 10.1177/1933719117707056
journals.sagepub.com/home/rsx

Abstract

We previously demonstrated decreased expression of key genes regulating cortisol biosynthesis in long-term hypoxic (LTH) sheep fetal adrenals compared to controls. We also showed that inhibition of the extracellular signal-regulated kinases (ERKs) with the mitogen-activated protein kinase (MEK)/ERK inhibitor UO126 limited adrenocorticotrophic (ACTH)-induced cortisol production in ovine fetal adrenocortical cells (FACs), suggesting a role for ERKs in cortisol synthesis. This study was designed to determine whether the previously observed decrease in LTH cytochrome P45011A1/cytochrome P450c17 (CYP11A1/CYP17) in adrenal glands was maintained in vitro, and whether ACTH alone with or without UO126 treatment had altered the expression of CYP11A1, CYP17, and steroidogenic acute regulatory protein (StAR) in control versus LTH FACs. Ewes were maintained at high altitude (3820 m) from ~40 days of gestation (dG). At 138 to 141 dG, fetal adrenal glands were collected from LTH (n = 5) and age-matched normoxic controls (n = 6). Fetal adrenocortical cells were challenged with ACTH (10^{-8} M) with or without UO126 ($10 \mu\text{M}$) for 18 hours. Media samples were collected for cortisol analysis and messenger RNA (mRNA) for CYP11A1, CYP17, and StAR was quantified by quantitative real-time polymerase chain reaction. Cortisol was higher in the LTH versus control ($P < .05$). StAR mRNA was decreased in LTH versus control ($P < .05$). UO126 alone had no effect on mRNA in either group. UO126 prevented the increase in CYP11A1 and CYP17 in control FACs. Basal CYP11A1 and CYP17 were not different in LTH versus control. ACTH increased CYP11A1 and CYP17 only in control FACs ($P < .05$). UO126 attenuated the ACTH response indicative of a role for ERK in CYP11A1 and CYP17 expression. ACTH may require additional factors in FACs to fully regulate StAR expression.

Keywords

CYP11A1, CYP17, fetus, hypoxia, StAR

Introduction

The late gestation ontogenic increase in fetal plasma cortisol resulting from adrenocortical maturation is critical for optimal fetal organ tissue maturation and growth, and in sheep, plays an essential role in parturition.^{1,2} Therefore, the precision of maturation and regulation of cortisol biosynthesis in the fetal adrenal gland are crucial for proper fetal development and timing of parturition.

Cortisol is synthesized from cholesterol through a series of enzymatic events in the ovine adrenal gland. A number of studies have shown that of these enzymatic steps, the primary enzymes that are rate limiting in the maturation of cortisol biosynthesis in the ovine fetal adrenal are the initial cleavage of cholesterol to pregnenolone via cytochrome P45011A1 (CYP11A1; side chain cleavage) and the subsequent 17 hydroxylation step (cytochrome P450c17; CYP17; 17 α hydroxylase).³⁻¹⁰ In addition, a key step in steroidogenesis in steroid

producing cells is the transfer of cholesterol from the outer to the inner mitochondrial membrane via steroidogenic acute regulatory protein (StAR)¹¹⁻¹⁶; as such, although not an enzymatic step, delivery of cholesterol to CYP11A1 via StAR may be regarded as the first rate-limiting step in steroidogenesis.

Oxygen delivery from the maternal circulation to the fetus is critical to achieve proper fetal growth and development. It is well accepted that hypoxia represents a major threat to the

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developing fetus often accompanying various situations such as preeclampsia, intra-uterine growth restriction, smoking, and high altitude.¹⁷⁻¹⁹ Hypoxia is also well recognized as a potent activator of the hypothalamo-pituitary-adrenocortical (HPA) stress axis.²⁰⁻²⁵ From a clinical perspective, not only women that live at altitude but also women that smoke have heart/lung disease or are anemic during pregnancy expose the fetus to hypoxia.²⁶ Although studies have centered at understanding the effects of acute hypoxia on the fetal HPA axis,²⁷⁻²⁹ much needs to be learned in terms of the in utero effects of moderate gestational long-term hypoxia (LTH).

Our laboratory has developed an ovine model of high-altitude-induced LTH and found that development under conditions of LTH has profound effects on the fetal HPA axis at all levels: the hypothalamic paraventricular nucleus, anterior pituitary, and adrenal gland.^{21,30-32} Key studies from our laboratory have clearly shown that the normal ontogenic late gestation rise in fetal plasma cortisol is preserved in the LTH fetus, similar to that of normoxic fetuses^{32,33} despite elevated basal plasma adrenocorticotrophic (ACTH) concentrations.²¹ Interestingly, the expression of key steroidogenic genes CYP11A1 and CYP17 as well as the ACTH receptor is reduced in the LTH fetal adrenal cortex, but ACTH-induced cortisol biosynthesis is paradoxically enhanced.³⁰⁻³² We also found that this was not the result of increased cyclic adenosine monophosphate (cAMP) production and/or protein kinase A (PKA).³⁴

Although, the function and regulatory mechanisms of the extracellular signal-regulated kinases (ERKs) are well recognized,³⁵⁻³⁹ there are no data in the literature other than our own published studies⁴⁰ regarding ERK1/2 function and regulation in responses to LTH in fetal adrenocortical cells (FACs). Extracellular signal-regulated kinases have been suggested to play a regulatory role in ACTH-induced steroidogenesis because of their potential to phosphorylate and thus activate StAR. We found that StAR messenger RNA (mRNA) is not different in the adrenal cortex of the LTH fetus while levels of the 30 kD form of StAR protein are elevated.³² The 30 kD form of StAR is considered the so-called spent or mature inactive isoform of StAR, undergoing proteolytic processing during the course of cholesterol transport to the inner mitochondrial membrane.⁴¹ This would suggest a compensatory mechanism in the LTH fetal adrenal cortex leading to increased activation of StAR stabilizing cortisol production in the prepartum period despite lowered expression of CYP11A1 and CYP17. Studies by Ferreira et al³⁹ demonstrated a major role for ERK1/2 in chronic ACTH-induced steroidogenesis in the rat adrenal gland. Additionally, studies by Poderoso et al⁴² showed that ERK1/2 are implicated in phosphorylation of StAR. Thus, regulation of StAR activity by ERK would have significant impact on cortisol production. Recently, we showed that ERK1/2 inhibition with UO126 decreased cortisol production in response to ACTH treatment in ovine FACs from both control and LTH fetuses, and the net decrease was significantly greater in the LTH group compared to control, suggesting a role for ERKs in cortisol synthesis.⁴⁰

Understanding the role of the mechanisms regulating the LTH ovine fetal adrenal cortex will provide knowledge into

the cellular and molecular mechanisms governing cortisol biosynthesis in the near-term sheep fetus as well as fetal adaptations occurring during development. We designed the present study to examine whether the observed decrease in LTH CYP11A1 and CYP17 in adrenal glands as previously reported was maintained in vitro FACs, and whether ACTH and/or ERK1/2 inhibition altered the expression of CYP11A1, CYP17, and StAR in the LTH when compared to the normoxic control FACs.

Methods

Animal Procedures

All procedures were conducted with approval of the Institutional Animal Care and Use Committee (Loma Linda University School of Medicine, Loma Linda, California). Time-dated pregnant sheep were transported to Barcroft Laboratory, White Mountain Research Station, Bishop, California, at an elevation of 3820 m at ~40 days of gestation (dG), where they were maintained until near term (~138 to 141 dG; term is 146 dG), at which time they were transferred from the high-altitude facility to Loma Linda University. Upon arrival, nitrogen infusion through a maternal tracheal catheter was used to maintain maternal arterial PO₂ levels similar to that observed at high altitude (~60 mm Hg) as previously described.^{30,31} At 138-141 dG, both LTH and normoxic control ewes were sedated with pentobarbital sodium, intubated, and maintained under general anesthesia with 1.5% to 2% halothane in oxygen. Fetuses were then delivered through a midline laparotomy, and the fetal adrenal glands were collected from LTH and normoxic control animals in ice-cold media M-199 (Sigma-Aldrich, St. Louis, Missouri), containing 2.2 g sodium bicarbonate (Sigma-Aldrich), 2.0 g bovine serum albumin (Sigma-Aldrich), and 0.1 g L-Glutamine (Sigma-Aldrich) as we have previously described.³⁴

Adrenocortical Cell Dispersion

The adrenal cortex was separated from the medulla, enzymatically dispersed with 40 mg collagenase type II (Worthington Biomedical, Lakewood, New Jersey), 40 mg of Polypep bovine protein digest (Sigma-Aldrich), and 100 μ L of DNase I (type IV; Sigma-Aldrich) dissolved in 10 mL of sodium Krebs buffer (0.4% collagenase). The resulting monodispersed FACs were aliquoted in duplicate (2.5×10^5 cells) into individual tubes with media M-199 and allowed to equilibrate for 2 hours prior to initiation of each study. Cell viability was confirmed by Trypan blue (Sigma-Aldrich) exclusion. All experiments were conducted on FACs at 37°C. All procedures were performed by methods which we have previously described and validated.³⁴

Experimental Protocol

Dispersed FACs were challenged with ACTH (10^{-8} M) with or without UO126 (Calbiochem, Billerica, Massachusetts) for 18 hours (250 000 cells/tube). The effect of ERK inhibition on the expression of key genes was studied in an additional set of

FACs pretreated 1 hour with UO126 (10 μ M) before ACTH stimulation for 18 hours. Media were collected at 0, 1, 2, 3 and 18 hours after the start of ACTH treatment for cortisol analysis and snap frozen in liquid nitrogen. At the end of the study, FACs in lysis buffer (RNA Later, Cat # AM7030, Ambion, Austin, TX, USA) were snap frozen in liquid nitrogen, and all samples were stored at -80°C until analysis.

Cortisol Assay

Cortisol was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) cortisol kit (Oxford Biomedical Research, Oxford, Michigan) that has been previously described and validated for use in our laboratory.^{33,43}

Quantitative Real-Time Polymerase Chain Reaction Analysis of CYP11A1, CYP17, and StAR mRNA Expression in Ovine FACs

Following isolation of total RNA from FACs (Cat. # AM1921, Ambion), the RNA was treated with DNase I (1 unit) at 37°C for 30 minutes, and DNase was removed via polymerase chain reaction (PCR) purification columns. Reverse transcription was performed using 500 ng of total input RNA per sample using oligo (dT₂₁) as the primer, and I-script (Bio-Rad, Hercules, California) as the reverse transcriptase in a reaction mixture of 20 μ L. The details of the quantitative real-time PCR have been previously described for our laboratory in detail.³² Real-time PCR was performed using complementary DNA (cDNA) generated from the first-strand synthesis reaction. All PCR reactions were performed in triplicate. For the qRT-PCR, SYBR Green (1X SYBR green master mix, Bio-Rad) was used as the fluorophore, and RT-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. A 3-step PCR was used: an initial denaturation step of 95°C for 10 minutes was performed to activate the hot-start Taq DNA polymerase followed by sequential cycles consisting of denaturation at 95°C for 45 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds. Cyclophilin was used as a “housekeeping” mRNA as we have previously validated³²; a total of 35 PCR cycles were performed and a melt curve analysis was conducted on each sample after the final cycle to assure that a single product was attained. For quantification, a synthetic single-stranded DNA standard was used to generate a standard curve for extrapolation of starting cDNA concentrations per reaction. Each standard point was run in duplicate and in the same PCR block as the unknowns as previously described.³² Extrapolation of unknowns from the standard curve was performed using Prism 3 (graph Pad Software INC, San Diego, California) predicting unknowns from the standard curve Ct values.

Table 1. Forward (Fw) and Reverse (Rv) Primer Sequences Used for Quantitative Real-Time PCR.

Gene	Primer Sequence	NCBI
CYP11A1		
Fw	5'-GGAGGATGTCAAGGCCAATA-3'	D50057
Rv	5'-TCTTGCTTATGTGCGCCTCT-3'	
CYP17		
Fw	5'-CATCAGAGAAGTGCTCCGAATCC-3'	AF251388
Rv	5'-TCCTGCTCCAAAGGGCAAGTAG-3'	
StAR		
Fw	5'-CAGAAGATTGGAAGACACGGTC-3'	AF290202
Rv	5'-AGGTGAGTTTGGTCTTGAGGG-3'	
CYCLO		
Fw	5'-CCATCGTGTGTCAAGGACTTCAT-3'	BT020966
Rv	5'-CTTGCCATCTAGCCAGGGTCTT-3'	

Abbreviations: CYCLO, cyclophilin; CYP11A1, cytochrome P45011A1; CYP17, 17 α -hydroxylase; Fw, forward; Rv, reverse; NCBI, National Center for Biotechnology Information; PCR, quantitative real-time polymerase chain reaction; StAR, steroidogenic acute regulatory protein

Statistical Analysis

Differences between LTH and normoxic control FACs were compared using analysis of variance with Bonferroni post hoc tests used where appropriate, and $P < .05$ was considered significant. All data are presented as means $\pm$ standard error of mean (SEM). Cortisol data are expressed as mean ng/mL/250 000 cells $\pm$ SEM.

Results

Cortisol Responses to ACTH

Basal cortisol secretion (Figure 1A) was greater in the LTH FACs when compared to the normoxic control group over the course of 18 hours in vitro ($P < .05$). In response to ACTH, there was a significant increase in cortisol biosynthesis in both control and LTH FACs (Figure 1B). However, in response to ACTH, cortisol production in the LTH FACs was significantly greater compared to control FACs (time and treatment effect, $P < .05$, at 2 hours [9.22 ± 1.52 vs 2.26 ± 0.88], 3 hours [13.78 ± 2.80 vs 2.77 ± 1.14], and 18 hours [20.08 ± 5.10 vs 5.73 ± 1.72], respectively).

Effect of UO126 on ACTH-Stimulated Cortisol Production

Pretreatment with UO126 significantly inhibited ACTH-stimulated cortisol production (time and treatment effect, $P < .05$) in both groups (Figure 1C) compared to ACTH treatment alone (Figure 1B). In the control FACs, UO126 pretreatment completely blocked cortisol production. UO126 treatment also reduced cortisol production in the LTH FACs compared to ACTH alone; however, there was still a significant increase in cortisol over time at 2, 3, and 18 hours following ACTH treatment.

Figure 1. Cortisol production in LTH and control FACS. Under basal conditions, (A) cortisol secretion was significantly higher in the LTH FACS ($n = 5$) when compared with control ($n = 6$) group ($P < .05$). ACTH (10 nM) stimulated cortisol biosynthesis (B) in both LTH and control group. However, the cortisol response was significantly greater in the LTH ($n = 5$) group compared with control ($n = 6$; $*P < .05$). Pretreatment with UO126 significantly inhibited ACTH-stimulated cortisol secretion in both groups (C) compared to ACTH alone ($P < .05$). FACS indicates fetal adrenocortical cells; LTH, long-term hypoxic.

Quantitative Real-Time PCR Analysis of mRNA Expression in Response to ACTH and UO126 Treatment

CYP11A1. There were no differences under basal conditions for CYP11A1 mRNA between control and LTH FACS after 18 hours in culture (Figure 2A). After 18 hours of ACTH treatment, CYP11A1 mRNA was significantly increased compared to basal ($P < .05$) in control (Figure 2A) but not in the LTH FACS. Although lower after 18 hours of UO126 treatment alone, there was no significant difference in CYP11A1 mRNA examined in either group (Figure 2A). Treatment of FACS with UO126 and ACTH for 18 hours ameliorated the increase in CYP11A1 mRNA (Figure 2A) in response to ACTH in the normoxic control FACS ($P < .05$) but had little effect on LTH FACS. Cyclophilin was not different between groups (Figure 2B).

CYP17. Under basal conditions, there were no differences for CYP17 mRNA between control and LTH FACS after 18 hours in culture (Figure 3). After 18 hours of ACTH treatment, CYP17 mRNA was significantly increased compared to basal

($P < .05$) in control (Figure 3) but not in the LTH FACS. However, in response to ACTH, CYP17 mRNA levels in the LTH FACS were at intermediate levels, being not different from either basal or ACTH-stimulated levels in the control FACS indicative of a minimal response to ACTH in the LTH FACS. Although lower after 18 hours of UO126 treatment alone, there was no significant difference in CYP17 mRNA examined in either group (Figure 3). Treatment of FACS with UO126 and ACTH for 18 hours ameliorated the increase in CYP17 mRNA (Figure 3) in response to ACTH in the normoxic control FACS ($P < .05$) but had little effect on LTH FACS. Cyclophilin was not different between groups (Figure 2B).

StAR. After 18 hours under basal conditions, StAR mRNA was lower ($P < .05$) in the LTH when compared to the normoxic control FACS (Figure 4). StAR mRNA levels were increased in response to 18-hour ACTH treatment in the normoxic control FACS (Figure 4) when compared to the LTH group ($P < .05$), and while levels of StAR mRNA were not significantly elevated in response to ACTH in the LTH FACS, the level was

Figure 2. CYP11A1 (A) and cyclophilin (B) mRNA in LTH and control fetal adrenocortical cells expressed as fg mRNA/12.5 ng cDNA 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. The effects of ERK1/2 inhibition on CYP11A1 were studied by pretreatment with UO126 (10 μ M) before 10^{-8} M ACTH stimulation. ACTH treatment increased CYP11A1 mRNA in control ($n = 6$) but not LTH ($n = 5$). Treatment with UO126 and ACTH prevented the increase in CYP11A1 mRNA in control FACs ($P < .05$). Cyclophilin was not different between groups (B). cDNA indicates complementary DNA; CYP11A1, cytochrome P45011A1; ERK, extracellular signal-regulated kinase; LTH, long-term hypoxic; mRNA, messenger RNA; PCR, polymerase chain reaction.

also not significantly different from that observed in control FACs in response to ACTH indicative of an intermediate response to ACTH as observed for CYP11A1 and CYP17. Although lower after 18 h of UO126 treatment alone, there was no significant difference in StAR mRNA examined in either group (Figure 4). Eighteen hours of UO126 and ACTH treatment resulted in a decrease in StAR mRNA (Figure 4) in the

Figure 3. CYP17 mRNA in LTH and control fetal adrenocortical cells expressed as fg mRNA/12.5 ng cDNA 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. The effects of ERK1/2 inhibition on CYP17 mRNA were studied by pretreatment with UO126 (10 μ M) before 10^{-8} M ACTH stimulation. ACTH increased CYP17 mRNA in control ($n = 6$) but not LTH ($n = 5$). Treatment with UO126 and ACTH prevented the increase in CYP17 mRNA in control FACs ($P < .05$). Cyclophilin was not different between groups (Figure 2B). CYP17 indicates cytochrome P450c17; ERK, extracellular signal-regulated kinase; LTH, long-term hypoxic; mRNA, messenger RNA; PCR, polymerase chain reaction.

Figure 4. StAR mRNA in LTH and control fetal adrenocortical cells expressed as fg mRNA/12.5 ng cDNA 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. The effects of ERK1/2 inhibition were studied by pretreatment with UO126 (10 μ M) before 10^{-8} M ACTH stimulation. StAR mRNA was decreased in LTH ($n = 6$) versus control ($n = 5$) under basal conditions, and following ACTH or ACTH + UO126 ($P < .05$). Cyclophilin was not different between groups (Figure 2B). cDNA, indicates complementary DNA; ERK, extracellular signal-regulated kinase; LTH, long-term hypoxic; mRNA, messenger RNA; PCR, polymerase chain reaction; StAR, steroidogenic acute regulatory protein.

LTH FACs when compared to the normoxic control group ($P < .05$). Cyclophilin was not different between groups (Figure 2B).

Discussion

Previous studies from our laboratory have demonstrated that the ovine fetus mounts a significant adaptive endocrine strategy under the potentially adverse conditions of LTH.⁴⁴ Despite higher basal plasma ACTH concentrations, the normal ontogeny of basal plasma cortisol is maintained.^{21,30,31} However, the LTH fetus exhibits enhanced cortisol biosynthesis in response to an acute secondary stressor, such as umbilical cord occlusion or hypotension.^{30,31} There are several key adaptive changes at the level of the fetal adrenal that likely contribute to these LTH fetuses being able to maintain normal basal plasma cortisol concentrations.

As previously reported, basal cortisol secretion (Figure 1A) was greater in the LTH FACs throughout the 18-hour culture period when compared to the normoxic control group. Consistent with our previous results, which were limited to an acute 60-minute *in vitro* response to ACTH,⁴⁰ 18 hours of ACTH treatment resulted in a greater cortisol production in LTH FACs compared to FACs from normoxic control fetuses with maximal cortisol output attained by 3 hours and sustained through 18 hours. UO126 eliminated ACTH-stimulated cortisol production in control FACs and significantly attenuated cortisol production in the LTH FACs compared to ACTH treatment alone. These findings confirm our previous results supporting a role for ERK1/2 in ACTH-induced cortisol biosynthesis in the ovine fetal adrenal cortex.

Our findings on CYP11A1 and CYP17 mRNA in the present study after 18 hours *in vitro* and under unstimulated conditions are in marked contrast to those we previously reported in adrenal cortex obtained at 138 to 141 dG.³² Considering the present results were derived from late-gestation FACs after 18 hours *in vitro*, the apparent discrepancy with our prior *in vitro* findings could be the result of several scenarios: (1) the removal of ACTH for 18 hours may have reduced expression of these key enzymes selectively in the control FACs, (2) the removal from the *in vitro* hypoxic conditions, or (3) the removal of an endogenous inhibitor present in the LTH group. With regard to the latter, we recently reported that infusion of a leptin receptor antagonist into late-gestation LTH fetal sheep increased CYP11A1 and CYP17 mRNA in the adrenal cortex to levels similar to that of the normoxic control group.⁴⁵

Levels of StAR in the LTH are more enigmatic. In our original report,³² we noted that adrenal cortical StAR mRNA was not different compared to control normoxic fetuses obtained from uninstrumented fetuses at 138 to 141 days and that total StAR protein followed a similar trend for the active forms of StAR with an increase in the spent 30 kD form. However, in a subsequent study, StAR mRNA was lower in adrenals obtained from LTH fetuses obtained after 4 days of saline infusion at 142 to 144 dG, just 2 to 4 days prior to expected delivery. Our present study, where StAR mRNA was reduced

in the LTH FACs after 18 hours *in vitro*, is more in line with our latter *in vitro* finding and may indicate that regulation of StAR in the LTH fetus is highly dynamic in these fetuses as term gestation approaches.

In the present study, we wanted to determine the role of ACTH in the regulation of the key steroidogenic enzymes, CYP11A1 and CYP17 as well as StAR in a controlled *in vitro* system. Additionally, we chose to assess the ability of UO126 to modulate ACTH-stimulated expression of these genes. This concept was based on the capability of UO126 to severely limit cortisol output in response to ACTH, which was especially evident over an 18-hour period versus 60 minutes since cortisol production over 18 hours likely depends at least in part on induction of key genes in the steroidogenic pathway, including StAR. Curiously, the results from our studies showed that 18 hours of ACTH treatment resulted in elevated CYP11A1 and CYP17 in control FACs but not LTH FACs. ACTH did not increase StAR in either group, although the levels of StAR achieved in response to ACTH were greater in control FACs compared to LTH cells.

It is difficult to reconcile the robust increase in CYP11A1 and CYP17 and the higher levels of StAR mRNA observed in the control FACs in response to ACTH and the significantly attenuated cortisol output observed in these cells in response to ACTH. We reported previously that the level of cAMP,³⁴ long identified as a major signal transduction component in the ACTH receptor-G α s-coupled signaling pathway,⁴⁶⁻⁴⁸ attained in response to ACTH is similar in LTH compared to control FACs. As such, this implies differences in signaling downstream from cAMP in the uncoupling of CYP/StAR expression and cortisol production in the control FACs or the enhanced cortisol output *in vitro* in the LTH FACs despite reduced expression of CYP/StAR in these cells. Studies by Sewer and Waterman⁴⁹ have elucidated a role for phosphoprotein phosphatases (PPs) in the steroidogenic response to cAMP using H295 R adrenocortical cells treated. This group showed that okadaic acid, a selective inhibitor of PP, results in an inhibitory effect at the level of transcription of the human CYP11A1 and CYP17 genes. In our present results, the low-level expression of CYP11A1 and CYP17 after ACTH treatment in the LTH group could also be reflective of adaptive changes under LTH conditions affecting steroidogenic factor-1 level of phosphorylation. Since StAR is also subject to kinase activation, PP activity could limit the delivery of cholesterol to the inner mitochondrial membrane in the control but not LTH FACs. This is supported by our previous observation of greater levels of 30 kD StAR in the adrenal cortex of the LTH fetus.³²

The role of the mitogen-activated protein kinase (MEK)-ERK signaling pathway in regulating cortisol biosynthesis remains largely unexplored and somewhat controversial. Peterson et al⁵⁰ clearly demonstrated the presence of ERK 1/2 in the adult ovine adrenal cortex. In adult bovine adrenocortical cells, ERK1/2 activation inhibits ACTH-induced steroidogenesis,⁵¹ and a negative effect of ACTH on ERK activity was also observed by Watanabe et al⁵² In contrast, in Y-1 adrenocortical

cells and ovarian granulosa cells, ERK1/2 promoted hormone-induced steroidogenesis.^{53,54} In adult rats, in vivo chronic treatment with ACTH increased pERK1/2 in the adrenal cortex and, as expected, corticosterone production, whereas MEK inhibition prevented both ACTH-induced ERK activation and the increase in corticosterone.^{39,55}

Based on the UO126 inhibition of cortisol production, we examined the role of ERK1/2 on ACTH-induced mRNA for CYP11A1 and CYP17. Treatment of FACs with UO126 and ACTH for 18 hours prevented the increase in CYP11A1 and CYP17 mRNA in the normoxic control only. Our results demonstrate that UO126 attenuated the ACTH response that is indicative of a role for ERK in CYP11A1 and CYP17 expression in the control but not LTH FACs. To our knowledge, we are the first to show that UO126 attenuated the ACTH response, indicative of a role for ERK in CYP11A1 and CYP17 expression. Thus, the present findings support a role for ERK1/2 activation in CYP11A1 and CYP17 expression during 18 hours of treatment with or without ACTH and UO126 in the FACs. In terms of treating the FACs for 18 hours with UO126 alone, the results showed no effect on any mRNA for CYP11A1, CYP17, and StAR.

Clearly, in the LTH FACs, ACTH is capable of providing a robust signal, largely via ERK1/2 activity, to stimulate cortisol production but is unable to convey a signal for increasing gene expression for key ACTH-regulated steroidogenic genes. Conversely, in the control FACs, ACTH was highly effective in increasing expression of CYP11A1 and CYP17 but was limited in its capacity to stimulate cortisol production. These findings point to complex signaling pathways that can uncouple expression of steroidogenic genes and actual cortisol production and that conditions of LTH during development can modulate this signaling schema.

Conclusions

To summarize our findings, we previously reported that under unstimulated basal conditions, freshly collected adrenal cortical tissue from LTH fetuses had lower mRNA levels of CYP11A1 and CYP17 and similar levels of StAR compared to normoxic controls. This is in marked contrast to the present study after 18 hours of culture. Together, these data suggest that this could be the result of removal of an in vivo inhibitor, perhaps leptin. ACTH increased CYP11A1 and CYP17 only in control FACs consistent with our previous observation of slower maturation of the adrenal cortex in response to LTH. UO126 attenuated the ACTH response indicative of a role for ERK in CYP11A1 and CYP17 expression. ACTH may require additional factors in FACs to fully regulate StAR expression. In this study, we further demonstrated that LTH FACs have adapted to the adverse conditions of LTH demonstrating an adaptive response and that sustained enhanced cortisol secretion is maintained for longer periods of time (18 hours) when compared to the normoxic control group. In addition to the classical cAMP/PKA pathway, ERK signaling is involved in part in ACTH-induced steroidogenesis possibly by playing a

more permissive role of integrating information from extracellular signals. The enhanced cortisol response coupled with the enhanced effect of ERK inhibition in the LTH group suggests that downstream signaling pathways are up regulated in response to LTH. The modulation of ERK signaling in the adrenal cortex has broad implications for other physiological systems in fetuses developing under conditions of sustained moderate hypoxia.

Acknowledgments

The authors thank Kimberly Hyatt and Krista Hanson for their technical help with this research.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Supported by National Institutes of Health Grants PO1HD31226 and R01-HD50620.

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