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LOMA LINDA UNIVERSITY
School of Medicine
in conjunction with the
Faculty of Graduate Studies

Long-Term Hypoxia Regulates Cortisol Biosynthesis in the
Ovine Fetal Adrenal Cortex

by

Vladimir Enrique Vargas

A Dissertation submitted in partial satisfaction of
the requirements for the degree of
Doctor of Philosophy in Physiology

December 2010

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

, Chairperson

Charles A. Ducusay, Professor of Physiology/Pharmacology and Obstetrics and Gynecology, Center for Perinatal Biology

Dean A. Myers, Professor and The John W. Records Chair
Section of Maternal-Fetal Medicine, Oklahoma University, Health Science Center

William J. Pearce, Professor of Physiology/Pharmacology, Center for Perinatal Biology

Steven M. Yellon, Professor of Physiology/Pharmacology and Obstetrics and Gynecology, Center for Perinatal Biology

Lubo Zhang, Professor of Physiology/ Pharmacology, Center for Perinatal Biology

ACKNOWLEDGEMENTS

I am grateful to my mentor Dr. Charles A. Ducsay, to whom I will always be in debt for everything he thought me during the process of transforming me into a scientific investigator, as well into a more complete person. The endurance, tenacity, disciplined, and dedication to the sciences imparted by my mentor will always be present in my life.

Also, I want to thank all the members of my guidance committee, Dr. Dean Myers, Dr. William Pearce, Dr. Steven Yellon, and Dr. Lubo Zhang, for their personal advice, guidance, and support.

I want to express my personal gratitude and many thanks to the personnel in our laboratory, “lab D”, especially to Kanchan Kaushal for all her guidance, support, and patience.

Finally, I also want to thank Loma Linda University School of Medicine, the Loma Linda University Graduate School, the Loma Linda University Physiology and Pharmacology Department, the Loma Linda University IMSD Program, and the Loma Linda University Center for Perinatal Biology for providing guidance, support, and facilities.

CONTENTS

Approval Page.....	iii
Acknowledgements.....	iv
Table of Contents.....	v
List of Figures.....	viii
List of Abbreviations.....	ix
Abstract.....	x
Chapter	
1. Introduction.....	1
Introduction.....	1
Background.....	3
The Fetal HPA axis.....	3
The role of the Fetal HPA axis in Parturition.....	6
The Fetal HPA axis and Hypoxia.....	7
HPA axis Responses to Acute Hypoxia.....	7
HPA axis Responses to Long-Term Hypoxia.....	8
Mechanisms of Cortisol Biosynthesis.....	11
The Role of the ACTH Receptor on Steroidogenesis.....	14
The Role of cAMP on Steroidogenesis.....	14
The Role of PKA on Steroidogenesis.....	15
The Role of StAR Protein on Steroidogenesis.....	16
The Role of Key Steroidogenic Enzymes on Cortisol Biosynthesis.....	18
The Role of CREB Protein on Steroidogenesis.....	20
The Role of ERK1/2 on Steroidogenesis.....	21
Significance of the Effects of the LTH on Cortisol Biosynthesis in Ovine FACs.....	23

2. Long-Term Hypoxia Enhances Cortisol Biosynthesis in the Near-Term Ovine Fetal Adrenal Cortical Cells.....	25
Abstract.....	26
Introduction.....	27
Methods.....	29
Animals Procedures	29
Adrenocortical Cell Dispersion.....	29
Cyclic 3,5-Adenosine Mono Phosphate and Cortisol Responses to ACTH.....	30
Effects of PKA Inhibition on Cortisol Biosynthesis.....	30
Effects of POMC and 22-kDa Pro-ACTH on Cortisol Biosynthesis.....	30
Cyclic 3,5-Adenosine Mono Phosphate Analysis.....	31
Cortisol Assay.....	32
Western Blot Analysis of StAR Protein.....	32
Statistical Analysis.....	33
Results.....	34
Cortisol and cAMP Responses to ACTH.....	34
Effects of PKA Inhibition on Cortisol Biosynthesis	34
StAR Protein Expression	35
Proopiomelanocortin (POMC) and 22-kDa Pro-ACTH	35
Discussion.....	46
References.....	54
3. Steroidogenic Acute Regulatory (StAR) Protein mRNA Expression in the Near-Term Ovine Fetal Adrenal Cortical Cells: Effects of Long-Term Hypoxia	58
Abstract.....	58
Introduction.....	59
Methods.....	61
Animals Procedures	61
Adrenocortical Cell Dispersion.....	61
Cortisol and StAR mRNA Responses to ACTH.....	62
qRT-PCR analysis of StAR mRNA in Ovine FACs.....	62
Statistical Analysis.....	63
Results.....	63
Cortisol Responses to ACTH	63
qRT-PCR analysis of StAR mRNA in Ovine FACs.....	64

Discussion.....	69
References.....	73
4. The Extracellular Signal-Regulated Kinases (ERK1/2) Signaling Pathway Plays a Role in Cortisol Secretion in the Near-Term Ovine Fetal Adrenal In Vitro: Effects of Long-Term Hypoxia.....	75
Abstract.....	76
Introduction.....	77
Methods.....	79
Animals Procedures	79
Adrenocortical Cell Dispersion	79
Cortisol Responses to ACTH Treatment	80
Effects of ERK1/2 Inhibition on Cortisol Biosynthesis.....	80
Cortisol Assay.....	81
Western Blot Analysis of ERK 1 and 2, and Phospho ERK 1 and 2.....	81
Statistical Analysis.....	82
Results.....	83
Cortisol Responses to ACTH and UO126 Treatment.....	83
ERK1/2 and Phospho ERK1/2 Protein expression in Response to ACTH and UO126 Treatment	83
Basal	83
ACTH treatment.....	84
Effects of UO126 on ACTH treatment	84
Discussion.....	93
References.....	98
5. Conclusions.....	102
References.....	109

FIGURES

Figures	Page
1.1 Schematic Diagram Illustrating Cortisol Biosynthesis Pathways in the Adrenal Cortex	13
2.1 <i>In Vitro</i> cortisol biosynthesis in FACs.....	36
2.2 cAMP generation in FACs.....	38
2.3 Effects of H-89 inhibition of PKA on cortisol biosynthesis in FACs.....	40
2.4 StAR Western blot.....	42
2.5 ACTH precursors and cortisol biosynthesis	44
3.1 Long-term <i>In Vitro</i> (3 hours) cortisol biosynthesis in FACs.....	65
3.2 StAR mRNA expression in LTH FACs	67
4.1 <i>In Vitro</i> cortisol biosynthesis in LTH FACs.....	85
4.2 Basal ERK1/2 and Phospho ERK1/2 Western blot.....	87
4.3 Effects of ACTH treatment on ERK1/2 and Phospho ERK1/2 Western blot	89
4.4 Effects of UO126 pre-treatment of FACs followed by ACTH treatment on ERK1/2 and Phospho ERK1/2 Western blot	91

ABBREVIATIONS

ACTH	Adrenocorticotropic Hormone
AVP	Arginine Vasopressin
cAMP	Cyclic Adenosine Mono Phosphate
CRF	Corticotrophin Releasing Factor
CYP11A1	Cholesterol Side-chain cleavage
CYP17	17 Alpha-hydroxylase
ERK1/2	Extracellular Signal Regulated Kinases
LTH	Long-Term hypoxia
PKA	Protein Kinase A
POMC	Proopiomelanocortin
PVN	Para Ventricular Nucleus
StAR	Steroidogenic Acute Regulatory Protein
UCO	Umbilical Cord Occlusion

ABSTRACT OF THE DISSERTATION

Long-Term Hypoxia Regulates Cortisol Biosynthesis in the Ovine Fetal Adrenal Cortex

by

Vladimir Enrique Vargas

Doctor of Philosophy, Graduate Program in Physiology
Loma Linda University, December 2010
Dr. Charles A. Ducusay, Chairperson

Previous studies demonstrated enhanced fetal adrenal cortisol biosynthesis following exposure to long-term hypoxia (LTH). The studies presented here were designed to determine the mechanism(s) regulating this important adaptive endocrine response. Ewes were maintained at high altitude (3,820 m) from approximately day 40 to 138-141 of gestation. Fetal adrenal glands were then collected from LTH and age-matched normoxic controls. Dispersed fetal adrenal cortical cells (FACs) were untreated, treated with ACTH, treated in combination with ACTH precursors (POMC and 22-kDa pro-ACTH), or pre-treated with H-89 and or UO126 followed by ACTH treatment. Following ACTH treatment, cortisol biosynthesis increased in both groups but values were significantly higher in the LTH FACs. Neither basal nor ACTH-stimulated cAMP levels differed between groups. H-89 inhibition of PKA decreased cortisol to levels observed in the un-stimulated FACs in both groups. StAR protein was higher in the LTH group under un-stimulated, and following ACTH treatment. ACTH precursors alone or in combination with ACTH did not affect cortisol synthesis in either group. StAR mRNA was not different between groups. Although UO126 inhibition of ERK decreased cortisol in both groups, the net decrease was greater in the LTH group in response to ACTH

treatment. Basal ERK1/2, and pERK1/2 were not different between groups. In response to ACTH treatment pERK1/2 was higher in the LTH FACs, however, pERK1/2 declined significantly in normoxic control but not LTH FACs. Total ERK1/2 did not differ between groups or after ACTH treatment. UO126 significantly reduced pERK 10 to 20 fold in both groups similarly at all time points. Together with previous *in vivo* data, these data indicate that enhanced cortisol output in the LTH group is the result of increased adrenal ACTH sensitivity. This enhanced sensitivity is not due to differences in cAMP, PKA and or ACTH precursors. StAR, and the ERKs appear to play a key role in the enhanced cortisol response to ACTH following LTH. These adaptive responses to LTH are critical in maintaining normal fetal growth and development and these data may have important clinical implications.

CHAPTER 1

INTRODUCTION

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis plays a key role in the response to stress during fetal development. Since fetal growth and development must be precisely orchestrated, any perturbation that can alter the general processes of maturation can initiate permanent changes to the fetus. Cortisol is critical to allow the fetus to mount an adaptive response to acute stressors. Under stressful conditions, glucocorticoid secretion leads to increase lipolysis, glycogenolysis, and protein catabolism, which ultimately result in increased plasma glucose. All of these metabolic responses are mediated by glucocorticoids. However, sustained levels of glucocorticoids can have negative effects on the fetus by suppressing anabolic processes resulting in hypertension, muscle atrophy and delayed in maturation and organ growth. Further, as clearly outlined by Barker and others (Barker, 2000; Barker et al., 2005; Hales et al., 2001; Osmond et al., 2000; Painter et al., 2005; Phillips et al., 2000), fetuses exposed to maternal stressors during the course of gestation are more vulnerable to potential diseases later in life.

One of the stressors known to affect fetal development is hypoxia. Although many studies have centered on understanding the effects of acute hypoxia, there is lack of data on the effects of long-term hypoxia (LTH) during fetal development. Previous studies from our laboratory have shown that in the sheep fetus, the adrenal gland has

adapted to conditions of LTH. We have shown that, while basal plasma adrenocorticotrophic (ACTH) levels are elevated, basal levels of plasma cortisol in fetuses exposed to LTH are similar to normoxic controls (Harvey et al., 1993; Myers et al., 2005a). In marked contrast, the LTH fetus is capable of enhanced cortisol biosynthesis in response to a secondary stressor compared to the normoxic control fetus (Adachi et al., 2004; Imamura et al., 2004). However, the mechanisms responsible for this enhanced cortisol response remain to be elucidated. A wide range of factors may play a role in this adaptive response to LTH including changes in the major ACTH/cyclic 3,5-adenosine mono phosphate (cAMP) signaling pathway, in cholesterol availability, and steroidogenic acute regulatory (StAR) protein as well as function of key steroidogenic enzymes. Regulation of adrenal cortisol biosynthesis during fetal development must be well coordinated in order to respond to stress while allow effective organ maturation and at least in the sheep, successful timing of delivery.

The work presented in this dissertation will serve to further elucidate the mechanisms involved in the regulation of cortisol biosynthesis during LTH in the sheep fetus and will test the general hypothesis that *LTH regulates key elements involved in cortisol biosynthesis in ovine fetal adrenocortical cells (FACs)*. The general hypothesis is in turn composed of two hypotheses that specifically address the mechanisms involved in cortisol biosynthesis in response to LTH in the near-term sheep fetus. **Hypothesis 1:** In response to ACTH treatment, ovine LTH FACs increase cortisol production *in vitro* when compared to the normoxic control by increasing activity of cAMP, protein kinase A (PKA), and expression and/or phosphorylation of steroidogenic acute regulatory (StAR) protein. Additionally, ACTH precursors may also play an inhibitory role on cortisol

secretion. **Hypothesis 2:** In response to ACTH treatment, the extracellular signal-regulated kinases (ERK1/2) exhibit an increased level of phosphorylation in LTH when compared to the normoxic control FACs.

To address the first hypothesis, multiple approaches were utilized. We designed the studies to measure the levels of cyclic cAMP in response to ACTH treatment in LTH vs. normoxic control FACs, also, by studying the role of PKA on cortisol secretion in the LTH FACs by means of inhibiting the kinase with a PKA selective inhibitor, and by determining protein expression of StAR. We also determined if LTH alters fetal adrenal cortical sensitivity to ACTH precursors proopiomelanocortin (POMC) and 22-kDa pro-ACTH. The second hypothesis was tested by studying the role of the extracellular signal-regulated kinases (ERK1/2) in regulating ACTH-induced steroidogenesis in the presence or absence of the selective MEK/ERK1/2 inhibitor UO126.

All of the studies were performed using our unique model of LTH during fetal development. Time dated pregnant sheep were maintained at an elevation of 3,820 m, from day 40 to near term (term in these animals is approximately 145 days' gestation) which reduced maternal PO₂ to ~60mmHg. On days 138-141 of gestation fetal adrenal glands were collected from normoxic control and LTH animals followed by adrenal cortical cell dispersion.

Background

The Fetal HPA Axis

An intact HPA axis in the near-term sheep fetus is critical for organ maturation and the cortisol surge before and during parturition (Challis et al., 1989; Liggins, 1976,

1994; Magyar et al., 1980). The hypothalamic paraventricular nucleus (PVN) is the site of biosynthesis and release of corticotropin-releasing factor (CRF) and arginine vasopressin (AVP). These neuropeptides act on the anterior pituitary to direct the processing of POMC to adrenocorticotrophic (ACTH) hormone. Studies by Bell et al. (Bell et al., 2005) showed that lesion of the PVN delayed the processing of POMC to ACTH in the anterior pituitary, leading to a reduction in fetal plasma ACTH levels. These data indicated that in the sheep fetus an intact PVN is crucial for maintaining processing of POMC to ACTH, and subsequent ACTH release to allow adrenocortical growth and maturation.

Growth and maturational effects of ACTH on the adrenal gland are mediated through autocrine/paracrine functions of growth factors such as the insulin like growth factors I and II (IGF-I and IGF-II), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF), (Mesiano et al., 1997). Further, studies by Myers et al. (Myers et al., 1992) demonstrated that bilateral lesion of the PVN in the sheep fetus decreased adrenal mRNA gene expression of both 17α -hydroxylase (P-450_{17 α}), and side-chain cleavage (P-450_{scc}). However, bilateral lesion of the fetal PVN did not affect gene expression of 11β -hydroxylase (P-450_{11 β}), 21 hydroxylase (P-450_{C21}), and 3β -hydroxysteroid dehydrogenase (3β -HSD). These studies showed that an intact PVN is essential in the regulation of 17α -hydroxylase (P-450_{17 α}) and side-chain cleavage (P-450_{scc}) on cortisol biosynthesis in the adrenal cortex. More specifically, the role of key steroidogenic enzymes in the adrenal mitochondria are to catalyze the reactions that culminate in cortisol biosynthesis, therefore, without an intact PVN, the normal rise in

fetal plasma ACTH is absent leading to a disruption in the steroidogenic enzymatic reactions resulting in delayed parturition in the sheep fetus.

In addition, several studies have investigated the role of ACTH and cortisol on adrenal gland function and cortisol secretion in the sheep fetus (Challis et al., 1985; Durand et al., 1981a; Durand et al., 1984; Manchester et al., 1983). Glickman and Challis (Glickman et al., 1980) studied dispersed fetal adrenal cells with ACTH treatment at 50, 100, 130 days of gestation, and at term. They found a positive cortisol response to ACTH treatment at all periods studied except at 100 days gestation. They concluded that as gestation progresses the sensitivity of the fetal adrenal cells to ACTH treatment increases until the end of gestation, and it may involve the activation of P450 17 α -hydroxylase. Other studies have also examined the role of ACTH in the activation of the P450 cholesterol side chain cleavage (Simpson et al., 1988).

Additional studies by Rose et al. (Rose et al., 1982) investigated plasma immunoreactive ACTH and cortisol concentrations in response to ACTH₁₋₂₄ infusion into the sheep fetus during the last three weeks of gestation. The study showed that although fetal plasma cortisol increased near-term, immunoreactive ACTH concentration did not increase concomitant with cortisol surge. The results demonstrated that fetal adrenal sensitivity to ACTH increases in the near-term sheep fetus. Another study by Castro et al. (Castro et al., 1992) investigated age-dependent (97 and 136 days gestation) differences in the levels of plasma immunoreactive ACTH compared to bioactive ACTH-like activity in fetal sheep. They found that the ratio of bioactive to immunoreactive ACTH was greater in the older near-term (136 days gestation) fetuses when compared to the

younger fetuses at 97 days gestation. Their research supports the concept that there is an age-dependent increase in ACTH bioactivity in the plasma of fetal sheep.

The Role of the Fetal HPA axis in Parturition

Numerous studies have focused on understanding the role of the fetal HPA axis on the control of parturition in the sheep (Antolovich et al., 1990; Antolovich et al., 1991; Antoni, 1986; Apostolakis et al., 1994; Brooks et al., 1988; Challis et al., 2000; Jones et al., 1977a; Wood, 1988, 1999). The classic studies of Liggins et al. (Liggins, 1968, 1969; Liggins et al., 1973) clearly showed that the sheep fetus plays an important role in the processes of parturition. Infusion of ACTH, and/or cortisol to ovine fetuses at 88-129 days of gestation resulted in premature delivery. In marked contrast, infusion of ACTH or cortisol to the pregnant ewe did not result in premature parturition. These studies clearly demonstrated the critical role of the fetal HPA axis in the process of parturition in the sheep.

Studies by McDonald and Nathanielsz (McDonald et al., 1991) defined the critical area of the hypothalamus that is involved in this regulatory process. Fetal sheep at 118-122 days gestation under went bilateral ablation of the paraventricular nucleus (PVN) resulting in reduced fetal adrenal weight, failure of the pre-partum ACTH and cortisol surge, and delayed parturition. In similar studies fetal sheep at 106-110 days gestation, Gluckman et al. (Gluckman et al., 1991) demonstrated that bilateral lesions of the PVN resulted in delayed parturition, however, fetal adrenal weight was normal in contrast to the studies performed by McDonald and Nathanielsz (McDonald et al., 1991). It is possible that the difference in adrenal weight observed by both groups might be the

result of the different gestational ages chosen to perform their respective studies. In both studies, ACTH secretion and cortisol surge before term are essential for delivery to occur, a process that could be compromised if the PVN is bilaterally lesioned. Additional studies showed that maturation of the HPA axis at the level of the anterior pituitary or hypothalamus plays a key role in the process of parturition (Wood et al., 1991) and bilateral adrenalectomy prevented the normal timing of parturition (Drost et al., 1968). Taken together, the sheep fetus HPA axis plays an important role in the processes leading to parturition.

The Fetal HPA Axis and Hypoxia

HPA axis Responses to Acute Hypoxia

A wide range of stressors has been shown to activate the fetal HPA axis, including environmental pollutants, protein deprivation, hypotension, hemorrhage, and oxygen insufficiency i.e., hypoxia. For example, the effects of acute hypoxia on the fetal HPA axis have been extensively studied (Akagi et al., 1990; Boddy et al., 1974a; Fisher et al., 1982; Giussani et al., 1996; Iwamoto et al., 1989; Jackson et al., 1987; Jones et al., 1977b; Robillard et al., 1981; Towell et al., 1987). Boddy et al. (Boddy et al., 1974b) performed experiments in catheterized fetal (96-145 days gestation) and adult pregnant sheep. The results demonstrated that after periods of acute hypoxia (60 minutes) in pregnant sheep, plasma ACTH and cortisol concentrations increased, whereas, fetal plasma ACTH concentrations increased sharply, but the cortisol surge that was seen in the pregnant ewe was absent in the fetus. Studies from Boddy et al. showed that in response to acute hypoxia fetal adrenal responsiveness to ACTH is greatly diminished,

when compared to the pregnant ewe. Other studies by Jones et al. (Jones et al., 1977b) investigated the effects of acute hypoxia (60 minutes) on plasma ACTH concentration and adrenal corticoid release at mid-term to late gestation fetal sheep (96-145 GA). The results showed gradual and small cortisol increases before 139 days gestation that may be of maternal origin, while fetal cortisol secretion after 139 days was greatly enhanced. The refractory responsiveness of the fetal adrenal to ACTH at mid-gestation may reflect the adaptive capacity of the fetal adrenal gland.

Additional studies have shown changes at the level of the HPA axis that brings about physiological adaptations in response to acute hypoxia, such as blood flow to fetal organs. For example, the brain stem, cerebellum, cerebrum, heart and adrenals received more blood flow when compared to other organs such as the pancreas, digestive tract, and kidney (Peeters et al., 1979). Recent studies by Fletcher et al. (Fletcher et al., 2006), showed that exposing the sheep fetus to periods of hypoxemia led to alterations in the fetal sheep HPA axis resulting in changes to basal plasma ACTH, cortisol concentrations, and increased fetal cardiovascular responses during the last two weeks of gestation.

HPA axis Responses to Long-Term Hypoxia

The pregnant sheep is a well-established model used to study developmental and stress responses of the fetal HPA axis as described above, and has been an effective model to study responses to acute stressors like hypoxia. However, far less is known regarding fetal HPA axis responses to LTH. Using a unique model of high altitude hypoxia during fetal development in the sheep, our laboratory has demonstrated a significant number of adaptive changes in the fetal HPA axis response to LTH (Ducsay,

1998; Ducsay et al., 2007; Monau et al., 2009; Myers et al., 2008; Root et al., 2008). For example, studies by Imamura et al. (Imamura et al., 2004) showed that LTH fetuses responded differently to a secondary stressor like umbilical cord occlusion (UCO) compared to normoxic controls. Although plasma ACTH concentrations increased and reached a similar peak in both groups following UCO, peak cortisol levels were higher in the LTH group. Similarly, in response to a milder stressor (hypotension), Adachi et al. (Adachi et al., 2004) showed an enhanced cortisol response in the LTH group. A key question is what is the mechanism(s) that are responsible for this adaptation at the level of the adrenal gland?

More recent studies from our group (Myers et al., 2005a) have shown that LTH increases POMC processing in the sheep fetus near term. There was reduced expression of ACTH precursors (POMC and 22-kDa pro-ACTH) and ACTH in the anterior pituitary while basal plasma immunoreactive ACTH₁₋₃₉ and ACTH precursors (POMC and 22-kDa pro-ACTH) were significantly increased in the LTH fetuses when compared to the normoxic control group. This observation showed that despite higher levels of basal plasma immunoreactive ACTH and ACTH precursors in the LTH fetuses, basal plasma cortisol production remained similar to the normoxic control group. The results of these studies suggested that the adrenal gland of the LTH fetus has decreased its sensitivity to ACTH and potentially to precursor molecules such as POMC and 22-kDa pro-ACTH. Also, data from adrenal gland tissue homogenates in LTH fetuses showed a reduction in the ACTH receptor mRNA, and a decrease in the expression of key steroidogenic enzymes P450 cholesterol side chain cleavage [CYP11A1] and P450 17 α -hydroxylase [CYP17] in the LTH fetuses when compared to the normoxic control group (Myers et al.,

2005b). Taken together, these data suggest that other elements at the level of the fetal adrenocortical cells (FACs) ACTH/cAMP signaling pathway may be responsible for the observed adaptive changes in cortisol biosynthesis under conditions of LTH.

Additional studies from our laboratory (Ducsay et al., 2006) showed that in response to LTH plasma leptin concentrations and leptin receptor expression are elevated in the near-term sheep fetus. Also, the mRNA for the major leptin OB-R the OB-Rb isoform is increased in the adrenal cortex of the LTH sheep fetus. For example, the role of leptin and its capacity to inhibit ACTH and cortisol secretion in fetal sheep, rats, and humans has been demonstrated (McMillen et al., 2004; Pralong et al., 1998), and in bovine adrenocortical cells (Bornstein et al., 1997), as well as the capacity to inhibit the HPA axis in mice and rats in response to stress (Heiman et al., 1997). Additionally, leptin has been shown to inhibit the pre-partum ACTH and cortisol secretion in fetal sheep resulting in longer gestation periods in the sheep (Yuen et al., 2004). Leptin is also implicated in regulating key steroidogenic enzymes involved in cortisol synthesis in bovine adrenocortical cells (Kruse et al., 1998). Moreover, studies by Forhead et al. (Forhead et al., 2002) demonstrated an inverse correlation of fetal PO_2 levels and plasma leptin concentrations in the near-term sheep fetus. Additional studies in differentiated adipocytes exposed to hypoxia results in enhanced leptin and stimulates the hypoxia-inducible factor-1 (HIF-1) cascade. Therefore our results and those of others as described previously indicate that leptin is capable of regulating the sheep fetus HPA axis and may play a role in ACTH-induced cortisol biosynthesis in the LTH sheep fetus, therefore, leptin may be considered a hypoxia inducible gene in the LTH sheep fetus. The role of

leptin on LTH sheep fetus has the potential to explain in part the capacity of the fetal adrenal to maintain normal cortisol levels despite higher plasma ACTH.

In other reports from our laboratory, Monau et al. (Monau et al., 2009; Monau et al., 2010) showed a significant regulatory role for nitric oxide on ACTH-induced cortisol biosynthesis in the ovine LTH FACs. The studies demonstrated that endothelial nitric oxide synthase (eNOS) was increased in the LTH adrenal cortex when compared to the normoxic control group. Also, treatment of the LTH FACs with nitric oxide inhibited ACTH-induced cortisol secretion. The results from these studies clearly revealed the complexity of the regulation of cortisol secretion under conditions of LTH.

Mechanisms of Cortisol Biosynthesis

Under normal physiologic conditions the synthesis of cortisol involves a series of enzymatic reactions that are carefully orchestrated. Specifically, ligand binding (ACTH) to specific ACTH-receptors along the membrane of the FACs leads to the activation of GTP-binding proteins (G-proteins) that in turn are coupled to the receptors. The G_s protein subunit activates the enzyme adenylyl cyclase, resulting in the conversion of ATP to cAMP. The active cAMP then stimulates Protein Kinase A (PKA), leading the kinase to phosphorylate various enzymes, intracellular proteins, and nuclear factors (Antoni, 2000; Daniel et al., 1998; Kamenetsky et al., 2006). Following PKA activation, cholesterol, a key substrate for glucocorticoid synthesis is then transferred to the inner mitochondrial membrane by the StAR protein. Cortisol is produced and released into the circulation through a series of enzymatic reactions involving key steroidogenic enzymes such as P450 cholesterol side chain cleavage [CYP11A1], and P450 17 α -hydroxylase

[CYP17], Figure 1.1 (Sewer et al., 2007; Stocco, 2001). For example, *in vitro* studies performed by Durand et al. (Durand et al., 1981a; Durand et al., 1981b) demonstrated that ACTH or cholera toxin induces steroidogenic activity that takes place at different levels in the pathway leading to cortisol synthesis.

Among the factors enhanced are changes at the cell membrane involving adenylate cyclase activation leading to cAMP synthesis, and activation of other downstream elements distal cAMP. Their findings also showed that ACTH infusion into the sheep fetus leads to maturation of the system that results in the steroidogenic response. Other elements down-stream cAMP activation involves the role of StAR protein as well as the key steroidogenic enzymes. Although, more specific studies have centered at delineating the mechanisms that govern maturation and activation of the steroidogenic capacity of the sheep fetal adrenal gland (Challis et al., 1986; Durand et al., 1985; Lye et al., 1984), and numerous studies have centered at delineating the biosynthesis and secretion of cortisol under normal conditions and or in response to acute stressors like hypoxia in the sheep fetus (Boddy et al., 1974b; Jackson et al., 1989; Jones et al., 1977b), there is a lack of data regarding cortisol biosynthesis in response to LTH in the sheep fetus. Our goal is to further elucidate the mechanisms that regulate cortisol biosynthesis in the near-term sheep fetus in response to LTH, and to our knowledge, we are the first to address this area of research. The key areas of regulation are outlined in Figure 1.1 and include, cAMP, PKA, StAR, ACTH precursors (POMC and 22-kDa pro-ACTH, and ERK1/2, all of which play an important role in the regulation of cortisol biosynthesis in the developing sheep fetus.

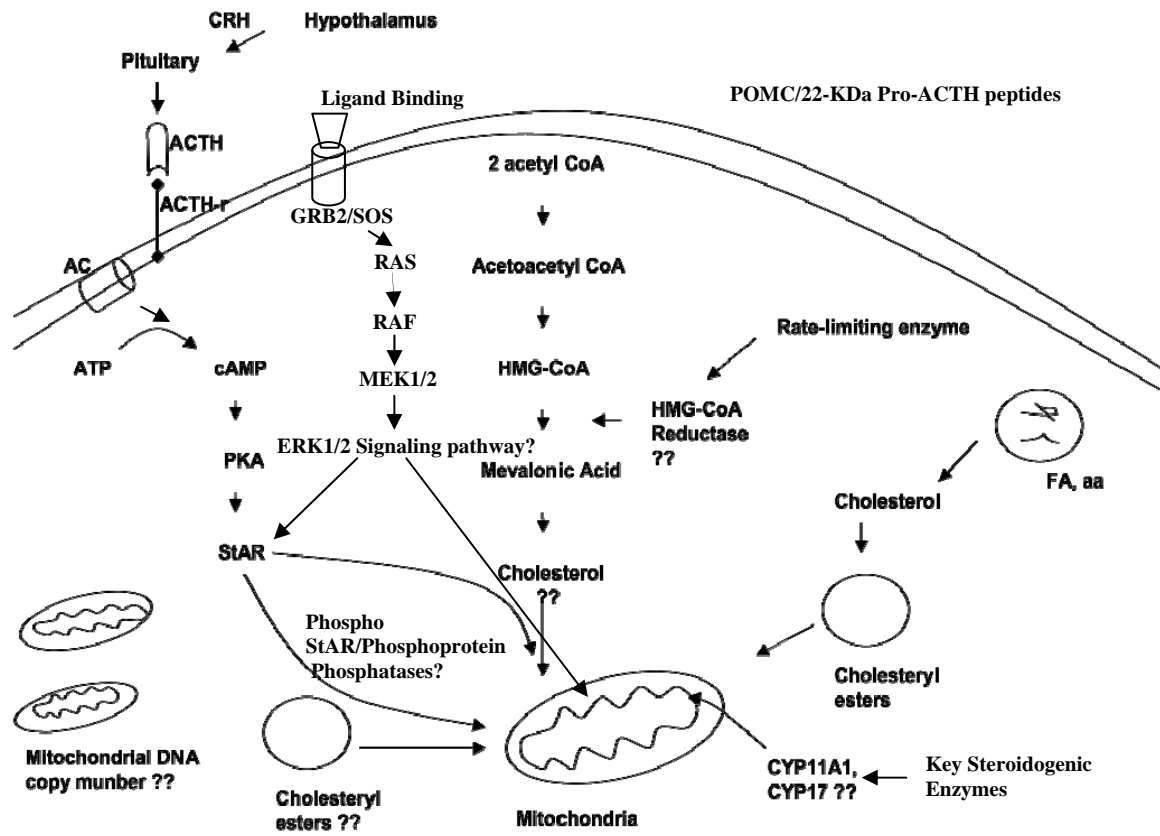


Figure 1.1 Schematic Diagram Illustrating Cortisol Biosynthesis Pathways in the Adrenal Cortex.

The Role of the ACTH Receptor on Steroidogenesis

Activation of the ACTH/cAMP signaling pathway that results in cortisol biosynthesis requires agonist-receptor binding at the level of the FAC membrane. The review of the literature identifies the ACTH receptor as a melanocortin receptor (type 2) that is better known as the MC₂ receptor. Hormone binding (ACTH) to the ACTH receptor has been studied in adult and fetal sheep (Carey et al., 2006; Edwards et al., 2002; Elias et al., 2000; Saez et al., 1984; Su et al., 2008), in humans (Mesiano et al., 1996), and to some extent in response to hypoxia (Fraser et al., 2001). However, the ACTH receptor has not been extensively investigated in sheep fetuses exposed to LTH during most of gestation. Data from our laboratory have demonstrated that in response to LTH, ACTH receptor mRNA levels are decreased when compared to the normoxic control group (Myers et al., 2005b). This finding was somewhat unexpected since we hypothesized that the elevated plasma ACTH, and enhanced cortisol secretion observed in the LTH fetal sheep in response to UCO treatment (Imamura et al., 2004) was in part mediated by an increase in ACTH receptor. Therefore, other mechanisms appear to be involved in the capacity of the LTH sheep fetus to respond with enhanced cortisol secretion. Since the regulatory mechanism does not appear to be at the level of the ACTH receptor itself, we then decided to study the intracellular mechanisms governing cortisol biosynthesis in the LTH sheep fetus.

The Role of cAMP on Steroidogenesis

It was not until 1958 that Sutherland and Rall (Rall et al., 1958; Sutherland et al., 1958) successfully fractionated and characterized cAMP from a variety of animal tissues.

The first report demonstrating cAMP accumulation in response to ACTH treatment came about using slices of bovine adrenal cortex (Haynes, 1958). Later studies by the same group revealed that cAMP led to corticoid production in sectioned rat adrenal glands (Haynes et al., 1959). Moreover, Grahame et al. (Grahame-Smith et al., 1967) carried out studies that revealed cAMP as the intracellular mediator of the actions of ACTH on rat adrenal quarters. Also, the increase in cAMP preceded the initiation of steroid biosynthesis, demonstrating that cAMP was necessary for steroid biosynthesis. In addition, the study demonstrated that treating rat adrenal tissue *in vitro* with cycloheximide, an inhibitor of protein synthesis, did not cause a decrease in cAMP levels in response to ACTH treatment. Therefore, the treatment with cycloheximide suggested that its site of action was past beyond the point of adenyl cyclase activation. However, evidence showing cAMP as the major second messenger molecule becoming activated in response to ACTH treatment and driving cortisol biosynthesis in the adrenal gland comes from tissues and animal models that were not exposed to the effects of LTH.

The studies in this dissertation will lend insight into the mechanisms governing cortisol biosynthesis in the ovine LTH FACs, and will demonstrate if the mechanisms governing cortisol biosynthesis under LTH conditions are the same, or at least in part similar to that under normal cortisol biosynthesis, and if exposure of the developing sheep fetus to LTH has affected the ACTH/cAMP signaling pathway.

The Role of PKA on Steroidogenesis

cAMP-dependent protein kinase A (PKA) activates intracellular proteins by the process of phosphorylation-dephosphorylation (Krebs et al., 1979; Walsh et al., 1968).

PKA has been shown expressed not only in Mammalian, but also in a wide variety of cell types (Kuo et al., 1969a, 1969b). Particularly in the adrenal cortex, PKA regulates cellular function of various proteins by the process of phosphorylation (Schimmer, 1995), however, PKA phosphorylating events are short lived and reversible culminating with dephosphorylation mediated by phosphoprotein phosphatases. These phosphatases (PP1, PP2A, and PP2B) are highly expressed in the adrenal and luteal cells (Cohen, 1989; Ford et al., 1996; Sayed et al., 1997), and post-translational modifications such as phosphorylation have shown to play a key role in the events leading to steroidogenesis particularly suggesting a regulatory event at the level of StAR, (Arakane et al., 1997; Fleury et al., 2004; LeHoux et al., 2004; Manna et al., 2005). However, not much work has been done in the area of phosphorylation-dephosphorylation events regulating steroidogenesis in the ovine LTH FACs. Although important, we will not investigate the role of phosphoprotein phosphatases, however, the role of PKA in ovine LTH FACs is key to gaining an insight into the regulatory mechanisms of cortisol biosynthesis in ovine LTH FACs. For this reason we will first explore the role of PKA inhibition on cortisol biosynthesis in the ovine LTH FACs. It is possible that due to the adverse conditions of LTH during development in the sheep fetus, PKA activity might have been increased in response to ACTH treatment to allow additional phosphorylation of key proteins involved in enhanced cortisol biosynthesis.

The Role of StAR Protein on Steroidogenesis

The principal function of StAR is to mediate the transfer of cholesterol from the outer to the inner mitochondrial membrane of glucocorticoid producing cells (Manna et

al., 2002; Manna et al., 2009; Miller, 2007; Papadopoulos et al., 2007; Stocco, 2001; Yamazaki et al., 2006) and is regarded as the first rate-limiting step in steroidogenesis. Recent data suggest that phosphoprotein phosphatases may play a role in the regulation of StAR protein expression and cortisol biosynthesis. Studies have shown that inhibition of PP1 and PP2A activities with calyculin A and Cantharin causes an inhibition of StAR protein expression, and a dose-response inhibition of steroidogenesis (Burns et al., 2000; Jones et al., 2000; Poderoso et al., 2002). Other reports have shown that inhibition of PP1 and PP2A activities with calyculin A, Okadaic Acid a selective inhibitor of PP2A, and Cantharin causes an inhibition at the level of transcription of key enzymes such as CYP11A1 (Sewer et al., 2002c). Arakane et al. (Arakane et al., 1997), demonstrated *in vitro*, that mutating potential sites of phosphorylation in the StAR protein reduces the capacity of COS-1 cell extracts to incorporate ³²P into StAR protein and showed that phosphorylation sites were located at serine 194/195. Moreover, the work of Orme-Johnson et al. (Orme-Johnson, 1990) found that StAR phosphorylation is initiated as a result of activating the ACTH/cAMP signaling pathway and subsequent phosphorylation of the protein (Alberta et al., 1989; Epstein et al., 1989; Pon et al., 1986).

The studies described above suggested that StAR protein expression might play a major regulatory role in the events leading to greater cortisol secretion in the ovine LTH FACs. Recent studies from our laboratory performed in whole adrenal gland tissue homogenates from LTH fetal adrenals, showed that the mature inactive (30 kDa) form of StAR protein was higher when compared to the normoxic control group (Myers et al., 2005b). We studied the role of StAR using the ovine LTH FACs to better gain understanding of the mechanisms by which StAR mediates transfer of cholesterol from

the outer to the inner mitochondrial membrane. Perhaps, there is more StAR protein available to transfer cholesterol in response to ACTH treatment in the LTH FACs.

The Role of Key Steroidogenic Enzymes on Cortisol Biosynthesis

Another important regulatory component involved in cortisol biosynthesis in the ovine adrenal gland are the steroidogenic enzymes P45011A1 (CYP11A1) and P450c17 (CYP17) (Sewer et al., 2002a; Simpson et al., 1988). Steroid hormones are necessary for proper development of secondary sex characteristics, regulation of metabolism, and homeostasis (Conley et al., 1997; Goto et al., 2006; Kersten, 2010; Stratakis et al., 2004). In steroidogenic tissues such as the adrenal cortex P45011A1 (CYP11A1) processes cholesterol into pregnenolone. The major role of P45011A1 is to catalyze a series of enzymatic reactions to produce 20 α hydroxylation, 22 α hydroxylation, and excision at the C20/22 bond junction, to transform the C27 cholesterol into C21 pregnenolone, P450c17 then hydroxylates pregnenolone at position 17 to produce 17-hydroxypregnenolone (Hiwatashi et al., 1979; Mitani et al., 1982; Simpson, 1979; Waterman et al., 1985). Therefore, the precise expression of key steroidogenic enzymes is highly regulated at different levels and proves to be necessary for successful steroid hormone expression (Miller, 2005; Simpson et al., 1987).

In studies by Simpson and Waterman (Simpson et al., 1988), and more recently by Ozbay et al. (Ozbay et al., 2006), and, Sewer and Waterman (Sewer et al., 2007; Sewer et al., 2001), it became clear that ACTH is necessary for the expression of these enzymes to initiate cortisol synthesis. Additional work by Tangalakis et al. (Tangalakis et al., 1989) have shown that gene expression of steroid hydroxylases P45011A1

(CYP11A1) and P450c17 (CYP17) increases in the adrenal gland as gestation progresses. However, the pattern of expression is lowest between 90-120 days of gestation when compared to 40-60 days, while gene expression is highest after 120 days of gestation. In another series of *in vivo* experiments the same group (Tangalakis et al., 1990) reported that ACTH infusion to sheep fetuses resulted in an increase in gene expression of P45011A1 (CYP11A1) and P450c17 (CYP17) between 90-120 days gestation. These studies showed that ACTH plays an important role on gene expression of key steroid hydroxylases in cortisol regulation and biosynthesis in the sheep fetus. In addition, other groups have centered at delineating the transcriptional machinery that regulates P45011A1 (CYP11A1) and P450c17 (CYP17) gene expression using different model systems other than the sheep fetus (Fluck et al., 2004; Gizard et al., 2001; Lin et al., 2001; Sewer et al., 2002d; Sher et al., 2007). More recently, studies from our laboratory have investigated the cortisol response to ACTH treatment in dispersed FACs *in vitro* in both LTH and normoxic control groups. Surprisingly, our results showed that the LTH group responded with a marked increase in cortisol secretion when compared to the control group (Monau et al., 2010). However, additional studies from our laboratory showed a decrease in the mRNA levels and protein expression of both P45011A1 (CYP11A1) and P450c17 (CYP17) enzymes in the LTH group (Myers et al., 2005b). Interestingly, other *in vivo* studies from our laboratory have shown no difference in basal plasma cortisol concentrations in both groups (Adachi et al., 2004; Imamura et al., 2004), despite basal plasma ACTH being significantly higher, in the LTH when compared to the normoxic control group (Myers et al., 2005a). A potential explanation for the apparent discrepancy could be found at the level of enzymatic activity of P45011A1 (CYP11A1) and P450c17

(CYP17). It is possible that although mRNA and protein expression levels are decreased there is still sufficient enzymatic activity to produce enough cortisol. To our knowledge, there are no data addressing the state of enzymatic activity of CYP11A1 and CYP17 following long-term hypoxia.

The Role of CREB Protein on Steroidogenesis

The cyclic-AMP response element (CREB)-binding protein is a 43 kDa phosphoprotein (Montminy et al., 1987) that is regarded as one of the best characterized stimulus-induced transcription factors that becomes activated in response to a variety of intracellular signaling mechanisms including PKA phosphorylation at serine 133. Moreover, CREB plays key roles in inflammation, metabolism, development, proliferation, differentiation, adaptive responses, and signal transduction (Johannessen et al., 2004; Shaywitz et al., 1999). Studies by Rosenberg et al. (Rosenberg et al., 2002) have demonstrated that CREB may play important roles in development of endocrine tissues. Additionally, another study pointed out differences in CREB protein expression in the fetal zone of normal human adrenal cortex when compared to the definitive zone (Rosenberg et al., 2003). These studies are important since it shows the role of CREB in endocrine tissues specially that of the fetal adrenal gland, which, our laboratory is currently performing research on. Also, CREB has been shown to become active in response to hypoxic stress in different cell lines (Meyuhas et al., 2008), and rat brain regions (Omata et al., 2008). Important to our studies, CREB/CRE modulator (CREM), which is a member of the CREB family, has been implicated in StAR protein expression, gene transcription, and steroidogenesis in MA-10 mouse Leydig tumor cells (Manna et

al., 2002; Manna et al., 2003). An important question is, what is the role of CREB on cAMP-induced transcriptional activity, and protein expression of StAR in the ACTH-induced cortisol synthesis in the ovine LTH FACs? In regards to CREB and StAR protein regulation in the ovine LTH FACs, not much is known, however, studies by Clem et al. (Clem et al., 2005) have shown the cAMP stimulates phospho CREB interaction with the StAR gene promoter region in mice. Therefore, the studies on CREB status in the ovine LTH FACs will shed important information in terms of the role of CREB in the mechanisms governing enhanced cortisol secretion in the ovine LTH FACs.

The Role of ERK1/2 on Steroidogenesis

The classical ACTH/cAMP signaling pathway is generally accepted as the major mechanism involved in Cortisol biosynthesis in mammalian species. Cortisol is important for proper organ development and growth, and plays an important role in the process of parturition in the sheep fetus. Our laboratory has previously found that development under conditions of long-term hypoxia (LTH) in the ovine fetus has profound effects on StAR protein, expression of key steroidogenic genes P45011A1 (CYP11A1) and P450c17 (CYP17), as well as ACTH-induced steroidogenesis in the adrenal gland (Imamura et al., 2004; Myers et al., 2005b). In light of these observations we then decided to investigate the extracellular signal-regulated kinases (ERK1/2) as a potential key regulatory mechanism in ACTH-induced steroidogenesis in the LTH FACs. Although, the function and regulatory mechanisms of ERK1/2 are well recognized (Boulton et al., 1991; Cobb et al., 1991a; Cobb et al., 1991b; Ferreira et al., 2004; Hoeflich et al., 2009; McNeill et al., 2005; Rocha et al., 2003; Seger et al., 1991; Torii et

al., 2004), there are no data in the literature regarding ERK1/2 function and regulation in responses to LTH FACs. We do know that ERK1/2 are responsive to extracellular signals (Blenis, 1993; Crews et al., 1992a; Forti et al., 2006; Katz et al., 2007; Ullrich et al., 1990), cAMP-dependent mechanisms (Faure et al., 1994; Frodin et al., 1994; Gyles et al., 2001; Sugden et al., 1997; Yarwood et al., 1996), as well as being phosphorylated by MEK1/2 (Alessandrini et al., 1992; Brott et al., 1993; Crews et al., 1992b; Le et al., 2001). Relevant to our area of study on the mechanisms regulating enhanced cortisol secretion in the ovine LTH FACs, is the role of StAR protein, since it plays a key role in the transfer of cholesterol from the outer to the inner mitochondrial membrane (Manna et al., 2002) in cells involved in steroidogenesis. Studies by Ferreira et al. (Ferreira et al., 2007) recently demonstrated a major role for phospho ERK1/2 activity in chronic ACTH-induced steroidogenesis in the rat adrenal gland. Additionally, studies reviewed by Poderoso et al. (Poderoso et al., 2009) shows that ERK1/2 are implicated in StAR phosphorylation. Moreover, phospho ERK1/2 are implicated to play a role in cAMP-induced transcription of the StAR gene, and the activation of key transcription factors including steroidogenic factor 1 (SF-1) in response to cAMP stimulation (Gyles et al., 2001). It is possible that in addition to the classical ACTH/cAMP signaling pathway, the MAPK/ERK signaling pathway may play a permissive regulatory role in the ovine LTH FACs. Information obtained from the ERK1/2 studies will highlight regulatory mechanisms by which ovine LTH FACs can activate additional adaptive changes to maintain normal cortisol production under less than ideal situations such as LTH, as well as the capacity to allow enhanced cortisol secretion in response to further hypoxic stress.

Defining the regulatory mechanisms of LTH on the ovine fetal adrenal cortex will provide an insight into the cellular and molecular events that govern cortisol biosynthesis in the near-term sheep fetus as well as fetal adaptations that takes place during development. In summary, the review of the literature highlights major areas of research interest that will be investigated. In general, our hypothesis states that ***LTH regulates key elements involved in cortisol biosynthesis in the ovine FACs.*** The proposed studies and experimental procedures are hypothesis driven. To our knowledge, there are no data addressing the key areas currently proposed under conditions of LTH in the ovine FACs. Taken together, the data generated from the proposed studies will further our understanding of the sheep fetus adaptations to LTH.

Significance of the Effects of LTH on Cortisol Biosynthesis in Ovine FACs

The work presented in this dissertation has both implications from a basic science stand point and a clinical perspective. From a basic science aspect the research aims to further investigate the intracellular mechanisms involved in the regulation of cortisol biosynthesis and metabolism in the ovine LTH FACs. Specifically, it will provide a more in depth study of the ACTH/cAMP signaling pathway, and the permissive role of ERK1/2 signaling pathway on cortisol biosynthesis. The results from this work will also help to dissect major areas that might be involved in the enhanced cortisol production in the sheep fetus in response to a secondary stressor like LTH. Such areas include the role of cAMP, PKA, StAR protein, POMC and 22-kDa pro-ACTH, and the role of ERK1/2 phosphorylation pathway. From a clinical standpoint, these studies will provide important insight into the possible mechanism(s) that might regulate fetal development near-term in

humans, as well as the role of cortisol on the processes leading to organ maturation and parturition. A better understanding of the adaptive changes during fetal development promises the potential for design of better antenatal drugs to enhance the well being of the compromised fetus developing under less than ideal conditions. Taken together, the data generated from the proposed studies will further our understanding of fetal endocrine and physiologic adaptations to LTH.

CHAPTER 2

LONG TERM HYPOXIA ENHANCES CORTISOL BIOSYNTHESIS IN NEAR TERM OVINE FETAL ADRENAL CORTICAL CELLS

Vladimir E. Vargas, B.S.¹, Kanchan M. Kaushal, M.S.¹, Tshepo R. Monau, Ph.D.¹ Dean
A. Myers, Ph.D.², & Charles A. Ducusay, Ph.D.¹

¹Center for Perinatal Biology, Loma Linda University School of Medicine, Loma Linda,
CA, 92350 and Department of Obstetrics and Gynecology, ²University of Oklahoma
Health Sciences Center, Oklahoma City, OK, 73190

Running Head: Long term hypoxia and fetal adrenal cortisol biosynthesis

Key Words: 22-kDa pro-ACTH, ACTH, cAMP, PKA, StAR, POMC

Correspondence:

Charles A. Ducusay, Ph.D.
Center for Perinatal Biology
School of Medicine, Loma Linda
University
Loma Linda, CA 92350
cducusay@som.llu.edu
(909) 558-4325, FAX (909) 558-4029

Supported by National Institutes of Health Grants PO1HD31226, R01HD51951 and
IMSD 2R 25GM060501-05

The Work Presented in this chapter has been published,
Vargas, V. E., Kaushal, K. M., Monau, T., Myers, D. A., & Ducusay, C. A. (2010). Long-
Term Hypoxia Enhances Cortisol Biosynthesis in Near-Term Ovine Fetal Adrenal
Cortical Cells. *Reprod Sci.* PMID: 21079237.

Abstract

This study was designed to determine the potential mechanism(s) of previously observed enhanced fetal cortisol secretion following exposure to long-term hypoxia (LTH). Pregnant ewes were maintained at high altitude (3,820 m) for approximately the last 100 days of gestation. Between days 138-141 of gestation, adrenal glands were collected from LTH and age-matched normoxic control fetuses. cAMP, cortisol and steroidogenic acute regulatory (StAR) protein were measured in response to ACTH stimulation. Cortisol responses to ACTH were also measured in the presence of either the PKA inhibitor H-89, POMC or 22-kDa pro-ACTH. Cortisol output was higher in the LTH group compared to the control, ($p < 0.05$) following ACTH treatment while the cAMP response was similar in both groups. Although PKA inhibition decreased cortisol production in both groups, however, no differences were observed between groups. Western analysis revealed a significant increase in protein expression for StAR in the LTH group ($p < 0.05$, compared to control). POMC and 22-kDa pro-ACTH did not alter the cortisol response to ACTH treatment. Results from the present study taken together with those of previous *in vivo* studies suggest that the enhanced cortisol output in the LTH group is not the result of differences in cAMP generation or PKA. We conclude that enhanced cortisol production in LTH adrenals is the result of enhanced protein expression of StAR and potential downstream signaling pathways.

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is a key component of the stress response during fetal development governing the production of cortisol from the adrenal cortex. Cortisol plays a pivotal role in lipolysis, glycogenolysis, and protein catabolism and in fetal sheep increased fetal adrenal cortisol biosynthesis during the last three weeks of gestation is essential for organ maturation. The classic studies of Liggins¹ showed fetal cortisol also plays an important role in the initiation of parturition in this species. However, prematurely elevated levels of glucocorticoids can have negative effects on the fetus by suppressing anabolic processes resulting in muscle atrophy and delayed maturation and organ growth.² Therefore, regulation of adrenal cortisol biosynthesis must be well coordinated for effective responses to stress as well as to allow effective organ maturation and successful timing of delivery.

A wide range of stressors activates the fetal HPA axis, one of the most potent being hypoxia. Although much is known about the effects of acute hypoxia on fetal HPA function,³⁻⁶ very little is known about the effects of long-term hypoxia (LTH) on the fetal HPA axis including cortisol biosynthesis. Using a unique model of high altitude hypoxia during gestation in sheep, our laboratory has demonstrated a significant number of adaptive changes in the fetal HPA-axis response to LTH. We have shown that LTH increases the processing of proopiomelanocortin (POMC) to ACTH in the anterior pituitary of fetal sheep near term⁷ resulting in elevated basal plasma ACTH₁₋₃₉ in the LTH fetuses compared to the normoxic control group. In addition, the LTH fetuses also exhibited elevated circulating levels of the ACTH precursors (POMC and 22-kDa pro-ACTH). Despite higher levels of basal plasma ACTH₁₋₃₉, basal plasma cortisol

concentrations in the LTH group remained the same as control and were accompanied with a decreased adrenocortical expression of key steroidogenic enzymes, P450 cholesterol side chain cleavage [CYP11A1] and P450 17 α -hydroxylase [CYP17], in the LTH fetuses when compared to the normoxic controls.⁸ Schwartz et al.,⁹ showed that POMC and 22-kDa pro-ACTH exert a significant inhibitory effect on ACTH-induced cortisol synthesis in ovine fetal adrenal cortical cells *in vitro*. These data suggest that under basal conditions, the elevated levels of ACTH precursors play a role in maintaining normal cortisol secretion despite elevated ACTH levels in the LTH fetus. However, despite these differences, LTH fetuses demonstrate an enhanced cortisol response to a secondary stressor such as umbilical cord occlusion¹⁰ or hypotension¹¹ compared to normoxic controls and as such are able to overcome a putative inhibitory signal governing the adaptive basal adrenocortical function.

A key question arising from these studies is what is the mechanism(s) that is responsible for this adaptation observed in the LTH fetus at the level of the adrenal gland? ACTH, via cAMP and its protein kinase (PKA) initiates steroidogenesis by liberating cholesterol via cholesterol esterase. PKA also activates steroidogenic acute regulatory (StAR) protein, transferring cholesterol to the inner mitochondrial membrane where CYP11A1 (P450 side chain-cleavage) metabolizes cholesterol to pregnenolone, the initial limiting step in steroidogenesis.^{12, 13} We designed the present study to test the hypothesis that the enhanced cortisol output following a secondary stressor in LTH fetuses is the result of enhanced activity of either the cyclic 3,5-adenosine mono phosphate (cAMP), protein kinase A (PKA), or the steroidogenic acute regulatory protein

(StAR) pathway. We also determined if the LTH alters fetal adrenal cortical sensitivity to the ACTH precursors POMC and 22-kDa pro-ACTH.

Methods

Animals Procedures

All procedures were conducted with approval of the Institutional Animal Care and Use Committees (Loma Linda University School of Medicine, Loma Linda, CA). Pregnant sheep were transported to Barcroft Laboratory White Mountain Research Station in Bishop, California at an elevation of 3,820 m at ~day 40 of gestation and maintained at this facility to near term (term = 146 days) at which time they were transferred to Loma Linda University. Upon arrival, hypoxia was maintained in these animals by nitrogen infusion through a maternal tracheal catheter to maintain maternal arterial PO₂ levels similar to that observed at high altitude (<60mmHg) as previously described.^{10,11} On days 138-141 of gestation both normoxic control and LTH ewes were sedated with pentobarbital sodium, intubated, and maintained under general anesthesia with 1.5-2% halothane in oxygen. Fetuses were then delivered through a midline laparotomy, and the fetal adrenal glands were collected in ice-cold media M-199 (Sigma-Aldrich, St. Louis, MO), containing 2.2 g Sodium Bicarbonate, 2.0 g bovine serum albumin and 0.1 g L-Glutamine.

Adrenocortical Cell Dispersion

The adrenal cortex was separated from the medulla, enzymatically dispersed in 0.4% collagenase solution containing 40 mg collagenase Type II (Worthington Biomedical, Lakewood, NJ), 40 mg of Polypep bovine protein digest (Sigma), and 100 µl

of DNase I (Type IV) from Sigma-Aldrich (dissolved in 10 ml of Sodium Krebs Buffer). Following dispersion, fetal adrenal cortical cells (FACs) were aliquoted (2.5×10^5 cells) into individual tubes or 24 well cell culture plates with media (M-199), and allowed to equilibrate for 2 hours prior to initiation of each study as required by each experimental protocol. Cell viability was confirmed by Trypan blue exclusion.

Cyclic 3,5-Adenosine Mono Phosphate and Cortisol Responses to ACTH

For assessment of cortisol responses to ACTH, FACs, in duplicate, were treated with 10 nmol/L ACTH and cells and media were collected at 0 min (baseline), 15, 30, and 60 min after treatment, frozen in liquid nitrogen and stored at -80°C until determination of cAMP and cortisol, respectively.

Effects of PKA Inhibition on Cortisol Biosynthesis

The role of the cAMP-dependent PKA on cortisol biosynthesis was determined by pre-treating FACs (2.5×10^5 cells/well; in triplicate) with the PKA inhibitor, H-89 ($5\mu\text{M}$) for 1 hour followed by ACTH treatment (100 pM). Media samples were collected at 0 min (baseline) and 1, hour after addition of ACTH. All media samples were frozen in liquid nitrogen for determination of cortisol.

Effects of POMC and 22-kDa Pro-ACTH on Cortisol Biosynthesis

Recombinant ovine POMC and 22-kDa pro-ACTH were produced as previously described.¹⁴ FACs (2.5×10^5 cells/well; in triplicate) were either untreated, or treated with either ACTH (100 pM), POMC (200 pM) or 22-kDa pro-ACTH (200 pM) alone or

ACTH plus either POMC or 22-kDa pro-ACTH. Media samples were collected at time 0 (baseline) and 1 hour after treatments, and then frozen in liquid nitrogen for determination of cortisol.

Cyclic 3,5-Adenosine Mono Phosphate Analysis

Upon retrieval from -80°C, tubes containing FACs were thawed in ice/water and the media was carefully removed from the pelleted cells. Tissue was homogenized using a pestle mortar for three minutes per sample, on ice, adding 100 µl ice-cold 6% Trichloroacetic acid (TCA) in 1.5 ml polypropylene tubes. Following homogenization, the tube and pestle tip were rinsed with 200 µl 6% TCA (2x) to obtain a final total volume of 600 µl. Samples were incubated for an additional 45 minutes. At the end of the incubation step, the samples were centrifuged at 2000 X g for 15 min at 4°C, the supernatant recovered and transferred to 50 ml conical tubes. The supernatant was extracted 3 times with 5 parts of water saturated diethyl ether. The upper ether layer was aspirated and discarded after each wash. After the ether was fully evaporated from the sample, 400µl of the supernatant was transferred into polypropylene tubes and dried under a stream of nitrogen at 60°C. Samples were stored at -80°C until the day of analysis. cAMP was analyzed using a cAMP [¹²⁵I] assay System Non-acetylation procedure radioimmunoassay (RIA) from GE HealthCare (Piscataway, NJ). On the day of assay the dried extracts were reconstituted in 1000 µl volume of assay buffer prior to analysis and the procedures for cAMP determination were followed as per manufacture's instructions.

Cortisol Assay

Cortisol was measured using a commercially available ELISA cortisol Kit (Oxford Biomedical Research, Oxford, MI) that has been previously described and validated for use in our laboratory.^{15, 16}

Western Blot Analysis of StAR Protein

StAR protein was analyzed from FACs collected at 0 (baseline) and 60 minutes for both normoxic control and LTH groups as described above. FACs were lysed in 100 μ l of lysis buffer (97.9% prelysis buffer [150 mM NaCl, 50 mM Tris-HCL, 10 mM EDTA, 0.01% Tween-20, 0.01% β -mercaptoethanol], 1.67% PMSF, 0.2% leupeptin, 0.1% DTT, 0.04% aprotinin [Sigma, St Louis, MO]) and left on ice for 40 minutes. Homogenates were centrifuged at 15,000 X g for 15 minutes at 4°C and the resulting supernatants aliquoted and stored at -80°C. Protein concentration was determined by using a BCA protein assay (Pierce Bioreagents, Rockford, IL) with bovine serum albumin as the standard.

Absorbance was measured at 595 nm on a Perkin Elmer (Waltham, MA) bioassay plate reader. StAR expression was determined by Western blotting using methods, which we have previously described and validated.^{7, 8} Briefly, protein samples were denatured for 5 minutes at boiling temperature, then, a total of 10 μ g of protein were loaded per lane. Protein samples were separated using 15% polyacrylamide gels (Bio-Rad, Hercules, CA) and subjected to electrophoresis (SDS PAGE) in running buffer containing 30 ml Tris/Glycine/SDS stock, and 270 ml distilled water at room temperature. Electrophoresis was performed at 121 volts for 55 minutes and protein transferred to nitrocellulose membranes using a Transblot cell apparatus (Bio-Rad) at 4°C, 270 mA for 2 hours. The

transfer buffer consisted of 80 ml of concentrated transfer buffer, 160 ml of methanol (analytical reagent grade), and 560 ml of distilled water. Nitrocellulose membranes were subsequently incubated with a rabbit polyclonal StAR primary antibody (Abcam, Product# ab3343). The primary antibody solution contained 5 % non-fat dry milk (0.5g) in 10 ml of Tris-Buffered Saline containing 0.1 % Tween 20 (TTBS). The primary antibody was added at a 1:1000 dilution (10 μ l of primary antibody in 10 ml blocking solution) and membranes incubated at 4°C overnight on a 3D rotator. The membranes were then washed 3 X 15 minutes each with TTBS washing buffer on a 3D rotator, and incubated with anti-rabbit IgG (Goat), HRP-labeled, secondary antibody (NEF812, PerkinElmer) solution at a 1:5000 dilution (10 μ l of secondary antibody in 50 ml blocking solution) for 75 minutes on a 3D rotator at 4°C. The membranes were then washed 3 X 15 minutes each with TTBS washing buffer on a 3D rotator, treated with chemiluminescence solution (Chemi-Glow, Alpha Innotech) prior to imaging with the FluorChem digital imaging system (Alpha Innotech, San Leandro, CA) as we have previously described.¹⁶ The integrated density values (I.D.V.) of the bands were used to compare normoxic control and LTH StAR expression. An internal positive standard prepared from whole fetal adrenal tissue was used to normalize StAR protein.

Statistical Analysis

Differences between normoxic control and LTH FACs were compared using ANOVA with Bonferroni post-tests used were appropriate and $p < 0.05$ was considered significant. All data are presented as means \pm SEM.

Results

Cortisol and cAMP Responses to ACTH

Basal cortisol secretion in control FACs remained relatively constant throughout the study period (Figure 2.1A). In contrast, in the LTH group, cortisol secretion increased steadily during the study period and was significantly greater than control ($p < 0.05$) at 30 and 60 min (Figure 2.1A). Following ACTH treatment, there was a significant increase in cortisol biosynthesis in both control and LTH FACs (Figure 2.1B). However cortisol output was significantly higher in the LTH group when compared to the control, ($p < 0.05$) at 15, 30 and 60 min. Basal cAMP levels were not different between groups (Figure 2.2A). In response to ACTH, there was a marked increase in cAMP levels in both control and LTH FACs. However, there was no difference in cAMP levels between control and LTH FACs at any of the time points measured.

Effects of PKA Inhibition on Cortisol Biosynthesis

Similar to the studies presented in Figure 2.1, the cortisol response to ACTH was significantly greater in the FACs from the LTH group compared to control at 1h (Figure 2.3). In response to H-89 inhibition of PKA, cortisol biosynthesis was decreased to levels observed in the un-stimulated FACs in both groups and the overall percent decrease in cortisol response with H-89 was similar in both control (28.7 ± 3.4) and LTH (23.2 ± 3.9) ($p > 0.05$).

StAR Protein Expression

Western analysis of StAR showed that the mature, inactive (30 kDa) form of StAR was significantly higher in the LTH group when compared to the normoxic control, ($p < 0.05$) under un-stimulated conditions (1.02 ± 0.07 vs. 0.59 ± 0.05 , LTH vs. control respectively), and at 60 min following ACTH treatment (1.26 ± 0.04 vs. 0.65 ± 0.05 , LTH vs. control respectively), integrated density values (Figure 2.4).

Proopiomelanocortin (POMC) and 22-kDa Pro-ACTH

POMC and 22-kDa pro-ACTH alone did not affect basal cortisol synthesis in either control or LTH FACs (Figure 2.5). In response to ACTH treatment, there was a significant increase in cortisol biosynthesis in both control and LTH FACs. However, the cortisol response in the LTH group was greater than control ($p < 0.01$). Simultaneous treatment with ACTH and either POMC or 22-kDa pro-ACTH, did not affect cortisol synthesis compared to ACTH treatment alone.

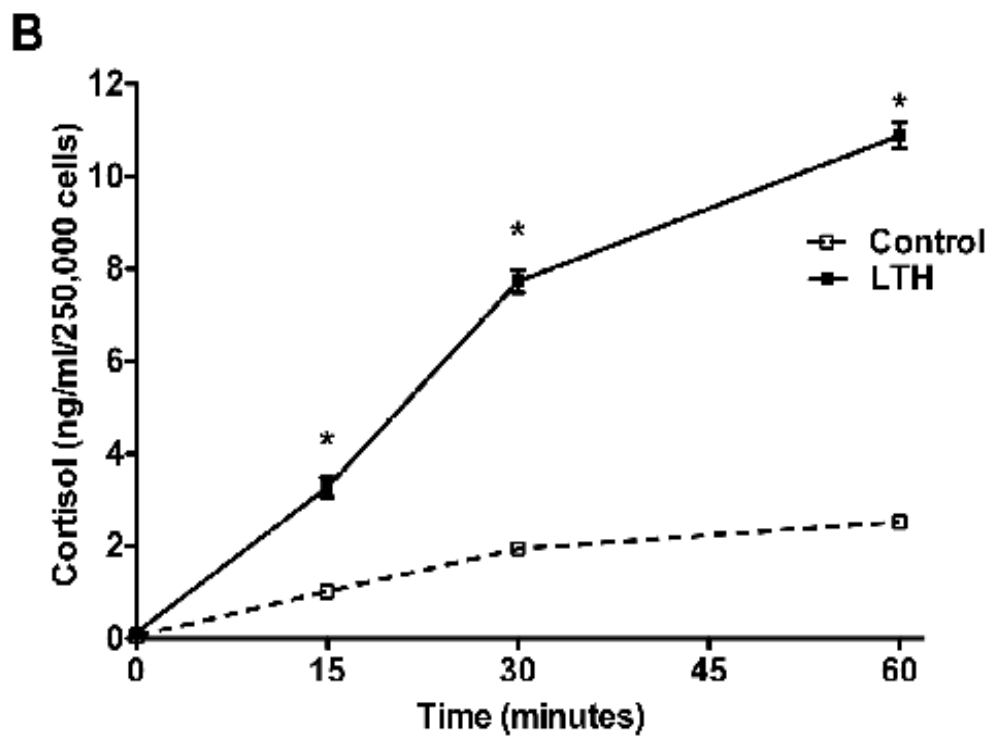
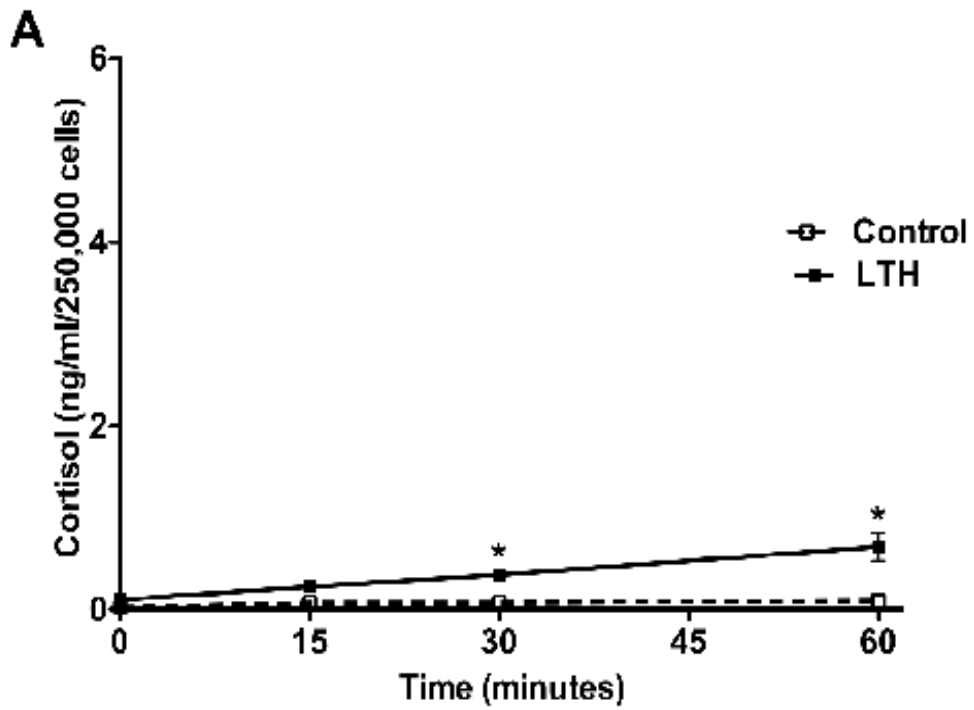


Figure 2.1 Time course of basal (A) and ACTH-stimulated (B) cortisol production in control and LTH FACs. Under basal conditions, cortisol output was significantly greater in the LTH group compared with control (* $p < 0.05$). Although ACTH (10nM) increased cortisol biosynthesis in both groups (n=6), the response was significantly greater in the LTH group compared to control ($p < 0.05$).

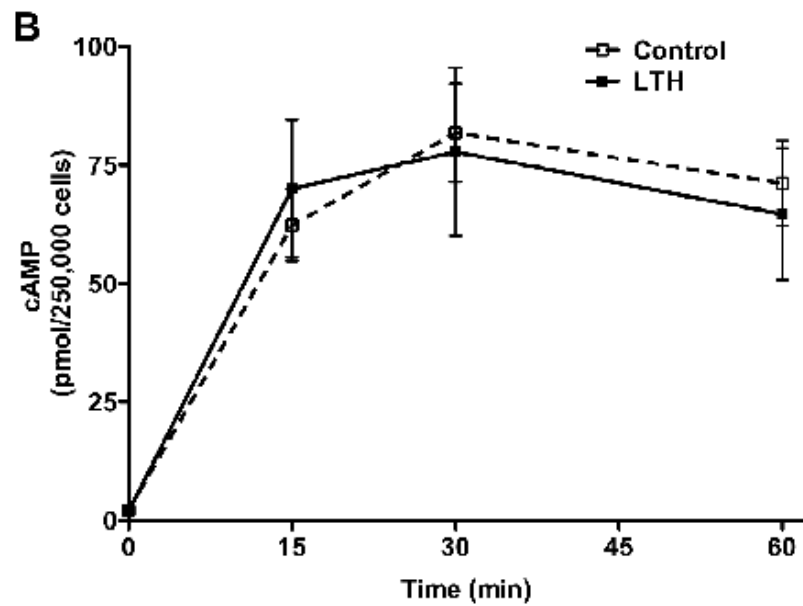
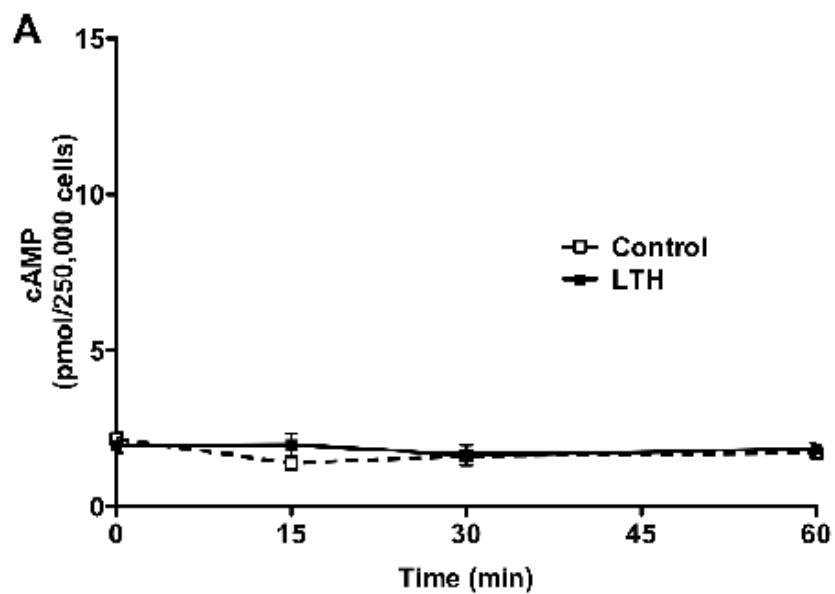


Figure 2.2 Time course of basal (A) and ACTH-stimulated (B) cAMP production in control and LTH FACs (n=6). There was no difference in either basal or ACTH-stimulated cAMP generation between control and LTH FACs.

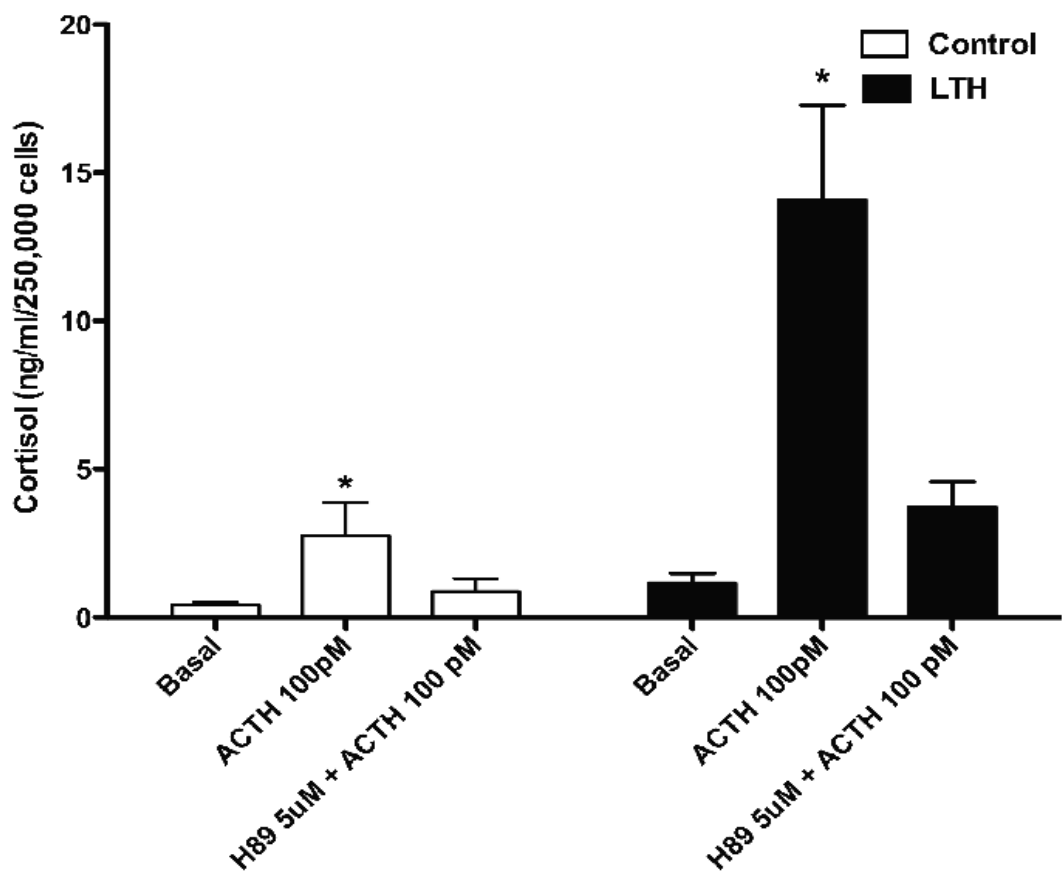


Figure 2.3 Effects of ACTH-stimulated cortisol production in response to PKA inhibition with H-89. In response to ACTH alone, cortisol secretion increased significantly in both groups (Control n = 6, LTH = 7) compared to basal cortisol secretion (*p<0.05). Pretreatment with H-89 returned cortisol secretion to basal levels.

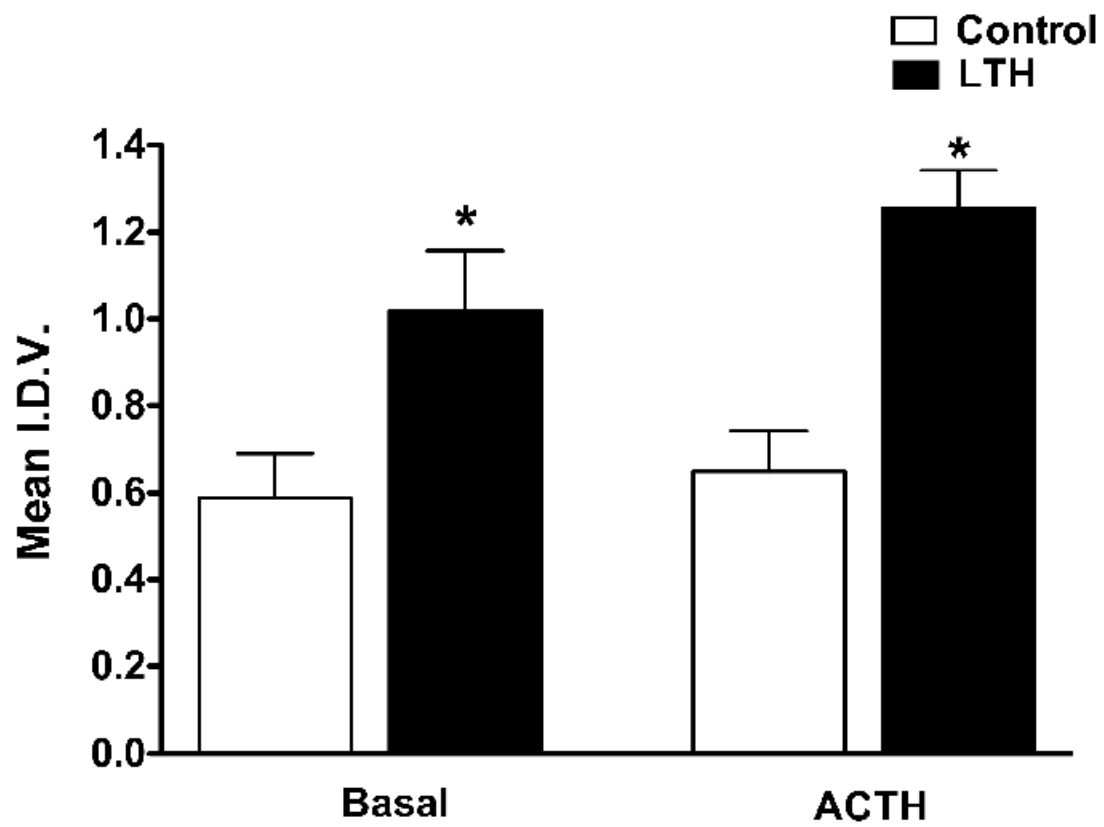


Figure 2.4 StAR protein expression in control and LTH FACs before and after ACTH treatment. Protein expression was significantly greater in LTH (n = 6) vs. control (n= 6) FACs under both basal conditions and ACTH stimulation (*p<0.05 compared to control).

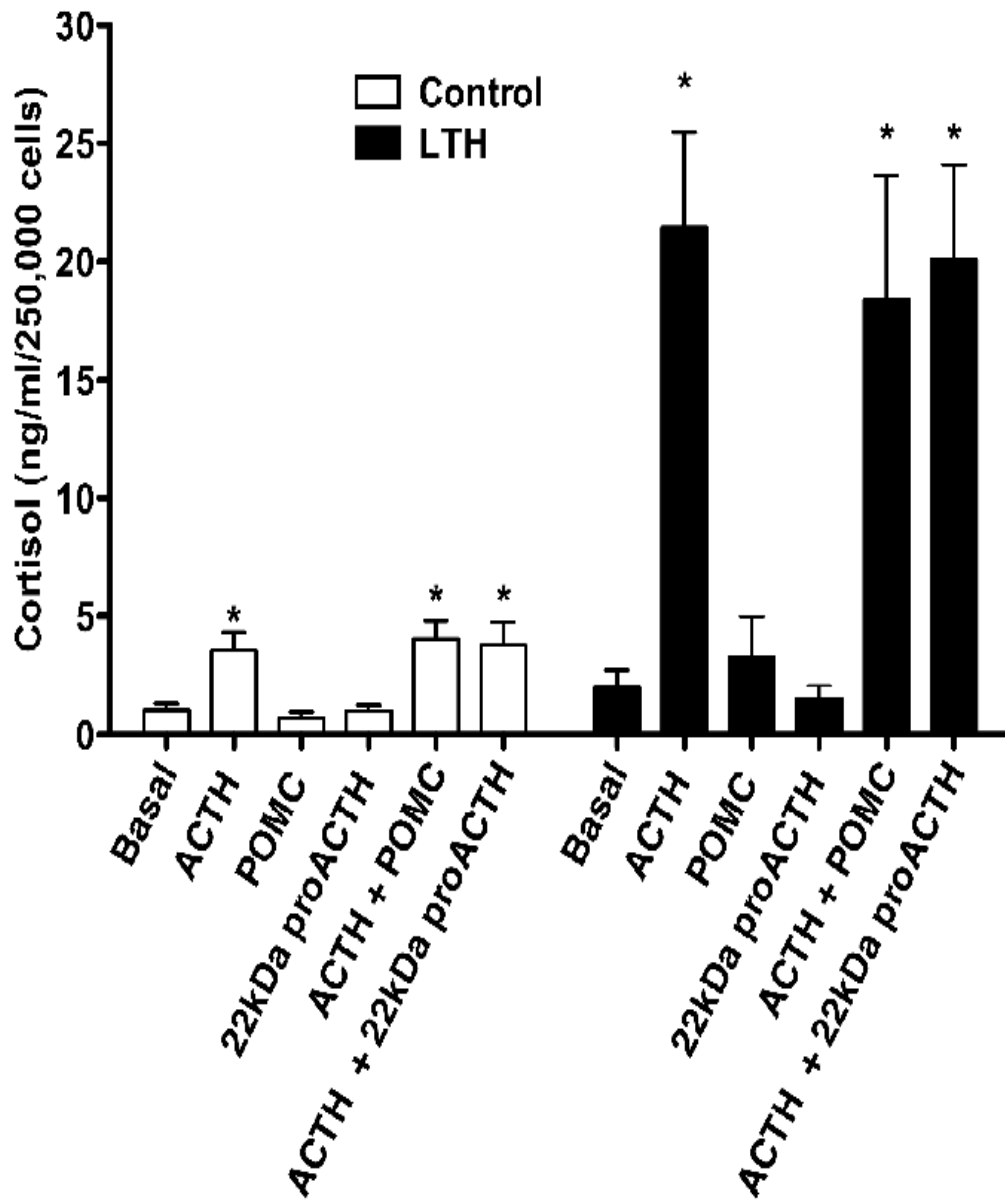


Figure 2.5 ACTH-stimulated cortisol production in control (n = 5) and LTH (n = 5) FACS in response to ACTH alone or simultaneous treatment with ACTH and either POMC or 22-kDa pro-ACTH. ACTH treatment significantly increased cortisol production in both groups which was unaffected by POMC or 22-kDa pro-ACTH (p<0.05 compared to basal).

Discussion

Our laboratory has previously demonstrated that LTH results in a significant number of adaptive changes in the fetal HPA-axis. Under basal conditions, despite higher plasma levels of immunoreactive ACTH₁₋₃₉ in the LTH fetus, cortisol concentrations are similar to normoxic controls.^{7, 11} However, in response to a secondary stressor such as umbilical cord occlusion, LTH fetuses exhibited an enhanced cortisol response compared to normoxic controls.¹⁰ Similarly, in response to a less severe secondary stressor (hypotension) the cortisol response was also enhanced in the LTH fetal sheep.¹¹ We also showed that LTH increases plasma ACTH precursors (POMC and 22-kDa pro-ACTH).⁷ Taken together, these results suggest that the fetal adrenal sensitivity to ACTH decreases in response to development under conditions of long term hypoxia preventing over- or early- activation of the fetal adrenal cortical maturation while the capacity to respond to acutely elevated ACTH during stress is enhanced. We designed the present study to address potential cellular mechanism(s) of the enhanced cortisol secretion in response to elevated ACTH and the response to the putative inhibitory ACTH precursors.

In an effort to elucidate these adaptive changes, we utilized an *in vitro*, primary cell culture system. An initial step was to determine the response of the LTH FACs to ACTH *in vitro*. There was a significant increase in cortisol biosynthesis in both control and LTH FACs in response to ACTH. However, the overall response in the LTH group was greater than control. Importantly, this response mimics what we previously observed in *in vivo* studies in response to secondary stressors.^{10, 11} Since we previously showed that mRNA for the ACTH receptor and mRNA and protein expression for the key steroidogenic enzymes CYP11A1 and CYP17 involved in cortisol synthesis are actually

reduced in response to LTH,⁸ it was apparent that other mechanisms are involved in this enhanced response to ACTH in the LTH group.

ACTH mediates its signal transduction primarily via activation of adenylate cyclase resulting in cAMP generation and downstream protein kinase A activation.^{17, 18} Since this is the first key step in this signaling pathway, we hypothesized that the LTH FACs would demonstrate an enhanced cAMP response. In the present study, however, cAMP levels increased during the first 30 minutes following ACTH stimulation, and remained elevated throughout the remainder of the experiment with values similar between groups (Figure 2.2B). Considering that we previously observed a nearly 50% decrease in ACTH receptor expression in the LTH fetal adrenal cortex,¹⁹ the retained capacity to produce cAMP compared to control fetuses would suggest that some aspect of adenylate cyclase activation has been increased to compensate for the lower ACTH receptor expression. This could be via increases in either $G_{\alpha s}$ expression or increases in adenylate cyclase itself. Alternatively, phosphodiesterase activity could be suppressed in the LTH FACs providing for elevated cAMP levels despite lowered ACTH receptor expression. In the present study, we did not use a phosphodiesterase inhibitor since we wanted to focus on cortisol production associated with physiological levels of cAMP in a manner similar to other studies.^{20, 21 22} Clearly, future studies will be needed to resolve differences in the expression and or coupling of $G_{\alpha s}$ /adenylate cyclase to the ACTH receptor and/or phosphodiesterase activity.

Since cAMP production was similar in LTH and control FACs, yet cortisol production elevated in LTH FACs, PKA itself might represent a site mediating the increased cortisol production in response to ACTH. In the present study we chose to

examine the role of PKA by utilizing the PKA inhibitor, H-89. The results from this study showed that inhibition of PKA in ovine FACs plays an important role in the regulation of ACTH induced cortisol synthesis consistent with adult adrenocortical cells. As observed in the cAMP study above, the cortisol response to ACTH alone was significantly greater in the LTH FACs compared to control (Figure 2.3). Importantly this same difference was observed when we used a more physiological dose of ACTH (100pM) and peak values were similar to when 10 nmol/L ACTH was used. In response to H89 inhibition of PKA the net decrease was significantly greater in the LTH group compared to control. However, this was the result of the enhanced cortisol response to ACTH alone. However, the overall percent inhibition of the cortisol response to ACTH was similar in both groups, suggesting that PKA activity is similar in control and LTH FACs. To our knowledge, these are the first studies to directly demonstrate the role of PKA in cortisol biosynthesis in the ovine fetal adrenal.

One of the initial steps in steroidogenesis is the transfer of cholesterol to the inner mitochondrial membrane via StAR²³ and is regarded as the first rate-limiting step in steroidogenesis. Hypoxia was previously shown to enhance StAR protein expression in neonatal rat adrenal glands.²⁴ Recent studies from our laboratory showed that StAR expression was greater in the LTH fetal adrenal cortical tissue when compared to control.⁸ This implicates StAR as a potential key target for the enhanced cortisol synthesis observed in the present study in the LTH FACs. These results suggested that StAR might play a more important role during the process of glucocorticoid synthesis under conditions of LTH. Since StAR protein is involved in the transfer of cholesterol from the outer to the inner mitochondrial membrane, elevated StAR may serve to provide more

cholesterol for processing to cortisol suggesting that enhanced cholesterol delivery may occur in these animals to compensate for lower CYP11A1 and CYP17 expression. In the present study, Western analysis showed that the mature, inactive (30 kDa) form of StAR was increased in the LTH group when compared to the normoxic control substantiating the *in vivo* findings. StAR expression was significantly elevated in LTH adrenocortical cells (both basal and after 1 hour of ACTH stimulation). In the absence of ACTH, there was elevated cortisol production in the LTH cells compared to control, likely reflecting basal cholesterol transport due to the higher StAR. In response to ACTH, the elevated StAR would facilitate the increased steroidogenesis observed in the LTH adrenocortical cells. Although phosphorylation of StAR via PKA is thought to activate StAR translocation and thus cholesterol transfer we were unable to evaluate differences in StAR phosphorylation between LTH and control FACs since there are currently no antibodies available for detecting phospho-StAR in ruminants. A role for StAR in the enhanced cortisol biosynthesis in LTH FACs is also supported by our prior observation that there are no differences in expression of downstream steroidogenic enzymes, CYP21⁸ and CYP11B1.²⁵ This would likely implicate early steps in cortisol biosynthesis that are the target of LTH. An intriguing aspect for future studies would also be to examine if differences exist in the LTH FACs in mitochondrial numbers and/or mitochondrial function.

An important characteristic of PKA is that its role in phosphorylation is short lived and reversible, with dephosphorylation mediated by phosphoprotein phosphatases.²⁶ There are various studies that have shown that phosphoprotein phosphatases (PP1, PP2A, and PP2B) are highly expressed in the adrenal and luteal cells²⁷⁻²⁹. Recent data suggest

that phosphoprotein phosphatases may play a role in the regulation of StAR protein expression and cortisol biosynthesis³⁰⁻³². Studies have shown that inhibition of PP1 and PP2A activities with calyculin A and Cantharidin causes an inhibition of StAR protein expression, and a dose-response inhibition of steroidogenesis. Additionally, other reports have shown that inhibition of PP1 and PP2A activities with calyculin A, Okadaic Acid a selective inhibitor of PP2A, and Cantharidin causes an inhibition at the level of transcription of key enzymes such as CYP11A1³³. It is possible that phosphorylation and dephosphorylation events may play a dual-specificity role in the regulation of steroidogenesis under conditions of LTH.

Although the study of phosphoprotein phosphatases was beyond the scope of the present study, this may be an area worthy of future investigation. It is possible that since StAR protein is increased in the LTH adrenocortical cells *in vitro*, then the phosphorylation state of StAR may have been higher. An increase in the amount of StAR protein would require addition of phosphate groups to drive the activity of the StAR protein. Several studies point to post-translational modifications such as phosphorylation as being involved in the events leading to steroidogenesis particularly suggesting a regulatory event at the level of steroidogenic acute regulatory protein (StAR),³⁴⁻³⁷. For instance, Arakane *et al.*³⁴, demonstrated *in vitro* that mutating potential sites of phosphorylation in the StAR protein reduces the capacity of COS-1 cell extracts to incorporate ³²P into StAR protein. The study showed the phosphorylation sites were located at serine 194/195. Furthermore, the work of Orme-Johnson *et al.*,³⁸ led to the discovery that StAR phosphorylation is initiated as a result of activating the ACTH-cAMP signaling pathway and subsequent phosphorylation of the protein³⁹.

A unique and unexpected finding of the present studies was that while basal cortisol production in control FACs was relatively constant over the one hour incubation period as expected, cortisol production increased in the LTH FACs during the one hour incubation period (Figure 1.1A). This observation suggested removal of an inhibitory factor found *in vivo* which limits basal cortisol production in these fetuses. Previous studies from Schwartz et al.⁹ showed that the ACTH precursors POMC and 22-kDa pro-ACTH exert a significant inhibitory action on cortisol synthesis in fetal but not adult adrenocortical cells from sheep. This study provided an indication that the capacity to respond to the ACTH precursors is regulated and unique to the fetal adrenal cortex. Since our previous *in vivo* studies demonstrated enhanced basal plasma concentrations of ACTH₁₋₃₉ as well as POMC and 22-kDa pro-ACTH in LTH fetuses, it seemed possible that the capacity to respond to the inhibitory ACTH precursors is enhanced in the LTH FACs. Thus, we hypothesized that POMC and 22-kDa pro-ACTH were the inhibitory factors that were removed in the *in vitro* setting.⁷

To test this hypothesis, we treated FACs from control and LTH fetuses with either ACTH alone or in combination with POMC or 22-kDa pro-ACTH in a manner similar to that previously described by Schwartz et al.,⁹. In marked contrast to results from the Schwartz study⁹, we found that neither of the peptides had an effect on cortisol biosynthesis in either control or LTH FACs (Figure 2.5). It is important to point out two key differences in their study and the present data. The first is that the majority of adrenals in their studies were collected from animals at an earlier gestational age than those used in the present study (138-141 dG). Given the noted lack of effect of the precursors on cortisol production in the adult adrenocortical cell as noted by Schwartz et

al.,⁹ it is likely that the ovine fetal adrenal becomes insensitive to the inhibitory effects of the ACTH precursors during the final maturational processes that occur in the adrenal cortex as term gestation approaches. Secondly, the ACTH precursors used in Schwartz's study were purified from pituitaries while in the present study, the precursors were recombinant ovine POMC and 22-kDa pro-ACTH. Perhaps the purification process used by Schwartz et al.,⁹ changed the biological activity from the original native molecules or that differences in glycosylation state in the recombinant proteins compared to those of pituitary origin may have altered their bioactivity. In either case, the ACTH precursors had no inhibitory effect in our study. It does not appear therefore that the observed enhanced precursor levels in LTH fetuses play a role in the regulation of cortisol biosynthesis during late gestation.

Other factors found in the circulation inhibit glucocorticoid biosynthesis. Yuen et al.⁴⁰ found that leptin infusion into the ovine fetus had an inhibitory effect on cortisol secretion *in vivo*. *In vitro* studies using rat and human adrenal cortical cells also demonstrated that leptin inhibited glucocorticoid production.⁴¹ We previously showed that *in vivo*, LTH ovine fetuses had plasma leptin levels approximately 3-fold higher than normoxic controls.⁴² Perhaps the removal of the higher level of leptin inhibition *in vitro* allowed greater basal expression of cortisol in the LTH group. This hypothesis is currently under investigation.

In summary, the developing LTH sheep fetus has adapted to the adverse conditions of chronic hypoxia. Data from the present study indicate that the ability of the LTH fetal adrenal to respond to a secondary stressor with enhanced cortisol production is not the result changes in cAMP, PKA or potential effects of POMC or 22-kDa pro-ACTH. StAR

protein expression however is a likely candidate mediating at least in part, the observed enhanced cortisol biosynthesis in the LTH FACs. Interestingly, the Extracellular Signal-Regulated Kinases (ERK1/2) have been shown to regulate cAMP-dependent cortisol production at the level of StAR gene transcription. We have recently shown that inhibition of the MEK/ERK pathway results in significant inhibition of cortisol biosynthesis in FACs.⁴³ We are currently investigating the role of ERK1/2 in the regulation of StAR in the LTH ovine fetal adrenal.

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CHAPTER 3
ADDENDUM TO CHAPTER 2
STEROIDOGENIC ACUTE REGULATORY (STAR) PROTEIN mRNA EXPRESSION
IN NEAR TERM OVINE FETAL ADRENAL CORTICAL CELLS: EFFECTS OF
LONG-TERM HYPOXIA

Abstract

We previously demonstrated enhanced cortisol secretion in the long-term hypoxic (LTH) fetal adrenocortical cells (FACs) that was not the result of differences in cyclic 3,5-adenosine mono phosphate (cAMP) and or protein kinase A (PKA). However, steroidogenic acute regulatory (StAR) protein expression was increased in the ovine LTH vs. the normoxic control FACs in response to ACTH treatment. The present study tested the hypothesis that StAR mRNA is increased in the ovine LTH when compared to the normoxic control FACs in response to ACTH treatment. Pregnant ewes were maintained at high altitude (3,820 m) for approximately the last 100 days of gestation. At 138-141 dG, fetal adrenal glands were collected from LTH and age-matched normoxic control fetuses, and dispersed adrenal cortical cells (2.5×10^5 cells/well; in duplicate) were challenged with 10^{-8} M ACTH. Cortisol output was higher in the LTH group compared to the control, ($p < 0.05$) following ACTH treatment. qRT-PCR analysis of StAR mRNA was not different between groups. However, there was a significant increase in StAR mRNA over time within each group in response to ACTH treatment, ($p < 0.05$). Results from the present study taken together with those of previous *in vivo* and *in vitro* studies

suggest that the enhanced cortisol output in the LTH group is not the result of enhanced StAR mRNA expression. The enhanced cortisol production in LTH adrenals is the result of downstream signaling pathways distal StAR mRNA expression, and may involve post-translational modifications at the level of StAR protein, and regulatory events at the level of the ERK signaling pathway.

Introduction

The study presented in this chapter on steroidogenic acute regulatory (StAR) protein mRNA expression in response to adrenocorticotrophic (ACTH) treatment in the ovine long-term hypoxic (LTH) fetal adrenocortical cells (FACs) is an addendum to chapter 2, and the data will become part of a later publication. Previous studies from our laboratory have shown important adaptations at the level of the sheep fetal adrenal cortex. For example, *in vivo* studies performed by Adachi et al. (Adachi et al., 2004) and Imamura et al. (Imamura et al., 2004) have shown that in response to a secondary stressor like umbilical cord occlusion (UCO), and or hypotension, cortisol secretion is enhanced in the LTH when compared to the normoxic control group despite no difference in basal plasma ACTH levels. In order to elucidate the mechanism(s) responsible for this enhanced cortisol secretion we also analyzed StAR protein, and key steroidogenic enzymes protein expression using whole adrenal tissue homogenates. The results showed increased StAR protein expression, and decreased P45011A1 (CYP11A1) and P450c17 (CYP17) enzymes protein expression (Myers et al., 2005b). These results suggested a significant regulatory role at the level of StAR protein on cortisol biosynthesis in the LTH FACs that may be responsible for the enhanced cortisol secretion in the LTH FACs.

At least in part, it appears that the enhanced cortisol secretion observed in the LTH FACs may be the result of increased transfer of cholesterol from the outer to inner mitochondrial membrane that can be accomplished by an increased in available StAR protein. Interestingly, current *in vitro* studies from our laboratory have shown important adaptations that have taken place throughout most of gestation in the ovine FACs under conditions of LTH (Vargas et al., 2010). The study showed that in response to ACTH treatment cortisol secretion in the LTH is enhanced when compared to the normoxic control FACs. The results from the study showed no significant differences in cyclic 3,5-adenosine mono phosphate (cAMP), protein kinase A (PKA), and or ACTH precursors (POMC and 22-kDa pro-ACTH). However, StAR protein expression was increased in the LTH when compared to the normoxic control FACs in response to ACTH treatment. An important question that remains to be addressed is, what is the state of StAR transcriptional activity in LTH FACs? It is possible that StAR mRNA is higher in the ovine LTH FACs to direct the synthesis of additional StAR protein to transfer more cholesterol to the inner mitochondrial membrane of the LTH FAC, allowing a compensatory mechanism that maintains enhanced cortisol secretion despite decreased steroidogenic enzyme expression. Taken together from the cumulative data on previous *in vivo* and *in vitro* experiments, and data from others (Gyles et al., 2001; Manna et al., 2009), the logical step to follow was to measure StAR mRNA expression. Therefore, we designed this study to test the hypothesis that StAR mRNA levels are higher in the ovine LTH when compared to the normoxic control FACs in response to ACTH treatment. The results from this study will provide insight into the mechanism(s) regulating cortisol biosynthesis in the LTH FACs.

Methods

Animal Procedures

All procedures were conducted with approval of the Institutional Animal Care and Use Committees (Loma Linda University School of Medicine, Loma Linda, CA). Pregnant sheep were transported to Barcroft Laboratory White Mountain Research Station in Bishop, California at an elevation of 3,820 m from ~day 40 of gestation to near term (term = 146 days) at which time they were transferred to Loma Linda University. Upon arrival, hypoxia was maintained in these animals by nitrogen infusion through a maternal tracheal catheter to maintain maternal arterial P_{O_2} levels similar to that observed at high altitude (<60mmHg) as previously described (Adachi et al., 2004; Imamura et al., 2004). On days 138-141 both LTH and normoxic control ewes were sedated with pentobarbital sodium, intubated, and maintained under general anesthesia with 1.5-2% halothane in oxygen. Fetuses were then delivered through a midline laparotomy, and the fetal adrenal glands were collected in ice-cold media M-199 (Sigma-Aldrich, St. Louis, MO), containing 2.2 g Sodium Bicarbonate, 2.0 g bovine serum albumin and 0.1 g L-Glutamine.

Adrenocortical Cell Dispersion

The adrenal cortex was separated from the medulla, enzymatically dispersed in 0.4% collagenase solution containing 40 mg collagenase Type II (Worthington Biomedical, Lakewood, NJ), 40 mg of Polypep bovine protein digest and 100 μ l of DNase I (Type IV) (Sigma-Aldrich,) dissolved in 10 ml of Sodium Krebs Buffer. The FACs were aliquoted (2.5×10^5 cells) into 24 well cell culture plates with media (M-199),

and allowed to equilibrate for 2 hours prior to initiation of the study as required by each experimental protocol. Cell viability was confirmed by Trypan blue exclusion.

Cortisol and StAR mRNA Responses to ACTH

For assessment of cortisol and StAR mRNA responses to ACTH, FACs, in duplicate, were treated with 10 nmol/L ACTH and tissue and media were collected at 3 hours (baseline) and at 3 hours after ACTH treatment, frozen in liquid nitrogen and stored at -80°C until determination of cortisol, and StAR mRNA expression.

qRT-PCR Analysis of StAR mRNA Expression in Ovine FACs

Total fetal adrenocortical RNA was isolated as per manufacture's instructions (RNAqueous-Micro, Ambion, Austin, TX) by treating frozen samples containing adrenal cortical cells in individual tubes (250,000 cells/tube; n=6). Prior to qRT-PCR, total RNA was treated with DNase I (1 unit) at 37°C for 30 minutes. Reverse transcription was performed using 350 ng of total input RNA per sample using oligo dT₂₁ as the primer, and I-script (Bio-Rad, Hercules, CA) as the reverse transcriptase in a reaction mixture of 20 µl. Real-time PCR was performed using 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 real-time PCR was performed utilizing a Bio-Rad iCycler equipped with the real-time optical fluorescent detection system. Primer sequences were as follows: b-StAR-Forward608 (5'-CAG AAG ATT GGA AAA GAC ACG GTC-3'), and b-StAR-Reverse857 (5'-AGG TGA GTT TGG TCC TTG AGG G-3'). A three 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 "housekeeping" mRNA; primer sequences were as follows: bovine cyclophilin forward primer (5'-CCA TCG TGT GAT CAA GGA CTT CAT-3'), and reverse primer (5'-CTT GCC ATC TAG CCA GGC AGT CTT-3'). 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. Extrapolation of unknowns from the standard curve was performed using prism 3 (graph Pad Software INC., san Diego, CA) predicting unknowns from the standard curve Ct values.

Statistical Analysis

Differences between LTH and normoxic control FACs were compared using ANOVA with Bonferroni post-tests used were appropriate and $p < 0.05$ was considered significant. All data are presented as means \pm SEM

Results

Cortisol Responses to ACTH

Basal cortisol secretion remained relatively constant throughout the study period and was not different between groups at 3 hours (Figure 3.1). Following ACTH treatment, there was a significant increase in cortisol biosynthesis in both control and LTH FACs (Figure 3.1). However, cortisol output was significantly higher in the LTH group at 3 hours following ACTH treatment, when compared to the control, ($p < 0.05$).

qRT-PCR Analysis of StAR mRNA in Ovine FACs

RT-PCR analysis of StAR mRNA expression showed no difference in the LTH when compared to the normoxic control FACs under un-stimulated conditions, and following ACTH treatment, (Figure 3.2). However, StAR mRNA revealed a significant increase over time within each group in response to ACTH treatment (* $p < 0.05$), when compared to basal levels, (24.38 ± 7.44 vs. 57.98 ± 3.54 , Basal vs. ACTH treatment, control), and (38.06 ± 2.95 vs. 62.35 ± 4.38 , Basal vs. ACTH treatment, LTH).

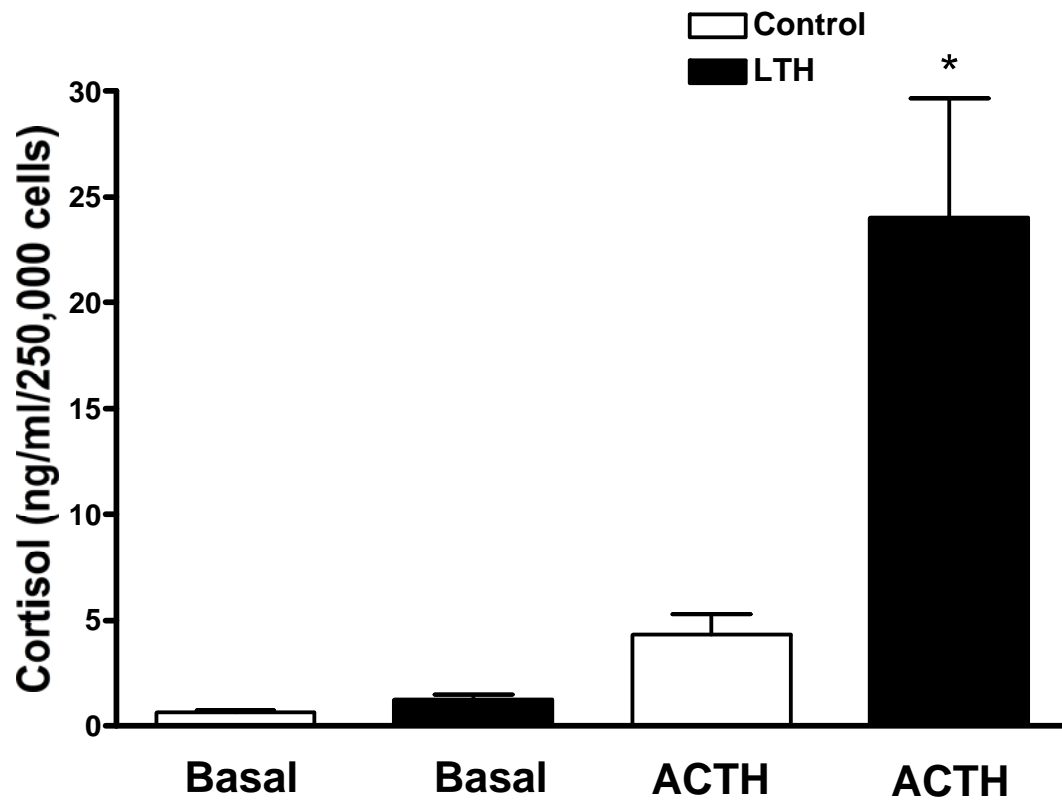


Figure 3.1 Basal and ACTH-stimulated cortisol production at the end of 3 hours in control and LTH FACS. Under basal conditions, cortisol output was not different between groups. Although ACTH (10nM) increased cortisol biosynthesis in both groups (n=6), the response was significantly greater in the LTH group compared to control (p<0.05).

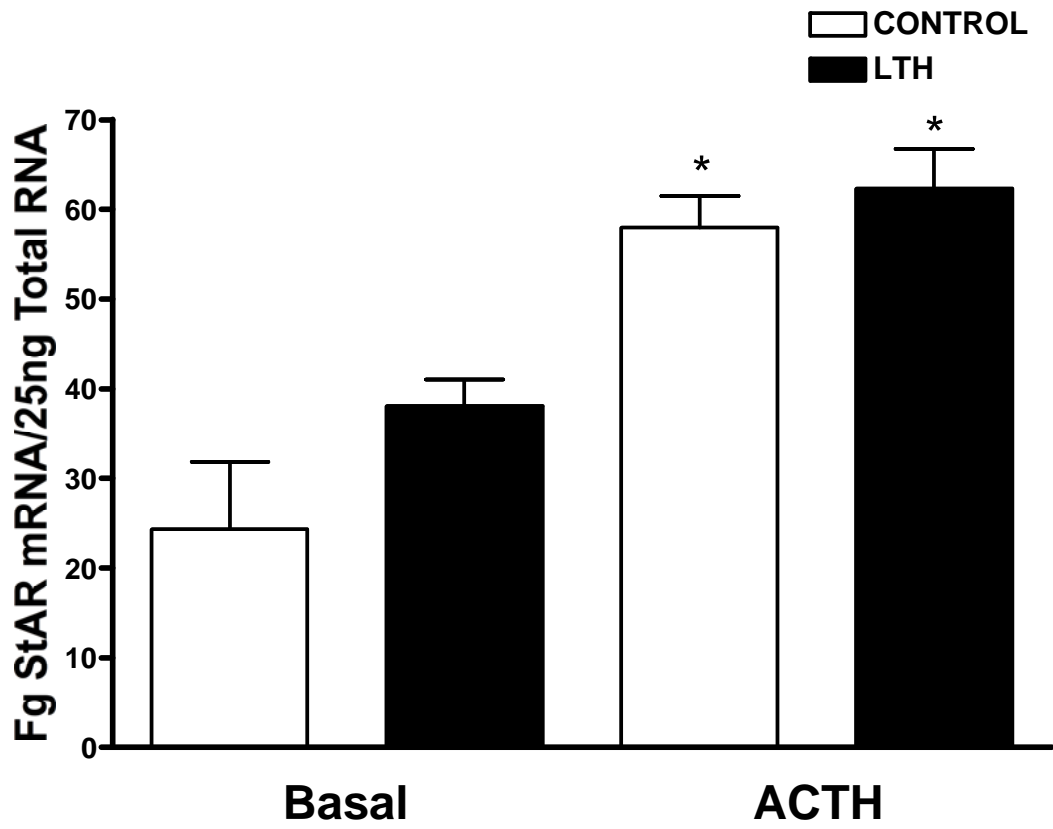


Figure 3.2 StAR mRNA gene expression in normoxic control and LTH FACs before and after 3 hours ACTH treatment. There was no difference in either basal or ACTH-stimulated mRNA expression between normoxic control (n= 4) and LTH (n = 5) FACs. StAR mRNA gene expression was significantly greater in each group in response to ACTH stimulation when compared to basal conditions (*p<0.05 compared to basal).

Discussion

The transfer of cholesterol from the outer to the inner mitochondrial membrane is an important regulatory event in cortisol biosynthesis in steroid producing cells (Christenson et al., 2000; Privalle et al., 1983; Stocco, 2000). A number of studies have shown that transfer of cholesterol and steroidogenesis could not be accomplished effectively without the aid of StAR protein (Manna et al., 2005; Miller, 2007; Papadopoulos et al., 2007; Stocco et al., 2005). However, little is known about the role of StAR on steroidogenic tissues under conditions of LTH.

Our laboratory has found significant adrenal adaptations to LTH with a decrease in expression of key steroidogenic enzymes P45011A1 (CYP11A1) and P450c17 (CYP17) (Myers et al., 2005b). Despite this difference compared to control adrenals, in response to secondary stressors (Adachi et al., 2004; Imamura et al., 2004), cortisol secretion was enhanced in the LTH when compared to the control group. A potential explanation for this enhanced cortisol secretion in the LTH FACs is the possibility of additional cholesterol in the LTH FACs and more StAR protein would be made available to transport cholesterol to the inner mitochondrial membrane allowing enhanced cortisol secretion despite decreased steroidogenic enzyme expression.

The results from the present study revealed that basal cortisol secretion remained relatively constant throughout the study period and was not different between groups at 3 hours (figure 3.1). These results are different from that reported previously (Vargas et al., 2010), however, a key point of the current study is that we measured basal cortisol at the end of 3 hours, whereas our previous studies focused on a more acute time course for up to 1 hour. It is possible that as time progresses the normoxic control basal cortisol

production parallels that of the LTH FACs. Following ACTH treatment, there was a significant increase in cortisol biosynthesis in both control and LTH FACs (Figure 3.1). However, cortisol output was significantly higher in the LTH group at 3 hours after ACTH treatment, when compared to the control FACs. These results are in agreement with our previous work that showed an enhanced cortisol secretion in response to ACTH treatment in the LTH when compared to the normoxic control FACs. We also showed that this enhanced cortisol secretion in the LTH group was not the result of differences in cAMP, and or PKA (Vargas et al., 2010).

StAR protein expression however, was increased in the LTH group when compared to the normoxic control FACs. Based on the finding that StAR protein expression was higher in the LTH when compared to the normoxic control FACs, we then decided to measure StAR mRNA gene expression. Since the interplay of hormones, and signaling pathways acting on the transcriptional machinery are so complex (Manna et al., 2009), we measured StAR mRNA at the end of 3 hours following ACTH treatment of the FACs. Contrary to our initial hypothesis, StAR mRNA gene expression was not different between control and LTH FACs. The results showed that Basal StAR mRNA expression had the tendency to be higher in the LTH when compared to the normoxic control FACs but the difference did not reach statistical significance. However, it is possible that basal StAR mRNA is higher in the LTH vs. normoxic control FACs, potentially reflecting additional StAR protein made available under basal conditions (Vargas et al., 2010). This would serve to transfer more cholesterol into the inner mitochondrial membrane to direct cortisol biosynthesis in the absence of ACTH

treatment and lower expression of key steroidogenic enzymes CYP11A1 and CYP17 hydroxylases.

StAR mRNA expression was not different in the LTH when compared to the normoxic control FACs following 3 hours of ACTH treatment, however, there was a significant increase of StAR mRNA transcriptional activity within each group over time (3 hours), reflecting the capacity of the adrenal cells to produce StAR protein and cortisol in both groups. The results from the present study on StAR mRNA are in agreement with our previous observations in whole adrenal tissue homogenates (Myers et al., 2005b). In these studies our laboratory used whole adrenal tissue homogenates instead of FACs, and the results showed that StAR mRNA expression was not different in the LTH when compared to the normoxic group. An important aspect about this study was the fact that the adrenal tissue homogenate was not treated with ACTH in either LTH or normoxic control group. Also, Western blot analysis on StAR protein expression using whole adrenal tissue homogenates demonstrated that the 30 kDa span form of StAR protein was higher in the LTH when compared to the normoxic control group. Together, the studies on FACs and whole tissue adrenal homogenates results demonstrated that StAR might be playing a more significant role in the enhanced cortisol secretion in response to ACTH treatment in the LTH ovine FACs.

Based on the current findings on a lack of effect of ACTH treatment to enhance StAR mRNA expression, other potential mechanisms might be involved. For example, what is the role of StAR transcriptional activity and the relationship between cAMP-dependent StAR gene transcription mediated through the extracellular signal-regulated kinases (ERK1/2), and or PKA-mediated CREB signaling pathway in the ovine LTH

FACs? Studies by Gyles et al. (Gyles et al., 2001) have clearly shown that StAR gene transcription mediated through cAMP-dependent mechanisms are regulated in part by ERK1/2, which in turn phosphorylate steroidogenic factor -1 (SF-1), leading to an increase in the transcriptional capacity of the StAR mRNA resulting in steroid synthesis. Our current results suggest that StAR gene transcriptional activity is turned on in both LTH and normoxic FACs. It is possible that StAR mRNA expression in the LTH FACs is mediated by extracellular signals suggesting a role on phosphorylation events mediated by ACTH treatment and activation of cAMP signaling pathways. However, the studies on transcriptional regulation of StAR expression are beyond the scope of the present study, however, they represent an important area of future research that will allow a better understanding of the mechanism regulating enhanced cortisol secretion in the ovine LTH FACs. Additional studies will be required to address this paradigm.

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CHAPTER 4

THE EXTRACELLULAR SIGNAL-REGULATED KINASES (ERK1/2) SIGNALING PATHWAY PLAYS A ROLE IN CORTISOL SECRETION IN THE NEAR TERM OVINE FETAL ADRENAL IN VITRO: EFFECTS OF LONG-TERM HYPOXIA

Vladimir E. Vargas, B.S.¹, Kanchan M. Kaushal, M.S.¹, Tshepo R. Monau, Ph.D.¹ Dean
A. Myers, Ph.D.², & Charles A. Ducsay, Ph.D.¹

¹Center for Perinatal Biology, Loma Linda University School of Medicine, Loma Linda,
CA, 92350 and Department of Obstetrics and Gynecology, ²University of Oklahoma
Health Sciences Center, Oklahoma City, OK, 73190

Running Head: Long term hypoxia and fetal adrenal cortisol biosynthesis

Key Words: 22- kDa pro-ACTH, ACTH, cAMP, PKA, StAR, POMC

Correspondence:

Charles A. Ducsay, Ph.D.
Center for Perinatal Biology
School of Medicine, Loma Linda University
Loma Linda, CA 92350
cducsay@som.llu.edu
(909) 558-4325, FAX (909) 558-4029

Supported by National Institutes of Health Grants PO1HD31226, R01HD51951 and
IMSD 2R 25GM060501-05

Abstract

This study assessed the role of the ERKs on the previously observed enhanced cortisol secretion in response to ACTH treatment in the long-term hypoxic (LTH) fetal adrenocortical cells (FACs). Ewes were taken to high altitude (3,820 m) from 40 to 138-141 dG. Animals were transported from high altitude to the lab, and reduced P_{O_2} was maintained by nitrogen infusion through a maternal tracheal catheter. At 138-141 dG, fetal adrenal glands were collected from LTH and control groups. Dispersed FACs (2.5×10^5 cells/tube; in duplicate), were challenged with 10^{-8} M ACTH. ERK inhibition was studied in additional cells pre-treated with UO126 for 1hr (10 μ M) before ACTH stimulation. Media, and FACs were collected at time 0 (baseline), 5, 10, 15, 30, and 60 min after ACTH stimulation for cortisol, and Western analysis of ERK1/2, and pERK1/2. Basal cortisol secretion was higher in the LTH compared with control. Following ACTH treatment cortisol was greater in the LTH group similarly. UO126 inhibited ACTH-mediated cortisol output in both groups. Under basal conditions, ERK1/2 and pERK1/2 protein were no different between groups. Following ACTH treatment, ERK 1/2 protein were no different between groups, however, pERK1/2 were higher in the LTH and levels declined in control only. UO126 (Time 0 min) had no effect on ERK 1/2 when compared to untreated levels. ACTH treatment resulted in decline of protein levels. UO126 resulted in 10 to 20 fold in reduction of protein in both groups. We conclude that basal ERK signaling in FACs is necessary for normal cortisol production. Sustained pERK in LTH induces greater cortisol production. ERKs are involved in part in ACTH-induced steroidogenesis possibly by playing a more permissive role of integrating information from extracellular signals.

Introduction

The classical adrenocorticotrophic (ACTH) hormone, 3,5-cyclic adenosine mono phosphate (cAMP) signaling pathway is generally accepted as the major mechanism regulating cortisol biosynthesis in the adrenal cortex (Daniel et al., 1998; Grahame-Smith et al., 1967; Rae et al., 1979; Rall et al., 1958; Sewer et al., 2002b; Sutherland et al., 1958). During gestation, cortisol is important for optimal fetal organ growth and maturation, and plays an essential role in the process of parturition in the sheep fetus (Liggins, 1994; Liggins et al., 1973). Our laboratory has developed a model of high altitude induced long-term hypoxia (LTH), and found that development under conditions of LTH has profound effects on the hypothalamic-pituitary-adrenal (HPA) axis (Adachi et al., 2004; Imamura et al., 2004; Myers et al., 2005a; Myers et al., 2005b). While expression of key steroidogenic genes P45011A1 (CYP11A1) and P450c17 (CYP17) as well as ACTH receptor mRNA is reduced in the adrenal cortex of the LTH fetus, ACTH-induced cortisol biosynthesis is paradoxically enhanced (Monau et al., 2010; Myers et al., 2005b). Recently, we demonstrated that the enhanced cortisol secretion observed in the LTH fetal adrenocortical cells (FACs) compared to normoxic controls was not the result of enhanced cAMP production and/or Protein kinase A (PKA) stimulation. However, expression of steroidogenic acute regulatory (StAR) protein was higher in the LTH vs. normoxic control FACs implicating enhanced cholesterol transport to the inner mitochondrial membrane as one potential mechanism for the enhanced cortisol production in response to ACTH treatment (Vargas et al., 2010).

Extracellular signal-regulated kinases (ERKs) have been suggested to play a regulatory role in ACTH-induced steroidogenesis because of their potential to

phosphorylate and thus activate StAR (Poderoso et al., 2008; Poderoso et al., 2009).

Although, the function and regulatory mechanisms of ERK1/2 are well recognized in a number of cell systems (Boulton et al., 1991; Cobb et al., 1991a; Cobb et al., 1991b; Ferreira et al., 2004; Hoeflich et al., 2009; Seger et al., 1991; Torii et al., 2004), the mechanisms of ERK1/2 activation in the sheep fetus adrenal cortex has not been studied. There is also a lack of data on the effects of LTH on ERK 1/2 function.

In general, ERK1/2 are activated by phosphorylation by MEK1/2 (Alessandrini et al., 1992; Brott et al., 1993; Crews et al., 1992b) which itself is activated by RAF. A variety of extracellular signals converge at RAF to stimulate ERK signaling (Blenis, 1993; Crews et al., 1992a; Faure et al., 1994; Frodin et al., 1994; Sugden et al., 1997; Ullrich et al., 1990; Yarwood et al., 1996). Relative to adrenal cortisol biosynthesis, studies by Ferreira et al. (Ferreira et al., 2007) recently demonstrated a major role for phospho ERK1/2 activation in chronic ACTH-induced steroidogenesis in the rat adrenal gland. Additionally, studies reviewed by Poderoso et al. (Poderoso et al., 2009) have shown that ERK1/2 are implicated in StAR phosphorylation. Moreover, phospho ERK1/2 activation are implicated in cAMP-induced steroidogenesis through transcription of the StAR gene, and the phosphorylation of key transcription factors including steroidogenic factor-1 (SF-1), (Gyles et al., 2001; Lehoux et al., 2003).

We designed the present study to examine the role of ERK1/2 signaling in our previously observed enhanced ACTH-stimulated cortisol production in LTH FACs. Specifically, we hypothesized that ERK1/2 phosphorylation is increased in LTH FACs in response to ACTH treatment when compared to levels in normoxic controls and that

blocking MEK function with UO126 would ameliorate the increased cortisol production in response to ACTH in the LTH FACs.

Methods

Animals Procedures

All procedures were conducted with approval of the Institutional Animal Care and Use Committees (Loma Linda University School of Medicine, Loma Linda, CA). Pregnant sheep were transported to Barcroft Laboratory White Mountain Research Station in Bishop, California at an elevation of 3,820 m from ~day 40 of gestation where they were maintained until near term (term = 146 days) at which time they were transferred to Loma Linda University. Upon arrival, hypoxia was maintained in the ewes by nitrogen infusion through a maternal tracheal catheter to maintain maternal arterial P_{O_2} levels similar to that observed at high altitude (<60mmHg) as previously described (Adachi et al., 2004; Imamura et al., 2004). On days 138-141 both LTH and normoxic control ewes were sedated with pentobarbital sodium, intubated, and maintained under general anesthesia with 1.5-2% halothane in oxygen. Fetuses were then delivered through a midline laparotomy, and the fetal adrenal glands were collected in ice-cold media M-199 (Sigma-Aldrich, St. Louis, MO), containing 2.2 g Sodium Bicarbonate, 2.0 g bovine serum albumin and 0.1 g L-Glutamine.

Adrenocortical Cell Dispersion

The adrenal cortex was separated from the medulla, enzymatically dispersed with 40 mg collagenase Type II (Worthington Biomedical, Lakewood, NJ), 40 mg of Polypep

bovine protein digest and 100 μ l of DNase I (Type IV) (Sigma-Aldrich,) dissolved in 10 ml of Sodium Krebs Buffer (0.4% collagenase final). The resulting mono-dispersed FACs were aliquoted (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 as required by each experimental protocol. Cell viability was confirmed by Trypan blue exclusion.

Cortisol Responses to ACTH Treatment

For assessment of cortisol responses to ACTH, FACs were treated with 10 nmol/L ACTH, and cells and media were collected separately at 0 min (baseline), 5, 10, 15, 30, and 60 min after treatment and frozen in liquid nitrogen and stored at -80°C for determination of cortisol.

Effects of ERK1/2 Inhibition on Cortisol Biosynthesis

Fetal adrenocortical cells (2.5×10^5 cells/well; in triplicate) were either untreated (basal cortisol secretion), or treated with ACTH (10 nmol/L) alone. The role of the ERKs in regulating ACTH-induced cortisol biosynthesis were determined by pre-treating FACs (2.5×10^5 cells/tube; in duplicate) with the MEK inhibitor, UO126 (10 μ M), for 1 hour followed by addition of 10 nmol/L ACTH. Media was collected at 0 (baseline), 5, 10, 15, 30, and 60 min and all samples were frozen in liquid nitrogen and stored at -80°C for determination of cortisol.

Cortisol Assay

Cortisol was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) cortisol kit (Oxford, MI) that has been previously described and validated for use in our laboratory (Ducsay *et al.*, 2009; Monau *et al.*, 2010).

Western Blot Analysis of ERK 1 and 2, and Phospho ERK 1 and 2

ERK1/2 protein expression was analyzed from FACs collected at 0 min (baseline), 5, 10, 15, 30, and 60 minutes for both LTH and normoxic control groups as described above. FACs were lysed in 150 μ l of lysis buffer (97.9% prelysis buffer [150 mM NaCl, 50 mM Tris-HCL, 10 mM EDTA, 0.01% Tween-20, 0.01% β -mercaptoethanol], 1.67% PMSF, 0.2% leupeptin, 0.1% DTT, 0.04% aprotinin (Sigma-Aldrich, St Louis, MO), 1mM sodium orthovanadate (Sigma-Aldrich), frozen in liquid nitrogen and stored at -80°C until Western blot analysis determination. Protein concentration was determined by BCA protein assay (Pierce Bioreagents, Rockford, IL) with bovine serum albumin as the standard. Absorbance was measured at 562 nm on a Synergy HT Multi-Detection Microplate reader (BioTek, Winooski, VT) using Gen5 software. Levels of ERK1/2 protein were determined by Western blotting using methods, which we have previously described and validated (Myers *et al.*, 2005a; Myers *et al.*, 2005b; Vargas *et al.*, 2010). Briefly, protein samples were denatured for 5 minutes at boiling temperature and a total of 3 (for ERK1/2) μ g of protein were loaded per lane. Proteins were subjected to SDS-PAGE using 10% polyacrylamide gels (Bio-Rad, Hercules, CA) at 200 Volts for 35 minutes until bands nearly reached the bottom of the gel. Proteins were transferred to

nitrocellulose membranes using a Transblot cell apparatus (Bio-Rad) at 4°C, 270 mA for 2 hours. Nitrocellulose membranes were incubated with commercially available rabbit monoclonal primary antibodies to total ERK1/2 (1:1000 dilution) and phospho ERK1/2 (1:2000 dilution; Cell Signaling, Danvers, MA) overnight in 5 % Bovine Serum Albumin (BSA) (Bio-Rad) in Tris-Buffered Saline containing 0.1% Tween 20 (TTBS). The membranes were incubated at 4°C with antibody solution overnight on a 3D rotator. Following the addition of ERK1/2 primary antibodies, the membranes were washed 3 X in 10 minutes total with TTBS washing buffer on a 3D rotator, and then subjected to incubation with secondary DyLight Goat Anti-rabbit antibody solution (Thermo Scientific, Rockford, IL) at a 1:10,000 dilution for ERK1/2 (1 hour) on a 3D rotator at room temperature. Following secondary antibody incubation the membranes were washed 3 X within 10 minutes each with TTBS washing buffer on a 3D rotator. The membranes were developed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). The Integrated Density Values (IDV) of the bands were used to compare LTH and normoxic control ERK1/2 expression. An internal positive standard prepared from fetal adrenal cells was used to normalize ERK1/2 protein.

Statistical Analysis

Differences between LTH and normoxic control FACs were compared using analysis of variance (ANOVA) with Bonferroni posttests used where appropriate and $P < .05$ was considered significant. All data are presented as means \pm standard error of mean (SEM).

Results

Cortisol Responses to ACTH and UO126 Treatment

Basal cortisol secretion (Figure 4.1A) was similar in both groups at the start of the experiment. However, by 30 min there was a divergence where levels became significantly higher in the LTH compared with normoxic control FACs (* $p < 0.05$) at 30min (1.28 ± 0.18 vs. 0.67 ± 0.04), and 60min (1.26 ± 0.26 vs. 0.62 ± 0.08). Following ACTH (10 nmol/L) treatment there was a significant increase in cortisol biosynthesis in both normoxic control and LTH FACs (Figure 4.1B), however, the response was significantly greater in the LTH group compared to control FACs, ($p < 0.01$) at 30min ($8.10 \pm 0.1.42$ vs. 3.19 ± 0.80), and 60min (12.43 ± 2.31 vs. 4.76 ± 1.13). Pre-treatment with UO126, inhibited ACTH-mediated cortisol secretion in both groups, however, the inhibition was significantly greater in the control (Figure 4.1C) compared to LTH FACs ($p < 0.01$) at 60min (0.80 ± 0.06 vs. 2.50 ± 0.58).

ERK1/2 and Phospho ERK1/2 Protein Expression in Response to ACTH and UO126 Treatment

Basal

Under untreated basal conditions, there were no differences in either ERK 1 (Figure 4.2A) or ERK 2 (Figure 4.2C) protein expression between control and LTH FACs across the course of the experiment. Likewise, levels of phospho-ERK 1 (Figure 4.2B) and phospho-ERK 2 (Figure 4.2D) protein did not differ between groups.

ACTH treatment

Following ACTH treatment, Western blot analysis revealed no differences in ERK1 (Figure 4.3A) protein in response to ACTH treatment in either control or LTH FACs. Likewise ERK 2 (Figure 4.3C) did not demonstrate any differences between groups although there was a trend toward higher levels in the LTH group that was not significant ($P < 0.07$). In marked contrast, in response to ACTH both phospho-ERK1 (Figure 4.3B) and phospho-ERK 2 (Figure 4.3D) were significantly higher over the course of the experiment in LTH vs. control FACs and levels declined significantly in control but not LTH FACs ($P < 0.05$).

Effects of UO126 on ACTH treatment

One hour of pre-incubation of FACs with UO126 (Time 0 min, Figure 4.4A,C) had no effect on ERK 1 or 2 when compared to untreated levels (Figure 4.2A,C). However, following the initiation of ACTH treatment, there was a significant decline ($P < 0.05$) in protein levels throughout the remainder of the study (Figure 4.4A, C). Further, UO126 resulted in a highly significant 10 to 20 fold in reduction in phospho ERK 1 and 2 protein in both groups similarly at all time points ($p < 0.01$ vs. untreated or ACTH alone).

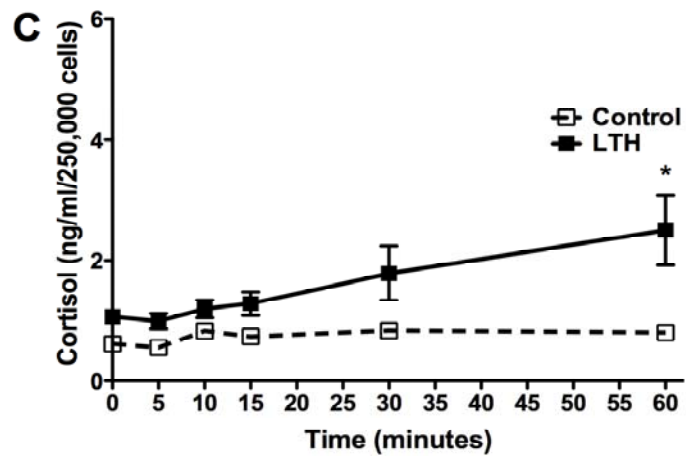
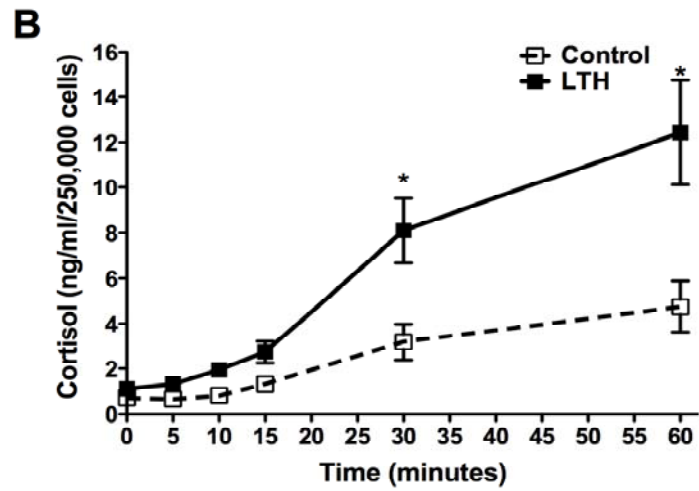
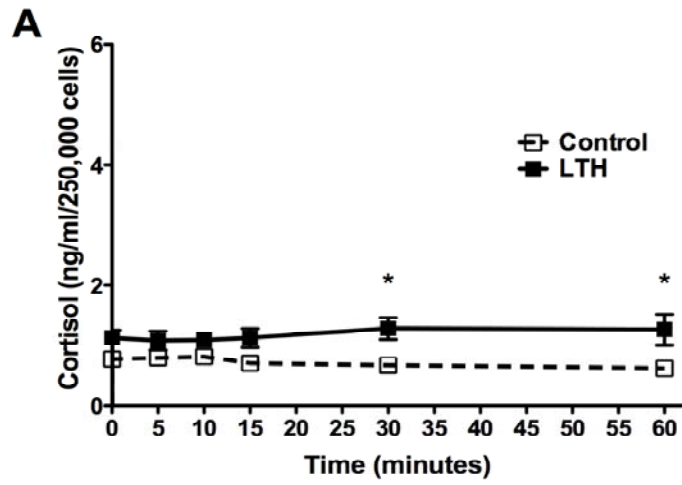


Figure 4.1 Time course of basal (A) ACTH-stimulated (B) and UO126 treatment + ACTH-stimulated (C) cortisol production in LTH and normoxic control FACs (n = 5 in each group). Under basal conditions, cortisol output was significantly greater in the LTH compared with normoxic control FACs (*p<0.05) at 30 and 60 min. Although ACTH (10 nmol/L) increased cortisol biosynthesis in both groups, the response was significantly greater in the LTH group compared to control FACs (p<0.05). Pretreatment with UO126 inhibited ACTH-stimulated cortisol secretion in both groups, however, the inhibition was significantly greater in the control compared to LTH FACs (p<0.05).

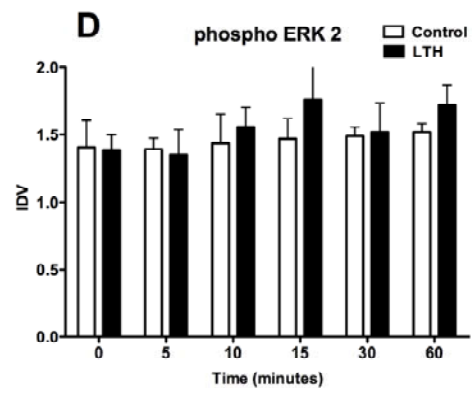
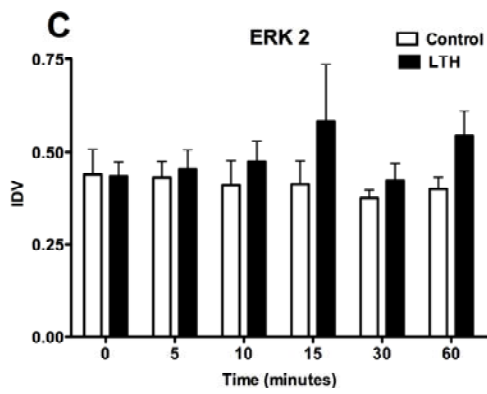
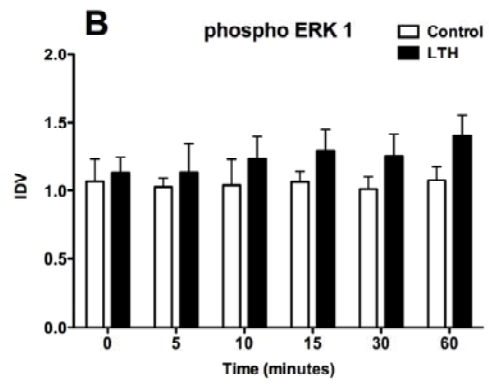
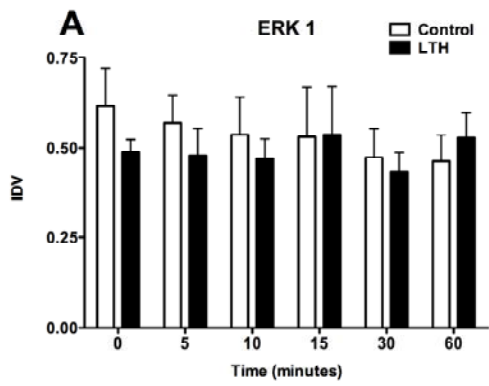


Figure 4.2 ERK1 (A), Phospho ERK1 (B), ERK 2 (C) and phospho ERK 2 (D) protein expression in control and LTH FACs (n = 5 in each group) under basal (untreated conditions). No differences between groups were observed across the time course of the experiment.

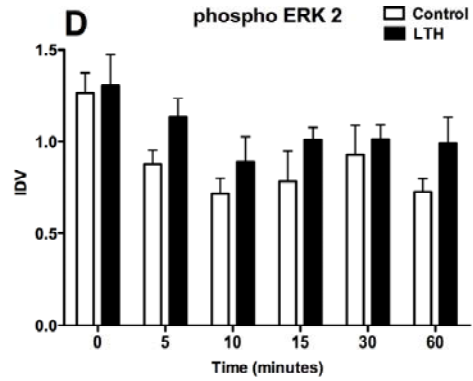
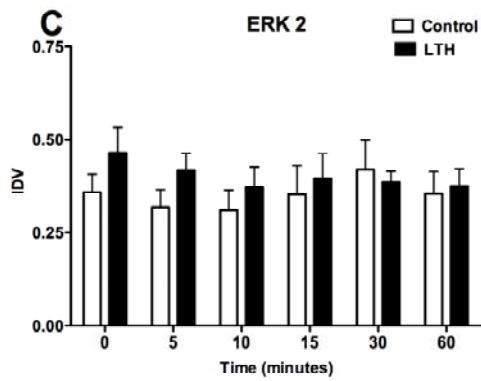
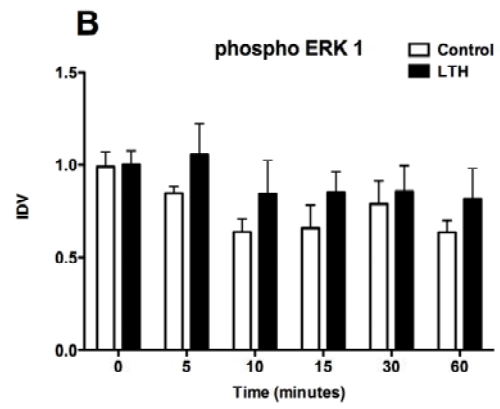
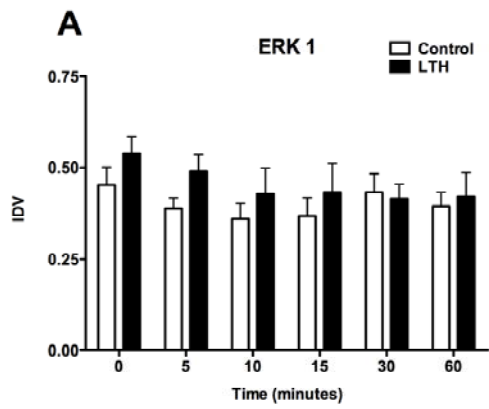


Figure 4.3 ERK1 (A), Phospho ERK1 (B), ERK 2 (C) and phospho ERK 2 (D) protein expression in control and LTH FACs (n = 5 in each group) following ACTH treatment (10 nmol/L) beginning at time 0. Phospho ERk 1 and 2 were significantly higher over the course of the experiment in LTH vs. control FACs and levels declined significantly in control but not LTH FACs ($p < 0.05$).

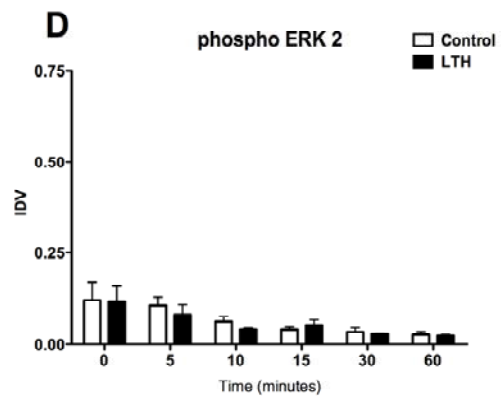
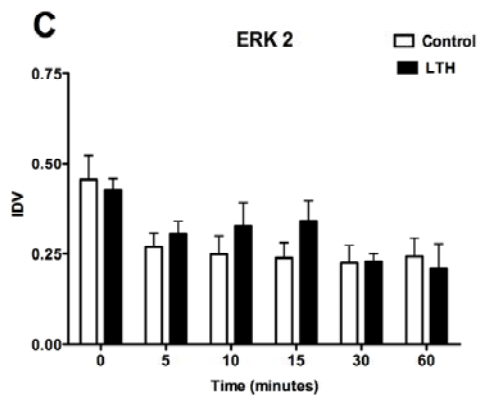
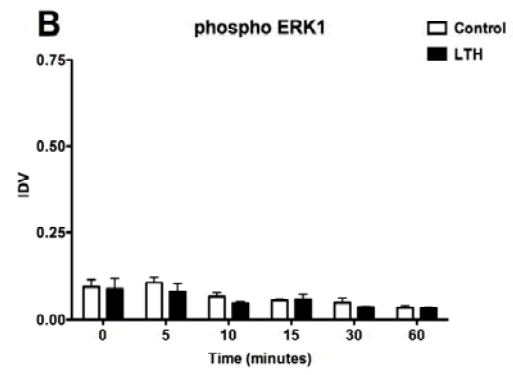
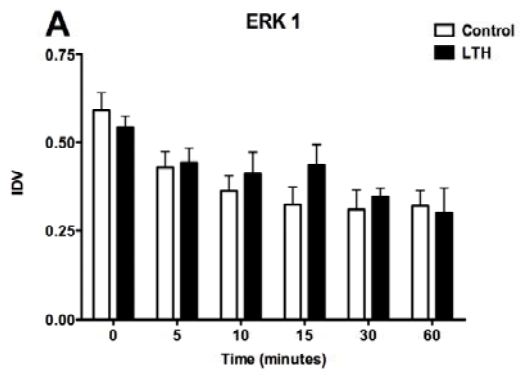


Figure 4.4 ERK1 (A), Phospho ERK1 (B), ERK 2 (C) and phospho ERK 2 (D) protein expression in control and LTH FACs (n = 5 in each group). Cells were pretreated with U0126 (10 μ M) for 1 h prior to ACTH treatment (10 nmol/L) beginning at time 0. A significant reduction (p<0.01) in ERK 1 and 2 was observed in both control and LTH following the initiation of ACTH treatment. Phospho ERK 1 and 2 protein expression was significantly reduced following U0126 treatment (p<0.01, compared to untreated) in both control and LTH FACs.

Discussion

The present study was designed to examine the potential role of ERK1/2 in ACTH stimulation of cortisol biosynthesis in the ovine fetal adrenal as well as the effect of LTH on this process. Key findings from the present study include: 1) ACTH-stimulated cortisol biosynthesis is significantly greater in LTH than control FACs; 2) the MEK inhibitor UO126 has a profound inhibitory effect on cortisol production in near term FACs, with a greater level of inhibition in control vs. LTH FACs; 3) ACTH induced a rapid (within 5-10 min), sustained decrease in pERK in control but not LTH FACs.

We have previously shown that the ovine fetus has mounted a significant adaptive endocrine strategy under the potentially adverse conditions of LTH. Despite higher basal plasma ACTH concentrations, normal basal plasma cortisol levels are maintained (Adachi et al., 2004; Imamura et al., 2004; Myers et al., 2005a). However, in response to a secondary stressor like umbilical cord occlusion or hypotension, the LTH fetus is capable of mounting an enhanced cortisol response (Adachi et al., 2004; Imamura et al., 2004). We have further defined several other key adaptive changes at the level of the fetal adrenal in response to LTH including decreased expression of CYP17 and CYP11A1 and the ACTH receptor with increased activation of StAR (Myers et al., 2005b).

Most recently, in an effort to elucidate the mechanism(s) responsible for the enhanced cortisol biosynthesis, we investigated the role of key elements of the ACTH/cAMP signaling pathway that are involved in cortisol biosynthesis in the LTH fetal FACs. The results from this study described in Chapter 2, showed that there were no apparent differences in cAMP or PKA stimulation that could explain the observed difference between control and LTH FACs. Importantly, StAR protein was higher in the

LTH group when compared to normoxic control group (Vargas et al., 2010). In light of these results, it is apparent that other signaling pathways play a regulatory role in the adaptive response of the fetal adrenal cortex to LTH.

One potential segment of this key signaling pathways are the extra cellular signal-regulated kinases 1/2. Although ERK1/2 are not well characterized in the adrenal cortex, Peterson et al., (Peterson *et al.*, 2001) clearly demonstrated the presence of these kinases in the adult ovine adrenal gland. Overall, it appears that in addition to PKA, ERKs may play a potential role in ACTH induced steroidogenesis (Hoeflich et al., 2009). However, the role of ERKs in regulating ACTH-induced steroidogenesis is controversial. In adult bovine adrenocortical cells, ERK1/2 activation inhibits ACTH-induced steroidogenesis (Cote et al., 1998), and a negative effect of ACTH was also observed by Watanabe et al. (Watanabe et al., 1997). In contrast, in mitotically active Y-1 adrenocortical cells and ovarian granulosa cells, ERK1/2 promoted hormone-induced steroidogenesis (Gyles et al., 2001; Le et al., 2001).

In the present study, consistent with our earlier findings (Vargas et al., 2010), ACTH stimulation resulted in greater cortisol production in the LTH FACs compared to normoxic controls (Figure 4.1B). Importantly, ERK1/2 inhibition with U0126 had a profound inhibitory effect on ACTH-stimulated cortisol biosynthesis in both groups. These results suggest that ERK1/2 facilitates cortisol biosynthesis in the ovine fetal adrenal. To determine the effect of ACTH on ERK1/2, we examined the levels of ERK1/2 and phospho-ERK1/2 under un-stimulated conditions and following ACTH stimulation with and without U0126. Under basal conditions, there were no differences in either ERK 1 or 2 in their phosphorylated forms between the two groups. Indeed, un-

stimulated levels of phospho-ERK1/2 were surprisingly high in both groups perhaps reflecting the mitotically active nature of the FACs. ACTH treatment had no effect on levels of ERK 1 or 2 in either group, however, ACTH decreased ERK1/2 phosphorylation in control but not LTH FACs. Considering that UO126 exerted a similar limiting action on ACTH-induced cortisol biosynthesis, the selective inhibition of ERK1/2 phosphorylation by ACTH in the control FACs may explain the lower level of cortisol production in these cells compared to those from the LTH animals. At present time it is unclear why ACTH did not exert the same inhibitory action on ERK phosphorylation in LTH FACs, however, there is evidence that hypoxia can affect ERK activation. Hypoxia activates ERK in neuronal nuclei of newborn piglets, (Mishra et al., 2004) and in endothelial cells (Zhu et al., 2003). The decreased in ERK phosphorylation in response to ACTH are in agreement with data from Sewer and Waterman (Sewer et al., 2003) indicating that ACTH stimulates dual specific phosphatases PP1 and PP2 that are capable of acting to deactivate ERK1/2. Thus the inability of ACTH to dephosphorylate pERK in LTH FACs may be responsible for the observed greater cortisol response in the LTH group compared to control.

As expected, treatment with UO126 resulted in a significant reduction in phospho-ERK 1 and 2 in both control and LTH FACs. Surprisingly, although UO126 pre-treatment did not alter ERK 1 and 2, in the absence of ACTH, there was a rapid and significant reduction in ERK1/2 in both control and LTH FACs following ACTH treatment in the presence of UO126. Unfortunately, due to a limited number of cells, a UO126 treatment alone group was not included. However, pre-treatment of FACs with UO126 for one hour prior to ACTH treatment had no effect on ERK 1/2 protein

expression and neither did ACTH treatment in the absence of the inhibitor. It appears that there is a synergistic effect of UO126 and ACTH on the degradation of ERK protein. At present, the mechanism for this effect is unknown, however, phosphorylation may serve to protect these kinases from proteosomal targeting.

ERK1/2 stimulation plays a role in up-regulation of StAR expression (Gyles et al., 2001). In our previous studies, we found that StAR protein expression is elevated in LTH FACs compared to control (Vargas et al., 2010) while mRNA levels did not differ (Chapter 3). The decrease in ERK phosphorylation in response to ACTH may therefore be responsible for the reduced levels of StAR found in the control group compared to LTH. It will be important to measure StAR protein expression following UO126 treatment.

The orphan nuclear receptor SF-1 is integral in the development of the adrenal cortex as well as cAMP (ACTH) induced expression of several key genes regulating cortisol biosynthesis including CYP17 and CYP11A1. SF-1 mediated expression of CYP17 has been demonstrated to be dependent on phosphorylation state. ERK signaling elevated SF-1 was associated with decreased cAMP induced CYP17 expression while ACTH-cAMP was associated with decreased phosphorylation of SF-1 (Sewer & Waterman, 2003b). The maintenance of phospho-ERK levels in response to ACTH in the LTH group could also play a role in our previously observed decrease in CYP17 and CYP11A1 in the fetal adrenal cortex

In summary, based on our present and previous findings, we propose that basal MEK-ERK signaling in FACs is necessary for normal cortisol production and that ACTH induced ERK dephosphorylation provides an intracellular feedback mechanism limiting

cortisol production in response to ACTH (stress). The inability of ACTH to dephosphorylate pERK in LTH FAs provides a mechanism via which ACTH induces greater cortisol production in LTH FAs. In addition to the classical cAMP/PKA pathway ERKs are 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 down stream signaling pathways are up-regulated in response to LTH.

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CHAPTER 5

CONCLUSIONS

Our studies on the cellular and molecular mechanism(s) that govern enhanced cortisol biosynthesis in the near-term ovine LTH fetus in response to secondary stressors revealed important adaptive changes in fetal adrenocortical cells (FACs). The studies evolved from previous findings from our laboratory in which plasma ACTH concentrations were increased in the LTH near-term fetus, and additional *in vivo* studies where the near-term fetus was challenged with stressors such as umbilical cord occlusion (UCO), and or hypotension (Adachi et al., 2004; Imamura et al., 2004; Myers et al., 2005a). Studies also showed that LTH resulted in a decrease in both the ACTH-receptor mRNA, as well as expression of both CYP11A1 and CYP17 enzymes, (Myers et al., 2005b). With this background in mind we developed the general hypothesis that *LTH regulates key elements involved in cortisol biosynthesis in ovine fetal adrenocortical cells (FACs)*. Our general hypothesis was in turn composed of two additional specific hypotheses that were the basis for the studies described in this dissertation. **Hypothesis 1:** In response to ACTH treatment, LTH FACs increase cortisol production when compared to the normoxic control by increasing cAMP and/or PKA activity and expression of StAR. We also examined the potential inhibitory role of ACTH precursors on cortisol secretion. **Hypothesis 2:** In response to ACTH treatment, ERK1/2 are activated to a greater degree in LTH vs. control FACs.

To test the first hypothesis, we used our *in vitro* primary cell culture system. The results from the study revealed important adaptations at the level of the ovine FACs. The data showed that under un-stimulated conditions basal cortisol output was greater in the LTH vs. the normoxic control FACs. These results were somewhat surprising since we did not expect cortisol to be higher in the LTH FACs in the absence of ACTH treatment. The elevated levels of basal cortisol can be explained in part by an increase in the amount of available cholesterol supply that might be already present in these cells as a result of LTH that in turn would require additional StAR protein synthesis in order to transfer more cholesterol to the inner mitochondrial membrane. Although, we have not performed studies addressing the actual cholesterol present in the adrenal cells, this is an attractive area that promises important findings and would contribute to our understanding of cortisol biosynthesis and secretion.

Importantly, our FACs in culture responded to “stress” levels of ACTH with an enhanced cortisol secretion in the LTH when compared to the normoxic control FACs. This result was in agreement with our previous *in vivo* observations in which umbilical cord occlusion and or hypotension resulted in an enhanced cortisol secretion in the LTH vs. the normoxic control group (Adachi et al., 2004; Imamura et al., 2004). To elucidate the mechanism(s) of enhanced cortisol biosynthesis we measured cAMP levels since this is the first key second messenger that becomes activated in response to ACTH treatment in steroid producing cells (Grahame-Smith et al., 1967; Sala et al., 1979). Contrary to our initial hypothesis, the elevated cortisol seen in our primary cell culture was not the result of differences in cAMP levels between control and LTH FACs. This indicated that other elements past the ACTH-receptor and distal cAMP appear involved in the enhanced

cortisol biosynthesis in the LTH FACs. One could argue that perhaps there was a difference in G-protein coupling or phosphodiesterase activity as possible regulatory mechanisms of cortisol biosynthesis (Durand et al., 1981b; Levitzki, 1987; Neer, 1995; Rae et al., 1979; Raymond, 1995). However, since the endpoint was no difference in cAMP levels between groups, we focused on other, more potentially important areas of the downstream signaling cascade.

Inhibition of PKA with H-89 resulted in a decrease in cortisol secretion in both groups, and the percent decrease was not different between groups. The inhibitory ACTH precursors POMC and 22-kDa pro-ACTH failed to alter cortisol secretion. Importantly, increased StAR protein expression was observed in the LTH FACs before and after ACTH stimulation when compared to control cells. Since StAR transfers intracellular cholesterol to the inner mitochondrial membrane (Fleury et al., 2004; LeHoux et al., 2004; Manna et al., 2005; Miller, 2007; Stocco, 2001) it seems likely that in response to LTH the adrenal gland has increased cholesterol storage in the form of cholesterol esters, resulting in more available precursor for cortisol biosynthesis.

In order to more clearly define the role of StAR protein on cortisol biosynthesis we need to develop additional studies focused on the phosphorylation of StAR protein (LeHoux et al., 2004) by PKA. It is possible that if the availability of cholesterol has increased in response to LTH then it would require additional StAR activation to transfer the steroid precursor. However, at present, there were no commercially available phospho StAR antibodies. A potential approach to circumvent the lack of phospho StAR antibodies includes adapting the studies of Arakane et al. (Arakane et al., 1997) into our LTH FACs model system to mutate conserved phosphorylation sites such as serine

194/195 to alanine on the StAR protein, and measuring the biological activity of the StAR protein as reflected in the capacity of PKA to phosphorylate the protein *in vitro*. Another possibility to study phospho-StAR activation is to perform antibody-antigen Immunoprecipitation (IP) studies. However, a limitation to this type of approach is the amount of fetal adrenocortical cells needed to perform IP. Since the number of cells required is large, this alone would place limitations on other potential experiments in our laboratory. Additional studies that might also shed new information in terms of the role of StAR on cortisol biosynthesis will include a focus on the phosphoprotein phosphatases (PP1, PP2A, and PP2B). These phosphatases have been shown to regulate cortisol biosynthesis (Burns et al., 2000; Jones et al., 2000; Sayed et al., 1997; Sewer et al., 2002c). It is possible that in response to ACTH treatment, the LTH ovine FACs phosphatases become inhibited in order to allow the phosphorylating potential of phospho-StAR to activate key intracellular elements that drive cortisol biosynthesis.

Since protein synthesis is highly dependent on the actual capacity of the transcription of the gene encoding StAR mRNA (Manna et al., 2009; Walsh et al., 2000) we expected changes at this level of regulation in response to LTH conditions. Our rationale was based on the studies of Gyles et al. (Gyles et al., 2001) that showed the role of the ERKs on cAMP-induced steroidogenesis through transcriptional activation of StAR mRNA expression. However, our results showed no differences in StAR mRNA expression between groups over time in response to ACTH treatment. As a result, it does not appear that differences in StAR mRNA are involved and therefore translational differences or post-translational modifications are critical.

We also explored the possibility of other signaling mechanisms that might play a somewhat more permissive role on steroidogenesis acting through cAMP-mediated events in response to extracellular signals. The studies on ERK1/2 revealed that inhibition of the upstream activator MEK1/2 with 10 μ M UO126 led to phosphorylation inhibition of ERK1/2 resulting in a cortisol decrease not only in the LTH but also in the normoxic control group. These results show for the first time, an obligatory role of ERKs in ACTH-induced cortisol secretion in the fetal adrenal. Western blot studies showed a significant decrease over time and in response to ACTH treatment in total phospho-ERK1/2 in the normoxic control group, whereas, total phospho-ERK1/2 did not change during the one hour study period in the LTH FACs. These results suggest that enhanced cortisol secretion in the LTH FACs could be in part explained by sustained phosphorylation of ERK1/2 allowing the ERKs to translocate to the nucleus to phosphorylate key transcription factors such as SF-1 to initiate StAR gene transcriptional activity.

It was also important to note that treating FACs with 10 μ M UO126 resulted in total phospho-ERK1/2 inhibition in both groups. An important future direction would be to examine the role of ERK phosphorylation on StAR activation. It is possible that StAR protein expression becomes more sensitive to the effects of inhibiting the ERKs with UO126 resulting in a decrease in StAR protein expression. Data of this type would further delineate the role of the ERKs at multiple levels of organization in the StAR protein, one at the level of StAR transcription, and another at the post-translational modification of StAR protein expression (Gyles et al., 2001).

Another important finding but puzzling was that pre-treating FACs for one hour with UO126 had no effect on ERK 1 and ERK 2 protein expression. However, following ACTH treatment, there was a rapid decreased of ERK 1 and ERK 2 protein expression. This finding suggests a synergistic effect of UO126 and ACTH that results in making the proteins more susceptible to degradation, and may involve a possible role for proteases in cortisol regulation in the LTH FACs. To address this possibility, one experimental approach would be to use the phosphatase inhibitor okadaic acid to determine the role of PP1 and PP2A on the LTH FACs. PP1 and PP2A are phosphatases that have been shown to play key roles on the phosphorylation state of the ERKs (Alessi et al., 1995; Belcher et al., 2005; Dougherty et al., 2005; Gotoh et al., 1990; Manfroid et al., 2001; Miyasaka et al., 1990; Mumby et al., 1993; Pei et al., 2003; Pullar et al., 2003; Silverstein et al., 2002; Sonoda et al., 1997; Ugi et al., 2002). If treatment of FACs with okadaic acid results in the activation of ERK 1 and ERK 2 proteins, and a lack of decrease in ERK1 and ERK2 protein expression, then, removal of phosphate groups by PP1 and PP2A could signal ERK 1 and ERK 2 proteins for degradation.

Taken together, the studies represent the first series of experiments addressing key components of the signaling pathways involved in cortisol biosynthesis under normal conditions and in response to stressors such as LTH in the ovine fetal adrenal gland. These studies demonstrated important adaptations that take place at the level of the fetal adrenal cortex in response to LTH. The enhanced cortisol secretion observed in the LTH is not the result of cAMP, PKA, ACTH precursors, and or StAR mRNA gene expression. StAR protein, and the ERK signaling pathway appear to be in part responsible for the enhanced cortisol release in the LTH FACs. Future research will center at elucidating the

mechanism(s) that regulates StAR protein and ERK 1 and ERK 2 in response to ACTH treatment on cortisol biosynthesis in the LTH FACs.

Although LTH itself is of fundamental physiologic importance, it also has significant clinical relevance. For instance, in pregnant women at altitude, the incidence of toxemia and growth retardation are elevated as well as many other serious conditions associated with increased perinatal morbidity. The fetuses of women who smoke during pregnancy along with those exposed to environmental pollution with carbon monoxide are also subjected to prolonged hypoxia. A number of other clinical conditions exist including preeclampsia, anemia, malnutrition, heart and lung disease or hemoglobinopathy in which the fetus may experience chronic hypoxic stress. Together, data from these studies have provided important insight into the basic mechanisms regulating cortisol biosynthesis under LTH conditions and may provide the groundwork for future translational studies.

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