6-2015

Long-Term Hypoxia Alters Ovine Fetal Adrenal eNOS and Cortisol Biosynthesis

Elizabeth Anne Newby

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

Long-Term Hypoxia Alters Ovine Fetal Adrenal eNOS and Cortisol Biosynthesis

by

Elizabeth Anne Newby

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

June 2015

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. Ducsay, Professor of Physiology

_____

Arlin Blood, Assistant Professor of Physiology

_____

Dean A. Myers, Professor, Maternal-Fetal Medicine, Oklahoma University, Health Science Center

_____

William J. Pearce, Professor of Physiology

_____

Lubo Zhang, Professor of Pharmacology

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Charles Ducsay, who provided me support and encouraged me throughout this journey. He pushed me to keep asking questions and look for the other side of what I found, and he showed me how to work in diverse and challenging environments while maintaining a sense of humor. For that I will always be grateful. Thank you to the rest of my committee members, Dr. Dean Myers, Dr. William. Pearce, Dr. Lubo Zhang, and Dr. Arlin Blood for their advice and support whenever I needed help.

To my Lab D members, thank you for your patience and support, and for giving me friendship and encouragement, especially during our many long days of work when we all felt a little crazy.

To my parents and siblings, thank you for loving me and believing in me and praying me through the rough patches. The messages and phone calls and visits always brought a smile to my face. To my California family, you guys are the best! Thank you for your love and friendship; you kept the homesickness at bay and gave me a sense of belonging that I will never forget. And finally, thanks to God who gives me strength and courage and faithfully guides my path. Without Him I would not have made it.

CONTENT

Approval Page iii

Acknowledgements iv

Table of Contents v

List of Figures ix

List of Tables xi

List of Abbreviations xii

Abstract xiii

Chapter

1. Fetal Endocrine and Metabolic Adaptations to Hypoxia 1

    Abstract 2
    Introduction 3
    Acute Hypoxia 6
    Chronic Hypoxia 9
    PAT and Leptin 13

        Metabolic Gene Expression 15

    Conclusions 17
    Nitric Oxide 20
    Hypoxia and NO Production 22
    Mechanisms of NO Disruption of Steroidogenesis 24

        Direct Effects 24
        Indirect Effects 24

    Mechanisms of eNOS Regulation 25
    Signaling Pathways and Steroidogenesis 26
    References 30

2. Adrenocorticotropic Hormone and PI3K/Akt Inhibition Reduce eNOS Phosphorylation and Increase Cortisol Biosyntehsis in Long-Term Hypoxic Ovine Fetal Adrenal Cortical Cells 47

    Abstract 48

Introduction 49
Materials and Methods 52

Animals 52
Cell Dispersion 53
Treatment Protocols 53

Effects of MEK/ERK1/2 Inhibition and ACTH Stimulation on Cortisol Biosynthesis and eNOS Phosphorylation 53
Effects of UO126 Pretreatment and ACTH, 22R-OHC, or WSC Stimulation on Cortisol Biosynthesis 54
Effects of PI3K/Akt Inhibition and ACTH Stimulation on Cortisol Biosynthesis and eNOS Phosphorylation 54

Cortisol Assay 55
Western Analysis 55
Statistical Analysis 56

Results 57

Effects of MEK/ERK1/2 Inhibition and ACTH Stimulation 57

Cortisol Production 57
Expression of eNOS 57
Phosphorylation of eNOS 58

Effects of UO126 and ACTH, 22R-OHC, and WSC Stimulation on Cortisol Production 58
Effects of PI3K/Akt Inhibition and ACTH Stimulation 58

Cortisol Production 58
Expression of eNOS 59
Phosphorylation of eNOS 59

Discussion 65
References 71

3. Effects of 8Bromo-cAMP and UO126 on Cortisol Biosynthesis and eNOS Phosphorylation in Ovine Long-Term Hypoxic Fetal Adrenocortical Cells 76

Abstract 76
Introduction 77
Materials and Methods 78

Animals 78
Cell Dispersion 79
Treatment Protocol 80

Effects of MEK/ERK1/2 inhibition and 8Bromo-cAMP stimulation on cortisol biosynthesis and eNOS phosphorylation 80

Cortisol Assay 81
Western Analysis 81
Statistical Analysis 82

Results 83

Effects of MEK/ERK1/2 Inhibition and 8Bromo-cAMP Stimulation 83

Cortisol Production 83
Expression of eNOS 83
Phosphorylation of eNOS 84

Discussion 87
References 90

4. Effects of A23187 and ACTH on Cortisol Biosynthesis and eNOS Phosphorylation in Ovine Long-Term Hypoxic Fetal Adrenocortical Cells 93

Abstract 93
Introduction 94
Materials and Methods 95

Animals 95
Cell Dispersion 96
Treatment Protocol 96

Effects of A23187 treatment and ACTH stimulation on eNOS phosphorylation and cortisol biosynthesis 96

Cortisol Assay 97
Western Analysis 97
Statistical Analysis 99

Results 99

Effects of A23187 and ACTH Stimulation 99

Cortisol Production 99
Expression of eNOS 100
Phosphorylation of eNOS 100

Discussion 103
References 106

5. Effects of Nitric Oxide on Cortisol Biosynthesis and mRNA Abundance of CYP11A1, CYP17, StAR, AND ACTH-R in Ovine Long-Term Hypoxic Fetal Adrenocortical Cells 111

Abstract 111
Introduction 112
Materials and Methods 114

Animals 114
Cell Dispersion 114
Treatment Protocol 115

Effects of NOS Inhibition, NO Supplementation, and ACTH Stimulation on Cortisol Biosynthesis and CYP11A1, CYP17, StAR, and ACTH-R mRNA Abundance 115

Cortisol Assay 116
qRT-PCR 116
Statistical Analysis 120

Results 120

Effects of NO on Cortisol Production 120
Effects of NO on mRNA Abundance of CYP11A1, CYP17, StAR, and ACTH-R 121

Discussion 127
References 133

6. Conclusions 140

References 149

FIGURES

Figures Page

Chapter 1

1. Schematic Diagram of eNOS Regulation in the LTH Adrenal Cortex.....28

Chapter 2

1. Effects of MEK/ERK1/2 Inhibition and ACTH on Cortisol Production.....60
2. Effects of MEK/ERK1/2 Inhibition and ACTH on eNOS Expression and Phosphorylation.....61
3. Effects of UO126 Pretreatment and ACTH, 22R-OHC, or WSC Stimulation on Cortisol Biosynthesis.....62
4. Effects of PI3K/Akt Inhibition and ACTH on Cortisol Production 63
5. Effects of PI3K/Akt Inhibition and ACTH on eNOS Expression and Phosphorylation 64

Chapter 3

1. Treatment Protocol Timeline 8Bromo-cAMP 80
2. Effects of MEK/ERK1/2 Inhibition and 8Bromo-cAMP on Cortisol Production.....85
3. Effects of MEK/ERK1/2 Inhibition and 8Bromo-cAMP on eNOS Expression and Phosphorylation.....86

Chapter 4

1. Treatment Protocol Timeline A23187 97
2. Effects of A23187 Treatment and ACTH on Cortisol Production 101
3. Effects of A23187 Treatment and ACTH on eNOS Expression and Phosphorylation 102

Chapter 5

1. Effects of DETA-NO, L-NAME, and ACTH on Cortisol Production 122

2. Effects of DETA-NO, L-NAME, and ACTH on CYP11A1 mRNA abundance 123

3. Effects of DETA-NO, L-NAME, and ACTH on CYP17 mRNA abundance 124

4. Effects of DETA-NO, L-NAME, and ACTH on StAR mRNA abundance 125

5. Effects of DETA-NO, L-NAME, and ACTH on ACTH-R mRNA abundance 126

TABLES

Tables Page

Chapter 5

1. Forward and Reverse Primer Sequences for qRT-PCR.....119

ABBREVIATIONS

22R-OHC	22R-Hydroxycholesterol
8Br	8Bromo-cAMP
A23187	Calcium Ionophore A23187
ACTH	Adrenocorticotropic Hormone
Ca^{2+}	Calcium
cAMP	Cyclic Adenosine Monophosphate
CYP11A1	P450 Cholesterol Sidechain Cleavage
CYP17	P450 17α-Hydroxylase
eNOS	Endothelial Nitric Oxide Synthase
FACs	Fetal Adrenocortical Cells
HPA	Hypothalamo-Pituitary-Adrenal Axis
LTH	Long-Term Hypoxia
NO	Nitric Oxide
peNOS	Endothelial Nitric Oxide Synthase Phosphorylation
PP2A	Protein Phosphatase 2A
StAR	Steroidogenic Acute Regulator Protein
UO	UO126
WSC	Water-Soluble Cholesterol
WT	Wortmannin

ABSTRACT OF THE DISSERTATION

Long-Term Hypoxia Alters Ovine Fetal Adrenal eNOS and Cortisol Biosynthesis

by

Elizabeth Anne Newby

Doctor of Philosophy, Graduate Program in Physiology
Loma Linda University, June 2015
Dr. Charles A. Ducsay, Chairperson

Maintaining normal levels of cortisol in response to chronic stress, while retaining the ability to respond to acute stress, is important for ensuring normal fetal growth and development. Long-term hypoxia (LTH) causes adaptations in the fetal hypothalamo-pituitary-adrenal (HPA) axis that maintain basal cortisol levels but enhance production in response to a secondary stress. Nitric oxide (NO), produced by endothelial nitric oxide synthase (eNOS) in the adrenal cortex, plays a significant role in regulating cortisol production in the LTH fetus. The production of NO is regulated by eNOS activity which can be altered via phosphorylation through key signaling pathways. In examining the effects of the MEK/ERK1/2, PI3K/Akt, and calcium signaling pathways, we found that the MEK/ERK1/2 pathway and calcium do not regulate eNOS phosphorylation (peNOS), but the PI3K/Akt pathway, along with ACTH, regulates peNOS in LTH fetal adrenocortical cells (FACs); inhibition of the PI3K/Akt pathway resulted in reduced peNOS and enhanced cortisol production in response to ACTH in LTH FACs. Defining the regulatory role of these pathways will enhance our understanding of how these adaptations to LTH impact the fetus.

CHAPTER ONE

FETAL ENDOCRINE AND METABOLIC ADAPTATIONS TO HYPOXIA

Elizabeth A. Newby,[1] Dean A. Myers,[2] and Charles A. Ducsay.[1]

[1]Center for Perinatal Biology, Loma Linda University, Loma Linda, CA 92350 and
[2]Department of Obstetrics and Gynecology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104.

Running Head: Fetal adaptations to hypoxia

Key Words: fetus, cortisol, adipose, hypoxia

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

Supported by National Institutes of Health Grants PO1HD31226, R01HD51951

Part of the work presented in this chapter has been submitted for publication to the American Journal of Physiology - Endocrinology and Metabolism.

Abstract

In utero, hypoxia is a significant, yet common, stress that perturbs homeostasis and can occur due to preeclampsia, preterm labor, maternal smoking, heart or lung disease, obesity, and high altitude. The fetus has the extraordinary capacity to respond to stress during development, This is mediated, in part, by the hypothalamic-pituitary-adrenal (HPA) axis and, more recently explored, changes in perirenal adipose tissue (PAT) in response to hypoxia. Obvious ethical considerations limit studies of the human fetus and fetal studies in the rodent model are limited due to size considerations and major differences in developmental landmarks. The sheep is a common model that has been used extensively to study the effects of both acute and chronic hypoxia on fetal development. In response to high-altitude induced, moderate long-term hypoxia (LTH), both the HPA axis and PAT adapt to preserve normal fetal growth and development, while allowing for responses to acute stress. LTH upregulates the HPA axis at the level of the hypothalamus and anterior pituitary yet maintains the normal ontogenic pattern of cortisol production during late gestation. Two mechanisms converge at the adrenal cortex that facilitate this divergent effect on fetal HPA function. In the PAT, LTH increases leptin production, which suppresses adrenocortical gene expression, while nitric oxide aids in maintaining acute cortisol production within the adrenal cortex. Although these adaptations appear beneficial during fetal development, they may become deleterious postnatally and into adulthood. This review will discuss some of the endocrine and metabolic adaptive changes that take place in response to hypoxia.

Introduction

Mammalian fetuses, and in particular fetuses from long gestational length pregnancies such as humans (primates) and ruminants, have the ability to respond to and/or adapt to stress during gestation to survive the potentially harsh intrauterine environment and continue to term. Post-birth, both the hypothalamic-pituitary-adrenal (HPA) axis, via cortisol, and the adrenomedullary/sympathetic nervous system (SNS), via catecholamines, serve as homeostatic regulators in response to acute and chronic stress. In larger mammalian fetuses, these systems exhibit maturation in late gestation and serve similar roles, providing the fetus with the means to respond to intrauterine stressors. While these responses to hypoxic stress may often be beneficial acutely, they have the potential to be deleterious, especially under sustained periods of hypoxic stress.

The influence of hypoxia during fetal development is of particular importance due to its potential to induce or "program" alterations in endocrinology and metabolism long after birth. It has been recognized for over two decades that an "adverse intrauterine environment" could lead to offspring predisposed to a variety of related disorders including cardiovascular, metabolic and obesity as adults. This so-called programming, also referred to as the "fetal origins of adult disease hypothesis", describes how an "adverse intrauterine environment" can trigger adaptive or maladaptive changes in the developing fetus to overcome the hostile conditions and survive (9, 11, 12, 157). Through epigenetic imprinting, these changes can lead to susceptibility of the fetus to acquire these cardiovascular and metabolic pathologies. Although the original hypothesis was largely derived from observations of offspring from malnourished or undernourished pregnancies, later studies have expanded on the impact of a variety of so-called intra-

uterine stressors. This has been reviewed in more detail by Godfrey (67) and Calkins (23).

Fetal hypoxia is a common stressor that occurs during pregnancy as the result of a variety of situations including maternal under or malnutrition, preeclampsia, preterm labor, smoking, heart or lung disease, obesity, and exposure to high altitude (13, 35, 46, 73, 94, 142, 160). Therefore, due to its prevalence, hypoxia likely plays a key role on the impact of an adverse intrauterine environment on the developing fetus. The impact of hypoxia on the fetus is dependent on a wide range of variables including gestational age, severity and duration of hypoxia, as well as confounders such as acidemia and hypercapnia.

When considering changes in response to hypoxic stress, the HPA axis is key due to its role in growth and maturation of the fetus. The HPA axis, through regulation of glucocorticoid biosynthesis, dictates differentiation and maturation of key organ systems including lung, liver, kidney, and regulation of metabolism including lipolysis, glycogenolysis, and protein catabolism (28, 116, 128). Acutely, activation of the HPA leads to a significant increase in cortisol (2, 16, 17, 83, 90), a glucocorticoid that plays a critical role in governing metabolism by influencing plasma glucose, lipid, and protein concentrations, as well as immune regulation, inflammation, and cardiovascular function. Under chronic stress conditions, cortisol production is associated with hyperglycemia, immune suppression, excess adipose deposition, bone loss, and hypertension (33, 158, 173). Therefore, the ability of the fetal HPA axis to adapt to limit cortisol production under conditions of chronic stress is crucial for maintaining normal development during gestation. The regulation of cortisol must be effectively coordinated to permit the late

gestation exponential rise in fetal plasma cortisol essential for fetal maturation, while permitting episodic cortisol production in response to acute stress.

Another key regulatory mediator influenced by hypoxia in the fetus is perirenal adipose tissue (PAT). In sheep, approximately 80% of fetal adipose tissue deposition occurs in the perirenal-abdominal region (171). During late gestation, fetal mass expands and adipose tissue develops and responds to hormonal and nutritional perturbations that can alter lipid storage and release, as well as induce secretion of leptin (152). Early changes in adipose function in response to hypoxia may play a role in fetal programming, due to the influence of leptin and gene expression on metabolic processes and the possible overlap between leptin and cortisol regulation.

For obvious ethical considerations, there is little data on the effect of hypoxia on endocrine and metabolic alterations in human fetuses. Additionally, although there are programming studies of the effects of hypoxia in rodents, due to the small size and developmental maturity of the fetus, they are not ideal for fetal endocrine and metabolic studies. Fetal studies have also been conducted in nonhuman primates, but they are limited due to the tremendous cost and lack of availability of animals. The sheep has become a major animal model for studying the impact of hypoxia on the developing fetus due to its relatively long gestational period, similarity of endocrine and physiological systems, and relative ease of fetal and maternal instrumentation.

Throughout this review, we will highlight key findings in relation to the impact of hypoxia on endocrine and metabolic responses of the fetus. Although as previously described, the majority of information has been derived from studies utilizing the ovine

fetus, wherever possible, we will draw correlates from human and non-human primate studies.

Acute Hypoxia

As described above, from a clinical perspective, fetal hypoxia can occur as a result of a wide range of maternal conditions. In an effort to mimic some of these conditions, multiple models of hypoxia have been developed. Acute hypoxia can be induced through maternal hypoxia (6, 37), blood flow restriction (182), or umbilical cord occlusion (UCO) (61, 69, 176), for a duration of a few minutes to several hours. In response to of acute hypoxia, there is a rapid release of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus which triggers adrenocorticotropic hormone (ACTH) secretion from the anterior pituitary followed by glucocorticoid production in the fetal adrenal cortex proportional to the degree and duration of hypoxia. This swift response of the HPA to acute stress emphasizes the critical role glucocorticoids play in homeostasis and limiting the physiological impact of stress on the fetus.

Several studies conducted in the fetal sheep examined the effects of acute hypoxemia induced by reduction in maternal oxygen or by reduction of uteroplacental blood flow by umbilical cord occlusion (UCO). Akagi and Challis showed that moderate maternal hypoxia (PO_2 reduced by 8.4 mmHg) for 1 hour increased fetal plasma AVP and ACTH in 106-117 days gestation (dG) fetuses (6). In a later gestation fetus (131 dG), Unno, et al. observed increased fetal plasma ACTH and cortisol concentrations following a 50% reduction in blood flow by UCO (176). Further, several studies found that acute

episodes (1-48h) of fetal hypoxemia (induced by maternal hypoxia or UCO) resulted in increased CRH mRNA in the fetal hypothalamus, proopiomelanocortin (POMC) mRNA in the fetal pituitary, and increased circulating AVP, ACTH, and cortisol concentrations in fetal plasma (7, 17, 19, 27, 90, 114, 145, 159, 169). While the response of the HPA axis to stress is best seen in the late gestation fetus, as the fetal HPA has matured and become fully responsive (54), changes in fetal plasma cortisol concentrations in response to acute hypoxemia have been reported in the ovine fetus as early as 120 dG (16). Together, the results of these studies exemplify the integrated response of the HPA axis to acute hypoxic stress.

Although the response to an acute hypoxic insult results in upregulation of the HPA, prolonged elevated cortisol levels lead to fetal growth restriction and, in ruminants, activation of the parturition cascade and early birth of small fetus (64, 164). To further examine the effects of hypoxia as a fetal stress, the response of the fetal HPA axis to repeated hypoxic perturbations or prolonged hypoxia over the course of several days has been investigated. Unno, et al. found that after repeated UCOs, fetal anterior pituitary responsiveness was maintained with increased levels of plasma ACTH released after each UCO, but adrenocortical responsiveness was blunted; despite elevated ACTH, cortisol levels remained similar to basal levels by the 12^{th} UCO (176). Green, et al. subjected 112-116 dG fetal sheep to repeated UCOs and saw increased plasma ACTH and cortisol concentrations but this response was attenuated after 4 days (69). These studies show that while fetal CRH/AVP and ACTH remained elevated in fetal plasma, cortisol returned to basal levels by the end of the hypoxic insult.

In its role as a glucocorticoid, cortisol regulates metabolism by influencing plasma glucose concentrations. Along with cortisol, plasma glucose levels and fetal growth and development are also regulated by insulin, a hormone secreted from pancreatic β-cells in response to increased plasma glucose concentrations that stimulates cellular uptake of glucose (53). Insulin secretion is tightly coupled to plasma glucose concentration, maintaining a relatively constant insulin-to-glucose ratio (I/G). However in response to an acute hypoxic challenge, several studies found that the fetus, had decreased insulin secretion (87, 187) accompanied by increased norepinephrine (NE) and epinephrine (E) secretion (36) and increased cortisol and corticosterone secretion (86). Further studies showed that hypoxic stress acts through an α_2-adrenergic mechanism to induce inhibition of insulin secretion (85, 87, 104, 167). The increase in glucocorticoid elevated plasma glucose and circulating catecholamines prevented hyperinsulinemia, but together resulted in hyperlactacemia and hypocarbia (107), showing a direct impact on fetal metabolism. In response to hypoxia, however, gluconeogenesis is initiated, and the excess lactate generated is used as a substrate for hepatic glucose production (107). This sympathoadrenal suppression of insulin secretion may act as a mechanism to conserve glucose and oxygen for essential organs such as the brain and heart (20, 62), but if sustained could result in reduced birth weight (85).

In the human fetus, Zamudio, et al. found that women living at high altitude experienced chronic hypoxia that resulted in IUGR, potentially initiated by fetal hypoglycemia; there was decreased circulating fetal glucose concentrations and consumption (190). This suggests altered placental metabolism that spares oxygen for fetal use but limits glucose availability for fetal growth. IUGR as a result of altered

glucose metabolism has also been reported in a rat model of hypoxia by Lueder, et al. (111). They observed that maternal exposure to 5 days of 10% ambient oxygen in the third trimester resulted in similar fetal plasma glucose concentrations between hypoxic and control but increased relative glucose utilization of hypoxic fetal tissues accompanied by acidosis, suggesting anaerobic metabolism and increased glycolysis in the hypoxic fetus.

In response to changes in metabolism, cortisol works to restore homeostasis to allow for the continued growth and development of the fetus. In the case of recurring acute hypoxic stress, continuous bursts of cortisol can become detrimental to fetal development and lead to a growth restricted fetus delivered pre-term. From these results, the ovine fetus has demonstrated an adaptation in the HPA axis where there is a dissociation in the response between the hypothalamic-pituitary axis and the adrenocortical response to brief repeated hypoxic stress or prolonged hypoxia over several days. While CRH/AVP and ACTH levels remain elevated, cortisol returns to basal levels to allow for normal growth and development of the fetus.

Chronic Hypoxia

Experimentally, chronic hypoxia (over days to weeks or even months) can be initiated early or late in gestation and can be induced through placental embolization (21, 58), placental restriction, secondary to nutrient restriction (51), or by high altitude resulting in moderate continuous hypoxia with normal pregnancy duration and no accompanying growth restriction (2, 78, 83, 92). Because the HPA axis matures in the latter third of gestation and increases in responsiveness as the fetus nears term (25, 101,

102, 141, 147, 150, 156, 177), studies often measure the effects of hypoxia in late gestation.

Gagnon, et al. examined the effects of fetal placental embolization (30% reduction in arterial PO_2) for 10 days in 122 dG sheep. It resulted in progressive hypoxemia with reduced fetal plasma ACTH but increased prostaglandin E_2 (PGE_2) and maintained cortisol (58, 131). This suggests that PGE_2 may be involved in an adaptation to maintain basal fetal cortisol levels when ACTH is reduced and indicates that additional factors other than ACTH play a role in regulating cortisol production in the ovine fetus.

To induce hypoxia by placental restriction (PR), Phillips, et al. performed caruncletomies prior to mating to reduce the number of placentomes formed in ewes. This resulted in gestational hypoxia, with fetal arterial PO_2 reduced by 30%. This highly successful model by the McMillen group allows for hypoxia throughout the entire course of gestation. However, the hypoxia is accompanied by nutrient restriction and IUGR. Due to PR, there was decreased POMC mRNA in the fetal pituitary and higher cortisol levels compared to control, despite similar levels of plasma ACTH at 140 dG (151). They hypothesized that the HPA axis adapts to operate at a new set point in the growth restricted fetus in response to nutrient restriction.

In the high altitude induced long-term hypoxic (LTH) ovine model, ewes are maintained at 3820 m beginning at approximately day 40 of gestation and continuing through to near term (139-141 dG, term is ~145, hypoxic fetal PO_2 ~18 mmHg, normoxic ~23 mmHg). In this model, the fetus has adapted to hypoxia such that pregnancies are of normal duration, fetuses are not growth restricted, and there is no accompanying acidosis (78, 92). Initial studies examining the effects of LTH on the ovine fetus showed that basal

immunoreactive (IR) ACTH and cortisol concentrations were similar to normoxic control fetuses (78, 133). However, subsequent studies revealed that LTH stimulated hypothalamic drive which enhanced expression of POMC and processing to ACTH with increased concentrations of $ACTH_{1-39}$ and key POMC precursors (POMC and 22 kDa ACTH) in plasma (133). Despite higher basal levels of $ACTH_{1-39}$, cortisol concentrations were not increased above normoxic controls in near term fetuses.

This dichotomy became even more interesting in response to a superimposed acute secondary stressor. Surprisingly, in the LTH fetuses in response to hypotension or UCO, both ACTH and cortisol increased, but the cortisol response was greater compared to the response in normoxic fetuses (2, 83, 132). Further studies by Myers, et al. demonstrated reduced expression of ACTH-R, CYP17, and CYP11A1 with no changes in CYP21 or StAR in the late gestation LTH fetal adrenal cortex compared to normoxic controls (135), suggesting that a reduced steroidogenic capacity in the LTH fetus may play a role in the disconnect between basal ACTH and cortisol levels. However, mechanisms must exist to allow for a heightened cortisol response to acute stress, despite the lowered expression of these key steroidogenic enzymes. The fetus has developed such that despite elevated basal plasma ACTH, normal ontogenic maturation of cortisol production is maintained and the prepartum exponential rise is preserved as well as the capacity to respond to an acute secondary stress. These adaptations indicate that the hypothalamic-pituitary portion of the axis responds to hypoxia as a stress by increasing the synthesis and release of ACTH secretagogues and activating the stress response However, adaptive responses at the level of the adrenal cortex suppress excess stimulation under basal conditions.

As described above, in the LTH fetal adrenal cortex, there is decreased expression of CYP11A1 and CYP17, two key enzymes mediating cortisol synthesis, as well as decreased ACTH receptor expression (135). The reduction of these factors would result in attenuated adrenal responsiveness to ACTH and limited cortisol production. Along with these changes, however, there is an increase in the spent form of steroidogenic acute regulator (StAR) protein (30 kDa), indicating increased transport of cholesterol into the inner mitochondrial membrane for the first step in cortisol biosynthesis. This could balance the adaptations of elevated basal plasma $ACTH_{1\text{-}39}$ but reduced adrenal responsiveness to maintain basal levels of plasma cortisol similar to those observed in normoxic fetuses. In the LTH adrenal cortex, there are no changes in SF-1 and DAX-1 expression, key transcription factors for ACTH-R and CYP11A1 and CYP17. This suggests that activation of these transcription factors is altered, possibly via phosphorylation state or increased recruitment of co-repressors (166).

The mechanisms involved in these adaptations have not been fully elucidated, however nitric oxide (NO) may play a major role in regulating cortisol production intracellularly in the LTH fetal adrenal cortex. Tsubaki, et al. examined adrenal tissue and observed increased expression of endothelial nitric oxide synthase (eNOS) in adrenal tissue that colocalizes with CYP17 in LTH fetuses suggesting NO plays a role in regulation of adrenal steroidogenesis (174). Monau, et al. also showed that eNOS is the dominant NOS isoform in the ovine fetal adrenal cortex, and eNOS mRNA and protein expression is increased in the LTH adrenal primarily in CYP17 expressing cells in the cortisol producing zona fasciculate (122). Subsequent studies by Monau, et al. showed that NO reduced ACTH-mediated cortisol production in LTH fetal adrenocortical cells

(FACs) in vitro, while inhibition of NOS activity increased cortisol production in LTH cells, with no effect on normoxic cells (123). Also, ACTH reduced eNOS activation via phosphorylation in LTH FACs (140), and NO-dependent inhibition of ACTH induced cortisol production in vitro FACs further supports the role of NO in regulating cortisol production in the LTH fetal adrenal (175). This may be possible by NO competing with the oxygen binding site of CYP11A1 and CYP17 (75, 174), disrupting the heme-oxygen complex attack by the enzyme on the steroid substrate. The increased release of NO under basal conditions would limit cortisol synthesis, while elevated ACTH release and signaling due to a secondary stress would inhibit NOS activity and remove NO inhibition, resulting in enhanced cortisol production in the LTH fetus. This provides a mechanism (NO) for the capability of the LTH fetus to overcome the reduced steroidogenic enzyme gene expression and mount an enhanced cortisol response to acute stressors. However the question remains as to what factor(s) is involved in the decreased expression of the key steroidogenic machinery in response to LTH?

PAT and Leptin

One factor that my play a role is leptin. This 16 kDa protein derived from adipose tissue is most widely recognized for its role in appetite regulation in the adult (5, 89). However, leptin has also been clearly demonstrated to regulate adrenal steroid biosynthesis. In adult bovine adrenocortical cells, leptin suppressed cortisol output in response to ACTH stimulation and this effect was mediated through a reduction in CYP17 and CYP11A1 expression (18, 99). Further, leptin is a hypoxia inducible gene (110). This adipocyte-derived hormone, like in human fetuses (108), circulates in the

fetal sheep and increases in abundance in perirenal adipocytes as gestation progresses (188, 189). As in other metabolic tissues, fetal PAT is influenced by maternal conditions and intrauterine stressors, such as hypoxia.

In sheep approximately 80% of fetal adipose tissue deposition occurs in the perirenal-abdominal region (152). Fetal PAT differentiation is initiated in mid gestation, and expands during late gestation with a concomitant increase in hormone receptor populations (152). Adipose tissue begins to develop and respond to hormonal and nutritional perturbations in the fetus which in turn affects lipid storage and release. Importantly, this adipose tissue depot serves as an endocrine organ with the production of leptin (152). Along with the intracellular regulation of cortisol production by NO in the fetal adrenal, extracellular regulation of cortisol and the fetal response to hypoxia may be regulated by leptin.

When infused into the late gestation ovine fetus, leptin attenuated the prepartum increase in fetal plasma ACTH and cortisol (80, 115, 189). Ducsay, et al. found that plasma leptin was elevated in the LTH fetus compared to normoxic controls, with PAT and placenta expressing higher levels of leptin mRNA (48). Also, OB-Ra (the inactive, short isoform) leptin receptor expression was reduced in the LTH hypothalamus while OB-Rb (the active, long-form) expression was increased in the adrenal (48), suggesting the potential for enhanced leptin activity in the fetal adrenal. Thus, leptin appears to be a hypoxia-inducible gene in the ovine fetus with the capacity to inhibit cortisol biosynthesis at the adrenocortical level.

Subsequent studies showed that StAR, ACTH-R, CYP11A1, and CYP17 expression were lower in the LTH fetus (47), and that a 96 hour leptin infusion into late

gestation spontaneously hypoxemic fetal sheep downregulated CYP21 mRNA, and ACTH-R and StAR mRNA and protein (168), indicating reduced adrenal responsiveness and a reduced capacity to produce cortisol. A 4-day infusion of a leptin receptor antagonist restored expression of CYP11A1 and CYP17 in the LTH fetus to levels similar to normoxic but did not affect fetal plasma ACTH or cortisol (47), demonstrating that LTH regulation of leptin can influence adrenal steroidogenic enzyme expression.

Although leptin plays a role in regulating the response of the HPA and adipose tissue to chronic stress, it works alongside cortisol and the adrenal to facilitate the fetal adaptation to hypoxia. Understanding the role of leptin in the intrauterine environment and the influence it has on the fetal HPA will help determine the long-term metabolic consequences of early life events and may include the ability of leptin to influence the development of obesity and its comorbidities.

Metabolic Gene Expression

Along with the production of leptin, other factors in adipose tissue are affected by hypoxia and may have a metabolic impact on the fetus. In the fetal sheep, as well as the human, PAT has classically been considered a brown fat deposit (brown adipose tissue, BAT). It expresses uncoupling protein 1 (UCP1) (26, 34, 42), which increases proton conduction of the inner mitochondrial membrane and catalyzes adaptive thermogenesis (24, 170). This enables the rapid generation of a significant amount of heat, and expression is most abundant in the newborn (170).

The fetal perirenal adipose depot in the LTH fetus, however, has been characterized with an unusual brown fat phenotype; there are mixed populations of

multilocular deposits, typical of white fat, and unilocular fat deposits, more common in brown fat. Leptin expression is more typical of white fat and it is equally distributed in unilocular and multilocular adipocytes with UCP1 staining distributed throughout the PAT. This unique phenotype has been termed “beige” fat; white adipose tissue (WAT, myf+5 lineage) expressing as BAT (myf-5) (26, 42, 70, 84, 149, 161). Within this tissue, Myers, et al. showed upregulation of UCP1, deiodinase 2 (DIO2), 11β hydroxysteroid dehydrogenase 1 (HSD11β1), peroxisome proliferator-activated receptor (PPAR) γ and PPAR coactivator (PGC) 1α mRNA in the LTH fetal adipose (134). As hallmarks of the brown fat phenotype, LTH appears to enhance brown fat functionality, and increased HSD11β1 and DIO2 would allow adipose tissue to increase the BAT phenotype without systemic increases in cortisol and triiodothyronine (T3) which would deleteriously impact fetal growth and organ function. Along with upregulated brown fat gene expression, Myers, et al. found increased mRNA of transcription factors that regulate expression of NRF2 and mtTFA, genes that govern mitochondrial function (136), further indicating a BAT phenotype.

The fetal adaptation to LTH in adipose tissue appears to involve increased leptin production and regulation of basal cortisol, as described above, as well as enhanced activation of adipose tissue. In the newborn, abdominal adipose is important for nonshivering thermogenesis and is regulated by UCP1. By increasing UCP1 expression, the fetus ensures adequate thermogenesis in the event of birth into oxygen limited conditions. UCP1 expression is regulated by cortisol and T3, and increases in HSD11β1 and DIO2 indicate increased capacity for local synthesis and regulation by these hormones in the adipose tissue. This enhanced brown fat phenotype in anticipation of

birth into a potentially hostile environment creates a balance between the upregulation of the HP axis while downregulating adrenal responsiveness to maintain basal levels.

These changes in the LTH fetus, however, are not maintained postnatally. After birth, LTH lambs lose their brown fat phenotype; Ducsay, et al. (49) and Symonds, et al. (170) showed that expression of UCP1, PGC1α, and PRDM16 decrease post birth, implying a lineage derived from WAT, not BAT. Although the beige fat phenotype initially is protective of adiposity, as the fetus expresses as brown fat, decreases in UCP1, PGC1α, and PRDM16 suggest a predisposition of the lamb to fat deposition. In the transition from fetus to neonate, there is a shift toward an enhanced white fat phenotype which may result in greater adiposity as the newborn matures; decreased BAT has been shown to result in obesity and related metabolic disorders that develop later in life (79, 84, 162).

The combined increased PAT expression and release of leptin, increased adrenocortical leptin receptor (OB-Rb) expression, and increased zona fasciculata-specific eNOS expression and activity (NO release) would limit the ability of elevated fetal plasma ACTH to stimulate cortisol production under basal conditions. Overcoming these mechanisms may allow for increased synthesis and release of cortisol in response to an acute secondary stressor.

Conclusions

The influence of hypoxia on the developing fetus has clearly been shown in the HPA and adipose tissue in the ovine model. A variety of other studies have shown changes in response to hypoxia in the macaques as well as the human. Hypoxia in the

human fetus has been associated with both maternal and fetal conditions including high altitude, maternal heart disease or pulmonary hypertension, preeclampsia, and placental insufficiency. These conditions often result in intrauterine growth restriction (IUGR), preterm delivery, or stillbirth (1, 63, 71, 73, 88, 100, 126, 139). Maternal smoking also leads to hypoxia in the human and has been associated with intrauterine growth restriction (8, 52, 96, 155, 181, 184); low birth weight is a significant risk factor for the development of obesity, hypertension, and type 2 diabetes (10, 13, 66, 67, 143, 163). Studies in a nonhuman primate model, Japanese macaques, show that a high fat diet reduces uterine volume blood flow, resulting in undernourished fetuses and an increased incidence of stillbirth (55). These studies show a dramatic effect of hypoxia on the growth potential of the fetus by either preventing full development, or predisposing the fetus to numerous detrimental disorders.

The sheep has emerged as a major model for studying the effects of hypoxia on the fetus. When challenged with an acute stress, the fetal HPA axis is activated to release cortisol to counteract the perturbation and return the fetus to homeostasis. Sympathoadrenal inhibition of insulin secretion in response to hypoxia ensures adequate glucose for essential functions to restore homeostasis. In the case of a chronic stress, such as long-term hypoxia, several studies have shown the remarkable ability of the fetus to adapt to circumvent growth restriction and preterm birth.

Hypoxia is a potent stressor that commonly affects the developing fetus and can cause adaptations in both the HPA axis as well as the adipose tissue. In the LTH fetus, the HPA adapts such that despite the upregulation of hypothalamic CRH/AVP and pituitary ACTH under basal conditions, adrenal production of cortisol is maintained at

normoxic levels. However in response to an acute secondary stressor, the production of cortisol is enhance beyond the stress response in normoxic controls. This proposes an adaptation of the system that maintains cortisol levels required for growth and development, but is combined with a programmed heightened response to acute stress. This mechanism may be mediated by NO production in adrenal cortical cells, but also by leptin production in fetal PAT. Both are capable of inhibiting cortisol synthesis, however the exact mechanisms are still undetermined. NO may interact with steroidogenic enzymes to reduce cortisol biosynthesis, while leptin may reduce adrenal responsiveness to ACTH.

In adipose tissue, there is a unique beige phenotype developed in response to chronic hypoxia. There is an upregulation of expression of BAT phenotypic genes, UCP1, DIO2, HSD11B1, PPARγ, and PGC1α that would ensure adequate nonshivering thermogenesis and indicate reduced adiposity. These genes, however, become downregulated after birth, shifting toward a WAT phenotype and predisposing the newborn to fat deposition. If the fetus were born into a hypoxic environment, this adaptation may be beneficial, but in a normoxic environment, this could have a significant detrimental life-long impact resulting in a variety of metabolic disorders including obesity and diabetes.

As briefly described above, NO plays a major role in steroidogenesis. Below is a detailed description of NO and the impact of hypoxia on NO production. It also describes the mechanisms of action of NO on steroidogenesis and mechanisms of regulation of eNOS.

Nitric Oxide

Nitric oxide (NO) is a diatomic free radical molecule that diffuses feely across cell membranes and is oxidized to nitrite (NO2-) and nitrate (NO3-) under physiological conditions (56, 93, 105). NO has a wide range of physiologic functions including smooth muscle relaxation and neurotransmission (124, 125, 180). NO is synthesized from L-arginine by a family of nitric oxide synthases (NOS) (82); constitutively expressed neuronal NOS (nNOS/NOS-I) and endothelial NOS (eNOS/NOS-III) and inducible NOS (iNOS/NOS-II). Regulation of eNOS and nNOS are Ca2+/calmodulin-dependent, while regulation of iNOS is Ca2+/calmodulin-independent (117). NO produced by eNOS and nNOS regulate physiologic functions while iNOS tends to be invoked in pathological situations.

Classically, NO-mediated cellular signaling is regulated via activation of soluble guanylate cyclase (sGC). In his review, Ignarro describes how NO binds to the heme group of guanylate cyclase to alter enzyme conformation and increase its activity (81). This leads to elevation of intracellular cyclic guanosine monophosphate (cGMP) followed by activation of protein kinase G (PKG). Although these actions of NO have been best studied in vascular relaxation, it has been shown that guanylate cyclase inhibitors do not fully block the vasorelaxant effects of NO, indicating a cGMP-independent component of NO activity (41).

Aside from vasorelaxation, a variety of cGMP-independent effects of NO have been studied, including the inhibition of steroidogenesis; Drewett et al., determined cGMP-independent NO inhibition of key rate-limiting steps in the steroidogenic pathway (45). Changes in NO have been shown to affect steroidogenesis in a variety of tissues

including inhibition in ovarian tissue of women (178), pigs (112, 113), rabbits (65, 186), and rats (120), while inhibition of NOS increased testosterone production in Leydig cells (44). In adult rat testis, immobilization stress increased NO and reduced the production of testosterone (97) and NO inhibited cortisol secretion (3). In the adrenal, NO inhibited basal, ACTH, and angiotensin II-induced aldosterone production in the adult rat (74, 75) and bovine adrenal cortical cells (76), while NOS inhibition increased aldosterone in humans (127). Also in the adrenal, NO donors decreased corticosterone production and NOS inhibition enhanced glucocorticoid output (38, 39). These studies show that changes in NO production affect steroidogenesis in multiple tissue types, including adrenal cells. In our lab, we have shown that NO inhibits cortisol biosynthesis and that inhibition of NOS enhances cortisol output in ovine LTH FACs (123). Together this suggests that the regulation of nitric oxide production may be important to the fetal adaptation to LTH.

Due to the short half-life of NO, the site and source of NO production must be close to the target cells for inhibition to occur. In the adrenal, nNOS has been shown to increase in the rat cortex following immobilization stress (95, 138), and eNOS expression has been demonstrated in the rate zona glomerulosa (40, 138) and fasciculata (40), as well as the adult sheep fasciculate (148). NOS mRNA was also detected in the near term rat pup (4). Our lab has identified robust eNOS, but minimal amounts of nNOS and iNOS expression in the ovine fetal adrenal, with the greatest density of eNOS in the cortisol producing zona fasciculata/reticularis area in both normoxic and LTH adrenal sections. We also found greater eNOS protein expression in the LTH compared to normoxic adrenals, colocalizing with CYP17, identifying eNOS expression in cortisol producing

cells (122). Together this implies that eNOS plays a major role in the fetal adaptation to LTH through direct interaction of NO within FACs.

Hypoxia and NO Production

Regulation of steroidogenesis by hypoxia has been clearly shown by Raff et al., (153, 154), and Hanke and Campbell demonstrated that reducing oxygen concentrations resulted in a lower threshold for NO-mediated inhibition of aldosterone synthesis in adult rat adrenals (74). Due to this regulation, NO represents a potential mechanism in the adrenocortical adaptation to LTH, and as the predominant NOS isoform in the ovine fetal adrenal cortex and increased in the LTH adrenal, eNOS is a potential target for adapted regulation of steroidogenesis in the LTH ovine fetus.

Hypoxia has a wide range of effects on NO production (and/or expression of NOS isoforms) in different animal models and tissues. Justice et al., showed that hypoxia increased eNOS expression and NO production in micro vessels in the heart of pigs (91), and Xiao found that LTH enhanced eNOS expression in ovine uterine arteries (185). In cerebral arteries, Williams et al., determined that hypoxia reduced eNOS expression but increased components of the NO/cGMP/PKG pathway, increasing vascular sensitivity to NO (183). Following hypoxia (5% O_2 for 7 days), Murata et al., observed reduced eNOS expression and function in cultured pulmonary arteries (130), and that hypoxia-induced pulmonary hypertension impaired the interaction of eNOS with its regulatory proteins, and thus reduced NO production (129). Thompson and Dong found that hypoxia may have divergent effects on eNOS expression, with decreased fetal eNOS expression but increased adult expression in guinea pig hearts (172). Hypoxia was shown by Chen and

Meyrick to stimulate eNOS-Hsp90 interaction and activate the PI3K/Akt pathway, leading to eNOS phosphorylation and increased NO production (30). These mixed results suggest that the effects of hypoxia on eNOS regulation and NO production may be tissue specific.

Hypoxia has also been shown to affect the regulatory pathways for both NO and cortisol synthesis. Mishra et al., showed that administration of a NOS inhibitor prevented hypoxia-induced phosphorylation of ERK in neuronal nuclei of newborn piglets, suggesting NO mediates ERK phosphorylation in response to hypoxia (119). Zhu et al., showed that hypoxia enhanced ERK phosphorylation in endothelial cells (192), and Onishi et al., showed that basal PKA activity in fetal hearts was increased in LTH fetuses compared with normoxic controls (144). These changes in regulatory signaling pathways could result in changes in steroid production.

Another signaling pathway that has been shown to be affected by hypoxia is through Akt. Decreased oxygen resulted in increased AMP-activation of AMPK, and hypoxia-induced AMPK/Akt-activation of eNOS was demonstrated in endothelial cells. Akt was also shown to be the dominant kinase involved in eNOS phosphorylation at Ser1177 in hypoxic endothelial cells, and direct AMPK phosphorylation of eNOS was suggested to occur under conditions of prolonged hypoxia (137). The Ser1177/79 residue on eNOS has been shown to be a substrate for both Akt (43, 57) and AMPK (31). Typically this serine residue is referred to Ser1177/79 as Ser1177 refers to the human residue while in the bovine/ovine it is Ser1179 (43, 57, 59). It was also shown that AMPK phosphorylates eNOS at Ser633 and signals NO bioavailability (32). Changes in

eNOS phosphorylation induced through these signaling pathways could affect eNOS activity and NO synthesis, and they could be influenced by oxygen levels.

Mechanisms of NO Disruption of Steroidogenesis

Direct Effects

An alternative mechanism to NO/cGMP signaling may be through NO-heme binding. Tsubaki et al., showed that NO is capable of competitively interacting with the heme-oxygen binding site, similar to guanylate cyclase, and binds to key steroidogenic enzymes CYP11A1 and CYP17 (174, 175). Peterson et al., suggested that because these P450 enzymes use several round of attack of the heme-oxygen complex on the steroid substrate, they may be more susceptible to NO inhibition than other enzymes (148).

Indirect Effects

Another potential mechanism of NO suppression of steroidogenesis is S-nitrosylation of key Cys residues in critical steroidogenic proteins. CYP11A1 and CYP17 are key steroidogenic enzymes have critical Cys residues in their actives sites. Modification of these Cys residues could play a role in their activity. Though largely unexplored in steroidogenic CYPs, Lee et al., reported CYP S-nitrosylation in liver (103). Zinc finger transcription factor SF-1, responsible for transcription of CYP11A1 and CYP17 and StAR (165, 191), is another target for NO-mediated S-nitrosylation. S-nitrosylation of Zn+ finger transcription factors results in the loss of Zn+ from the DNA binding pocket, disrupting their function as transcriptional activators (60, 98).

Mechanisms of eNOS Regulation

Regulation of eNOS can occur through post-translational mechanism including protein-protein interactions and phosphorylation (50). Interaction with Ca^{2+}/calmodulin can activate eNOS (117), as well as interaction with Caveolin-1 (Cav-1) and heat shock protein 90 (Hsp90) (29, 30, 68). Gratton et al., showed that Cav-1 keeps eNOS in an inactive state at the caveolae while inhibiting eNOS translocation and/or the stimulatory actions of calmodulin binding. Hsp90 binds soluble eNOS and may disrupt the Cav-1/eNOS complex, enhancing NOS activity (68).

Another method of eNOS regulation is through phosphorylation. There are multiple signaling pathways that may be involved in the phosphorylation of eNOS including MEK/ERK1/2 and PI3K/Akt, as well as Ca^{2+}/calmodulin, AMPK, PKA, PKC, and ERKs (14, 15, 30-32). We have already shown that basal Akt and ERK1/2 phosphorylation are elevated in the LTH fetal adrenal , and that regulation of ERK1/2 is able to affect cortisol production (179). The role of ERK1/2 on NOS expression and activity is controversial but evident in various tissues. Cale and Bird showed that inhibition of ERK1/2 upregulated ATP-stimulated eNOS activity but inhibited ATP-stimulated activity in COS-7 cells (22), while Chen and Meyrick showed that MEK/ERK1/2 inhibition enhanced eNOS activity in porcine pulmonary arteries (30). A variety of other studies have shown that MEK/ERK1/2 pathway inhibition reduces eNOS phosphorylation and NO production in multiple cell types (29, 109, 118, 121). Studies have also shown that eNOS phosphorylation can be altered through the PI3K/Akt pathway (57, 77, 118).

At present however, the potential role of these signaling pathways in eNOS regulation and NO production have not been explored. More importantly, information on the novel role of hypoxia in this regulatory process is lacking. This gap in our knowledge has served as the major focus of our current work.

Signaling Pathways and Steroidogenesis

ERK1/2 stimulation has been shown to affect steroidogenesis through upregulation of StAR expression (72), and we have shown that inhibition of ERK1/2 reduces cortisol production in fetal adrenocortical cells, with a greater effect in LTH FACs (179). It has also been shown that inhibition of the PI3K/Akt pathway reduced stimulated steroid production in multiple cell types including ovaries, testes, and adrenals (106, 146).

In the LTH fetus, we showed that NOS activity was significantly greater in LTH vs normoxic and that ACTH treatment significantly reduced NOS activity in LTH with no effect in normoxic, returning activity to levels observed in normoxic (123). Together with NO-stimulated inhibition of cortisol synthesis and enhanced cortisol in response to NOS inhibition, this may be a mechanism of regulating cortisol responses under conditions of LTH. Upregulation of adrenal eNOS in the LTH group may be responsible for enhanced basal NOS activity which would exert an inhibitory effect on basal cortisol production overcoming low level stimulation by elevated basal ACTH. Stress levels of ACTH would decrease NOS activity, enhancing cortisol output. The mechanisms involved in LTH regulation of adrenal eNOS and associated NO along with the

mechanisms via which NO modulates cortisol production may include key signaling pathways MEK/ERK1/2 and PI3K/Akt.

Based on the work in our laboratory and that of others, the following model of the potential role of NO in the regulation of cortisol biosynthesis under conditions of LTH has been developed (**Figure 1**).

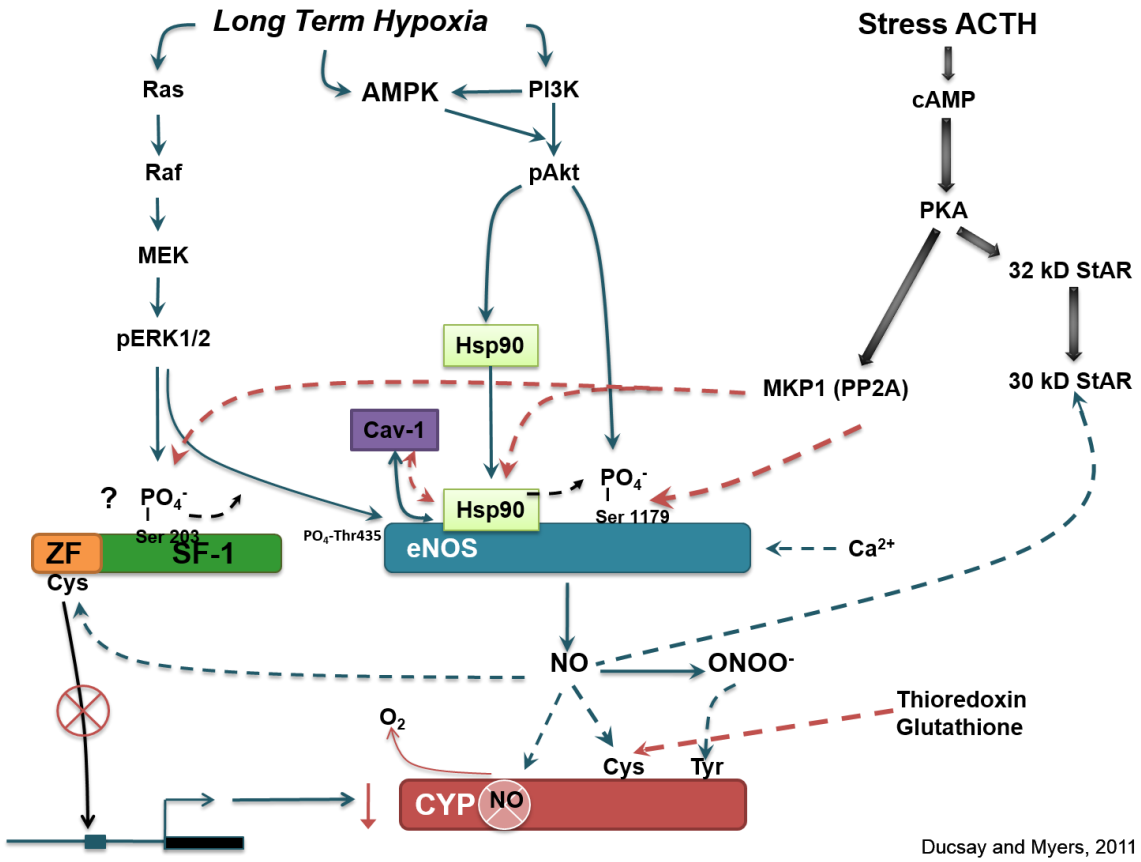

Figure 1. Schematic diagram of eNOS regulation in the LTH adrenal cortex. LTH results in increased activation of PI3K, AMPK, and Ras signaling pathways. Activation of PI3K and/or AMPK results in elevated pAkt/Akt which we hypothesize results in increased activity of eNOS via phosphorylation of eNOS at Ser1177/79 and dissociation of Cav-1 and recruitment of the eNOS activator, Hsp90. Activated eNOS results in NO generation leading to Cys S-nitrosylation of critical target proteins governing cortisol synthesis (CY11A1, CYP17), and SF-1, which regulates CYP11A1/17 transcription. We hypothesize that S-nitrosylation of Cys residues in the DNA binding domain of SF-1 reduces its capacity to interact with specific SF-1 cis elements in the promoter regions of CYP11A1/17 while nitrosylation of CYP11A1/17 decreases enzyme activity. These hypotheses are consistent with decreased cortisol synthesis and CYP expression observed in the adrenal cortex of LTH fetal sheep in spite of elevated basal plasma ACTH. However, under conditions of a secondary stressor, large increases in ACTH (~10-20 fold over basal levels) override the inhibitory effects of NO and result in enhanced cortisol production compared to normoxic.

Under basal conditions, despite increased hypothalamic drive, cortisol output remains normal in the LTH fetus. In response to a secondary stressor, this biochemical/molecular "brake" on cortisol production can be overridden, allowing the LTH fetus to mount an enhanced response. NO, through regulation of NOS may be one of the key factors involved in adapted regulation of cortisol in response to LTH. The following studies were designed to define the mechanisms involved in this adaptive response. This project addressed the role of key signaling pathways MEK/ERK1/2 and PI3K/Akt, as well as Ca^{2+}, governing eNOS activity in the ovine fetal adrenal, the effect of NO on key steroidogenic enzymes, and the role of eNOS in cortisol biosynthesis in the fetal adaptation to LTH. The expression and phosphorylation of eNOS at Ser1177/79, one primary phosphorylation site governing eNOS activity, and cortisol production was investigated in response to MEK/ERK1/2 or PI3K/Akt pathway inhibition, or Ca^{2+} induction accompanied by secondary stress stimulation with ACTH. NO supplementation and eNOS inhibition were used in combination with ACTH stimulation to determine the role of NO on the expression of CYP11A1, CYP17, StAR, and ACTH-R mRNA as well as cortisol production in both normoxic and LTH FACs.

References

1. ACOG technical bulletin. Pulmonary disease in pregnancy. Number 224--June 1996. American College of Obstetricians and Gynecologists. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics* 54: 187-196, 1996.

2. **Adachi K, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters ovine fetal endocrine and physiological responses to hypotension. *American journal of physiology Regulatory, integrative and comparative physiology* 287: R209-217, 2004.

3. **Adams ML, Nock B, Truong R, and Cicero TJ**. Nitric oxide control of steroidogenesis: endocrine effects of NG-nitro-L-arginine and comparisons to alcohol. *Life sciences* 50: Pl35-40, 1992.

4. **Afework M, Tomlinson A, and Burnstock G**. Distribution and colocalization of nitric oxide synthase and NADPH-diaphorase in adrenal gland of developing, adult and aging Sprague-Dawley rats. *Cell Tissue Res* 276: 133-141, 1994.

5. **Ahima RS, Saper CB, Flier JS, and Elmquist JK**. Leptin regulation of neuroendocrine systems. *Frontiers in neuroendocrinology* 21: 263-307, 2000.

6. **Akagi K, and Challis JR**. Hormonal and biophysical responses to acute hypoxemia in fetal sheep at 0.7-0.8 gestation. *Canadian journal of physiology and pharmacology* 68: 1527-1532, 1990.

7. **Akagi K, and Challis JR**. Threshold of hormonal and biophysical responses to acute hypoxemia in fetal sheep at different gestational ages. *Canadian journal of physiology and pharmacology* 68: 549-555, 1990.

8. **Andres RL, and Day MC**. Perinatal complications associated with maternal tobacco use. *Seminars in neonatology : SN* 5: 231-241, 2000.

9. **Barker DJ**. Adult consequences of fetal growth restriction. *Clinical obstetrics and gynecology* 49: 270-283, 2006.

10. **Barker DJ**. In utero programming of chronic disease. *Clinical science (London, England : 1979)* 95: 115-128, 1998.

11. **Barker DJ**. Obesity and early life. *Obesity reviews : an official journal of the International Association for the Study of Obesity* 8 Suppl 1: 45-49, 2007.

12. **Barker DJ, Bagby SP, and Hanson MA**. Mechanisms of disease: in utero programming in the pathogenesis of hypertension. *Nature clinical practice Nephrology* 2: 700-707, 2006.

13. **Barker DJ, and Clark PM**. Fetal undernutrition and disease in later life. *Reviews of reproduction* 2: 105-112, 1997.

14. **Bernier SG, Haldar S, and Michel T**. Bradykinin-regulated interactions of the mitogen-activated protein kinase pathway with the endothelial nitric-oxide synthase. *The Journal of biological chemistry* 275: 30707-30715, 2000.

15. **Bird IM, Sullivan JA, Di T, Cale JM, Zhang L, Zheng J, and Magness RR**. Pregnancy-Dependent Changes in Cell Signaling Underlie Changes in Differential Control of Vasodilator Production in Uterine Artery Endothelial Cells. *Endocrinology* 141: 1107-1117, 2000.

16. **Bocking AD, McMillen IC, Harding R, and Thorburn GD**. Effect of reduced uterine blood flow on fetal and maternal cortisol. *Journal of developmental physiology* 8: 237-245, 1986.

17. **Boddy K, Jones CT, Mantell C, Ratcliffe JG, and Robinson JS**. Changes in plasma ACTH and corticosteroid of the maternal and fetal sheep during hypoxia. *Endocrinology* 94: 588-591, 1974.

18. **Bornstein SR, Uhlmann K, Haidan A, Ehrhart-Bornstein M, and Scherbaum WA**. Evidence for a novel peripheral action of leptin as a metabolic signal to the adrenal gland: leptin inhibits cortisol release directly. *Diabetes* 46: 1235-1238, 1997.

19. **Boshier DP, Holloway H, and Liggins GC**. Effects of cortisol and ACTH on adrenocortical growth and cytodifferentiation in the hypophysectomized fetal sheep. *Journal of developmental physiology* 3: 355-373, 1981.

20. **Boyle DW, Meschia G, and Wilkening RB**. Metabolic adaptation of fetal hindlimb to severe, nonlethal hypoxia. *The American journal of physiology* 263: R1130-1135, 1992.

21. **Boyle JW, Lotgering FK, and Longo LD**. Acute embolization of the uteroplacental circulation: uterine blood flow and placental CO diffusing capacity. *Journal of developmental physiology* 6: 377-386, 1984.

22. **Cale JM, and Bird IM**. Inhibition of MEK/ERK1/2 signalling alters endothelial nitric oxide synthase activity in an agonist-dependent manner. *The Biochemical journal* 398: 279-288, 2006.

23. **Calkins K, and Devaskar SU**. Fetal origins of adult disease. *Current problems in pediatric and adolescent health care* 41: 158-176, 2011.

24. **Cannon B, and Nedergaard J**. Brown adipose tissue: function and physiological significance. *Physiol Rev* 84: 277-359, 2004.

25. **Carey LC, Su Y, Valego NK, and Rose JC**. Infusion of ACTH stimulates expression of adrenal ACTH receptor and steroidogenic acute regulatory protein

mRNA in fetal sheep. *American journal of physiology Endocrinology and metabolism* 291: E214-220, 2006.

26. **Casteilla L, Forest C, Robelin J, Ricquier D, Lombet A, and Ailhaud G**. Characterization of mitochondrial-uncoupling protein in bovine fetus and newborn calf. *The American journal of physiology* 252: E627-636, 1987.

27. **Challis JR, Fraher L, Oosterhuis J, White SE, and Bocking AD**. Fetal and maternal endocrine responses to prolonged reductions in uterine blood flow in pregnant sheep. *American journal of obstetrics and gynecology* 160: 926-932, 1989.

28. **Challis JRG, Matthews SG, Gibb W, and Lye SJ**. Endocrine and paracrine regulation of birth at term and preterm. *Endocrine reviews* 21: 514-550, 2000.

29. **Chen DB, Bird IM, Zheng J, and Magness RR**. Membrane estrogen receptor-dependent extracellular signal-regulated kinase pathway mediates acute activation of endothelial nitric oxide synthase by estrogen in uterine artery endothelial cells. *Endocrinology* 145: 113-125, 2004.

30. **Chen JX, and Meyrick B**. Hypoxia increases Hsp90 binding to eNOS via PI3K-Akt in porcine coronary artery endothelium. *Laboratory investigation; a journal of technical methods and pathology* 84: 182-190, 2004.

31. **Chen Z-P, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, and Kemp BE**. AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Letters* 443: 285-289, 1999.

32. **Chen Z, Peng I-C, Sun W, Su M-I, Hsu P-H, Fu Y, Zhu Y, DeFea K, Pan S, Tsai M-D, and Shyy JY-J**. AMP-Activated Protein Kinase Functionally Phosphorylates Endothelial Nitric Oxide Synthase Ser633. *Circulation Research* 104: 496-505, 2009.

33. **Chrousos GP**. The role of stress and the hypothalamic-pituitary-adrenal axis in the pathogenesis of the metabolic syndrome: neuro-endocrine and target tissue-related causes. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* 24 Suppl 2: S50-55, 2000.

34. **Clarke L, Buss DS, Juniper DT, Lomax MA, and Symonds ME**. Adipose tissue development during early postnatal life in ewe-reared lambs. *Experimental physiology* 82: 1015-1027, 1997.

35. **Cnattingius S, Bergstrom R, Lipworth L, and Kramer MS**. Prepregnancy weight and the risk of adverse pregnancy outcomes. *The New England journal of medicine* 338: 147-152, 1998.

36. **Cohen WR, Piasecki GJ, Cohn HE, Susa JB, and Jackson BT**. Sympathoadrenal responses during hypoglycemia, hyperinsulinemia, and hypoxemia in the ovine fetus. *The American journal of physiology* 261: E95-102, 1991.

37. **Cohn HE, Sacks EJ, Heymann MA, and Rudolph AM**. Cardiovascular responses to hypoxemia and acidemia in fetal lambs. *American journal of obstetrics and gynecology* 120: 817-824, 1974.

38. **Cymeryng CB, Dada LA, Colonna C, Mendez CF, and Podesta EJ**. Effects of L-arginine in rat adrenal cells: involvement of nitric oxide synthase. *Endocrinology* 140: 2962-2967, 1999.

39. **Cymeryng CB, Dada LA, and Podesta EJ**. Effect of nitric oxide on rat adrenal zona fasciculata steroidogenesis. *The Journal of endocrinology* 158: 197-203, 1998.

40. **Cymeryng CB, Lotito SP, Colonna C, Finkielstein C, Pomeraniec Y, Grión N, Gadda L, Maloberti P, and Podestá EJ**. Expression of Nitric Oxide Synthases in Rat Adrenal Zona Fasciculata Cells. *Endocrinology* 143: 1235-1242, 2002.

41. **Denninger JW, and Marletta MA**. Guanylate cyclase and the ·NO/cGMP signaling pathway. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1411: 334-350, 1999.

42. **Devaskar SU, Anthony R, and Hay W, Jr.** Ontogeny and insulin regulation of fetal ovine white adipose tissue leptin expression. *American journal of physiology Regulatory, integrative and comparative physiology* 282: R431-438, 2002.

43. **Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, and Zeiher AM**. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399: 601-605, 1999.

44. **Dobashi M, Fujisawa M, Yamazaki T, Okuda Y, Kanzaki M, Tatsumi N, Tsuji T, Okada H, and Kamidono S**. Inhibition of steroidogenesis in Leydig cells by exogenous nitric oxide occurs independently of steroidogenic acute regulatory protein (star) mRNA. *Archives of andrology* 47: 203-209, 2001.

45. **Drewett JG, Adams-Hays RL, Ho BY, and Hegge DJ**. Nitric oxide potently inhibits the rate-limiting enzymatic step in steroidogenesis. *Molecular and Cellular Endocrinology* 194: 39-50, 2002.

46. **Ducsay CA**. Fetal and maternal adaptations to chronic hypoxia: prevention of premature labor in response to chronic stress. *Comparative biochemistry and physiology Part A, Molecular & integrative physiology* 119: 675-681, 1998.

47. **Ducsay CA, Furuta K, Vargas VE, Kaushal KM, Singleton K, Hyatt K, and Myers DA**. Leptin receptor antagonist treatment ameliorates the effects of long-term maternal hypoxia on adrenal expression of key steroidogenic genes in the ovine

fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 304: R435-442, 2013.

48. **Ducsay CA, Hyatt K, Mlynarczyk M, Kaushal KM, and Myers DA**. Long-term hypoxia increases leptin receptors and plasma leptin concentrations in the late-gestation ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 291: R1406-1413, 2006.

49. **Ducsay CA, Newby EA, Cato C, Singleton K, and Myers DA**. Long term hypoxia during gestation alters perirenal adipose tissue in the lamb: a trigger for adiposity? *J Devel Origins of Health and Disease* 4: 1194, 2013.

50. **Dudzinski DM, and Michel T**. Life history of eNOS: Partners and pathways. *Cardiovascular research* 75: 247-260, 2007.

51. **Dyer JL, McMillen IC, Warnes KE, and Morrison JL**. No evidence for an enhanced role of endothelial nitric oxide in the maintenance of arterial blood pressure in the IUGR sheep fetus. *Placenta* 30: 705-710, 2009.

52. **England LJ, Kendrick JS, Gargiullo PM, Zahniser SC, and Hannon WH**. Measures of maternal tobacco exposure and infant birth weight at term. *American journal of epidemiology* 153: 954-960, 2001.

53. **Fowden AL**. The role of insulin in fetal growth. *Early human development* 29: 177-181, 1992.

54. **Fraser M, Braems GA, and Challis JR**. Developmental regulation of corticotrophin receptor gene expression in the adrenal gland of the ovine fetus and newborn lamb: effects of hypoxia during late pregnancy. *The Journal of endocrinology* 169: 1-10, 2001.

55. **Frias AE, Morgan TK, Evans AE, Rasanen J, Oh KY, Thornburg KL, and Grove KL**. Maternal high-fat diet disturbs uteroplacental hemodynamics and increases the frequency of stillbirth in a nonhuman primate model of excess nutrition. *Endocrinology* 152: 2456-2464, 2011.

56. **Fukuto JM**. Chemistry of nitric oxide: biologically relevant aspects. *Advances in pharmacology (San Diego, Calif)* 34: 1-15, 1995.

57. **Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, and Sessa WC**. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399: 597-601, 1999.

58. **Gagnon R, Murotsuki J, Challis JR, Fraher L, and Richardson BS**. Fetal sheep endocrine responses to sustained hypoxemic stress after chronic fetal placental embolization. *The American journal of physiology* 272: E817-823, 1997.

59. **Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, Figeys D, Harrison DG, Berk BC, Aebersold R, and Corson MA**. Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *The Journal of biological chemistry* 274: 30101-30108, 1999.

60. **Garbán HJ, Márquez-Garbán DC, Pietras RJ, and Ignarro LJ**. Rapid nitric oxide-mediated S-nitrosylation of estrogen receptor: Regulation of estrogen-dependent gene transcription. *Proceedings of the National Academy of Sciences of the United States of America* 102: 2632-2636, 2005.

61. **Gardner DS, Fletcher AJ, Fowden AL, and Giussani DA**. A novel method for controlled and reversible long term compression of the umbilical cord in fetal sheep. *J Physiol* 535: 217-229, 2001.

62. **Gardner DS, Jamall E, Fletcher AJ, Fowden AL, and Giussani DA**. Adrenocortical responsiveness is blunted in twin relative to singleton ovine fetuses. *J Physiol* 557: 1021-1032, 2004.

63. **Giussani DA, Phillips PS, Anstee S, and Barker DJ**. Effects of altitude versus economic status on birth weight and body shape at birth. *Pediatric research* 49: 490-494, 2001.

64. **Gluckman PD**. Editorial: nutrition, glucocorticoids, birth size, and adult disease. *Endocrinology* 142: 1689-1691, 2001.

65. **Gobbetti A, Boiti C, Canali C, and Zerani M**. Nitric oxide synthase acutely regulates progesterone production by in vitro cultured rabbit corpora lutea. *The Journal of endocrinology* 160: 275-283, 1999.

66. **Godfrey KM, and Barker DJ**. Fetal nutrition and adult disease. *The American journal of clinical nutrition* 71: 1344s-1352s, 2000.

67. **Godfrey KM, and Barker DJ**. Fetal programming and adult health. *Public health nutrition* 4: 611-624, 2001.

68. **Gratton J-P, Fontana J, O'Connor DS, García-Cardeña G, McCabe TJ, and Sessa WC**. Reconstitution of an Endothelial Nitric-oxide Synthase (eNOS), hsp90, and Caveolin-1 Complex in Vitro : Evidence that hsp90 Facilitates Calmodulin Stimulated Displacement of eNOS from Caveolin-1. *Journal of Biological Chemistry* 275: 22268-22272, 2000.

69. **Green LR, Kawagoe Y, Fraser M, Challis JR, and Richardson BS**. Activation of the hypothalamic-pituitary-adrenal axis with repetitive umbilical cord occlusion in the preterm ovine fetus. *Journal of the Society for Gynecologic Investigation* 7: 224-232, 2000.

70. **Guerra C, Koza RA, Yamashita H, Walsh K, and Kozak LP**. Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. *The Journal of clinical investigation* 102: 412-420, 1998.

71. **Guy ES, Kirumaki A, and Hanania NA**. Acute asthma in pregnancy. *Critical care clinics* 20: 731-745, x, 2004.

72. **Gyles SL, Burns CJ, Whitehouse BJ, Sugden D, Marsh PJ, Persaud SJ, and Jones PM**. ERKs Regulate Cyclic AMP-induced Steroid Synthesis through Transcription of the Steroidogenic Acute Regulatory (StAR) Gene. *Journal of Biological Chemistry* 276: 34888-34895, 2001.

73. **Hameed A, Karaalp IS, Tummala PP, Wani OR, Canetti M, Akhter MW, Goodwin I, Zapadinsky N, and Elkayam U**. The effect of valvular heart disease on maternal and fetal outcome of pregnancy. *Journal of the American College of Cardiology* 37: 893-899, 2001.

74. **Hanke CJ, and Campbell WB**. Endothelial cell nitric oxide inhibits aldosterone synthesis in zona glomerulosa cells: modulation by oxygen. *American Journal of Physiology - Endocrinology And Metabolism* 279: E846-E854, 2000.

75. **Hanke CJ, Drewett JG, Myers CR, and Campbell WB**. Nitric oxide inhibits aldosterone synthesis by a guanylyl cyclase-independent effect. *Endocrinology* 139: 4053-4060, 1998.

76. **Hanke CJ, O'Brien T, Pritchard KA, Jr., and Campbell WB**. Inhibition of adrenal cell aldosterone synthesis by endogenous nitric oxide release. *Hypertension* 35: 324-328, 2000.

77. **Harris MB, Blackstone MA, Sood SG, Li C, Goolsby JM, Venema VJ, Kemp BE, and Venema RC**. Acute activation and phosphorylation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors. *American journal of physiology Heart and circulatory physiology* 287: H560-566, 2004.

78. **Harvey LM, Gilbert RD, Longo LD, and Ducsay CA**. Changes in ovine fetal adrenocortical responsiveness after long-term hypoxemia. *The American journal of physiology* 264: E741-747, 1993.

79. **Himms-Hagen J, Cui J, Danforth E, Jr., Taatjes DJ, Lang SS, Waters BL, and Claus TH**. Effect of CL-316,243, a thermogenic beta 3-agonist, on energy balance and brown and white adipose tissues in rats. *The American journal of physiology* 266: R1371-1382, 1994.

80. **Howe DC, Gertler A, and Challis JR**. The late gestation increase in circulating ACTH and cortisol in the fetal sheep is suppressed by intracerebroventricular infusion of recombinant ovine leptin. *The Journal of endocrinology* 174: 259-266, 2002.

81. **Ignarro LJ**. Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annual review of pharmacology and toxicology* 30: 535-560, 1990.

82. **Ignarro LJ**. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society* 53: 503-514, 2002.

83. **Imamura T, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters endocrine and physiologic responses to umbilical cord occlusion in the ovine fetus. *Journal of the Society for Gynecologic Investigation* 11: 131-140, 2004.

84. **Ishibashi J, and Seale P**. Medicine. Beige can be slimming. *Science (New York, NY)* 328: 1113-1114, 2010.

85. **Jackson BT, Cohn HE, Morrison SH, Baker RM, and Piasecki GJ**. Hypoxia-induced sympathetic inhibition of the fetal plasma insulin response to hyperglycemia. *Diabetes* 42: 1621-1625, 1993.

86. **Jackson BT, Morrison SH, Cohn HE, and Piasecki GJ**. Adrenal secretion of glucocorticoids during hypoxemia in fetal sheep. *Endocrinology* 125: 2751-2757, 1989.

87. **Jackson BT, Piasecki GJ, Cohn HE, and Cohen WR**. Control of fetal insulin secretion. *American journal of physiology Regulatory, integrative and comparative physiology* 279: R2179-2188, 2000.

88. **Jensen GM, and Moore LG**. The effect of high altitude and other risk factors on birthweight: independent or interactive effects? *American journal of public health* 87: 1003-1007, 1997.

89. **Jequier E**. Leptin signaling, adiposity, and energy balance. *Annals of the New York Academy of Sciences* 967: 379-388, 2002.

90. **Jones CT, Boddy K, Robinson JS, and Ratcliffe JG**. Developmental changes in the responses of the adrenal glands of foetal sheep to endogenous adrenocorticotrophin, as indicated by hormone responses to hypoxaemia. *The Journal of endocrinology* 72: 279-292, 1977.

91. **Justice JM, Tanner MA, and Myers PR**. Endothelial cell regulation of nitric oxide production during hypoxia in coronary microvessels and epicardial arteries. *Journal of Cellular Physiology* 182: 359-365, 2000.

92. **Kamitomo M, Longo LD, and Gilbert RD**. Right and left ventricular function in fetal sheep exposed to long-term high-altitude hypoxemia. *The American journal of physiology* 262: H399-405, 1992.

93. **Kerwin JF, Jr., Lancaster JR, Jr., and Feldman PL**. Nitric oxide: a new paradigm for second messengers. *Journal of medicinal chemistry* 38: 4343-4362, 1995.

94. **Keyes LE, Armaza JF, Niermeyer S, Vargas E, Young DA, and Moore LG**. Intrauterine growth restriction, preeclampsia, and intrauterine mortality at high altitude in Bolivia. *Pediatric research* 54: 20-25, 2003.

95. **Kishimoto J, Tsuchiya T, Emson PC, and Nakayama Y**. Immobilization-induced stress activates neuronal nitric oxide synthase (nNOS) mRNA and protein in hypothalamic-pituitary-adrenal axis in rats. *Brain research* 720: 159-171, 1996.

96. **Kolas T, Nakling J, and Salvesen KA**. Smoking during pregnancy increases the risk of preterm births among parous women. *Acta obstetricia et gynecologica Scandinavica* 79: 644-648, 2000.

97. **Kostić T, Andrić S, Kovačević R, and Marić D**. The involvement of nitric oxide in stress-impaired testicular steroidogenesis. *European Journal of Pharmacology* 346: 267-273, 1998.

98. **Kroncke KD**. Zinc finger proteins as molecular targets for nitric oxide-mediated gene regulation. *Antioxidants & redox signaling* 3: 565-575, 2001.

99. **Kruse M, Bornstein SR, Uhlmann K, Paeth G, and Scherbaum WA**. Leptin down-regulates the steroid producing system in the adrenal. *Endocrine research* 24: 587-590, 1998.

100. **Kumar R**. Prenatal factors and the development of asthma. *Current opinion in pediatrics* 20: 682-687, 2008.

101. **Le Roy C, Li JY, Stocco DM, Langlois D, and Saez JM**. Regulation by adrenocorticotropin (ACTH), angiotensin II, transforming growth factor-beta, and insulin-like growth factor I of bovine adrenal cell steroidogenic capacity and expression of ACTH receptor, steroidogenic acute regulatory protein, cytochrome P450c17, and 3beta-hydroxysteroid dehydrogenase. *Endocrinology* 141: 1599-1607, 2000.

102. **Lebrethon MC, Naville D, Begeot M, and Saez JM**. Regulation of corticotropin receptor number and messenger RNA in cultured human adrenocortical cells by corticotropin and angiotensin II. *The Journal of clinical investigation* 93: 1828-1833, 1994.

103. **Lee C-M, Kim B-Y, Li L, and Morgan ET**. Nitric Oxide-dependent Proteasomal Degradation of Cytochrome P450 2B Proteins. *Journal of Biological Chemistry* 283: 889-898, 2008.

104. **Leos RA, Anderson MJ, Chen X, Pugmire J, Anderson KA, and Limesand SW**. Chronic exposure to elevated norepinephrine suppresses insulin secretion in fetal sheep with placental insufficiency and intrauterine growth restriction. *American journal of physiology Endocrinology and metabolism* 298: E770-778, 2010.

105. **Lewis RS, and Deen WM**. Kinetics of the reaction of nitric oxide with oxygen in aqueous solutions. *Chemical research in toxicology* 7: 568-574, 1994.

106. **Light A, and Hammes SR**. Membrane receptor cross talk in steroidogenesis: recent insights and clinical implications. *Steroids* 78: 633-638, 2013.

107. **Limesand SW, Rozance PJ, Smith D, and Hay WW, Jr.** Increased insulin sensitivity and maintenance of glucose utilization rates in fetal sheep with placental insufficiency and intrauterine growth restriction. *American journal of physiology Endocrinology and metabolism* 293: E1716-1725, 2007.

108. **Linnemann K, Malek A, Sager R, Blum WF, Schneider H, and Fusch C**. Leptin production and release in the dually in vitro perfused human placenta. *The Journal of clinical endocrinology and metabolism* 85: 4298-4301, 2000.

109. **Liu S, and Rockey DC**. Cicletanine stimulates eNOS phosphorylation and NO production via Akt and MAP kinase/Erk signaling in sinusoidal endothelial cells. *American journal of physiology Gastrointestinal and liver physiology* 305: G163-171, 2013.

110. **Lolmede K, Durand de Saint Front V, Galitzky J, Lafontan M, and Bouloumie A**. Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* 27: 1187-1195, 2003.

111. **Lueder FL, Kim SB, Buroker CA, Bangalore SA, and Ogata ES**. Chronic maternal hypoxia retards fetal growth and increases glucose utilization of select fetal tissues in the rat. *Metabolism: clinical and experimental* 44: 532-537, 1995.

112. **Masuda M, Kubota T, and Aso T**. Effects of nitric oxide on steroidogenesis in porcine granulosa cells during different stages of follicular development. *European journal of endocrinology / European Federation of Endocrine Societies* 144: 303-308, 2001.

113. **Masuda M, Kubota T, Karnada S, and Aso T**. Nitric oxide inhibits steroidogenesis in cultured porcine granulosa cells. *Mol Hum Reprod* 3: 285-292, 1997.

114. **Matthews SG, and Challis JR**. Levels of pro-opiomelanocortin and prolactin mRNA in the fetal sheep pituitary following hypoxaemia and glucocorticoid treatment in late gestation. *The Journal of endocrinology* 147: 139-146, 1995.

115. **McMillen IC, Muhlhausler BS, Duffield JA, and Yuen BS**. Prenatal programming of postnatal obesity: fetal nutrition and the regulation of leptin synthesis and secretion before birth. *The Proceedings of the Nutrition Society* 63: 405-412, 2004.

116. **Meaney MJ, Viau V, Bhatnagar S, Betito K, Iny LJ, O'Donnell D, and Mitchell JB**. Cellular mechanisms underlying the development and expression of individual differences in the hypothalamic-pituitary-adrenal stress response. *J Steroid Biochem Mol Biol* 39: 265-274, 1991.

117. **Michel T, and Feron O**. Nitric oxide synthases: which, where, how, and why? *The Journal of clinical investigation* 100: 2146-2152, 1997.

118. **Mineo C, Yuhanna IS, Quon MJ, and Shaul PW**. High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases. *The Journal of biological chemistry* 278: 9142-9149, 2003.

119. **Mishra OP, Zubrow AB, and Ashraf QM**. Nitric oxide-mediated activation of extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) during hypoxia in cerebral cortical nuclei of newborn piglets. *Neuroscience* 123: 179-186, 2004.

120. **Mitsube K, Mikuni M, Matousek M, and Brannstrom M**. Effects of a nitric oxide donor and nitric oxide synthase inhibitors on luteinizing hormone-induced ovulation in the ex-vivo perfused rat ovary. *Human reproduction (Oxford, England)* 14: 2537-2543, 1999.

121. **Molinari C, Uberti F, Grossini E, Vacca G, Carda S, Invernizzi M, and Cisari C**. 1alpha,25-dihydroxycholecalciferol induces nitric oxide production in cultured endothelial cells. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 27: 661-668, 2011.

122. **Monau TR, Vargas VE, King N, Yellon SM, Myers DA, and Ducsay CA**. Long-term hypoxia increases endothelial nitric oxide synthase expression in the ovine fetal adrenal. *Reproductive sciences (Thousand Oaks, Calif)* 16: 865-874, 2009.

123. **Monau TR, Vargas VE, Zhang L, Myers DA, and Ducsay CA**. Nitric oxide inhibits ACTH-induced cortisol production in near-term, long-term hypoxic ovine fetal adrenocortical cells. *Reproductive sciences (Thousand Oaks, Calif)* 17: 955-962, 2010.

124. **Moncada S, and Higgs EA**. Endogenous nitric oxide: physiology, pathology and clinical relevance. *European journal of clinical investigation* 21: 361-374, 1991.

125. **Moncada S, Palmer RM, and Higgs EA**. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109-142, 1991.

126. **Mortola JP, Frappell PB, Aguero L, and Armstrong K**. Birth weight and altitude: a study in Peruvian communities. *The Journal of pediatrics* 136: 324-329, 2000.

127. **Muldowney JA, 3rd, Davis SN, Vaughan DE, and Brown NJ**. NO synthase inhibition increases aldosterone in humans. *Hypertension* 44: 739-745, 2004.

128. **Munck A, Guyre PM, and Holbrook NJ**. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine reviews* 5: 25-44, 1984.

129. **Murata T, Sato K, Hori M, Ozaki H, and Karaki H**. Decreased Endothelial Nitric-oxide Synthase (eNOS) Activity Resulting from Abnormal Interaction between eNOS and Its Regulatory Proteins in Hypoxia-induced Pulmonary Hypertension. *Journal of Biological Chemistry* 277: 44085-44092, 2002.

130. **Murata T, Yamawaki H, Hori M, Sato K, Ozaki H, and Karaki H**. Hypoxia impairs endothelium-dependent relaxation in organ cultured pulmonary artery. *Eur J Pharmacol* 421: 45-53, 2001.

131. **Murotsuki J, Challis JR, Johnston L, and Gagnon R**. Increased fetal plasma prostaglandin E2 concentrations during fetal placental embolization in pregnant sheep. *American journal of obstetrics and gynecology* 173: 30-35, 1995.

132. **Myers DA, Bell P, Mlynarczyk M, and Ducsay CA**. Long term hypoxia alters plasma ACTH 1-39 and ACTH precursors in response to acute cord occlusion in the ovine fetus. *Journal of the Society for Gynecologic Investigation* 11: 249A, 2004.

133. **Myers DA, Bell PA, Hyatt K, Mlynarczyk M, and Ducsay CA**. Long-term hypoxia enhances proopiomelanocortin processing in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 288: R1178-1184, 2005.

134. **Myers DA, Hanson K, Mlynarczyk M, Kaushal KM, and Ducsay CA**. Long-term hypoxia modulates expression of key genes regulating adipose function in the late-gestation ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 294: R1312-1318, 2008.

135. **Myers DA, Hyatt K, Mlynarczyk M, Bird IM, and Ducsay CA**. Long-term hypoxia represses the expression of key genes regulating cortisol biosynthesis in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 289: R1707-1714, 2005.

136. **Myers DA, Hyatt K, Mlynarczyk M, Kaushal KM, and Ducsay CA**. Term hypoxia increases expression of transcription factors governing mitochondrial function and replication in peri-renal adipose tissue in the late gestation ovine fetus. *Reproductive sciences (Thousand Oaks, Calif)* 16: 331A, 2009.

137. **Nagata D, Mogi M, and Walsh K**. AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress. *The Journal of biological chemistry* 278: 31000-31006, 2003.

138. **Natarajan R, Lanting L, Bai W, Bravo EL, and Nadler J**. The role of nitric oxide in the regulation of aldosterone synthesis by adrenal glomerulosa cells. *J Steroid Biochem Mol Biol* 61: 47-53, 1997.

139. **Ness RB, and Sibai BM**. Shared and disparate components of the pathophysiologies of fetal growth restriction and preeclampsia. *American journal of obstetrics and gynecology* 195: 40-49, 2006.

140. **Newby EA, Kaushal KM, Myers DA, and Ducsay CA**. Adrenocorticotropic Hormone and PI3K/Akt Inhibition Reduce eNOS Phosphorylation and Increase Cortisol Biosynthesis in Long-Term Hypoxic Ovine Fetal Adrenal Cortical Cells. *Reproductive sciences (Thousand Oaks, Calif)* 2015.

141. **Nicol MR, Wang H, Ivell R, Morley SD, Walker SW, and Mason JI**. The expression of steroidogenic acute regulatory protein (StAR) in bovine adrenocortical cells. *Endocrine research* 24: 565-569, 1998.

142. **Nordentoft M, Lou HC, Hansen D, Nim J, Pryds O, Rubin P, and Hemmingsen R**. Intrauterine growth retardation and premature delivery: the influence of maternal smoking and psychosocial factors. *American journal of public health* 86: 347-354, 1996.

143. **Ong KK, and Dunger DB**. Perinatal growth failure: the road to obesity, insulin resistance and cardiovascular disease in adults. *Best practice & research Clinical endocrinology & metabolism* 16: 191-207, 2002.

144. **Onishi J, Browne VA, Kono S, Stiffel VM, and Gilbert RD**. Effects of long-term high-altitude hypoxia and troponin I phosphorylation on cardiac myofilament calcium responses in fetal and nonpregnant sheep. *Journal of the Society for Gynecologic Investigation* 11: 1-8, 2004.

145. **Ozolins IZ, Young IR, and McMillen IC**. Surgical disconnection of the hypothalamus from the fetal pituitary abolishes the corticotrophic response to intrauterine hypoglycemia or hypoxemia in the sheep during late gestation. *Endocrinology* 130: 2438-2445, 1992.

146. **Paul S, Pramanick K, Kundu S, Roy Moulik S, Pal P, and Mukherjee D**. Involvement of PI3 kinase and MAP kinase in IGF-I and insulin-induced ovarian steroidogenesis in common carp Cyprinus carpio. *General and comparative endocrinology* 181: 98-106, 2013.

147. **Penhoat A, Jaillard C, and Saez JM**. Regulation of bovine adrenal cell corticotropin receptor mRNA levels by corticotropin (ACTH) and angiotensin-II (A-II). *Mol Cell Endocrinol* 103: R7-10, 1994.

148. **Peterson JK, Moran F, Conley AJ, and Bird IM**. Zonal Expression of Endothelial Nitric Oxide Synthase in Sheep and Rhesus Adrenal Cortex. *Endocrinology* 142: 5351-5363, 2001.

149. **Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, and Nedergaard J**. Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a

population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. *The Journal of biological chemistry* 285: 7153-7164, 2010.

150. **Phillips ID, Ross JT, Owens JA, Young IR, and McMillen IC**. The peptide ACTH(1-39), adrenal growth and steroidogenesis in the sheep fetus after disconnection of the hypothalamus and pituitary. *J Physiol* 491 (Pt 3): 871-879, 1996.

151. **Phillips ID, Simonetta G, Owens JA, Robinson JS, Clarke IJ, and McMillen IC**. Placental restriction alters the functional development of the pituitary-adrenal axis in the sheep fetus during late gestation. *Pediatric research* 40: 861-866, 1996.

152. **Poulos SP, Hausman DB, and Hausman GJ**. The development and endocrine functions of adipose tissue. *Mol Cell Endocrinol* 323: 20-34, 2010.

153. **Raff H, Ball DL, and Goodfriend TL**. Low oxygen selectively inhibits aldosterone secretion from bovine adrenocortical cells in vitro. *American Journal of Physiology - Endocrinology And Metabolism* 256: E640-E644, 1989.

154. **Raff H, Bruder ED, and Group tSLsMCATS**. Steroidogenesis in human aldosterone-secreting adenomas and adrenal hyperplasias: effects of hypoxia in vitro. *American Journal of Physiology - Endocrinology And Metabolism* 290: E199-E203, 2006.

155. **Robinson JS, Moore VM, Owens JA, and McMillen IC**. Origins of fetal growth restriction. *European journal of obstetrics, gynecology, and reproductive biology* 92: 13-19, 2000.

156. **Rose JC, Meis PJ, Urban RR, and Greiss FC, Jr.** In vivo evidence for increased adrenal sensitivity to adrenocorticotropin-(1-24) in the lamb fetus late in gestation. *Endocrinology* 111: 80-85, 1982.

157. **Roseboom TJ, van der Meulen JH, Ravelli AC, Osmond C, Barker DJ, and Bleker OP**. Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. *Mol Cell Endocrinol* 185: 93-98, 2001.

158. **Rosmond R**. Role of stress in the pathogenesis of the metabolic syndrome. *Psychoneuroendocrinology* 30: 1-10, 2005.

159. **Rurak DW**. Plasma vasopressin levels during hypoxaemia and the cardiovascular effects of exogenous vasopressin in foetal and adult sheep. *The Journal of Physiology* 277: 341-357, 1978.

160. **Salafia CM, Vogel CA, Bantham KF, Vintzileos AM, Pezzullo J, and Silberman L**. Preterm delivery: correlations of fetal growth and placental pathology. *American journal of perinatology* 9: 190-193, 1992.

161. **Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scime A, Devarakonda S, Conroe HM, Erdjument-Bromage H, Tempst P, Rudnicki MA, Beier DR, and Spiegelman BM**. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* 454: 961-967, 2008.

162. **Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, Ishibashi J, Cohen P, Cinti S, and Spiegelman BM**. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. *The Journal of clinical investigation* 121: 96-105, 2011.

163. **Seckl JR**. Glucocorticoid programming of the fetus; adult phenotypes and molecular mechanisms. *Mol Cell Endocrinol* 185: 61-71, 2001.

164. **Seckl JR**. Glucocorticoids and small babies. *The Quarterly journal of medicine* 87: 259-262, 1994.

165. **Sewer MB, and Waterman MR**. ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. *Microscopy research and technique* 61: 300-307, 2003.

166. **Sewer MB, and Waterman MR**. cAMP-dependent Protein Kinase Enhances CYP17 Transcription via MKP-1 Activation in H295R Human Adrenocortical Cells. *Journal of Biological Chemistry* 278: 8106-8111, 2003.

167. **Sperling MA, Christensen RA, Ganguli S, and Anand R**. Adrenergic modulation of pancreatic hormone secretion in utero: studies in fetal sheep. *Pediatric research* 14: 203-208, 1980.

168. **Su Y, Carey LC, Rose JC, and Pulgar VM**. Leptin Alters Adrenal Responsiveness by Decreasing Expression of ACTH-R, StAR, and P450c21 in Hypoxemic Fetal Sheep. *Reproductive Sciences* 19: 1075-1084, 2012.

169. **Sug-Tang A, Bocking AD, Brooks AN, Hooper S, White SE, Jacobs RA, Fraher LJ, and Challis JR**. Effects of restricting uteroplacental blood flow on concentrations of corticotrophin-releasing hormone, adrenocorticotrophin, cortisol, and prostaglandin E2 in the sheep fetus during late pregnancy. *Canadian journal of physiology and pharmacology* 70: 1396-1402, 1992.

170. **Symonds ME, Budge H, Perkins AC, and Lomax MA**. Adipose tissue development--impact of the early life environment. *Progress in biophysics and molecular biology* 106: 300-306, 2011.

171. **Symonds ME, and Stephenson T**. Maternal nutrition and endocrine programming of fetal adipose tissue development. *Biochemical Society transactions* 27: 97-103, 1999.

172. **Thompson LP, and Dong Y**. Chronic hypoxia decreases endothelial nitric oxide synthase protein expression in fetal guinea pig hearts. *Journal of the Society for Gynecologic Investigation* 12: 388-395, 2005.

173. **Tsigos C, and Chrousos GP**. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *Journal of psychosomatic research* 53: 865-871, 2002.

174. **Tsubaki M, Hiwatashi A, Ichikawa Y, and Hori H**. Electron paramagnetic resonance study of ferrous cytochrome P-450scc-nitric oxide complexes: effects of cholesterol and its analogues. *Biochemistry* 26: 4527-4534, 1987.

175. **Tsubaki M, Ichikawa Y, Fujimoto Y, Yu NT, and Hori H**. Active site of bovine adrenocortical cytochrome P-450(11) beta studied by resonance Raman and electron paramagnetic resonance spectroscopies: distinction from cytochrome P-450scc. *Biochemistry* 29: 8805-8812, 1990.

176. **Unno N, Giussani DA, Hing WK, Ding XY, Collins JH, and Nathanielsz PW**. Changes in adrenocorticotropin and cortisol responsiveness after repeated partial umbilical cord occlusions in the late gestation ovine fetus. *Endocrinology* 138: 259-263, 1997.

177. **Valego NK, Su Y, Carey LC, Young SF, Tatter SB, Wang J, and Rose JC**. Hypothalamic-pituitary disconnection in fetal sheep blocks the peripartum increases in adrenal responsiveness and adrenal ACTH receptor expression. *American journal of physiology Regulatory, integrative and comparative physiology* 289: R410-r417, 2005.

178. **Van Voorhis BJ, Dunn MS, Snyder GD, and Weiner CP**. Nitric oxide: an autocrine regulator of human granulosa-luteal cell steroidogenesis. *Endocrinology* 135: 1799-1806, 1994.

179. **Vargas VE, Kaushal KM, Monau TR, Myers DA, and Ducsay CA**. Extracellular signal-regulated kinases (ERK1/2) signaling pathway plays a role in cortisol secretion in the long-term hypoxic ovine fetal adrenal near term. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 304: R636-R643, 2013.

180. **Warner TD, Mitchell JA, Sheng H, and Murad F**. Effects of cyclic GMP on smooth muscle relaxation. *Advances in pharmacology (San Diego, Calif)* 26: 171-194, 1994.

181. **Wideroe M, Vik T, Jacobsen G, and Bakketeig LS**. Does maternal smoking during pregnancy cause childhood overweight? *Paediatric and perinatal epidemiology* 17: 171-179, 2003.

182. **Wilkening RB, and Meschia G**. Fetal oxygen uptake, oxygenation, and acid-base balance as a function of uterine blood flow. *The American journal of physiology* 244: H749-755, 1983.

183. **Williams JM, White CR, Chang MM, Injeti ER, Zhang L, and Pearce WJ**. Chronic hypoxic decreases in soluble guanylate cyclase protein and enzyme activity are age dependent in fetal and adult ovine carotid arteries. *Journal of applied physiology (Bethesda, Md : 1985)* 100: 1857-1866, 2006.

184. **Williams LA, Evans SF, and Newnham JP**. Prospective cohort study of factors influencing the relative weights of the placenta and the newborn infant. *BMJ (Clinical research ed)* 314: 1864-1868, 1997.

185. **Xiao D, Bird IM, Magness RR, Longo LD, and Zhang L**. Upregulation of eNOS in pregnant ovine uterine arteries by chronic hypoxia. *American Journal of Physiology - Heart and Circulatory Physiology* 280: H812-H820, 2001.

186. **Yamauchi J, Miyazaki T, Iwasaki S, Kishi I, Kuroshima M, Tei C, and Yoshimura Y**. Effects of nitric oxide on ovulation and ovarian steroidogenesis and prostaglandin production in the rabbit. *Endocrinology* 138: 3630-3637, 1997.

187. **Yates DT, Macko AR, Chen X, Green AS, Kelly AC, Anderson MJ, Fowden AL, and Limesand SW**. Hypoxaemia-induced catecholamine secretion from adrenal chromaffin cells inhibits glucose-stimulated hyperinsulinaemia in fetal sheep. *J Physiol* 590: 5439-5447, 2012.

188. **Yuen BS, McMillen IC, Symonds ME, and Owens PC**. Abundance of leptin mRNA in fetal adipose tissue is related to fetal body weight. *The Journal of endocrinology* 163: R11-14, 1999.

189. **Yuen BS, Owens PC, Symonds ME, Keisler DH, McFarlane JR, Kauter KG, and McMillen IC**. Effects of leptin on fetal plasma adrenocorticotropic hormone and cortisol concentrations and the timing of parturition in the sheep. *Biology of reproduction* 70: 1650-1657, 2004.

190. **Zamudio S, Torricos T, Fik E, Oyala M, Echalar L, Pullockaran J, Tutino E, Martin B, Belliappa S, Balanza E, and Illsley NP**. Hypoglycemia and the origin of hypoxia-induced reduction in human fetal growth. *PloS one* 5: e8551, 2010.

191. **Zhao H, Li Z, Cooney AJ, and Lan ZJ**. Orphan nuclear receptor function in the ovary. *Frontiers in bioscience : a journal and virtual library* 12: 3398-3405, 2007.

192. **Zhu Y, Sun Y, Xie L, Jin K, Sheibani N, and Greenberg DA**. Hypoxic induction of endoglin via mitogen-activated protein kinases in mouse brain microvascular endothelial cells. *Stroke; a journal of cerebral circulation* 34: 2483-2488, 2003.

CHAPTER TWO

ADRENOCORTICOTROPIC HORMONE AND PI3K/AKT INHIBITION REDUCE ENOS PHOSPHORYLATION AND INCREASE CORTISOL BIOSYNTHESIS IN LONG-TERM HYPOXIC OVINE FETAL ADRENAL CORTICAL CELLS

Elizabeth A. Newby[1], Kanchan M. Kaushal[1], Dean A. Myers[2] and Charles A. Ducsay[1].

[1]Center for Perinatal Biology, Loma Linda University, Loma Linda, CA 92350 and
[2]Department of Obstetrics and Gynecology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104.

Running Head: Long term hypoxia and adrenal eNOS

Key Words: sheep, adrenocorticotropic hormone, hypoxia

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

Supported by National Institutes of Health Grants PO1HD31226, R01HD51951

The work presented in this chapter has been published,
Newby, E. A., Kaushal, K. M., Myers, D. A., & Ducsay, C. A. (2015). Adrenocorticotropic Hormone and PI3K/Akt Inhibition Reduce eNOS Phosphorylation and Increase Cortisol Biosynthesis in Long-Term Hypoxic Ovine Fetal Adrenal Cortical Cells. Reprod Sci. PMID: 25656500.

Abstract

This study was designed to determine the role of the MEK/ERK1/2 and PI3K/Akt pathways in cortisol production and endothelial nitric oxide synthase (eNOS) phosphorylation (peNOS) in the ovine fetal adrenal in response to long-term hypoxia (LTH). Pregnant ewes were maintained at high altitude (3820 m) for the last 100 days of gestation (dGa). At 138 to 142 dGa, fetal adrenal cortical cells (FACs) were collected from LTH and age-matched normoxic fetuses. Cortisol production and peNOS were measured in response to pretreatment with the MEK/ERK1/2 pathway inhibitor UO126 (UO) and adrenocorticotropic hormone (ACTH) stimulation. UO126 reduced ACTH-stimulated cortisol in both normoxic and LTH FACs. UO126 alone or in combination with ACTH reduced peNOS in the normoxic group, while ACTH alone or ACTH + UO inhibited peNOS in LTH FACs. Additionally, cortisol was measured in response to pretreatment with UO and treatment with 22R-hydroxycholesterol (22R-OHC) or water-soluble cholesterol (WSC) with and without ACTH stimulation. UO126 had no effect on 22R-OHC–treated cells, but reduced cortisol in cells treated with WSC and/or ACTH. Cortisol and peNOS were also measured in response to pretreatment with PI3K/Akt pathway inhibitor Wortmannin (WT) and ACTH stimulation. Wortmannin further increased cortisol under ACTH-stimulated conditions and, like ACTH, reduced peNOS in LTH but not normoxic FACs. Together, these data suggest that in LTH FACs MEK/ERK1/2 does not regulate peNOS but that UO acts downstream from eNOS, possibly at cholesterol transport, to affect cortisol production in LTH FACs, while the PI3K/Akt pathway, along with ACTH, regulates peNOS and plays a role in the fetal adaptation to LTH in FACs.

Introduction

Hypoxia is a potent stressor that activates the hypothalamo-pituitary-adrenal (HPA) axis, and acutely, leads to a significant increase in cortisol (1-3, 19, 20). Since cortisol is involved in lipolysis, glycogenolysis, and protein catabolism, a sustained elevation of plasma cortisol concentrations can suppress anabolic processes resulting in muscle atrophy and delayed maturation and organ growth (26, 34). Therefore, under conditions of chronic stress, the ability of the fetal HPA axis to adapt to limit cortisol production is crucial for maintaining normal development during gestation. The regulation of cortisol must also be effectively coordinated to permit the late gestation exponential rise in fetal plasma cortisol essential for fetal maturation, while permitting episodic cortisol production in response to acute stress.

In our laboratory, we have clearly shown the ability of the fetal HPA axis to adapt to the chronic stress of long-term moderate gestational hypoxia (LTH). In this model, the fetus develops under high altitude induced (3820 m from approximately day 40 of gestation) moderate hypoxia (fetal PO_2 ~17-19 mmHg vs. ~21-23 mmHg normoxic controls). Under conditions of LTH, the fetus maintains normal basal plasma cortisol concentrations despite elevated adrenocorticotropic hormone (ACTH) (35). Although we found no differences in cyclic adenosine monophosphate (cAMP) production or protein kinase A (PKA) activation either basally or in response to ACTH in LTH fetal adrenocortical cells (FACs), we did observe decreased expression of CYP11A1 and CYP17, two key steroidogenic P450 enzymes, as well as decreased ACTH receptor expression (36). When combined, this may contribute to maintaining basal cortisol levels in the LTH fetus. In contrast to basal conditions, the LTH fetus displays a heightened

cortisol response to an acute secondary stressor compared to normoxic fetuses (1, 19). Thus, the LTH fetus has developed a mechanism of regulation that maintains basal plasma cortisol, despite elevated basal ACTH, but allows the fetus to overcome this suppression for an enhanced cortisol response to an acute secondary stress.

One possible effector of cortisol regulation in this system is nitric oxide (NO), a diatomic free radical gas with a variety of physiological functions that is produced from L-arginine by NO synthases (NOSs) (18, 33). We have previously shown that NO inhibits ACTH-stimulated cortisol production in LTH ovine FACs and that endothelial NOS (eNOS) inhibition enhances LTH FAC cortisol biosynthesis (32). We have also demonstrated that eNOS is the most abundant isoform of NOS in the ovine fetal adrenal cortex, and that expression of eNOS is enhanced in LTH adrenals compared to normoxic controls (31), consistent with the observed dissociation between elevated plasma ACTH and normal basal output of cortisol. However, in line with enhanced ACTH and stress-stimulated cortisol production in the LTH fetus, ACTH treatment significantly reduced eNOS activity in LTH FACs compared to normoxic (32).

A key mechanism involved in the regulation of eNOS is phosphorylation at a serine activation site. In the human, it is serine residue 1177, and in the bovine/ovine it is serine residue 1179, typically referred to as Ser1177/79 (10, 14, 15). A reduction in phosphorylation at this residue could be the result of either decreased kinase activation or active dephosphorylation (4, 7, 16). Both of these potential mechanisms would lead to a reduction in NOS activity, with a resultant decrease in NO production. This in turn would allow for increased cortisol biosynthesis under ACTH-stimulated conditions in the LTH fetal adrenal. Phosphorylation at this site may be affected by different cell signaling

pathways including MEK/ERK1/2 and PI3K/Akt. Various studies have shown that inhibition of the MEK/ERK1/2 pathway with UO126 (UO) reduces eNOS phosphorylation and NO production in a variety of cell types (5, 23, 28, 30). Other studies have shown that the PI3K/Akt pathway also targets eNOS at Ser1177/79 in endothelial cells (14, 17, 28). The roles of the MEK/ERK1/2 and the PI3K/Akt pathways in eNOS activation and in the context of LTH in ovine FACs have yet to be examined. However, we have shown that NO clearly inhibits basal and ACTH-induced cortisol synthesis in ovine FACs (32). Paradoxically, we observed that inhibition of MEK/ERK1/2 signaling with UO did not have the predicted enhancement of basal or ACTH-induced cortisol synthesis but rather inhibited ACTH-induced cortisol synthesis (43), suggesting that while MEK/ERK1/2 may target eNOS in these cells, it has additional pathways that may predominate in regulating cortisol synthesis.

In light of these findings, UO has also been shown to block steroidogenesis in both granulosa (9) and Leydig (25, 38) cells. This may be through effects on steroidogenic acute regulatory (StAR) protein, which transports cholesterol into the mitochondria (8, 21, 24, 39-41); inhibition of StAR activity would prevent cortisol biosynthesis. These studies showed that UO inhibited synthesis of steroid in both stimulated cells and cells supplemented with water-soluble cholesterol (WSC), both of which require cholesterol transport into the mitochondria for steroidogenesis. However, steroid production was unaffected in cells treated with 22R-hydroxycholesterol (22R-OHC), a mitochondrial membrane permeable form of cholesterol that does not require facilitative transport. Therefore, although MEK/ERK1/2 may have a major role in

cholesterol transport in FACs, the role of LTH on this process has not yet been determined in ovine FACs.

The present study was designed to 1) determine if either the MEK/ERK1/2 or PI3K/Akt pathways regulate eNOS phosphorylation in FACs, 2) address the role of MEKERK1/2 in facilitating cholesterol transport to the mitochondria, and 3) determine the potential adaptive alterations in these pathways in response to LTH.

Materials and Methods

Animals

Time-dated pregnant ewes were maintained at the Barcroft Laboratory White Mountain Research Station (3820m, maternal PO2 ~ 60mmHg) from approximately day 40 of gestation to near term (term ≅ 146 days). Following transportation to the laboratory, hypoxia was maintained by nitrogen infusion through a maternal tracheal catheter as previously described (1, 12, 19, 32, 42). Age-matched, normoxic ewes served as controls. On days 138-142 of gestation, ewes were sedated and maintained under general anesthesia while fetuses were delivered through midline laparotomy. Procedures were performed as previously described in detail (29). 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 (BSA), and 0.1 g L-glutamine for cell dispersion and subsequent study. All procedures were conducted with the approval of the Institutional Animal Care and Use Committees (Loma Linda University School of Medicine, Loma Linda, CA).

Cell Dispersion

Procedures for collection of FACs were similar to those we previously described.(42, 43) Briefly, fetal adrenal glands were divided in half along the longitudinal axis and the cortex was separated from the medulla. The cortical tissue was minced and enzymatically dispersed with 40 mg collagenase Type II (Worthington Biomedical, Lakewood, NJ), 40 mg of Polypep bovine protein digest (Sigma-Aldrich) and 100 µl of DNAse I (Type IV) (Sigma-Aldrich) dissolved in 10 ml of Sodium Krebs Buffer (0.4% collagenase). The resulting mono-dispersed FACs were aliquoted into individual tubes with media (M-199), and allowed to equilibrate for 2 hours at 37°C prior to initiation of each study as required by each experimental protocol. Cell viability was confirmed by Trypan blue exclusion.

Treatment Protocols

Effects of MEK/ERK1/2 Inhibition and ACTH Stimulation on Cortisol Biosynthesis and eNOS Phosphorylation

FACs from normoxic (n=7) and LTH (n=5) fetuses, aliquoted at $7.5x10^5$ cells/1mL, were untreated, pretreated with MEK/ERK1/2 inhibitor UO126 (UO, 10 µM) for 1 hour, or stimulated with ACTH (100 pM), with and without UO pretreatment. Media and cells were collected at 0 (baseline), 10, 20, and 60 minutes after stimulation. Media was immediately frozen in liquid nitrogen, and stored at -80°C until determination of cortisol. Cells were lysed in 80 µL of lysis buffer (93% prelysis buffer [1 mM Trizma Base, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% Triton X-100, 0.5% Igepal CO-630, 20 mM NaF], 1% 100 mM phenylmethanesulfonyl fluoride [PMSF], 1% Protease

Inhibitor Cocktail [PIC, Sigma, St Louis, Missouri], 5% 20 mM sodium orthovanadate), frozen in liquid nitrogen, and stored at -80°C until analysis.

Effects of UO126 Pretreatment and ACTH, 22R-OHC, or WSC Stimulation on Cortisol Biosynthesis

This experiment was designed to examine the interaction between inhibition of the MEK/ERK1/2 pathway with UO126 and cholesterol transport across the mitochondrial membrane. FACs from normoxic (n=6) and LTH (n=7) fetuses, aliquoted at $2.5x10^5$ cells/mL, were either untreated, pretreated with UO (10 µM) for 1 hour, or stimulated with ACTH (100 pM) with and without UO pretreatment, treated with membrane permeable 22R-hydroxycholesterol (22R-OHC, 10 µM), with and without UO pretreatment, treated with water-soluble cholesterol (WSC, 10 µM), with and without UO pretreatment, or a combined stimulation of ACTH and 22R-OHC treatment or ACTH and WSC treatment with and without UO pretreatment. The membrane permeable form, 22R-OHC does not require transport across the mitochondrial membrane whereas WSC is transport dependent. Media was collected at 60 minutes after stimulation and stored as described above for later cortisol analysis.

Effects of PI3K/Akt Inhibition and ACTH Stimulation on Cortisol Biosynthesis and eNOS Phosphorylation

FACs from normoxic (n=7) and LTH (n=9) fetuses, aliquoted at $7.5x10^5$ cells/1mL, were either untreated, pretreated with PI3K/Akt inhibitor Wortmannin (WT, 10 nM) for 1 hour, or stimulated with ACTH (100 pM), with and without WT

pretreatment. Media and cells were collected at 0 (baseline), 10, 20, and 60 minutes after stimulation and stored as described above. We chose to use Wortmannin instead of LY294002 because in preliminary studies, the LY compound dramatically reduced FAC viability.

Cortisol Assay

Cortisol was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) cortisol kit (Oxford Biomedical Research, Oxford, MI) that has been previously described and validated for use in our laboratory (13, 32, 36).

Western Analysis

Endothelial nitric oxide synthase protein was analyzed from FACs collected at 0 (baseline), 10, 20, and 60 minutes for both normoxic and LTH groups, described above. Samples were thawed and protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, Illinois) with BSA as the standard. Absorbance was measured at 595 nm on a BioTek Synergy HT Multi-Mode Microplate Reader (Winooski, Vermont).

Endothelial nitric oxide protein phosphorylation was determined by Western blotting using methods we have previously described and validated.(35, 36) Briefly, protein samples were denatured for 5 minutes at boiling temperature and 20 µg of protein were loaded per lane. Protein samples were separated using 7.5% polyacrylamide gels (Bio-Rad, Hercules, CA) and subjected to electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) using a Transblot cell apparatus (Bio-Rad).

To determine the level of eNOS protein phosphorylation, the membranes were incubated with a rabbit monoclonal phospho-eNOS (Ser1177) (C9C3) primary antibody (Cell Signaling, Product#9570) in 10 mL 5% BSA TBST solution (1:1000) overnight at 4°C. Membranes were then incubated with goat anti-rabbit polyclonal secondary antibody (ThermoScientific, Product#35571) in 10 mL 5% BSA TBST solution (1:10000) for 90 minutes, washed, and imaged with a Licor Odyssey scanner at 700 nm. The relative optical densities (ROD) of the bands were used to compare normoxic to LTH phosphorylated eNOS (peNOS) protein expression. An internal positive standard prepared from whole fetal adrenal tissue was used to normalize peNOS protein.

To determine the level of eNOS protein expression, the membranes were first stripped of phosphorylated antibody and incubated with mouse anti-eNOS primary antibody (BD Transduction, Product# 610296) in 10 mL 5% NFDM TBST solution (1:250) overnight at 4°C. Membranes were then incubated with goat anti-mouse polyclonal secondary antibody (Thermo Scientific, Product# 35518) in 10 mL 5% NFDM TBST solution (1:20000) for 90 minutes, washed, and imaged with a Licor Odyssey scanner at 800 nm. The RODs of the bands were used to compare normoxic to LTH eNOS protein expression. An internal positive standard prepared from whole fetal adrenal tissue was used to normalize eNOS protein as we have previously described in our laboratory (31, 43).

Statistical Analysis

Descriptive statistics are presented as mean ± standard error. Data analysis was performed using repeated measures analysis of variance (ANOVA) with 1 between-

subject factor (treatment) and 1 within-subject factor (time) stratified by oxygenation level (normoxic or LTH). The main effect tested was in vitro treatment (WT or UO). For the cholesterol study, 2-way ANOVA was used with oxygenation level (normoxic or LTH) tested across cholesterol treatment. Alpha was set at .05 significance level. Post hoc tests were adjusted using the Bonferroni method. Statistical analyses were performed using IBM SPSS Statistics (Version 22; IBM Corporation, 2013).

Results

Effects of MEK/ERK1/2 Inhibition and ACTH Stimulation

Cortisol Production

There were no differences observed in cortisol production from either control or LTH FACs that were pretreated with UO compared to the untreated cells, and cortisol levels remained relatively constant throughout the 60 minutes of study (**Figure 1**). There was a significant increase in cortisol output from both normoxic (4.49 +/- 0.89 ng/mL) and LTH (13.25 +/- 0.78 ng/mL) FACs; in control normoxic FACs, cortisol was significantly elevated by 60 minutes after ACTH stimulation ($p<0.05$), while a significant increase in cortisol output was observed in LTH FACs in response to ACTH by 10 minutes. Pretreatment with UO inhibited the stimulated increase in cortisol in both normoxic and LTH FACs compared to ACTH alone ($p<0.05$).

Expression of eNOS

Treatment with ACTH and UO pretreatment had no effect on expression of eNOS protein in either LTH or control FACs (**Figure 2A**).

Phosphorylation of eNOS

In normoxic FACs, all three treatments resulted in a similar reduction in peNOS. In contrast, LTH FACs demonstrated a significant reduction in peNOS in the ACTH treated groups (UO + ACTH or ACTH alone) compared to untreated FACs ($p<0.05$). This reduction was similar in both groups (**Figure 2B**).

Effects of UO126 and ACTH, 22R-OHC, and WSC Stimulation on Cortisol Production

Treatment with either ACTH and 22R-OHC, or ACTH and WSC resulted in enhanced cortisol production in FACs from both normoxic and LTH groups compared to respective untreated controls ($p<0.05$) while the effect was greater in the LTH group compared to the normoxic group ($p<0.05$) (Figure 3). Pretreatment with UO blocked the ACTH and WSC stimulated increase but had no effect on cortisol production in cells stimulated with 22R-OHC in either normoxic or LTH FACs (**Figure 3**).

Effects of PI3K/Akt Inhibition and ACTH Stimulation

Cortisol Production

Pretreatment with WT alone had no effect on cortisol biosynthesis compared to untreated control FACs in both normoxic and LTH FACs, and cortisol levels remained relatively constant throughout the 60 minutes of study (**Figure 4**). There was a significant increase in cortisol production by 60 minutes ($p<0.05$) in cells stimulated with ACTH in both normoxic (7.54 +/- 1.37 ng/mL) and LTH (8.85 +/- ng/mL) FACs. ACTH cells pretreated with WT demonstrated significantly enhanced cortisol production in both

normoxic (12.42 +/- 1.96 ng/mL) and LTH (26.18 +/- 4.48 ng/mL) compared to ACTH alone ($p<0.05$).

Expression of eNOS

No differences were observed in eNOS expression between treatment groups in both normoxic and LTH FACs (**Figure 5A**).

Phosphorylation of eNOS

There were no significant changes in phosphorylation between treatment groups in the normoxic FACs (**Figure 5B**). In LTH FACs, there was a significant reduction in phosphorylation with WT pretreatment, ACTH stimulation, and combined pretreatment and stimulation compared to untreated FACs ($p<0.05$). This reduction was similar, with no differences observed among WT, ACTH, or WT+ACTH groups.

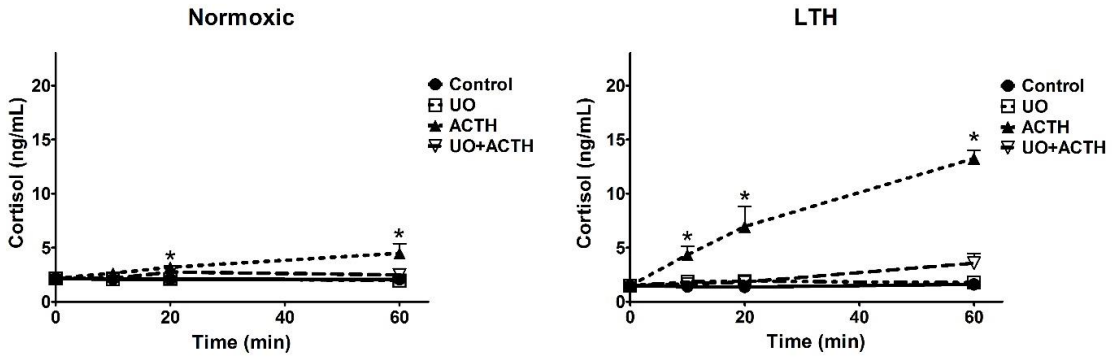

Figure 1. Time course of cortisol production in normoxic and LTH FACs with MEK/ERK1/2 inhibition and ACTH stimulation. Treatment with ACTH (100pM) increased cortisol production in both normoxic and LTH FACs. Pretreatment with UO (10μM) had no effect on basal cortisol, but prevented increased cortisol biosynthesis in response to ACTH stimulation in both normoxic and LTH FACs. (Normoxic n=7, LTH n=5) Values represent mean values ± SEM. *$p<0.05$ compared to time 0. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; UO, UO126; ACTH, adrenocorticotropic hormone.

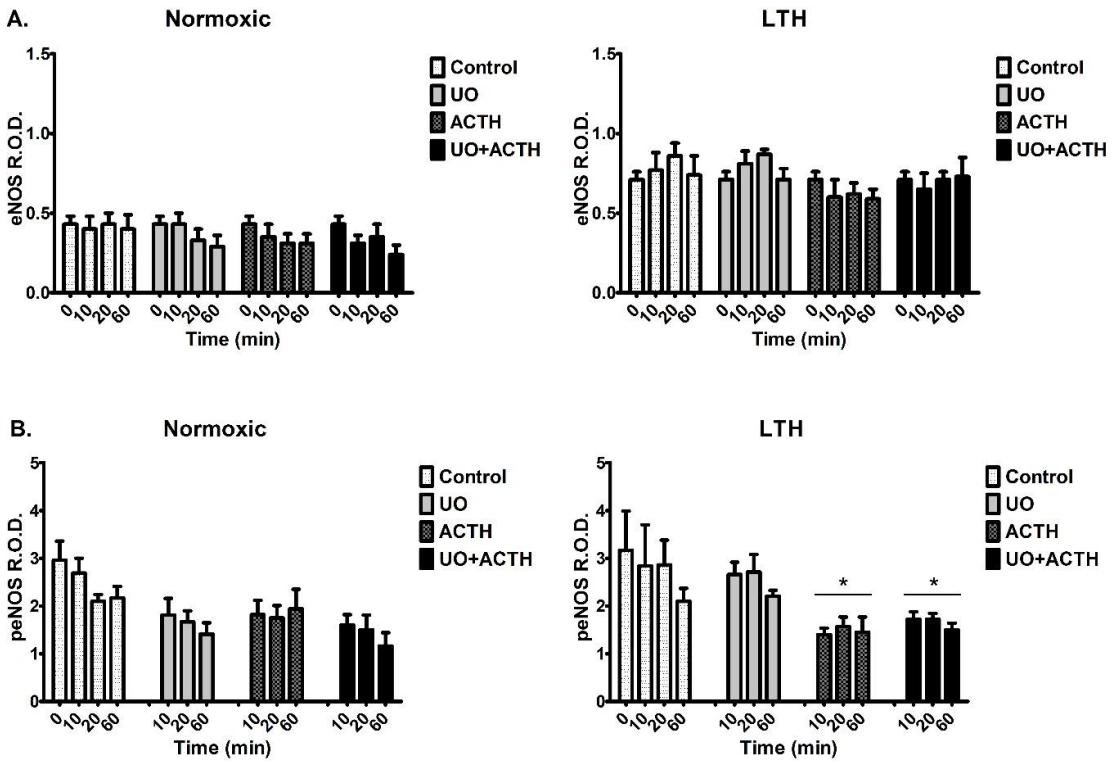

Figure 2. Protein expression (A) and phosphorylation (B) of eNOS in response to MEK/ERK1/2 inhibition and ACTH stimulation in normoxic and LTH FACs as determined by Western analysis. Pretreatment with UO (10μM) with and without ACTH (100pM) stimulation had no effect on eNOS in both normoxic and LTH FACs. Pretreatment with UO (10μM) had no effect on peNOS in both normoxic and LTH FACs. Treatment with ACTH had no effect on peNOS in normoxic FACs but decreased peNOS in LTH FACs ($p<0.05$ compared to untreated LTH FACs; Normoxic n=7, LTH n=5). Values represent mean values ± SEM. *$p<0.05$ compared to control. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; eNOS, endothelial nitric oxide synthase; peNOS, phosphorylated endothelial nitric oxide synthase; ROD, relative optical density; UO, UO126; ACTH, adrenocorticotropic hormone.

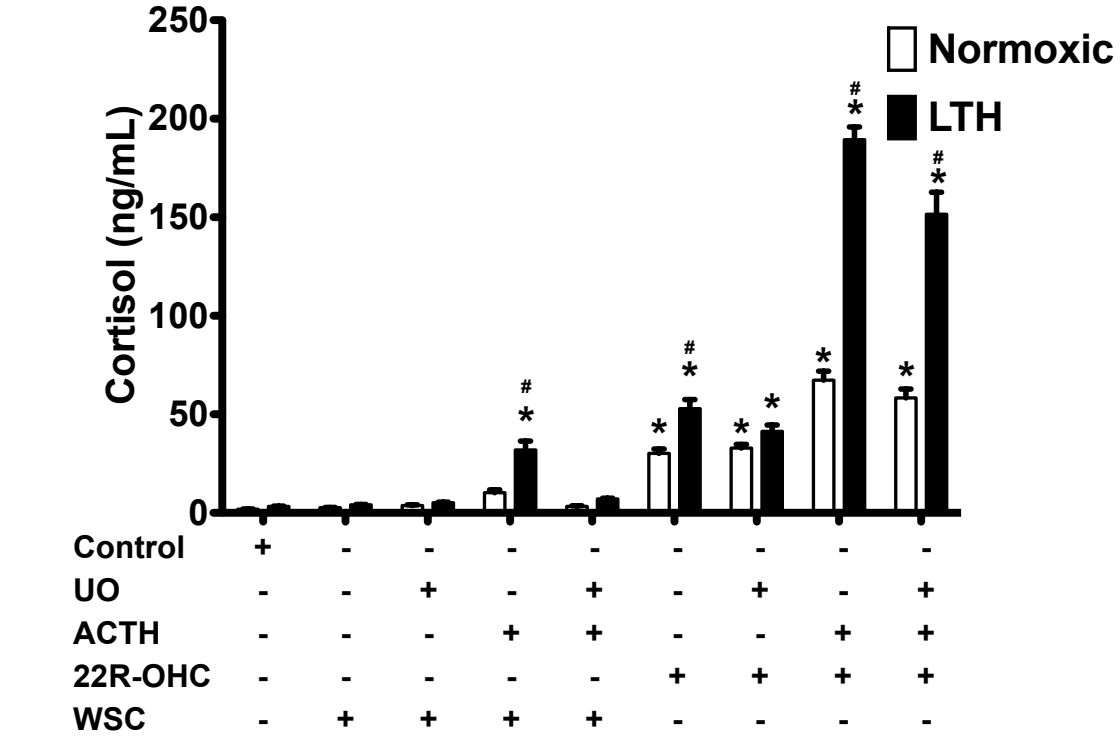

Figure 3. Cortisol production in response to UO126 pretreatment and 22R-OHC or WSC stimulation with or without ACTH in normoxic and LTH FACs. ACTH (100pM), 22R-OHC (10μM), and WSC (10μM) stimulation increased cortisol production in both normoxic and LTH FACs, with a greater increase in dual stimulated cells. Pretreatment with UO blocked cortisol increase in cells stimulated with ACTH and WSC but had no effect on cells stimulated with 22R-OHC in both normoxic and LTH FACs. (Normoxic n=6, LTH n=7) Values represent mean values ± SEM. *$p<0.05$ compared to untreated control, #$p<0.05$ compared to normoxic. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; C, Control; UO, UO126; ACTH, adrenocorticotropic hormone; 22R-OHC, 22R-hydroxycholesterol; WSC, water-soluble cholesterol.

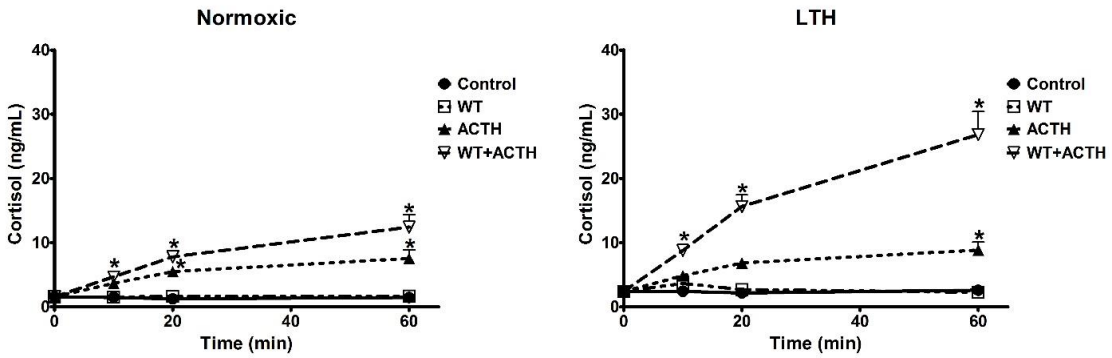

Figure 4. Time course of cortisol production in normoxic and LTH FACs with PI3K/Akt inhibition and ACTH stimulation. Treatment with ACTH (100pM) increased cortisol production in both normoxic and LTH FACs. Pretreatment with WT (10nM) had no effect on basal cortisol but enhanced cortisol biosynthesis in response to ACTH stimulation in both normoxic and LTH. (Normoxic n=7, LTH n=9) Values represent mean values ± SEM. *$p<0.05$ compared to time 0. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; WT, Wortmannin; ACTH, adrenocorticotropic hormone.

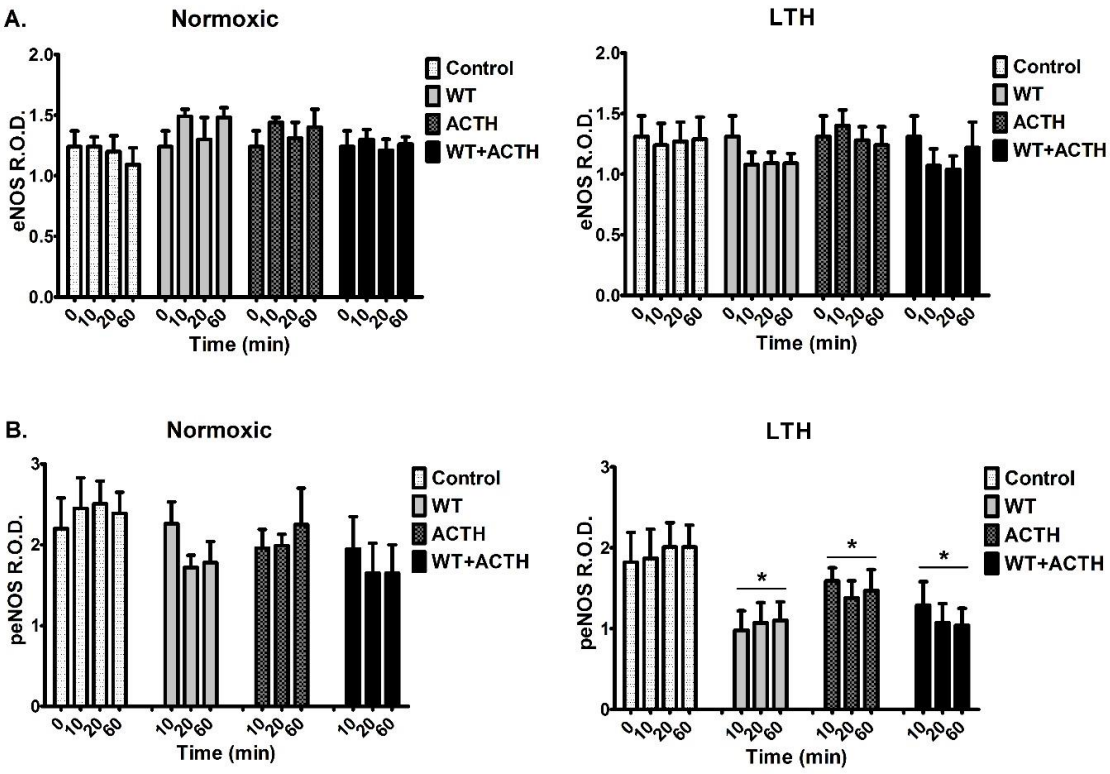

Figure 5. Protein expression (A) and phosphorylation (B) of eNOS in response to PI3K/Akt inhibition and ACTH stimulation in normoxic and LTH FACs as determined by Western analysis. Pretreatment with WT (10nM) with and without ACTH (100pM) stimulation had no effect on eNOS in both normoxic and LTH FACs. Pretreatment with WT (10nM) with and without ACTH (100pM) stimulation had no effect on peNOS in normoxic FACs but reduced peNOS in LTH FACs. (Normoxic n=7, LTH n=9) Values represent mean values ± SEM. *$p<0.05$ compared to control. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; eNOS, endothelial nitric oxide synthase; peNOS, phosphorylated endothelial nitric oxide synthase; ROD, relative optical density; WT, Wortmannin; ACTH, adrenocorticotropic hormone.

Discussion

The regulation of cortisol is a crucial component of fetal development due to its involvement in catabolic processes; chronically high levels of cortisol can suppress anabolic processes, which can prevent normal tissue growth and maturation of the fetus (26, 34). The ovine fetus demonstrates a divergent adaptive response of the HPA axis to LTH. At the level of the hypothalamus and anterior pituitary, there is clearly an activation of the stress response with enhanced proopiomelanocortin (POMC) processing to ACTH coupled with elevated basal plasma ACTH levels in LTH fetuses compared to normoxic controls (35). There is also a distinct up regulation of the ACTH response to AVP compared to CRH in the LTH fetuses (13). In contrast to the hypothalamic and pituitary responses, we showed that basal adrenal cortisol biosynthesis is normal despite the elevated plasma ACTH.(1, 11, 19, 35) Surprisingly, the LTH fetus responds more robustly, with enhanced cortisol production to a secondary stressor compared to the normoxic fetus (1, 19), suggesting an adaptation in the HPA axis that maintains normal basal levels but allows for enhanced production in response to stress.

We previously reported that NO inhibits ACTH-stimulated cortisol production, while eNOS inhibition enhanced cortisol biosynthesis in LTH ovine FACs (32). We also found that endothelial NOS (eNOS) is the most abundant adrenal cortical NOS isoform and LTH enhanced not only expression of eNOS (31), but also NOS activity compared to normoxic controls (32). Activity of eNOS can be influenced by a number of factors including substrate availability, protein-protein interactions, and post-translational modification via phosphorylation (6). In this study we address the ability of the cell signaling kinase pathways MEK/ERK1/2 and PI3K/Akt to regulate eNOS activity via

phosphorylation of Ser1177/79 to alter NO production that, in turn, could then affect cortisol biosynthesis. We also addressed if the site of action of MEK/ERK1/2 on cortisol synthesis in FACs was up- or downstream of cholesterol translocation into the mitochondria, the site of the first rate-limiting step in steroidogenesis, since we previously observed a major inhibition in ACTH-induced cortisol synthesis in FACs (43).

In a previous study we showed MEK/ERK1/2 inhibition reduced cortisol output in FACs in response to stimulation with ACTH (43). In the present study, as in our previously published report (43), we found that MEK/ERK1/2 inhibition with UO126 (UO) prevented the increase of cortisol in response to ACTH in both normoxic and LTH FACs. While ACTH-stimulated cortisol production was inhibited in both normoxic and LTH FACs, the ability of UO to almost completely prevent the enhanced cortisol production in response to ACTH in the LTH FACs indicates that the adaptive response seen in the LTH fetus is dependent upon MEK/ERK1/2 signaling. In the present study, we also explored the effect of UO on eNOS expression or phosphorylation. UO126 alone did not affect eNOS expression. In normoxic FACs, all 3 treatments (UO, ACTH and ACTH + UO) reduced peNOS similarly over time. This suggests that ACTH itself is not responsible for changes in eNOS phosphorylation in normoxic FACs. In contrast, in LTH FACs, UO alone had no effect on peNOS. As predicted, however, ACTH significantly reduced peNOS, and the same effect was observed with ACTH in the presence of UO. Together with the cortisol data, these findings demonstrate that MEK/ERK1/2 signaling, while playing a role in the adaptive increase in ACTH-stimulated cortisol production in the LTH adrenal cortex, does so through a pathway not involving MEK/ERK1/2 mediated phosphorylation of eNOS.

Results from the MEK/ERK1/2 experiment also demonstrated the divergent effects of ACTH on cortisol and peNOS in LTH FACs; ACTH increased cortisol while decreasing peNOS which supports our hypothesis that eNOS is involved in the fetal adaptation to LTH and that regulation of eNOS phosphorylation alters NO production, which then affects cortisol biosynthesis in LTH FACs. These results show that while inhibition of the MEK/ERK1/2 pathway with UO was able to prevent the increase in cortisol in cells stimulated with ACTH, it had no effect on peNOS suggesting that UO works through a different mechanism to inhibit cortisol production.

Inhibition of steroidogenesis by UO has been shown in both granulosa (9) and Leydig (25, 38) cells. These studies demonstrated that although stimulated synthesis of steroid was inhibited by UO, when cells were stimulated with 22R-hydroxycholesterol (22R-OHC), a membrane permeable form of cholesterol, steroid production was unaffected. This suggests UO was blocking steroidogenesis by preventing cholesterol translocation into the mitochondria, a process carried out by steroidogenic acute regulatory (StAR) protein. StAR, classically regulated by PKA via cAMP (39), is responsible for transporting cholesterol to the inner mitochondrial membrane for conversion from cholesterol to pregnenolone by CYP11A1 (P450scc) (8, 21, 24, 40, 41), the rate-limiting step for cortisol biosynthesis. Inhibition of StAR activity would halt steroidogenesis by eliminating the substrate. In this study, we stimulated FACs with 22R-OHC, a substrate that does not require transport, to examine the effects of UO on cholesterol translocation. We found that UO blocked the cortisol increase observed in cells stimulated with ACTH and water-soluble cholesterol (WSC), which both require cholesterol transport across the membrane, however UO had no effect on the 22R-OHC

stimulated increase. Together this suggests that UO may be able to block cholesterol transport to limit cortisol production and demonstrates that an adaptation does occur in response to LTH to allow for increased cortisol in response to a secondary stressor.

Although it is evident from our prior studies that eNOS is involved in the fetal adaptation to LTH and that ACTH stimulation decreases peNOS while increasing cortisol, our finding in the present study that inhibition of MEK/ERK1/2 did not have an effect on peNOS suggests that another pathway may play a role in the phosphorylation state of peNOS. Inhibition of the PI3K/Akt pathway has been implicated in inhibition of stimulated steroid production in multiple steroidogenic cell types (22, 37) and has also been shown to reduce eNOS phosphorylation in endothelial cells followed by decreased NO production (14, 17, 28). We examined the role of the PI3K/Akt pathway by stimulating FACs with ACTH, with and without pretreatment with PI3K/Akt pathway inhibitor WT, and examined cortisol and eNOS protein expression and phosphorylation in both normoxic and LTH groups. Pretreatment with WT had no effect alone but enhanced ACTH-stimulated cortisol synthesis above ACTH alone. This suggests that the PI3K/Akt pathway differentially regulates cortisol biosynthesis in LTH FACs and may work to prevent even higher levels of cortisol in LTH FACs under stimulated conditions.

We also found that PI3K/Akt inhibition did not affect eNOS expression in either normoxic or LTH FACs and had no effect on peNOS in normoxic FACs. However, WT, and ACTH as we had previously seen, significantly reduced peNOS in LTH FACs, suggesting the involvement of the PI3K/Akt pathway in the fetal adaptation to LTH; PI3K/Akt inhibition with WT reduced peNOS and allowed for an even greater increase in cortisol in ACTH-stimulated LTH FACs. This further supports the idea that

phosphorylation of eNOS is closely linked to cortisol synthesis in the LTH fetus while other mechanisms play a more dominant role in normoxic adrenals. It also suggests that while ACTH stimulates cortisol biosynthesis in both normoxic and LTH FACs, it may also interact with the PI3K/Akt pathway in LTH FACs resulting in the observed enhanced cortisol production.

A possible intermediary between ACTH and eNOS is protein phosphatase 2A (PP2A). PP2A has been shown to be capable of dephosphorylating eNOS and the inhibition of PP2A increases peNOS in endothelial cells (16, 27), however the effects of LTH on this system are unexplored. Preliminary data from our lab shows significantly greater PP2A expression in the LTH adrenal cortex compared to normoxic tissue (unpublished results) suggesting the involvement of PP2A in the fetal adaptation to LTH. If ACTH increases PP2A activity, combined with greater PP2A expression, it would reduce peNOS, thereby reducing NO production and effectively limiting the inhibition of NO on cortisol production in LTH FACs.

Taken together, the results from the present studies as well as our previous work (31, 32, 42, 43) indicate that LTH has profound adaptive effects on the fetal adrenal cortex. NO, produced by eNOS, may play an important role in this adaptation in the LTH fetus. The results from this study show that while the MEK/ERK pathway is involved in cortisol biosynthesis, as evidenced by UO inhibition of cholesterol transport in response to ACTH stimulation, it is not involved in the differential regulation of eNOS phosphorylation. These results also show that both the PI3K/Akt pathway and ACTH differentially regulate peNOS. Collectively, these data suggest that the PI3k/Akt pathway and ACTH regulation of eNOS phosphorylation in LTH fetal adrenal cortical cells may

be key components of the fetal adaptation in cortisol biosynthesis. Combined with the higher levels of eNOS protein in the LTH adrenals, the PI3K/Akt pathway and ACTH may work congruently in LTH FACs to regulate eNOS activity via phosphorylation; the PI3K/Akt pathway maintains peNOS to allow NO to be produced while ACTH stimulation overrides the PI3K/Akt pathway to reduce peNOS and limit NO production. Together, these mechanisms would preserve normal cortisol levels under basal conditions but also allow for the robust increase in cortisol observed in stimulated LTH FACs when compared to normoxic controls.

References

1. **Adachi K, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters ovine fetal endocrine and physiological responses to hypotension. *American journal of physiology Regulatory, integrative and comparative physiology* 287: R209-217, 2004.

2. **Bocking AD, McMillen IC, Harding R, and Thorburn GD**. Effect of reduced uterine blood flow on fetal and maternal cortisol. *Journal of developmental physiology* 8: 237-245, 1986.

3. **Boddy K, Jones CT, Mantell C, Ratcliffe JG, and Robinson JS**. Changes in plasma ACTH and corticosteroid of the maternal and fetal sheep during hypoxia. *Endocrinology* 94: 588-591, 1974.

4. **Cale JM, and Bird IM**. Inhibition of MEK/ERK1/2 signalling alters endothelial nitric oxide synthase activity in an agonist-dependent manner. *The Biochemical journal* 398: 279-288, 2006.

5. **Chen DB, Bird IM, Zheng J, and Magness RR**. Membrane estrogen receptor-dependent extracellular signal-regulated kinase pathway mediates acute activation of endothelial nitric oxide synthase by estrogen in uterine artery endothelial cells. *Endocrinology* 145: 113-125, 2004.

6. **Chen JX, and Meyrick B**. Hypoxia increases Hsp90 binding to eNOS via PI3K-Akt in porcine coronary artery endothelium. *Laboratory investigation; a journal of technical methods and pathology* 84: 182-190, 2004.

7. **Chen Z, Peng I-C, Sun W, Su M-I, Hsu P-H, Fu Y, Zhu Y, DeFea K, Pan S, Tsai M-D, and Shyy JY-J**. AMP-Activated Protein Kinase Functionally Phosphorylates Endothelial Nitric Oxide Synthase Ser633. *Circulation Research* 104: 496-505, 2009.

8. **Daniel PB, Walker WH, and Habener JF**. Cyclic AMP signaling and gene regulation. *Annual review of nutrition* 18: 353-383, 1998.

9. **Dewi DA, Abayasekara DR, and Wheeler-Jones CP**. Requirement for ERK1/2 activation in the regulation of progesterone production in human granulosa-lutein cells is stimulus specific. *Endocrinology* 143: 877-888, 2002.

10. **Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, and Zeiher AM**. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399: 601-605, 1999.

11. **Ducsay CA**. Fetal and maternal adaptations to chronic hypoxia: prevention of premature labor in response to chronic stress. *Comparative biochemistry and physiology Part A, Molecular & integrative physiology* 119: 675-681, 1998.

12. **Ducsay CA, Hyatt K, Mlynarczyk M, Root BK, Kaushal KM, and Myers DA**. Long-term hypoxia modulates expression of key genes regulating adrenomedullary function in the late gestation ovine fetus. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 293: R1997-R2005, 2007.

13. **Ducsay CA, Mlynarczyk M, Kaushal KM, Hyatt K, Hanson K, and Myers DA**. Long-term hypoxia enhances ACTH response to arginine vasopressin but not corticotropin-releasing hormone in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 297: R892-899, 2009.

14. **Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, and Sessa WC**. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399: 597-601, 1999.

15. **Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, Figeys D, Harrison DG, Berk BC, Aebersold R, and Corson MA**. Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *The Journal of biological chemistry* 274: 30101-30108, 1999.

16. **Greif DM, Kou R, and Michel T**. Site-specific dephosphorylation of endothelial nitric oxide synthase by protein phosphatase 2A: evidence for crosstalk between phosphorylation sites. *Biochemistry* 41: 15845-15853, 2002.

17. **Harris MB, Blackstone MA, Sood SG, Li C, Goolsby JM, Venema VJ, Kemp BE, and Venema RC**. Acute activation and phosphorylation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors. *American journal of physiology Heart and circulatory physiology* 287: H560-566, 2004.

18. **Ignarro LJ**. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society* 53: 503-514, 2002.

19. **Imamura T, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters endocrine and physiologic responses to umbilical cord occlusion in the ovine fetus. *Journal of the Society for Gynecologic Investigation* 11: 131-140, 2004.

20. **Jones CT, Boddy K, Robinson JS, and Ratcliffe JG**. Developmental changes in the responses of the adrenal glands of foetal sheep to endogenous adrenocorticotrophin, as indicated by hormone responses to hypoxaemia. *The Journal of endocrinology* 72: 279-292, 1977.

21. **Kamenetsky M, Middelhaufe S, Bank EM, Levin LR, Buck J, and Steegborn C**. Molecular details of cAMP generation in mammalian cells: a tale of two systems. *Journal of molecular biology* 362: 623-639, 2006.

22. **Light A, and Hammes SR**. Membrane receptor cross talk in steroidogenesis: recent insights and clinical implications. *Steroids* 78: 633-638, 2013.

23. **Liu S, and Rockey DC**. Cicletanine stimulates eNOS phosphorylation and NO production via Akt and MAP kinase/Erk signaling in sinusoidal endothelial cells. *American journal of physiology Gastrointestinal and liver physiology* 305: G163-171, 2013.

24. **Manna PR, Dyson MT, Eubank DW, Clark BJ, Lalli E, Sassone-Corsi P, Zeleznik AJ, and Stocco DM**. Regulation of steroidogenesis and the steroidogenic acute regulatory protein by a member of the cAMP response-element binding protein family. *Molecular endocrinology (Baltimore, Md)* 16: 184-199, 2002.

25. **Martinelle N, Holst M, Soder O, and Svechnikov K**. Extracellular signal-regulated kinases are involved in the acute activation of steroidogenesis in immature rat Leydig cells by human chorionic gonadotropin. *Endocrinology* 145: 4629-4634, 2004.

26. **Meaney MJ, Viau V, Bhatnagar S, Betito K, Iny LJ, O'Donnell D, and Mitchell JB**. Cellular mechanisms underlying the development and expression of individual differences in the hypothalamic-pituitary-adrenal stress response. *J Steroid Biochem Mol Biol* 39: 265-274, 1991.

27. **Michell BJ, Chen Z, Tiganis T, Stapleton D, Katsis F, Power DA, Sim AT, and Kemp BE**. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *The Journal of biological chemistry* 276: 17625-17628, 2001.

28. **Mineo C, Yuhanna IS, Quon MJ, and Shaul PW**. High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases. *The Journal of biological chemistry* 278: 9142-9149, 2003.

29. **Mlynarczyk M, Imamura T, Umezaki H, Kaushal KM, Zhang L, and Ducsay CA**. Long-term hypoxia changes myometrial responsiveness and oxytocin receptors in the pregnant ewe: differential effects on longitudinal versus circular smooth muscle. *Biology of reproduction* 69: 1500-1505, 2003.

30. **Molinari C, Uberti F, Grossini E, Vacca G, Carda S, Invernizzi M, and Cisari C**. 1alpha,25-dihydroxycholecalciferol induces nitric oxide production in cultured endothelial cells. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 27: 661-668, 2011.

31. **Monau TR, Vargas VE, King N, Yellon SM, Myers DA, and Ducsay CA**. Long-term hypoxia increases endothelial nitric oxide synthase expression in the ovine fetal adrenal. *Reproductive sciences (Thousand Oaks, Calif)* 16: 865-874, 2009.

32. **Monau TR, Vargas VE, Zhang L, Myers DA, and Ducsay CA**. Nitric oxide inhibits ACTH-induced cortisol production in near-term, long-term hypoxic ovine fetal adrenocortical cells. *Reproductive sciences (Thousand Oaks, Calif)* 17: 955-962, 2010.

33. **Moncada S, Palmer RM, and Higgs EA**. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109-142, 1991.

34. **Munck A, Guyre PM, and Holbrook NJ**. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine reviews* 5: 25-44, 1984.

35. **Myers DA, Bell PA, Hyatt K, Mlynarczyk M, and Ducsay CA**. Long-term hypoxia enhances proopiomelanocortin processing in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 288: R1178-1184, 2005.

36. **Myers DA, Hyatt K, Mlynarczyk M, Bird IM, and Ducsay CA**. Long-term hypoxia represses the expression of key genes regulating cortisol biosynthesis in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 289: R1707-1714, 2005.

37. **Paul S, Pramanick K, Kundu S, Roy Moulik S, Pal P, and Mukherjee D**. Involvement of PI3 kinase and MAP kinase in IGF-I and insulin-induced ovarian steroidogenesis in common carp Cyprinus carpio. *General and comparative endocrinology* 181: 98-106, 2013.

38. **Renlund N, Jo Y, Svechnikova I, Holst M, Stocco DM, Soder O, and Svechnikov K**. Induction of steroidogenesis in immature rat Leydig cells by interleukin-1alpha is dependent on extracellular signal-regulated kinases. *Journal of molecular endocrinology* 36: 327-336, 2006.

39. **Richards JS**. New signaling pathways for hormones and cyclic adenosine 3',5'-monophosphate action in endocrine cells. *Molecular endocrinology (Baltimore, Md)* 15: 209-218, 2001.

40. **Sewer MB, and Waterman MR**. ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. *Microscopy research and technique* 61: 300-307, 2003.

41. **Stocco DM**. StAR protein and the regulation of steroid hormone biosynthesis. *Annual review of physiology* 63: 193-213, 2001.

42. **Vargas VE, Kaushal KM, Monau T, Myers DA, and Ducsay CA**. Long-term hypoxia enhances cortisol biosynthesis in near-term ovine fetal adrenal cortical cells. *Reproductive sciences (Thousand Oaks, Calif)* 18: 277-285, 2011.

43. **Vargas VE, Kaushal KM, Monau TR, Myers DA, and Ducsay CA**. Extracellular signal-regulated kinases (ERK1/2) signaling pathway plays a role in cortisol secretion in the long-term hypoxic ovine fetal adrenal near term. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 304: R636-R643, 2013.

CHAPTER THREE

EFFECTS OF 8BROMO-CAMP AND UO126 ON CORTISOL BIOSYNTHESIS AND ENOS PHOSPHORYLATION IN OVINE LONG-TERM HYPOXIC FETAL ADRENOCORTICAL CELLS

Abstract

Previously we have demonstrated enhanced cortisol biosynthesis in long-term hypoxic (LTH) fetal adrenocortical cells (FACs) in response to stress levels of ACTH that was not a result of differences in cyclic 3,5-adenosine mono phosphate (cAMP) or protein kinase A (PKA). We did show that inhibition of the MEK/ERK1/2 pathway with UO126 (UO) was able to prevent increased cortisol in both normoxic and LTH FACs but the mechanism was unknown. This study was designed to determine the role of cAMP stimulation, using the analog 8Bromo-cAMP (8Br), and MEK/ERK1/2 pathway inhibition on cortisol production and eNOS phosphorylation (peNOS) in the ovine fetal adrenal in response to long term hypoxia (LTH). Pregnant ewes were maintained at high altitude (3820m) for approximately the last 100 days of gestation (dGa). At 138-142 dGa, fetal adrenal cortical cells (FACs) were collected from LTH and age matched normoxic ovine fetuses. Cortisol production and peNOS were measured in response to 8Br stimulation and pretreatment with MEK/ERK1/2 pathway inhibitor UO. Neither 8Br nor UO affected peNOS but UO reduced 8Br-stimulated cortisol in normoxic and LTH FACs suggesting that cAMP and the MEK/ERK1/2 pathway are not involved in regulating eNOS phosphorylation in the LTH ovine fetal adrenal.

Introduction

The study presented in this chapter on the effects of 8Bromo-cAMP (8Br) stimulation and MEK/ERK1/2 inhibition with UO126 (UO) on cortisol biosynthesis and eNOS phosphorylation in ovine long-term hypoxic (LTH) fetal adrenocortical cells (FACs) was conducted as an adjunct to the studies presented in the previous published chapter.

The fetus has the ability to adapt the hypothalamic-pituitary-adrenal (HPA) axis to the chronic stress of long-term moderate gestational hypoxia (LTH). In response to conditions of LTH, the fetus maintains normal basal plasma cortisol concentrations, despite elevated levels of adrenocorticotropic hormone (ACTH) (17). However, unlike basal conditions, the LTH fetus has a heightened cortisol response to acute secondary stressors compared to normoxic fetuses (1, 11). While we found no differences in cyclic adenosine monophosphate (cAMP) production or protein kinase A (PKA) activation basally or in response to ACTH in LTH fetal adrenocortical cells (FACs) (23), CYP11A1 and CYP17, two key steroidogenic P450 enzymes, and ACTH receptor (ACTH-R) were decreased (18). These changes may contribute to the ability of the LTH fetus to maintain basal cortisol levels despite elevated ACTH, however the mechanism of regulation that allows the fetus to overcome this suppression with enhanced cortisol production in response to a secondary stress is still undefined.

In our previous studies, we found that inhibition of the MEK/ERK1/2 signaling pathway with UO126 reduced ACTH-induced cortisol production in both normoxic and LTH FACs (24), and that MEK/ERK1/2 signaling does not affect cortisol through regulation of eNOS phosphorylation; inhibition with UO126 did not alter peNOS in

either normoxic or LTH FACs and did not differentially limit ACTH-stimulated cortisol production between normoxic and LTH FACs (19). Together these results suggest that the MEK/ERK1/2 pathway affects cortisol production and acts through a mechanism other than NO to impact cortisol steroidogenesis. Classically, ACTH-stimulated cortisol biosynthesis is regulated via 3,5-cAMP/PKA activation of steroidogenic acute regulatory (StAR) protein (7, 21). We found that enhanced ACTH-stimulated cortisol production in LTH FACs is not a result of increased cAMP production and/or PKA activation, however, expression of StAR protein, responsible for cholesterol transport in to the inner mitochondrial membrane for the initial rate limiting step in steroidogenesis, was increased in LTH FACs compared to normoxic controls (23). These results indicate that while MEK/ERK1/2 signaling is involved in cortisol biosynthesis, the adaptive mechanism dissociating cortisol production from elevated ACTH lies downstream from ACTH signal transduction. This study was designed to (1) assess whether activation of steroidogenesis via membrane permeable cAMP analog 8Bromo-cAMP (8Br) is altered by MEK/ERK1/2 pathway inhibition with UO, and (2) determine whether 8Br activation of steroidogenesis regulates eNOS phosphorylation in FACs.

Materials and Methods

Animals

Time-dated pregnant ewes were maintained at the Barcroft Laboratory White Mountain Research Station (3820m, maternal PO_2 ~ 60mmHg) from approximately 40 days gestational age (dGa) to near term (term ≅ 146 days). Following transportation to the laboratory, hypoxia was maintained by nitrogen infusion through a maternal tracheal

catheter as previously described (1, 9, 11, 15, 19, 23). Age-matched, normoxic ewes served as controls. On 138 to 142 dGa, ewes were sedated and maintained under general anesthesia while fetuses were delivered through midline laparotomy. Procedures were performed as previously described in detail (13). Fetal adrenal glands were collected in ice-cold media M-199 (Sigma-Aldrich, St. Louis, Missouri), containing 2.2 g sodium bicarbonate, 2.0 g bovine serum albumin, and 0.1 g L-glutamine for cell dispersion and subsequent study. All procedures were conducted with the approval of the Institutional Animal Care and Use Committees (Loma Linda University School of Medicine, Loma Linda, CA).

Cell Dispersion

Procedures for collection of FACs were similar to those we described previously (19, 23, 24). Briefly, fetal adrenal glands were divided in half along the longitudinal axis and the cortex was separated from the medulla. The cortical tissue was minced and enzymatically dispersed with 40 mg collagenase Type II (Worthington Biomedical, Lakewood, NJ), 40 mg of Polypep bovine protein digest (Sigma-Aldrich) and 100 µl of DNAse I (Type IV; Sigma-Aldrich) dissolved in 10 mL of Sodium Krebs Buffer (0.4% collagenase). The resulting monodispersed FACs were aliquoted into individual tubes with media (M-199), and allowed to equilibrate for 2 hr at 37°C prior to initiation of the study as required by the experimental protocol. Cell viability was confirmed by Trypan blue exclusion.

Treatment Protocol

Effects of MEK/ERK1/2 inhibition and 8Bromo-cAMP stimulation on cortisol biosynthesis and eNOS phosphorylation

FACs, aliquoted at $7.5x10^5$ cells/mL, were untreated, pretreated with MEK/ERK1/2 inhibitor UO126 (UO, 10 µM) for 1 hour, or stimulated with 8Bromo-cAMP (8Br, 10 mM), with and without UO pretreatment. Media and cells were collected at 0 (baseline), 10, 20, and 60 minutes after stimulation (**Figure 1**). Media were immediately frozen in liquid nitrogen, and stored at -80°C until determination of cortisol. Cells were lysed in 80 µL of lysis buffer (93% prelysis buffer [1 mmol/L Trizma Base, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1% Triton X-100, 0.5% Igepal CO-630, 20 mmol/L NaF], 1% 100 mmol/L phenylmethanesulfonyl fluoride, 1% Protease Inhibitor Cocktail [Sigma, St Louis, Missouri], 5% 20 mmol/L sodium orthovanadate), frozen in liquid nitrogen, and stored at -80°C until analysis.

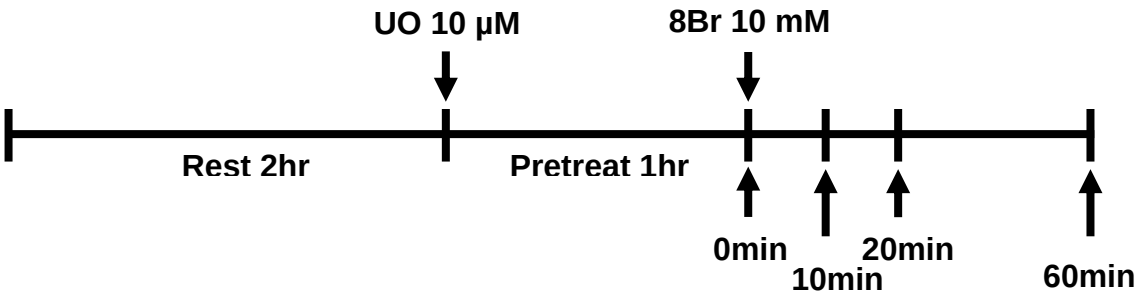

Figure 1. Timeline for treatment protocol 8Bromo-cAMP. UO, UO126; 8Br, 8Bromo-cAMP.

Cortisol Assay

Cortisol was measured using a commercially available enzyme-linked immunosorbent assay cortisol kit (Oxford Biomedical Research, Oxford, Michigan) that has been previously described and validated for use in our laboratory (10, 15, 18).

Western Analysis

Endothelial NOS protein was analyzed from FACs collected at 0 (baseline), 10, 20, and 60 min for both normoxic and LTH groups, described earlier. Samples were thawed and protein concentration was determined using a bicinchoninic acid protein assay (Thermo Scientific, Rockford, Illinois) with BSA as the standard. Absorbance was measured at 595 nm on a BioTek Synergy HT Multi-Mode Microplate Reader (Winooski, Vermont).

Endothelial NOS protein phosphorylation was determined by Western blotting using methods we have previously described and validated (17, 18). Briefly, protein samples were denatured for 5 minutes at boiling temperature and a total of 20 µg of protein were loaded per lane. Protein samples were separated using 7.5% polyacrylamide gels (Bio-Rad, Hercules, California) and subjected to electrophoresis (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, Massachusetts) using a Transblot cell apparatus (Bio-Rad).

To determine the level of eNOS protein phosphorylation, the membranes were incubated with a rabbit monoclonal phospho-eNOS (Ser1177; C9C3) primary antibody (Cell Signaling, Product#9570) in 10 mL 5% BSA Tris-buffered saline with Tween 20

(TBST) solution (1:1000) overnight at 4°C. Membranes were then incubated with goat anti-rabbit polyclonal secondary antibody (ThermoScientific, Product#35571) in 10 mL 5% BSA TBST solution (1:10000) for 90 minutes, washed, and imaged with a Licor Odyssey Infrared Imaging System (LI-COR Bio-sciences, Lincoln, Nebraska) at 700 nm. The relative optical densities (ROD) of the bands were used to measure normoxic and LTH phosphorylated eNOS (peNOS) protein expression. An internal positive standard prepared from whole fetal adrenal tissue was used to normalize peNOS protein.

To determine the level of eNOS protein expression, the membranes were first stripped of phosphorylated antibody and incubated with mouse anti-eNOS primary antibody (BD Transduction, Product# 610296) in 10 mL 5% nonfat dry milk (NFDM) TBST solution (1:250) overnight at 4°C. Membranes were then incubated with goat anti-mouse polyclonal secondary antibody (Thermo Scientific, Product# 35518) in 10 mL 5% NFDM TBST solution (1:20000) for 90 minutes, washed, and imaged with a Licor Odyssey Infrared Imaging System (LI-COR Bio-sciences, Lincoln, Nebraska) at 800 nm. The RODs of the bands were used to measure normoxic and LTH eNOS protein expression. An internal positive standard prepared from whole fetal adrenal tissue was used to normalize eNOS protein as we have previously described in our laboratory (14, 24).

Statistical Analysis

Descriptive statistics are presented as mean ± standard error. Data analysis was performed using two-way analysis of variance (ANOVA) with 1 between-subject factor (treatment) and 1 within-subject factor (time) stratified by oxygenation level (normoxic

or LTH). The main effect tested was in vitro treatment (UO). Alpha was set at .05 significance level. Post hoc tests were adjusted using the Bonferroni method. Statistical analyses were performed GraphPad Prism 5 (Version 5.04; GraphPad Software, Inc., 2010).

Results

Effects of MEK/ERK1/2 Inhibition and 8Bromo-cAMP Stimulation

Cortisol Production

There were no differences observed in cortisol production from either normoxic (n=6) or LTH (n=6) FACs that were pretreated with UO compared to the untreated cells, and cortisol levels remained relatively constant throughout the 60 minutes of study (**Figure 2**). There was a significant increase in cortisol output from both normoxic and LTH FACs; in control normoxic FACs, cortisol was significantly elevated by 60 minutes after 8Br stimulation ($p<0.05$) compared to time 0, while a significant increase in cortisol output was observed in LTH FACs in response to 8Br by 10 minutes compared to time 0. The maximal increase in cortisol production was approximately 15 fold in the LTH FACs compared to approximately 5 fold in the control cells. Pretreatment with UO inhibited the 8Br-stimulated increase in cortisol in both normoxic and LTH FACs compared to 8Br alone ($p<0.05$).

Expression of eNOS

Treatment with 8Br and UO pretreatment had no effect on expression of eNOS protein in either normoxic or LTH FACs compared to control (**Figure 3A**).

Phosphorylation of eNOS

Treatment with 8Br and UO pretreatment had no effect on phosphorylation of eNOS protein in either normoxic or LTH FACs compared to control (**Figure 3B**).

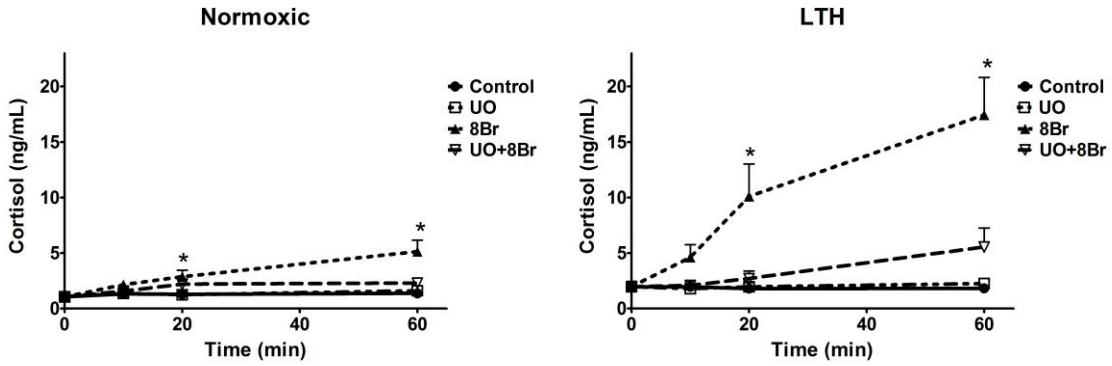

Figure 2. Time course of cortisol production in normoxic and LTH FACs with MEK/ERK1/2 inhibition and 8Bromo-cAMP stimulation. Treatment with 8Br (10mM) increased cortisol production in both normoxic and LTH FACs. UO126 (10µM) pretreatment had no effect on basal cortisol, but prevented increased cortisol biosynthesis in response to 8Br stimulation in both normoxic and LTH. (Normoxic n=6, LTH n=6) Values represent mean values ± SEM. *$p<0.05$ compared to time 0. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; UO, UO126; 8Br, 8Bromo-cAMP.

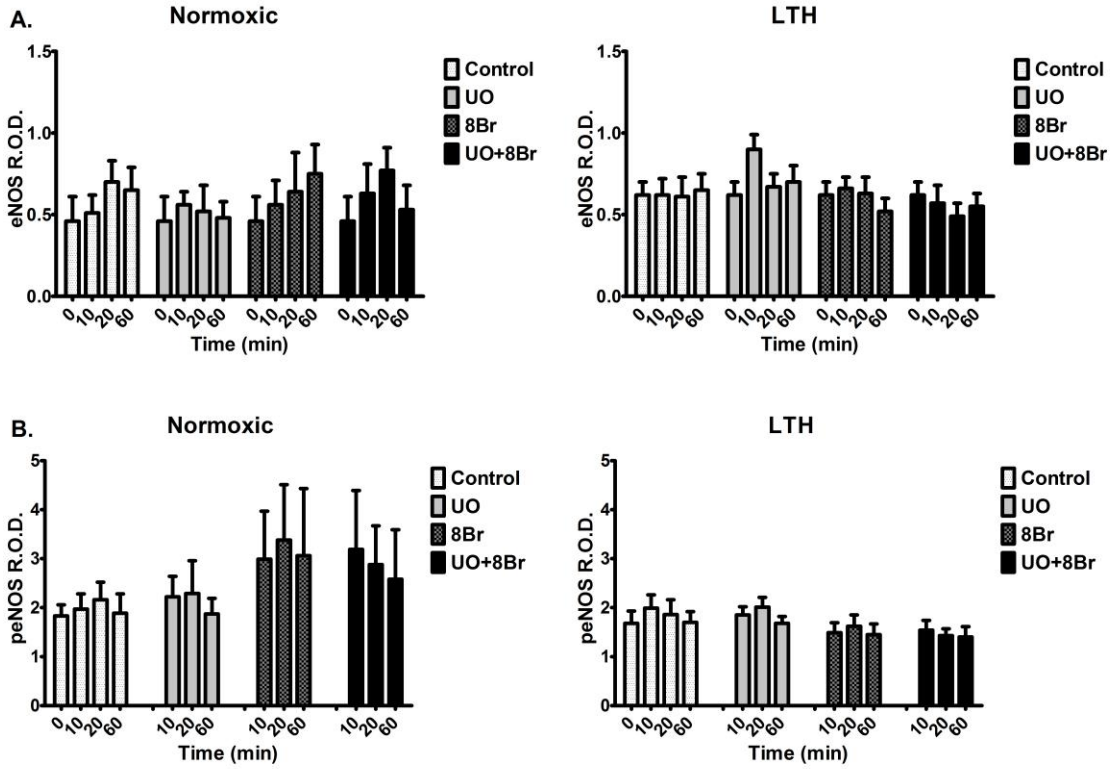

Figure 3. Protein expression (A) and phosphorylation (B) of eNOS in response to MEK/ERK1/2 inhibition and 8Bromo-cAMP stimulation in normoxic and LTH FACs as determined by Western analysis. Pretreatment with UO (10μM) with and without 8Br (10mM) stimulation had no effect on either eNOS or peNOS in both normoxic and LTH FACs compared to control. (Normoxic n=6, LTH n=6) Values represent mean values ± SEM. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; eNOS, endothelial nitric oxide synthase; peNOS, phosphorylated endothelial nitric oxide synthase; ROD, relative optical density; UO, UO126; 8Br, 8Bromo-cAMP.

Discussion

Cortisol regulation is a critical component of fetal development due to its involvement tissue growth and maturation; chronically high levels of glucocorticoids can suppress normal anabolic processes necessary for fetal growth (3, 12, 16). In the LTH ovine fetus, there is an adaptive response of the HPA axis to upregulate the hypothalamic-anterior pituitary portion with increased release of CRH and AVP (10), as well as elevated basal plasma ACTH and enhanced processing of POMC to ACTH (17). In contrast, expression of key steroidogenic enzymes (CYP11A1 and CYP17) as well as expression of ACTH receptor mRNA are reduced (18), and basal adrenal cortisol levels remain normal, despite elevated ACTH (1, 8, 11, 17). And in response to a secondary stressor, the LTH fetus produces enhanced levels of cortisol compared to the normoxic fetus (1, 11). Enhanced cortisol secretion in LTH FACs was not the result of increased cAMP production and/or protein kinas A (PKA) stimulation, however expression of steroidogenic acute regulatory protein was greater in LTH compared to normoxic FACs (23). Together, this suggests an adaptation in the HPA axis that maintains normal basal levels of cortisol required for fetal development, but allows for enhanced cortisol production in response to a secondary stress.

Cortisol production in the adrenal cortex is classically known to be regulated by signaling of ACTH via 3,5-cAMP, which activates PKA liberation of cholesterol and activation of StAR, and StAR transfers cholesterol to the inner mitochondrial membrane for conversion into pregnenolone by CYP11A1, a major rate limiting step cortisol biosynthesis (2, 7, 20-22). In a previous study, we also showed that cortisol production can be regulated by nitric oxide (NO); NO inhibited ACTH-stimulated cortisol

production and endothelial nitric oxide synthase (eNOS) inhibition enhanced cortisol synthesis in LTH ovine FACs (15). Further, we demonstrated that LTH enhanced eNOS expression (14) and NOS activity in LTH compared to normoxic adrenals (15). We more recently found that MEK/ERK1/2 inhibition with UO126 (UO) reduced ACTH-stimulated cortisol production in both normoxic and LTH FACs (19, 24), but had no effect on eNOS phosphorylation (peNOS) (19). Unlike UO, stimulation of cortisol production with ACTH reduced peNOS in LTH FACs and enhanced cortisol production above normoxic levels (19).

In the present study, we examined the effects of UO inhibition on 8Bromo-cAMP (8Br) stimulated cortisol production and 8Br stimulation on peNOS to determine the involvement of cAMP in the fetal adaptation to LTH. 8Br is a cAMP analog that does not require a membrane receptor; it diffuses through the membrane to activate PKA and stimulate cortisol production. In rat adrenal zona fasiculata cells, 8Br was shown to increase corticosterone biosynthesis (5, 6), and treatment of Y1 mouse adrenocortical cells with 8Br increased StAR protein and mRNA in a PKA-dependent manner (4). In our FACs, we found that, similar to ACTH in chapter 2, 8Br enhanced cortisol production in both normoxic and LTH FACs, consistent with cAMP/PKA pathway regulation, with LTH levels greater than those achieved in normoxic FACs. Also similar to the previous study, UO reduced cortisol output in response to 8Br stimulation in both normoxic and LTH FACs, indicating that UO inhibition of cortisol synthesis is downstream of cAMP/PKA signaling. Unlike ACTH, however, 8Br-stimulated cortisol production did not elicit as great of an enhancement in LTH FACs, possibly due to the time required to reach stimulatory intracellular concentrations, and 8Br had no effect on

peNOS in both normoxic and LTH FACs, suggesting that ACTH dephosphorylation is not cAMP dependent and that ACTH regulates acute cortisol production through alternative signaling mechanisms activated by LTH that induce phosphatase activity.

Taken together, the results from the present study as well as our previous work (14, 15, 23, 24) indicate that LTH has profound adaptive effects on the fetal adrenal cortex; activation of the cAMP/PKA pathway enhances cortisol production in ovine LTH FACs. The results from this study show that while the MEK/ERK1/2 pathway and cAMP are involved in cortisol biosynthesis, as evidenced by UO inhibition and 8Bromo-cAMP stimulation of cortisol production, they are not involved in the differential regulation of eNOS phosphorylation. 8Br had similar effects as ACTH and in response to UO on cortisol in LTH and normoxic FACs, supporting the role of cAMP in cortisol synthesis in FACs, but UO inhibition seems to be downstream of cAMP, as 8Br stimulation was able to enhance cortisol synthesis but UO effectively limited cortisol production in both normoxic and LTH FACs. However, 8Br had no effect on peNOS, indicating the activation of alternative mechanisms by ACTH to induce phosphatase activity. Although 8Br stimulation did enhance cortisol in LTH FACs to levels above those observed in the normoxic FACs, the use of this analog was not able to replicate the results of ACTH-stimulated reduction in peNOS. This suggests that this cAMP analog does not effectively replicate the actions of the native cAMP, or that ACTH activates an alternative mechanism outside of the classical cAMP/PKA signaling pathway to affect both cortisol and eNOS phosphorylation.

References

1. **Adachi K, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters ovine fetal endocrine and physiological responses to hypotension. *American journal of physiology Regulatory, integrative and comparative physiology* 287: R209-217, 2004.

2. **Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, Stocco DM, and Strauss JF**. Phosphorylation of Steroidogenic Acute Regulatory Protein (StAR) Modulates Its Steroidogenic Activity. *Journal of Biological Chemistry* 272: 32656-32662, 1997.

3. **Challis JRG, Matthews SG, Gibb W, and Lye SJ**. Endocrine and paracrine regulation of birth at term and preterm. *Endocrine reviews* 21: 514-550, 2000.

4. **Clark BJ, Ranganathan V, and Combs R**. Steroidogenic acute regulatory protein expression is dependent upon post-translational effects of cAMP-dependent protein kinase A. *Mol Cell Endocrinol* 173: 183-192, 2001.

5. **Cymeryng CB, Dada LA, Colonna C, Mendez CF, and Podesta EJ**. Effects of L-arginine in rat adrenal cells: involvement of nitric oxide synthase. *Endocrinology* 140: 2962-2967, 1999.

6. **Cymeryng CB, Dada LA, and Podesta EJ**. Effect of nitric oxide on rat adrenal zona fasciculata steroidogenesis. *The Journal of endocrinology* 158: 197-203, 1998.

7. **Daniel PB, Walker WH, and Habener JF**. Cyclic AMP signaling and gene regulation. *Annual review of nutrition* 18: 353-383, 1998.

8. **Ducsay CA**. Fetal and maternal adaptations to chronic hypoxia: prevention of premature labor in response to chronic stress. *Comparative biochemistry and physiology Part A, Molecular & integrative physiology* 119: 675-681, 1998.

9. **Ducsay CA, Hyatt K, Mlynarczyk M, Root BK, Kaushal KM, and Myers DA**. Long-term hypoxia modulates expression of key genes regulating adrenomedullary function in the late gestation ovine fetus. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 293: R1997-R2005, 2007.

10. **Ducsay CA, Mlynarczyk M, Kaushal KM, Hyatt K, Hanson K, and Myers DA**. Long-term hypoxia enhances ACTH response to arginine vasopressin but not corticotropin-releasing hormone in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 297: R892-899, 2009.

11. **Imamura T, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters endocrine and physiologic responses to umbilical cord occlusion in the ovine fetus. *Journal of the Society for Gynecologic Investigation* 11: 131-140, 2004.

12. **Meaney MJ, Viau V, Bhatnagar S, Betito K, Iny LJ, O'Donnell D, and Mitchell JB**. Cellular mechanisms underlying the development and expression of individual differences in the hypothalamic-pituitary-adrenal stress response. *J Steroid Biochem Mol Biol* 39: 265-274, 1991.

13. **Mlynarczyk M, Imamura T, Umezaki H, Kaushal KM, Zhang L, and Ducsay CA**. Long-term hypoxia changes myometrial responsiveness and oxytocin receptors in the pregnant ewe: differential effects on longitudinal versus circular smooth muscle. *Biology of reproduction* 69: 1500-1505, 2003.

14. **Monau TR, Vargas VE, King N, Yellon SM, Myers DA, and Ducsay CA**. Long-term hypoxia increases endothelial nitric oxide synthase expression in the ovine fetal adrenal. *Reproductive sciences (Thousand Oaks, Calif)* 16: 865-874, 2009.

15. **Monau TR, Vargas VE, Zhang L, Myers DA, and Ducsay CA**. Nitric oxide inhibits ACTH-induced cortisol production in near-term, long-term hypoxic ovine fetal adrenocortical cells. *Reproductive sciences (Thousand Oaks, Calif)* 17: 955-962, 2010.

16. **Munck A, Guyre PM, and Holbrook NJ**. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine reviews* 5: 25-44, 1984.

17. **Myers DA, Bell PA, Hyatt K, Mlynarczyk M, and Ducsay CA**. Long-term hypoxia enhances proopiomelanocortin processing in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 288: R1178-1184, 2005.

18. **Myers DA, Hyatt K, Mlynarczyk M, Bird IM, and Ducsay CA**. Long-term hypoxia represses the expression of key genes regulating cortisol biosynthesis in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 289: R1707-1714, 2005.

19. **Newby EA, Kaushal KM, Myers DA, and Ducsay CA**. Adrenocorticotropic Hormone and PI3K/Akt Inhibition Reduce eNOS Phosphorylation and Increase Cortisol Biosynthesis in Long-Term Hypoxic Ovine Fetal Adrenal Cortical Cells. *Reproductive sciences (Thousand Oaks, Calif)* 2015.

20. **Pon LA, Hartigan JA, and Orme-Johnson NR**. Acute ACTH regulation of adrenal corticosteroid biosynthesis. Rapid accumulation of a phosphoprotein. *The Journal of biological chemistry* 261: 13309-13316, 1986.

21. **Sewer MB, and Waterman MR**. Adrenocorticotropin/cyclic adenosine 3',5'-monophosphate-mediated transcription of the human CYP17 gene in the adrenal cortex is dependent on phosphatase activity. *Endocrinology* 143: 1769-1777, 2002.

22. **Stocco DM**. StAR protein and the regulation of steroid hormone biosynthesis. *Annual review of physiology* 63: 193-213, 2001.

23. **Vargas VE, Kaushal KM, Monau T, Myers DA, and Ducsay CA**. Long-term hypoxia enhances cortisol biosynthesis in near-term ovine fetal adrenal cortical cells. *Reproductive sciences (Thousand Oaks, Calif)* 18: 277-285, 2011.

24. **Vargas VE, Kaushal KM, Monau TR, Myers DA, and Ducsay CA**. Extracellular signal-regulated kinases (ERK1/2) signaling pathway plays a role in cortisol secretion in the long-term hypoxic ovine fetal adrenal near term. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 304: R636-R643, 2013.

CHAPTER FOUR

EFFECTS OF A23187 AND ACTH ON CORTISOL BIOSYNTHESIS AND ENOS PHOSPHORYLATION IN OVINE LONG-TERM HYPOXIC FETAL ADRENOCORTICAL CELLS

Abstract

In the long-term hypoxic (LTH) fetus, we have shown that cortisol production is enhanced in response to secondary stress, while basal levels remain normal despite elevated basal ACTH. Nitric oxide (NO) may be a major regulator of this mechanism as we have shown that NO inhibits cortisol production in LTH fetal adrenocortical cells (FACs). We have also shown that endothelial nitric oxide synthase (eNOS) expression is greater in the LTH fetal adrenal, and that NOS activity is reduced by ACTH treatment in LTH FACs. This study was designed to determine the role of calcium signaling on eNOS phosphorylation (peNOS) and subsequent cortisol production in the ovine FACs in response to long term hypoxia (LTH). Pregnant ewes were maintained at high altitude (3820m) for approximately the last 100 days of gestation (dGa). At 138-142 dGa, fetal adrenal cortical cells (FACs) were collected from LTH and age matched normoxic ovine fetuses. Cortisol production and peNOS were measured in response to pretreatment with calcium ionophore A23187 and ACTH stimulation. A23187 had no effect on cortisol production or peNOS in both normoxic and LTH FACs, suggesting that calcium signaling does not play a major role in regulating cortisol or eNOS phosphorylation in the ovine fetal adrenal.

Introduction

Long-term hypoxia (LTH) in the fetus causes adaptations that lead to increased basal plasma concentrations of ACTH, however cortisol levels remain normal under basal conditions (37). In response to a secondary stress, cortisol production is enhanced in the LTH fetus beyond levels achieved in the normoxic (3, 24), suggesting a mechanism of cortisol regulation that maintains normal cortisol levels under basal conditions but allows for a heightened output in response to stress. The mechanism for this adaptation may be mediated by nitric oxide (NO) produced from endothelial nitric oxide synthase (NOS).

Previous work from our lab has shown that eNOS expression was greater in LTH fetal adrenals (34), and that NO inhibited ACTH-stimulated cortisol production while NOS inhibition enhanced cortisol synthesis in LTH FACs (35). This suggests that eNOS and subsequent NO production plays an important role in regulating cortisol biosynthesis in response to a secondary stress in the fetus.

The regulation of eNOS activity has been shown to occur through post-translational mechanisms including phosphorylation (13), protein-protein interactions with caveolin-1 (Cav-1) and heat shock protein 90 (Hsp90) (7, 8, 22), and interaction with calcium (Ca^{2+})/calmodulin (CaM) (31). Calmodulin is an allosteric activator of eNOS and increases in intracellular Ca^{2+} concentrations promote the dissociation of eNOS with the inhibitory protein Cav-1 and association of eNOS with CaM (27, 29, 39, 45). The activated eNOS-CaM complex synthesizes NO until Ca^{2+} decreases and CaM dissociates, followed by reformation of the eNOS-Cav-1 complex (1, 25, 27, 30, 40).

This mechanism of Ca^{2+} regulation of eNOS has been well characterized in endothelial cells, but not as well explored in steroidogenic cells, especially the fetal

adrenal, and studies on the effects of Ca^{2+} on eNOS phosphorylation (peNOS) are limited. Further, the role of Ca^{2+} regulation of peNOS under hypoxic conditions is uncertain. In endothelial cells, treatment with calcium ionophore A23187 increased peNOS and NO production (4, 23, 51), while Ca^{2+} chelators and calcium free media prevented stimulated increases in peNOS (15, 21). Taken together, these studies suggest that Ca^{2+} plays an important role in regulating peNOS. This study was designed to examine the effects of elevating intracellular calcium with calcium ionophore A23187 on peNOS and subsequent cortisol biosynthesis to determine the role of calcium in regulating fetal eNOS and cortisol production in LTH ovine FACs.

Materials and Methods

Animals

Time-dated pregnant ewes were maintained at the Barcroft Laboratory White Mountain Research Station (3820m, maternal PO2 ~ 60mmHg) from approximately 40 dGa to near term (term ≅ 146 days). Following transportation to the laboratory, hypoxia was maintained by nitrogen infusion through a maternal tracheal catheter as previously described (3, 11, 24, 35, 41, 49). Age-matched, normoxic ewes served as controls. On 138 to 142 dGa, ewes were sedated and maintained under general anesthesia while fetuses were delivered through midline laparotomy. Procedures were performed as previously described in detail (33). Fetal adrenal glands were collected in ice-cold media M-199 (Sigma-Aldrich, St. Louis, Missouri), containing 2.2 g sodium bicarbonate, 2.0 g bovine serum albumin, and 0.1 g L-glutamine for cell dispersion and subsequent study.

All procedures were conducted with the approval of the Institutional Animal Care and Use Committees (Loma Linda University School of Medicine, Loma Linda, CA).

Cell Dispersion

Procedures for collection of FACs were similar to those we described previously (41, 49, 50). Briefly, fetal adrenal glands were divided in half along the longitudinal axis and the cortex was separated from the medulla. The cortical tissue was minced and enzymatically dispersed with 40 mg collagenase Type II (Worthington Biomedical, Lakewood, NJ), 40 mg of Polypep bovine protein digest (Sigma-Aldrich) and 100 µl of DNAse I (Type IV; Sigma-Aldrich) dissolved in 10 mL of Sodium Krebs Buffer (0.4% collagenase). The resulting monodispersed FACs were aliquoted into individual tubes with media (M-199), and allowed to equilibrate for 2 hr at 37°C prior to initiation of the study as required by the experimental protocol. Cell viability was confirmed by Trypan blue exclusion.

Treatment Protocol

Effects of A23187 treatment and ACTH stimulation on eNOS phosphorylation and cortisol biosynthesis

FACs, aliquoted at $7.5x10^5$ cells/mL, were untreated, pretreated with calcium ionophore A23187 (3.3 µM) for 1 hour, or stimulated with ACTH (100 pM), with and without A23187 pretreatment. Media and cells were collected at 0 (baseline), 10, 20, and 60 minutes after stimulation. Media were immediately frozen in liquid nitrogen, and stored at -80°C until determination of cortisol. Cells were lysed in 80 µL of lysis buffer

(93% prelysis buffer [1 mmol/L Trizma Base, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1% Triton X-100, 0.5% Igepal CO-630, 20 mmol/L NaF], 1% 100 mmol/L phenylmethanesulfonyl fluoride, 1% Protease Inhibitor Cocktail [Sigma, St Louis, Missouri], 5% 20 mmol/L sodium orthovanadate), frozen in liquid nitrogen, and stored at -80°C until analysis.

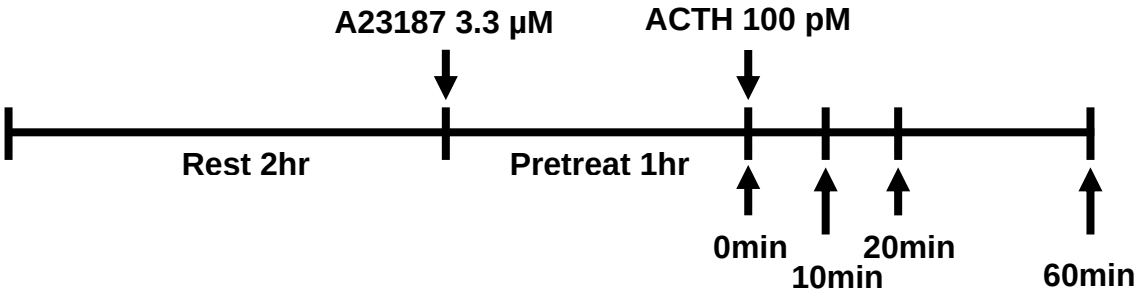

Figure 1. Timeline for treatment protocol A23187. ACTH, adrenocorticotropic hormone; A23187, calcium ionophore A23187.

Cortisol Assay

Cortisol was measured using a commercially available enzyme-linked immunosorbent assay cortisol kit (Oxford Biomedical Research, Oxford, Michigan) that has been previously described and validated for use in our laboratory (12, 35, 38).

Western Analysis

Endothelial NOS protein was analyzed from FACs collected at 0 (baseline), 10, 20, and 60 min for both normoxic and LTH groups, described earlier. Samples were thawed and protein concentration was determined using a bicinchoninic acid protein assay (Thermo Scientific, Rockford, Illinois) with BSA as the standard. Absorbance was

measured at 595 nm on a BioTek Synergy HT Multi-Mode Microplate Reader (Winooski, Vermont).

Endothelial NOS protein expression and phosphorylation was determined by Western blotting using methods we have previously described and validated (37, 38). Briefly, protein samples were denatured for 5 minutes at boiling temperature and a total of 20 µg of protein were loaded per lane. Protein samples were separated using 7.5% polyacrylamide gels (Bio-Rad, Hercules, California) and subjected to electrophoresis (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, Massachusetts) using a Transblot cell apparatus (Bio-Rad).

To determine the level of eNOS phosphorylation, the membranes were incubated with a rabbit monoclonal phospho-eNOS (Ser1177; C9C3) primary antibody (Cell Signaling, Product#9570) in 10 mL 5% BSA Tris-buffered saline with Tween 20 (TBST) solution (1:1000) overnight at 4°C. Membranes were then incubated with goat anti-rabbit polyclonal secondary antibody (ThermoScientific, Product#35571) in 10 mL 5% BSA TBST solution (1:10000) for 90 minutes, washed, and imaged with a Licor Odyssey Infrared Imaging System (LI-COR Bio-sciences, Lincoln, Nebraska) at 700 nm. The relative optical densities (ROD) of the bands were used to measure normoxic and LTH phosphorylated eNOS (peNOS) protein expression. An internal positive standard prepared from whole fetal adrenal tissue was used to normalize peNOS protein.

To determine the level of eNOS protein expression, the membranes were first stripped of phosphorylated antibody and incubated with mouse anti-eNOS primary antibody (BD Transduction, Product# 610296) in 10 mL 5% nonfat dry milk (NFDM)

TBST solution (1:250) overnight at 4°C. Membranes were then incubated with goat anti-mouse polyclonal secondary antibody (Thermo Scientific, Product# 35518) in 10 mL 5% NFDM TBST solution (1:20000) for 90 minutes, washed, and imaged with a Licor Odyssey Infrared Imaging System (LI-COR Bio-sciences, Lincoln, Nebraska) at 800 nm. The RODs of the bands were used to measure normoxic and LTH eNOS protein expression. An internal positive standard prepared from whole fetal adrenal tissue was used to normalize eNOS protein as we have previously described in our laboratory (34, 50).

Statistical Analysis

Descriptive statistics are presented as mean ± standard error. Data analysis was performed using two-way analysis of variance (ANOVA) with 1 between-subject factor (treatment) and 1 within-subject factor (time) stratified by oxygenation level (normoxic or LTH). The main effect tested was in vitro treatment (A23187). Alpha was set at .05 significance level. Post hoc tests were adjusted using the Bonferroni method. Statistical analyses were performed GraphPad Prism 5 (Version 5.04; GraphPad Software, Inc., 2010).

Results

Effects of A23187 and ACTH Stimulation

Cortisol Production

ACTH significantly increased cortisol production in both normoxic and LTH FACs. A23187 did not affect cortisol output in both normoxic and LTH FACs, with and

without ACTH stimulation (**Figure 2**).

Expression of eNOS

Treatment with A23187 and ACTH had no effect on expression of eNOS protein in either normoxic or LTH FACs compared to control (**Figure 3A**).

Phosphorylation of eNOS

Treatment with A23187 and ACTH had no effect on phosphorylation of eNOS protein in either normoxic or LTH FACs compared to control (**Figure 3B**).

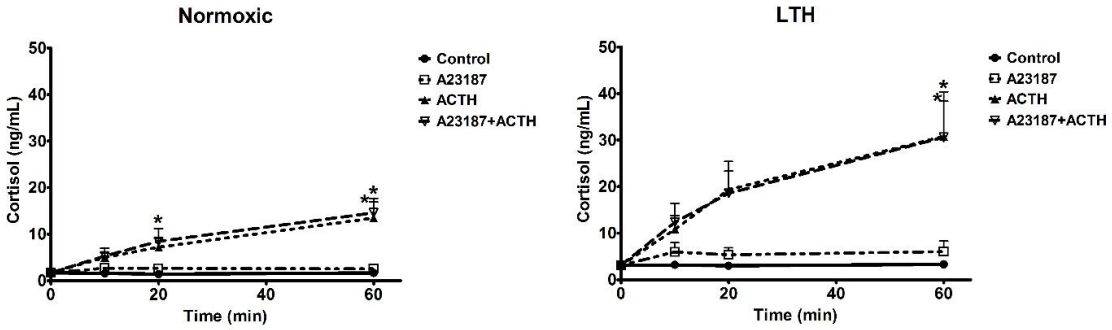

Figure 2. Time course of cortisol production in normoxic and LTH FACs with calcium ionophore A23187 pretreatment and ACTH stimulation. Treatment with ACTH (100pM) increased cortisol production in both normoxic and LTH FACs. A23187 (3.3µM) pretreatment had no effect on basal or ACTH-induced cortisol biosynthesis in both normoxic and LTH. (Normoxic n=5, LTH n=5) Values represent mean values ± SEM. *$p<0.05$ compared to time 0. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; ACTH, adrenocorticotropic hormone; A23187, calcium ionophore A23187.

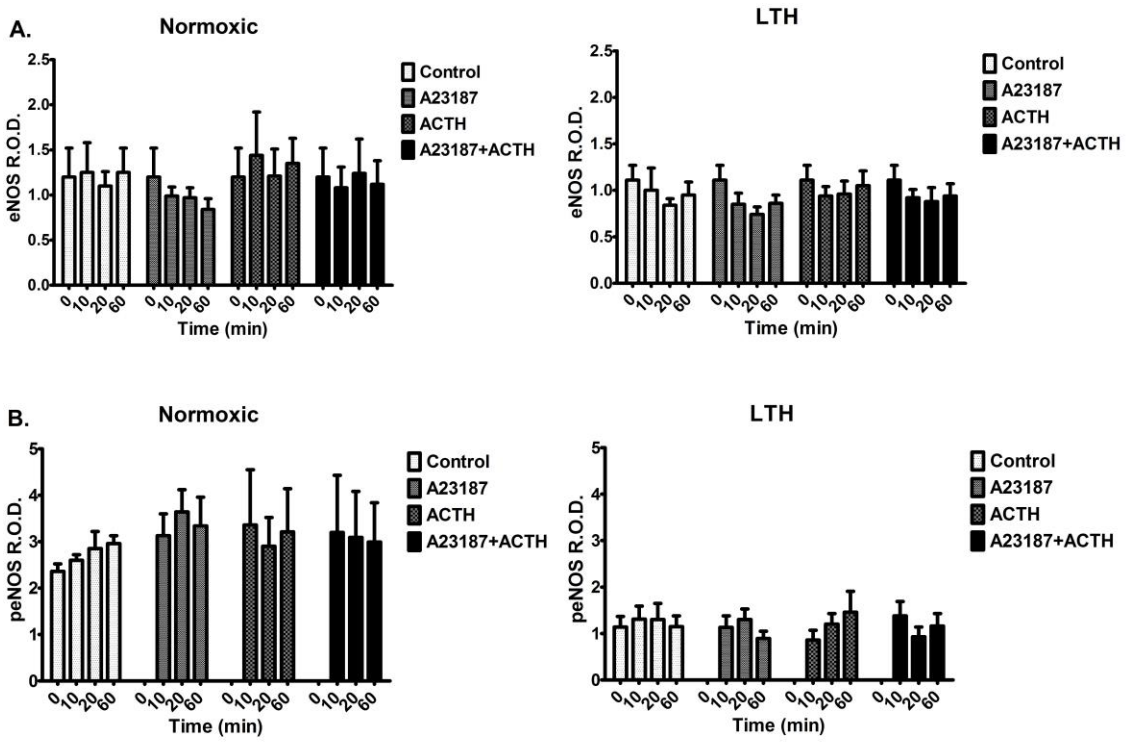

Figure 3. Protein expression (A) and phosphorylation (B) of eNOS in response to calcium ionophore A23187 and ACTH stimulation in normoxic and LTH FACs as determined by Western analysis. Pretreatment with A23187 (3.3µM) with and without ACTH (100pM) stimulation had no effect on eNOS or peNOS in both normoxic and LTH FACs compared to control. (Normoxic n=5, LTH n=5) Values represent mean values ± SEM. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; eNOS, endothelial nitric oxide synthase; peNOS, phosphorylated endothelial nitric oxide synthase; ROD, relative optical density; ACTH, adrenocorticotropic hormone; A23187, calcium ionophore A23187.

Discussion

The regulation of cortisol biosynthesis in the fetus is critical due to its involvement in growth and maturation, and chronically high levels can suppress the normal processes necessary for development (6, 28, 36). The hypothalamic-pituitary-adrenal (HPA) axis of the long-term hypoxic (LTH) fetus undergoes significant adaptation resulting in maintenance of basal levels of cortisol, despite elevated ACTH (37), and enhanced cortisol biosynthesis in response to a secondary stress (3, 24). This adaptation may be mediated by nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS); we have shown that eNOS expression was greater in LTH fetal adrenals (34), and that NO inhibited ACTH-stimulated cortisol production while NOS inhibition enhanced cortisol synthesis in LTH fetal adrenocortical cells (FACs) (35). Therefore, eNOS regulation of NO production appears to play a critical role in regulating cortisol biosynthesis in response to stress in the LTH fetus.

Regulation of eNOS has been shown to occur through post-translational mechanisms including phosphorylation (9, 10, 13, 17, 18, 32), protein-protein interactions with caveolin-1 (Cav-1) and heat shock protein 90 (Hsp90) (7, 8, 19, 20, 22), and interaction with calcium (Ca^{2+})/calmodulin (CaM) (16, 31). In our lab, we have shown that although there is significant co-localization of eNOS with regulatory proteins Hsp90 and Cav-1 present in the fetal adrenal, there were no changes in co-localization of either protein with eNOS in the LTH fetus compared to normoxic controls (46). This indicates that Cav-1/Hsp90 regulation of eNOS is likely not an important part of the fetal adaptation to LTH and suggests that other methods are involved. Calcium is a possible regulator involved in managing eNOS activity. Increases in intracellular Ca^{2+}

concentrations ($[Ca^{2+}]_i$) have been shown to activate eNOS by inducing dissociation of eNOS with Cav-1 and association with CaM, initiating NO production (27, 29, 39, 45). Decreases in $[Ca^{2+}]_i$ were followed by CaM dissociation and reformation of the eNOS-Cav-1 complex, halting NO synthesis (1, 25, 27, 30, 40), showing that eNOS activity is dependent on $[Ca^{2+}]_i$.

This mechanism of Ca^{2+} regulation of eNOS has been well characterized in endothelial cells (1, 2, 16, 25, 27, 30, 40, 44), but not as well explored in steroidogenic cells, especially the fetal adrenal, and studies on the effects of Ca^{2+} on eNOS phosphorylation (peNOS) are limited. Further, the role of Ca^{2+} regulation of peNOS under hypoxic conditions is uncertain. Studies have shown that in bovine aortic endothelial cells (BAECs) and COS-7 transfected cells, treatment with calcium ionophore A23187 increased peNOS at Ser1177 and NO production (4, 23), while treatment of endothelial cells with Ca^{2+} chelators prevented VEGF-mediated peNOS and NO synthesis (21). In porcine aortic endothelial cells (PAECs), bradykinin-induced peNOS was reduced in calcium-free media (15), and treatment with A23187 increased NO in pregnant ovine uterine arteries, inducing relaxation that was enhanced in hypoxic tissues compared to normoxic (51). Together these results suggest a possible role for Ca^{2+} regulation of peNOS that may be affected by LTH.

In this study we investigated the role of intracellular calcium on peNOS and cortisol production in the LTH ovine FACs. We found that while ACTH increased cortisol production, as we have previously described (35, 41, 49, 50), pretreatment of FACs with calcium ionophore A23187 had no effect on eNOS expression and phosphorylation, and subsequent ACTH-stimulated cortisol production was not affected

in both normoxic and LTH FACs. This implies that $[Ca^{2+}]_i$ does not play a major role in the fetal adaptation to LTH, and may not contribute to eNOS regulation in the fetal adrenal.

It has been suggested that eNOS regulation may be tissue specific (14), and thus the lack of calcium influence in the fetal adrenal may be a result of this specificity in regulation. It could also be a difference in regulation based on the type of agonist; the requirement of calcium for NO production has been shown to vary depending on the type of agonist. A rise in calcium is necessary in response to acetylcholine (43) or vascular endothelial growth factor (42), however fluid shear stress (26), estrogen (5), and insulin (48) do not require increases in calcium for NO production. In these cases, it is likely that CaM is bound, as it is an essential regulator of electron flux required for NO generation (2), but that it's affinity is not as affected by calcium concentrations. The negative charge of the phosphate could permit greater activation of eNOS without changing CaM affinity at varying calcium levels (10, 17, 32, 47), thus generating NO even at lower calcium/CaM concentrations. Together these data suggest that the lack of changes in peNOS and cortisol as a result of calcium ionophore A23187 treatment could be tissue specific. Calcium may not be a major regulator of eNOS in FACs and thus increases in intracellular calcium may not have an impact on eNOS activity.

References

1. **Abu-Soud HM, and Stuehr DJ**. Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proceedings of the National Academy of Sciences of the United States of America* 90: 10769-10772, 1993.

2. **Abu-Soud HM, Yoho LL, and Stuehr DJ**. Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer. *The Journal of biological chemistry* 269: 32047-32050, 1994.

3. **Adachi K, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters ovine fetal endocrine and physiological responses to hypotension. *American journal of physiology Regulatory, integrative and comparative physiology* 287: R209-217, 2004.

4. **Cale JM, and Bird IM**. Inhibition of MEK/ERK1/2 signalling alters endothelial nitric oxide synthase activity in an agonist-dependent manner. *The Biochemical journal* 398: 279-288, 2006.

5. **Caulin-Glaser T, Garcia-Cardena G, Sarrel P, Sessa WC, and Bender JR**. 17 beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca2+ mobilization. *Circ Res* 81: 885-892, 1997.

6. **Challis JRG, Matthews SG, Gibb W, and Lye SJ**. Endocrine and paracrine regulation of birth at term and preterm. *Endocrine reviews* 21: 514-550, 2000.

7. **Chen DB, Bird IM, Zheng J, and Magness RR**. Membrane estrogen receptor-dependent extracellular signal-regulated kinase pathway mediates acute activation of endothelial nitric oxide synthase by estrogen in uterine artery endothelial cells. *Endocrinology* 145: 113-125, 2004.

8. **Chen JX, and Meyrick B**. Hypoxia increases Hsp90 binding to eNOS via PI3K-Akt in porcine coronary artery endothelium. *Laboratory investigation; a journal of technical methods and pathology* 84: 182-190, 2004.

9. **Chen Z-P, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, and Kemp BE**. AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Letters* 443: 285-289, 1999.

10. **Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, and Zeiher AM**. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399: 601-605, 1999.

11. **Ducsay CA, Hyatt K, Mlynarczyk M, Root BK, Kaushal KM, and Myers DA**. Long-term hypoxia modulates expression of key genes regulating adrenomedullary function in the late gestation ovine fetus. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 293: R1997-R2005, 2007.

12. **Ducsay CA, Mlynarczyk M, Kaushal KM, Hyatt K, Hanson K, and Myers DA**. Long-term hypoxia enhances ACTH response to arginine vasopressin but not corticotropin-releasing hormone in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 297: R892-899, 2009.

13. **Dudzinski DM, and Michel T**. Life history of eNOS: Partners and pathways. *Cardiovascular research* 75: 247-260, 2007.

14. **Feron O, Belhassen L, Kobzik L, Smith TW, Kelly RA, and Michel T**. Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. *The Journal of biological chemistry* 271: 22810-22814, 1996.

15. **Fleming I, Fisslthaler B, Dimmeler S, Kemp BE, and Busse R**. Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ Res* 88: E68-75, 2001.

16. **Forstermann U, Pollock JS, Schmidt HH, Heller M, and Murad F**. Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 88: 1788-1792, 1991.

17. **Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, and Sessa WC**. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399: 597-601, 1999.

18. **Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, Figeys D, Harrison DG, Berk BC, Aebersold R, and Corson MA**. Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *The Journal of biological chemistry* 274: 30101-30108, 1999.

19. **Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, and Sessa WC**. Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* 392: 821-824, 1998.

20. **Garcia-Cardena G, Fan R, Stern DF, Liu J, and Sessa WC**. Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *The Journal of biological chemistry* 271: 27237-27240, 1996.

21. **Gelinas DS, Bernatchez PN, Rollin S, Bazan NG, and Sirois MG**. Immediate and delayed VEGF-mediated NO synthesis in endothelial cells: role of PI3K, PKC and PLC pathways. *British journal of pharmacology* 137: 1021-1030, 2002.

22. **Gratton J-P, Fontana J, O'Connor DS, García-Cardeña G, McCabe TJ, and Sessa WC**. Reconstitution of an Endothelial Nitric-oxide Synthase (eNOS), hsp90, and Caveolin-1 Complex in Vitro : Evidence that hsp90 Facilitates Calmodulin Stimulated Displacement of eNOS from Caveolin-1. *Journal of Biological Chemistry* 275: 22268-22272, 2000.

23. **Hirata K, Kuroda R, Sakoda T, Katayama M, Inoue N, Suematsu M, Kawashima S, and Yokoyama M**. Inhibition of endothelial nitric oxide synthase activity by protein kinase C. *Hypertension* 25: 180-185, 1995.

24. **Imamura T, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters endocrine and physiologic responses to umbilical cord occlusion in the ovine fetus. *Journal of the Society for Gynecologic Investigation* 11: 131-140, 2004.

25. **Ju H, Zou R, Venema VJ, and Venema RC**. Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. *The Journal of biological chemistry* 272: 18522-18525, 1997.

26. **Kuchan MJ, and Frangos JA**. Role of calcium and calmodulin in flow-induced nitric oxide production in endothelial cells. *The American journal of physiology* 266: C628-636, 1994.

27. **Marletta MA**. Nitric oxide synthase: aspects concerning structure and catalysis. *Cell* 78: 927-930, 1994.

28. **Meaney MJ, Viau V, Bhatnagar S, Betito K, Iny LJ, O'Donnell D, and Mitchell JB**. Cellular mechanisms underlying the development and expression of individual differences in the hypothalamic-pituitary-adrenal stress response. *J Steroid Biochem Mol Biol* 39: 265-274, 1991.

29. **Michel JB, Feron O, Sacks D, and Michel T**. Reciprocal regulation of endothelial nitric-oxide synthase by Ca2+-calmodulin and caveolin. *The Journal of biological chemistry* 272: 15583-15586, 1997.

30. **Michel JB, Feron O, Sase K, Prabhakar P, and Michel T**. Caveolin versus calmodulin. Counterbalancing allosteric modulators of endothelial nitric oxide synthase. *The Journal of biological chemistry* 272: 25907-25912, 1997.

31. **Michel T, and Feron O**. Nitric oxide synthases: which, where, how, and why? *The Journal of clinical investigation* 100: 2146-2152, 1997.

32. **Michell BJ, Griffiths JE, Mitchelhill KI, Rodriguez-Crespo I, Tiganis T, Bozinovski S, de Montellano PR, Kemp BE, and Pearson RB**. The Akt kinase signals directly to endothelial nitric oxide synthase. *Current biology : CB* 9: 845-848, 1999.

33. **Mlynarczyk M, Imamura T, Umezaki H, Kaushal KM, Zhang L, and Ducsay CA**. Long-term hypoxia changes myometrial responsiveness and oxytocin receptors

in the pregnant ewe: differential effects on longitudinal versus circular smooth muscle. *Biology of reproduction* 69: 1500-1505, 2003.

34. **Monau TR, Vargas VE, King N, Yellon SM, Myers DA, and Ducsay CA**. Long-term hypoxia increases endothelial nitric oxide synthase expression in the ovine fetal adrenal. *Reproductive sciences (Thousand Oaks, Calif)* 16: 865-874, 2009.

35. **Monau TR, Vargas VE, Zhang L, Myers DA, and Ducsay CA**. Nitric oxide inhibits ACTH-induced cortisol production in near-term, long-term hypoxic ovine fetal adrenocortical cells. *Reproductive sciences (Thousand Oaks, Calif)* 17: 955-962, 2010.

36. **Munck A, Guyre PM, and Holbrook NJ**. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine reviews* 5: 25-44, 1984.

37. **Myers DA, Bell PA, Hyatt K, Mlynarczyk M, and Ducsay CA**. Long-term hypoxia enhances proopiomelanocortin processing in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 288: R1178-1184, 2005.

38. **Myers DA, Hyatt K, Mlynarczyk M, Bird IM, and Ducsay CA**. Long-term hypoxia represses the expression of key genes regulating cortisol biosynthesis in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 289: R1707-1714, 2005.

39. **Nathan C, and Xie QW**. Nitric oxide synthases: roles, tolls, and controls. *Cell* 78: 915-918, 1994.

40. **Nathan C, and Xie QW**. Regulation of biosynthesis of nitric oxide. *The Journal of biological chemistry* 269: 13725-13728, 1994.

41. **Newby EA, Kaushal KM, Myers DA, and Ducsay CA**. Adrenocorticotropic Hormone and PI3K/Akt Inhibition Reduce eNOS Phosphorylation and Increase Cortisol Biosynthesis in Long-Term Hypoxic Ovine Fetal Adrenal Cortical Cells. *Reproductive sciences (Thousand Oaks, Calif)* 2015.

42. **Papapetropoulos A, Garcia-Cardena G, Madri JA, and Sessa WC**. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *The Journal of clinical investigation* 100: 3131-3139, 1997.

43. **Peach MJ, Loeb AL, Singer HA, and Saye J**. Endothelium-derived vascular relaxing factor. *Hypertension* 7: I94-100, 1985.

44. **Pollock JS, Forstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, and Murad F**. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells.

Proceedings of the National Academy of Sciences of the United States of America 88: 10480-10484, 1991.

45. **Stuehr DJ**. Structure-function aspects in the nitric oxide synthases. *Annual review of pharmacology and toxicology* 37: 339-359, 1997.

46. **Tanaka H, Kaushal KM, Wilson SM, Myers DA, and Ducsay CA**. Long-term hypoxia does not alter co-localization of heat shock protein 90 or caveolin-1 with eNOS in the ovine fetal adrenal. *Reproductive sciences (Thousand Oaks, Calif)* 19(suppl 258A): 2012.

47. **Tran QK, Leonard J, Black DJ, Nadeau OW, Boulatnikov IG, and Persechini A**. Effects of combined phosphorylation at Ser-617 and Ser-1179 in endothelial nitric-oxide synthase on EC50(Ca2+) values for calmodulin binding and enzyme activation. *The Journal of biological chemistry* 284: 11892-11899, 2009.

48. **Tsukahara H, Gordienko DV, Tonshoff B, Gelato MC, and Goligorsky MS**. Direct demonstration of insulin-like growth factor-I-induced nitric oxide production by endothelial cells. *Kidney international* 45: 598-604, 1994.

49. **Vargas VE, Kaushal KM, Monau T, Myers DA, and Ducsay CA**. Long-term hypoxia enhances cortisol biosynthesis in near-term ovine fetal adrenal cortical cells. *Reproductive sciences (Thousand Oaks, Calif)* 18: 277-285, 2011.

50. **Vargas VE, Kaushal KM, Monau TR, Myers DA, and Ducsay CA**. Extracellular signal-regulated kinases (ERK1/2) signaling pathway plays a role in cortisol secretion in the long-term hypoxic ovine fetal adrenal near term. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 304: R636-R643, 2013.

51. **Xiao D, Bird IM, Magness RR, Longo LD, and Zhang L**. Upregulation of eNOS in pregnant ovine uterine arteries by chronic hypoxia. *American Journal of Physiology - Heart and Circulatory Physiology* 280: H812-H820, 2001.

CHAPTER FIVE

EFFECTS OF NITRIC OXIDE ON CORTISOL BIOSYNTHESIS AND MRNA ABUNDANCE OF CYP11A1, CYP17, STAR, AND ACTH-R IN OVINE LONG-TERM HYPOXIC FETAL ADRENOCORTICAL CELLS

Abstract

We previously showed a decrease in key steroidogenic enzymes CYP11A1 and CYP17, as well as a decrease in ACTH receptor (ACTH-R) in the ovine fetal adrenal that may contribute to the ability of the LTH fetus to maintain basal cortisol levels despite elevated ACTH. We have also shown an increase in steroidogenic acute regulatory (StAR) protein in LTH fetal adrenocortical cells (FACs) that would allow for an enhanced response to a secondary stress. And we have shown the ability of NO to inhibit ACTH-induced cortisol production. This study was designed to determine the role of nitric oxide (NO), using DETA-NO, an NO donor, and L-NAME, a NOS inhibitor, in altering steroidogenic capacity by measuring cortisol production and mRNA of CYP11A1, CYP17, StAR, and ACTH-R. Pregnant ewes were maintained at high altitude (3820m) for approximately the last 100 days of gestation (dGa). At 138-142 dGa, fetal adrenal cortical cells (FACs) were collected from LTH and age matched normoxic ovine fetuses and treated over the course of 96h at 37°C and 5% CO_2. Cortisol production and mRNA were measured in response to DETA-NO, L-NAME, and/or ACTH treatment. DETA-NO and L-NAME did not alter cortisol or mRNA abundance of CYP11A1, CYP17, StAR, and ACTH-R with and without ACTH treatment in both normoxic and LTH FACs. ACTH treatment significantly increased cortisol production in both

normoxic and LTH FACs, and normoxic cortisol concentrations were greater than LTH. ACTH increased mRNA abundance in both normoxic and LTH FACs, but was not affected by DETA-NO or L-NAME treatment. Together these results suggest that ACTH treatment is able to overcome NO inhibition of steroidogenesis over 96h, and NO does not affect steroidogenesis at the level of mRNA abundance of key steroidogenic enzymes CYP11A1 and CYP17 or StAR and ACTH-R in the LTH fetal adrenal.

Introduction

Hypoxia is a common fetal stressor that leads to adaptations in the ovine fetal adrenal. In response to long-term moderate gestational hypoxia (LTH), the fetus maintains normal basal plasma cortisol concentrations, despite elevated basal levels of adrenocorticotropic hormone (ACTH) (45). However, the LTH fetus has an enhanced cortisol response to acute secondary stressors compared to normoxic fetuses (1, 28). This suggests an adaptation in the HPA axis that prevents early and excess cortisol production, but allows for increased cortisol production in response to a secondary stress.

Regulation of cortisol is important for fetal development due to its involvement in tissue growth and maturation (8, 36, 44), and cortisol biosynthesis is regulated through a series of enzymatic steps. Steroidogenesis involves the key rate-limiting enzymes cytochrome P450 side-chain cleavage (CYP11A1) and cytochrome P450 17α-hydroxylase (CYP17). Additionally, steroidogenic acute regulatory (StAR) protein and ACTH receptor (ACTH-R) are other necessary components. ACTH initiates steroidogenesis through activation of ACTH-R (38). StAR is responsible for cholesterol transport in to the inner mitochondrial membrane, the initial rate limiting step in

steroidogenesis, where CYP11A1 converts cholesterol into pregnenolone (3, 9, 37, 38, 52, 61). CYP17 continues cortisol biosynthesis by mediating the 17α-hydroxylation of pregnenolone to 17α-hydroxypregnenolone and progesterone 17α-hydroxyprogesterone (25).

Under basal conditions, we have previously shown that despite elevated plasma ACTH, expression of CYP11A1 and CYP17 and ACTH-R are decreased in the adrenal gland of the LTH fetus (46), while expression of steroidogenic acute regulatory protein was greater in LTH compared to normoxic fetal adrenocortical cells (FACs) (68). We also observed that under acute secondary stress in vivo, cortisol production is greater in LTH fetuses than normoxic controls (1, 28). This is paralleled by in vitro studies that demonstrate enhanced cortisol biosynthesis in response to "stress" levels of ACTH in LTH FACs (69). Taken together, these data suggest that levels of CYP11A1, CYP17, and ACTH-R are adequate to carry out cortisol biosynthesis in the LTH fetal adrenal, but that there is an inhibitory mechanism under basal conditions on these biosynthetic proteins that is overridden to induce enhanced cortisol in response to a secondary stressor.

A possible mediator of biosynthetic activity in the LTH fetal adrenal is nitric oxide (NO), a diatomic free radical molecule with a variety of physiological functions and produced from L-arginine by NO synthases (NOSs) (27, 42). NO has clearly been shown to inhibit CYP expression (17, 59) and activity (11, 13, 23) in adrenal cells. Studies from our own laboratory showed that NO inhibited ACTH-stimulated cortisol while NOS inhibition enhanced cortisol output in LTH FACs (41) . Thus, NO appears to play a role in regulating cortisol biosynthesis in the LTH fetal adrenal.

This study was designed to address the role of extended NO exposure, via NO donor DETA-NO, or removal, via NOS inhibitor L-NAME, on CYP11A1, CYP17, StAR, and ACTH-R mRNA abundance in normoxic and LTH FACs. We also determined the effects of altered NO exposure on cortisol biosynthesis.

Materials and Methods

Animals

Time-dated pregnant ewes were maintained at the Barcroft Laboratory White Mountain Research Station (3820m, maternal PO2 ~ 60mmHg) from approximately day 40 of gestation to near term (term = 146 days). Following transportation to the laboratory, hypoxia was maintained by nitrogen infusion through a maternal tracheal catheter as previously described (1, 15, 28, 41, 50, 68). Age-matched, normoxic ewes served as controls. On days 138-142 of gestation, ewes were sedated and maintained under general anesthesia while fetuses were delivered through midline laparotomy. Procedures were performed as previously described in detail (40). Fetal adrenal glands were collected in ice-cold media DMEM (Sigma-Aldrich, St. Louis, MO), containing 3.2 g sodium bicarbonate for cell dispersion and subsequent study. All procedures were conducted with the approval of the Institutional Animal Care and Use Committees (Loma Linda University School of Medicine, Loma Linda, CA).

Cell Dispersion

Fetal adrenal glands were divided in half along the longitudinal axis and the cortex was separated from the medulla. The cortical tissue was minced and enzymatically

dispersed with 40 mg collagenase Type II (Worthington Biomedical, Lakewood, NJ), 40 mg of Polypep bovine protein digest (Sigma-Aldrich) and 100 µl of DNAse I (Type IV) (Sigma-Aldrich) dissolved in 10 ml of Sodium Krebs Buffer (0.4% collagenase). The resulting mono-dispersed FACs were aliquoted $2.5x10^5$ cells/mL into 24 well plates with media (DMEM-FBS 5%), and incubated for 24h at 37°C and 5% CO_2 prior to initiation of the study as required by the experimental protocol. FBS was added to DMEM cell media to provide the necessary factors require for cell growth. Cell viability was confirmed by Trypan blue exclusion. All procedures were performed as previously described and validated for our laboratory (50, 69).

Treatment Protocol

Effects of NOS Inhibition, NO Supplementation, and ACTH Stimulation on Cortisol Biosynthesis and CYP11A1, CYP17, StAR, and ACTH-R mRNA Abundance

To examine the effects of NO on mRNA abundance and cortisol production, we used the NO donor (Z)-1-[2-(2-aminoethyl-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) (26), and NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME). Continuous exposure to NO donors is potentially cytotoxic, therefore DETA-NO was chosen for this experiment because we have previously shown that this NO donor does not affect cell viability during cell culture (24). It also has a long half-life and the chosen dose (20uM) maintains physiological levels of NO (4, 7, 53). The dose of L-NAME used was that found to be effective in a previous study (41).

After the 24h incubation, all media was removed from each well, labeled 0h post treatment, and immediately frozen in liquid nitrogen and stored at -80C until

determination of cortisol. Following the initial media collection, FACs were either untreated, treated with NOS inhibitor L-NAME (1 mM), treated with NO donor DETA-NO (20 µM), or treated with stress levels of ACTH (100 pM), with and without L-NAME or DETA-NO in DMEM-FBS, and returned to the incubator at 37°C and 5% CO_2. Media, along with the appropriate agonist or antagonist were replaced every 24 h for a total of 96 h post treatment and collected media was immediately frozen in liquid nitrogen, and stored at -80°C until determination of cortisol. At the end of the 96h treatment period, cells were lysed in 250 µL Denaturation Solution (Ambion) per well for 20 min, rinsed with 200 µL Denaturation Solution, frozen in liquid nitrogen, and stored at -80C until analysis.

Cortisol Assay

Cortisol was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) cortisol kit (Oxford Biomedical Research, Oxford, MI) that has been previously described and validated for use in our laboratory (16, 41, 46).

qRT-PCR

Quantitative real-time PCR analysis. Quantitative real-time (qRT) PCR was used to quantify the mRNA for CYP17, CYP11A1, StAR, and ACTH-R (MC2). Total RNA was prepared from adrenal cortical cells (n=5 for normoxic and LTH) with an RNA preparation kit as per the manufacturer's instructions (Qiagen). Before qRT-PCR, total RNA (1 µg) was treated with DNase I (1 U) at 37°C for 60 min and DNase was removed via PCR purification columns. Reverse transcription was performed using 1 µg of total

RNA per sample, oligo (dT21) as the primer, and Superscript II (Invitrogen) as reverse transcriptase. The details of the qRT PCR have been previously described for our laboratory in detail (45, 49). For all genes of interest, real-time PCR was also performed using control reverse-transcription reactions in which the reverse transcriptase was purposely omitted.

Real-time PCR was performed using cDNA generated from the first-strand synthesis reaction. All PCRs were performed in triplicate. For CYP11A1, CYP17, ACTH-R, and cyclophilin, 50 ng cDNA/PCR reaction were used. For the qRT-PCR, Sybr green (1x Sybr green master mix; Quanta Biosciences, Gaithersburg, Maryland) was utilized as the fluorophore, and real-time PCR was performed utilizing a Bio-Rad iCycler equipped with the real-time optical fluorescent detection system. The primer sequences used are listed in **Table 1**; the primers were derived from cDNA sequences available at the National Center for Biotechnology Information (ovine CYP17: AF251388; ovine CYP11A1: D50057; ovine StAR: AF290202; ovine ACTH receptor: NM_001009442; bovine cyclophilin B: BT020966). A three- step PCR was used: an initial denaturation step of 95°C for 1.5 min to activate the hot-start Taq DNA polymerase, followed by sequential cycles consisting of denaturation at 95°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. A total of 35 PCR cycles were performed. qRT-PCR was performed for each sample (in triplicate) for cyclophilin as a control mRNA using the identical first-strand cDNA used for quantification of mRNA for the gene of interest and in the same PCR run as for the gene of interest to circumvent any between-run variation. Cyclophilin was used as a “housekeeping” mRNA, since we previously found that cyclophilin mRNA is not glucocorticoid responsive and does not change in expression in

adrenal cells in vitro in response to a variety of stimuli, including ACTH. For quantification purposes, a synthetic, double-stranded DNA standard was used to generate a standard curve for extrapolation of starting cDNA concentrations per reaction using the Ct (threshold at which the fluorescence of each PCR reaction increased above baseline) values for standards to create a linear standard curve (100, 10, 1, 0.1, 0.01, and 0.001 pg of standard cDNA). Extrapolation of unknowns from the standard curve was performed using Prism 3 (GraphPad Software, San Diego, CA), predicting unknowns from the standard curve Ct values.

Table 1. Forward and reverse primer sequences used for quantitative real-time PCR

Gene		*Primer Sequence*	*NCBI*
CYP17	Fw	5'-CATCAGAGAAGTGCTCCGAATCC-3'	AF251388
	Rv	5'-TCCTGCTCCAAAGGGCAAGTAG-3'	
CYP11A1	Fw	5'- GGAGGATGTCAAGGCCAATA-3'	D50057
	Rv	5'- TCTTGCTTATGTCGCCCTCT-3'	
StAR	Fw	5'-CAGAAGATTGGAAAAGACACGGTC-3'	AF290202
	Rv	5'-AGGTGAGTTTGGTCCTTGAGGG-3'	
ACTH-R	Fw	5'-ATGAAACACATTCTCAATCTG-3'	NM_001009442
	Rv	5'-AACGTTTTCCAAAATCTTGTAC-3'	
CYCLO	Fw	5'-CCATCGTGTGATCAAGGACTTCAT-3'	BT020966
	Rv	5'-CTTGCCATCTAGCCAGGCAGTCTT-3'	

Fw, forward; Rv, reverse; CYCLO, cyclophilin; CYP17, 17a-hydroxylase; CYP11A1, cholesterol side-chain cleavage; StAR, steroidogenic acute regulatory protein; ACTH-R, adrenocorticotropic hormone receptor; NCBI, National Center for Biotechnology Information.

Statistical Analysis

Descriptive statistics are presented as mean ± standard error. Data analysis for cortisol was performed using two-way analysis of variance (ANOVA) with 1 between-subject factor (treatment) and 1 within-subject factor (time) stratified by oxygenation level (normoxic or LTH). Cortisol levels are reported as log transformed due to the large scale of change between control untreated and ACTH treatment. Data analysis for mRNA abundance was performed using two-way ANOVA with 1 between-subject factor (oxygenation level) and 1 within-subject factor (treatment). Alpha was set at .05 significance level. Post hoc tests were adjusted using the Bonferroni method. Statistical analyses were performed GraphPad Prism 5 (Version 5.04; GraphPad Software, Inc., 2010).

Results

Effects of NO on Cortisol Production

There were no differences observed in cortisol production from either normoxic (n=5) or LTH (n=5) FACs that were treated with DETA-NO or L-NAME compared to untreated cells, and cortisol levels remained relatively constant throughout the 96h time course (**Figure 1**). In normoxic FACs, ACTH treatment significantly increased cortisol output by 24h and remained significantly elevated throughout the 96h time course compared to untreated FACs. In LTH FACs, ACTH treatment significantly increased cortisol output by 48h but production fell off by 96h. DETA-NO and L-NAME treatment did not significantly affect ACTH-stimulated cortisol for the entire course of treatment in either normoxic or LTH FACs. Although not significant, there was a trend for cortisol

production from normoxic FACs to be higher than LTH FACs in response to ACTH (838.64 +/- 359.57 Normoxic vs 573.39 +/- 310.11 LTH ACTH only; 834.30 +/- 385.94 Normoxic vs 260.99 +/- 74.60 LTH ACTH + L-NAME; 1000.62 +/- 444.35 Normoxic vs 430.08 +/- 131.92 LTH ACTH + DETA-NO).

Effects of NO on mRNA Abundance of CYP11A1, CYP17, StAR, and ACTH-R

DETA-NO and L-NAME had no significant effects on mRNA abundance of CYP11A1, CYP17, StAR, and ACTH-R in both normoxic and LTH FACs (**Figures 2-5**). ACTH treatment increased expression in both normoxic and LTH FACs. There were no significant changes in basal mRNA abundance between normoxic and LTH FACs.

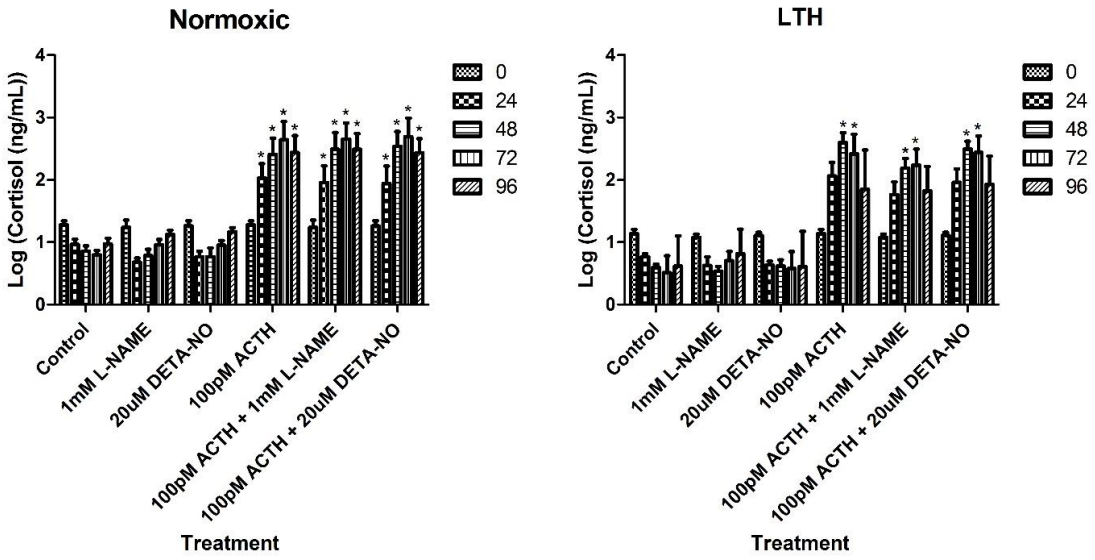

Figure 1. Time course of cortisol production in normoxic and LTH FACs with NO donor DETA-NO and NOS inhibitor L-NAME treatment, with and without ACTH stimulation. Treatment with ACTH (100pM) increased cortisol production in both normoxic and LTH FACs. DETA-NO (20µM) and L-NAME (1mM) had no effect on cortisol, in the presence and absence of ACTH in both normoxic and LTH. (Normoxic n=5, LTH n=5) Values represent mean values ± SEM. *$p<0.05$ compared to time control. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; DETA-NO, (Z)-1-[2-(2-aminoethyl-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; L-NAME, N(G)-nitro-L-arginine methyl ester; ACTH, adrenocorticotropic hormone.

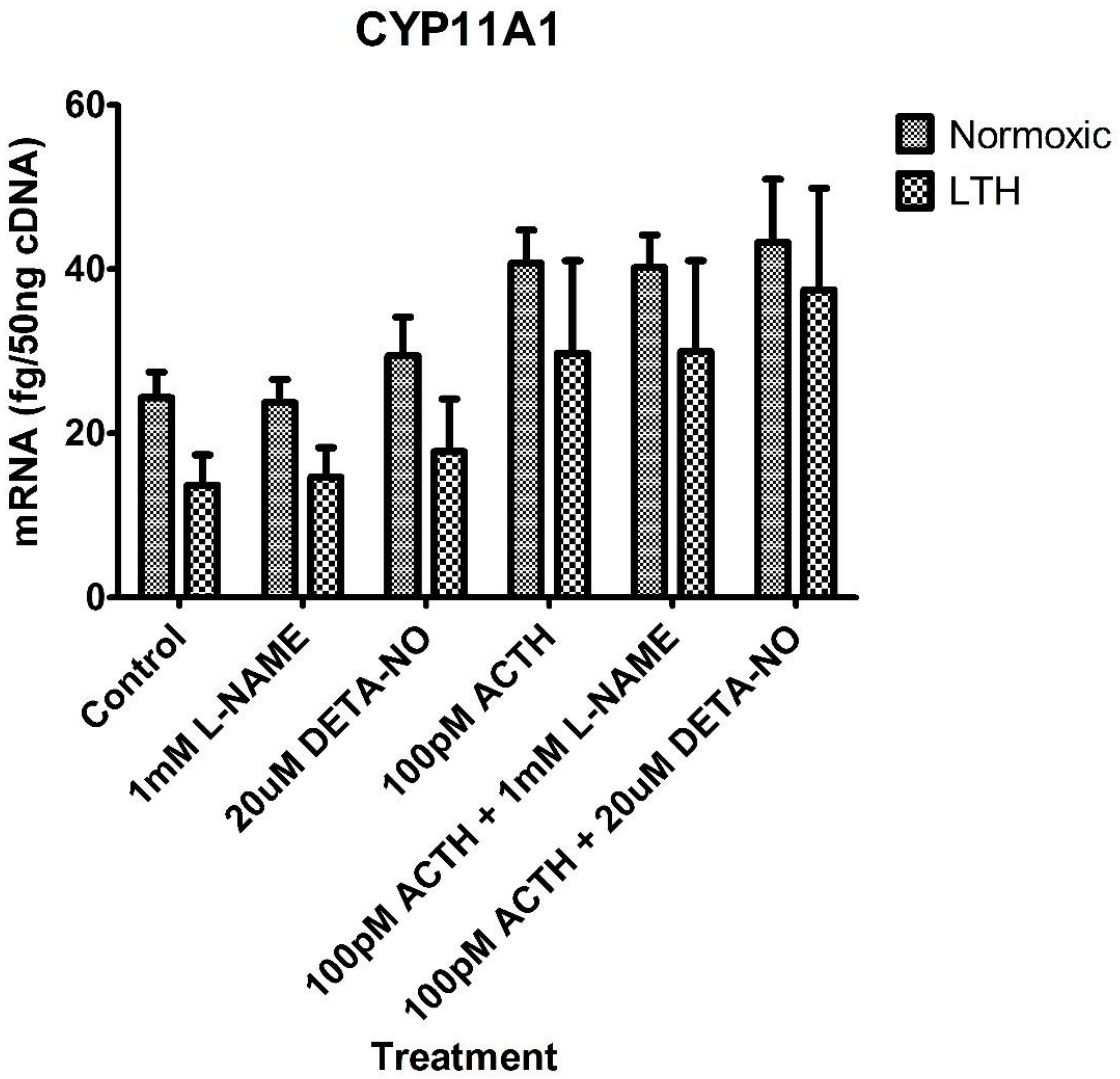

Figure 2. mRNA abundance of CYP11A1 in normoxic and LTH FACs in response to NO donor DETA-NO and NOS inhibitor L-NAME treatment, with and without ACTH stimulation. Treatment with ACTH (100pM) increased mRNA in both normoxic and LTH FACs compared to control. DETA-NO (20µM) and L-NAME (1mM) had no effect on mRNA, in the presence and absence of ACTH in both normoxic and LTH. (Normoxic n=5, LTH n=5) Values represent mean values ± SEM. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; DETA-NO, (Z)-1-[2-(2-aminoethyl-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; L-NAME, N(G)-nitro-L-arginine methyl ester; ACTH, adrenocorticotropic hormone.

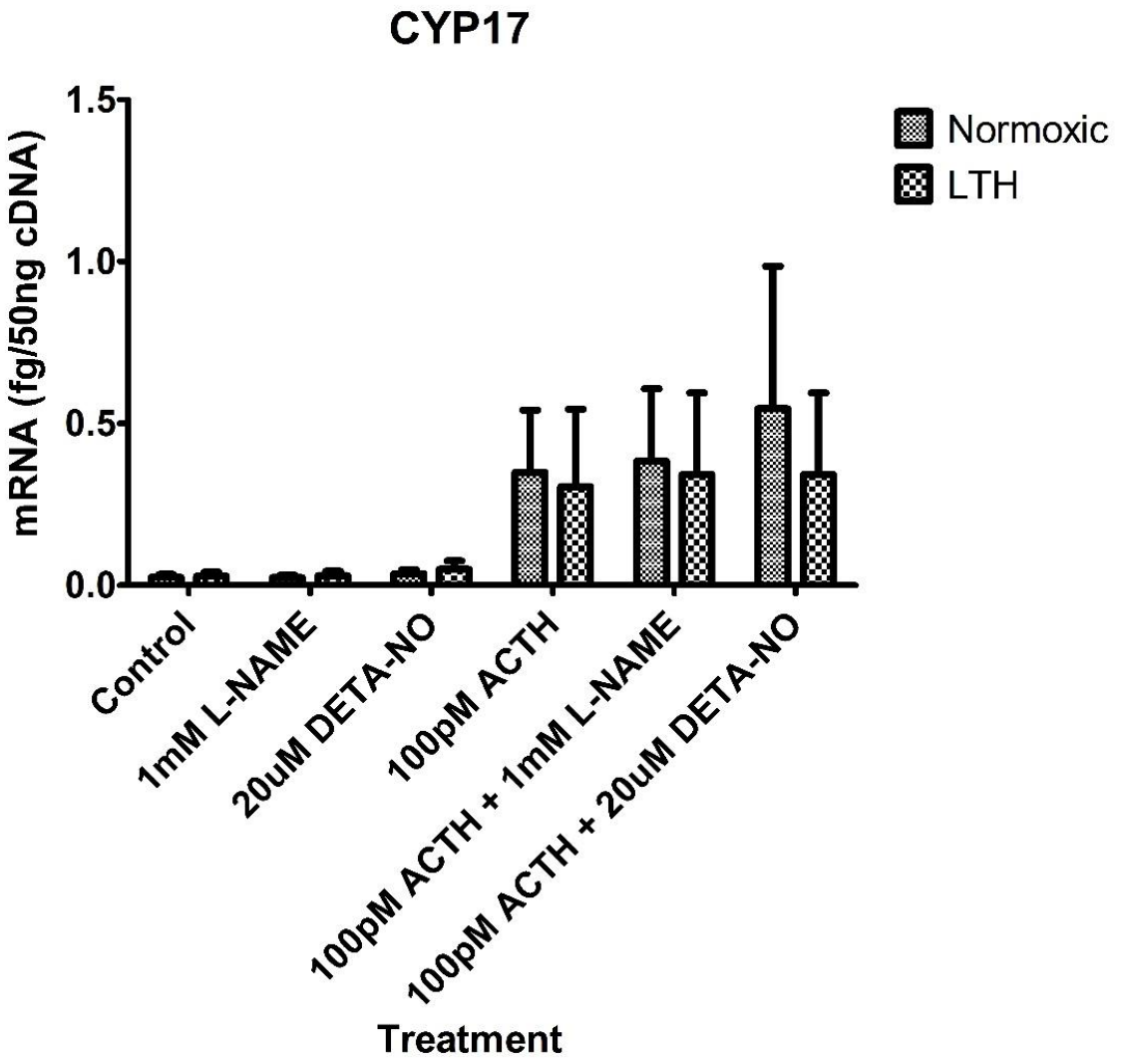

Figure 3. mRNA abundance of CYP17 in normoxic and LTH FACs in response to NO donor DETA-NO and NOS inhibitor L-NAME treatment, with and without ACTH stimulation. Treatment with ACTH (100pM) increased mRNA in both normoxic and LTH FACs compared to control. DETA-NO (20μM) and L-NAME (1mM) had no effect on mRNA, in the presence and absence of ACTH in both normoxic and LTH. (Normoxic n=5, LTH n=5) Values represent mean values ± SEM. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; DETA-NO, (Z)-1-[2-(2-aminoethyl-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; L-NAME, N(G)-nitro-L-arginine methyl ester; ACTH, adrenocorticotropic hormone.

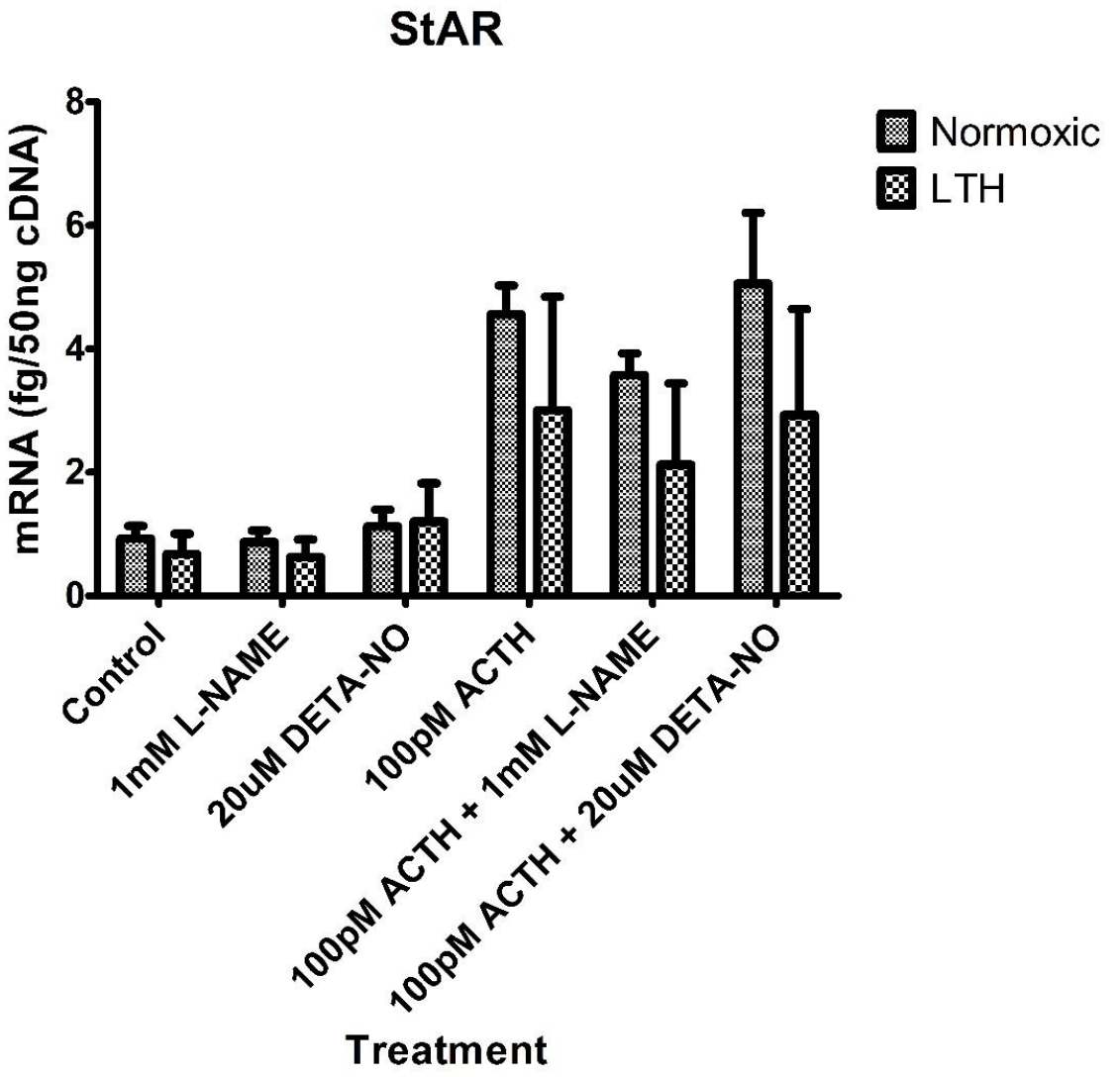

Figure 4. mRNA abundance of StAR in normoxic and LTH FACs in response to NO donor DETA-NO and NOS inhibitor L-NAME treatment, with and without ACTH stimulation. Treatment with ACTH (100pM) increased mRNA in both normoxic and LTH FACs compared to control. DETA-NO (20µM) and L-NAME (1mM) had no effect on mRNA, in the presence and absence of ACTH in both normoxic and LTH. (Normoxic n=5, LTH n=5) Values represent mean values ± SEM. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; DETA-NO, (Z)-1-[2-(2-aminoethyl-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; L-NAME, N(G)-nitro-L-arginine methyl ester; ACTH, adrenocorticotropic hormone.

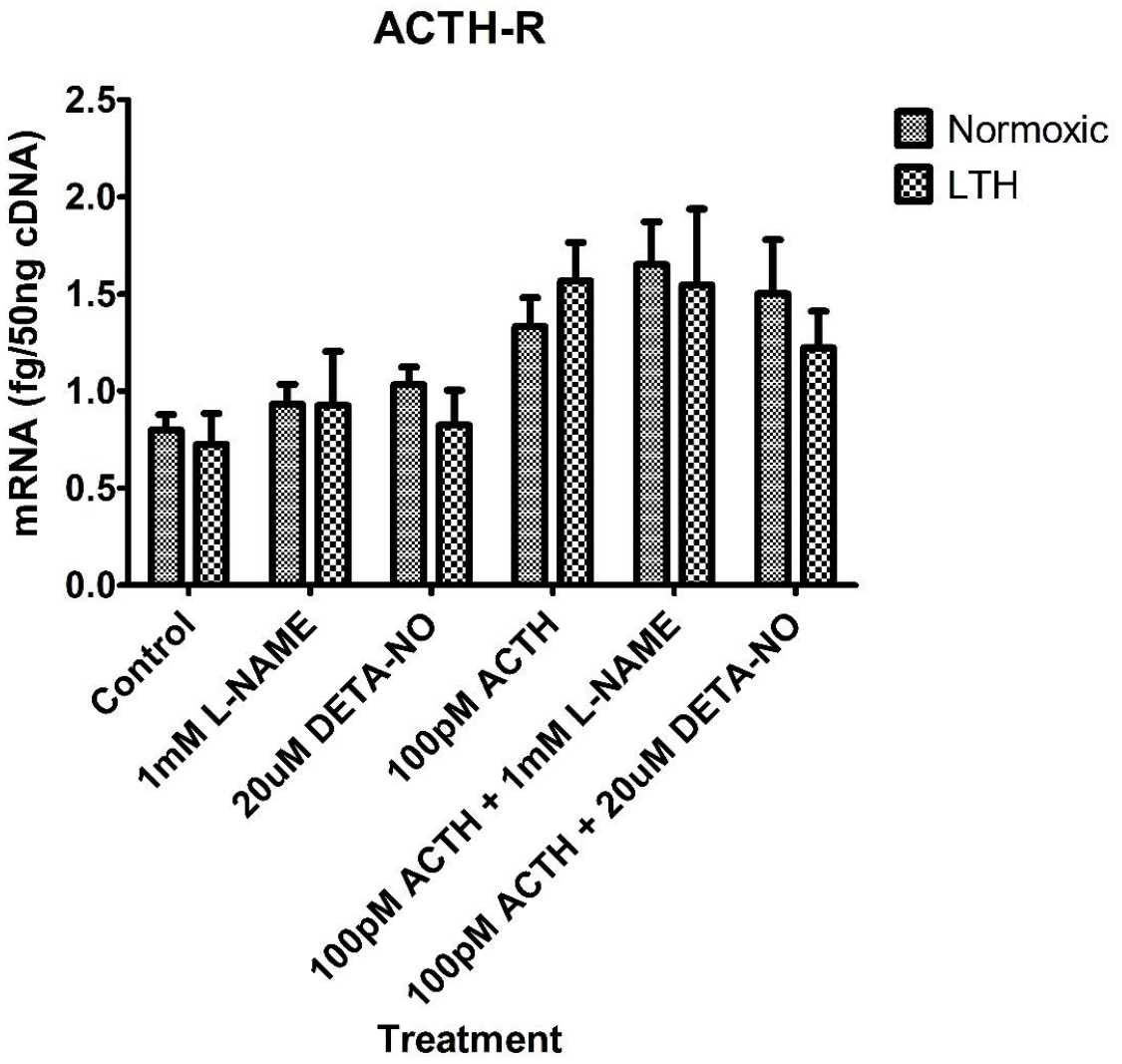

Figure 5. mRNA abundance of ACTH-R in normoxic and LTH FACs in response to NO donor DETA-NO and NOS inhibitor L-NAME treatment, with and without ACTH stimulation. Treatment with ACTH (100pM) increased mRNA in both normoxic and LTH FACs compared to control. DETA-NO (20μM) and L-NAME (1mM) had no effect on mRNA, in the presence and absence of ACTH in both normoxic and LTH. (Normoxic n=5, LTH n=5) Values represent mean values ± SEM. FACs, fetal adrenocortical cells; LTH, long-term hypoxia; DETA-NO, (Z)-1-[2-(2-aminoethyl-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; L-NAME, N(G)-nitro-L-arginine methyl ester; ACTH, adrenocorticotropic hormone.

Discussion

In response to conditions of long-term moderate gestational hypoxia (LTH), the fetus has the ability to adapt the hypothalamic-pituitary-adrenal (HPA) axis to preserve normal growth and development. Under basal conditions, normal basal plasma cortisol concentrations are maintained, despite elevated levels of adrenocorticotropic hormone (ACTH) (45). However, the LTH fetus has a heightened cortisol response to acute secondary stressors compared to normoxic fetuses (1, 28). There is also reduced expression of key steroidogenic enzymes CYP11A1 (P450 side chain cleavage) and CYP17 (P450 17α-hydroxylase), as well as reduced ACTH receptor (ACTH-R) mRNA in the LTH adrenal cortex (46), but expression of steroidogenic acute regulatory (StAR) protein is greater in LTH compared to normoxic fetal adrenocortical cells (FACs) (68). Together these changes suggest an adaptive response to LTH to prevent excessive cortisol production that would restrict fetal growth, but allow for enhanced cortisol production in response to a secondary stress.

A possible effector on cortisol production in this system is nitric oxide (NO). Aside from its well established role in the vascular system, NO has also been shown to inhibit steroidogenesis in a variety of tissues. NO reduced steroidogenesis in ovarian tissue of women (67), pigs (34, 35), rabbits (19, 73), and rats (39), and NO also reduced testosterone (30) and cortisol secretion (2) in the adult rat. Inhibition of NOS increased testosterone in the Leydig cells (14) and increased aldosterone production in humans (43). In the adrenal, NO inhibited basal, ACTH, and angiotensin II-induced aldosterone production in zona glomerulosa cells of adult rat adrenal cortex transfected with eNOS (21, 22) and adult bovine zona glomerulosa cells (23). In rat zona fasciculata cells, NO

donors decreased both unstimulated and ACTH-stimulated corticosterone production, and NOS inhibition enhanced glucocorticoid output (11, 12). In our lab, we have shown that NO inhibits cortisol biosynthesis and that inhibition of NOS enhances cortisol output in ovine LTH FACs (41), therefore nitric oxide may play an important role in the fetal adaptation to LTH in regulating cortisol production.

This study investigated the effects of NO on cortisol production and mRNA abundance of key steroidogenic enzymes CYP11A1 and CYP17, as well as StAR and ACTH-R in ovine FACs to determine a potential point of action of NO. Cortisol biosynthesis in the adrenal is initiated by ACTH activation of ACTH-R (38). Subsequent signaling leads to StAR-mediated translocation of cholesterol from the outer to the inner mitochondrial membrane, providing substrate, where CYP11A1 converts cholesterol into pregnenolone (3, 9, 52, 55, 56). CYP17 continues cortisol biosynthesis by mediating the 17α-hydroxylation of pregnenolone to 17α-hydroxypregnenolone and progesterone to 17α-hydroxyprogesterone (25). Changes in these proteins would directly affect the ability of the fetus to produce cortisol.

Interestingly we found that treatment with either the NO donor DETA-NO or NOS inhibition with L-NAME over 96h did not affect cortisol production compared to untreated cells in both normoxic and LTH FACs, however ACTH stimulation significantly increased cortisol output in normoxic FACs by 24h and in LTH FACs by 48h compared to untreated cells (Figure 1). ACTH-stimulated cortisol biosynthesis was not significantly affected by either DETA-NO or L-NAME treatment in both normoxic and LTH FACs, however this could be due to sustained stimulation of the cells with stress levels of ACTH. ACTH has been shown to increase the expression of CYP11A1

and CYP17 in the fetal adrenal (10, 18, 47, 48, 54, 57, 63), and elevated ACTH in the fetal sheep was shown to be accompanied by increased 30 kDa StAR (72), the inactive spent form, suggesting enhanced translocation of cholesterol to the inner mitochondrial membrane. Together, the increased levels of ACTH through continuous treatments in this study would result in increased levels of these enzymes followed by increased production of cortisol. These increases in gene expression could overcome NO inhibition to produce cortisol in response to stress.

We also found that cortisol output from normoxic FACs tended to reach higher levels than those in LTH FACs in response to ACTH. This could be due to the lack of cholesterol availability in the media. The production of steroid hormones is dependent on the availability of cholesterol within the steroidogenic cells. Sources of cholesterol include from the serum high density lipoproteins (HDLs) or low density lipoprotein (LDL) (20, 31), or de novo synthesis from acetate. Steroidogenesis occurs when cholesterol is mobilized from cellular stores to the outer mitochondrial membrane, via cholesterol esterase (5, 6, 64), or taken up from the plasma, followed by the transfer from the outer to the inner mitochondrial membrane by StAR (29, 33, 62). We have recently shown that LDL receptor and HMG-CoA Reductase mRNA was increased in whole adrenal cortex in the LTH fetus compared to normoxic control (unpublished results), however basal and ACTH stimulated cortisol output was lower in LTH compared to control in FACs over the course of 36h (70). This suggests that although LTH adrenals seem to have increased capacity for LDL uptake and de novo synthesis of cholesterol from acetate, there may be a defect in LDL transport in the LTH fetal adrenal cortex compared to normoxic controls.

Our earlier acute studies indicated a significant inhibitory effect of NO on cortisol biosynthesis in the LTH FACs (41). With this in mind, we wanted to determine if NO had a potentially longer lasting effect and altered gene expression of key proteins involved in cortisol production. In the present study we found that treatment with DETA-NO or L-NAME both with or without ACTH for 96h had no significant effect on mRNA abundance of CYP11A1, CYP17, StAR, and ACTH-R in both normoxic and LTH FACs (Figure 2). ACTH treatment, however, increased mRNA in both normoxic and LTH FACs, but unlike our previous study (46), levels in untreated cells were not different in LTH compared to normoxic. This could be due to a lack of basal ACTH stimulation; as we have previously shown, ACTH is required for normal CYP11A1 and CYP17 expression (47, 48). Together these results suggest that NO does not play a significant role in the regulation of CYP11A1, CYP17, StAR, and ACTH-R mRNA, and that changes in mRNA are not responsible for the inhibition of acute ACTH-induced cortisol production by NO that we have previously observed (41).

The effects of NO on steroidogenesis, including cortisol production, have been well established, but the mechanism of action is still uncertain. In this study we showed that NO does not affect mRNA abundance of key steroidogenic enzymes and proteins, but NO may alter steroidogenesis by other post-translational mechanisms. One possibility is that NO may act directly on the CYP enzymes, through competitive interaction at the heme-oxygen binding site of CYP11A1 and CYP17 (22), or indirectly through S-nitrosylation of CYP11A1 and CYP17 (60). It has been shown that NO is capable of interacting with the heme-oxygen binding site of CYP11A1 and CYP17 (65, 66), and because these enzymes use several rounds of attack of the heme-oxygen complex on the

steroid substrate (51), they may be more sensitive to NO inhibition than other enzymes. It has also been demonstrated that decreased oxygen concentrations resulted in a lower threshold for NO-mediated inhibition of aldosterone synthesis in adult rat adrenals (21), suggesting that the LTH fetus may be more susceptible to NO inhibition of cortisol.

NO may also act through S-nitrosylation of key Cys residues in the active sites on CYP11A1 and CYP17, which could lead to suppressed steroidogenic activity and decreased cortisol production. S-nitrosylation has been unexplored in steroidogenic CYPs but it has been shown in the liver to affect both CYP transcription through zinc finger transcription interaction (71), as well as reduction in CYP expression (32). S-nitrosylation has also been proposed as the mechanisms responsible for the NO-induced inhibition of aromatase activity in granulosa cells (58), and inhibition of corticosterone production in rat adrenocortical cells (12). These interactions of NO on CYP11A1 and CYP17 activity may lead to inhibition of acute ACTH-induced steroidogenesis following NO treatment in LTH FACs.

The HPA axis of the fetal sheep undergoes significant adaptations in response to development under conditions of LTH. These changes include increased circulating basal ACTH, but reduced expression of CYP11A1, CYP17, and ACTH-R and normal plasma cortisol concentrations. Together, these adaptations suggest heightened activation of the hypothalamic-pituitary arm of the HPA axis, but reduced adrenocortical capacity to respond to ACTH. This divergent adaptation may serve to limit cortisol production in the basal state yet allow increased production of cortisol when needed during acute secondary stress. The mechanisms involved in this adaptation are not yet fully understood, however nitric oxide appears to still play a role. Although NO has been

shown to inhibit ACTH-induced cortisol production, the results from this study show that NO does not affect mRNA abundance of key steroidogenic enzymes CYP11A1 and CYP17, as well as StAR and ACTH-R. The lack of change in mRNA suggests that the effects of NO on cortisol biosynthesis are downstream from transcription, perhaps affecting enzyme activity through competition at the heme-oxygen binding site or through S-nitrosylation in the activation sites of the CYP enzymes. Future work should investigate the possibility of S-nitrosylation of the CYP enzymes as a mechanism for NO-induced cortisol inhibition.

References

1. **Adachi K, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters ovine fetal endocrine and physiological responses to hypotension. *American journal of physiology Regulatory, integrative and comparative physiology* 287: R209-217, 2004.

2. **Adams ML, Nock B, Truong R, and Cicero TJ**. Nitric oxide control of steroidogenesis: endocrine effects of NG-nitro-L-arginine and comparisons to alcohol. *Life sciences* 50: Pl35-40, 1992.

3. **Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, Stocco DM, and Strauss JF**. Phosphorylation of Steroidogenic Acute Regulatory Protein (StAR) Modulates Its Steroidogenic Activity. *Journal of Biological Chemistry* 272: 32656-32662, 1997.

4. **Beckman JS**. Parsing the effects of nitric oxide, S-nitrosothiols, and peroxynitrite on inducible nitric oxide synthase-dependent cardiac myocyte apoptosis. *Circ Res* 85: 870-871, 1999.

5. **Behrman HR, and Amstrong DT**. Cholesterol esterase stimulation by luteinizing hormone in luteinized rat ovaries. *Endocrinology* 85: 474-480, 1969.

6. **Behrman HR, and Greep RO**. Hormonal dependence of cholesterol ester hydrolase in the corpus luteum and adrenal. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme* 4: 206-209, 1972.

7. **Brown GC**. Nitric oxide and mitochondrial respiration. *Biochimica et biophysica acta* 1411: 351-369, 1999.

8. **Challis JRG, Matthews SG, Gibb W, and Lye SJ**. Endocrine and paracrine regulation of birth at term and preterm. *Endocrine reviews* 21: 514-550, 2000.

9. **Churchill PF, and Kimura T**. Topological studies of cytochromes P-450scc and P-45011 beta in bovine adrenocortical inner mitochondrial membranes. Effects of controlled tryptic digestion. *The Journal of biological chemistry* 254: 10443-10448, 1979.

10. **Coulter CL, Ross JT, Owens JA, Bennett HP, and McMillen IC**. Role of pituitary POMC-peptides and insulin-like growth factor II in the developmental biology of the adrenal gland. *Archives of physiology and biochemistry* 110: 99-105, 2002.

11. **Cymeryng CB, Dada LA, Colonna C, Mendez CF, and Podesta EJ**. Effects of L-arginine in rat adrenal cells: involvement of nitric oxide synthase. *Endocrinology* 140: 2962-2967, 1999.

12. **Cymeryng CB, Dada LA, and Podesta EJ**. Effect of nitric oxide on rat adrenal zona fasciculata steroidogenesis. *The Journal of endocrinology* 158: 197-203, 1998.

13. **Cymeryng CB, Lotito SP, Colonna C, Finkielstein C, Pomeraniec Y, Grión N, Gadda L, Maloberti P, and Podestá EJ**. Expression of Nitric Oxide Synthases in Rat Adrenal Zona Fasciculata Cells. *Endocrinology* 143: 1235-1242, 2002.

14. **Dobashi M, Fujisawa M, Yamazaki T, Okuda Y, Kanzaki M, Tatsumi N, Tsuji T, Okada H, and Kamidono S**. Inhibition of steroidogenesis in Leydig cells by exogenous nitric oxide occurs independently of steroidogenic acute regulatory protein (star) mRNA. *Archives of andrology* 47: 203-209, 2001.

15. **Ducsay CA, Hyatt K, Mlynarczyk M, Root BK, Kaushal KM, and Myers DA**. Long-term hypoxia modulates expression of key genes regulating adrenomedullary function in the late gestation ovine fetus. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 293: R1997-R2005, 2007.

16. **Ducsay CA, Mlynarczyk M, Kaushal KM, Hyatt K, Hanson K, and Myers DA**. Long-term hypoxia enhances ACTH response to arginine vasopressin but not corticotropin-releasing hormone in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 297: R892-899, 2009.

17. **Eum H-A, Yeom D-H, and Lee S-M**. Role of nitric oxide in the inhibition of liver cytochrome P450 during sepsis. *Nitric Oxide* 15: 423-431, 2006.

18. **Glickman JA, and Challis JR**. The changing response pattern of sheep fetal adrenal cells throughout the course of gestation. *Endocrinology* 106: 1371-1376, 1980.

19. **Gobbetti A, Boiti C, Canali C, and Zerani M**. Nitric oxide synthase acutely regulates progesterone production by in vitro cultured rabbit corpora lutea. *The Journal of endocrinology* 160: 275-283, 1999.

20. **Gwynne JT, and Mahaffee DD**. Rat adrenal uptake and metabolism of high density lipoprotein cholesteryl ester. *The Journal of biological chemistry* 264: 8141-8150, 1989.

21. **Hanke CJ, and Campbell WB**. Endothelial cell nitric oxide inhibits aldosterone synthesis in zona glomerulosa cells: modulation by oxygen. *American Journal of Physiology - Endocrinology And Metabolism* 279: E846-E854, 2000.

22. **Hanke CJ, Drewett JG, Myers CR, and Campbell WB**. Nitric oxide inhibits aldosterone synthesis by a guanylyl cyclase-independent effect. *Endocrinology* 139: 4053-4060, 1998.

23. **Hanke CJ, O'Brien T, Pritchard KA, Jr., and Campbell WB**. Inhibition of adrenal cell aldosterone synthesis by endogenous nitric oxide release. *Hypertension* 35: 324-328, 2000.

24. **He J, Xiao Y, and Zhang L**. Cocaine-mediated apoptosis in bovine coronary artery endothelial cells: role of nitric oxide. *The Journal of pharmacology and experimental therapeutics* 298: 180-187, 2001.

25. **Hough D, Cloete SW, Storbeck K, Swart AC, and Swart P**. Cortisol production in sheep is influenced by the functional expression of two cytochrome P450 17alpha-hydroxylase/17,20-lyase (CYP17) isoforms. *Journal of animal science* 91: 1193-1206, 2013.

26. **Hrabie JA, Klose JR, Wink DA, and Keefer LK**. New nitric oxide-releasing zwitterions derived from polyamines. *The Journal of Organic Chemistry* 58: 1472-1476, 1993.

27. **Ignarro LJ**. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society* 53: 503-514, 2002.

28. **Imamura T, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters endocrine and physiologic responses to umbilical cord occlusion in the ovine fetus. *Journal of the Society for Gynecologic Investigation* 11: 131-140, 2004.

29. **Jefcoate CR, McNamara BC, Artemenko I, and Yamazaki T**. Regulation of cholesterol movement to mitochondrial cytochrome P450scc in steroid hormone synthesis. *J Steroid Biochem Mol Biol* 43: 751-767, 1992.

30. **Kostić T, Andrić S, Kovačević R, and Marić D**. The involvement of nitric oxide in stress-impaired testicular steroidogenesis. *European Journal of Pharmacology* 346: 267-273, 1998.

31. **Kovanen PT, Goldstein JL, Chappell DA, and Brown MS**. Regulation of low density lipoprotein receptors by adrenocorticotropin in the adrenal gland of mice and rats in vivo. *The Journal of biological chemistry* 255: 5591-5598, 1980.

32. **Lee C-M, Kim B-Y, Li L, and Morgan ET**. Nitric Oxide-dependent Proteasomal Degradation of Cytochrome P450 2B Proteins. *Journal of Biological Chemistry* 283: 889-898, 2008.

33. **Liscum L, and Dahl NK**. Intracellular cholesterol transport. *J Lipid Res* 33: 1239-1254, 1992.

34. **Masuda M, Kubota T, and Aso T**. Effects of nitric oxide on steroidogenesis in porcine granulosa cells during different stages of follicular development. *European journal of endocrinology / European Federation of Endocrine Societies* 144: 303-308, 2001.

35. **Masuda M, Kubota T, Karnada S, and Aso T**. Nitric oxide inhibits steroidogenesis in cultured porcine granulosa cells. *Mol Hum Reprod* 3: 285-292, 1997.

36. **Meaney MJ, Viau V, Bhatnagar S, Betito K, Iny LJ, O'Donnell D, and Mitchell JB**. Cellular mechanisms underlying the development and expression of individual differences in the hypothalamic-pituitary-adrenal stress response. *J Steroid Biochem Mol Biol* 39: 265-274, 1991.

37. **Miller WL**. StAR search--what we know about how the steroidogenic acute regulatory protein mediates mitochondrial cholesterol import. *Molecular endocrinology (Baltimore, Md)* 21: 589-601, 2007.

38. **Miller WL, and Auchus RJ**. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine reviews* 32: 81-151, 2011.

39. **Mitsube K, Mikuni M, Matousek M, and Brannstrom M**. Effects of a nitric oxide donor and nitric oxide synthase inhibitors on luteinizing hormone-induced ovulation in the ex-vivo perfused rat ovary. *Human reproduction (Oxford, England)* 14: 2537-2543, 1999.

40. **Mlynarczyk M, Imamura T, Umezaki H, Kaushal KM, Zhang L, and Ducsay CA**. Long-term hypoxia changes myometrial responsiveness and oxytocin receptors in the pregnant ewe: differential effects on longitudinal versus circular smooth muscle. *Biology of reproduction* 69: 1500-1505, 2003.

41. **Monau TR, Vargas VE, Zhang L, Myers DA, and Ducsay CA**. Nitric oxide inhibits ACTH-induced cortisol production in near-term, long-term hypoxic ovine fetal adrenocortical cells. *Reproductive sciences (Thousand Oaks, Calif)* 17: 955-962, 2010.

42. **Moncada S, Palmer RM, and Higgs EA**. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109-142, 1991.

43. **Muldowney JA, 3rd, Davis SN, Vaughan DE, and Brown NJ**. NO synthase inhibition increases aldosterone in humans. *Hypertension* 44: 739-745, 2004.

44. **Munck A, Guyre PM, and Holbrook NJ**. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine reviews* 5: 25-44, 1984.

45. **Myers DA, Bell PA, Hyatt K, Mlynarczyk M, and Ducsay CA**. Long-term hypoxia enhances proopiomelanocortin processing in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 288: R1178-1184, 2005.

46. **Myers DA, Hyatt K, Mlynarczyk M, Bird IM, and Ducsay CA**. Long-term hypoxia represses the expression of key genes regulating cortisol biosynthesis in the

near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 289: R1707-1714, 2005.

47. **Myers DA, McDonald TJ, and Nathanielsz PW**. Effect of bilateral lesions of the ovine fetal hypothalamic paraventricular nuclei at 118-122 days of gestation on subsequent adrenocortical steroidogenic enzyme gene expression. *Endocrinology* 131: 305-310, 1992.

48. **Myers DA, McDonald TJ, and Nathanielsz PW**. Effect of placement of dexamethasone adjacent to the ovine fetal paraventricular nucleus on adrenocortical steroid hydroxylase messenger ribonucleic acid. *Endocrinology* 131: 1329-1335, 1992.

49. **Myers DA, Trinh JV, and Myers TR**. Structure and function of the ovine type 1 corticotropin releasing factor receptor (CRF1) and a carboxyl-terminal variant. *Mol Cell Endocrinol* 144: 21-35, 1998.

50. **Newby EA, Kaushal KM, Myers DA, and Ducsay CA**. Adrenocorticotropic Hormone and PI3K/Akt Inhibition Reduce eNOS Phosphorylation and Increase Cortisol Biosynthesis in Long-Term Hypoxic Ovine Fetal Adrenal Cortical Cells. *Reproductive sciences (Thousand Oaks, Calif)* 2015.

51. **Peterson JK, Moran F, Conley AJ, and Bird IM**. Zonal Expression of Endothelial Nitric Oxide Synthase in Sheep and Rhesus Adrenal Cortex. *Endocrinology* 142: 5351-5363, 2001.

52. **Pon LA, Hartigan JA, and Orme-Johnson NR**. Acute ACTH regulation of adrenal corticosteroid biosynthesis. Rapid accumulation of a phosphoprotein. *The Journal of biological chemistry* 261: 13309-13316, 1986.

53. **Schmidt K, Desch W, Klatt P, Kukovetz WR, and Mayer B**. Release of nitric oxide from donors with known half-life: a mathematical model for calculating nitric oxide concentrations in aerobic solutions. *Naunyn-Schmiedeberg's archives of pharmacology* 355: 457-462, 1997.

54. **Sewer MB, and Waterman MR**. ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. *Microscopy research and technique* 61: 300-307, 2003.

55. **Simpson ER, and Boyd GS**. The cholesterol side-chain cleavage system of bovine adrenal cortex. *European journal of biochemistry / FEBS* 2: 275-285, 1967.

56. **Simpson ER, and Boyd GS**. The cholesterol side-chain cleavage system of the adrenal cortex: a mixed-function oxidase. *Biochemical and biophysical research communications* 24: 10-17, 1966.

57. **Simpson ER, and Waterman MR**. Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. *Annual review of physiology* 50: 427-440, 1988.

58. **Snyder GD, Holmes RW, Bates JN, and Van Voorhis BJ**. Nitric oxide inhibits aromatase activity: Mechanisms of action. *The Journal of Steroid Biochemistry and Molecular Biology* 58: 63-69, 1996.

59. **Stadler J, Trockfeld J, Schmalix WA, Brill T, Siewert JR, Greim H, and Doehmer J**. Inhibition of cytochromes P4501A by nitric oxide. *Proceedings of the National Academy of Sciences* 91: 3559-3563, 1994.

60. **Stamler JS, Lamas S, and Fang FC**. Nitrosylation: The Prototypic Redox-Based Signaling Mechanism. *Cell* 106: 675-683, 2001.

61. **Stocco DM**. StAR protein and the regulation of steroid hormone biosynthesis. *Annual review of physiology* 63: 193-213, 2001.

62. **Stocco DM, and Clark BJ**. Regulation of the acute production of steroids in steroidogenic cells. *Endocrine reviews* 17: 221-244, 1996.

63. **Tangalakis K, Coghlan JP, Crawford R, Hammond VE, and Wintour EM**. Steroid hydroxylase gene expression in the ovine fetal adrenal gland following ACTH infusion. *Acta endocrinologica* 123: 371-377, 1990.

64. **Trzeciak WH, and Boyd GS**. The effect of stress induced by ether anaesthesia on cholesterol content and cholesteryl-esterase activity in rat-adrenal cortex. *European journal of biochemistry / FEBS* 37: 327-333, 1973.

65. **Tsubaki M, Hiwatashi A, Ichikawa Y, and Hori H**. Electron paramagnetic resonance study of ferrous cytochrome P-450scc-nitric oxide complexes: effects of cholesterol and its analogues. *Biochemistry* 26: 4527-4534, 1987.

66. **Tsubaki M, Ichikawa Y, Fujimoto Y, Yu NT, and Hori H**. Active site of bovine adrenocortical cytochrome P-450(11) beta studied by resonance Raman and electron paramagnetic resonance spectroscopies: distinction from cytochrome P-450scc. *Biochemistry* 29: 8805-8812, 1990.

67. **Van Voorhis BJ, Dunn MS, Snyder GD, and Weiner CP**. Nitric oxide: an autocrine regulator of human granulosa-luteal cell steroidogenesis. *Endocrinology* 135: 1799-1806, 1994.

68. **Vargas VE, Kaushal KM, Monau T, Myers DA, and Ducsay CA**. Long-term hypoxia enhances cortisol biosynthesis in near-term ovine fetal adrenal cortical cells. *Reproductive sciences (Thousand Oaks, Calif)* 18: 277-285, 2011.

69. **Vargas VE, Kaushal KM, Monau TR, Myers DA, and Ducsay CA**. Extracellular signal-regulated kinases (ERK1/2) signaling pathway plays a role in cortisol

secretion in the long-term hypoxic ovine fetal adrenal near term. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 304: R636-R643, 2013.

70. **Vargas VE, Newby EA, Ducsay CA, Kaushal KM, Hyatt K, Singleton K, and Myers DA**. Effect of low density (LDL) and or high density (HDL) lipoprotein on cortisol production in ovine fetal adrenal cortical cells (FACs): effect of long-term hypoxia (LTH). *J Soc Repro Investig* 22: 170-171A, 2015.

71. **Vossen C, and Erard M**. Down-regulation of nuclear receptor DNA-binding activity by nitric oxide--HNF4 as a model system. *Medical science monitor : international medical journal of experimental and clinical research* 8: Ra217-220, 2002.

72. **Warnes KE, McMillen IC, Robinson JS, and Coulter CL**. Differential actions of metyrapone on the fetal pituitary-adrenal axis in the sheep fetus in late gestation. *Biology of reproduction* 71: 620-628, 2004.

73. **Yamauchi J, Miyazaki T, Iwasaki S, Kishi I, Kuroshima M, Tei C, and Yoshimura Y**. Effects of nitric oxide on ovulation and ovarian steroidogenesis and prostaglandin production in the rabbit. *Endocrinology* 138: 3630-3637, 1997.

CHAPTER SIX

CONCLUSIONS

Hypoxia is a common fetal stress that can occur during pregnancy due to maternal malnutrition, smoking, heart or lung disease, obesity, preeclampsia, or exposure to high altitude (3, 19, 27, 38, 47, 69). The fetus synthesizes cortisol from the fetal adrenal as part of the hypothalamus-pituitary-adrenal axis (HPA) response to stress (1, 6, 7, 45, 46). Regulation of cortisol is important for normal fetal development due to its involvement in growth and organ maturation, as well as regulation of plasma glucose, lipid, and protein concentrations (10, 56, 66). Through studies in our lab, we have shown that the fetus has the ability to adapt to chronic stress during the course of gestation. When exposed to moderate gestational hypoxia during the last hundred days of gestation (long-term hypoxia, LTH), basal levels of cortisol remain similar to levels in the normoxic fetus, despite elevated levels of ACTH (67), and when challenged with a secondary stressor, the LTH fetus responds with a higher output of cortisol than the normoxic fetus (1, 45). We also found that expression of two key steroidogenic P450 enzymes, CYP11A1 and CYYP17, was decreased along with the ACTH receptor (ACTH-R) in LTH FACs (68), and that StAR expression was increased (82). Together, these changes suggest an adaptation in the fetal adrenal that maintains normal basal levels of cortisol required for fetal development, but allows for enhanced cortisol in response to a secondary stress. These adaptations may be mediated through NO produced by eNOS in the adrenocortical cells.

The effects of nitric oxide have been well established in adult steroidogenic tissues and cells, with increases in NO leading to reduced steroidogenesis (21, 26, 36, 39-41, 51, 54, 55, 61, 81, 85) and inhibition of NOS resulting in increased steroid production (20, 26, 65). This key role of NO has been demonstrated in a variety of species, including human and rat, however there are very few studies on the effects of NO in the fetus, as well as limited studies on the regulation of NOS in non-endothelial cells. In our lab, we have studied the effects of NO on ovine fetal adrenal cortical cells (FACs) as well as the effects of exposure to LTH. We found that NO inhibits cortisol biosynthesis, while inhibition of NOS enhances cortisol output in ovine LTH FACs (64). We have also shown that eNOS expression is upregulated in LTH FACs (63), suggesting that NO via eNOS may play a role in the fetal adaptation to LTH.

Regulation of eNOS can occur through multiple post-translational mechanisms including phosphorylation via MEK/ERK1/2 and PI3K/Akt signaling, as well as calcium/calmodulin (4, 5, 12-14, 28). Changes in phosphorylation of eNOS at serine activation site 1177 in human, 1179 in bovine/ovine (25, 33, 34), could lead to altered NOS activity and subsequent NO production that could ultimately have an effect on cortisol biosynthesis. This brought us to our general hypothesis that adaptations in cortisol production in the LTH fetus are mediated by eNOS-derived NO. Specifically, we investigated the role of key signaling pathways MEK/ERK1/2, PI3K/Akt, and calcium in regulating eNOS phosphorylation (peNOS) at Ser1177/79 and the subsequent effects of changes in these pathways on cortisol production in ovine LTH FACs. We also determined the effects of NO on the expression of key steroidogenic enzymes CYP11A1 and CYP17, as well as steroidogenic proteins StAR and ACTH-R and the potential

effects on cortisol biosynthesis. Through studying the role of these key signaling pathways on peNOS and the impact of NO on the CYPs, StAR, and ACTH-R along with their effects on cortisol biosynthesis in FACs, we wanted to better understand the influence of LTH in the ovine fetus and adaptations that may occur in the fetal adrenal response to LTH as a chronic stressor during pregnancy.

A variety of studies have shown that inhibition of the MEK/ERK1/2 pathway with UO126 (UO) reduces peNOS and NO production (11, 52, 60, 62), and a previous study from our lab showed that MEK/ERK1/2 inhibition with UO resulted in decreased ACTH-stimulated cortisol output in FACs (83). In chapter 2, we determined that the MEK/ERK1/2 signaling pathway is involved in the adaptive increase in ACTH-stimulated cortisol in LTH FACs, however this effect is not mediated through peNOS; UO treatment did not reduce peNOS. Instead, it is likely that MEK/ERK1/2 signaling is more important for cholesterol transport in the mitochondria as inhibition of this pathway with UO prevented the increase in cortisol observed in response to ACTH treatment, but had no effect on ACTH-stimulated cortisol levels in the presence of membrane permeable 22R-hydroxycholesterol (22R-OHC). These findings are consistent with other studies that have shown that UO blocks steroidogenesis in both granulosa (24) and Leydig cells (53, 71) but does not affect steroid production in cells treated with 22R-OHC.

Another signaling pathway that has been shown to target eNOS at Ser1177/79 in endothelial cells (33, 42, 60) is the PI3K/Akt pathway. In our FACs we found that, unlike the MEK/ERK1/2 pathway, the PI3K/Akt pathway played a significant role in regulating peNOS as well as cortisol production in LTH FACs. In response to PI3K/Akt pathway inhibition with wortmannin, ACTH-stimulated cortisol was elevated above levels

produced by ACTH stimulation alone, and peNOS was reduced compared to untreated cells. Together this suggests that peNOS is closely linked to cortisol biosynthesis in the LTH fetus, and that the PI3K/Akt pathway works in the LTH fetus to prevent even higher levels of cortisol under stimulating conditions.

In both these experiments we demonstrated divergent effects of ACTH on cortisol and peNOS in the LTH FACs; treatment with ACTH increased cortisol while decreasing peNOS, supporting our hypothesis that eNOS is involved in the fetal adaptation to LTH. When combined with higher levels of eNOS protein in LTH adrenals that we previously observed (63) and the ability of NO to inhibit cortisol production in LTH FACs (64), the PI3K/Akt pathway and ACTH may work together in LTH FACs to regulate peNOS; PI3K/Akt signaling maintains peNOS and NO production while ACTH stimulation overrides PI3K/Akt signaling to reduce peNOS and limit NO. This mechanism would preserve normal basal cortisol levels but allow for a robust cortisol response in stressed FACs in LTH compared to normoxic controls.

In a concurrent study with chapter 2, chapter 3 assessed the role cAMP activation of steroidogenesis via membrane permeable analog 8Bromo-cAMP (8Br). ACTH-stimulation of cortisol classically occurs through 3,5-cAMP/PKA activation of StAR protein (23, 76), and 8Br has been shown to effectively increase glucocorticoids in adrenal cells (17, 20, 21), supporting cAMP/PKA regulation. Because 8Br is membrane permeable, it bypasses ACTH-activation of the ACTH-R and stimulates PKA directly to determine if the fetal adaptation to LTH is cAMP-dependent. Consistent with previous studies using ACTH, 8Br treatment enhanced cortisol production in LTH FACs above levels observed in normoxic controls. We also found that MEK/ERK1/2 pathway

inhibition with UO reduced 8Br-stimulated cortisol output, further supporting the involvement of the MEK/ERK1/2 pathway in cortisol production and indicating that UO inhibition of cortisol synthesis is downstream of cAMP/PKA signaling. However, 8Br treatment had no effect on peNOS, suggesting that the use of this analog was not able to replicate the actions of endogenous cAMP, or that ACTH-mediated dephosphorylation of eNOS is not cAMP dependent and that alternative signaling mechanisms are activated to induce phosphatase activity in LTH FACs.

A possible intermediary between ACTH and eNOS is protein phosphatase 2A (PP2A). PP2A has been shown to be capable of dephosphorylating eNOS and the inhibition of PP2A with okadaic acid increases peNOS in endothelial cells (37, 57), however the effects of LTH on this system are unexplored. Preliminary data from our lab shows significantly greater PP2A expression in the LTH adrenal cortex compared to normoxic tissue (unpublished results) suggesting the involvement of PP2A in the fetal adaptation to LTH. If ACTH increases PP2A activity, combined with greater PP2A expression, it would reduce peNOS, thereby reducing NO production and effectively limiting the inhibition of NO on cortisol production in LTH FACs. Further studies examining the activity of PP2A in the fetal adrenal cortex could help determine the mechanism responsible for ACTH-mediated reduction in peNOS.

Calcium regulation of eNOS activity has been well characterized in endothelial cells; increases in intracellular calcium using calcium ionophore A23187 increased peNOS and NO production (8, 43, 84), and calcium chelators and calcium free media prevented stimulated increases in peNOS (31, 35). The effects of calcium on steroidogenic cells have not yet been explored, and the impact of hypoxia on calcium

regulation of eNOS is uncertain. Chapter 4 investigated the effects of increasing intracellular calcium in FACs and we found that peNOS and cortisol were unaffected; both basal and ACTH-stimulated levels of peNOS and cortisol did not change in response to elevated calcium. This suggests that calcium does not play a major role in regulating fetal adrenal eNOS and is not involved in the fetal adaptation to LTH. The lack of involvement of calcium in the regulation of eNOS may be due to tissue specificity (30), or the requirement of calcium may be reduced due to the type of agonist in FACs (9, 80).

Cortisol biosynthesis in the adrenal requires a series of enzymatic steps initiated by ACTH signaling through the ACTH receptor (ACTH-R) to activate steroidogenic acute regulatory (StAR) protein (59). Following activation, StAR transports cholesterol to the inner mitochondrial membrane where the key rate-limiting enzyme cytochrome P450 side-chain cleavage (CYP11A1) cleaves cholesterol into pregnenolone (2, 16, 58, 59, 70, 78). Cytochrome P450 17α-hydroxylase (CYP17) then continues cortisol biosynthesis by mediating the 17α-hydroxylation of pregnenolone to 17α-hydroxypregnenolone and progesterone 17α-hydroxyprogesterone (44). In adrenal cells, NO has been shown to inhibit CYP expression (29, 77) and activity (20, 22, 41), which would limit cortisol production, and previous studies from our lab found that NO inhibited ACTH-stimulated cortisol while NOS inhibition enhanced cortisol output in LTH FACs (64). For chapter 5, we sought to determine a potential point of action for NO and any longer lasting effects in gene expression. We found that extended NO exposure, via NO donor DETA-NO, or removal, via NOS inhibitor L-NAME, had no effect on mRNA abundance of key steroidogenic enzymes CY11A1 and CYP17, and key steroidogenic proteins StAR and ACTH-R. In contrast to our acute studies (64), there were also no significant changes in

ACTH-induced cortisol production in response to NO exposure or removal. This suggests that the effects of NO are downstream from transcription and may inhibit cortisol biosynthesis through competition at the heme-oxygen binding site or through S-nitrosylation in the CYP enzyme activation sites. We have recently found that NO induces S-nitrosylation of CYP11A1 in LTH FACs (unpublished results). Although these data are from a limited number of animals, the continuation of this work will be important in determining the mechanism of action for NO in inhibiting cortisol production as a part of the fetal adrenal adaptation to LTH.

Taken together, these studies address the potential adaptations in the role of NO and the regulation of its production via peNOS in response to LTH in the ovine fetal adrenal gland under basal and stressed conditions. In investigating the signaling pathways involved in regulating peNOS, we found that the PI3K/Akt pathway may work to maintain basal levels of cortisol through peNOS-derived NO. Stimulation of FACs with stress levels of ACTH could override this signal, possibly through activation of PP2A, to reduce peNOS and NO inhibition, allowing for the enhanced levels of cortisol produced in LTH FACs. Future studies could investigate the activity of PP2A through use of specific inhibitors or activators to see if changes in PP2A activity affect peNOS and subsequent cortisol production. This would further elucidate the adaptations in signaling mechanisms involved in regulating peNOS in the LTH fetal adrenal, and if there are any differential effects in LTH compared to normoxic. Although NO does not seem to have any effects on gene expression of key steroidogenic proteins, the inhibitory abilities of NO could be through direct actions on the proteins via S-nitrosylation or competition with O_2 for the heme-oxygen binding site. Continuation of our current studies on the

effects of NO on S-nitrosylation of CYP11A1 could provide further insight into the adaptive mechanisms responsible for the changes observed in cortisol production in the LTH fetus.

Many fetal and maternal conditions including maternal malnutrition, smoking, heart or lung disease, obesity, preeclampsia, or exposure to high altitude (3, 19, 27, 38, 47, 69) can result in varying degrees of hypoxia that can adversely affect the developing fetus and increase perinatal morbidity and mortality. In response to hypoxic stress, the fetus synthesizes cortisol from the fetal adrenal as part of the hypothalamus-pituitary-adrenal axis (HPA) (1, 6, 7, 45, 46). Due to its involvement in growth and organ maturation, as well as its effects on metabolism, regulation of cortisol is important for normal fetal development (10, 56, 66).When exposed to hypoxic conditions for an extended period of time, the fetus can adapt in order to preserve normal growth and development. We have shown that basal levels of cortisol remain similar to levels in the normoxic fetus, despite elevated levels of ACTH (67), and when challenged with a secondary stressor, the LTH fetus responds with a higher output of cortisol than the normoxic fetus (1, 45). We suspect this ability of the fetus is due to NO produced by eNOS and that the regulation of eNOS activity is altered in response to LTH.

Because NO has such potent effects on steroidogenesis and involvement in the fetal adaptation to LTH, its therapeutic use should be considered carefully. Currently, inhaled NO (iNO) is used clinically as a treatment in the neonatal intensive care unit (NICU) for premature infants with respiratory distress syndrome (75) and newborns with persistent pulmonary hypertension (18, 48, 50) to induce selective pulmonary vasodilation (32) and reduce the incidence of chronic lung disease and death. In the

newborn lamb, it has been reported that iNO reversed hypoxic pulmonary vasoconstriction (72), and increased oxygenation (86) and survival (87), and in the human, iNO rapidly increased oxygenation in infants with severe hypoxemia and pulmonary-artery hypertension, without causing systemic hypotension (50, 73). Although iNO is rapidly metabolized in the lung (49), limiting the direct effects of NO on the adrenal, the potential systemic effects through increases in oxygen could influence steroidogenesis and alter cortisol production in the LTH adapted newborn. If the newborn has adapted the HPA as a fetus to lower levels of oxygen in response to LTH, suddenly increases in systemic oxygen through the use of iNO could have negative consequences by superseding NO regulation of cortisol in the adrenal and increasing cortisol output. If high levels of cortisol are sustained, they could interfere with the HPA response to acute stressors associated with extrauterine life and be detrimental to the newborn's growth and development, as elevated cortisol is associated with hyperglycemia, immune suppression, excess adipose deposition, bone loss, and hypertension (15, 74, 79). Understanding the mechanisms of action for the regulation of NO and cortisol production are important for determining the adaptations in the fetal HPA in response to LTH, and they can provide insight into better care for at risk pregnancies. Determining the long-term effects of reduced oxygen can also inform on the potential outcomes in the LTH newborn of iNO-treatment induced increases in systemic oxygen currently being used in the NICU.

References

1. **Adachi K, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters ovine fetal endocrine and physiological responses to hypotension. *American journal of physiology Regulatory, integrative and comparative physiology* 287: R209-217, 2004.

2. **Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, Stocco DM, and Strauss JF**. Phosphorylation of Steroidogenic Acute Regulatory Protein (StAR) Modulates Its Steroidogenic Activity. *Journal of Biological Chemistry* 272: 32656-32662, 1997.

3. **Barker DJ, and Clark PM**. Fetal undernutrition and disease in later life. *Reviews of reproduction* 2: 105-112, 1997.

4. **Bernier SG, Haldar S, and Michel T**. Bradykinin-regulated interactions of the mitogen-activated protein kinase pathway with the endothelial nitric-oxide synthase. *The Journal of biological chemistry* 275: 30707-30715, 2000.

5. **Bird IM, Sullivan JA, Di T, Cale JM, Zhang L, Zheng J, and Magness RR**. Pregnancy-Dependent Changes in Cell Signaling Underlie Changes in Differential Control of Vasodilator Production in Uterine Artery Endothelial Cells. *Endocrinology* 141: 1107-1117, 2000.

6. **Bocking AD, McMillen IC, Harding R, and Thorburn GD**. Effect of reduced uterine blood flow on fetal and maternal cortisol. *Journal of developmental physiology* 8: 237-245, 1986.

7. **Boddy K, Jones CT, Mantell C, Ratcliffe JG, and Robinson JS**. Changes in plasma ACTH and corticosteroid of the maternal and fetal sheep during hypoxia. *Endocrinology* 94: 588-591, 1974.

8. **Cale JM, and Bird IM**. Inhibition of MEK/ERK1/2 signalling alters endothelial nitric oxide synthase activity in an agonist-dependent manner. *The Biochemical journal* 398: 279-288, 2006.

9. **Caulin-Glaser T, Garcia-Cardena G, Sarrel P, Sessa WC, and Bender JR**. 17 beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca2+ mobilization. *Circ Res* 81: 885-892, 1997.

10. **Challis JRG, Matthews SG, Gibb W, and Lye SJ**. Endocrine and paracrine regulation of birth at term and preterm. *Endocrine reviews* 21: 514-550, 2000.

11. **Chen DB, Bird IM, Zheng J, and Magness RR**. Membrane estrogen receptor-dependent extracellular signal-regulated kinase pathway mediates acute activation of endothelial nitric oxide synthase by estrogen in uterine artery endothelial cells. *Endocrinology* 145: 113-125, 2004.

12. **Chen JX, and Meyrick B**. Hypoxia increases Hsp90 binding to eNOS via PI3K-Akt in porcine coronary artery endothelium. *Laboratory investigation; a journal of technical methods and pathology* 84: 182-190, 2004.

13. **Chen Z-P, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, and Kemp BE**. AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Letters* 443: 285-289, 1999.

14. **Chen Z, Peng I-C, Sun W, Su M-I, Hsu P-H, Fu Y, Zhu Y, DeFea K, Pan S, Tsai M-D, and Shyy JY-J**. AMP-Activated Protein Kinase Functionally Phosphorylates Endothelial Nitric Oxide Synthase Ser633. *Circulation Research* 104: 496-505, 2009.

15. **Chrousos GP**. The role of stress and the hypothalamic-pituitary-adrenal axis in the pathogenesis of the metabolic syndrome: neuro-endocrine and target tissue-related causes. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* 24 Suppl 2: S50-55, 2000.

16. **Churchill PF, and Kimura T**. Topological studies of cytochromes P-450scc and P-45011 beta in bovine adrenocortical inner mitochondrial membranes. Effects of controlled tryptic digestion. *The Journal of biological chemistry* 254: 10443-10448, 1979.

17. **Clark BJ, Ranganathan V, and Combs R**. Steroidogenic acute regulatory protein expression is dependent upon post-translational effects of cAMP-dependent protein kinase A. *Mol Cell Endocrinol* 173: 183-192, 2001.

18. **Clark RH, Kueser TJ, Walker MW, Southgate WM, Huckaby JL, Perez JA, Roy BJ, Keszler M, and Kinsella JP**. Low-dose nitric oxide therapy for persistent pulmonary hypertension of the newborn. Clinical Inhaled Nitric Oxide Research Group. *The New England journal of medicine* 342: 469-474, 2000.

19. **Cnattingius S, Bergstrom R, Lipworth L, and Kramer MS**. Prepregnancy weight and the risk of adverse pregnancy outcomes. *The New England journal of medicine* 338: 147-152, 1998.

20. **Cymeryng CB, Dada LA, Colonna C, Mendez CF, and Podesta EJ**. Effects of L-arginine in rat adrenal cells: involvement of nitric oxide synthase. *Endocrinology* 140: 2962-2967, 1999.

21. **Cymeryng CB, Dada LA, and Podesta EJ**. Effect of nitric oxide on rat adrenal zona fasciculata steroidogenesis. *The Journal of endocrinology* 158: 197-203, 1998.

22. **Cymeryng CB, Lotito SP, Colonna C, Finkielstein C, Pomeraniec Y, Grión N, Gadda L, Maloberti P, and Podestá EJ**. Expression of Nitric Oxide Synthases in Rat Adrenal Zona Fasciculata Cells. *Endocrinology* 143: 1235-1242, 2002.

23. **Daniel PB, Walker WH, and Habener JF**. Cyclic AMP signaling and gene regulation. *Annual review of nutrition* 18: 353-383, 1998.

24. **Dewi DA, Abayasekara DR, and Wheeler-Jones CP**. Requirement for ERK1/2 activation in the regulation of progesterone production in human granulosa-lutein cells is stimulus specific. *Endocrinology* 143: 877-888, 2002.

25. **Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, and Zeiher AM**. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399: 601-605, 1999.

26. **Dobashi M, Fujisawa M, Yamazaki T, Okuda Y, Kanzaki M, Tatsumi N, Tsuji T, Okada H, and Kamidono S**. Inhibition of steroidogenesis in Leydig cells by exogenous nitric oxide occurs independently of steroidogenic acute regulatory protein (star) mRNA. *Archives of andrology* 47: 203-209, 2001.

27. **Ducsay CA**. Fetal and maternal adaptations to chronic hypoxia: prevention of premature labor in response to chronic stress. *Comparative biochemistry and physiology Part A, Molecular & integrative physiology* 119: 675-681, 1998.

28. **Dudzinski DM, and Michel T**. Life history of eNOS: Partners and pathways. *Cardiovascular research* 75: 247-260, 2007.

29. **Eum H-A, Yeom D-H, and Lee S-M**. Role of nitric oxide in the inhibition of liver cytochrome P450 during sepsis. *Nitric Oxide* 15: 423-431, 2006.

30. **Feron O, Belhassen L, Kobzik L, Smith TW, Kelly RA, and Michel T**. Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. *The Journal of biological chemistry* 271: 22810-22814, 1996.

31. **Fleming I, Fisslthaler B, Dimmeler S, Kemp BE, and Busse R**. Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ Res* 88: E68-75, 2001.

32. **Frostell C, Fratacci MD, Wain JC, Jones R, and Zapol WM**. Inhaled nitric oxide. A selective pulmonary vasodilator reversing hypoxic pulmonary vasoconstriction. *Circulation* 83: 2038-2047, 1991.

33. **Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, and Sessa WC**. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399: 597-601, 1999.

34. **Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, Figeys D, Harrison DG, Berk BC, Aebersold R, and Corson MA**. Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the

phosphatidylinositol 3-kinase inhibitor LY294002. *The Journal of biological chemistry* 274: 30101-30108, 1999.

35. **Gelinas DS, Bernatchez PN, Rollin S, Bazan NG, and Sirois MG**. Immediate and delayed VEGF-mediated NO synthesis in endothelial cells: role of PI3K, PKC and PLC pathways. *British journal of pharmacology* 137: 1021-1030, 2002.

36. **Gobbetti A, Boiti C, Canali C, and Zerani M**. Nitric oxide synthase acutely regulates progesterone production by in vitro cultured rabbit corpora lutea. *The Journal of endocrinology* 160: 275-283, 1999.

37. **Greif DM, Kou R, and Michel T**. Site-specific dephosphorylation of endothelial nitric oxide synthase by protein phosphatase 2A: evidence for crosstalk between phosphorylation sites. *Biochemistry* 41: 15845-15853, 2002.

38. **Hameed A, Karaalp IS, Tummala PP, Wani OR, Canetti M, Akhter MW, Goodwin I, Zapadinsky N, and Elkayam U**. The effect of valvular heart disease on maternal and fetal outcome of pregnancy. *Journal of the American College of Cardiology* 37: 893-899, 2001.

39. **Hanke CJ, and Campbell WB**. Endothelial cell nitric oxide inhibits aldosterone synthesis in zona glomerulosa cells: modulation by oxygen. *American Journal of Physiology - Endocrinology And Metabolism* 279: E846-E854, 2000.

40. **Hanke CJ, Drewett JG, Myers CR, and Campbell WB**. Nitric oxide inhibits aldosterone synthesis by a guanylyl cyclase-independent effect. *Endocrinology* 139: 4053-4060, 1998.

41. **Hanke CJ, O'Brien T, Pritchard KA, Jr., and Campbell WB**. Inhibition of adrenal cell aldosterone synthesis by endogenous nitric oxide release. *Hypertension* 35: 324-328, 2000.

42. **Harris MB, Blackstone MA, Sood SG, Li C, Goolsby JM, Venema VJ, Kemp BE, and Venema RC**. Acute activation and phosphorylation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors. *American journal of physiology Heart and circulatory physiology* 287: H560-566, 2004.

43. **Hirata K, Kuroda R, Sakoda T, Katayama M, Inoue N, Suematsu M, Kawashima S, and Yokoyama M**. Inhibition of endothelial nitric oxide synthase activity by protein kinase C. *Hypertension* 25: 180-185, 1995.

44. **Hough D, Cloete SW, Storbeck K, Swart AC, and Swart P**. Cortisol production in sheep is influenced by the functional expression of two cytochrome P450 17alpha-hydroxylase/17,20-lyase (CYP17) isoforms. *Journal of animal science* 91: 1193-1206, 2013.

45. **Imamura T, Umezaki H, Kaushal KM, and Ducsay CA**. Long-term hypoxia alters endocrine and physiologic responses to umbilical cord occlusion in the ovine fetus. *Journal of the Society for Gynecologic Investigation* 11: 131-140, 2004.

46. **Jones CT, Boddy K, Robinson JS, and Ratcliffe JG**. Developmental changes in the responses of the adrenal glands of foetal sheep to endogenous adrenocorticotrophin, as indicated by hormone responses to hypoxaemia. *The Journal of endocrinology* 72: 279-292, 1977.

47. **Keyes LE, Armaza JF, Niermeyer S, Vargas E, Young DA, and Moore LG**. Intrauterine growth restriction, preeclampsia, and intrauterine mortality at high altitude in Bolivia. *Pediatric research* 54: 20-25, 2003.

48. **Kinsella JP, and Abman SH**. Inhaled nitric oxide: current and future uses in neonates. *Seminars in perinatology* 24: 387-395, 2000.

49. **Kinsella JP, McQueston JA, Rosenberg AA, and Abman SH**. Hemodynamic effects of exogenous nitric oxide in ovine transitional pulmonary circulation. *The American journal of physiology* 263: H875-880, 1992.

50. **Kinsella JP, Neish SR, Shaffer E, and Abman SH**. Low-dose inhalation nitric oxide in persistent pulmonary hypertension of the newborn. *Lancet* 340: 819-820, 1992.

51. **Kostić T, Andrić S, Kovačević R, and Marić D**. The involvement of nitric oxide in stress-impaired testicular steroidogenesis. *European Journal of Pharmacology* 346: 267-273, 1998.

52. **Liu S, and Rockey DC**. Cicletanine stimulates eNOS phosphorylation and NO production via Akt and MAP kinase/Erk signaling in sinusoidal endothelial cells. *American journal of physiology Gastrointestinal and liver physiology* 305: G163-171, 2013.

53. **Martinelle N, Holst M, Soder O, and Svechnikov K**. Extracellular signal-regulated kinases are involved in the acute activation of steroidogenesis in immature rat Leydig cells by human chorionic gonadotropin. *Endocrinology* 145: 4629-4634, 2004.

54. **Masuda M, Kubota T, and Aso T**. Effects of nitric oxide on steroidogenesis in porcine granulosa cells during different stages of follicular development. *European journal of endocrinology / European Federation of Endocrine Societies* 144: 303-308, 2001.

55. **Masuda M, Kubota T, Karnada S, and Aso T**. Nitric oxide inhibits steroidogenesis in cultured porcine granulosa cells. *Mol Hum Reprod* 3: 285-292, 1997.

56. **Meaney MJ, Viau V, Bhatnagar S, Betito K, Iny LJ, O'Donnell D, and Mitchell JB**. Cellular mechanisms underlying the development and expression of individual differences in the hypothalamic-pituitary-adrenal stress response. *J Steroid Biochem Mol Biol* 39: 265-274, 1991.

57. **Michell BJ, Chen Z, Tiganis T, Stapleton D, Katsis F, Power DA, Sim AT, and Kemp BE**. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *The Journal of biological chemistry* 276: 17625-17628, 2001.

58. **Miller WL**. StAR search--what we know about how the steroidogenic acute regulatory protein mediates mitochondrial cholesterol import. *Molecular endocrinology (Baltimore, Md)* 21: 589-601, 2007.

59. **Miller WL, and Auchus RJ**. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine reviews* 32: 81-151, 2011.

60. **Mineo C, Yuhanna IS, Quon MJ, and Shaul PW**. High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases. *The Journal of biological chemistry* 278: 9142-9149, 2003.

61. **Mitsube K, Mikuni M, Matousek M, and Brannstrom M**. Effects of a nitric oxide donor and nitric oxide synthase inhibitors on luteinizing hormone-induced ovulation in the ex-vivo perfused rat ovary. *Human reproduction (Oxford, England)* 14: 2537-2543, 1999.

62. **Molinari C, Uberti F, Grossini E, Vacca G, Carda S, Invernizzi M, and Cisari C**. 1alpha,25-dihydroxycholecalciferol induces nitric oxide production in cultured endothelial cells. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 27: 661-668, 2011.

63. **Monau TR, Vargas VE, King N, Yellon SM, Myers DA, and Ducsay CA**. Long-term hypoxia increases endothelial nitric oxide synthase expression in the ovine fetal adrenal. *Reproductive sciences (Thousand Oaks, Calif)* 16: 865-874, 2009.

64. **Monau TR, Vargas VE, Zhang L, Myers DA, and Ducsay CA**. Nitric oxide inhibits ACTH-induced cortisol production in near-term, long-term hypoxic ovine fetal adrenocortical cells. *Reproductive sciences (Thousand Oaks, Calif)* 17: 955-962, 2010.

65. **Muldowney JA, 3rd, Davis SN, Vaughan DE, and Brown NJ**. NO synthase inhibition increases aldosterone in humans. *Hypertension* 44: 739-745, 2004.

66. **Munck A, Guyre PM, and Holbrook NJ**. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine reviews* 5: 25-44, 1984.

67. **Myers DA, Bell PA, Hyatt K, Mlynarczyk M, and Ducsay CA**. Long-term hypoxia enhances proopiomelanocortin processing in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 288: R1178-1184, 2005.

68. **Myers DA, Hyatt K, Mlynarczyk M, Bird IM, and Ducsay CA**. Long-term hypoxia represses the expression of key genes regulating cortisol biosynthesis in the near-term ovine fetus. *American journal of physiology Regulatory, integrative and comparative physiology* 289: R1707-1714, 2005.

69. **Nordentoft M, Lou HC, Hansen D, Nim J, Pryds O, Rubin P, and Hemmingsen R**. Intrauterine growth retardation and premature delivery: the influence of maternal smoking and psychosocial factors. *American journal of public health* 86: 347-354, 1996.

70. **Pon LA, Hartigan JA, and Orme-Johnson NR**. Acute ACTH regulation of adrenal corticosteroid biosynthesis. Rapid accumulation of a phosphoprotein. *The Journal of biological chemistry* 261: 13309-13316, 1986.

71. **Renlund N, Jo Y, Svechnikova I, Holst M, Stocco DM, Soder O, and Svechnikov K**. Induction of steroidogenesis in immature rat Leydig cells by interleukin-1alpha is dependent on extracellular signal-regulated kinases. *Journal of molecular endocrinology* 36: 327-336, 2006.

72. **Roberts JD, Jr., Chen TY, Kawai N, Wain J, Dupuy P, Shimouchi A, Bloch K, Polaner D, and Zapol WM**. Inhaled nitric oxide reverses pulmonary vasoconstriction in the hypoxic and acidotic newborn lamb. *Circ Res* 72: 246-254, 1993.

73. **Roberts JD, Polaner DM, Lang P, and Zapol WM**. Inhaled nitric oxide in persistent pulmonary hypertension of the newborn. *Lancet* 340: 818-819, 1992.

74. **Rosmond R**. Role of stress in the pathogenesis of the metabolic syndrome. *Psychoneuroendocrinology* 30: 1-10, 2005.

75. **Schreiber MD, Gin-Mestan K, Marks JD, Huo D, Lee G, and Srisuparp P**. Inhaled nitric oxide in premature infants with the respiratory distress syndrome. *The New England journal of medicine* 349: 2099-2107, 2003.

76. **Sewer MB, and Waterman MR**. Adrenocorticotropin/cyclic adenosine 3',5'-monophosphate-mediated transcription of the human CYP17 gene in the adrenal cortex is dependent on phosphatase activity. *Endocrinology* 143: 1769-1777, 2002.

77. **Stadler J, Trockfeld J, Schmalix WA, Brill T, Siewert JR, Greim H, and Doehmer J**. Inhibition of cytochromes P4501A by nitric oxide. *Proceedings of the National Academy of Sciences* 91: 3559-3563, 1994.

78. **Stocco DM**. StAR protein and the regulation of steroid hormone biosynthesis. *Annual review of physiology* 63: 193-213, 2001.

79. **Tsigos C, and Chrousos GP**. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *Journal of psychosomatic research* 53: 865-871, 2002.

80. **Tsukahara H, Gordienko DV, Tonshoff B, Gelato MC, and Goligorsky MS**. Direct demonstration of insulin-like growth factor-I-induced nitric oxide production by endothelial cells. *Kidney international* 45: 598-604, 1994.

81. **Van Voorhis BJ, Dunn MS, Snyder GD, and Weiner CP**. Nitric oxide: an autocrine regulator of human granulosa-luteal cell steroidogenesis. *Endocrinology* 135: 1799-1806, 1994.

82. **Vargas VE, Kaushal KM, Monau T, Myers DA, and Ducsay CA**. Long-term hypoxia enhances cortisol biosynthesis in near-term ovine fetal adrenal cortical cells. *Reproductive sciences (Thousand Oaks, Calif)* 18: 277-285, 2011.

83. **Vargas VE, Kaushal KM, Monau TR, Myers DA, and Ducsay CA**. Extracellular signal-regulated kinases (ERK1/2) signaling pathway plays a role in cortisol secretion in the long-term hypoxic ovine fetal adrenal near term. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 304: R636-R643, 2013.

84. **Xiao D, Bird IM, Magness RR, Longo LD, and Zhang L**. Upregulation of eNOS in pregnant ovine uterine arteries by chronic hypoxia. *American Journal of Physiology - Heart and Circulatory Physiology* 280: H812-H820, 2001.

85. **Yamauchi J, Miyazaki T, Iwasaki S, Kishi I, Kuroshima M, Tei C, and Yoshimura Y**. Effects of nitric oxide on ovulation and ovarian steroidogenesis and prostaglandin production in the rabbit. *Endocrinology* 138: 3630-3637, 1997.

86. **Zayek M, Cleveland D, and Morin FC, 3rd**. Treatment of persistent pulmonary hypertension in the newborn lamb by inhaled nitric oxide. *The Journal of pediatrics* 122: 743-750, 1993.

87. **Zayek M, Wild L, Roberts JD, and Morin FC, 3rd**. Effect of nitric oxide on the survival rate and incidence of lung injury in newborn lambs with persistent pulmonary hypertension. *The Journal of pediatrics* 123: 947-952, 1993.