Adrenocorticotrophic Hormone and PI3K/Akt Inhibition Reduce eNOS Phosphorylation and Increase Cortisol Biosynthesis in Long-Term Hypoxic Ovine Fetal Adrenal Cortical Cells

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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 adrenocorticotrophic 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.

Keywords

sheep, adrenocorticotrophic hormone, hypoxia

Introduction

Hypoxia is a potent stressor that activates the hypothalamo-pituitary-adrenal (HPA) axis and, acutely, leads to a significant increase in cortisol.¹⁻⁵ Since cortisol is involved in lipolysis, glycogenolysis, and protein catabolism, a sustained elevation in plasma cortisol concentrations can suppress anabolic processes resulting in muscle atrophy and delayed maturation and organ growth.^{6,7} 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 40 days of gestation [dGa]) moderate hypoxia (fetal $PO_2 \sim 17$ -19 vs ~ 21 -23 mm Hg normoxic controls). Under conditions of LTH, the fetus maintains normal basal plasma cortisol concentrations, despite elevated adrenocorticotrophic hormone (ACTH).⁸ Although we found no differences in cyclic adenosine monophosphate (cAMP) production or protein kinase A (PKA)

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activation either basally or in response to ACTH in LTH fetal adrenocortical cells (FACs), we did observe decreased expression of CYP11A1 and CYP17, 2 key steroidogenic P450 enzymes, as well as decreased ACTH receptor expression.⁹ 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.^{1,2} 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 which is produced from L-arginine by NO synthases (NOSs).^{10,11} 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.¹² 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,¹³ 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.¹²

A key mechanism involved in the regulation of eNOS is phosphorylation at a serine activation site. In human, it is serine residue 1177, and in the bovine/ovine it is serine residue 1179, typically referred to as Ser1177/79.¹⁴⁻¹⁶ A reduction in phosphorylation at this residue could be the result of either decreased kinase activation or active dephosphorylation.¹⁷⁻¹⁹ 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.²⁰⁻²³ Other studies have shown that the PI3K/Akt pathway also targets eNOS at Ser1177/79 in endothelial cells.^{14,21,24} The roles of MEK/ERK1/2 and 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.¹² 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,²⁵ 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 granulosa²⁶ and Leydig^{27,28} cells. This may be through effects on steroidogenic acute regulatory (StAR) protein, which transports cholesterol into the mitochondria²⁹⁻³⁴; 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 whether either the MEK/ERK1/2 or the PI3K/Akt pathways regulate eNOS phosphorylation in FACs, (2) address the role of MEK/ERK1/2 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 (3820 m, maternal $PO_2 \sim 60$ mm Hg) from approximately 40 dGa to near term (term $\cong 146$ days). Following transportation to the laboratory, hypoxia was maintained by nitrogen infusion through a maternal tracheal catheter as described previously.^{1,2,12,35,36} 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 described previously in detail.³⁷ 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 (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, California).

Cell Dispersion

Procedures for collection of FACs were similar to those we described previously.^{25,36} 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, New Jersey), 40 mg of Polypep bovine protein digest (Sigma-Aldrich), and 100 μ L of DNase I (type IV; Sigma-Aldrich) dissolved in 10 mL of Sodium Krebs Buffer (0.4% collagenase). The resulting mono-dispersed FACs were aliquoted into individual tubes with

media (M-199) and allowed to equilibrate for 2 hr 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. Fetal adrenal cortical cells from normoxic ($n = 7$) and LTH ($n = 5$) fetuses, aliquoted at 7.5×10^5 cells/mL, were untreated, pretreated with MEK/ERK1/2 inhibitor UO126 (UO, 10 μ mol/L) for 1 hr, or stimulated with ACTH (100 pmol/L), with and without UO 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 μ L of lysis buffer (93% pre-lysis 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.

Effects of UO126 pretreatment and ACTH, 22R-OHC, or WSC stimulation on cortisol. 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. Fetal adrenal cortical cells from normoxic ($n = 6$) and LTH ($n = 7$) fetuses, aliquoted at 2.5×10^5 cells/mL, were either untreated, pretreated with UO (10 μ mol/L) for 1 hour, or stimulated with ACTH (100 pmol/L) with and without UO pretreatment, treated with membrane permeable 22R-OHC (10 μ mol/L), with and without UO pretreatment, treated with WSC (10 μ mol/L), 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 were collected 60 minutes after stimulation and stored as described earlier for later cortisol analysis.

Effects of PI3K/Akt inhibition and ACTH stimulation on cortisol biosynthesis and eNOS phosphorylation. Fetal adrenal cortical cells from normoxic ($n = 7$) and LTH ($n = 9$) fetuses, aliquoted at 7.5×10^5 cells/mL, were untreated, pretreated with PI3K/Akt inhibitor Wortmannin (WT, 10 nmol/L) for 1 hour, or stimulated with ACTH (100 pmol/L), with and without WT pretreatment. Media and cells were collected at 0 (baseline), 10, 20, and 60 minutes after stimulation and stored as described earlier. We chose to use WT 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 cortisol kit (Oxford Biomedical Research, Oxford, Michigan) that has been previously described and validated for use in our laboratory.^{9,12,38}

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 phosphorylation was determined by Western blotting using methods we have previously described and validated.^{8,9} Briefly, protein samples were denatured for 5 minutes at boiling temperature and 20 μ g 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 of 5% BSA TBST solution (1:10 000) 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 (RODs) 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 of 5% NFDM TBST solution (1:20 000) 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.^{13,25}

Figure 1. Time course of cortisol production in normoxic and LTH FACs with MEK/ERK1/2 inhibition and ACTH stimulation. Stimulation with ACTH (100 pmol/L) increased cortisol production in both normoxic and LTH FACs. UO126 (10 μ mol/L) pretreatment 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 $\pm$ standard error of the means (SEMs). * $P < .05$ compared to time 0, # $P < .05$ compared to UO + ACTH. FACs indicates fetal adrenocortical cells; LTH, long-term hypoxia; UO, UO126; ACTH, adrenocorticotrophic hormone.

Statistical Analysis

Descriptive statistics are presented as mean $\pm$ 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 ($P < .05$), while a significant increase in cortisol output was observed in LTH FACs in response to ACTH by 10 minutes. Pretreatment with UO inhibited the ACTH-stimulated increase in cortisol in both normoxic and LTH FACs compared to ACTH alone ($P < .05$).

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 3 treatments resulted in a similar reduction in p-eNOS. In contrast, LTH FACs demonstrated a significant reduction in p-eNOS in the ACTH-treated groups (UO + ACTH or ACTH alone) compared to untreated FACs ($P < .05$). 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 ($P < .05$), while the effect was greater in the LTH group compared to the normoxic group ($P < .05$; 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 either normoxic or 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 ($P < .05$) in cells stimulated with ACTH in both normoxic (7.54 ± 1.37) ng/mL and LTH (8.85 ± 1.25 ng/mL) FACs. Adrenocorticotrophic hormone-stimulated 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 ($P < .05$).

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 (10 μ mol/L) with and without ACTH (100 pmol/L) stimulation had no effect on eNOS in either normoxic or LTH FACs. Pretreatment with UO and treatment with ACTH reduced on peNOS in normoxic FACs. Pretreatment with UO had no effect on peNOS in LTH FACs. Treatment with ACTH with and without UO pretreatment reduced peNOS in LTH FACs (Normoxic $n = 7$, LTH $n = 5$). Values represent mean values $\pm$ standard error of the means (SEMs). * $P < .05$ compared to control. FACs indicates 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, adrenocorticotrophic hormone.

Expression of eNOS. No differences were observed in expression of eNOS protein 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 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 ($P < .05$). This reduction was similar, with no difference observed among WT, ACTH, or WT + ACTH groups.

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.^{6,7} 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 processing to ACTH coupled with elevated basal plasma ACTH levels in LTH fetuses compared to normoxic controls.⁸ There is also a distinct upregulation of the ACTH response to AVP compared to CRH in LTH fetuses.³⁸ In contrast to the hypothalamic and pituitary responses, we showed that basal adrenal cortisol biosynthesis is normal, despite the elevated plasma ACTH.^{1,2,8,39} Surprisingly, the LTH fetus responds more robustly, with enhanced cortisol production to a secondary stressor compared to the normoxic fetus,^{1,2} suggesting an adaptation in the HPA axis that maintains normal basal levels but allows for enhanced production in response to stress.

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 (100 pmol/L), 22R-OHC (10 μ mol/L), and WSC (10 μ mol/L) 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 $\pm$ standard error of the means (SEMs). * $P < .05$ compared to untreated control, # $P < .05$ compared to normoxic. FACs indicates fetal adrenocortical cells; LTH, long-term hypoxia; C, Control; UO, UO126; ACTH, adrenocorticotrophic hormone; 22R-OHC, 22R-hydroxycholesterol; WSC, water-soluble cholesterol.

We previously reported that NO inhibited ACTH-stimulated cortisol production, while eNOS inhibition enhanced cortisol biosynthesis in LTH ovine FACs.¹² We also found that eNOS is the most abundant adrenal cortical NOS isoform and LTH enhanced not only expression of eNOS¹³ but also NOS activity compared to normoxic controls.¹² Activity of eNOS can be influenced by a number of factors including substrate availability, protein-protein interactions, and posttranslational modification via phosphorylation.⁴⁰ 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 whether 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.²⁵

In a previous study, we showed MEK/ERK1/2 inhibition reduced cortisol output in FACs in response to stimulation with ACTH.²⁵ In the present study, as in our previously published report,²⁵ we found that MEK/ERK1/2 inhibition with UO126 (UO) prevented the increase in 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 granulosa²⁶ and Leydig^{27,28} cells. These studies demonstrated that although stimulated synthesis of steroid was inhibited by UO, when cells were stimulated with 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 StAR protein. Steroidogenic acute regulatory protein, classically regulated by PKA via cAMP,³⁰ is responsible for transporting cholesterol to the inner mitochondrial membrane for conversion from cholesterol to pregnenolone by CYP11A1 (P450_{scc}),^{29,31-34} 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 WSC, both of which 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.

Figure 4. Time course of cortisol production in normoxic and LTH FACS with PI3K/Akt inhibition and ACTH stimulation. Stimulation with ACTH (100 pmol/L) increased cortisol production in both normoxic and LTH FACS. WT (10 nmol/L) pretreatment 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 $\pm$ standard error of the means (SEMs). * $P < .05$ compared to time 0, # $P < .05$ compared to ACTH alone. FACS indicates fetal adrenocortical cells; LTH, long-term hypoxia; WT, Wortmannin; ACTH, adrenocorticotrophic hormone.

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 in LTH FACS 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^{41,42} and has also been shown to reduce eNOS phosphorylation in endothelial cells followed by decreased NO production.^{14,21,24} 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). Protein phosphatase 2A has been shown to be capable of dephosphorylating eNOS, and the inhibition of PP2A increases peNOS in endothelial cells^{18,43}; however, the effects of LTH on this system are unexplored. Preliminary data from our laboratory show significantly greater expression of PP2A 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^{12,13,25,36} indicate that LTH has profound adaptive effects on the fetal adrenal cortex. Nitric oxide, 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 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 FACS 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

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 (10 nmol/L) with and without ACTH (100 pmol/L) stimulation had no effect on eNOS in both normoxic and LTH FACs. Pretreatment with WT (10 nmol/L) with and without ACTH (100 pmol/L) 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 $\pm$ standard error of the means (SEMs). * $P < .05$ compared to time 0. FACs indicates 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, adrenocorticotrophic hormone.

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.

Declaration of Conflicting Interests

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References

- Imamura T, Umezaki H, Kaushal KM, Ducsay CA. Long-term hypoxia alters endocrine and physiologic responses to umbilical cord occlusion in the ovine fetus. *J Soc Gynecol Investig.* 2004; 11(3):131-140.
- Adachi K, Umezaki H, Kaushal KM, Ducsay CA. Long-term hypoxia alters ovine fetal endocrine and physiological responses to hypotension. *Am J Physiol Regul Integr Comp Physiol.* 2004; 287(1):R209-R217.
- Bocking AD, McMillen IC, Harding R, Thorburn GD. Effect of reduced uterine blood flow on fetal and maternal cortisol. *J Dev Physiol.* 1986;8(4):237-245.
- Boddy K, Jones CT, Mantell C, Ratcliffe JG, Robinson JS. Changes in plasma ACTH and corticosteroid of the maternal and fetal sheep during hypoxia. *Endocrinology.* 1974;94(2): 588-591.
- Jones CT, Boddy K, Robinson JS, Ratcliffe JG. Developmental changes in the responses of the adrenal glands of foetal sheep to endogenous adrenocorticotrophin, as indicated by hormone responses to hypoxaemia. *J Endocrinol.* 1977;72(3):279-292.
- Meaney MJ, Viau V, Bhatnagar S, et al. Cellular mechanisms underlying the development and expression of individual

- differences in the hypothalamic–pituitary–adrenal stress response. *J Steroid Biochem Mol Biol.* 1991;39(2):265-274.
7. Munck A, Guyre PM, Holbrook NJ. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr Rev.* 1984;5(1):25-44.
 8. Myers DA, Bell PA, Hyatt K, Mlynarczyk M, Ducsay CA. Long-term hypoxia enhances proopiomelanocortin processing in the near-term ovine fetus. *Am J Physiol Regul Integr Comp Physiol.* 2005;288(5):R1178-R1184.
 9. Myers DA, Hyatt K, Mlynarczyk M, Bird IM, Ducsay CA. Long-term hypoxia represses the expression of key genes regulating cortisol biosynthesis in the near-term ovine fetus. *Am J Physiol Regul Integr Comp Physiol.* 2005;289(6):R1707-R1714.
 10. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev.* 1991; 43(2):109-142.
 11. Ignarro LJ. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *J Physiol Pharmacol.* 2002; 53(4 pt 1):503-514.
 12. Monau TR, Vargas VE, Zhang L, Myers DA, Ducsay CA. Nitric oxide inhibits ACTH-induced cortisol production in near-term, long-term hypoxic ovine fetal adrenocortical cells. *Reprod Sci.* 2010;17(10):955-962.
 13. Monau TR, Vargas VE, King N, Yellon SM, Myers DA, Ducsay CA. Long-term hypoxia increases endothelial nitric oxide synthase expression in the ovine fetal adrenal. *Reprod Sci.* 2009;16(9):865-874.
 14. Fulton D, Gratton JP, McCabe TJ, et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature.* 1999;399(6736):597-601.
 15. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature.* 1999;399(6736): 601-605.
 16. Gallis B, Corthals GL, Goodlett DR, et al. 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. *J Biol Chem.* 1999;274(42):30101-30108.
 17. Chen Z, Peng I-C, Sun W, et al. Amp-activated protein kinase functionally phosphorylates endothelial nitric oxide synthase ser633. *Circ Res.* 2009;104(4):496-505.
 18. Greif DM, Kou R, Michel T. Site-specific dephosphorylation of endothelial nitric oxide synthase by protein phosphatase 2a: evidence for crosstalk between phosphorylation sites. *Biochemistry.* 2002;41(52):15845-15853.
 19. Cale JM, Bird IM. Inhibition of mek/erk1/2 signalling alters endothelial nitric oxide synthase activity in an agonist-dependent manner. *Biochem J.* 2006;398(2):279-288.
 20. Chen DB, Bird IM, Zheng J, 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.* 2004;145(1):113-125.
 21. Mineo C, Yuhanna IS, Quon MJ, Shaul PW. High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and map kinases. *J Biol Chem.* 2003; 278(11):9142-9149.
 22. Molinari C, Uberti F, Grossini E, et al. 1 α ,25-dihydroxycholecalciferol induces nitric oxide production in cultured endothelial cells. *Cell Physiol Biochem.* 2011;27(6): 661-668.
 23. Liu S, Rockey DC. Cicletanine stimulates ENOS phosphorylation and no production via Akt and MAP kinase/ERK signaling in sinusoidal endothelial cells. *Am J Physiol Gastrointest Liver Physiol.* 2013;305(2):G163-G171.
 24. Harris MB, Blackstone MA, Sood SG, et al. Acute activation and phosphorylation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors. *Am J Physiol Heart Circ Physiol.* 2004;287(2):H560-H566.
 25. Vargas VE, Kaushal KM, Monau TR, Myers DA, 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. *Am J Physiol Regul Integr Comp Physiol.* 2013;304(8):R636-R643.
 26. Dewi DA, Abayasekara DR, Wheeler-Jones CP. Requirement for ERK1/2 activation in the regulation of progesterone production in human granulosa-lutein cells is stimulus specific. *Endocrinology.* 2002;143(3):877-888.
 27. Martinelle N, Holst M, Soder O, Svechnikov K. Extracellular signal-regulated kinases are involved in the acute activation of steroidogenesis in immature rat Leydig cells by human chorionic gonadotropin. *Endocrinology.* 2004;145(10):4629-4634.
 28. Renlund N, Jo Y, Svechnikova I, et al. Induction of steroidogenesis in immature rat Leydig cells by interleukin-1 α is dependent on extracellular signal-regulated kinases. *J Mol Endocrinol.* 2006;36(2):327-336.
 29. Daniel PB, Walker WH, Habener JF. Cyclic AMP signaling and gene regulation. *Annu Rev Nutr.* 1998;18:353-383.
 30. Richards JS. New signaling pathways for hormones and cyclic adenosine 3',5'-monophosphate action in endocrine cells. *Mol Endocrinol.* 2001;15(2):209-218.
 31. Kamenetsky M, Middelhaufe S, Bank EM, Levin LR, Buck J, Steegborn C. Molecular details of camp generation in mammalian cells: a tale of two systems. *J Mol Biol.* 2006;362(4):623-639.
 32. Manna PR, Dyson MT, Eubank DW, et al. Regulation of steroidogenesis and the steroidogenic acute regulatory protein by a member of the camp response-element binding protein family. *Mol Endocrinol.* 2002;16(1):184-199.
 33. Sewer MB, Waterman MR. ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. *Microsc Res Tech.* 2003;61(3):300-307.
 34. Stocco DM. Star protein and the regulation of steroid hormone biosynthesis. *Annu Rev Physiol.* 2001;63:193-213.
 35. Ducsay CA, Hyatt K, Mlynarczyk M, Root BK, Kaushal KM, Myers DA. Long-term hypoxia modulates expression of key genes regulating adrenomedullary function in the late gestation ovine fetus. *Am J Physiol Regul Integr Comp Physiol.* 2007; 293(5):R1997-R2005.
 36. Vargas VE, Kaushal KM, Monau T, Myers DA, Ducsay CA. Long-term hypoxia enhances cortisol biosynthesis in near-term ovine fetal adrenal cortical cells. *Reprod Sci.* 2011;18(3):277-285.

37. Mlynarczyk M, Imamura T, Umezaki H, Kaushal KM, Zhang L, Ducsay CA. Long-term hypoxia changes myometrial responsiveness and oxytocin receptors in the pregnant ewe: differential effects on longitudinal versus circular smooth muscle. *Biol Reprod.* 2003;69(5):1500-1505.
38. Ducsay CA, Mlynarczyk M, Kaushal KM, Hyatt K, Hanson K, Myers DA. Long-term hypoxia enhances ACTH response to arginine vasopressin but not corticotropin-releasing hormone in the near-term ovine fetus. *Am J Physiol Regul Integr Comp Physiol.* 2009;297(3):R892-R899.
39. Ducsay CA. Fetal and maternal adaptations to chronic hypoxia: prevention of premature labor in response to chronic stress. *Comp Biochem Physiol A Mol Integr Physiol.* 1998;119(3):675-681.
40. Chen JX, Meyrick B. Hypoxia increases HSP90 binding to ENOS via PI3K-Akt in porcine coronary artery endothelium. *Lab Invest.* 2004;84(2):182-190.
41. Light A, Hammes SR. Membrane receptor cross talk in steroidogenesis: recent insights and clinical implications. *Steroids.* 2013;78(6):633-638.
42. Paul S, Pramanick K, Kundu S, Roy Moulik S, Pal P, Mukherjee D. Involvement of PI3 kinase and map kinase in IGF-I and insulin-induced ovarian steroidogenesis in common carp cyprinus carpio. *Gen Comp Endocrinol.* 2013;181:98-106.
43. Michell BJ, Chen Z, Tiganis T, et al. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase c and the cAMP-dependent protein kinase. *J Biol Chem.* 2001;276(21):17625-17628.