Leptin Alters Adrenal Responsiveness by Decreasing Expression of ACTH-R, StAR, and P450c21 in Hypoxemic Fetal Sheep

Reproductive Sciences
19(10) 1075-1084
© The Author(s) 2012
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1933719112442246
<http://rs.sagepub.com>

Yixin Su, MD^{1,2}, Luke C. Carey, PhD^{1,2},
James C. Rose, PhD^{1,2,3}, and Victor M. Pulgar, PhD^{1,2}

Abstract

The late gestation increase in adrenal responsiveness to adrenocorticotropin (ACTH) is dependent upon the upregulation of the ACTH receptor (ACTH-R), steroidogenic acute regulatory protein (StAR), and steroidogenic enzymes in the fetal adrenal. Long-term hypoxia decreases the expression of these and adrenal responsiveness to ACTH in vivo. Leptin, an adipocyte-derived hormone which attenuates the peripartum increase in fetal plasma cortisol is elevated in hypoxic fetuses. Therefore, we hypothesized that increases in plasma leptin will inhibit the expression of the ACTH-R, StAR, and steroidogenic enzymes and attenuate adrenal responsiveness in hypoxic fetuses. Spontaneously hypoxemic fetal sheep (132 days of gestation, PO₂ ~ 15 mm Hg) were infused with recombinant human leptin (n = 8) or saline (n = 7) for 96 hours. An ACTH challenge was performed at 72 hours of infusion to assess adrenal responsiveness. Plasma cortisol and ACTH were measured daily and adrenals were collected after 96 hours infusion for messenger RNA (mRNA) and protein expression measurement. Plasma cortisol concentrations were lower in leptin-compared with saline-infused fetuses (14.8 ± 3.2 vs 42.3 ± 9.6 ng/mL, $P < .05$), as was the cortisol:ACTH ratio (0.9 ± 0.074 vs 46 ± 1.49 , $P < .05$). Increases in cortisol concentrations were blunted in the leptin-treated group after ACTH₁₋₂₄ challenge ($F = 12.2$, $P < .0001$). Adrenal ACTH-R, StAR, and P450c21 expression levels were reduced in leptin-treated fetuses ($P < .05$), whereas the expression of Ob-Ra and Ob-Rb leptin receptor isoforms remained unchanged. Our results indicate that leptin blunts adrenal responsiveness in the late gestation hypoxemic fetus, and this effect appears mediated by decreased adrenal ACTH-R, StAR, and P450c21 expression.

Keywords

leptin, adrenal responsiveness, ACTH-R, StAR

Introduction

In fetal sheep, the prepartum increase in circulating cortisol is required for the differentiation and maturation of key fetal organs such as the fetal lung, liver, kidney, and brain and for the normal timing of parturition and the successful transition to extrauterine life.¹ This increase is dependent upon an increase in fetal adrenal responsiveness to adrenocorticotropin (ACTH). Many factors are presumably involved in regulating this increase in responsiveness; leptin may be one of these factors.

Leptin is well known to influence food intake and energy expenditure,² and increasing evidence suggests that leptin also influences hypothalamic–pituitary–adrenal (HPA) axis activity.³ For instance, chronic administration of leptin to *ob/ob* mice decreases plasma corticosterone levels.⁴ In addition, leptin inhibits the release of corticotropin releasing hormone (CRH) from the hypothalamus in vitro and blunts plasma ACTH and corticosterone responses to restraint in vivo stress in adult rodents.⁵

Gestation-related changes in plasma leptin and effects on the HPA axis have also been reported. Specifically, plasma

leptin concentrations have been found to increase in the late gestation fetus⁶ along with the expression of leptin in perirenal adipocytes from fetal sheep.⁷ Furthermore, it has been shown that the late gestation increases in plasma ACTH and cortisol in the fetal sheep are suppressed by intracerebroventricular or intravenous infusions of leptin.^{6,8} The mechanisms underlying the suppressive effects of leptin have not been established.

Among the elements important for the prepartum increase in fetal adrenal steroid production are increases in the expression

¹ Departments of Obstetrics and Gynecology, Wake Forest School of Medicine, Winston-Salem, NC, USA

² Center for Research in Obstetrics and Gynecology, Wake Forest School of Medicine, Winston-Salem, NC, USA

³ Departments of Physiology and Pharmacology, Wake Forest School of Medicine, Winston-Salem, NC, USA

Corresponding Author:

James C. Rose, Department of Obstetrics and Gynecology, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA

Email: jimrose@wakehealth.edu

of the ACTH receptor (ACTH-R) and steroidogenic acute regulatory protein (StAR). The ACTH-R is a 7-transmembrane-spanning G-protein-coupled receptor located in the zona fasciculata of the adrenal gland, whereas StAR transports the steroid precursor cholesterol from the outer to the inner mitochondrial membrane for further processing. We and others have reported that responsiveness of the fetal sheep adrenal gland to ACTH and the expression of adrenal ACTH-R, StAR, P450_{scc} (cytochrome p450 side-chain cleavage), and P450_{c21} (steroid 21-hydroxylase) increase in late pregnancy, resulting in increased glucocorticoid production. We have also shown that if the peripartum increases in ACTH-R and StAR expression are blocked, adrenal responsiveness is inhibited.⁹⁻¹¹

Evidence of regulation of fetal endocrine responses by hypoxemia has been obtained in an ovine mild long-term hypoxemia model (MLTH) among others. Mild long-term hypoxemia model fetuses display relatively normal¹² or slightly elevated¹³ basal ACTH and normal basal cortisol levels but have a blunted cortisol response to an ACTH challenge¹²; this is likely the result of the lower expression of ACTH-R, P450_{scc}, and P450_{c17} (17 α -hydroxylase/17,20-lyase) steroidogenic enzymes observed in MLTH fetuses.¹⁴ Chronic hypoxemia also alters the leptin system with a reported increase in plasma leptin and the adrenal leptin receptor Ob-Rb messenger RNA (mRNA) in MLTH.¹⁵

Given the upregulation of adrenal leptin receptors by hypoxemia and the importance of ACTH-R, StAR, and steroidogenic enzymes in mediating adrenal responses, we hypothesized that increased leptin levels would reduce ACTH-R, StAR, P450_{scc}, and P450_{c21} expression and suppress adrenal responses to ACTH in the late gestation mildly hypoxemic fetal sheep. To test this hypothesis, we infused late gestation, mildly hypoxemic fetal sheep with leptin and examined the effects of leptin infusion on (1) adrenal responsiveness by giving an ACTH challenge; (2) basal levels of plasma cortisol and ACTH concentrations; and (3) ACTH-R, StAR, P450_{scc}, and P450_{c21} expression. We also measured leptin receptor Ob-Ra and Ob-Rb isoform expression in the fetal adrenal to determine whether leptin downregulates its receptor. Our results suggest that the mechanism by which leptin impairs fetal adrenal responsiveness to ACTH is related to a suppression of the ACTH-R, StAR, and P450_{c21} expression.

Materials and Methods

Animals and Surgery

A total of 15 pregnant ewes (obtained from local breeders) were used in this study. Sheep were housed in straw-lined pens with ad libitum access to food and water. Ewes were fasted 24 hours prior to surgery which was performed under general anesthesia. Aseptic techniques were used to insert fetal femoral arterial and venous and amniotic catheters. Bolus gentamicin (80 mg; Abbott Laboratories, North Chicago, Illinois) and ampicillin (500 mg; American Pharmaceutical Partners, Schaumburg, Illinois) in isotonic saline were administered to

the ewe for 2 days after surgery. All procedures used in this study were approved by the Animal Care and Use Committee at Wake Forest University School of Medicine.

Leptin Infusion

Approximately 5 days after surgery (at 132 days of gestational age), fetuses were intravenously infused with recombinant human leptin (R&D Systems, Abingdon, UK) in saline at a dose intended to deliver approximately 20 μ g/kg per hour over 96 hours. This dose was selected based on results from a previous study showing that 20 μ g/h leptin affects ovarian steroidogenesis in sheep.¹⁶ The use of human leptin is supported by evidence of its bioactivity in sheep. Human and ovine leptin are highly conserved and no differences have been reported in their biological activity.^{17,18} Human leptin has been utilized in studies of ovarian function in sheep,^{16,19} and we have recently shown that human leptin inhibits directly adrenocortical steroidogenesis in ovine adrenocortical cells from adult sheep.²⁰

Control fetuses were infused with saline only. On the first day leptin infusion started at 0900 hours, blood samples were collected before infusion started, at 1000, and 1400 hours. On each subsequent day, the blood samples were collected daily at 0900 hours for measurement of plasma ACTH, cortisol, and leptin concentrations. Blood gases and pH (ABL5 blood gas analyzer; Radiometer, Copenhagen, Denmark) were monitored daily during the experiment.

At 72 hours of infusion, after the daily blood sample, a bolus of ACTH was administered (ACTH₁₋₂₄, 0.5 mL, 100 ng/kg body weight; Cortrosyn, Organon Inc, West Orange, New Jersey) to assess adrenal responsiveness, and blood samples were taken at 15 minutes, 30 minutes, 1 hour, 6 hours, and 24 hours thereafter. The dose of 100 ng/kg has been demonstrated to maximally elevate cortisol concentrations in fetuses of a similar gestational age.²¹

After 96 hours (24 hours after ACTH bolus) of continuous leptin or saline infusion, ewes and fetuses were killed with an overdose of intravenous sodium pentobarbitone (85 mg/kg), and fetal adrenals were removed and snap frozen for later ACTH-R, StAR, and leptin receptor mRNA and protein analysis.

Adrenocorticotropin and Cortisol Radioimmunoassay

Fetal plasma immunoreactive ACTH concentrations were measured using a radioimmunoassay kit (MP Biomedical LLC, Orangeburg, New York) according to the manufacturer's instructions. The sensitivity of measurement was 5.7 pg/mL. The intra- and inter-assay coefficients of variation were 7.1% and 9.5%, respectively. According to the manufacturer, the cross-reactivity of the rabbit anti-human ACTH₁₋₃₉ is 100% with ACTH₁₋₂₄, <1% with β -endorphin, α -melanocyte-stimulating hormone, α -lipotrophin, and β -lipotrophin. The sensitivity of the assay was 9 pg/mL. The intra-assay coefficient of variation was < 7.4%.

Plasma cortisol concentrations were measured using the ImmuChen ¹²⁵I Cortisol Radioimmunoassay Kit (MP Biomedicals) according to the manufacturer's instructions. The sensitivity

Table 1. Primers and Probes Used in Real-Time PCR Assays^a

Gene	Forward Primer	Reverse Primer	Probe Sequence
ACTH-R	GTATGAAAACATCAACAGTACAGCAAGAA	AAAACTCCGACAATGGATACTGTGA	FAM-CTGCTGTGATTTTGCC-MGB
StAR	GCCCCACCTGCATGGT	GAGTTTGGTCCTTGAGGGACTTC	FAM-CCGCCCCCTGGCTG-MGB
P450scc	GCAGAGATACCTGAAAGTGA	GGCATAGATGGCCACTTGCA	FAM-TTCCTGCCAAGACACTGT-MGB
P450c21	CGGTGGCCTTCTACTTCAC	TCTCGATCCAACCTCCTGAA	FAM-CACCCCGAGATTCACT-MGB
Ob-Ra	CCCCGAGGAAAGTTCACCTATG	TGGTGGCACTCTTGCTCATT	FAM-ACGCAGTGTACTGCTGC-MGB
Ob-Rb	GGAGACAGCCCTCTGTTAAATATGC	TGAGCTGTTTATAAGCCCTTGCT	FAM-CCTCCTCGGCTTCACC-MGB
GAPDH	GCATCGTGGAGGGACTTATGAC	GGCCATCCACAGTCTTCTG	FAM-ACGCCATCACTGCCACCT-MGB

Abbreviations: ACTH-R, adrenocorticotrophic hormone receptor; PCR, polymerase chain reaction; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450, family 11, subfamily A, polypeptide 1; P450c21, cytochrome P450 21-hydroxylase; Ob-Ra, leptin receptor short form; Ob-Rb, leptin receptor long form; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

^a Nucleotide sequences are 5'-3'.

of measurement was 0.7 ng/mL. The intra- and inter-assay coefficients of variation were 6.8% and 9.5%, respectively.

Leptin Enzyme-Linked Immunosorbent Assay

Plasma leptin concentrations were measured with a commercial enzyme-linked immunosorbent assay ([ELISA] kit; Multi-species Leptin Assay, Linco Research, St Charles, Missouri) using recombinant ovine leptin standards. Calibration curves made using recombinant ovine leptin standards and the recombinant human leptin standards (provided with the kit) were similar. This kit has previously been reported to detect ovine leptin,¹⁵ and no differences have been observed in biological activity between ovine and human leptin.^{22,23} The sensitivity of measurement was 1 ng/mL of recombinant ovine standard. The intra- and interassay coefficients of variation were 8.7% and 8.1%, respectively.

Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction

The relative abundance of ACTH-R, StAR, P450scc, P450c21, and Ob-Ra and Ob-Rb mRNA transcripts in fetal adrenal cortex were measured by quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) using TaqMan PCR. Primers of PCR were designed from the sheep sequences with Genbank accession numbers: AF116874 (*MC2R* gene for ACTH-R), AF290202 (*STAR* gene for StAR), S65754 (*CYP11A1* gene for P450scc), M92837 (*CYP21D* gene for P450c21), AY278244 (Ob-Ra gene for leptin receptor short form), and U62124 (Ob-Rb gene for leptin receptor long form), respectively. Glyceraldehyde 3-phosphate dehydrogenase (Genbank accession number U94889) was used as control. Total RNA of 1 µg was reverse transcribed in a 20 µL reaction mixture using an ABI High Capacity complementary DNA (cDNA) Archive kit, according to the manufacturer's instructions (Applied Biosystems, Foster City, California). The reaction contained 1× RT buffer, 100 µmol/L of each deoxynucleoside triphosphate, 1× random primer, and 100 U of reverse transcriptase. The reaction was carried out at 25°C for 10 minutes then at 37°C for 2 hours.

Control reactions were those in which the RT enzyme or the target RNA was omitted from the reaction.

Taqman PCR was performed on the cDNA samples using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). For each gene tested (see Table 1), PCR was carried out in multiplex mode, with every 20 µL reaction containing 2 µL of cDNA reaction, 1× Taqman universal PCR master mix, 250 nmol/L of a gene-specific primer, 250 nmol/L of FAM (fluorescein amidite)-labeled fluorogenic Taqman probe, and 2.5 U of TaqMan enzymes. The thermal cycling conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. An increase in fluorescence was obtained at the annealing and extension step at 60°C.

The relative level of expression of each gene in the samples was determined using the relative $2^{-\Delta\Delta C_t}$ expression method as previously described.²⁴ After the linear range of amplification (threshold cycle, C_t) was determined for the genes of interest, it was normalized against an endogenous GAPDH control and then against the untreated control sample, which served as the calibrator. The value of the relative level of expression for the gene of interest represents 2 independent reactions performed in triplicate.

Isolation of Adrenal Membrane and Mitochondrial Protein

To isolate cell membranes, adrenal cortex tissue was homogenized in cold 50 mmol/L Tris HCL (pH 7.5), 0.1 mmol/L EDTA, 0.5 mmol/L DTT, and protease inhibitor cocktail (1:200 diluted; Sigma, St Louis, Missouri). Homogenates were centrifuged for 8 minutes at 2000g at 4°C. The resultant supernatant was centrifuged for 20 minutes at 10 000g at 4°C. Finally the supernatant was centrifuged for a further 1 hour at 100 000g at 4°C to pellet the cellular membrane.

To isolate mitochondria, adrenal cortex tissue was first homogenized in cold sucrose buffer containing 50 mmol/L Tris HCL, 0.25 mol/L sucrose, 0.1 mmol/L EDTA (pH 7.4), and protease inhibitor cocktail (1:200 diluted: P8340, Sigma). Adrenal homogenates were centrifuged at 600g for 30 minutes at 4°C. The resultant supernatant was centrifuged at 9000g for 30 minutes at 4°C to pellet the mitochondria.

Adrenocorticotropin Receptor, StAR, and Ob-R Western Blot Analysis

Protein samples (50 μ g of either cellular membranes for ACTH-R and Ob-R or mitochondrial for StAR) were loaded and separated on 12% SDS-polyacrylamide gel. Proteins were transferred onto a polyvinylidene fluoride membrane and blocked with 5% nonfat dry milk in 0.05% Tween 20 in Tris-buffered saline for 60 minutes at room temperature before incubation with the rabbit polyclonal antibodies against ACTH-R, StAR, or Ob-R (Santa Cruz Biotechnology, Santa Cruz, California), immunoreactive proteins were visualized using the enhanced chemiluminescence method as described by the manufacturer and exposed to Amersham Hyperfilm ECL.

Statistical Analysis

All numerical data are presented as mean $\pm$ standard error of the mean. Differences between means were evaluated using the unpaired Student *t* test or analysis of variance (ANOVA) with Newman-Keuls multiple test, as appropriate. Significance was set at the .05 level.

Results

Fetal body weights at postmortem were 3956 ± 30 g ($n = 8$) in the leptin-infused fetuses and 3816 ± 40 g ($n = 7$) in the control fetuses. There was no difference in total adrenal weights between leptin-infused (0.46 ± 0.04 g) and control (0.49 ± 0.04 g) fetuses. Fetal blood gases were similar between the groups at each time point. Before infusion, the pH, PCO₂, and PO₂ values were, respectively, 7.37 ± 0.03 , 44.2 ± 1.4 mm Hg, and 16.4 ± 1.1 mm Hg in leptin-infused fetuses and 7.32 ± 0.04 , 46.6 ± 3.0 mm Hg, and 14.1 ± 1.9 mm Hg in control fetuses. After 96 hours of infusion, the pH, PCO₂, and PO₂ values were, respectively, 7.37 ± 0.02 , 48 ± 0.5 mm Hg, and 15.4 ± 1.07 mm Hg in leptin-infused fetuses and 7.4 ± 0.02 , 47.9 ± 0.7 mm Hg, and 14.0 ± 1.1 mm Hg in control fetuses.

Plasma Leptin Concentrations

There were significant effects of treatment ($F = 159.1$ $P < .0001$), time ($F = 11.11$, $P < .0001$), and interaction ($F = 11.68$, $P < .0001$) for plasma leptin concentrations (Figure 1). Plasma leptin concentrations were significantly higher than preinfusion concentrations by the second day of leptin infusion ($P < .05$) vs control fetuses and remained significantly elevated for the duration of the experiment.

Plasma ACTH and Cortisol Concentrations

For plasma ACTH concentrations, there were no within- or between-group differences during the 96 hours of infusion period (Figure 2A). In contrast, for plasma cortisol concentrations, there were effects of time ($F = 5.21$, $P < .001$) and a

Figure 1. Plasma Leptin levels in fetuses receiving an infusion of vehicle (O, $n = 7$) or Leptin (●, $n = 8$) for 96 hours. Asterisks denote significant differences ($P < .05$) between saline- and leptin-infused groups.

Figure 2. Plasma ACTH (A), Cortisol (B), and ratio of Cortisol:ACTH (C) in fetuses receiving an infusion of vehicle (O, $n = 7$) or leptin (●, $n = 8$) for 96 hours. Asterisks denote significant differences ($P < .05$) between saline- and leptin-infused groups. ACTH indicates adrenocorticotropin.

time and treatment interaction ($F = 4.56$, $P < .003$) between the groups (Figure 2B). The ratio of plasma cortisol:ACTH concentration was significantly lower in leptin-infused group compared with saline-infused group; there were effects

Figure 3. Plasma ACTH (A), Cortisol (B), and ratio of Cortisol:ACTH (C) before and after ACTH₁₋₂₄ challenge in fetuses receiving an infusion of vehicle (O, n = 7) or leptin (●, n = 8) for 96 hours. Asterisks denote significant differences ($P < .05$) between saline- and leptin-infused groups.

of treatment ($F = 11.1$, $P < .001$) and time ($F = 2.44$, $P = .05$; Figure 2C).

Adrenal Response to ACTH₁₋₂₄

There was a tendency for plasma ACTH concentrations to increase in leptin-infused or control fetuses after the ACTH₁₋₂₄ challenge, but the change was not significant ($F = 1.9$, $P = .1$; Figure 3A). Cortisol concentrations increased significantly ($F = 12.2$, $P < .0001$). This increase was greater in the control group than in the leptin infusion group ($F = 12.4$, $P = .0008$; Figure 3B). The ratio of cortisol:ACTH concentration was significantly higher ($F = 14.9$, $P = .0003$) in saline-infused group at 30 minutes after ACTH challenge compared with leptin-infused group (Figure 3C).

Leptin Receptor Expression

There were no statistically significant differences in leptin receptor short (Ob-Ra) or long (Ob-Rb) isoforms in both

mRNA (Figure 4 A, B) and protein levels (Figure 4 C, D) in adrenal glands of control and leptin-infused animals.

P450_{scc} and P450_{c21} mRNA Expression

P450_{c21} mRNA expression levels were significantly lower in leptin-infused fetuses compared with control fetuses ($P < .05$; Figure 5) while P450_{scc} tended to be reduced ($P > .05$).

ACTH-R and StAR Protein Expression

Adrenal cortical ACTH-R (Figure 6) and StAR (Figure 7) mRNA and protein expression levels were significantly lower in leptin-infused fetuses compared with control fetuses ($P < .05$).

Discussion

This study was designed to determine whether increasing plasma leptin levels in the spontaneously hypoxemic fetus attenuates adrenal responsiveness to ACTH and the mechanisms potentially responsible for any attenuation.

We found that leptin, infused into the hypoxemic late gestation sheep fetus blunts adrenal cortisol responsiveness to ACTH and inhibits adrenal expression of ACTH-R, StAR, and P450_{c21}. These results suggest that the leptin-related inhibition of the fetal cortisol response to ACTH is a consequence of decreased ACTH-R, StAR, and steroidogenic enzyme P450_{c21} expression in the fetal adrenal gland.

With an average PO₂ of 15 mm Hg, animals in both of our experimental groups were spontaneously hypoxemic. The effects of hypoxemia on the HPA axis, and more specifically on adrenocortical responses, have been shown dependent on the duration and intensity of the insult; fetal hypoxemia does not change^{25,12} or activates the HPA axis.²⁶⁻³³ In our study, the levels of ACTH and cortisol prior to any manipulation were not different between groups and were similar to the levels we and others have reported previously in normoxic fetuses.^{34,13} Thus, the mild, spontaneous hypoxemia in our animals was insufficient to induce activation of baseline cortisol secretion. However, the response to ACTH in the mildly hypoxic, saline-infused animals in our present study is substantially less than the responses to ACTH in similar aged normoxic fetuses we reported previously³⁴ (23 ± 3 ng/mL present study vs 45 ± 6 ng/mL earlier study; $P < .01$), consistent with the observations in MLTH fetuses.¹² It has been suggested that this loss of responsiveness is related to an increase in fetal plasma leptin and leptin receptors in the adrenal.¹⁵ The spontaneous hypoxemia in our animals induces a moderate increase in plasma leptin (~ 1.5 ng/mL) compared to values observed in normoxic animals¹⁵ which agree with the above suggestion.

The effects of MLTH on adrenal responsiveness appear to involve alterations in adrenocortical steroidogenic capacity including reduced expression of the ACTH receptor and the steroidogenic enzymes P450_{c17} and P450_{scc} with no detectable changes in P450_{c21} or StAR expression.¹⁴ In our study,

Figure 4. Effect of leptin infusion on leptin receptor short (Ob-Ra) or long (Ob-Rb) isoforms in both mRNA (A, B) and protein levels (C, D) in adrenal glands of saline- and leptin-infused animals ($P > .05$). ACTH indicates adrenocorticotropin; mRNA, messenger RNA.

leptin infusion downregulated the expression of ACTH-R. In addition, StAR mRNA and protein and also P450c21 mRNA were reduced which suggests that supraphysiological plasma leptin concentrations affect some different points in the adrenal glucocorticoid biosynthetic pathway than those influenced by the smaller increases produced by hypoxemia.¹⁴ Overall these findings suggest a diminished responsiveness to ACTH (decreased ACTH receptor expression) coupled with a decreased capacity of the hypoxemic leptin-treated fetal adrenal to synthesize cortisol (lower StAR and P450c21). Reduced expression of ACTH-R and StAR protein would induce a reduction in the cholesterol levels available to the mitochondrial P450scc enzyme, whereas reduced expression of P450c21 would interfere with the steps from 17α -hydroxyprogesterone to 11-deoxycortisol and progesterone to 11-deoxycorticosterone essential for cortisol and aldosterone production. Acting in concert, these changes could easily attenuate cortisol responses to ACTH.

The other result of the leptin induced changes in the adrenal was an inhibition in the peripartum increase in fetal plasma cortisol. This has been reported previously in normoxic fetuses when the plasma leptin levels were raised to concentrations similar to what our infusions achieved⁶; however, it contrasts with an earlier study in which lower doses of leptin did not alter fetal plasma cortisol levels.³⁵ Our protocol of infusion produced steady-state plasma leptin concentrations higher than those reported by Forhead et al.³⁶ These levels of plasma leptin may be necessary to suppress basal cortisol concentrations.

It appears that leptin can modulate HPA axis activity at both central and peripheral levels. Leptin receptors have been

Figure 5. Effect of leptin infusion on P450c21 (A) and P450scc (B) mRNA expression. P450c21 mRNA was significantly reduced in the Leptin-infused fetuses (■, $n = 7$) compared with saline-infused (□, $n = 8$). Asterisks denote significant differences ($P < .05$) between saline- and leptin-infused groups. ACTH indicates adrenocorticotropin; mRNA, messenger RNA.

identified in the hypothalamus³⁶⁻³⁹ and leptin has also been implicated in the modulation of neurotransmitter levels. Diabetic rats treated with leptin display normalization in serum

Figure 6. Effect of Leptin infusion on ACTH-R mRNA (A) and protein (B) expression. ACTH-R mRNA and protein were significantly reduced in the Leptin-infused fetuses (■, $n = 7$) compared with saline-infused (□, $n = 8$). Asterisks denote significant differences ($P < .05$) between saline- and leptin-infused groups. ACTH indicates adrenocorticotropin; mRNA, messenger RNA.

corticosterone and a concomitant reduction in norepinephrine (NE) levels in the PVN (paraventricular nucleus).⁴⁰ The stimulation of CRH transcription by direct administration of NE into the PVN⁴¹ and the dramatic suppression of the stress response in animals with lesions of the noradrenergic innervations to the hypothalamus⁴² together with the reduction in NE levels caused by leptin treatment suggest a NE-mediated central role for leptin in the HPA axis modulation. The ACTH levels in turn are known to regulate adrenal steroidogenesis. Specifically, previous in vitro and in vivo studies showed that ACTH positively regulates adrenal responsiveness, upregulating ACTH-R and StAR mRNA expression in the fetal sheep adrenal.^{9,43-48}

In the present study, we did not detect a significant increase in immunoreactive ACTH 15 minutes after a bolus injection. Since the antibody used recognized the ACTH amino acid sequence 5 to 18 on the human ACTH molecule, it is plausible that binding to additional ACTH peptides makes it difficult to discriminate the increase in ACTH after ACTH₁₋₂₄ injection. Perhaps a more likely explanation relates to the half-life of the peptide. The half-life reported for injected ACTH in fetal sheep is around 1 minute,⁴⁹ probably making any significant increase in ACTH difficult to detect in our first sample taken about 15 half-lives after the injection.

Leptin exerts its function by binding to the leptin receptor (Ob-R) and several splice variants of Ob-R (Ob-Ra through

Figure 7. Effect of leptin infusion on StAR mRNA (A) and protein (B) expression. StAR mRNA and protein were significantly reduced in the leptin-infused fetuses (■, $n = 7$) compared with saline-infused (□, $n = 8$). Asterisks denote significant differences ($P < .05$) between saline- and leptin-infused groups. StAR indicates steroidogenic acute regulatory protein; mRNA, messenger RNA.

Ob-Rf) have been detected in mouse, human, and rat; these isoforms differ in the length of their cytoplasmic domain with the long Ob-Rb isoform displaying full signaling capacity.⁵⁰ Downregulation of the leptin receptor has been observed after incubation of rat adrenal slices with leptin.⁵¹ Considering that our protocol of infusion produced plasma concentrations of leptin well above the normal physiological range, we examined the effects of leptin infusion on the expression of the short (Ob-Ra ~90-100 kDa) and long (Ob-Rb ~120-130 kDa) isoforms of the leptin receptor. Our results indicating no differences in leptin receptor mRNA and protein expression between control and leptin-treated animals suggested that the leptin transduction pathway, at least at the level of receptor expression, is not altered after 96 hours of leptin infusion. Thus, the effect of leptin does not represent withdrawal of a leptin-induced function in the adrenal because of receptor downregulation. The mechanisms by which leptin regulates its receptor in adrenal glands are unclear and remain to be determined.

Evidence of the mechanisms by which leptin modifies gene expression obtained in the human adrenocortical NCI-H295 tumor cell line indicates that leptin inhibits P450scc expression through activation of a cyclic adenosine monophosphate (cAMP)-degrading pathway that down-regulates *CYP11A1* promoter activity,⁵² how these mechanisms are operating in

vivo in the whole animal and the role of the leptin receptor in the ovine adrenal⁵⁴ warrant further investigation.

In terms of enzymatic activity effects modulating adrenal steroidogenesis, the increased expression of endothelial nitric oxide synthase in adrenal tissue from MLTH fetuses and its colocalization with P450c17 suggest a role for nitric oxide (NO) in the regulation of adrenal steroidogenesis.⁵⁵ This was recently confirmed with the reported NO-dependent inhibition of ACTH-induced cortisol production *in vitro* in adrenocortical cells from MLTH fetuses.⁵⁶ It has been recognized that diatomic gases such as CO and NO competitively interact with the oxygen-binding site of heme-containing cytochrome enzymes, including the steroidogenic rate-limiting enzymes P450scc,⁵⁷ aldosterone synthase P450 (P450aldo),⁵⁸ and P450c17.⁵⁹ Due to the multistep nature of the conversion of cholesterol to pregnenolone by P450scc (involving three distinct chemical reactions), and the 2 activities of P450c17 (hydroxylase and lyase),⁶⁰ these chemical steps in steroidogenesis may be greatly affected by NO-heme interaction, as previously proposed.⁶¹ It is conceivable that leptin acts via NO to diminish adrenal responses in both of our experimental groups. Further experiments are needed to understand the interplay between hypoxemia-induced NO and leptin on the regulation of fetal endocrine responses.

Perspectives and Significance

Our results suggest a role for leptin in modulating adrenal function in the hypoxic fetus. We demonstrated that an increase in circulating leptin concentration in the late gestation hypoxemic sheep fetus reduces adrenal responsiveness to ACTH, and these effects most probably result from direct downregulation of adrenal ACTH-R, StAR, and P450c21 expression. How leptin mediates its effects on gene expression and on enzymatic activities of the adrenal steroidogenic pathways remains to be investigated. Leptin is becoming an important factor in the intrauterine hormonal environment able to influence the development of obesity and its comorbidities,⁶² and the study of the interactions of leptin and the fetal HPA axis will clarify the possible role/roles of leptin in determining early life events that may have long-term metabolic consequences.

Declaration of Conflicting Interests

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

Funding

This research was supported by National Institute of Child Health and Human Development Grant HD-11210.

References

- Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. *Endocr Rev*. 2000; 21(5):514-550.
- Jéquier E. Leptin signaling, adiposity, and energy balance. *Ann NY Acad Sci*. 2002;967:379-388.
- Ahima RS, Saper CB, Flier JS, Elmquist JK. Leptin regulation of neuroendocrine systems. *Front Neuroendocrinol*. 2000;21(3):263-307.
- Ahima RS, Prabakaran D, Mantzoros C, et al. Role of leptin in the neuroendocrine response to fasting. *Nature*. 1996;382(6588): 250-252.
- Heiman ML, Ahima RS, Craft LS, Schoner B, Stephens TW, Flier JS. Leptin inhibition of the hypothalamic-pituitary-adrenal axis in response to stress. *Endocrinology*. 1997;138(9):3859-3863.
- Yuen BSJ, Owens PC, Symonds ME, et al. Effects of leptin on fetal plasma adrenocorticotrophic hormone and cortisol concentrations and the timing of parturition in the sheep. *Biol Reprod*. 2004; 70(6):1650-1657.
- Yuen BS, McMillen C, Symonds ME, Owens PC. Abundance of leptin mRNA in fetal adipose tissue is related to fetal body weight. *J Endocrinol*. 1999;163(3):R11-R14.
- Howe DC, Gertler A, Challis JR. The late gestation increase in circulating ACTH and cortisol in the fetal sheep is suppressed by intracerebroventricular infusion of recombinant ovine leptin. *J Endocrinol*. 2002;174(2):259-266.
- Carey LC, Su Y, Valego NK, Rose JC. Infusion of ACTH stimulates expression of adrenal ACTH receptor and steroidogenic acute regulatory protein mRNA in fetal sheep. *Am J Physiol Endocrinol Metab*. 2006;291(2):E214-E220.
- Valego NK, Su Y, Carey LC, et al. Hypothalamic-pituitary disconnection in fetal sheep blocks the peripartum increases in adrenal responsiveness and adrenal ACTH receptor expression. *Am J Physiol Regul Integr Comp Physiol*. 2005;289(2): R410-R417.
- Phillips ID, Ross JT, Owens JA, Young IR, McMillen IC. The peptide ACTH(1-39), adrenal growth and steroidogenesis in the sheep fetus after disconnection of the hypothalamus and pituitary. *J Physiol*. 1996;491(pt 3):871-879.
- Harvey LM, Gilbert RD, Longo LD, Ducasay CA. Changes in ovine fetal adrenocortical responsiveness after long-term hypoxemia. *Am J Physiol Endocrinol Metab*. 1993;264(5 pt 1):E741-E747.
- Myers DA, Bell PA, Hyatt K, Mlynarczyk M, Ducasay 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.
- Myers DA, Hyatt K, Mlynarczyk M, Bird IM, Ducasay 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.
- Ducasay CA, Hyatt K, Mlynarczyk M, Kaushal KM, Myers DA. Long-term hypoxia increases leptin receptors and plasma leptin concentrations in the late-gestation ovine fetus. *Am J Physiol Regul Integr Comp Physiol*. 2006;291(5):R1406-R1413.
- Kendall NR, Gutierrez CG, Scaramuzzi RJ, et al. Direct *in vivo* effects of leptin on ovarian steroidogenesis in sheep. *Reproduction*. 2004;128(6):757-765.
- Dyer CJ, Simmons JM, Matteri RL, Keisler DH. cDNA cloning and tissue-specific gene expression of ovine leptin, NPY-Y1

- receptor, and NPY-Y2 receptor. *Domest Anim Endocrinol.* 1997; 14(5):295-303.
18. Gertler A, Simmons J, Keisler DH. Large-scale preparation of biologically active recombinant ovine obese protein (leptin). *FEBS Lett.* 1998;422(2):137-140.
19. Munoz-Gutierrez M, Findlay PA, Adam CL, et al. The ovarian expression of mRNAs for aromatase, IGF-I receptor, IGF-binding protein-2, -4 and -5, leptin and leptin receptor in cycling ewes after three days of leptin infusion. *Reproduction.* 2005; 130(6):869-881.
20. Su Y, Figueroa JP, Rose JC. Antenatal betamethasone (Beta) exposure enhances leptin induced inhibition of steroidogenic acute regulatory protein (StAR) and ACTH-receptor expression in adult ovine adrenocortical cells [Abstract]. *Endocr Rev* 2010; 31(3 Suppl 1):S692.
21. Block WA Jr, Draper ML, Rose JC, Schwartz J. Maturation of cortisol responses to adrenocorticotrophic hormone in twin fetal sheep in vivo. *Am J Obstet Gynecol.* 1999;181(2):498-502.
22. Delavaud C, Bocquier F, Chilliard Y, et al. Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma leptin concentration assessed by a specific RIA in sheep. *J Endocrinol.* 2000;165(2):519-526.
23. Ehrhardt RA, Slepatis RM, Siegal-Willott J, Van Amburgh ME, Bell AW, Boisclair YR. Development of a specific radioimmunoassay to measure physiological changes of circulating leptin in cattle and sheep. *J Endocrinol.* 2000;166(3):519-528.
24. Applied Biosystems. Relative Quantitation of Gene Expression. User Bulletin #2. ABI PRISM 7700 Sequence Detection System. 2001:11-5.
25. Kerr DR, Castro MI, Valego NK, Rawashdeh NM, Rose JC. Corticotropin and cortisol responses to corticotropin-releasing factor in the chronically hypoxemic ovine fetus. *Am J Obstet Gynecol.* 1992;167(6):1686-1690.
26. 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.
27. Boshier DP, Holloway H, Liggins GC. Effects of cortisol and ACTH on adrenocortical growth and cytodifferentiation in the hypophysectomized fetal sheep. *J Dev Physiol.* 1981;3(6):355-373.
28. Robinson JS, Kingston EJ, Jones CT, Thorburn GD. Studies on experimental growth retardation in sheep. The effect of removal of a endometrial caruncles on fetal size and metabolism. *J Dev Physiol.* 1979;1(5):379-398.
29. 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.
30. Challis JR, Fraher L, Oosterhuis J, White SE, Bocking AD. Fetal and maternal endocrine responses to prolonged reductions in uterine blood flow in pregnant sheep. *Am J Obstet Gynecol.* 1989; 160(4):926-932.
31. Gagnon R, Challis J, Johnston L, Fraher L. Fetal endocrine responses to chronic placental embolization in the late-gestation ovine fetus. *Am J Obstet Gynecol.* 1994;170(3):929-938.
32. Murotsuki J, Gagnon R, Matthews SG, Challis JR. Effects of long-term hypoxemia on pituitary-adrenal function in fetal sheep. *Am J Physiol Endocrinol Metab.* 1996;271(4 pt 1):E678-E685.
33. Gardner DS, Fletcher AJW, Bloomfield MR, Fowden AL, Giussani DA. Effects of prevailing hypoxaemia, acidaemia or hypoglycaemia upon the cardiovascular, endocrine and metabolic responses to acute hypoxaemia in the ovine fetus. *J Physiol.* 2002; 540(pt 1):351-366.
34. Rose JC, Meis PJ, Urban RB, Greiss FC. *In vivo* evidence for increased adrenal sensitivity to adrenocorticotropin-(1-24) in the lamb fetus late in gestation. *Endocrinology.* 1982;111(1): 80-85.
35. Hsu HT, Chang YC, Chiu YN, Liu CL, Chang KJ, Guo IC. Leptin interferes with adrenocorticotropin/3',5'-cyclic adenosine monophosphate (cAMP) signaling, possibly through a Janus kinase 2-phosphatidylinositol 3-kinase/Akt-phosphodiesterase 3-cAMP pathway, to down-regulate cholesterol side-chain cleavage cytochrome P450 enzyme in human adrenocortical NCI-H295 cell line. *J Clin Endocrinol Metab.* 2006;91(7):2761-2769.
36. Forhead AJ, Lamb CA, Franko KL, et al. Role of leptin in the regulation of growth and carbohydrate metabolism in the ovine fetus during late gestation. *J Physiol.* 2008;586(9):2393-2403.
37. Mercer JG, Hoggard N, Williams LM, et al. Coexpression of leptin receptor and preproneuropeptide Y mRNA in arcuate nucleus of mouse hypothalamus. *J Neuroendocrinol.* 1996;8(10):733-735.
38. Zamorano PL, Mahesh VB, De Sevilla LM, Chorch LP, Bhat GK, Brann DW. Expression and localization of the leptin receptor in endocrine and neuroendocrine tissues of the rat. *Neuroendocrinology.* 1997;65(3):223-232.
39. Dyer CJ, Simmons JM, Matteri RL, Keisler DH. Leptin receptor mRNA is expressed in ewe anterior pituitary and adipose tissues and is differentially expressed in hypothalamic regions of well-fed and feed-restricted ewes. *Domest Anim Endocrinol.* 1997; 14(2):119-128.
40. Luoh SM, Di Marco F, Levin N, et al. Cloning and characterization of a human leptin receptor using a biologically active leptin immunoadhesin. *J Mol Endocrinol.* 1997;18(1):77-85.
41. Clark KA, MohanKumar SMJ, Kasturi BS, MohanKumar PS. Effects of central and systemic administration of leptin on neurotransmitter concentrations in specific areas of the hypothalamus. *Am J Physiol Regul Integr Comp Physiol.* 2006;290(2): R306-R312.
42. Itoi K, Helmreich DL, Lopez-Figueroa MO, Watson SJ. Differential regulation of corticotropin-releasing hormone and vasopressin gene transcription in the hypothalamus by norepinephrine. *J Neurosci.* 1999;19(13):5464-5472.
43. Szafarczyk A, Malaval F, Laurent A, Gibaud R, Assenmacher I. Further evidence for a central stimulatory action of catecholamines on adrenocorticotropin release in the rat. *Endocrinology.* 1987;121(3):883-892.
44. Le Roy C, Li JY, Stocco DM, Langlois D, Saez JM. Regulation by adrenocorticotropin (ACTH), angiotensin II, transforming growth factor- β , and insulin-like growth factor I of bovine adrenal cell steroidogenic capacity and expression of ACTH receptor, steroidogenic acute regulatory protein, cytochrome P450c17, and β -hydroxysteroid dehydrogenase. *Endocrinology.* 2000; 141(5):1599-1607.
45. Lebrethon MC, Naville D, Begeot M, Saez JM. Regulation of corticotropin receptor number and messenger RNA in cultured

- human adrenocortical cells by corticotropin and angiotensin II. *J Clin Invest*. 1994;93(4):1828-1833.
46. Liu J, Heikkilä P, Kahri AI, Voutilainen R. Expression of the steroidogenic acute regulatory protein mRNA in adrenal tumors and cultured adrenal cells. *J Endocrinol*. 1996;150(1):43-50.
47. Nicol MR, Wang H, Ivell R, Morley SD, Walker SW, Mason JI. The expression of steroidogenic acute regulatory protein (StAR) in bovine adrenocortical cells. *Endocr Res*. 1998;24(3-4):565-569.
48. Penhoat A, Jaillard C, Saez JM. Regulation of bovine adrenal cell corticotropin receptor mRNA levels by corticotropin (ACTH) and angiotensin-II (A-II). *Mol Cell Endocrinol*. 1994;103(1-2):R7-R10.
49. Mountjoy KG, Bird IM, Rainey WE, Cone RD. ACTH induces up-regulation of ACTH receptor mRNA in mouse and human adrenocortical cell lines. *Mol Cell Endocrinol*. 1994;99(1):R17-R20.
50. Jones CT, Luther E, Ritchie JW, Worthington D. The clearance of ACTH from the plasma of adult and fetal sheep. *Endocrinology*. 1975;96(1):231-234.
51. Sweeney G. Leptin signalling. *Cell Signal*. 2002;14(8):655-663.
52. Tena-Sempere M, Pinilla L, Gonzalez LC, Casanueva FF, Diéguez C, Aguilar E. Homologous and heterologous down-regulation of leptin receptor messenger ribonucleic acid in rat adrenal gland. *J Endocrinol*. 2000;167(3):479-486.
53. 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.
54. Monau TR, Vargas VE, Lubo Z, 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.
55. Tsubaki M, Hiwatashi A, Ichikawa Y, Hori H. Electron paramagnetic resonance study of ferrous cytochrome P-450_{scc}-nitric oxide complexes: effects of cholesterol and its analogues. *Biochemistry*. 1987;26(14):4527-4534.
56. Tsubaki M, Ichikawa Y, Fujimoto Y, Yu NT, 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-450_{scc}. *Biochemistry*. 1990;29(37):8805-8812.
57. Nakajin S, Hall PF. Side chain cleavage of C21 steroids by testicular microsomal cytochrome P-450 (17-hydroxylase/lyase): involvement of heme. *J Steroid Biochem*. 1983;19(3):1345-1348.
58. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev*. 2011;32(1):81-151.
59. Peterson JK, Moran F, Conley AJ, Bird IM. Zonal expression of endothelial nitric oxide synthase in sheep and rhesus adrenal cortex. *Endocrinology*. 2001;142(12):5351-5363.
60. Alexe DM, Syridou G, Petridou ET. Determinants of early life leptin levels and later life degenerative outcomes. *Clin Med Res*. 2006;4(4):326-335.