Periconceptional undernutrition in sheep accelerates maturation of the fetal hypothalamic-pituitary-adrenal axis in late gestation.

Abbreviated title: Periconceptional nutrition and the fetal HPAA

Frank H. Bloomfield[*†], Mark H. Oliver[*], Paul Hawkins[*], Alison C. Holloway[†‡], Melanie Campbell[†], Peter D. Gluckman[*], Jane E. Harding[*] and John R.G. Challis[†].

[*]Liggins Institute, University of Auckland, Private Bag 92019, Auckland, New Zealand, [†]Departments of Physiology and Obstetrics and Gynaecology, University of Toronto, Toronto, Ontario, Canada. [‡]Current address: Department of Obstetrics and Gynaecology, McMaster University, Hamilton, Ontario, Canada.

Address for correspondence: Dr Frank Bloomfield, Liggins Institute, University of Auckland, Private Bag 92019, Auckland, New Zealand. Tel: (+64 9) 3737599 x 86107, Fax: (+64 9) 3737597, Email: f.bloomfield@auckland.ac.nz

Reprint requests: Dr Frank Bloomfield

Key words: fetal HPA axis, maternal nutrition, programming

Abstract

We have investigated the effects of moderate maternal periconceptional undernutrition from 60 d before to 30 d after mating on fetal hypothalamic-pituitary-adrenal axis (HPAA) function in late gestation. Ewes were sampled regularly during the period of undernutrition for circulating hormone levels. Vascular catheters were inserted into ewes and their singleton fetuses at 112 d gestation (term, 145 d), and fetal $ACTH_{1-24}$ and metyrapone challenge tests were performed at 127 and 128 d. Post mortems were performed at 132 d. Fetuses of undernourished ewes (UN, n = 12) had elevated baseline cortisol concentrations ($P < 0.05$) compared with fetuses of *ad libitum* fed ewes (N, n = 10). There were no differences between groups in fetal responses to ACTH challenge, but only UN fetuses demonstrated ACTH and 11-deoxycortisol responses to metyrapone ($P < 0.05$). UN fetuses had increased mRNA levels for proopiomelanocortin and prohormone convertase (PC)-1, but not PC-2, in the *pars intermedia* of the pituitary gland ($P < 0.05$). Glucocorticoid receptor mRNA levels were not different between groups in pituitary or hypothalamus. Maternal cortisol and ACTH levels during undernutrition were profoundly suppressed ($P < 0.001$), rather than elevated, in UN ewes. Furthermore, the normal pregnancy rise in maternal serum progesterone concentrations was delayed in undernourished mothers. These data demonstrate that events around the time of conception have profound effects on fetal HPA development in late gestation, and that factors other than fetal exposure to excess glucocorticoids may be important.

Introduction

The fetal hypothalamic-pituitary-adrenal axis (HPAA) plays a vital role in fetal maturation during late gestation (1). In all species studied to date, there is a rise in circulating cortisol concentrations before birth (2) which is responsible for the maturation of organs and metabolic pathways in preparation for life *ex utero*. This prepartum cortisol surge is also important in the processes leading to parturition and, in some species such as the sheep, is essential for the onset of the labour (3-5). During the prepartum cortisol surge, rather than the classical feedback loop, there is a feed-forward loop between cortisol and ACTH, allowing cortisol and ACTH concentrations to rise concomitantly. The mechanism underlying this feed-forward loop is not understood.

Cortisol crosses the placenta, and the fetal HPAA is therefore also exposed to maternal glucocorticoids (6). Inappropriate exposure of the developing fetus to maternal glucocorticoids has been proposed as a mechanism for fetal programming (7, 8), and administration of synthetic glucocorticoids to pregnant ewes and guinea pigs results in altered HPAA function and hypertension in the offspring (9-11). Undernutrition also results in altered HPAA function in the offspring in several species (12-14), and has also been hypothesized to exert these effects via excessive fetal glucocorticoid exposure (15). This could occur as a result of the stress of undernutrition leading to elevated maternal glucocorticoids, which are then transferred across the placenta (16), or as a result of reduced placental inactivation of active to inactive glucocorticoid (14). Undernourished rat dams have been reported to have elevated glucocorticoid levels, along with growth restriction and altered HPAA function in the pups (15). Maternal adrenalectomy with corticosterone replacement at basal levels resulted in pups that were still growth restricted but did not have altered

HPAA function, suggesting that it may be fetal exposure to excessive glucocorticoid rather than growth restriction *per se* that results in fetal programming. However, many experimental paradigms of maternal undernutrition have not reported maternal glucocorticoid concentrations, and in undernourished sheep decreased maternal cortisol levels have also been reported (17).

We have recently reported that moderate periconceptional undernutrition in the sheep, from 60 days before until 30 days after mating (term is 145 days in this breed), programmes the fetal glucose/insulin axis, with altered insulin responses to glucose but not arginine (18). In a parallel cohort of animals allowed to deliver normally, we demonstrated precocious activation of the fetal HPAA and preterm birth (19). Here, we report studies of dynamic fetal HPAA function testing in late gestation, and measurements of maternal circulating hormone concentrations during the period of undernutrition, attempting to identify the level of the HPAA at which activation occurred and possible mechanisms underlying that activation.

To confirm the hypothesis that undernutrition exposes the fetus to excess maternal glucocorticoid, we measured maternal cortisol and ACTH concentrations before, during and after undernutrition. The period of undernutrition encompassed the period when maintenance of pregnancy depends upon progesterone production by the corpus luteum, the function of which has been reported to be altered in undernutrition (20), and also covered the period of initial placental development and attachment in the sheep (21). Elevated progesterone concentrations around the time of conception have also been reported to have profound effects on fetal and placental growth (22, 23). Luteal progesterone production is in turn partly regulated by ovine placental lactogen (oPL) (24, 25), an hormone produced by the binucleate cells of the ovine placenta. Ovine PL levels are nutritionally regulated (26), with concentrations in the ewe falling

with maternal food restriction (27). During the period of histiotrophic nutrition of the developing embryo, before the placenta has fully attached and developed, oPL levels correlate with uterine gland hypertrophy (the source of histiotrophic nutrients) (28, 29). In late gestation, oPL concentrations are correlated with both placental and fetal weights (30), and a role for oPL in nutrient partitioning between the mother and fetus has been postulated (31-33). We therefore also measured maternal progesterone and oPL concentrations throughout pregnancy.

Methods

Animals: After acclimatization to a concentrate feed designed by us for prolonged feeding of ruminants, non-pregnant Romney ewes were randomly assigned to maintenance feeds ("N"; concentrates at 3-4% of body weight per day) or undernutrition ("UN"; 2 days fast followed by concentrates at 1-2% of body weight per day) from 60 days prior to mating as described previously (18). The UN regimen was designed to reduce maternal bodyweight by 10-15% (19). A fortnight before mating with Dorset rams, the estrous cycle of all ewes was synchronized (34). Undernutrition was continued until 30 days after mating in UN ewes. Thereafter all ewes were fed maintenance feeds. Only singleton-bearing ewes were used in this experiment, and there were 10 N ewes and 12 UN ewes. From –60 d gestation until transport to the laboratory at 105 d gestation, maternal blood samples were withdrawn by jugular puncture at fortnightly intervals.

Following acclimatization to laboratory conditions for 7 days, surgery was performed for the implantation of chronic indwelling vascular catheters in fetal and maternal arteries and veins as described previously (18). On day 127 of gestation fetuses

underwent an $ACTH_{1-24}$ stimulation test, and on day 128 a metyrapone test was performed. Both the $ACTH_{1-24}$ and the metyrpone tests were performed in the morning on all animals, with the baseline samples taken between 08:00 and 10:00 prior to feeding. Metyrapone blocks 11β hydroxylase (P450C11β1), the enzyme that catalyses the conversion of 11-deoxycortisol to cortisol, the final step in cortisol biosynthesis. Thus, the normal response to metyrapone is a fall in circulating cortisol concentrations, followed by a rise in ACTH concentrations in response to a decrease in negative feedback, and a secondary rise in 11 deoxycortisol as ACTH drives the adrenal gland to produce more cortisol, synthesis of which is blocked by metryapone. For the ACTH stimulation test, paired fetal and maternal arterial blood samples were withdrawn –15, 0, 5, 10, 15, 30, 60 and 120 minutes after an intravenous bolus to the fetus of 0.3 μg of $ACTH_{1-24}$ (Sigma Chemical, St Louis, MO, USA). For the metyrapone test, similar paired samples were withdrawn –15, 0, 30, 60 and 120 minutes after fetal intravenous injection of 30 mg of metyrapone (Sigma Chemical) over 1 minute. All samples were collected onto ice and processed for later hormone analysis as described previously (12). Cortisol and ACTH concentrations from the four baseline samples of these two tests were averaged to provide a basal cortisol and ACTH concentration for each fetus. However, in the analysis of each test, only the baseline samples from that test were used. At 131 d gestation, ewes were killed with an overdose of pentobarbitone. Fetal tissues were rapidly dissected, frozen over dry ice and stored at –80°C until analysed. Experiments were approved by the institutional animal ethics committee.

Hormone analysis*:* ACTH was measured by a commercial RIA kit which detects $ACTH_{1-24}$ (Diasorin, Stillwater, MN, USA) and which has been previously validated

for use in the sheep (35). Intra- and inter-assay CVs were 7.9 and 7.2% respectively. Cortisol was measured by an in-house radioimmunoassay (12). Briefly, cortisol was extracted from 100 μL plasma with 4 mL of diethyl ether with an efficiency of >95%. After reconstitution with phosphate buffered saline (PBS) and gelatin, samples were incubated in duplicate overnight at 4ºC in the presence of cortisol antibody and ^{3}H-cortisol (Amersham Life Sciences, Arlington Heights, IL, USA). The cortisol antibody was generated in this laboratory and had cross-reactivities as follows: <0.1% with cortisone, 21-deoxycortisone, progesterone, 17α–hydroxypregnenolone and pregnanediol, 1.6% with 11-deoxycortisol and 0.2% with 11β–hydroxyprogesterone. Bound and free cortisol were separated by incubation and centrifugation with dextran-coated neutral charcoal (Dextran T70, Pharmacia Fine Chemicals, Baie, D'Ufe, PQ; Norit A, Fisher Scientific, Toronto, Canada), and the supernatant mixed with 4 mL scintillation cocktail and counted. Two volumes of sample were assayed and parallelism was observed. Intra- and inter-assay coefficients of variation (CV) were 5.0 and 6.9% respectively and the detection limit was 0.2 ng/mL. 11 deoxycortisol was measured using a commercial double antibody RIA kits (ICN Pharmaceuticals, Inc., Costa Mesa, CA) after extraction and threefold concentration of plasma samples with diethyl ether. (intra-assay CV 9.2%; detection limit 0.016 ng/mL). Progesterone was measured by an in-house radioimmunoassay following extraction with diethyl ether as described previously (12) (intra-assay CV 5.4%; detection limit 1.5 ng/mL). Ovine placental lactogen was analysed by RIA as described previously (26) (intra- and inter-assay CVs 6.7 and 9.4% respectively).

***In-situ* hybridization:** We followed the method we have described previously, with minor modifications (36). Briefly, 12 μm sections of frozen pituitaries and

hypothalami were sectioned using a cryostat (Jung CM 300, Leica Instruments, Nussloch, Germany) and mounted onto pre- coated slides (Superfrost, Fisher Scientific, Nepean, Ontario, Canada), fixed in 4% paraformaldehyde for 5 minutes, rinsed in phosphate-buffered saline (PBS; 2 x 1 min) and dehydrated in an ascending alcohol series. 45-mer oligonucleotide probes complementary to sequences of the porcine proopiomelanocortin (POMC) (37) and prohormone convertase (PC)-1 and –2 genes (38, 39), the ovine glucocorticoid receptor (GR) (40), bovine prolactin (PRL) (36), ovine arginine vasopressin (AVP) (41) and CRH genes (42) and a 42-mer oligonucleotide probe complementary to bases of the ovine urocortin (UCN) gene (43) were labeled using terminal deoxynucleotidyl transferase (Pharmacia Biotech, Baie d'Urfe, PQ, Canada) and [α-^{35}S]dATP (NEN Du Pont Canada, Mississauga, ON, Canada). The sections were hybridized overnight in a moist chamber (42°C) with the radiolabeled probes. After hybridization, the sections were washed and exposed to autoradiographic film (Biomax, Kodak, Rochester, NY) with ^{14}C standards (American Radiochemical, St Louis, MO, USA). Control 45-mer antisense or nonsense sequence oligonucleotide probes were included with each probe to assess nonspecific hybridization. The autoradiograms were then analyzed using computerized image analysis software (Imaging Research, St. Catherines, ON, Canada). The relative optical densities (ROD) of hybridized probes were assessed using a minimum of 12 sections for each animal. For pituitary sections, analysis of the pars intermedia and pars distalis was undertaken separately. For hypothalamic sections, values represent total paraventricular nucleus (PVN) signal.

Statistical analysis: Baseline fetal and maternal hormone concentrations were compared by an unpaired Student's t test after confirming parametric distribution.

Fetal cortisol area under the curve (AUC) following $ACTH_{1-24}$ injection, and fetal ACTH, 11 deoxycortisol and cortisol AUC following metyrapone injection were compared by unpaired Student's t test. The acute fetal cortisol response to metyrapone injection was analysed by a paired Student's t test. Maternal ACTH, cortisol and progesterone levels during the period of nutritional manipulation (-60 to +30 days from conception) and from the end of nutritional manipulation onwards were analysed separately with repeated measures ANOVA. For the *in situ* hybridizations, RODs were compared by Student's t test, if necessary following log transformation to achieve parametric distribution. We regarded P values of < 0.05 as statistically significant. Data are expressed as mean±S.E.

Results

There were no significant differences between groups in fetal weight (UN, 3.99±0.09 Kg; N, 4.28±0.21 Kg; $P > 0.1$) or adrenal weight (UN, 491±5 mg; N, 448±3 mg; $P > 0.7$). Adrenal weights expressed per unit body weight were also not significantly different (data not shown). Overall, there was a statistically significant, although not very strong, positive correlation between adrenal weight and baseline circulating cortisol concentration ($r^2 = 0.26$, $P = 0.03$). The association was stronger in the UN fetuses than in the N fetuses, but no longer statistically significant (UN, $r^2 = 0.3$, $P = 0.1$; N, $r^2 = 0.1$, $P = 0.5$).

Baseline HPAA function: Baseline fetal cortisol concentrations (the mean of the two baseline samples taken on d 127 and the two taken on d 128 for each animal) were significantly higher in fetuses of undernourished ewes (2.5±0.4 vs 1.3±0.1 ng/mL, $P < 0.02$; Fig. 1). Fetal baseline ACTH concentrations were not significantly different between groups (UN: 29.5±3.1 pg/mL, N: 29.0±3.7 pg/mL; $P > 0.9$). In UN fetuses

there was a significant positive correlation between plasma cortisol (F) and ACTH concentrations (F = 0.3+0.05*ACTH; $r^2 = 0.7$, $P < 0.001$), whereas in N fetuses there was a non-significant negative correlation (F = 2.06-0.03*ACTH; $r^2 = 0.28$, $P = 0.11$). Maternal cortisol concentrations at baseline were not significantly different between groups (UN: 3.0±0.5 ng/mL, N: 2.1±0.3 ng/mL; $P > 0.1$).

HPAA stimulation tests: Following injection of $ACTH_{1-24}$ there was no difference between groups in peak or AUC values for ACTH or cortisol (Fig. 1).

Prior to administration of metyrapone, fetal 11-deoxycortisol concentrations were below the detection limit of the assay and are reported as the minimum detectable dose (0.016 ng/mL). Following metyrapone injection, only UN fetuses showed a significant fall in circulating cortisol levels and rises in ACTH and 11 deoxycortisol concentrations (Fig. 2). Only two N fetuses had a detectable rise in 11 deoxycortisol, and this was minimal. The AUC for both ACTH and 11 deoxycortisol were significantly elevated in UN fetuses (Fig. 2). UN fetuses with the highest baseline cortisol concentrations had the greatest fall in cortisol following metyrapone administration, and the greatest rise in 11-deoxycortisol concentrations (data not shown). Eight N fetuses and two UN fetuses failed to respond to metyrapone with a fall in cortisol or rise in 11-deoxycortisol concentrations.

***In situ* hybridization:** There were no differences between groups in CRH ($P = 0.29$) or AVP ($P = 0.09$) mRNA levels in the PVN of the hypothalamus (Fig. 3). In the pituitary gland, POMC mRNA levels were upregulated in UN fetuses in the *pars intermedia* ($P < 0.05$), but not in the *pars distalis* (Fig. 3). PC-1 ($P < 0.05$), but not PC-2, mRNA levels were also upregulated in the *pars intermedia* (Fig. 2). Regression analysis demonstrated a significant association between POMC and PC-1 mRNA levels in fetuses ($R^2 = 0.3$, $P < 0.05$), suggesting that those fetuses that

upregulated POMC expression also upregulated PC-1 expression. GR mRNA levels in the *pars distalis* were not significantly different between groups and there was no significant correlation between GR mRNA levels and basal circulating cortisol concentration (r^2=0.16 for N fetuses, P = 0.3; r^2 = 0.05 for UN fetuses, P = 0.55). Interestingly, we did not find evidence of GR message in the *pars intermedia* (Fig. 3). We also investigated mRNA levels of other pituitary hormones that have corticotrophic action. Urocortin and prolactin mRNA levels were not significantly different between groups (data not shown).

Maternal hormones during undernutrition: Prior to commencing nutritional manipulation, maternal plasma concentrations of cortisol, ACTH and progesterone were not significantly different between groups (Fig. 4). During undernutrition, the maternal HPAA was profoundly suppressed, with ACTH concentrations approximately half, and cortisol concentrations almost a quarter, of those in the control group (P < 0.001; Fig. 4). Following cessation of undernutrition cortisol concentrations returned to control values and were subsequently not different between groups (Fig 4). ACTH concentrations returned to values slightly, but significantly, above control values and remained elevated (Fig. 4).

Maternal progesterone concentrations were not significantly different between groups during the period of undernutrition. In the *ad libitum* fed ewes, progesterone concentrations rose steadily throughout the study (Fig. 4). However, in UN ewes progesterone concentrations plateaued following cessation of undernutrition, and remained significantly lower than in control ewes until day 93 of gestation (Fig. 4).

Maternal plasma concentrations of ovine placental lactogen (oPL) were not different for most of the study period but began to diverge in late gestation, with values in UN

ewes falling and becoming significantly less than those in the N ewes as delivery approached (Fig. 4).

Discussion

The fetal HPAA plays a crucial role in the onset of parturition in the sheep (3, 44). In the human, a prepartum glucocorticoid surge also occurs and is thought to be important both in the preparation of the fetus for extra-uterine life and in the processes of parturition, although the precise role of glucocorticoids in the latter is less clear in the human (5). The underlying mechanisms that determine when this cortisol surge takes place, and thus when parturition will occur, have not been elucidated.

During fetal life the ovine adrenal gland has three phases of activity. Early in gestation it is capable of secreting cortisol, but then goes through a quiescent phase between approximately 90 and 120 days gestation (45, 46). This phase is characterized by low mRNA levels of P450c17 (47). From 120 days onwards, there is a steady and gradual increase in cortisol production by the adrenal gland, and this is thought to be in response to increasing ACTH secretion from the pituitary (48).

The studies we report here provide evidence that the time of adrenal maturation may be regulated, in part, by maternal nutritional status in the periconceptional period, despite the fact that the moderate nutritional restriction ended almost 100 days before we tested HPAA function. We have previously reported that this moderate nutritional restriction also resulted in preterm birth, probably as a direct consequence of the advanced fetal HPAA maturation (19). The results of the ACTH stimulation test demonstrate that the adrenal cortisol response was not altered by periconceptional undernutrition, and suggest that the pathway for cortisol biosynthesis was equally

responsive to $ACTH_{1-24}$ stimulation in UN and N fetuses. However, only the UN fetuses demonstrated a significant response to metyrapone, as evidenced by a significant fall in fetal cortisol levels and a rise in 11-deoxycortisol levels and ACTH. A positive response to a metyrapone test necessitates successful inhibition of 11β hydroxylase, demonstrated by a fall in circulating cortisol concentrations and a rise in 11 deoxycortisol concentrations, and an intact negative feedback loop, demonstrated by a rise in ACTH concentrations. The presence of a response to the metryapone test in all but two of the UN fetuses, but absence of a response in all but two of the N fetuses (in which only a minimal response was seen), is consistent with advanced maturation of the fetal HPAA in the UN group. Furthermore, greater falls in cortisol concentrations following metyrapone were seen in fetuses that had the highest basal concentrations, and in two UN fetuses with low cortisol concentrations, no fall (and no rise in 11-deoxycortisol) was detected. This suggests that development of the fetal HPAA with respect to the negative feedback response is a continuum, but that some kind of maturational threshold may need to be reached before a negative feedback response is detectable.

The most consistent explanation for the findings of similar responses to $ACTH_{1-24}$ injection but different responses to the metyrapone challenge, is an alteration in negative feedback sensitivity in UN fetuses. However, we cannot exclude the possibility that the differences in responses to the metyrapone test could be due to technical limitations at the very low glucocorticoid concentrations seen in the fetus. The cross-reactivity of the cortisol antibody used in the cortisol assay with 11-deoxycortisol could have led to some measurement error when cortisol concentrations are very low and 11 deoxycortisol concentrations are high after metyrapone. There is,

however, no reason why this should be the case in UN fetuses but not in N fetuses. It is also possible that there was inadequate inhibition of 11β hydroxylase in N fetuses but not in UN fetuses, although there is no reason to suppose that this was the case given that the metyrapone challenge was the same in all fetuses and there were no significant differences between groups in fetal weight or adrenal weight. Finally, the circulating cortisol in the N fetuses could be largely of maternal origin and therefore would not fall following fetal metyrapone administration. This would not invalidate the conclusion that the fetal HPAA is more mature in UN fetuses, although the conclusion that the feedback axes are different would be more tenuous. However, we believe that the most likely explanation for our failure to demonstrate a fall in circulating cortisol concentrations in N fetuses following metyrapone adminstration reflects a limitation of the sensitivity of the assay in detecting small changes in cortisol at such low concentrations, particularly when some of the circulating cortisol is of maternal origin. The fact that a small increase in 11 deoxycortisol area under the curve following metyrapone was seen in two N fetuses would be consistent with this.

Before 120 days gestation in the fetal sheep, adrenalectomy does not influence fetal ACTH or cortisol concentrations. However, after this time, fetal ACTH concentrations increase markedly, suggesting that the fetal pituitary sensitivity to cortisol negative feedback is increasing (49). Negative feedback of the HPAA occurs at pituitary level and above via the glucocorticoid receptor. Although we did not find a difference in GR mRNA levels in the PD, we could not find evidence of GR expression in the PI. This is consistent with a previous report in late gestation ovine fetuses (50), and may suggest that ACTH secretion from the PI in fetal life is not under normal negative feedback control. Furthermore, POMC mRNA levels were

increased in the *pars intermedia*, although not in the *pars distalis*. Differential regulation of POMC expression in the PI and PD following hypoxia in late gestation fetal sheep has been reported previously (50, 51). We also report up-regulation of PC-1, but not PC-2, in the PI. If these changes in expression are matched with changes in enzyme activity, then this could be expected to result in increased ACTH production from the PI in UN fetuses. This would be consistent with previous studies reporting abundant expression of immunoreactive ACTH in the fetal PI, in contrast to very sparse staining in the adult PI (52). A recent study demonstrated that in placentally-restricted, chronically hypoxemic ovine fetuses, a population of non-CRH target pituitary corticotrophs emerged in late gestation that secreted high basal levels of ACTH *in vitro* (53). We did not collect pituitary cells for culture in our experiment, and are therefore unable to determine whether a similar change in pituitary corticotroph cell population may contribute to the findings in our study. We did, however, investigate other potential routes of corticotrophic stimulation in the pituitary. Urocortin, a CRH-like peptide, has been shown to stimulate ACTH release from the pituitary and placenta (54, 55), and urocortin mRNA is expressed in the pituitary and in human fetal membranes and endometrium (56, 57). We did not find any difference in urocortin mRNA levels between groups. Similarly, mRNA levels of prolactin, which, in common with POMC mRNA levels is under dopaminergic control (58), were not different between groups.

We speculate that the concurrent rises in ACTH and cortisol seen in late gestation prior to parturition may be due to ACTH secretion from the PI, which is not under normal negative feedback control and may be the site of the feed-forward loop seen in during the prepartum period. If so, this could explain how UN fetuses can demonstrate a negative feedback response to metyrapone, mediated by GR in the *pars*

distalis, but also demonstrate evidence of a feed-forward loop, mediated by the *pars intermedia*. The presence of a feed-forward loop in these fetuses is supported by a significant positive correlation between cortisol and ACTH concentrations in UN fetuses, but a negative non-significant correlation in N fetuses. It is also consistent with our finding of an early prepartum surge in cortisol and ACTH, culminating in early delivery, in the cohort of UN fetuses which were allowed to proceed to normal delivery (19).

Contrary to the prevailing hypothesis which proposes fetal exposure to elevated maternal glucocorticoids as a fundamental mechanism for fetal programming (7, 15, 59), we report a profound suppression of the maternal HPA axis in response to a chronic, moderate nutritional insult. The difference in maternal glucocorticoid response to undernutrition between our study and others may be a reflection of the duration of undernutrition, with short periods of more severe undernutrition resulting in an increase in maternal, and fetal, glucocorticoid concentrations (16), but prolonged chronic moderate undernutrition (as in our study) resulting in adaptive down-regulation. Indeed, our results are consistent with studies from the 1940s and 1960s in fasted non-pregnant ewes and rats, in which adrenal and pituitary weights were reduced and morphology of the glands suggested atrophy (60-62). Those studies did not measure circulating hormone concentrations. However, a recent study in which pregnant ewes were undernourished from 28 to 80 days of gestation also reported reduced maternal circulating cortisol concentrations (17).

On cessation of undernutrition in our study, maternal cortisol concentrations returned to normal, although ACTH levels returned to a level slightly, but significantly, higher than baseline and remained elevated for several weeks. We do not have detailed hormonal profiles covering the period that undernourished ewes were refed at 30 days

gestation. Administration of the synthetic glucocorticoid dexamethasone to pregnant ewes at a similar point in gestation (28 days), permanently altered blood pressure of the offspring, whereas dexamethasone given at 64 days had no effect (11, 63). These findings may suggest that the period around 30 days gestation, when the fetal adrenal gland is just beginning to develop glucocorticoid secretory capacity, may represent a critical window during development when the fetal HPAA is very sensitive to glucocorticoid concentrations and can be permanently reset by changes in those concentrations. We cannot exclude the possibility that the fetuses in our study may have been exposed to a change in the level of glucocorticoid exposure at a critical time, either as a result of a cortisol "spike" upon maternal refeeding, or simply as a result of the change from low to normal levels of maternal cortisol. However, our results do suggest that the maternal glucocorticoid response to undernutrition may differ with different experimental paradigms, and that effects seen in the fetus cannot necessarily be assumed to be consequent upon increased fetal glucocorticoid exposure without some measure of glucocorticoid concentrations in the mother and/or fetus.

An alternative possible explanation for the altered fetal development in late gestation that we report here and elsewhere (18, 19) may lie in altered placental function. We do not have direct measurements of placental function, but the lower circulating levels of ovine placental lactogen (oPL), a somatotrophic hormone produced by the placenta, in late gestation and the lower progesterone concentrations during the first half of gestation in UN ewes, at the time that the placenta is attaching, suggest that there may be alterations in placental function after periconceptional undernutrition. Various aspects of placental function could interact with the fetal HPAA, including placental POMC production, 11ß-HSD-II activity, and local prostaglandin concentrations (64-66).

It is also possible that the lower progesterone concentrations in early gestation in UN ewes could affect fetal development in other ways. Kleemann *et al* have demonstrated that only 3 days of progesterone treatment around the time of conception in sheep can dramatically alter local hormone production and thus embryonic, fetal and placental growth (22, 23). In human pregnancies the appearance of pinopodes, morphological structures thought to be important in blastocyst attachment and uterine recipiency, is associated with concentrations of progesterone and progesterone receptors (67-69). In early gestation progesterone is produced by the corpus luteum and, in sheep, this has shown to be regulated in part by oPL (24, 25, 70). Luteal progesterone production has also been shown to be reduced by nutritional restriction in pigs (20).

It is clear that there may be complex interactions between maternal nutrition and the maternal hormonal environment, both systemic and local, around the time of conception that could have profound effects on the development of the embryo and placenta. Our data have demonstrated that periconceptional undernutrition alters development of the fetal HPAA many months later in a manner that is consistent with accelerated maturation. Our previous finding of preterm birth following periconceptional undernutrition in a similar cohort of sheep (19), supports this conclusion. Further investigations are necessary to determine the mechanisms by which these changes occur, the stage of development at which they occur, and also whether they have significance for health in adult life.

Acknowledgements

This work was supported by the Health Research Council of New Zealand, the Canadian Institutes for Health Research and the Basser Travelling Fellowship awarded by the Royal Australasian College of Physicians (to F.H.B.).

References

1. **Liggins GC** 1994 The role of cortisol in preparing the fetus for birth. Reprod Fertil Dev 6:141-150
2. **Fowden AL, Li J, Forhead AJ** 1998 Glucocorticoids and the preparation for life after birth: are there long-term consequences of the life insurance? Proc Nutr Soc 57:113-122
3. **Liggins GC** 1968 Premature parturition after infusion of corticotrophin or cortisol into foetal lambs. J Endocrinol 42:323-329
4. **Liggins GC, Fairclough RJ, Grieves SA, Kendall JZ, Knox BS** 1973 The mechanism of initiation of parturition in the ewe. Recent Prog Hormone Res 29:111-150
5. **Challis JRG, Matthews SG, Gibb W, Lye SJ** 2000 Endocrine and paracrine regulation of birth at term and preterm. Endocr Rev 21:514-550
6. **Hennessy DP, Coghlan JP, Hardy KJ, Scoggins BA, Wintour EM** 1982 The origin of cortisol in the blood of fetal sheep. J Endocrinol 95:71-9
7. **Benediktsson R, Lindsay RS, Noble J, Seckl JR, Edwards CRW** 1993 Glucocorticoid exposure *in utero:* a new model for adult hypertension. Lancet 341:339-341
8. **Welberg LAM, Seckl JR** 2001 Prenatal stress, glucocorticoids and the programming of the brain. J Neuroendocrinol 13:113-128
9. **Sloboda DM, Moss TJ, Gurrin LC, Newnham JP, Challis JR** 2002 The effect of prenatal betamethasone administration on postnatal ovine hypothalamic-pituitary-adrenal function. J Endocrinol 172:71-81

10. **Dean F, Yu C, Lingas R, Matthews SG** 2001 Prenatal glucocorticoid modifies hypothalamo-pituitary-adrenal regulation in prepubertal guinea pigs. Neuroendocrinology 73:194-202

11. **Dodic M, May CN, Wintour EM, Coghlan JP** 1998 An early prenatal exposure to excess glucocorticoid leads to hypertensive offspring in sheep. Clin Sci 94:149-155

12. **Bloomfield FH, Oliver MH, Giannoulias CD, Gluckman PD, Harding JE, Challis JR** 2003 Brief undernutrition in late-gestation sheep programs the hypothalamic-pituitary-adrenal axis in adult offspring. Endocrinology 144:2933-2940

13. **Lingas R, Matthews SG** 2001 A short period of maternal nutrient restriction in late gestation modifies pituitary-adrenal function in adult guinea pig offspring. Neuroendocrinology 73:302-311

14. **Langley-Evans SC, Phillips GJ, Benediktsson R, Gardner DS, Edwards CR, Jackson AA, Seckl JR** 1996 Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. Placenta 17:169-172

15. **Edwards LJ, Coulter CL, Symonds ME, McMillen IC** 2001 Prenatal undernutrition, glucocorticoids and the programming of adult hypertension. Clin Exp Pharmacol Physiol 28:938-941

16. **Lesage J, Bondeau B, Grino M, Breant B, Dupouy JP** 2001 Maternal undernutrition during late gestation induces fetal overexposure to glucocorticoids and intrauterine growth retardation, and disturbs the hypothalamo-pituitary-adrenal axis in the newborn rat. Endocrinology 142:1692-1702

17. **Bispham J, Gopalakrishnan GS, Dandrea J, Wilson V, Budge H, Keisler DH, Broughton Pipkin F, Stephenson T, Symonds ME** 2003 Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. Endocrinology 144:3575-3585

18. **Oliver MH, Hawkins P, Breier B, van Zijl PL, Sargison S, Harding JE** 2001 Maternal undernutrition during the periconceptual period increases plasma taurine levels and insulin response to glucose but not arginine in the late gestational fetal sheep. Endocrinology 142:4576-4579

19. **Bloomfield FH, Oliver MH, Hawkins P, Campbell M, Phillips DJ, Gluckman PD, Challis JRG, Harding JE** 2003 A periconceptional nutritional origin for non-infectious preterm birth. Science 300:606

20. **Mao J, Treacy BK, Almeida RCL, Novak S, Dixon WT, Foxcroft GR** 2001 Feed restriction and insulin treatment affect subsequent luteal function in the immediate postovulatory period in pigs: progesterone production *in vivo* and messenger ribonucleic acid expression for key steroidogenic enzymes. Biol Reprod 64:359-367

21. **Guillomot M** 1995 Cellular interactions during implantation in domestic ruminants. J Reprod Fertil Suppl 49:39-51

22. **Kleemann DO, Walker SK, Hartwich KM, Fong L, Seamark RF, Robinson JS, Owens JA** 2001 Fetoplacental growth in sheep administered progesterone during the first three days of pregnancy. Placenta 22:14-23

23. **Kleemann DO, Walker SK, Seamark RF** 1994 Enhanced fetal growth in sheep administered progesterone during the first three days of pregnancy. J Reprod Fertil 102:411-7

24. **Wierzchos E, Gregoraszczuk EL, Zieba D, Murawski M, Gertler A** 2000 Placental lactogen not growth hormone and prolactin regulates secretion of progesterone in vitro by the 40-45 day ovine corpus luteum of pregnancy. Folia Biol (Krakow) 48:19-24

25. **Gregoraszczuk EL, Zieba D, Wierzchos E, Murawski M, Gertler A** 2000 Pacental lactogen as a regulator of luteal cells function during pregnancy in sheep. Theriogenology 53:877-885

26. **Oliver MH, Harding JE, Breier BH, Evans PC, Gluckman PD** 1992 The nutritional regulation of circulating placental lactogen in fetal sheep. Pediatr Res 31:520-523

27. **Brinsmead MW, Bancroft BJ, Thorburn GD, Waters MJ** 1981 Fetal and material ovine placental lactogen during hyperglycaemia, hypoglycaemia and fasting. J Endocrinol 90:337-43

28. **Noel S, Herman A, Johnson GA, Gray CA, Stewart MD, Bazer FW, Gertler A, Spencer TE** 2003 Ovine placental lactogen specifically binds to endometrial glands of the ovine uterus. Biol Reprod 68:772-80

29. **Stewart MD, Johnson GA, Gray CA, Burghardt RC, Schuler LA, Joyce MM, Bazer FW, Spencer TE** 2000 Prolactin receptor and uterine milk protein expression in the ovine endometrium during the estrous cycle and pregnancy. Biol Reprod 62:1779-89

30. **Schoknecht PA, Nobrega SN, Petterson JA, Ehrhardt RA, Slepetis R, Bell AW** 1991 Relations between maternal and fetal plasma concentrations of placental lactogen and placental and fetal weights in well-fed ewes. J Anim Sci 69:1059-1063

31. **Grumbach MM, Kaplan SL, Sciarra JJ, Burr IM** 1968 Chorionic growth hormone-prolactin (CGP): secretion, disposition, biologic activity in man, and postulated function as the "growth hormone" of the 2d half of pregnancy. Ann N Y Acad Sci 148:501-31

32. **Ryan EA, Enns L** 1988 Role of gestational hormones in the induction of insulin resistance. J Clin Endocrinol Metab 67:341-7

33. **Thordarson G, McDowell GH, Smith SV, Iley S, Forsyth IA** 1987 Effects of continuous intravenous infusion of an ovine placental extract enriched in placental lactogen on plasma hormones, metabolites and metabolite biokinetics in non-pregnant sheep. J Endocrinol 113:277-83

34. **Wheaton JE, Carlson KM, Windels HF, Johnston LJ** 1993 CIDR: A new progesterone-releasing intravaginal device for induction of estrus and cycle control in sheep and goats. Anim Reprod Sci 33:127-141

35. **Jeffray TM, Matthews SG, Hammond GL, Challis JRG** 1998 Divergent changes in plasma ACTH and pituitary POMC mRNA after cortisol administration to the late gestation ovine fetus. Am J Physiol 274:E417-E425

36. **Matthews SG, Han X, Lu F, Challis JRG** 1994 Developmental changes in the distribution of pro-opiomelanocortin and prolactin mRNA in the pituitary of the ovine fetus and lamb. J Mol Endocrinol 13:175-185

37. **Gossard FJ, Chang AC, Cohen SN** 1986 Sequence of the cDNA encoding porcine pro-opiomelanocortin. Biochim Biophys Acta 866:68-74

38. **Dai G, Smeekens SP, Steiner DF, McMurtry JP, Kwok SC** 1995 Characterization of multiple prohormone convertase PC1/3 transcripts in porcine ovary. Biochim Biophys Acta 1264:1-6

39. **Seidah NG, Fournier H, Boileau G, Benjannet S, Rondeau N, Chretien M** 1992 The cDNA structure of the porcine pro-hormone convertase PC2 and the comparative processing by PC1 and PC2 of the N-terminal glycopeptide segment of porcine POMC. FEBS Lett 310:235-9

40. **Yang K, Hammond GL, Challis JR** 1992 Characterization of an ovine glucocorticoid receptor cDNA and developmental changes in its mRNA levels in the fetal sheep hypothalamus, pituitary gland and adrenal. J Mol Endocrinol 8:173-80

41. **Matthews SG, Challis JRG** 1995 Regulation of CRH and AVP mRNA in the developing ovine hypothalamus: effects of stress and glucocorticoids. Am J Physiol 268:E1096-E1107

42. **Matthews SG, Heavens RP, Sirinathsinghji DJ** 1991 Cellular localization of corticotropin releasing factor mRNA in the ovine brain. Brain Res Mol Brain Res 11:171-6

43. **Cepoi D, Sutton S, Arias C, Sawchenko P, Vale WW** 1999 Ovine genomic urocortin: cloning, pharmacologic characterization, and distribution of central mRNA. Brain Res Mol Brain Res 68:109-18

44. **Liggins GC** 1969 Premature delivery of foetal lambs infused with glucocorticoids. J Endocrinol 45:515-523

45. **Glickman JA, Challis JR** 1980 The changing response pattern of sheep fetal adrenal cells throughout the course of gestation. Endocrinology 106:1371-6

46. **Wintour EM** 1984 Developmental aspects of the hypothalamic-pituitary-adrenal axis. J Dev Physiol 6:291-9

47. **Tangalakis K, Coghlan JP, Connell J, Crawford R, Darling P, Hammond VE, Haralambidis J, Penschow J, Wintour EM** 1989 Tissue distribution

and levels of gene expression of three steroid hydroxylases in ovine fetal adrenal glands. Acta Endocrinol (Copenh) 120:225-32

48. **Tangalakis K, Coghlan JP, Crawford R, Hammond VE, Wintour EM** 1990 Steroid hydroxylase gene expression in the ovine fetal adrenal gland following ACTH infusion. Acta Endocrinol (Copenh) 123:371-7

49. **Wintour EM, Coghlan JP, Hardy KH, Hennessy DP, Lingwood BE, Scoggins BA** 1980 Adrenal corticosteroids and immunoreactive ACTH in chronically cannulated ovine foetuses with bilateral adrenalectomy. Acta Endocrinol (Copenh) 95:546-52

50. **Green LR, Kawagoe Y, Fraser M, Challis JRG, Richardson BS** 2000 Activation of the hypothalamic-pituitary-adrenal axis with repetitive umbilical cord occlusion in the preterm ovine fetus. J Soc Gynecol Invest 7:224-232

51. **Braems GA, Matthews SG, Challis JRG** 1996 Differential regulation of proopiomelanocortin messenger ribonucleic acid in the pars distalis and pars intermedia of the pituitary gland after prolonged hypoxemia in fetal sheep. Endocrinology 137:2731-2738

52. **Perry RA, Mulvogue HM, McMillen IC, Robinson PM** 1985 Immunohistochemical localization of ACTH in the adult and fetal sheep pituitary. J Dev Physiol 7:397-404

53. **Butler TG, Schwartz J, McMillen IC** 2002 Differential effects of the early and late intrauterine environment on corticotrophic cell development. J Clin Invest 110:783-791

54. **Holloway AC, Howe DC, Chan G, Clifton VL, Smith R, Challis JR** 2002 Urocortin: a mechanism for the sustained activation of the HPA axis in the late-gestation ovine fetus? Am J Physiol Endocrinol Metab 283:E165-71

55. **Petraglia F, Florio P, Benedetto C, Marozio L, Di Blasio AM, Ticconi C, Piccione E, Luisi S, Genazzani AR, Vale W** 1999 Urocortin stimulates placental adrenocorticotropin and prostaglandin release and myometrial contractility in vitro. J Clin Endocrinol Metab 84:1420-3

56. **Florio P, Arcuri F, Ciarmela P, Runci Y, Romagnoli R, Cintorino M, Di Blasio AM, Petraglia F** 2002 Identification of urocortin mRNA and peptide in the human endometrium. J Endocrinol 173:R9-14

57. **Petraglia F, Florio P, Gallo R, Simoncini T, Saviozzi M, Di Blasio AM, Vaughan J, Vale W** 1996 Human placenta and fetal membranes express human urocortin mRNA and peptide. J Clin Endocrinol Metab 81:3807-10

58. **Munemura M, Cote TE, Tsuruta K, Eskay RL, Kebabian JW** 1980 The dopamine receptor in the intermediate lobe of the rat pituitary gland: pharmacological characterization. Endocrinology 107:1676-83

59. **Edwards CRW, Benediktsson R, Lindsay RS, Seckl JR** 1993 Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension? Lancet 341:355-357

60. **Keenan DM, McManus WR, Scoggins BA, Myers K, Freer M** 1968 The effect of undernutrition on the activity of the adrenal cortex of Merino wethers. Res Vet Sci 9:573-577

61. **Mulinos MG, Pomerantz L** 1942 Pseudo-hypophysectomy. A condition resembling hypophysectomy produced by malnutrition. Endocrinology 26:493-504

62. **Jackson CM** 1932 Structural changes when growth is suppressed by undernourishment in the albino rat. Am J Anat 51:347-379

63. **Gatford KL, Wintour EM, De Blasio MJ, Owens JA, Dodic M** 2000 Differential timing for programming of glucose homoeostasis, sensitivity to insulin and blood pressure by in utero exposure to dexamethasone in sheep. Clin Sci (Lond) 98:553-560

64. **Young IR, Deayton JM, Hollingworth SA, Thorburn GD** 1996 Continuous intrafetal infusion of prostaglandin E2 prematurely activates the hypothalamo-pituitary-adrenal axis and induces parturition in sheep. Endocrinology 137:2424-31

65. **McKeown KJ, Challis JR, Small C, Adamson L, Bocking AD, Fraser M, Rurak D, Riggs KW, Lye SJ** 2000 Altered fetal pituitary-adrenal function in the ovine fetus treated with RU486 and meloxicam, an inhibitor of prostaglandin synthase-II. Biol Reprod 63:1899-904

66. **Unno N, Wu WX, Wong CH, Bennett PR, Shinozuka N, Nathanielsz PW** 1998 Prostaglandin regulation of fetal plasma adrenocorticotropin and cortisol concentrations in late-gestation sheep. Biol Reprod 58:514-9

67. **Stavreus-Evers A, Nikas G, Sahlin L, Eriksson H, Landgren BM** 2001 Formation of pinopodes in human endometrium is associated with the concentrations of progesterone and progesterone receptors. Fertil Steril 76:782-791

68. **Develioglu OH, Hsiu JG, Nikas G, Toner JP, Oehninger S, Jones HW, Jr.** 1999 Endometrial estrogen and progesterone receptor and pinopode expression in stimulated cycles of oocyte donors. Fertil Steril 71:1040-7

69. **Nikas G, Develioglu OH, Toner JP, Jones HWJ** 1999 Endometrial pinopodes indicate a shift in the window of receptivity in IVF cycles. Hum Reprod 14:787-792

70. **Gregoraszczuk EL, Gertler A, Futoma E** 2000 Effect of exogenous ovine placental lactogen on basal and prostaglandin-stimulated progesterone production by porcine luteal cells. Acta Vet Hung 48:199-208

FIGURE LEGENDS FOR FIGURES WITH BAR GRAPHS

FIG. 1. ACTH (*top*) and cortisol (*bottom*) responses to a synacthen ($ACTH_{1-24}$) challenge. *Inset histograms* represent AUC. Values are mean ± SEM. The *arrow* denotes the time of ACTH injection. ● and *black bars*, UN (n = 12). □ and *white bars*, N (n = 10).

FIG. 2. Cortisol (*top*), 11 deoxycortisol (*middle*) and ACTH (*bottom*) responses to a metyrapone challenge. *Inset histograms* represent AUC. Values are mean ± SEM. The *arrow* denotes the time of metyrapone injection. ● and *black bars*, UN (n = 12). □ and *white bars*, N (n = 10). $^{*}P < 0.05$ *vs* N fetuses, $^{\dagger}P < 0.05$ *vs* baseline cortisol value.

FIG. 3. Representative *in situ* hybridization images with semi-quantitative densitometric analysis (*histograms*) of CRH and AVP mRNA levels in the paraventricular nucleus (PVN) of the hypothalamus (*top*) and of POMC, PC-1, PC-2 and GR in the pituitary (*bottom*). Signal in the pituitary was analysed separately for the *pars distalis* (PD) and the *pars intermedia* (PI). Values are mean ± SEM. *Left column* and *white bars*, N (n = 9). *Middle column* and *black bars*, UN (n = 9). *Right column,* sense controls. $^{*}P < 0.05$ *vs* N.

FIG. 4. Maternal circulating cortisol (*top left*), ACTH (*top right*), progesterone (*bottom left*) and ovine placental lactogen (*bottom right*) concentrations. Values are mean ± SEM. T he *black bar* represents the period of undernutrition. ●, UN ewes (n = 12). □, N ewes (n = 10). $^{***}P < 0.001$ *vs* N ewes.

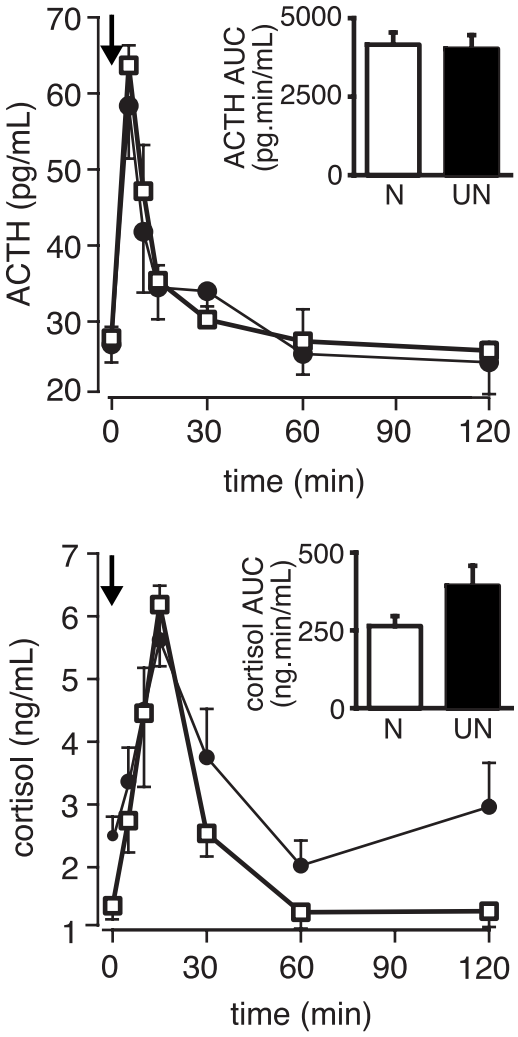
ALTERNATIVE FIGURE LEGENDS FOR FIGURES WITH POINT GRAPHS

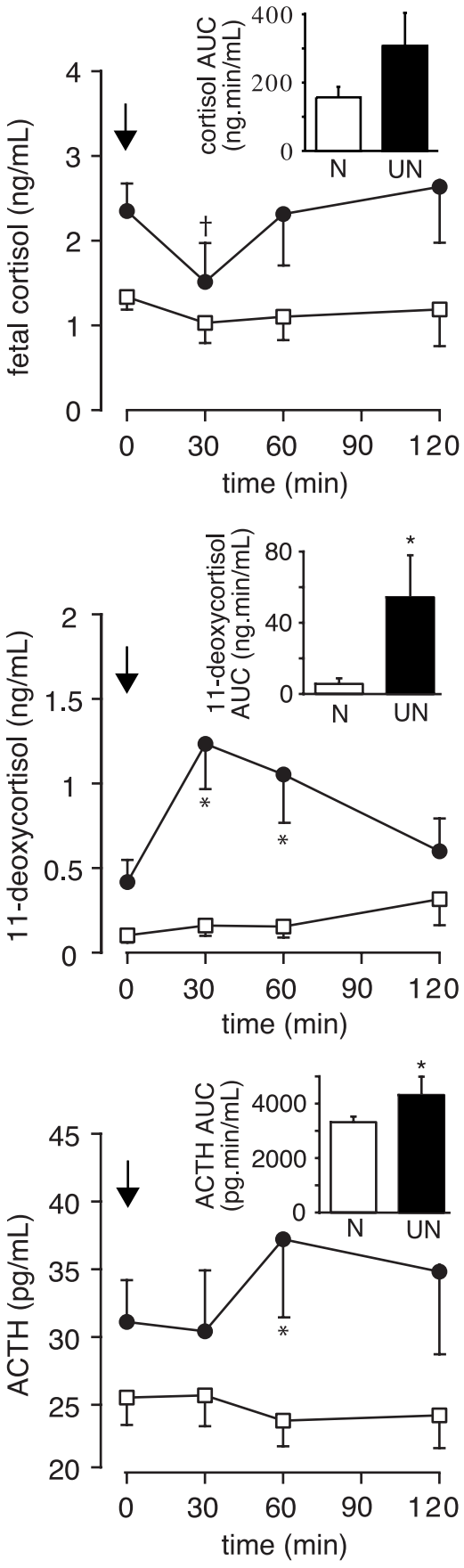
FIG. 1. ACTH (*top*) and cortisol (*bottom*) responses to a synacthen ($ACTH_{1-24}$) challenge. Values are mean ± SEM. The *arrow* denotes the time of ACTH injection. *Inset graphs* represent area under the curve for individual animals with the mean represented by a line. ●, UN (n = 12). ☐, N (n = 10).

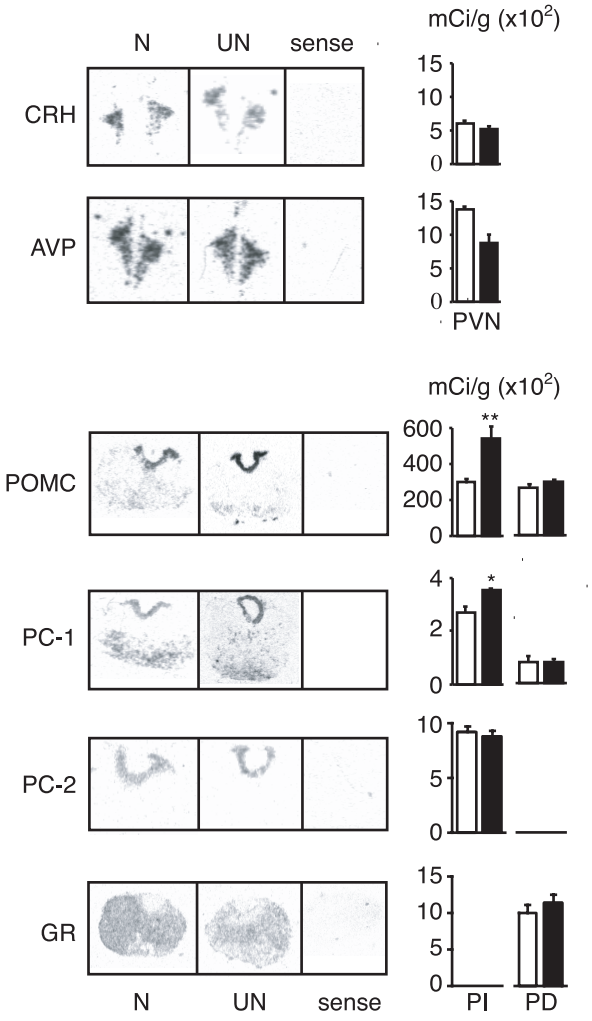
FIG. 2. Cortisol (*top*), 11 deoxycortisol (*middle*) and ACTH (*bottom*) responses to a metyrapone challenge. Values are mean ± SEM. The *arrow* denotes the time of metyrapone injection. *Inset graphs* represent area under the curve for individual animals with the median represented by a line. ●, UN (n = 12). ☐, N (n = 10). $^{*}P <$ 0.05 *vs* N fetuses, † $P < 0.05$ *vs* baseline cortisol value.

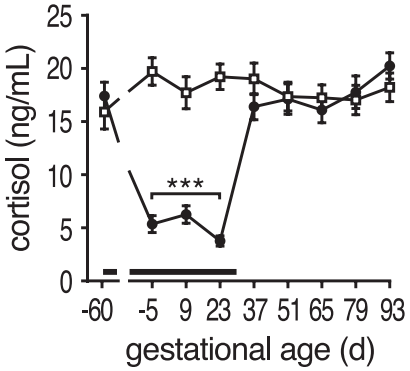
FIG. 3. Representative *in situ* hybridization images with semi-quantitative densitometric analysis (*point graphs*) of CRH and AVP mRNA levels in the paraventricular nucleus (PVN) of the hypothalamus (*top*) and of POMC, PC-1, PC-2 and GR in the pituitary (*bottom*). Signal in the pituitary was analysed separately for the *pars distalis* (PD) and the *pars intermedia* (PI). In *point graphs* individual animals are shown with medians. *Left column* and ☐, N (n = 9). *Middle column* and ●, UN (n = 9). *Right column,* sense controls. $^{*}P < 0.05$ *vs* N.

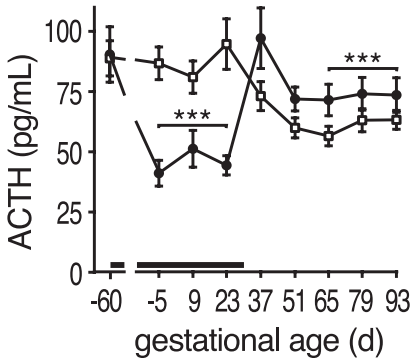
FIG. 4. Maternal circulating cortisol (*top left*), ACTH (*top right*), progesterone (*bottom left*) and ovine placental lactogen (*bottom right*) concentrations. Values are mean ± SEM. T he *black bar* represents the period of undernutrition. ●, UN ewes (n = 12). ☐, N ewes (n = 10). $^{***}P < 0.001$ *vs* N ewes.

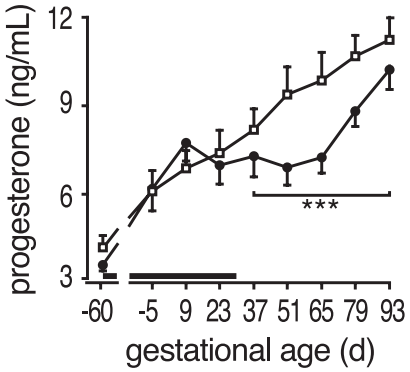

70
60
50
40
30
20
ACTH (pg/mL)
0
30
60
90
120
time (min)
5000
2500
0
ACTH AUC
(pg.min/mL)
N
UN
7
6
5
4
3
2
1
cortisol (ng/mL)
0
30
60
90
120
time (min)
500
250
0
cortisol AUC
(ng.min/mL)
N
UN

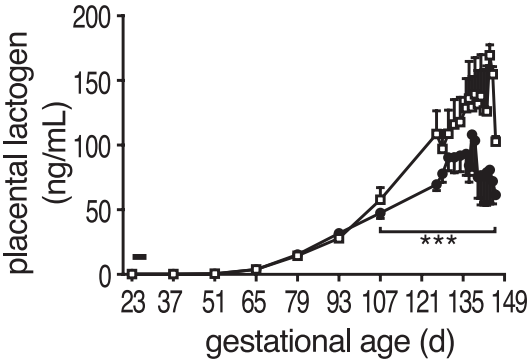

fetal cortisol (ng/mL)
4
3
2
1
0
0
30
60
90
120
time (min)
+
cortisol AUC (ng.min/mL)
400
200
0
N
UN
11-deoxycortisol (ng/mL)
2
1.5
1
0.5
0
*
*
11-deoxycortisol AUC (ng.min/mL)
80
40
0
*
N
UN
ACTH (pg/mL)
45
40
35
30
25
20
*
ACTH AUC (pg.min/mL)
4000
2000
0
*
N
UN

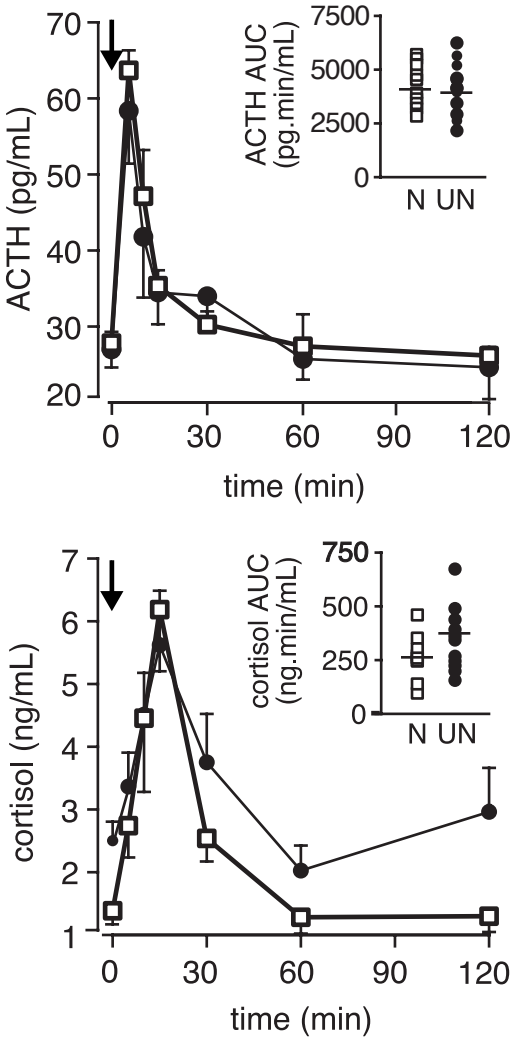

N
UN
sense
CRH
AVP
mCi/g (x10²)
15
10
5
0
PVN
POMC
PC-1
PC-2
GR
600
400
200
4
2
**
*
PI
PD

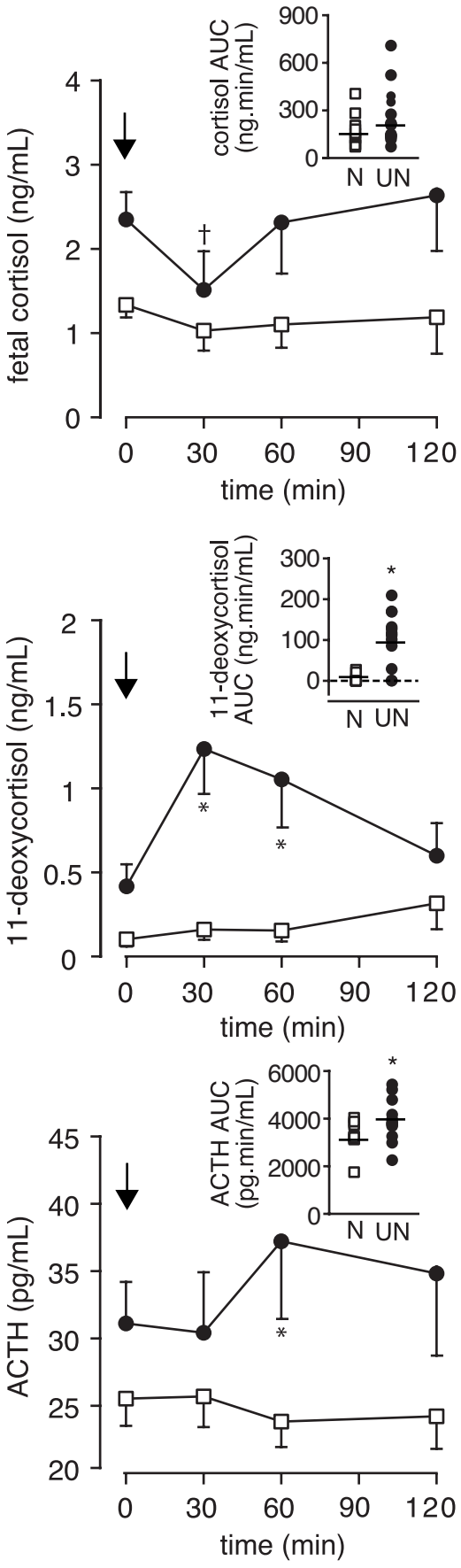

cortisol (ng/mL)
25
20
15
10
5
0
***
-60 -5 9 23 37 51 65 79 93
gestational age (d)

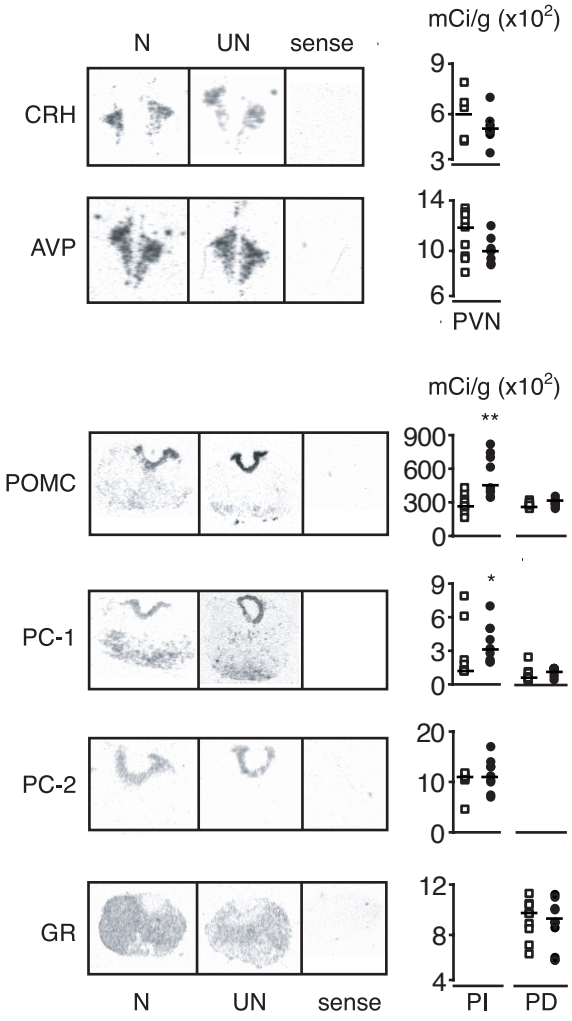

ACTH (pg/mL)
100
75
50
25
0
***
***
-60 -5 9 23 37 51 65 79 93
gestational age (d)


progesterone (ng/mL)
12
9
6
3
***
-60 -5 9 23 37 51 65 79 93
gestational age (d)


placental lactogen (ng/mL)
200
150
100
50
0
***
23 37 51 65 79 93 107 121 135 149
gestational age (d)


ACTH (pg/mL)
70
60
50
40
30
20
0
30
60
90
120
time (min)
ACTH AUC
(pg.min/mL)
7500
5000
2500
0
N UN
cortisol (ng/mL)
7
6
5
4
3
2
1
0
30
60
90
120
time (min)
cortisol AUC
(ng.min/mL)
750
500
250
0
N UN


fetal cortisol (ng/mL)
4
3
2
1
0
0
30
60
90
120
time (min)
+
cortisol AUC (ng.min/mL)
900
600
300
0
N
UN
11-deoxycortisol (ng/mL)
2
1.5
1
0.5
0
*
*
11-deoxycortisol AUC (ng.min/mL)
300
200
100
0
*
N
UN
ACTH (pg/mL)
45
40
35
30
25
20
*
ACTH AUC (pg.min/mL)
6000
4000
2000
0
*
N
UN


N
UN
sense
CRH
AVP
mCi/g (x10²)
9
6
3
14
10
6
PVN
POMC
PC-1
PC-2
GR
mCi/g (x10²)
900
600
300
0
**
9
6
3
0
*
20
10
0
12
8
4
N
UN
sense
PI
PD