0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(7):2933–2940

doi: 10.1210/en.2003-0189

Brief Undernutrition in Late-Gestation Sheep Programs the Hypothalamic-Pituitary-Adrenal Axis in Adult Offspring

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Reduced size at birth in humans has been associated with altered function of the hypothalamic-pituitary-adrenal (HPA) axis in childhood and adult life. Experimentally, maternal undernutrition has also been associated with altered fetal HPA function. However, the relationship between birth size, fetal nutrition, and adult pathophysiology is not clear. We recently have reported that glucose tolerance, blood pressure, and IGF-I levels in adult sheep were more closely associated with birth weight than with nutritional insult in late gestation or with current weight. Here, we report adult HPA function in the same group of animals.

Pregnant ewes were severely undernourished for 10 d (UN10) or 20 d (UN20) from 105 d gestation (term, 146 d), or were *ad libitum*-fed controls. At 30 months, female offspring were subjected to an insulin tolerance test and a CRH plus arginine vasopressin (AVP) challenge. UN20 lambs were lighter at birth, but there were no significant differences in weight at 30 months. Adult UN10 ewes had an increased ACTH response to both CRH+AVP challenge and insulin tolerance test, but no differences in cortisol response. UN10 ewes also demonstrated elevated 11-deoxycortisol concentrations, but lower progesterone concentrations, in response to CRH+AVP challenge. In contrast, the responses of UN20 ewes to these challenges were not different from *ad libitum* controls. Protein levels of $P450_{c17}$ and $P450_{11\beta 1}$ were not significantly different among groups.

We conclude that brief maternal undernutrition for 10 d, but not 20 d, in late gestation alters HPA function in adult offspring. In contrast to our previous findings, these HPA effects are independent of birth weight and current weight, suggesting that different mechanisms may be involved in programming different physiological axes. (*Endocrinology* 144: 2933–2940, 2003)

THE FETAL ORIGINS of adult disease hypothesis was developed around epidemiological observations that predisposition to a number of adult diseases, such as cardiovascular disease, stroke, type II diabetes, and Syndrome X, is associated with decreasing birth weight across the normal birth weight spectrum (1). It has been proposed that an adverse fetal environment, in which fetal nutrient demand outstrips supply, leads to permanent alterations in fetal homeostatic mechanisms (2, 3). Thus, small size at birth may not be causally linked to postnatal disease risk. Rather, birth size may be a surrogate marker for underlying events that result both in reduced birth weight and in programming of postnatal physiology. Consistent with this, we have shown previously that lambs may be born of similar size following quite different intrauterine growth trajectories in response to maternal undernutrition (4, 5). We, and others, have also shown that maternal nutritional insults that have no effect on birth weight can have pronounced effects on fetal metabolism (6), blood pressure (7), and hormonal responses (8), including alterations in fetal hypothalamic-pituitary-adrenal (HPA) axis function (9, 10).

One mechanism by which maternal undernutrition may influence both fetal growth and homeostasis is via altered HPA function. Maternal undernutrition results in increased circulating cortisol concentrations in pregnant sheep (11). Protein undernutrition in rat dams reduces placental activity of 11β-hydroxysteroid dehydrogenase (HSD) type II, which converts active cortisol to inactive cortisone, thus protecting the fetus from maternal circulating glucocorticoids (12). Exposure of the fetus to excess glucocorticoids, either by maternal administration of synthetic glucocorticoids that are not substrates for 11β-HSD II or by inhibiting the activity of 11β-HSD II with carbenoxolone, has been shown to reduce fetal growth and result in elevated blood pressure, altered HPA function, and altered glucose homeostasis in the offspring (13–18). Thus maternal undernutrition may result in fetal programming at least in part via perturbation of the maternal and fetoplacental HPA axes.

Alterations in HPA function have also been reported in humans born small. Low birth weight is associated with increased urinary glucocorticoid excretion in children (19) and with elevated basal plasma cortisol concentrations (20) and greater adrenocortical responsiveness to ACTH in adults (21). This altered HPA function has in turn been proposed to play an important role in programming of disease risk, be-

Abbreviations: ACTH-R, ACTH receptor; AUC, area under the curve; AVP, arginine vasopressin; CBC, cortisol binding capacity; CI, confidence interval(s); CV, coefficient(s) of variation; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; HSD, 11β-hydroxysteroid dehydrogenase; ITT, insulin tolerance test; M_W, molecular weight; UN10, undernourished for 10 d; UN20, undernourished for 20 d.

cause persistently altered HPA function may itself contribute to the pathogenesis of diseases such as hypertension and diabetes (20).

The aims of this study were therefore to investigate the effects of a maternal nutritional insult, birth weight, and current weight on HPA function in adult offspring of undernourished ewes. It has been shown previously that 10 d of maternal undernutrition in late gestation results in slowing of fetal growth that resumes upon refeeding, whereas after 19 d undernutrition the slower growth appears to be irreversible (22). We therefore studied two nutritional groups, one undernourished for 10 d (UN10) and one for 20 d (UN20), in an attempt to further clarify the relative importance of the nutritional insult *vs.* fetal growth. We have recently reported that adult glucose tolerance, blood pressure, and IGF-I levels were more strongly related to birth weight than to nutritional insult in this flock of ewes (23). However, if fetal undernutrition programs the fetal HPA axis, which itself plays a role in reduced fetal growth and also in programming of other organ systems, then effects in the HPA axis may be more closely related to the nutritional insult than to birth weight. We therefore hypothesized that maternal undernutrition in late gestation would result in altered HPA function in adult offspring, and that this altered function would be related to the nutritional insult independent of size at birth.

Materials and Methods

Romney ewes were mated with Dorset rams following synchronization of estrus with the use of intravaginal progesterone release devices (24). Only ewes found to be carrying singleton lambs by ultrasound at 60 d gestation were used for these experiments. Throughout early and midgestation, ewes remained on pasture but received 100–200 g of feed concentrates daily (Multifeed sheep nuts, NRM Ltd., Newmarket, Auckland, New Zealand) as a supplement and also to acclimatize them to this feed. The concentrate diet had 10 MJ/kg metabolizable energy and 12.9% crude protein. At 95 d gestation, ewes were placed in separate indoor pens, fed the concentrate diet, and allowed free access to water and barley straw of low nutritional value. After 10 d acclimatization, ewes were allocated to treatment groups using random number tables. Control ewes were fed *ad libitum* (13–15 MJ/d) from 105 d, whereas restricted ewes were undernourished for either 10 d (UN10, 0.3 MJ/d until refeeding at 115 d) or 20 d (UN20, 0.3 MJ/d until 115 d then 0.5 MJ/d until refeeding at 125 d). Barley straw rations were increased during periods of restriction to provide bulk and maintain rumen function. At 125 d, ewes were returned to supplemented pasture feeding in preparation for lambing. Ewes were bred in three groups, with the three treatment groups drawn evenly from among the breeding groups.

Soon after birth, lambs (females, n = 13 per group) were ear tagged, weighed, and measured. They were then returned to pasture with their mothers. Because of the logistical difficulties of keeping a large flock of uncastrated rams on the farm, the rams were killed at 22 months of age. At 30 months of age, female offspring (n = 11 per group) were bought back into the laboratory for testing of HPA function after synchronization of estrus with the use of intravaginal progesterone release devices to ensure that changes in circulating steroid hormones due to individual variations in the estrous cycle did not confound interpretation of the studies. Ewes were housed in individual metabolic cages with free access to food and water, in a laboratory with temperature control (16 ± 1 C; humidity, 50 ± 10%) and a 12-h light, 12-h dark cycle. After 1-wk acclimatization, ewes were weighed and fasted overnight before surgery. Under general anesthesia (2% halothane in O_2), catheters were placed in a femoral artery and vein via the tarsal vessels. Intramuscular antibiotics were administered perioperatively (5 ml streptopen, 250 mg procaine penicillin/250 mg dihydrostreptomycin sulfate, Pittman Moore Ltd., Upper Hutt, New Zealand). Two weeks after surgery, HPA response to physiological stress was tested by an insulin tolerance test (ITT). Ewes were fasted overnight and then injected iv with insulin (0.15 U/kg body weight, Humulin R, Eli Lilly, Indianapolis, IN). Heparinized arterial blood samples were taken −10, 0, 2.5, 5, 7, 10, 20, 30, 40, 50, 60, 70, 80, 90, 120, 150, and 180 min from insulin injection. Ewes were refed after the completion of the sampling. All blood samples were collected on ice then centrifuged at 3000 rpm. Plasma aliquots were stored at −20 C until analysis. One week after the ITT, ewes were again fasted overnight before receiving an iv injection of 0.5 μg/kg body weight ovine CRH and 0.1 μg/kg arginine vasopressin (AVP; Bachem, Torrance, CA). The ITT and CRH+AVP tests were always done between 0800 and 0900 h to minimize any impact from circadian variations in circulating ACTH and cortisol concentrations. Arterial blood samples were taken at −10, 0, 10, 20, 30, 60, 90, 120, 180, and 240 min after CRH+AVP injection and handled as above. After completion of the CRH+AVP challenge, ewes were euthanized with an overdose of pentobarbitone, and the adrenal glands were frozen over dry ice. Experiments were approved by the Animal Ethics Committee of the University of Auckland.

RIAs

Cortisol was measured in duplicate by RIA after extraction of plasma samples with diethyl ether (25). Briefly, cortisol was extracted from 100 μl plasma with 4 ml of diethyl ether with an efficiency of more than 95%. After reconstitution with PBS and gelatin, samples were incubated overnight at 4 C in the presence of cortisol antibody and ^{3}H-cortisol (Amersham Life Sciences, Arlington Heights, IL). The cortisol antibody was generated in this laboratory and had cross-reactivities as follows: less than 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'Urfé, Québec, Canada; Norit A, Fisher Scientific, Toronto, Ontario, Canada), and the supernatant was mixed with 4 ml scintillation cocktail and counted. Intra- and interassay coefficients of variation (CV) were 5.1 and 7.2%, respectively. ACTH was measured by a commercial RIA kit (DiaSorin, Inc., Stillwater, MN) previously validated for use in the sheep (26) with intra- and interassay CV of 8.6 and 6.9%, respectively. Androstenedione and 11-deoxycortisol were measured by commercial coated tube (androstenedione, Diagnostic Systems Laboratories Inc., Webster, TX) or double antibody (11-deoxycortisol, ICN Pharmaceuticals, Inc., Costa Mesa, CA) RIA kits after extraction and 3-fold concentration of plasma samples with diethyl ether. Minimal detectable concentrations were 0.011 and 0.016 ng/ml, respectively, and the interassay CV were 9.2 and 9.3%, respectively.

Cortisol binding capacity (CBC) was measured by a modification of the saturation binding assay (27, 28). In brief, duplicate 50-μl aliquots of plasma were added to 10 × 75 mm borosilicate tubes containing 10,000 cpm [^{3}H]cortisol and 16 ng cold cortisol. Additional duplicates were aliquoted as above into tubes containing excess (1 μg) cold cortisol. Tubes were incubated at 37 C for 30 min, followed by 12–18 h at 4 C. Bound and free cortisol were separated using dextran-coated charcoal in tricine buffer [0.15 M (pH 7.4)]. Tubes were centrifuged at 1500 × *g* for 10 min at 4 C, and 100 μl of the supernatant was added to 4 ml scintillation fluid and counted. This assay does not measure cortisol binding to albumin because this complex dissociates rapidly after addition of charcoal, and the unbound cortisol is adsorbed and precipitated with the charcoal upon centrifugation.

CBC was calculated from the percentage of bound [^{3}H]cortisol minus percentage of nonspecific binding, multiplied by the total [cortisol] (endogenous in 50 μl plasma plus unlabeled cold in each tube plus [^{3}H]cortisol added) and corrected to 1 ml plasma. Inter- and intraassay CV were 3.8 and 5.7%, respectively.

Western blot analysis

Adrenal tissue was homogenized (PT200 homogenizer, Polytron, Kinematica AG, Lucerne, Switzerland) in the presence of Complete Mini EDTA-free Protease inhibitors (Böhringer Mannheim Biochemicals, Laval, Québec, Canada), 100 μM sodium orthovanadate, and RIPA lysis buffer. After centrifugation at 15,000 × *g* at 4 C for 10 min, protein content was assayed in duplicate by the Bradford protein assay (29).

Protein standards and 100 μg protein were separated by electrophoresis on 10% polyacrylamide gels at 80 V for 20 min, then 150 V for 1 h. Control samples from an unrelated adrenal gland were loaded onto each gel. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Mississauga, Canada) at 110 V for 1.5 h at 4 C. Equal loading and transfer were confirmed by staining with S-Ponceau (Sigma, St. Louis, MO). After washes with PBS, nonspecific binding was blocked by overnight incubation with 5% skimmed milk at 4 C. Blots were then incubated with primary antibody ($P450_{C17}$ 1:300, $P450_{11\beta1}$ 1:100 dilution; Chemicon International Inc., Temecula, CA) for 1 h. After PBS plus Tween washes, blots were incubated with secondary antisera [antirabbit ($P450_{C17}$) or antimouse ($P450_{11\beta1}$) IgG horseradish peroxidase conjugate, Amersham Life Sciences] at a 1:2000 dilution. Specific protein bands were detected with the Amersham Electrochemiluminescence Detection System (ECL, Amersham Life Sciences), and the blots were exposed to film (X-Omat Blue XB-1, Eastman Kodak Co., Rochester, NY). Films were scanned into a Macintosh computer, and the signals were measured using Scion Image 1.62c Mac software (Scion Corp., Frederick, MD). For $P450_{C17}$, two bands were detected, and the major band running close to the 47.5 molecular weight (M_W) marker was analyzed. For $P450_{11\beta1}$, a single band running halfway between the 47.5 and 62 M_W markers was analyzed. All signals are expressed as optical densities relative to the control signal.

Statistical analysis

Data are presented as mean ± SEM. Nonparametric data were log-transformed. Cortisol and ACTH responses at each time point of the CRH+AVP challenge were analyzed using multiple regression analysis, with breeding group, birth weight, current weight, and treatment group as covariates. Regression coefficients for UN10 and UN20 groups against *ad libitum* were plotted with their 95% confidence intervals (CI) for each time point. Where the 95% CI did not cross zero, a significant response was found at the 5% level. Responses to the ITT were analyzed in a similar manner, with the glucose concentration at each time point added as an additional covariate. Total area under the curve (AUC) was calculated for plasma ACTH and cortisol responses to both the CRH+AVP and ITT challenges using SigmaStat (SPSS, Inc., Chicago, IL). Multiple regression analysis, using the same variables as above, was then performed with significance at the 5% level. Androstenedione and 11-deoxycortisol concentrations and the Western blots were analyzed by ANOVA with the Games-Howell *post hoc* test if a significant effect was found. Statview 5.0.1 (SAS Institute, Cary, NC) was used for all statistical analyses unless otherwise stated.

Results

UN20 lambs were lighter than *ad libitum* controls at birth (Table 1), but there were no significant differences in ewe weight at 30 months of age (Table 1). Detailed anthropomorphic data are reported elsewhere, along with descriptions of maternal feed intake during the nutritional manipulation (30). Baseline cortisol concentrations in UN10 ewes were significantly lower than in *ad libitum* controls (−2.1 ng/ml; 95% CI, −3.8 to −0.4; $P = 0.02$). Values in UN20 ewes were intermediate and were not significantly different from *ad libitum* or UN10 ewes (Table 1). Baseline cortisol concentrations were not affected by birth weight or by current weight. Baseline ACTH concentrations were not significantly different among groups, although they tended to be higher in UN10 ewes ($P = 0.06$; Table 1). Baseline ACTH concentrations decreased with increasing current weight (−0.7 pg/ml·kg current weight; 95% CI, −1.2 to −0.1; $P = 0.03$) but tended to increase with increasing birth weight (+5.7 pg/ml birth weight; 95% CI, −0.2 to 11.7; $P = 0.06$).

ACTH concentrations and AUC in response to both the CRH+AVP ($P < 0.05$; Fig. 1) and the ITT challenges ($P < 0.05$; Fig. 2) were greater in UN10 ewes than in *ad libitum* or UN20 ewes but were unaffected by birth weight and current weight. Cortisol concentrations and AUC were not significantly different among groups in response to either the CRH+AVP or ITT challenge (Figs. 1 and 2). However, cortisol AUC decreased with increasing current weight in the ITT (−25.3 ng/min/ml per kg current weight; 95% CI, −45.2 to −5.3; $P = 0.02$) with no effect of birth weight. There was no effect of either current weight or birth weight in cortisol AUC in response to the CRH+AVP challenge.

Baseline CBC was not significantly different among groups (Table 1), did not change during the CRH+AVP challenge (data not shown), and was unaffected by birth weight or current weight. Baseline progesterone concentrations were not different among groups (Table 1). In response to the CRH+AVP challenge, progesterone concentrations fell in the UN10 group ($P = 0.006$; Fig. 3, *top*). This fall was affected by both current weight and birth weight, decreasing further as current weight increased (−0.01 ng/ml·kg current weight; 95% CI, −0.01 to −0.0004; $P = 0.0001$) but increasing as birth weight increased (+0.06 ng/ml·kg birth weight; 95% CI, 0.02–0.1; $P = 0.008$). Baseline 11-deoxycortisol concentrations were higher in UN10 and UN20 ewes compared with controls ($P < 0.05$; Table 1), but were unaffected by birth weight or current weight. Androstenedione concentrations were not significantly different among groups (Table 1), and were unaffected by birth weight or current weight. Both 11-deoxycortisol and androstenedione concentrations increased during the CRH+AVP challenge (Fig. 3, *middle* and *bottom*). The 11-deoxycortisol peak concentration was higher in UN10 ewes than *ad libitum* controls, but this was not statistically significant ($P = 0.07$; Fig. 3, *middle*). However, the 11-deoxycortisol AUC was greater in UN10 ewes ($P < 0.05$; Fig. 3, *middle*). There were no significant differences between

TABLE 1. Ewe birth weight, weight at 30 months, and baseline concentrations of ACTH, CBC, and steroids

	Ad libitum	UN10	UN20
Birth weight (kg)	5.6 ± 0.2	5.1 ± 0.2	4.8 ± 0.2[a]
Weight at 30 months (kg)	74 ± 2	72 ± 2	69 ± 2
Baseline concentrations:			
Cortisol (ng/ml)	5.4 ± 0.8	3.4 ± 0.4[a]	4.6 ± 0.5
ACTH (pg/ml)	25.5 ± 2.4	31.2 ± 3.3[b]	28.8 ± 2.8
CBC (ng/ml)	15.0 ± 1.7	15.8 ± 1.9	20.4 ± 2.4
Progesterone (ng/ml)	0.41 ± 0.04	0.41 ± 0.03	0.44 ± 0.04
11-Deoxycortisol (pg/ml)	50 ± 1	57 ± 2[a]	59 ± 3[a]
Androstenedione (ng/ml)	0.47 ± 0.1	0.44 ± 0.09	0.50 ± 0.07

n = 11 per group, except for birth weight, where n = 13 per group. Values are mean ± SEM.
[a] $P < 0.05$; [b] $P = 0.06$ *vs. ad libitum*.

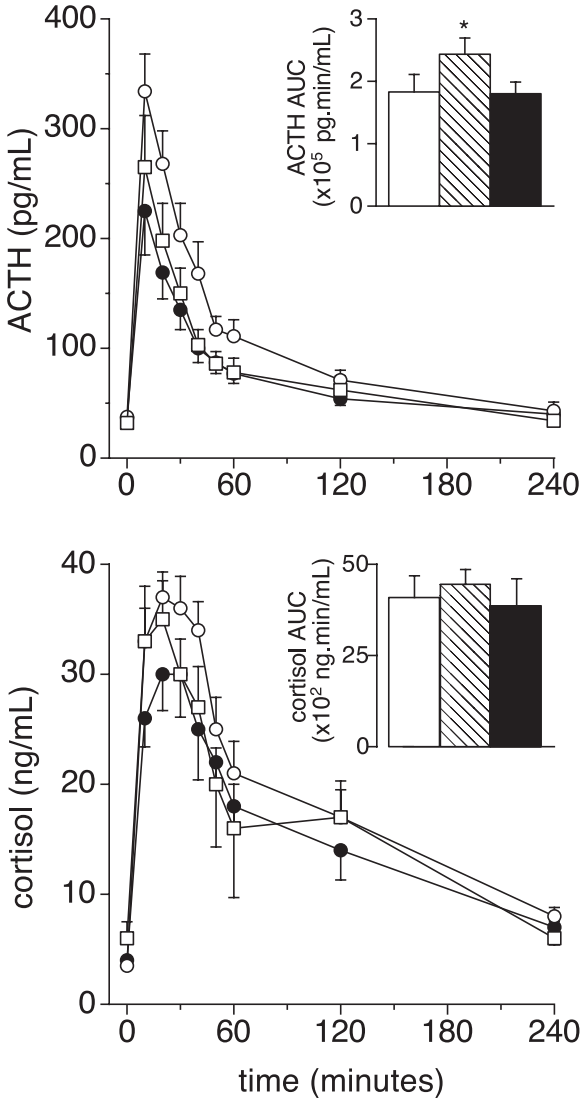

FIG. 1. ACTH (*top*) and cortisol (*bottom*) responses to the CRH+AVP challenge. *Inset histograms* represent AUC. Values are mean ± SEM; n = 11 per group. □ and *white bars*, *Ad libitum*; ○ and *hatched bars*, UN10; ● and *black bars*, UN20. *, $P < 0.05$ *vs. ad libitum* and UN20 groups.

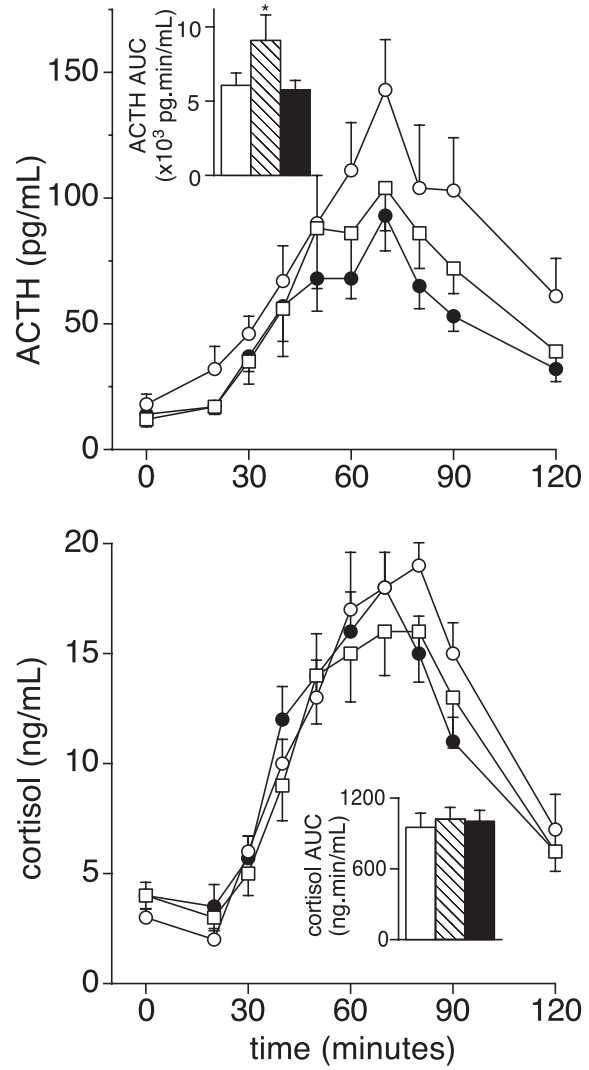

FIG. 2. ACTH (*top*) and cortisol (*bottom*) responses to an ITT. *Inset histograms* represent AUC. Values are mean ± SEM; n = 11 per group. □ and *white bars*, *Ad libitum*; ○ and *hatched bars*, UN10; ● and *black bars*, UN20. *, $P < 0.05$ *vs. ad libitum* and UN20 groups.

groups in androstenedione concentrations, and no effect of birth weight or current weight on concentrations of either 11-deoxycortisol or androstenedione.

$P450_{C17}$ and $P450_{11B1}$ protein levels measured by Western blot analysis were not significantly different among groups (Fig. 4).

Discussion

The data reported here demonstrate that a brief period of maternal undernutrition in late gestation results in altered regulation of the HPA axis in adult life. Furthermore, this effect is largely independent of birth weight and current weight. This is in contrast to our recent report of the glucose/insulin and IGF axes and blood pressure in the same animals, where birth weight, rather than nutritional group, had the greatest influence on adult physiology (23). This suggests that different metabolic and homeostatic processes are programmed via different mechanisms in fetal life.

It is already apparent that the timing of the programming insult is important in determining the fetal response. Human epidemiological data from the Dutch Hunger Winter demonstrated that famine around the time of conception and during the first half of pregnancy resulted in an increased risk in later life of glucose intolerance (31) and obesity (32), whereas exposure to famine in the latter half of pregnancy led to an increased risk of an atherogenic lipid profile and coronary heart disease (33–35). In sheep, administration of dexamethasone to the ewe for only 48 h at d 27–28 of gestation resulted in hypertension in late gestation that persisted into postnatal life, yet the same dose given at 64 d had no effect (15). Interestingly, glucose tolerance was not altered in the fetuses of ewes treated at either 27 or 64 d (36), demonstrating programming of hypertension independently of glucose tolerance. The epidemiological data from the Dutch Famine also provide evidence for independent program-

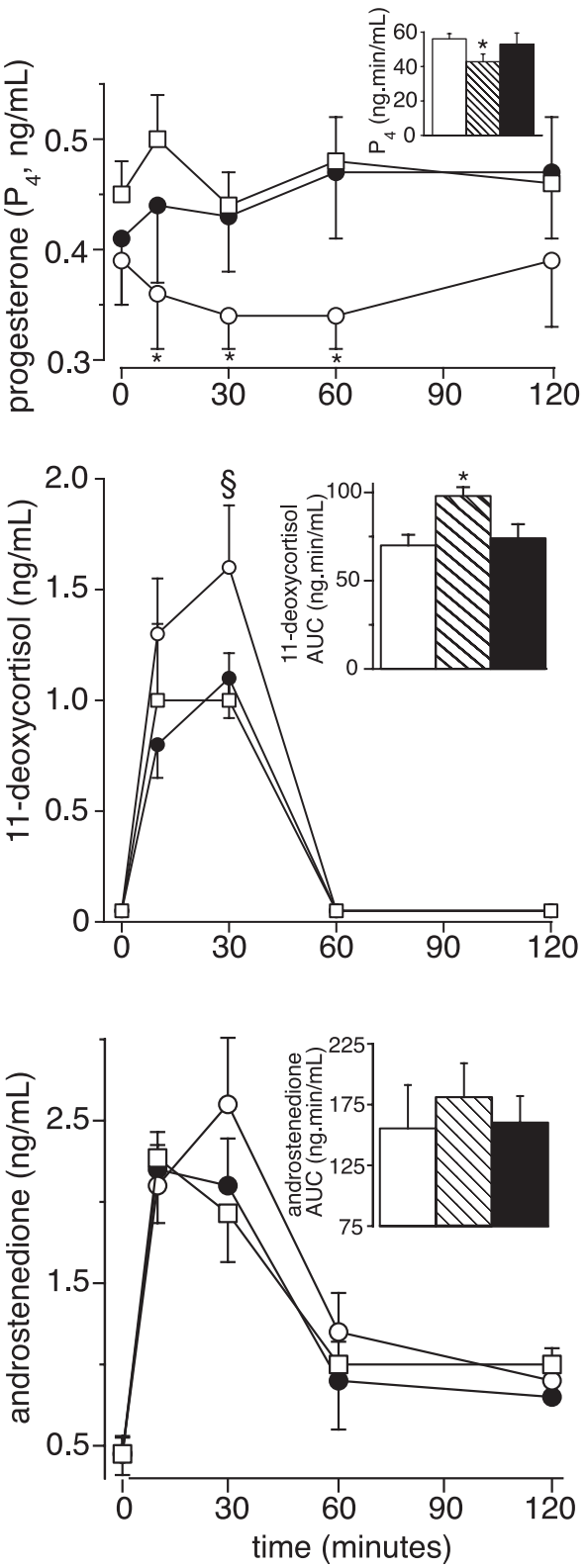

FIG. 3. Responses of progesterone (*top*), 11-deoxycortisol (*middle*), and androstenedione (*bottom*) concentrations to the CRH+AVP challenge. The *inset histograms* represent the AUC. Values are mean ± SEM; n = 11 per group. □ and *white bars*, *Ad libitum*; ○ and *hatched bars*, UN10; ● and *black bars*, UN20. *, $P < 0.05$; §, $P = 0.07$ *vs. ad libitum* group.

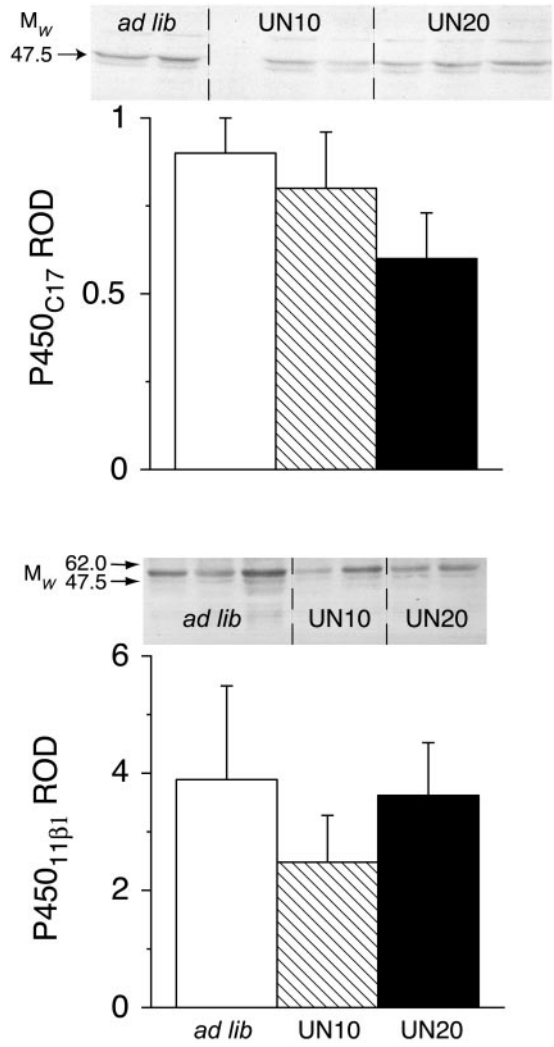

FIG. 4. Representative Western blots for $P450_{c17}$ (*top*) and $P450_{11\beta1}$ (*bottom*) protein, with histograms representing semiquantitative analysis of protein levels. Values are mean ± SEM; n = 11 per group. *White bars*, *Ad libitum*; *hatched bars*, UN10; *black bars*, UN20. There were no statistically significant differences between groups.

ming of different metabolic and homeostatic processes. Thus, it seems likely that a single mechanism, such as exposure to excess, or inappropriate, concentrations of glucocorticoids, cannot explain all aspects of fetal programming.

Here, we provide evidence that the duration of the insult is also important in programming postnatal physiology, independent of birth weight. We might have expected that the offspring of ewes undernourished for longer would demonstrate more profound programming effects. However, they did not demonstrate any alteration of HPA function as adults, despite being lighter at birth than controls. Rather, fetuses whose mothers were undernourished for 10 d, and which were not different in birth weight from controls, were the ones that went on to demonstrate altered HPA function as adults. Adrenal steroidogenic enzyme mRNA levels in a subset of fetuses from the cohort of ewes undernourished in this study that were killed at 125 d gestation were reduced in UN10 fetuses compared with UN20 fetuses (37), although

neither group was significantly different from *ad libitum* controls.

One possible explanation for the differences in UN10, but not UN20, offspring is that a brief (10 d) period of severe undernutrition in late gestation leads to a down-regulation of the HPA axis as an adaptive mechanism. A more prolonged insult (20 d) may result in secondary adaptive mechanisms resulting in up-regulation, as the fetus prepares itself for possible imminent delivery in the face of a hostile intrauterine environment that has persisted beyond some threshold. Undernutrition for 30 d in late gestation sheep has been shown to result in an elevation of maternal circulating cortisol concentrations during the first 10 d (11). After 10 d, maternal cortisol concentrations tended to be lower in undernourished ewes, although this was not statistically significant. It is, however, supported by older studies in sheep that have demonstrated suppression of pituitary and adrenal growth with chronic undernutrition (38–40). Furthermore, in the subset of fetuses killed at 125 d gestation in the cohort we describe here, maternal plasma and umbilical cord plasma cortisol concentrations were reduced after 20 d undernutrition (37).

An alternative, more speculative, explanation is that the difference between UN10 and UN20 offspring may be due to differences in growth patterns *in utero*. Although we did not insert fetal growth catheters in this experiment, and so are unable to report on the precise growth response of the individual animals to the nutritional insult, Mellor and Murray (22) previously described slowing of fetal growth with 10 d maternal undernutrition in late gestation, followed by a rapid return to previous growth rate upon refeeding. However, if undernutrition was continued beyond 19 d, fetal growth rate failed to recover when the ewes were refed (22). We have also previously reported slowing of fetal growth during a brief, acute nutritional insult, with recovery following relief of the insult (5). Furthermore, we have demonstrated that the fetal growth response depends on prior growth rate, and that this in turn is influenced by maternal nutritional status around the time of conception (4). In the study that we report here, the nutritional status of the ewes around the time of conception was carefully monitored, and all ewes received supplementation with specially formulated pellet feed (30). Consequently, there was little variation in nutritional status of ewes, and this is substantiated by the absence of breeding group as a statistically significant variable in any of the multivariate analyses we performed.

However, there are increasing numbers of reports that catch-up growth in postnatal life may amplify the effects of fetal programming (41–43). This phenomenon has also been described in experimental studies (44, 45). We speculate that in our study there was *in utero* catch-up growth in fetuses exposed to 10 d of undernutrition, but not in those exposed to 20 d. We further speculate that this *in utero* catch-up growth may reveal the programmed phenotype. The potential importance of catch-up growth *in utero* requires further study, because this would have implications for any potential future treatment strategies aimed at intrauterine growth-restricted fetuses.

The finding of an accentuated ACTH response to hypoglycemia induced by the ITT as well as to the CRH+AVP challenge strongly suggests that the observed changes are physiologically relevant, although it is not clear whether they would potentially contribute to increases in disease risk such as has been proposed in relation to the human epidemiological studies (3). Glucose concentrations during the ITT were not significantly different between groups (23) and were included as a covariate in the analyses. The absence of any difference in cortisol response suggests altered sensitivity at the adrenal level. The lower baseline cortisol and higher ACTH concentrations in UN10 ewes may reflect altered ACTH receptor (ACTH-R) levels, and we did find lower ACTH-R mRNA levels in a cohort of UN10 fetuses killed at 125 d gestation compared with UN20 fetuses (37). Alterations in ACTH-R levels or in postreceptor mechanisms could account for both the reduced basal cortisol concentrations and the lack of elevated cortisol concentrations in the face of higher ACTH concentrations.

The higher baseline 11-deoxycortisol concentrations and the higher 11-deoxycortisol AUC and statistically significant fall in progesterone after CRH+AVP injection in UN10 ewes suggest that there may also be alterations in adrenal enzyme activity in the UN10 ewes. The lower $P450_{11\beta1}$ protein levels in this group, although not statistically different, would support this, as would the reduced mRNA levels for adrenal steroidogenic enzymes that we have found in the UN10 *vs.* UN20 fetuses killed at 125 d gestation (37).

Previous studies investigating programming of the fetal HPA axis have demonstrated altered glucocorticoid receptor (GR) mRNA expression in the brain, suggesting that some of the effects on the HPA axis may be mediated via altered negative feedback. A short period of maternal undernutrition in late-gestation guinea pigs reduced GR mRNA levels in the pituitary of female adult offspring, and basal cortisol levels were increased (46). Prenatal dexamethasone in guinea pigs also reduced GR mRNA levels in the hippocampus of prepubertal female offspring. However, in these animals basal cortisol levels were not altered and cortisol response to isolation stress was attenuated (47). It is interesting to note that in both of these studies these effects were specific to females; the male offspring often demonstrated the opposite effect. We were only able to study females for logistical reasons and so are unable to comment on any gender-specific effects. However, we did not find any effect of gender on glucose tolerance in these same animals at 5 months of age (23). In late-gestation fetal sheep exposed to mild maternal undernutrition for the first half of pregnancy, GR mRNA levels were reduced by 40% in the anterior pituitary (48). ACTH and cortisol responses to stimulation with exogenous CRH+AVP, ACTH, or acute isocapnic hypoxemia were reduced (9, 10). These studies are therefore inconsistent in their findings of alterations in GR mRNA levels in the pituitary, but they do suggest that there may be an element of dissociation between ACTH and cortisol levels. Such a dissociation may occur because of altered populations of CRH-target ACTH-secreting cells in the pituitary as has been reported following carunclectomy and hypoxemia in the sheep (49), or it may be secondary to alterations at the adrenal level. Unfortunately, we were unable to measure GR mRNA levels in the brain in this study.

In summary, we have demonstrated that a brief period of

undernutrition in late gestation results in altered HPA function in adult offspring. These changes are evident in response to physiological stimuli such as hypoglycemia, as well as in response to formal challenges of the HPA axis with CRH+AVP, and they appear to involve resistance to ACTH and/or altered steroidogenesis at the level of the adrenal gland. Furthermore, the changes are independent of birth weight or current weight, but are dependent upon the duration of undernutrition. This may be related to fetal adaptations to more prolonged *vs.* briefer periods of severe undernutrition, or possibly to fetal growth patterns and catch-up growth *in utero*. These data provide clear evidence that a nutritional insult before birth can permanently alter postnatal physiology independent of size at birth and that different physiological axes are programmed in different ways in response to an antenatal insult.

Acknowledgments

We acknowledge the technical assistance of Sam Rossenrode and Toni Smith-Wong, and also the assistance of Dr. D. M. Sloboda. The $P450_{C17}$ antibody was kindly donated by Dr. F. Labrie (Laval University, Québec, Canada).

Received February 10, 2003. Accepted April 3, 2003.

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These studies were funded by grants from the Wellcome Trust, the Health Research Council of New Zealand, the Canadian Institutes of Health Research, the New Zealand Lottery Grants Board, the Auckland Medical Research Foundation, and the Basser Traveling Fellowship from the Royal Australasian College of Physicians (to F.H.B.).

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