Effect of periconceptual undernutrition and gender on hypothalamic–pituitary–adrenal axis function in young adult sheep

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Abstract

Glucocorticoids are proposed to act as intermediary factors that transcribe the developmental programming sequelae of maternal nutrient restriction (NR). Periconceptual undernutrition of sheep markedly activates fetal hypothalamic–pituitary–adrenal (HPA) axis activity leading to preterm birth, while transient undernutrition during late gestation in sheep programs adult HPA axis function. To date, no study has examined resting or stimulated HPA axis function in young adult offspring following a periconceptual nutritional challenge. In the present study, 20 ewes were either periconceptually undernourished (50% metabolisable energy requirements from days 1 to 30 gestation; NR, $n=8$) or fed to control levels (100% requirement; controls, $n=12$) to term (147 days gestation). Ewes were blood sampled remotely at 2 and 30 days using automated blood sampling equipment. Thereafter, offspring (controls, $n=6/6$ males/females; NR, $n=4/4$ males/females) were reared to 1 year of age and on separate days received either an i.v. corticotrophin-releasing hormone (CRH; $0.5 \mu\text{g/kg}$) and vasopressin (AVP; $0.1 \mu\text{g/kg}$) challenge or a synthetic ACTH i.v. bolus (Synacthen; $1.25 \mu\text{g/kg}$), and blood samples were taken (manually and remotely) at appropriate intervals for measurement of plasma ACTH and cortisol accordingly. Resting plasma cortisol, assessed remotely, was similar in ewes during

undernutrition (control 18.3 ± 1.4 vs NR 23.4 ± 1.9 nmol/l) and in offspring at 4 months of age (control male 17.6 ± 2.9 ; control female 17.2 ± 0.4 , NR male 16.5 ± 3.1 , NR female 21.7 ± 4.0 nmol/l). At 12 months of age, however, resting plasma cortisol was significantly increased in NR females (control male 28.0 ± 1.5 , control female 32.9 ± 9 , NR male 32 ± 7 , NR female 53 ± 10 nmol/l, $F 5.7$, $P=0.02$) despite no difference in plasma ACTH concentration. There was an interaction between nutritional group and gender for both the pituitary and adrenal responses to CRH and AVP, i.e. for controls, females exhibited increased plasma ACTH or cortisol relative to males but for NR this trend was either not present or reversed. The adrenocortical response to synthetic ACTH was gender-dependent only, being greater in female offspring. Combined CRH and AVP provoked a transient hypertension and marked bradycardia in all animals, irrespective of dietary group or gender and could be effectively reproduced by an AVP bolus alone. In conclusion, the present study has shown that periconceptual undernutrition of sheep has only a minor influence on HPA axis function in their young adult offspring when considered alongside the effect of gender *per se*.

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Introduction

Intrauterine stress has been implicated as an intermediary factor for a range of adult onset morbidities, such as obesity, hypertension and syndrome X (consisting of the pentad of hypertension, hyperinsulinism, dyslipidemia, obesity and cardiovascular disease). This has become known as fetal and infant developmental ‘programming’; whereby a stimulus or insult occurring during a vulnerable or sensitive period of growth and development has lasting effects on the structure and function of tissues and body systems (Lucas *et al.* 1999).

One such insult commonly shown to have lasting, ‘programming’ effects on the fetus is maternal nutrient restriction (NR; Langley-Evans 2004, Symonds *et al.* 2005). The adult manifestations of a prenatally programmed insult are varied and most likely related to the duration, timing and relative severity of the insult (Gardner *et al.* 2005) as discussed many decades ago (McCance & Widdowson 1974, Widdowson & McCance 1975). Nevertheless, it would appear that the fundamental biological processes that underpin developmental plasticity are reasonably conserved in mammals at least, for example, late gestation insults in rats, sheep and humans

lead to poor glycaemic control in the resultant adult offspring (Nyirenda *et al.* 2001, Roseboom *et al.* 2001, Gardner *et al.* 2005).

Glucocorticoid hormones have many metabolic and cardiovascular actions on the body, reflecting the almost ubiquitous tissue distribution of their receptors (Dallman *et al.* 1993, Sapolsky *et al.* 2000). In the fetus, these actions are clearly age-dependent but, in general, intrauterine glucocorticoids (GC; of fetal and/or maternal origin) promote functional maturation over, or at the expense of, functional differentiation (Fowden *et al.* 1998, Fowden & Forhead 2004). Thus, GC in high doses may retard intrauterine growth (Redmond 1979), but rescue babies threatened with preterm labour and respiratory complications (Cummings *et al.* 1989, Ballard & Ballard 1996). GC have long been thought to act as intermediary factors that transcribe the developmental programming sequelae of maternal NR (Langley-Evans *et al.* 1996) and there is much evidence in humans and animal models to suggest such a role (Langley-Evans 1997, Phillips *et al.* 1998, Reynolds *et al.* 2001, 2003). In sheep, the change in maternal cortisol with undernutrition appears age-dependent, being elevated with late gestation undernutrition (Edwards & McMillen 2001) and reduced with early to mid-gestation undernutrition (Bispham *et al.* 2003). Interestingly, periconceptual undernutrition has been shown to markedly activate fetal hypothalamic–pituitary–adrenal (HPA) axis activity in late gestation leading to preterm birth in sheep (Bloomfield *et al.* 2003b). However, to date, only the effects of late gestation undernutrition on adult HPA axis function (Bloomfield *et al.* 2003a) have been examined. No study has examined resting or stimulated HPA axis function in adult offspring following a periconceptual nutritional challenge. Furthermore, in this study, we propose to pilot, in sheep, a novel remote sampling technique (Goddard *et al.* 1998, Sakkinen *et al.* 2004) for characterisation of maternal cortisol concentrations during the period of undernutrition and of resting plasma cortisol in the young offspring to avoid potential artefacts associated with manual restraint and sampling.

In addition, sex-specific regulation of the fetal (Giussani *et al.* 2001) and adult (Bloomfield *et al.* 2003a) HPA axis *per se* has been noted previously (for review, see Matthews *et al.* 2004). Furthermore, it is clear that responses to challenges that have been shown to incur developmental consequences exhibit a distinct sex-specific bias. For example, after maternal restraint stress in rats during late gestation (resulting in maternal HPA axis activation), only the adult female offspring are affected; males appear largely unaffected (McCormick *et al.* 1995). Prenatal alcohol exposure again leads to an increase in adult female, but not male, HPA axis activity (Lee & Rivier 1996).

Hence, the present study was established to address the hypothesis that periconceptual undernutrition of sheep elevates adult HPA axis sensitivity. It is proposed that the programming of adult HPA axis responses would also exhibit a distinct sex-specific bias. These twin hypotheses were addressed as follows: (1) the resting plasma concentrations of

adrenocorticotrophin (ACTH) and cortisol were analysed together with (2) ACTH and cortisol responses to a bolus dose of corticotrophin-releasing hormone (CRH) and vasopressin (AVP). (3) To separate pituitary from adrenal responsiveness we also, on a different day, administered a separate bolus dose of synthetic ACTH. Given the cardiopressor actions of AVP in high concentrations (Cowley 1988) and that periconceptual undernutrition has been shown to influence blood pressure regulation (Gardner *et al.* 2004), then the fourth aim of the study was to examine the cardiovascular responses to a CRH and AVP bolus.

Materials and Methods

Animals

All procedures were performed under the UK Animals (Scientific Procedures) Act, 1986. Twenty Blue-faced Leicester × Swaledale ewes were used in the study. All ewes were of similar age (7–8 months), body weight and fat distribution as assessed from physical characteristics in the lumbar spine and loin. Ewes were mated with a single ram and within 12 h of mating were individually housed and randomly assigned to one of two groups: (1) ewes were fed to adequately meet 100% metabolisable energy (ME) requirements as defined by the Agricultural and Food Research Council (AFRC) throughout gestation to term (term 147 days gestational age; controls $n=12$) and (2) ewes were fed to 50% AFRC ME requirements from days 1 to 30 gestation and thereafter to 100% ME requirement to term (nutrient restricted, NR; $n=8$). All ewes were bedded on wood shavings and fed a diet consisting of chopped hay (ME content of 8.9 MJ/kg dry matter (DM) and a digestible crude protein (DCP) content of 45 g/kg DM) and a barley-based concentrate (ME 8.9 MJ/kg DM, DCP 218 g/kg DM). All ewes were weighed and body condition was scored at fortnightly intervals and the nutritional regimen for each individual ewe was adjusted according to gestation age (to allow for growth of the conceptus) and twinning ($n=6$ controls, $n=3$ NR). At term, lambs were delivered naturally and birth weights recorded. For twin-bearing ewes, only one lamb was kept with the ewe during lactation to avoid the confounding influence of differences in nutrient intake and its effects on postnatal growth rate. In the control group ($n=12$), there were six males and six females and for NR ($n=8$), the sex ratio was four males and four females. The offspring were ewe reared until weaning at 3 months and thereafter grass-fed until aged 1 year. During this time, the weights and growth of individual lambs were recorded monthly. At 1 year of age, all sheep were group-housed indoors for 1 week prior to surgery. For 24 h prior to surgery all food, but not water, was withdrawn from the animals. Anaesthesia was induced with sodium thiopentone (20 mg/kg *i.v.* intraval sodium; Rhone Mérieux, Dublin, Ireland) and maintained with 1–2% halothane in 50:50 O₂/N₂O. Carotid and jugular catheters were inserted into each sheep and the incision closed.

All sheep received a dose of long-acting antibiotic (15 mg/kg i.m. amoxycillin (Duphamox); Fort Dodge Animal Health Ltd, Southampton, UK) and analgesia (1 mg/kg flunixin meglumine (Finadyne); Shering-Plough, Kenilworth, UK) postoperatively. Catheter patency was maintained by daily flushing with heparinised saline (50 IU heparin/ml). All sheep had established normal feeding patterns within 1–2 h after surgery and showed no visible signs of discomfort for the duration of the experimental period.

Experimental protocols

Remote blood sampling of pregnant ewes and their offspring

The automated blood-sampling equipment (ABSE) was used as described previously (Goddard *et al.* 1998). In brief, the ABSE is mounted in a small rucksack on the ewes back and programmed with a microprocessor to sample (5 ml from connected jugular vein catheter) at hourly intervals for 14 h from 0900 h. The unit automatically withdraws a set quantity of blood into a heparinised syringe and flushes the line with 1 ml heparinised NaCl after each sample. A total of 14 samples may be taken at prespecified intervals. The following day, the unit was removed and blood samples spun at 3000 r.p.m. for 10 min for decanting of resultant plasma. Pregnant ewes were sampled at days 2 and 30 of gestation, i.e. to cover the start and end of the maternal undernutrition period, while offspring were ABSE sampled at 4 months of age.

HPA axis function in offspring of periconceptionally undernourished ewes

A period of 2–4 days postoperative recovery was allowed before any experiment being performed. The investigator was blinded to the dietary origin of the sheep prior to any experiment or plasma analysis being performed. The experiments were conducted on separate days with 1–2 days interval between challenges and the order in which sheep received the challenges was randomised.

Experiment 1: CRH/AVP challenge. Baseline samples were taken at –30 and –15 min prior to a bolus i.v. injection of CRH (0.5 µg/kg) and vasopressin (AVP; 0.1 µg/kg) at time 0. Thereafter, regular blood samples were withdrawn at +5, 15, 30, 45, 60, 90, 120, 150 and 180 min. All blood samples were collected into chilled heparinised tubes, centrifuged for 5 min at 4 °C and, after decanting of the supernatant plasma, were stored at –20 °C for further analysis of plasma ACTH and cortisol concentrations. Before (30 min) and during this experiment, the arterial catheter was connected to a precalibrated pressure transducer (SensorNor 840; S 4925) attached at heart level and linked to a data acquisition system (Po-Ne-Mah; Version 3, Linton Instruments, Diss, Norfolk, UK). Analogue signals for real-time systolic, diastolic, mean arterial pressure and heart rate were recorded at 1-s intervals, digitised and stored on an Excel spreadsheet for further analysis. From these data, pulse pressure (systolic–diastolic) was derived.

Experiment 2: ACTH challenge. Two baseline blood samples (2 ml) were withdrawn before all sheep were administered a bolus dose of synthetic ACTH (Synacthen 1.25 µg/kg, Abbott Laboratories) at time 0. Thereafter, regular blood samples were withdrawn at +5, 15, 30, 60, 120 and 150 min. Blood samples were treated as described for Experiment 1 for further analysis of plasma cortisol concentration only.

Experiment 3: AVP challenge. To address whether the cardiovascular responses to combined CRH and AVP (Experiment 1) could be elicited by AVP alone, a proportion of sheep ($n=4$) received an identical AVP bolus (i.v., 0.1 µg/kg in 0.9% NaCl) and cardiovascular variables recorded as described previously.

Biochemical analyses

Cortisol An RIA was used for the quantitative measurement of total cortisol in plasma (Coat-a-Count, DPC, Caernarfon, Wales) as described previously (Edwards *et al.* 2001b, Bispham *et al.* 2003). In brief, the kit contained cortisol antibody coated tubes, ^{125}I cortisol and cortisol standards. Suitable dilutions were made of standards to improve accuracy at the lower end of the standard scale; in total seven standards varying from 0 to 276 nmol/l were used. Duplicate 25 µl plasma samples were incubated with ^{125}I cortisol in each tube for 45 min at 37 °C, decanted and counted for 1 min/tube with a gamma counter. The detection limit, defined as the apparent concentration at 95% B/B_0 , was 0.2 µg/dl. The intra- and interassay coefficients of variation were 5.6 and <10%.

ACTH An RIA was used for the quantitative measurement of ACTH in plasma (Diasorin, Slough, UK) as described previously (Gardner *et al.* 2001). The kit contained ACTH calibrators, rabbit anti-ACTH serum, ^{125}I ACTH, Precipitating Complex (GAR-PPT) and ACTH standards. In brief, duplicate 100 µl samples, 200 µl ACTH antiserum and 200 µl tracer were combined and incubated for 16–24 h at 2–8 °C. After this time, the precipitating complex was added to separate the bound/unbound tracer and the samples incubated for a further 15 min at 20–25 °C, centrifuged and decanted. The precipitate of each tube was counted for 1 min using a gamma counter. The minimum detectable concentration was 15 pg/tube. The intraassay coefficient of variation was 11.8%. The interassay coefficient of variation was not measured because only two assays were performed. However, routinely the same assay has an interassay variation of 10–15% in our hands (Gardner *et al.* 2001).

Plasma concentrations for both cortisol and ACTH were calculated from interpolation of a semi-log standard curve with B/B_0 (%) on the y -axis and either ACTH or cortisol on the x -axis.

Microscopy

At least 2 days after the ACTH challenge, all sheep were injected i.v. with a terminal anaesthetic (20 mg/kg sodium

pentobarbitone) and body and adrenal weights were determined. In a proportion of these sheep, one adrenal gland was fixed in 10% formaldehyde and, within 2 days, embedded in paraffin wax for sectioning (male $n=8$, female $n=5$). Each adrenal gland was cut in half along its longitudinal axis and between 4 and 8 $\times$ 8 μ m sections from the midline were taken with a microtome, mounted on individual slides and stained with haematoxylin and eosin. Adrenocortical and adrenomedullary areas (mm^2) were then determined on each section with a calibrated eye-piece graticule under low-power (4 $\times$) light microscopy. At least six measurements were taken from each of the adrenal sections.

Statistical analysis

All data are expressed as means $\pm$ S.E.M. unless otherwise stated. Baseline values and concentrations measured after each challenge were analysed as absolute concentrations and concentrations as a deviation from baseline (i.e. minus average baseline concentrations). With the baseline set at 0, the values for each hormone as a change from baseline were graphically represented using Prism (GraphPad version 3, San Diego, CA, USA). In addition to average resting concentrations for each hormone, other derived variables were peak cortisol and ACTH concentration achieved and absolute cortisol and ACTH secretion (measured as the area above fasting plasma cortisol and ACTH concentration under the plasma cortisol and ACTH curve; cortisol/ACTH_{AUC}). Derived (dependent) variables were then statistically tested using SPSS v14.0 by one- or two-way repeated measures ANOVA with nutritional group and gender as fixed effects. For all comparisons, statistical significance was accepted when $P < 0.05$.

Results

Maternal data

Details of maternal weight gain/loss during the period of undernutrition are as described previously (Gardner *et al.* 2004). Plasma concentrations of maternal cortisol were similar on days 2 and 30 of gestation in both control and NR ewes, and for clarity these data are presented in Fig. 1 at 30 days only, i.e. the last day of undernutrition when the greatest difference in maternal plasma cortisol would be expected. In addition, over the 14-h ABSE sampling period, there was no evidence of an ultradian rhythm in maternal plasma cortisol.

Offspring data

All sheep gave birth at the appropriate stage for this breed ($\sim 147 \pm 2$ days). Details of birth weights and postnatal growth rates are as described previously (Gardner *et al.* 2004, 2005). At 1 year of age, there was no difference in body weight between the two nutritional groups, but weights were higher in males relative to females (control male 57 ± 5 , control female 43 ± 2 kg, NR male 57 ± 4 , NR female 45 ± 2 kg, F 14.0,

Figure 1 Resting plasma cortisol in control and nutrient-restricted (NR) ewes at 30 days of gestation. Values are means $\pm$ S.E.M. for controls ($n=6$) and NR ($n=4$) sheep. Baseline blood samples were taken hourly over a 14-h period beginning at 1800 h using automated blood-sampling equipment. There were no effects of nutritional group on the resting plasma concentration of cortisol.

$P=0.001$). At 4 months of age, resting plasma cortisol was similar in all groups regardless of prenatal nutritional experience or sex (control male 17.6 ± 2.9 , control female 17.2 ± 0.4 , NR male 16.5 ± 3.1 , NR female 21.7 ± 4.0 nmol/l). Repeated ABSE sampling revealed no effect of time in any of the groups (Fig. 2; data for control vs NR included only for clarity).

Experiment 1: CRH and AVP challenge

ACTH and cortisol. Analysis of resting plasma ACTH concentrations revealed no effect of nutritional group, but a trend for an interaction between group and gender (control male 68 ± 9 , control female 130 ± 39 ; NR male 103 ± 32 ,

Figure 2 Resting plasma cortisol in control and nutrient-restricted (NR) male/female sheep at 4 months of age. Values are means $\pm$ S.E.M. for control male ($n=6$), control female ($n=6$), NR male ($n=4$) and NR female ($n=4$) sheep. Baseline blood samples were taken hourly over a 14-h period beginning at 1800 h using automated blood-sampling equipment. There were no effects of nutritional group or gender on the resting plasma concentration of cortisol.

NR female 65 ± 9 pg/ml; $F 3.6$, $P=0.07$). For baseline plasma cortisol, there was again no effect of nutritional group, but a significant effect of gender (control male 28.0 ± 1.5 , control female 32.9 ± 9 ; NR male 32 ± 7 , NR female 53 ± 10 nmol/l; $F 5.7$; $P=0.02$). For the ACTH:cortisol ratio, there was a borderline significance for an effect of group ($F 4.71$, $P=0.046$) and a trend for an interaction between group $\times$ gender (control male 2.04 ± 0.38 , control female 1.24 ± 0.26 ; NR male 2.29 ± 1.22 , NR female 3.68 ± 0.46 nmol/[pmol per l^{-1}]; $F 3.1$, $P=0.09$). Upon administration of the CRH and AVP bolus, all individuals exhibited a significant rise in ACTH and cortisol (Fig. 3A and B; $P<0.001$). For ACTH, the response was similar for group and gender, but there was a significant ($F 24.3$, $P<0.001$) interaction between group $\times$ gender, i.e. for controls females exhibited increased plasma ACTH relative to males, but for NR this trend was reversed (control male 7415 ± 1222 , control female $18\,378 \pm 2575$; NR male $13\,142 \pm 417$, NR female 6709 ± 2082 AUC units; Fig. 3A). For cortisol, there

was a trend for an effect of group ($P=0.06$), but significant effects of gender ($F 5.3$, $P=0.03$) and the gender $\times$ group ($F 12.1$, $P=0.003$) interaction (control male $10\,751 \pm 1717$, control female $21\,998 \pm 2006$; NR male $13\,877 \pm 528$, NR female $11\,648 \pm 2753$ AUC units; Fig. 3B).

Blood pressure and heart rate. Analysis of resting mean arterial blood pressure and heart rate revealed no significant effect of nutritional group or gender (systolic blood pressure: control male 96 ± 3 , control female 103 ± 5 ; NR male 102 ± 1 , NR female 95 ± 5 mmHg; heart rate: control male 103 ± 5 , control female 107 ± 2 ; NR male 93 ± 8 , NR female 108 ± 6 beats/min). Administration of the CRH and AVP challenge elicited significant hypertension and bradycardia. However, the magnitude of these pressor and chronotropic responses was similar in all individuals. Figure 4 illustrates the responses in control and NR offspring only for clarity.

Figure 3 Plasma ACTH and cortisol after a combined bolus dose of CRH and AVP in control and nutrient-restricted (NR) male/female sheep at 1 year of age. Values are means $\pm$ s.e.m. for control male ($n=6$), control female ($n=6$), NR male ($n=4$) and NR female ($n=4$) sheep. Baseline blood samples were taken at -30 and -15 min prior to a bolus i.v. injection of CRH ($0.5 \mu\text{g/kg}$) and vasopressin (AVP; $0.1 \mu\text{g/kg}$) at time 0. Thereafter, regular blood samples were withdrawn at $+5$, 15 , 30 , 45 , 60 , 90 , 120 , 150 and 180 min. For ACTH, there was a significant ($F 24.3$, $P<0.001$) interaction between group $\times$ gender. For cortisol, there was a trend for an effect of group ($P=0.06$), but significant effects of gender ($F 5.3$, $P=0.03$) and the gender $\times$ group ($F 12.1$, $P=0.003$) interaction.

Figure 4 Δ mean arterial blood pressure (A) and heart rate (B) response to a combined CRH and AVP bolus in control and nutrient-restricted (NR) sheep at 1 year of age. Values are means $\pm$ S.E.M. for control ($n=12$) and NR ($n=8$) sheep. CRH and AVP elicited a significant ($P<0.001$) hypertension and sustained bradycardia in all sheep. There was no effect of periconceptual undernutrition or gender on the cardiovascular response to CRH and AVP.

Experiment 2: ACTH challenge

Cortisol. Analysis of the AUC for stimulated plasma cortisol concentrations after a bolus injection of synthetic ACTH indicated no effect of nutritional group, but a strong trend for an effect of gender (control male $11\,137 \pm 951$, control female $17\,831 \pm 2689$; NR male $11\,772 \pm 1738$, NR female $13\,629 \pm 3013$ AUC units; $F\,3.99$, $P=0.06$; Fig. 5). In addition, there was a significant effect of gender, but not nutritional group, on the peak cortisol level achieved during the challenge (control male 159 ± 7 , control female 240 ± 17 ; NR male 158 ± 29 , NR female 183 ± 26 nmol/l, $F\,7.44$, $P=0.01$).

Experiment 3: AVP challenge In four control sheep, administration of AVP alone elicited a similar significant, transient, hypertension (an increase of 10–20 mmHg) and sustained bradycardia (a decrease of ~ 40 beats/min) as produced with a combined CRH and AVP bolus (Fig. 6 vs Fig. 4).

Adrenal size and morphology. Absolute adrenal weights were similar between groups and sexes (control male 2.91 ± 0.39 , control female 3.38 ± 0.36 ; NR male 3.57 ± 0.50 , NR female 2.92 ± 0.25 g) and with the exception of control females, were proportionate to body weight (control male

49.6 ± 4.3 , control female 78.1 ± 6.5 ; NR male 62.7 ± 9.4 , NR female 64.4 ± 6.4 g/kg $\times 10^3$). In a subset of these sheep ($n=8$ males and $n=5$ females), as a proportion of total adrenal size, there was a trend for females to have a reduced cortical size when expressed as a percentage of total adrenal area (control male 75.9 ± 0.6 , control female 71.9 ± 7.2 ; NR male 75.1 ± 1.6 , NR female $67.8 \pm 4.4\%$; $F\,3.95$, $P=0.07$).

Discussion

The main finding of our study is that periconceptual undernutrition of sheep has no effect on maternal plasma cortisol and little lasting effect on resting HPA axis sensitivity of the young adult offspring, *in vivo* but when stimulated, nutritional group $\times$ gender interactions are observed. The overriding influence on HPA axis function in the present study is the sex of the adult being studied. In addition, this study has highlighted the potential for using remote blood sampling as a means to accurately gauge resting HPA axis function in sheep, represented only through measurement of plasma cortisol.

GC have, from early in the genesis of the programming of health and disease hypothesis, been thought to act as intermediary factors that transcribe the developmental programming sequelae of maternal undernutrition. From the

Figure 5 Plasma cortisol after a bolus dose of synthetic ACTH in control and nutrient-restricted (NR) male/female sheep at 1 year of age. Values are means $\pm$ S.E.M. for control male ($n=6$), control female ($n=6$), NR male ($n=4$) and NR female ($n=4$) sheep. There was no effect of nutritional group, but a strong trend for an effect of gender on the AUC ($F 3.99$, $P=0.06$) during the ACTH challenge.

early rodent studies (Gardner *et al.* 1998) to later large animal models (Challis *et al.* 2000, Edwards *et al.* 2001a, Bloomfield *et al.* 2004), a role for increased GC exposure or altered regulation of the HPA axis in fetal through to adult life, after variations in the maternal diet, has been proposed and intensively studied. However, for a number of reasons outlined below, a causal relationship has proved difficult to ascertain. For example, first, there are two HPA axes to consider (maternal and fetal), each of which show homeostatic regulation through gestation, i.e. the pregnant state alters HPA axis function *per se* and this changes as gestation

progresses. Furthermore, the fetal HPA axis develops increased function with gestation from a tonic dependence on maternal GC to gradual autonomy (Challis & Brooks 1989). Secondly, the placenta in some species can both produce corticotrophin-releasing factor, stimulating fetal HPA axis development (Florino *et al.* 2002), and further act as a control point for maternal GC entry into the fetal compartment through the expression and activity of the two 11β -hydroxysteroid dehydrogenase (11β -HSD) isoforms 1 and 2 (Seckl 1997). In addition, further levels of complexity in the axis exist in the form of binding globulins, which prevent access of GC to the GC receptor (Challis *et al.* 1995) and the developing regional-specific tissue expression of 11β -HSD within the fetus (Seckl & Chapman 1997, Whorwood *et al.* 2001). Consequently, the initial response of maternal–fetal HPA axes to maternal (perhaps fetal) undernutrition has only been partially described and appears gestational age-dependent. Thus, early to mid-gestation undernutrition reduces maternal resting plasma cortisol levels (Bispham *et al.* 2003), whereas late gestation undernutrition appears to elevate them (Edwards & McMillen 2001). In the present study, we have shown that undernutrition (50% estimated requirement) of the ewe for the first 30 days of pregnancy has no effect on the resting plasma cortisol concentration when assessed without external interference. This is important, as it has recently been demonstrated that a greater level of undernutrition in sheep (fed to 30% control intake), for a longer period (–60 to +30 days gestation), reduces maternal plasma cortisol during the challenge (Jaquiere *et al.* 2006).

To date, the effects of such maternal–fetal nutritional challenges on HPA axis function have largely been explored in the short term, i.e. in the fetus itself, rather than in the resultant adult offspring. In the fetus, periconceptional undernutrition enhances fetal ACTH production in late gestation (Edwards & McMillen 2002) to such an extent that marked preterm birth is induced in 50% of the offspring (Bloomfield *et al.* 2003b, 2004). In contrast, moderate undernutrition postconception, but restricted to early–mid-gestation, has the opposite effect, blunting HPA axis

Figure 6 Δ mean arterial blood pressure and heart rate response to an AVP bolus in four sheep at 1 year of age. Values are means $\pm$ S.E.M. for four control sheep. AVP alone elicited a significant but transient hypertension and sustained bradycardia.

sensitivity to a CRH and AVP stress test (Hawkins *et al.* 1999). When undernutrition is confined to late gestation alone then increased fetal plasma cortisol has been observed (Edwards & McMillen 2001). In the adult, follow-up of HPA axis function after maternal undernutrition has only been considered when the period(s) of undernutrition are confined to late gestation alone (Bloomfield *et al.* 2003a). Here, a short (10 days), but not longer (20 days) nutritional challenge programmed an increased ACTH response in female offspring to an identical CRH and AVP challenge as used in the present study (Bloomfield *et al.* 2003a). The present study is the first to report on juvenile and adult HPA axis activity in male and female offspring after early gestation, postconception undernutrition. We show no effect on resting plasma cortisol concentrations, assessed remotely, during juvenile life, but increased resting plasma cortisol in females, relative to males, in the resultant young adult offspring despite no difference in plasma ACTH. This apparent increase in resting adrenocortical sensitivity in females is exacerbated by periconceptional undernutrition, i.e. the ACTH:cortisol ratio is greatest in female NR. The stimulated pituitary response reveals significant gender $\times$ nutritional group interactions, i.e. for controls, females exhibit increased plasma ACTH or cortisol relative to males, but for NR this trend is either not present or reversed. However, the specific adrenocortical response to synthetic ACTH was gender-dependent only, being greater in female offspring.

It is interesting to note that the plasma concentrations of cortisol in juvenile offspring in the present study are much lower than those observed at 6–7 months, when the sheep were studied under more controlled experimental conditions in a metabolic crate. Clearly, this may be either an age-related effect or an effect of manual sampling. Concentrations at one year of age are similar to those observed in one other study in adult sheep under similar conditions (Bloomfield *et al.* 2003a). Given that the ABSE has been extensively trialled in deer to show a stimulatory effect of manual sampling (Goddard *et al.* 1998, Sakkinen *et al.* 2004) then we would suggest that sheep, perhaps less so than deer, do show enhanced HPA axis activity with manual sampling and this explains the higher plasma cortisol at one year in the present study. Clearly, however, this elevated activity is particularly apparent in female offspring that were periconceptionally undernourished.

Many previous studies have established sex-specific differences in the regulation of the HPA axis. The differences occur at virtually all levels of the axis, from feedback in the brain (Owen & Matthews 2003) to the adrenal gland itself (Silva *et al.* 2002, van Lier *et al.* 2003). In the sheep, Canny *et al.* (1999) specifically examined gender-related differences in HPA axis function *in vitro* and found increased adrenal steroidogenic potential and corticotrophin sensitivity in female relative to male sheep, a difference that appeared to occur at a postreceptor level. The present study supports these findings in the intact animal and confirms similar previous observations

(Roelfsema *et al.* 1993, Andrew *et al.* 1998, Silva *et al.* 2002, van Lier *et al.* 2003). We show that female sheep *per se* have a relatively greater cortisol response to a specifically adrenal orientated challenge. The increased appearance of cortisol in the circulation after a synthetic ACTH bolus was not related to adrenal size, and thus steroidogenic capacity, since the area occupied by the cortex was proportionately smaller in female sheep. Also increased storage of a labile, readily released form of cortisol in females is unlikely as there is little storage of steroid within the zona fasciculata (Robinson *et al.* 1983). Rather, in females there would appear to be either: (1) increased adrenocortical synthetic potential, (2) enhanced sensitivity to extra-adrenal trophic hormones (Canny *et al.* 1999) or (3) reduced sensitivity to central feedback mechanisms (Matthews *et al.* 2004). Of the potential candidate hormones, the greater oestrogen presence in females relative to males is the most likely (Handa *et al.* 1994, Lee & Rivier 1996, Ogilvie & Rivier 1997). Indeed, in hypogonadal males and females, i.e. genetically male or female but resting similar steroid hormone levels, specific differences in the adrenal responses exist, but are reversed; males show greater adrenal cortisol output (Roca *et al.* 2005). It is interesting to note that the reverse is also true in fetal life, i.e. female sheep fetuses *in utero* have relatively blunted HPA axis responses to hypoxic stress (Giussani *et al.* 2001).

In the present study, we observed a group $\times$ sex interaction for the appearance of ACTH in the plasma after administering a CRH and AVP bolus. In control sheep, the trend was for greater ACTH output in females relative to males, but this was reversed when those sheep were exposed to a nutritionally restricted diet during early gestation. The mechanism for this effect is currently unknown. There are a number of contradictory reports on gender differences in ACTH output after various stressors that invariably lie with the nature of the stressor employed and the species studied. Staying with the sheep as an experimental model, the analysis of POMC in corticotrophs revealed increased expression in males relative to females but, in stark contrast, increased actual ACTH output in females relative to males (Canny *et al.* 1999), illustrating that even in isolated corticotrophs *in vitro*, there is a marked complexity of function between the sexes.

Finally, high concentrations of AVP have been shown to have clear cardiopressor actions, hence its name (Cowley 1988). However, a similar independent role for ACTH in high concentrations has also been proposed (Whitworth *et al.* 2001). In the present study, we clearly show that AVP alone is capable of exerting equivalent cardiopressor responses to combined CRH and AVP, indicating that it is AVP rather than the combination of AVP and high ACTH produced after the CRH and AVP bolus, which is responsible for the cardiovascular responses observed in the present study.

In conclusion, our study has first illustrated a novel means for assessing true resting cortisol status in unrestrained, non-manually sampled sheep. Secondly, the plane of maternal nutrition around the time of conception and implantation can have effects on the function and sensitivity of the offspring's HPA axis as young adult sheep. The nature of these effects,

however, are sexually dimorphic, with female sheep appearing to show enhanced adrenal steroidogenic potential, despite no obvious adrenal morphological differences relative to males. The study provides further clear data that females respond differently to stress than males and that the periconceptual nutritional environment may influence this response.

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References

- Andrew R, Phillips DI & Walker BR 1998 Obesity and gender influence cortisol secretion and metabolism in man. *Journal of Clinical Endocrinology and Metabolism* **83** 1806–1809.
- Ballard RA & Ballard PL 1996 Antenatal hormone therapy for improving the outcome of the preterm infant. *Journal of Perinatology* **16** 390–396.
- 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.
- Bloomfield FH, Oliver MH, Giannoulas CD, Gluckman PD, Harding JE & Challis JR 2003a Brief undernutrition in late-gestation sheep programs the hypothalamic–pituitary–adrenal axis in adult offspring. *Endocrinology* **144** 2933–2940.
- Bloomfield FH, Oliver MH, Hawkins P, Campbell M, Phillips DJ, Gluckman PD, Challis JR & Harding JE 2003b A periconceptual nutritional origin for noninfectious preterm birth. *Science* **300** 606.
- Bloomfield FH, Oliver MH, Hawkins P, Holloway AC, Campbell M, Gluckman PD, Harding JE & Challis JR 2004 Periconceptual undernutrition in sheep accelerates maturation of the fetal hypothalamic–pituitary–adrenal axis in late gestation. *Endocrinology* **145** 4278–4285.
- Canny BJ, O'Farrell KA, Clarke IJ & Tilbrook AJ 1999 The influence of sex and gonadectomy on the hypothalamic–pituitary–adrenal axis of the sheep. *Journal of Endocrinology* **162** 215–225.
- Challis JR & Brooks AN 1989 Maturation and activation of hypothalamic–pituitary–adrenal function in fetal sheep. *Endocrine Reviews* **10** 182–204.
- Challis JR, Berdusco ET, Jeffray TM, Yang K & Hammond GL 1995 Corticosteroid-binding globulin (CBG) in fetal development. *Journal of Steroid Biochemistry and Molecular Biology* **53** 523–527.
- Challis J, Sloboda D, Matthews S, Holloway A, Alfaidy N, Howe D, Fraser M & Newnham J 2000 Fetal hypothalamic–pituitary–adrenal (HPA) development and activation as a determinant of the timing of birth, and of postnatal disease. *Endocrine Research* **26** 489–504.
- Cowley AW Jr 1988 Vasopressin and blood pressure regulation. *Clinical Physiology and Biochemistry* **6** 150–162.
- Cummings JJ, D'Eugenio DB & Gross SJ 1989 A controlled trial of dexamethasone in preterm infants at high risk for bronchopulmonary dysplasia. *The New England Journal of Medicine* **320** 1505–1510.
- Dallman MF, Strack AM, Akana SF, Bradbury MJ, Hanson ES, Scribner KA & Smith M 1993 Feast and famine: critical role of glucocorticoids with insulin in daily energy flow. *Frontiers in Neuroendocrinology* **14** 303–347.
- Edwards LJ & McMillen IC 2001 Maternal undernutrition increases arterial blood pressure in the sheep fetus during late gestation. *Journal of Physiology* **533** 561–570.
- Edwards LJ & McMillen IC 2002 Impact of maternal undernutrition during the periconceptual period, fetal number, and fetal sex on the development of the hypothalamo–pituitary–adrenal axis in sheep during late gestation. *Biology of Reproduction* **66** 1562–1569.
- Edwards LJ, Coulter CL, Symonds ME & McMillen IC 2001a Prenatal undernutrition, glucocorticoids and the programming of adult hypertension. *Clinical and Experimental Pharmacology & Physiology* **28** 938–941.
- Edwards LJ, Symonds ME, Warnes KE, Owens JA, Butler TG, Jurisicic A & McMillen IC 2001b Responses of the fetal pituitary–adrenal axis to acute and chronic hypoglycemia during late gestation in the sheep. *Endocrinology* **142** 1778–1785.
- Florio P, Severi FM, Ciarmela P, Fiore G, Calonaci G, Merola A, De Felice C, Palumbo M & Petraglia F 2002 Placental stress factors and maternal–fetal adaptive response: the corticotropin-releasing factor family. *Endocrine* **19** 91–102.
- Fowden AL & Forhead AJ 2004 Endocrine mechanisms of intrauterine programming. *Reproduction* **127** 515–526.
- Fowden AL, Li J & Forhead AJ 1998 Glucocorticoids and the preparation for life after birth: are there long-term consequences of the life insurance? *Proceedings of the Nutrition Society* **57** 113–122.
- Gardner DS, Jackson AA & Langley-Evans SC 1998 The effect of prenatal diet and glucocorticoids on growth and systolic blood pressure in the rat. *Proceedings of the Nutrition Society* **57** 235–240.
- Gardner DS, Fletcher AJ, Fowden AL & Giussani DA 2001 Plasma adrenocorticotropin and cortisol concentrations during acute hypoxemia after a reversible period of adverse intrauterine conditions in the ovine fetus during late gestation. *Endocrinology* **142** 589–598.
- Gardner DS, Pearce S, Dandrea J, Walker R, Ramsay MM, Stephenson T & Symonds ME 2004 Peri-implantation undernutrition programs blunted angiotensin II evoked baroreflex responses in young adult sheep. *Hypertension* **43** 1290–1296.
- Gardner DS, Tingey K, Van Bon BW, Ozanne SE, Wilson V, Dandrea J, Keisler DH, Stephenson T & Symonds ME 2005 Programming of glucose–insulin metabolism in adult sheep after maternal undernutrition. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **289** R947–R954.
- Giussani DA, Fletcher AJ & Gardner DS 2001 Sex differences in the pituitary–adrenocortical response to acute hypoxia in the ovine fetus during late gestation. *Journal of Endocrinology* **2** P62.
- Goddard PJ, Gaskin GJ & Macdonald AJ 1998 Automatic blood sampling equipment for use in studies of animal physiology. *Animal Science* **66** 769–775.
- Handa RJ, Burgess LH, Kerr JE & O'Keefe JA 1994 Gonadal steroid hormone receptors and sex differences in the hypothalamo–pituitary–adrenal axis. *Hormones and Behavior* **28** 464–476.
- Hawkins P, Steyn C, McGarrigle HH, Saito T, Ozaki T, Stratford LL, Noakes DE & Hanson MA 1999 Effect of maternal nutrient restriction in early gestation on development of the hypothalamic–pituitary–adrenal axis in fetal sheep at 0.8–0.9 of gestation. *Journal of Endocrinology* **163** 553–561.
- Jaquieri AL, Oliver MH, Bloomfield FH, Connor KL, Challis JR & Harding JE 2006 Fetal exposure to excess glucocorticoid is unlikely to explain the effects of periconceptual undernutrition in sheep. *Journal of Physiology* **572** 109–118.
- Langley-Evans SC 1997 Intrauterine programming of hypertension by glucocorticoids. *Life Sciences* **60** 1213–1221.
- Langley-Evans SC 2004 Experimental models of hypertension and cardiovascular disease. In *Fetal Nutrition and Adult Disease: Programming of Chronic Disease through Fetal Exposure to Undernutrition*, pp. 129–156. Ed. SC Langley-Evans. Wallingford, UK: CAB International.
- 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.

- Lee S & Rivier C 1996 Gender differences in the effect of prenatal alcohol exposure on the hypothalamic-pituitary-adrenal axis response to immune signals. *Psychoneuroendocrinology* **21** 145–155.
- Lucas A, Fewtrell MS & Cole TJ 1999 Fetal origins of adult disease—the hypothesis revisited. *British Medical Journal* **319** 245–249.
- Matthews SG, Owen D, Kalabis G, Banjanin S, Setiawan EB, Dunn EA & Andrews MH 2004 Fetal glucocorticoid exposure and hypothalamic-pituitary-adrenal (HPA) function after birth. *Endocrine Research* **30** 827–836.
- McCance RA & Widdowson EM 1974 The determinants of growth and form. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **185** 1–17.
- McCormick CM, Smythe JW, Sharma S & Meaney MJ 1995 Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats. *Developmental Brain Research* **84** 55–61.
- Nyirenda MJ, Welberg LA & Seckl JR 2001 Programming hyperglycaemia in the rat through prenatal exposure to glucocorticoids—fetal effect or maternal influence? *Journal of Endocrinology* **170** 653–660.
- Ogilvie KM & Rivier C 1997 Gender difference in hypothalamic-pituitary-adrenal axis response to alcohol in the rat: activational role of gonadal steroids. *Developmental Brain Research* **766** 19–28.
- Owen D & Matthews SG 2003 Glucocorticoids and sex-dependent development of brain glucocorticoid and mineralocorticoid receptors. *Endocrinology* **144** 2775–2784.
- Phillips DI, Barker DJ, Fall CH, Seckl JR, Whorwood CB, Wood PJ & Walker BR 1998 Elevated plasma cortisol concentrations: a link between low birth weight and the insulin resistance syndrome? *Journal of Clinical Endocrinology and Metabolism* **83** 757–760.
- Redmond GP 1979 Effect of drugs on intrauterine growth. *Clinics in Perinatology* **6** 5–19.
- Reynolds RM, Walker BR, Syddall HE, Whorwood CB, Wood PJ & Phillips DI 2001 Elevated plasma cortisol in glucose-intolerant men: differences in responses to glucose and habituation to venepuncture. *Journal of Clinical Endocrinology and Metabolism* **86** 1149–1153.
- Reynolds RM, Syddall HE, Walker BR, Wood PJ & Phillips DI 2003 Predicting cardiovascular risk factors from plasma cortisol measured during oral glucose tolerance tests. *Metabolism* **52** 524–527.
- Robinson PM, Comline RS, Fowden AL & Silver M 1983 Adrenal cortex of fetal lamb: changes after hypophysectomy and effects of Synacthen on cytoarchitecture and secretory activity. *Experimental Physiology* **68** 15–27.
- Roca CA, Schmidt PJ, Deuster PA, Danaceau MA, Altemus M, Putnam K, Chrousos GP, Nieman LK & Rubinow DR 2005 Sex-related differences in stimulated hypothalamic-pituitary-adrenal axis during induced gonadal suppression. *Journal of Clinical Endocrinology and Metabolism* **90** 4224–4231.
- Roelfsema F, van den BG, Frolich M, Veldhuis JD, van Eijk A, Buurman MM & Etman BH 1993 Sex-dependent alteration in cortisol response to endogenous adrenocorticotropin. *Journal of Clinical Endocrinology and Metabolism* **77** 234–240.
- Roseboom TJ, van der Meulen JH, Ravelli AC, Osmond C, Barker DJ & Bleker OP 2001 Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. *Molecular and Cellular Endocrinology* **185** 93–98.
- Sakkinen H, Tornbeg J, Goddard PJ, Eloranta E, Ropstad E & Saarela S 2004 The effect of blood sampling method on indicators of physiological stress in reindeer (*Rangifer tarandus tarandus*). *Domestic Animal Endocrinology* **26** 87–98.
- Sapolsky RM, Romero LM & Munck AU 2000 How do glucocorticoids influence stress responses?, Integrating permissive, suppressive, stimulatory, and preparative actions *Endocrine Reviews* **21** 55–89.
- Seckl JR 1997 Glucocorticoids, feto-placental 11 beta-hydroxysteroid dehydrogenase type 2, and the early life origins of adult disease. *Steroids* **62** 89–94.
- Seckl JR & Chapman KE 1997 Medical and physiological aspects of the 11beta-hydroxysteroid dehydrogenase system. *European Journal of Biochemistry* **249** 361–364.
- Silva C, Ines LS, Nour D, Straub RH & da Silva JA 2002 Differential male and female adrenal cortical steroid hormone and cortisol responses to interleukin-6 in humans. *Annals of the New York Academy of Sciences* **966** 68–72.
- Symonds ME, Budge H, Stephenson T & Gardner DS 2005 Experimental evidence for long-term programming effects of early diet. *Advances in Experimental Medicine and Biology* **569** 24–32.
- van Lier E, Perez-Clariget R & Forsberg M 2003 Sex differences in cortisol secretion after administration of an ACTH analogue in sheep during the breeding and non-breeding season. *Animal Reproduction Science* **79** 81–92.
- Whitworth JA, Schyvens CG, Zhang Y, Mangos GJ & Kelly JJ 2001 Glucocorticoid-induced hypertension: from mouse to man. *Clinical and Experimental Pharmacology and Physiology* **28** 993–996.
- Whorwood CB, Firth KM, Budge H & Symonds ME 2001 Maternal undernutrition during early to midgestation programs tissue-specific alterations in the expression of the glucocorticoid receptor, 11 β -hydroxysteroid dehydrogenase isoforms, and type 1 angiotensin II receptor in neonatal sheep. *Endocrinology* **142** 2854–2864.
- Widdowson EM & McCance RA 1975 A review: new thoughts on growth. *Pediatric Research* **9** 154–156.

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