Alterations in placental 11 β -hydroxysteroid dehydrogenase (11 β HSD) activities and fetal cortisol:cortisone ratios induced by nutritional restriction prior to conception and at defined stages of gestation in ewes

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Abstract

In the placenta, cortisol is inactivated by NADP⁺- and NAD⁺-dependent isoforms of 11 β -hydroxysteroid dehydrogenase (11 β HSD). Decreased placental 11 β HSD activities have been implicated in intrauterine growth restriction (IUGR) and fetal programming of adult diseases. The objective of this study was to investigate whether placental 11 β HSD activities and fetal plasma cortisol:cortisone ratios could be affected by nutritional restriction of ewes (70% maintenance diet) throughout gestation, for specific stages of gestation, or prior to mating. Chronic nutritional restriction from day 26 of gestation onwards decreased NAD⁺-dependent 11 β HSD activities by $52 \pm 4\%$ and $45 \pm 6\%$ on days 90 and 135 of gestation respectively. Although the decreases in enzyme activities were associated with fetal IUGR, the cortisol:cortisone ratio in fetal plasma was unaffected by chronic nutritional restriction throughout pregnancy. Nutritional restriction confined to early (days 26–45), mid- (days 46–90) and late gestation (days 91–135), or the 30 days prior to mating, had no significant effect on NAD⁺-dependent, placental 11 β HSD activities, nor was there evidence of IUGR. However, nutritional restriction at each stage of pregnancy and prior to mating was associated with significant decreases in the fetal plasma cortisol:cortisone ratio (3.2 ± 0.7 in control fetuses; 1.0 to 1.6 in fetuses carried by nutritionally restricted ewes). We conclude that nutritional restriction of pregnant ewes for more than 45 consecutive days can significantly decrease NAD⁺-dependent placental 11 β HSD activities in association with IUGR. While the cortisol:cortisone ratio in fetal plasma is sensitive to relatively acute restriction of nutrient intake, even prior to mating, this ratio does not reflect direct *ex vivo* measurements of placental 11 β HSD activities.

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Introduction

In a wide range of potential target cells, glucocorticoid actions are moderated by isoforms of 11 β -hydroxysteroid dehydrogenase (11 β HSD) which catalyse the interconversion of active 11 β -hydroxysteroids (cortisol and corticosterone) with their inert 11-ketosteroid metabolites (cortisone and 11-dehydrocorticosterone respectively) (see Michael *et al.* 2003 and references therein). Two biochemically distinct isoforms of 11 β HSD have been cloned. 11 β HSD1, which is ubiquitously expressed, is an NADP(H)-dependent enzyme which appears to act preferentially as an 11-ketosteroid reductase (11KSR), generating active glucocorticoid from cortisone/11-dehydrocorticosterone. In contrast, 11 β HSD2 is a high-affinity, 11 β -dehydrogenase enzyme which protects mineralocorticoid receptors from illicit

occupation by glucocorticoids in aldosterone target cells (Michael *et al.* 2003).

Within the placenta, both isoforms of 11 β HSD appear to fulfil important roles in controlling passage of glucocorticoid between the maternal and fetal circulations (Lopez-Bernal & Craft 1981, Pepe *et al.* 1996, Benediktsson *et al.* 1997, Dodds *et al.* 1997, Yang 1997, Burton & Waddell 1999). The current model of human placental function is that, in the decidua, placental extravillous trophoblast, chorion trophoblasts and amnion epithelial cells, 11 β HSD1 acts to increase the local concentration of cortisol, limiting the inflammatory response to the fetal allograft. In contrast, 11 β HSD2, localised to the placental syncytiotrophoblast, inactivates glucocorticoids, limiting passage of cortisol from the maternal to the fetal circulation (Krozowski *et al.* 1995, Stewart *et al.* 1995, Sun *et al.*

1997). Studies of cortisol–cortisone conversion in the human placenta have indicated an association between impaired placental inactivation of cortisol by 11 β HSD2 and intrauterine growth retardation (IUGR) (Edwards *et al.* 1993, Shams *et al.* 1998). In view of the current focus on fetal origins of adult disease and the fetal-programming ('Barker') hypothesis linking IUGR with increased risk of cardiovascular disease and diabetes (Barker & Clark 1997, Godfrey 1998, McMillen *et al.* 2001), the endocrine and paracrine control of placental 11 β HSD activities is currently under investigation in several independent laboratories.

Within the ovine placenta, both cloned isoforms of 11 β HSD are expressed and appear to play important physiological roles, although these may not be the same as those in the human, pig and rat. For example, although 11 β HSD2 is presumed to act in the sheep (as in other species) as a barrier to cortisol derived from the maternal circulation, the placental inactivation of cortisol decreases in the latter stages of gestation in marked contrast to the increase seen as pregnancy progresses in most other species (reviewed by Michael *et al.* 2003). The complete absence of 11 β HSD2 activity from the term ovine placenta (Whorwood *et al.* 2001) almost certainly reflects suppression of 11 β HSD2 expression by the pre-term rise in fetal cortisol output (Clarke *et al.* 2002). In the ovine placenta, the reductive activity of 11 β HSD1 at term in the face of declining 11 β HSD2 activity has been implicated in a paracrine glucocorticoid–prostaglandin feed-forward loop that may drive parturition (Whittle *et al.* 2001).

Recently, Whorwood *et al.* (2001) reported that nutritional restriction of ewes in early to mid-gestation (days 28–77 of gestation; term = 147 days) resulted in decreased expression of mRNA encoding 11 β HSD2 in the ovine placenta and in fetal tissues (adrenal glands and kidneys) at mid-gestation without any significant change in the placental expression of 11 β HSD1 mRNA. In their study, Whorwood *et al.* estimated NADP(H)- and NAD⁺-dependent 11 β HSD activities in fetal liver and perirenal adipose tissue, and in the fetal kidneys respectively, but did not establish whether any changes occurred to glucocorticoid metabolism within the placenta. The primary aim of the studies reported herein was to establish, therefore, whether NADP⁺- and NAD⁺-dependent 11 β HSD activities, measured *ex vivo* in ovine placentomes, could be influenced by nutritional restriction at defined stages of ovine pregnancy, including the periconceptual period. The secondary aim of this research was to establish whether plasma cortisol and cortisone concentrations, and the ratio of cortisol:cortisone in fetal plasma, reflected the direct assessments of placental 11 β HSD activities at the corresponding stages of gestation.

Materials and Methods

Experimental protocols

All studies were performed as described previously (Osgerby *et al.* 2002) in accordance with the UK Animals (Scientific Procedures) Act 1986 and with the approval of the Royal Veterinary College Experimental Ethics Committee. Each experiment was conducted during the normal seasonal breeding cycle, using multiparous Welsh Mountain ewes housed individually throughout the study under natural light at ambient temperature on straw bedding with *ad libitum* access to drinking water. (Experimental series A was performed 2 years prior to experimental series B; that is, in different breeding seasons.)

In both of the experimental series described below, the body condition score for each ewe was standardised to 2.5 prior to the commencement of the experiment (UK Ministry of Agriculture, Fisheries and Food 1996). All ewes were initially fed (at 0800 h and 1600 h) with a complete diet of pelleted sheep nuts that supplied 10.8 MJ metabolizable energy and 150 g crude protein per kg dry matter. The amount of diet provided to each ewe was calculated on an individual basis to provide 100% of their daily maintenance requirements based on criteria published by the UK Meat and Livestock Commission (MLC) (1988). After 30 days' acclimatisation, the ewes were synchronised for oestrus by the withdrawal of vaginal sponges impregnated with medroxyprogesterone acetate (60 mg Veramix; Upjohn, Crawley, UK), which had been inserted 12 days previously. Sponge withdrawal was accompanied by a single intramuscular injection of a luteolytic dose of the prostaglandin F_{2 α} analogue, cloprostenol (0.5 ml Estrumate; Schering-Plough Animal Health, Uxbridge, UK). Synchronisation was staggered for batches of ewes (randomised for treatment group) so that all ewes could be presented to the same fertile ram 48 h after sponge withdrawal/cloprostenol injection (hence minimising genotypic variation between pregnancies). In each case, day 0 of gestation was indicated by the first day at which ewes exhibited an obvious raddle mark on their rump (reflective of mating by the ram). Pregnancy was confirmed at presumptive day 16 of gestation by measuring plasma progesterone concentrations with a commercial enzyme immunoassay kit (Ridgeway Science Limited, Rodmore Mill Farm, Alvington, UK). All pregnancies were confirmed to be singleton pregnancies on day 60 of gestation by transabdominal ultrasound scans.

In experimental series A, 18 Welsh Mountain ewes carrying singleton pregnancies were randomly allocated to two experimental groups on day 22 of gestation. Ewes in the control group ($n = 8$) continued to receive 100% of their daily maintenance requirements throughout their pregnancies. Ewes allocated to the chronically undernourished group ($n = 10$) were weaned over 4 days onto 70% of their daily maintenance requirements, and remained on this lower plane of nutrition from day 26 of gestation

onwards. The amounts fed to each ewe were reviewed and adjusted each week in accordance with UK MLC guidelines. Serial samples of plasma were obtained from the jugular vein of each ewe on days 1, 20, 27, 41, 55, 69, 76, 83, 90, 97, 111, 125 and 132 of gestation. Ewes were humanely killed by captive bolt and exsanguination on either day 90 or day 135 of gestation (four control ewes and five undernourished ewes on each day), these days having been selected to represent periods of established placental growth and rapid fetal growth respectively (Barcroft 1946, Ehrhardt & Bell 1995). (It was not feasible to allow ewes to proceed to term since previous authors have reported a complete loss of 11 β HSD2 expression and activity in the term ovine placenta attributable to the pre-term rise in fetal cortisol output (Whorwood *et al.* 2001, Clarke *et al.* 2002).) Immediately after death, the gravid uteri were excised via a mid-line incision, and the placentomes were removed, snap frozen in liquid nitrogen-tempered isopentane (Merck, UK) and stored at -20°C pending *ex vivo* assay of 11 β HSD activities in the presence of excess NADP $^{+}$ or NAD $^{+}$. Fetuses were recovered, and fetal plasma samples (1–5 ml) were collected and stored (at -20°C) before measuring several fetal growth parameters, as previously described (Osgerby *et al.* 2002).

In experimental series B, 19 pregnant Welsh Mountain ewes were randomly allocated to a total of five experimental groups. The control ewes in experimental group B1 ($n = 4$) were maintained on 100% maintenance diet before mating and throughout pregnancy. Ewes in experimental groups B2 ($n = 3$), B3 ($n = 4$) and B4 ($n = 3$) were each restricted to a dietary intake calculated to provide 70% of the maintenance diet in early, mid- and late gestation (days 26–45, 46–90 or 91–135 of gestation) respectively. Ewes in these experimental groups were fed a 100% maintenance diet both before mating and at all unrestricted stages of pregnancy. Ewes in experimental group B5 ($n = 5$) were fed a restricted 70% diet in the 30 days prior to mating, but were provided with a 100% maintenance diet throughout pregnancy. In experimental series B, all animals were killed by captive bolt and exsanguination on day 135 of gestation, irrespective of experimental group number. Fetal blood samples and placentomes were collected and stored as for experimental series A.

Placentome 11 β HSD activities

Rates of cortisol oxidation by 11 β HSD were assayed *ex vivo* by standard radiometric conversion. For each ewe, enzyme activities were assessed (in triplicate) in a single placentome, selected at random irrespective of the anatomical structure of that placentome or its position within the uterus. Immediately prior to assay, placentomes were thawed and thin sagittal sections of tissue were cut from the centre of each placentome. For each placentome, 0.5 g of tissue was rapidly homogenised in 9 ml hypotonic

Tris–EDTA lysis buffer (Rusvai & Naray-Fejes-Toth 1993, Sewell *et al.* 1998, Thompson *et al.* 2000). After restoring isotonicity by addition of 1 ml KCl (1.5 mol/l) (Merck, UK), the homogenate was centrifuged at 250 *g* for 20 min at 4°C and the supernatant decanted into a fresh glass tube. From this supernatant, 100 μl volumes were transferred to nine glass culture tubes, to each of which was added 700 μl phosphate-buffered saline (PBS) (Life Technologies, UK). Triplicate tubes were also prepared as assay blanks containing 100 μl BSA solution (1 mg/ml prepared in PBS) in place of tissue homogenate. Each tube was then preincubated for 30 min at 37°C in a gyratory waterbath. To initiate enzyme assays, triplicate tubes received 100 μl PBS containing no exogenous cofactor, 4 mmol/l NADP $^{+}$ or 4 mmol/l NAD $^{+}$ (Sigma, UK), and 100 μl PBS containing 0.5 μCi [^3H]cortisol plus unlabelled cortisol (to a final steroid concentration of 100 nmol/l). Tubes were then returned to the waterbath for 1 h, after which reactions were terminated by the addition to each tube of 2 ml ice-cold chloroform. The assay duration and the tissue concentration of the placentome homogenates were each selected as optimal assay conditions under which the net oxidation of cortisol to cortisone conformed to first-order enzyme kinetics.

Following centrifugation at 1000 *g* for 30 min at 4°C , the aqueous phases were aspirated and the organic extracts were evaporated to dryness under nitrogen at 40°C . The steroid residues were resuspended in 20 μl ethyl acetate containing 1 mmol/l cortisol and 1 mmol/l cortisone (Sigma, UK), and were resolved by thin-layer chromatography (TLC) using Silica 60 TLC plates (Merck, UK) in an atmosphere of 92:8 (v/v) chloroform:95% (v/v) ethanol (Merck, UK). After quantification of [^3H]cortisol and [^3H]cortisone with a Bioscan 200 TLC radiochromatogram scanner (LabLogic, Sheffield, UK), 11 β HSD activities were calculated as pmol cortisol oxidised to cortisone over 1 h and standardised per mg protein, the protein concentration of each placentome homogenate having been determined by the Biorad assay (Bradford 1976, Rosa *et al.* 1980).

With the assay as described herein, multiple placentomes harvested at the same time from the same uterus in a given pregnancy exhibited comparable enzyme activities irrespective of their position in the uterus or their precise anatomical structure (in terms of proportions of fetal versus maternal tissue). Within a pregnancy, the coefficient of variation (CV) for NAD $^{+}$ -dependent 11 β HSD activities measured in different placentomes was only 13% (as compared with a CV of 27% for single placentomes derived from different ewes), there being no significant influence of position of the placentomes within the uterus (ANOVA $P > 0.6$).

Steroid radioimmunoassays

Total cortisol and cortisone concentrations (that is, the sum of the free steroid and protein-bound steroid concentrations) were measured in maternal and fetal plasma

samples by specific radioimmunoassays (Moore *et al.* 1985, Wood *et al.* 1996). The cortisol RIA had a range of 30–2000 nmol/l with intra- and interassay coefficients of variation of <7% and <8% respectively. The cortisone RIA had a range of 4–500 nmol/l with intra- and interassay coefficients of variation of <8% and <10% respectively. The antibodies used for these RIAs each exhibited under 0.1% cross-reactivity with progesterone.

Statistics

All statistical evaluations were performed with GraphPad Prism2 software (San Diego, CA, USA). For experimental series A, changes in the maternal plasma cortisol and cortisone concentrations, and in the cortisol:cortisone ratios, were analysed through gestation by two-way analysis of variance (ANOVA) (day of gestation in the first dimension and the nutritional status of the ewes in the second dimension) followed by linear regression analyses. All other data were analysed by one-way ANOVA followed by either Dunnett's or Bonferroni's multiple comparisons, as appropriate. In all cases, $P < 0.05$ was accepted to indicate statistical significance.

Results

Effects of chronic gestational restriction of nutrient intake

In experimental series A, chronic restriction of nutrient intake throughout gestation was associated with significant decreases in NAD^+ -dependent $11\beta\text{HSD}$ activities ($P < 0.001$) relative to placentomes from matched control ewes on days 90 and 135 of gestation (Fig. 1A). In contrast, oxidation of cortisol in the presence of NADP^+ was unaffected by chronic nutritional restriction (Fig. 1B) and did not differ significantly from the basal enzyme activities measured in the absence of exogenous cofactors on either day 90 or 135 (data not shown). On both days of gestation, NAD^+ -dependent $11\beta\text{HSD}$ activities were four- to sixfold higher than the basal enzyme activities in the same tissue homogenates (Fig. 1).

Between days 27 and 132 of gestation, the concentrations of cortisol and cortisone in maternal plasma did not change significantly in either control ewes or those with chronic restriction of nutrient intake ($R^2 \leq 0.151$, $F \leq 1.602$, $P > 0.2$) (Fig. 2A and B). In control ewes, the maternal plasma cortisol:cortisone ratios declined steadily from a mean value of 5.4 ± 1.5 on day 27 of gestation to a nadir of 1.7 ± 0.2 on day 111 of pregnancy ($R^2 = 0.544$, $F = 10.71$, $P < 0.01$; Fig. 2C). The plasma cortisol:cortisone ratios in nutritionally restricted ewes declined in parallel from a peak of 5.0 ± 1.1 on day 27 to 2.0 ± 0.5 on day 111 of gestation ($R^2 = 0.533$, $F = 10.25$, $P < 0.02$; Fig. 2C), there being no further impact of nutritional restriction on the decline in maternal plasma cortisol:cortisone ratios.

Figure 1 $11\beta\text{HSD}$ activities measured *ex vivo* in placentomes recovered from control ewes fed 100% maintenance diet through gestation (Cont) or ewes fed 70% maintenance diet (UN) from day 26 of gestation until death (day 90 or day 135 of gestation, as indicated on the figure). Enzyme activities were measured over 1 h in the presence of 100 nmol/l cortisol plus 400 $\mu\text{mol/l}$ NAD^+ (panel A) or NADP^+ (panel B). Each data point represents the mean $\pm$ S.E.M. for 3–5 independent placentomes, each taken at random from a different ewe. (Values for n in each data set are indicated in square brackets). *** $P < 0.01$ vs control placentomes on the corresponding day of gestation with the appropriate enzyme cofactor.

Although the absolute cortisol and cortisone concentrations in fetal plasma increased between days 90 and 135 of gestation, the cortisol:cortisone ratios in fetal plasma did not differ significantly between days 90 and 135 of gestation, nor did they differ between ewes maintained on the 100% vs 70% diets (Table 1). However, these ratios in fetal plasma (1.7 ± 0.3) were consistently and significantly lower than the ratio in the corresponding maternal plasma samples (3.9 ± 1.2 ; $P < 0.05$).

At day 135 of gestation, fetuses recovered from chronically undernourished ewes showed evidence of IUGR. Specifically, fetuses carried by chronically undernourished ewes tended to be lighter than control fetuses (3886 ± 238 g vs 4395 ± 99 g respectively; $P < 0.1$), and have a shorter thoracic girth (33.7 ± 0.6 cm vs 37.3 ± 0.8 cm respectively; $P < 0.01$), a shorter umbilical

Figure 2 Maternal plasma concentrations of cortisol (panel A), cortisone (panel B) and cortisol:cortisone ratios (panel C) across different days of gestation in ewes fed 100% maintenance diet throughout gestation (solid line) or ewes fed 70% maintenance diet from day 26 of gestation until death on day 90 or day 135 of gestation (broken line). Each data point represents the mean $\pm$ S.E.M. value for up to 10 ewes per time point.

Table 1 Plasma cortisol:cortisone ratios for fetuses carried by ewes fed either a 100% maintenance diet throughout gestation (control) or a 70% maintenance diet from day 26 of gestation until death on day 90 or 135 of gestation (chronically undernourished). Data are means $\pm$ S.E.M. for five or four independent plasma samples (as indicated in square brackets) each obtained from a different fetus.

Day of gestation	Nutritional status of ewe	
	Control	Chronically undernourished
Day 90 [n]	[5]	[5]
Plasma cortisol (nmol/l)	12 $\pm$ 4	8 $\pm$ 0
Plasma cortisone (nmol/l)	8 $\pm$ 2	8 $\pm$ 4
Cortisol:cortisone ratio	1.9 $\pm$ 0.8	1.8 $\pm$ 0.5
Day 135 [n]	[4]	[4]
Plasma cortisol (nmol/l)	30 $\pm$ 9	23 $\pm$ 3
Plasma cortisone (nmol/l)	21 $\pm$ 3	15 $\pm$ 2
Cortisol:cortisone ratio	1.4 $\pm$ 0.4	1.6 $\pm$ 0.2

There were no significant differences between experimental groups.

girth (33.6 ± 0.7 cm vs 37.8 ± 0.7 cm respectively; $P < 0.01$), shorter humerus bones (7.4 ± 0.1 cm vs 7.7 ± 0.1 cm respectively; $P < 0.05$) and shorter scapulae (6.5 ± 0.1 cm vs 6.1 ± 0.1 cm respectively; $P < 0.05$). (Further information on the effects of these diets on fetal development has been published by Osgerby *et al.* 2002.)

Effects of limited restriction of nutrient intake

In experimental series B, restriction of nutrient intake in early, mid- or late pregnancy had no significant effect on either basal or NAD⁺-dependent 11 β HSD activities measured in placentomes isolated on day 135 of gestation. Although the concentrations of cortisol and cortisone in fetal plasma were similarly unaffected by a transient restriction in nutrient intake at a defined stage of gestation (Fig. 3A and B), nutritional restriction at each stage of pregnancy was associated with a significant

decrease in the fetal plasma cortisol:cortisone ratio, as compared with fetuses carried by control ewes on the 100% maintenance diet throughout pregnancy (Fig. 3C).

Preconceptual restriction of nutrient intake in the 30 days prior to mating caused a similar decrease in the fetal cortisol:cortisone ratio at day 135 of gestation (Fig. 3C). However, this decrease in fetal steroid ratios did not achieve statistical significance with respect to control fetuses ($P < 0.1$) and occurred without any significant alteration in placentome 11 β HSD activities, as compared with those ewes fed the 100% maintenance diet prior to mating and throughout gestation.

Limited restriction of nutrient intake at defined stages of pregnancy and prior to mating had no significant impact on any of the measured indices of fetal growth (Table 2), nor did it result in any significant effect on the total weight

of placentomes in each experimental group (Table 2; ANOVA $P > 0.6$).

Discussion

Recent studies by Whorwood *et al.* (2001) have suggested that IUGR in pregnant ewes with restricted nutrient intake in early to mid-gestation may involve decreases in the expression and activity of the placental 11 β HSD2 enzyme at mid-gestation. Interestingly, Whorwood *et al.* also found the levels of 11 β HSD2 mRNA to be undetectable in the ovine placenta at term (day 147).

In experimental series A of the present study, we found that limiting nutrient intake to 70% of that required for normal maintenance from day 26 of gestation until death

Figure 3 Fetal plasma concentrations of cortisol (panel A), cortisone (panel B) and cortisol:cortisone ratios (panel C) on day 135 of gestation from control ewes fed 100% maintenance diet through gestation (Control) or ewes fed 70% maintenance diet between days 26 and 45 of gestation (Early), between days 46 and 90 of gestation (Mid) and between days 91 and 135 of gestation (Late), or for 30 days prior to mating (Pre). Each data point represents the mean $\pm$ S.E.M. for 3–5 fetuses. (Values for n in each data set are indicated in square brackets.) * $P < 0.05$, ** $P < 0.01$ vs fetal plasma cortisol:cortisone ratios in control fetuses.

Table 2 Placental weights and growth parameters on day 135 of gestation for fetuses carried by ewes fed either a 100% maintenance diet prior to and throughout gestation (control) or a 70% maintenance diet from days 26–45 of gestation (early restricted), days 46–90 of gestation (mid-restricted) and days 91–135 of gestation (late restricted), or for 30 days prior to mating (preconception restricted). Data are means $\pm$ S.E.M. for 3–5 independent observations (indicated for each group in square brackets).

	Nutritional status of ewe				
	Control [4]	Early restricted [5]	Mid-restricted [3]	Late restricted [4]	Preconception restricted [4]
Total weight of placentomes (g)	573 $\pm$ 45	511 $\pm$ 51	505 $\pm$ 71	527 $\pm$ 38	487 $\pm$ 51
Fetal weight (g)	4400 $\pm$ 293	4514 $\pm$ 298	4381 $\pm$ 321	4353 $\pm$ 142	4396 $\pm$ 148
Crown–rump length (cm)	47.5 $\pm$ 0.9	46.8 $\pm$ 0.7	47.3 $\pm$ 1.8	45.8 $\pm$ 0.6	45.9 $\pm$ 0.7
Thoracic girth (cm)	35.6 $\pm$ 0.9	35.7 $\pm$ 0.8	34.8 $\pm$ 0.2	35.3 $\pm$ 0.9	35.5 $\pm$ 0.9
Umbilical girth (cm)	37.8 $\pm$ 1.5	38.4 $\pm$ 1.2	37.8 $\pm$ 1.4	37.9 $\pm$ 0.8	37.8 $\pm$ 1.3
Forelimb length (cm)	19.1 $\pm$ 0.3	20.0 $\pm$ 0.4	19.0 $\pm$ 0.6	19.0 $\pm$ 0.4	19.0 $\pm$ 0.8
Ponderal index (g/cm ³)	0.041 $\pm$ 0.002	0.044 $\pm$ 0.002	0.041 $\pm$ 0.002	0.046 $\pm$ 0.001	0.046 $\pm$ 0.002

There were no significant differences between groups within each row; one-way ANOVA $P > 0.05$.

was associated with significant decreases in the NAD⁺-dependent oxidation of cortisol by ovine placentomes at both days 90 and 135 of gestation. The use of a particular pyridine nucleotide *in vitro* cannot be used to discriminate absolutely between the activities of the two cloned 11 β HSD isoforms; although 11 β HSD2 has an absolute requirement for NAD⁺ as an oxidative cofactor, 11 β HSD1 will use either NAD⁺ or NADP⁺ to support the oxidation of cortisol to cortisone. However, for two reasons, we believe that NAD⁺-dependent oxidation of cortisol in the present study reflected placental 11 β HSD2 activities. Firstly, on both days 90 and day 135, the level of NAD⁺-dependent cortisol oxidation was fivefold greater than that measured in the presence of NADP⁺. Second, the differences in NAD⁺-dependent cortisol oxidation observed between control placentomes and those recovered from nutritionally restricted ewes were not reflected by any differences in cortisol metabolism in the presence of NADP⁺. Hence, we conclude that the approximate 50% decrease in placental expression of 11 β HSD2 mRNA, as reported by Whorwood *et al.* (2001), appears to equate to a corresponding 50% decrease in the activity of this enzymatic barrier to transplacental transfer of cortisol on both days 90 and 135 of gestation. According to previous measurements of 11 β HSD2 expression and activities in the fetal kidney and adrenal glands (Whorwood *et al.* 2001), the transcriptional decrease in 11 β HSD2 activity and expression following nutrient restriction during gestation may not be confined to the placenta.

In experimental series A, levels of NAD⁺-dependent cortisol oxidation were the same on days 135 and 90 of gestation. This observation contrasts with the previous finding that 11 β HSD2 mRNA is undetectable in both the fetal and maternal components of term ovine placentae (Whorwood *et al.* 2001). There are three possible explanations of this apparent discrepancy. Firstly, it is possible that the decline in placental expression of 11 β HSD2 mRNA does not occur until the last week of pregnancy; that is, after day 135 of gestation. Second, the half-life of the 11 β HSD2 protein may allow 11 β HSD2 activity to persist in ovine placentomes despite declining expression of

the relevant mRNA transcript(s). Third, we cannot exclude the possibility that we were evaluating the activity of a third, as yet uncloned NAD⁺-dependent isoform of 11 β HSD in the ovine placenta.

We have previously reported that restriction of nutrient intake throughout pregnancy in the ewe results in a profound remodelling of the ovine placenta. Specifically, there is overgrowth of the fetal compartment of the placenta which comes to dominate the maternal contribution to this fetomaternal interface (Osgerby *et al.* 2002). This could have been relevant to our current study, since 11 β HSD2 appears to be confined to the fetal compartment of the placenta, with predominant expression of 11 β HSD1 in the maternal compartment (Krozowski *et al.* 1995, Stewart *et al.* 1995, Sun *et al.* 1997, Whorwood *et al.* 2001). However, we found no significant difference in NAD⁺-dependent cortisol oxidation by placentomes of different structural types. Moreover, enzyme activities were independent of the position within the uterus at which the placentomes were attached.

After establishment that chronic restriction of nutrient intake throughout gestation can compromise 11 β HSD2 activities in ovine placentomes, experimental series B determined whether 11 β HSD2 activities in late gestation could be programmed by nutritional restriction at a defined stage of gestation, or possibly even prior to conception. NAD⁺-dependent 11 β -dehydrogenase activities measured on day 135 of gestation were unaffected by nutritional restriction on days 26–45, 46–90 or 91–135 of gestation, and were also unaltered by restriction of nutrient intake for 30 days prior to mating. These observations suggest that the decreases in NAD⁺-dependent placental oxidation of cortisol reported in experimental series A may require a sustained restriction of nutrient intake that either must last for a minimum duration (50 days according to the findings of Whorwood *et al.* 2001) or must span two or more defined stages of pregnancy. Nutrient restriction confined to early, mid- or even late gestation does not appear to alter placental 11 β HSD activities on day 135 of gestation.

In all experiments, the fetal plasma cortisol:cortisone ratios were consistently lower than those in matched maternal plasma samples. This suggests that the fetal ratio of active to inert 11-oxosteroids either may be altered during the transplacental passage of glucocorticoids from the maternal to the fetal circulation, or may be modulated by 11 β HSD activities within the fetus. In support of the latter postulate, in both experimental series, there was no relationship between the predominant NAD⁺-dependent 11 β -dehydrogenase activities and the ratios of cortisol:cortisone in fetal plasma. In experimental series A, chronic nutritional restriction from day 26 of gestation onward resulted in a 50% decrease in placental 11 β HSD2 activities without altering fetal cortisol:cortisone ratios. Conversely, in experimental series B, nutritional restriction at each defined stage of gestation significantly decreased the fetal plasma cortisol:cortisone ratio at day 135 of gestation without affecting NAD⁺-dependent cortisol metabolism in the placentomes at this stage of pregnancy. This lack of correspondence between placental enzyme activities and fetal plasma cortisol:cortisone ratios calls into question the widespread assumption that the ratio of cortisol:cortisone in the fetal circulation is dependent on the level of cortisol oxidation within the placenta as this glucocorticoid passes from the maternal to the fetal circulation. Instead, the fetal ratio of active 11 β -hydroxysteroids to inert 11-ketosteroids in late gestation may depend upon the maturation of the fetal hypothalamo-pituitary-adrenal (HPA) axis and/or the expression and activity of 11 β HSD isoforms within the fetus (rather than the placenta). This view is consistent with recent studies of plasma cortisol concentrations in the fetal lamb (Edwards *et al.* 2001, Edwards & McMullen 2002).

To the best of our knowledge, this is the first report of discord between the ratio of cortisol:cortisone in fetal plasma and direct measurements of 11 β HSD in the placenta. Hence, we also considered the absolute concentrations of cortisol and cortisone within the fetal plasma samples. On the basis of the model that placental 11 β HSD activity dictates the level of cortisol delivered to the fetus from the maternal circulation, decreases in placental 11 β HSD activity should be reflected by increased cortisol and decreased cortisone concentrations in the fetal plasma. However, in experimental series A, the decrease in NAD⁺-dependent cortisol oxidation following chronic nutritional restriction was not associated with any significant changes in plasma cortisol or cortisone concentrations within the fetus. These data reinforce our central finding that the concentrations of cortisol and cortisone within the fetus, and indeed the fetal plasma cortisol:cortisone ratio, do not depend solely on placental 11 β HSD2 activities, as determined by measuring cortisol oxidation in the presence of excess NAD⁺ on days 90 or 135 of gestation. We would contend that the absolute levels of cortisol and cortisone within fetal plasma, and the fetal plasma cortisol:cortisone ratios, also reflect the intrinsic activity of the fetal HPA axis and local metabolism of cortisol by 11 β HSD within fetal tissues.

In evaluating our data, we note that the ratios of cortisol:cortisone within the fetal plasma were higher in the control animals for experimental series A than in those control fetuses for experimental series B. In making this observation, we would emphasise that these experiments were conducted in different breeding seasons, separated by 2 years, and that all ewes were housed under natural light and at ambient temperature (rather than under conditions of controlled light and temperature). Since environmental variables such as light intensity and temperature can profoundly influence both maternal and fetal physiology, particularly with respect to the activity of the HPA axis, we propose that differences in the prevalent conditions between the breeding seasons resulted in different plasma cortisol:cortisone ratios for the control fetuses in the two experimental series. Hence, while we are confident that it is valid to compare endocrine measurements across experimental groups within each experiment (since all ewes within an experiment were pregnant at the same time), it would not appear to be valid to compare values between experiments conducted in different seasons.

In interpreting our data, we should also inject a note of caution with regard to the numbers of animals in each experimental group. While randomisation was applied throughout the study (for example, in terms of assigning animals, timing of sample collection and selection of individual placentomes), we must concede that the limited sample numbers inevitably restrict the power of all statistical tests performed in this study.

We do not yet have an explanation for why nutritional restriction in the ewe for 30 days prior to mating had a tendency to suppress the fetal cortisol:cortisone ratio some 135 days after conception. However, Bloomfield *et al.* (2003) have recently reported that periconceptual restriction of nutrient intake in the ewe alters the subsequent maturation of the fetal HPA axis, as reflected in the timing of parturition. Such findings indicate that the pattern of hormone synthesis and metabolism in the fetus and associated placenta may be influenced by nutritional status affecting the development of the early embryo, possibly via effects on the oocyte prior to fertilisation or on the uterine environment at the time of implantation.

In summary, we have demonstrated that nutritional restriction of ewes can compromise the activity of 11 β HSD2 in the ovine placenta. This restriction needs to occur for more than 45 days continuously if the decrease in placental 11 β HSD2 is to be significant and to be associated with IUGR. Moreover, neither the ratio of cortisol:cortisone in fetal plasma (which appears to be sensitive to relatively acute nutritional restriction) nor the absolute concentrations of these steroids in fetal plasma appear to reflect direct assessments of placental 11 β HSD2 activities.

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