The late gestation increase in circulating ACTH and cortisol in the fetal sheep is suppressed by intracerebroventricular infusion of recombinant ovine leptin

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Abstract

The obese gene product leptin, originally characterised as an adipocyte hormone coordinating the behavioural and neuroendocrine responses to starvation, is expressed in fetal adipocytes and placental trophoblast cells and is present in the fetal circulation. Concentrations of leptin in fetal blood correlate with fetal bodyweight and fat mass. In post-natal life, leptin conveys information about calorie intake and the state of adipose tissue energy stores, and plasma leptin levels are generally inversely correlated with hypothalamo–pituitary adrenal (HPA) activity. Late fetal life is characterised by increasing HPA activity that prepares the fetus for extrauterine life and initiates the endocrine cascade leading to parturition. We have investigated the hypothesis that leptin in the fetal circulation can inhibit the fetal HPA axis, thereby providing a mechanism by which the fetus can determine the fine timing of parturition as long as it is adequately nourished and growing appropriately. Here we show that a 5-day intracerebroventricular infusion of leptin to the sheep fetus in late gestation inhibits the pre-parturient rise in ACTH and cortisol concentrations, and that this seems to be a centrally mediated effect.

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Introduction

The obese gene product leptin is present in the fetal circulation, and is expressed in fetal adipocytes (Yuen *et al.* 1999) and placental trophoblast cells (Hoggard *et al.* 1997, Henson *et al.* 1998, 1999). The physiological function of leptin in the fetal circulation is unknown. One uncontrolled study in the near term sheep fetus reports that acute intracerebroventricular infusion of leptin stimulates fetal swallowing and urine flow (Roberts *et al.* 2001). In post-natal life, leptin is intimately concerned with coordinating the behavioural and endocrine responses to energy restriction (Rosenbaum *et al.* 1997, Barb *et al.* 1999). Feed restriction is associated with a fall in expression of leptin mRNA in adipose tissue, and a decline in circulating leptin concentrations. Infusion of leptin inhibits feeding behaviour in fasted and fed animals, increases insulin sensitivity to glucose (Ogawa *et al.* 1999, Shimomura *et al.* 1999) and prevents the fasting-induced decline in gonadotrophin secretion (Casanueva & Dieguez 1999, Cunningham *et al.* 1999, Nagatani *et al.* 2000) and increase in adrenal corticosteroid secretion (Ahima *et al.* 1996). There is evidence for an interaction between leptin and the hypothalamo–pituitary adrenal (HPA) axis (Casanueva & Dieguez 1999). Corticosteroids increase leptin expression in cultured adipocytes (Russell *et al.* 1998). Infusion of leptin inhibits fasting- and stress-induced increases in corticosterone (Ahima *et al.* 1996, Heiman *et al.* 1997) and, whereas leptin is a satiety signal, corticosteroids generally stimulate appetite (Solano & Jacobson 1999, Wooldridge *et al.* 2001).

In late gestation, increasing activity of the fetal HPA axis is crucial for initiating events that lead to birth and for fetal preparation for extrauterine life (Challis *et al.* 2000). The mechanisms driving the increase in fetal HPA activity are poorly understood. Metabolic signals related to the ability to sustain fetal growth may be important. In a sense, all fetal growth is metabolically constrained (Vatnick *et al.* 1991, Gluckman *et al.* 1992) and increasing fetal size in late gestation places greater demands on maternal metabolism and transplacental transfer of nutrients (Schneider 1996). Poor maternal weight gain, fetal growth restriction and maternal fasting have all been identified as factors associated with premature delivery in humans and

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domestic species (Silver 1990, Ott 1993, Hediger *et al.* 1995, McMillen *et al.* 1995). Cord blood concentrations of leptin correlate with birthweight and fat mass (Jaquet *et al.* 1998, Geary *et al.* 1999), suggesting that leptin might signal information to the fetal HPA axis about fetal metabolic status. A fetus that is relatively well supplied with metabolic substrate for continued growth would be expected to have a higher plasma leptin concentration that in turn will inhibit the fetal HPA axis and delay parturition.

We hypothesised that leptin would suppress the fetal HPA axis *in utero*. Accordingly, we have investigated the effects of 120 h continuous intracerebroventricular infusion of recombinant ovine leptin on HPA axis activity in the late gestation sheep fetus. Fetuses were challenged with corticotrophin-releasing hormone (CRH) and vasopressin (AVP) to determine pituitary sensitivity after 96 h of leptin infusion. Intravenous glucose tolerance tests were administered to the fetuses to determine whether leptin infusion led to altered insulin resistance in the fetus.

Materials and Methods

Animals

Fetuses of time-mated mixed breed sheep were prepared with jugular, carotid and lateral ventricle cannulae on day 125–130 gestation (term 145 ± 2) as previously described (Challis *et al.* 1981). Vascular catheters were flushed daily with heparinised saline and fetal acid–base status and blood gases were monitored on a daily basis. Ewes were provided with food and water and allowed to feed *ad libitum*. They were housed in rooms with a 12 h light:12 h darkness cycle. The day before the start of the infusion period, ewes were placed into individual metabolism crates allowing limited forwards and backwards movement. The study was performed using protocols approved by the University of Toronto, Animal Study Review Board, according to the Guidelines of the Canadian Council for Animal Care (CCAC).

Leptin treatment

Fetuses received a continuous intracerebroventricular infusion of 20 μg/h recombinant ovine leptin (Gertler *et al.* 1998) ($n=6$) or cerebrospinal fluid vehicle ($n=4$) from day 135 to 140 gestation, infused at a rate of 120 μl/h.

Basal hormone secretion and challenge tests

Basal hormone secretion was assessed over a 4-h period starting at 0800 h on day 135 and again on day 140 after 120 h continuous infusion of intracerebroventricular leptin or vehicle. Samples (1 ml) were withdrawn every 10 min from the fetal carotid artery and volume replaced with heparinised saline. On both days at the end of the 4-h basal sampling period, a 4 g intravenous glucose challenge was given directly to the fetus and further blood samples withdrawn at 10, 20, 30, 60, 90 and 120 min. Arterial blood gases and haematocrit were monitored hourly during the sampling period. Additional daily samples were withdrawn at 0800, 1400 and 2200 h throughout the experiment. A pituitary challenge with 4 μg CRH + 4 μg AVP was administered at 0800 h on the morning of day 139 after 96 h continuous intracerebroventricular infusion of leptin or vehicle. Blood samples were separated immediately and plasma stored at −70 °C until analysis.

Hormone assays

Plasma concentrations of adrenocorticotrophin (ACTH) were measured by a commercially available radioimmunoassay kit (Diasorin, Stillwater, MN, USA) as previously described (Norman *et al.* 1985). Intra-assay and interassay coefficients of variation were 8% and 12% respectively, and the assay limit of sensitivity was 6 pg/ml. Cortisol was measured after extraction with diethyl ether (Challis *et al.* 1981). Intra-assay and interassay coefficients of variation were 9% and 12% respectively. Plasma leptin concentration was measured by kit (multispecies leptin assay, Linco Research, St Charles, MO, USA) using recombinant ovine leptin standards. This kit has previously been reported to detect ovine leptin (Delavaud *et al.* 2000, Ehrhardt *et al.* 2000). The combined intra- and interassay coefficient of variation was 8% and the assay limit of sensitivity was 1 ng/ml of recombinant ovine standard. Plasma insulin concentration was determined by kit (Linco, rat insulin) using rat insulin as standard. The combined intra- and interassay coefficient of variation was 8% and the assay limit of sensitivity 0·2 ng/ml. Glucose was determined by glucometer (Glucometer Elite, Bayer Inc., Ontario, Canada). The combined intra- and interassay coefficient of variation on pooled fetal plasma was 3%.

Statistical analysis

Values are presented as means ± S.E.M. for the number of animals studied. Individual profiles of ACTH and cortisol were analysed for pulsatile secretion using the Munro program (Skinner *et al.* 1995). Briefly, a rolling average of local nadirs is used to create a baseline from which pulses are detected as deviations of at least three standard deviations. Mean baseline, pulse amplitude, interpulse interval and average hormone concentrations were calculated for each animal before and at the end of the leptin infusion. Systematic differences between the groups were determined by analysis of the variance with *post hoc t*-test. The significance level was set at 5%. For pituitary and glucose challenge tests, basal, peak response and area under the curve were calculated and compared.

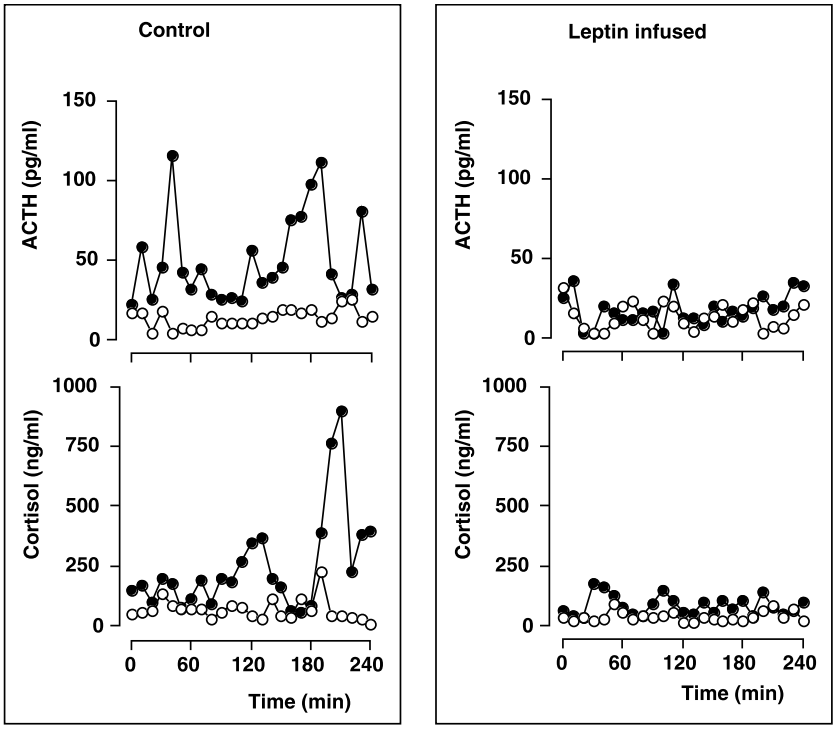

Figure 1 Montage showing ACTH (upper panels) and cortisol (lower panels) concentrations in two representative fetuses receiving an intracerebroventricular infusion of either vehicle (left-hand panel) or leptin (right-hand panel) for 5 days from day 135 to day 140 gestation. Hormone concentrations during the 4-h sampling period on day 135 are shown as open circles and during repeat sampling on day 140 as filled circles.

Results

Effect of leptin on basal HPA axis activity

Basal ACTH and cortisol concentrations were assessed on the morning of day 135 of gestation (prior to commencing intracerebroventricular infusion of recombinant leptin or vehicle) and again on the morning of day 140 (after 5 days of continuous treatment). The profiles of ACTH and cortisol concentrations in two representative fetuses are shown in Fig. 1.

The concentrations of both hormones in samples withdrawn every 10 min during a 4-h period between 0800 and 1200 h on days 135 and 140 were subjected to pulse analysis (Skinner *et al.* 1995). Figure 2 shows pulse amplitude, basal (nadir) and mean concentrations for ACTH and cortisol. Pulses of ACTH, detected as deviations from local baseline (the average of all local minima over a 60-min epoch) of more than three assay coefficients of variation, increased in amplitude between day 135 and day 140 in control fetuses. Basal (nadir) concentrations also increased, but there was no change in pulse frequency. At day 140, leptin infusion significantly abrogated the increase in ACTH pulse amplitude (ANOVA, treatment by time interaction: $P<0{\cdot}01$) and basal and mean ACTH concentrations were reduced significantly. Pulse frequency was unchanged by leptin treatment. Similarly, the gestation-dependent increases in pulse amplitude, basal and mean cortisol concentrations were significantly less in leptin-treated fetuses at day 140 compared with control fetuses (ANOVA, treatment by time interaction: $P<0{\cdot}01$).

Effect of leptin on pituitary sensitivity to CRH and AVP

The ACTH response (peak ACTH concentration and area under curve) to a bolus injection of CRH and AVP given after 96 h of continuous leptin infusion was not significantly different from that of control fetuses (Fig. 3) (ANOVA, main effect of treatment: $P>0{\cdot}05$). Basal ACTH prior to CRH and AVP challenge was significantly lower in the leptin-infused fetuses (ANOVA, main effect of treatment: $P<0{\cdot}01$). Basal cortisol concentrations were lower in the leptin-treated fetuses, but the adrenal response to CRH and AVP challenge (peak cortisol and area under the curve) did not differ between groups (ANOVA, $P<0{\cdot}01$).

Effect of leptin on glucose homeostasis

Plasma glucose and insulin concentrations following 1 g/kg fetal intravenous glucose challenge are shown in

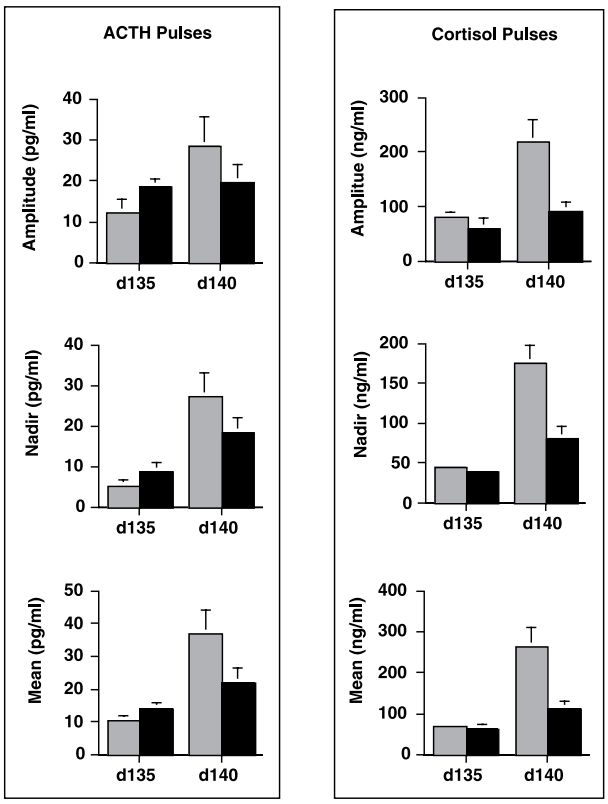

Figure 2 Summary of pulse analysis of plasma ACTH and cortisol concentrations during the 4-h sampling period on day 135 before, and on day 140 after 5 days of continuous intracerebroventricular infusion of vehicle (stippled bars) or leptin (solid bars). Pulse amplitude, nadir and mean hormone concentration are shown in upper, middle and lower panels respectively. Values are means ± S.E.M. ACTH and cortisol pulse amplitude, nadir (baseline) and mean concentration increased from day 135 to 140. The rise in amplitude, nadir and mean concentration was significantly less in leptin-infused fetuses (ANOVA: treatment by time interaction, $P<0{\cdot}01$).

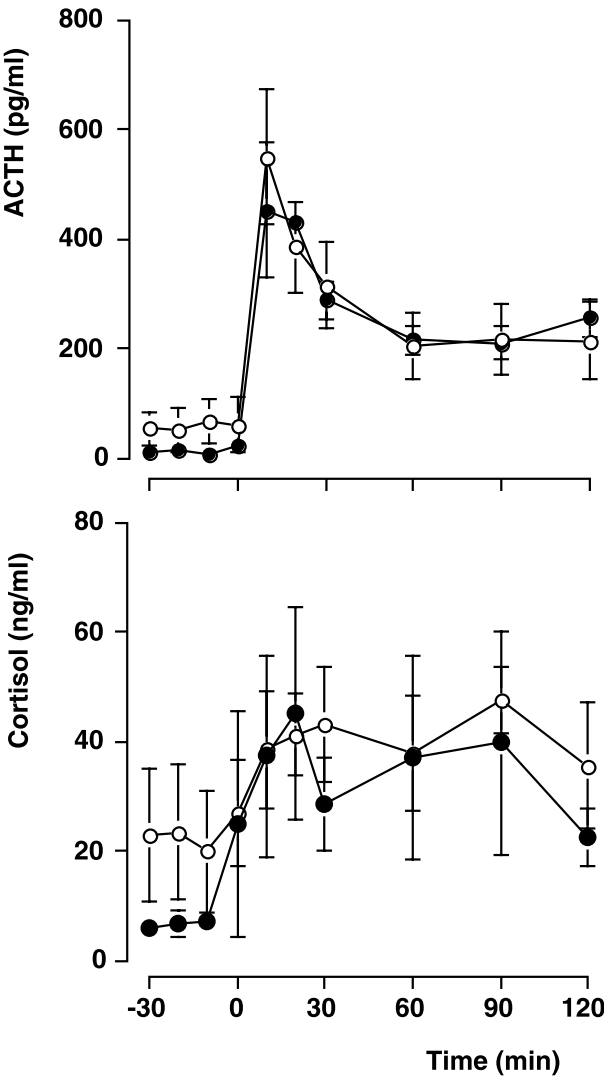

Figure 3 Plasma ACTH (upper panel) and cortisol (lower panel) concentrations after challenge with 4 µg corticotrophin releasing hormone and 4 µg vasopressin after 96 h of continuous intracerebroventricular infusion of vehicle (○) or leptin (●). Values are means ± S.E.M.

Fig. 4. Intracerebroventricular leptin infusion had no effect on basal glucose or insulin concentrations. Following intravenous glucose challenge, plasma insulin concentrations peaked with an approximate 20-min lag on peak glucose concentrations. There were no significant differences between groups in glucose or insulin peak or area under the insulin curve, or in the regression of peak insulin on peak glucose. There was no change in ACTH or cortisol concentrations during the glucose challenge.

Plasma leptin concentrations

Plasma leptin concentration was below the assay limit of sensitivity from 135 to 140 days in control fetuses, but was detected in the plasma of all leptin-infused fetuses (Fig. 5).

Discussion

We hypothesised that exogenous leptin would suppress the fetal HPA axis in late gestation. Our results demonstrate that continuous intracerebroventricular infusion of recombinant ovine leptin for 5 days abrogated the normal rise in plasma ACTH and cortisol concentrations that occur prior to parturition in the near term fetal sheep. Leptin infusion, however, despite elevating peripheral leptin concentrations and lowering cortisol, did not alter basal glucose or insulin concentrations. Similarly, the insulin response and glucose clearance after intravenous glucose challenge did not differ.

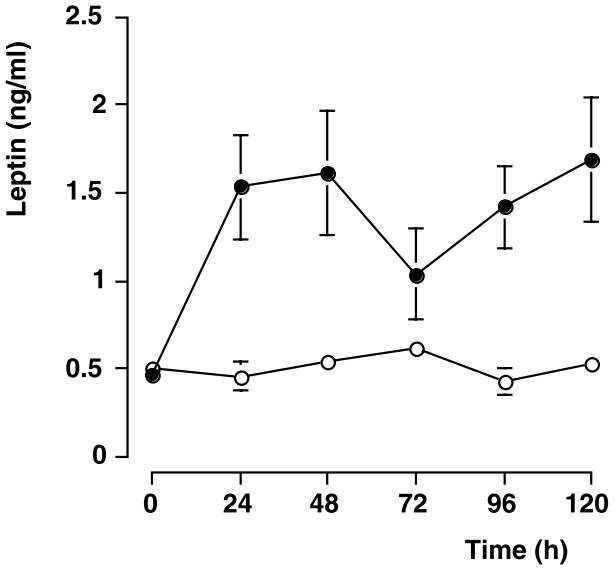

Figure 4 Daily leptin concentrations in fetal plasma during intracerebroventricular infusion of vehicle (○) or recombinant ovine leptin (●). Values are means ± S.E.M. Plasma leptin was significantly elevated by 24 h in fetuses receiving intracerebroventricular infusion of leptin, and remained elevated throughout the infusion.

In these experiments, leptin was administered intracerebroventricularly in order to achieve a significant elevation of leptin in the vicinity of the hypothalamic nuclei. Subsequently, the drainage of cerebrospinal fluid through lymphatics and arachnoid granulations allowed centrally administered leptin to enter the systemic circulation, as has been shown for other peptides (Mollanji *et al.* 2001). Infusing recombinant ovine leptin at a rate of 20 μg/h intracerebroventricularly, we have achieved plasma levels of leptin similar to those reported in adult sheep (Blache *et al.* 2000, Delavaud *et al.* 2000, Ehrhardt *et al.* 2000, Nagatani *et al.* 2000, Morrison *et al.* 2001, Thomas *et al.* 2001). Plasma leptin concentrations in the near term sheep fetus have been reported in the range of 400 pg/ml (Buchbinder *et al.* 2001, Forhead *et al.* 2002), unfortunately below the detection limit of the assay we have used. The leptin concentration achieved in the peripheral circulation in the treated fetuses is increased around three- to fourfold above that expected in control fetuses. Elevations of leptin in response to acute rises in cortisol, for example in association with hypoxaemia, however, might achieve similar high plasma concentrations. Nonetheless, actual hypothalamic levels may not be elevated to the same magnitude. Intracerebroventricularly administered leptin penetrates adjacent brain

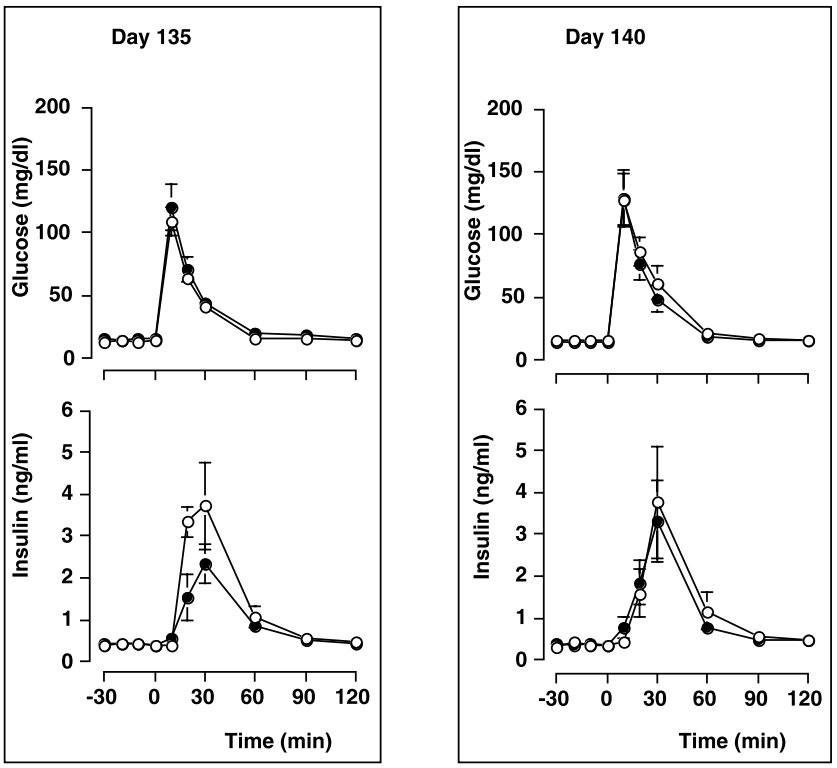

Figure 5 Plasma glucose (upper panels) and insulin (lower panels) during intravenous glucose challenge (4 g) on day 135 (left panel) and day 140 gestation (right panel). Hormone concentrations in fetuses receiving intracerebroventricular infusion of vehicle are shown as open circles and leptin as filled circles. Values are means ± S.E.M.

parenchyma poorly (Maness *et al.* 1998). Furthermore, transport of circulating leptin across the blood–brain barrier seems to be saturable (Ramsey *et al.* 1998), such that there is a 100- to 200-fold difference between plasma and cerebrospinal fluid concentrations (Dotsch *et al.* 1997, Blache *et al.* 2000).

Despite significant elevations of peripheral leptin and a decrease in cortisol, we found no change in basal glucose or insulin concentrations. As shown previously, fetal insulin responses were monophasic (Philipps *et al.* 1978). The glucose clearance and insulin response to glucose challenge did not change with gestation or leptin treatment. Leptin has been reported both to increase and decrease skeletal muscle sensitivity to insulin in rodents (Sweeney *et al.* 2001, Yaspelkis *et al.* 2001). In culture, high doses of leptin markedly reduced insulin-stimulated glucose uptake (Sweeney *et al.* 2001). In rodents, leptin also increases insulin sensitivity to glucose (Ogawa *et al.* 1999, Shimomura *et al.* 1999) and glucose disposal (Kamohara *et al.* 1997) by increasing uptake into brown adipose tissue. However, fetal brown adipose tissue is functionally immature until shortly before birth. Others find that in adult sheep, infusion of leptin had no effect on basal plasma insulin levels (Morrison *et al.* 2001).

In the fetal sheep, we found that exogenous leptin inhibits the HPA axis. The activation of fetal HPA activity in late gestation is well characterised and the increase in mean ACTH and cortisol concentrations in control fetuses is in agreement with previous reports. The amplitude and frequency of ACTH and cortisol pulse are in the range previously reported by others (Brooks & Challis 1991, Apostolakis *et al.* 1992, Canny *et al.* 1998), but we found an increase in ACTH and cortisol pulse amplitude from day 135 to day 140. Others examining the period 140–142 days could not demonstrate a change in pulsatile ACTH or cortisol characteristics (Apostolakis *et al.* 1992). We found that intracerebroventricular infusion of leptin at a rate similar to that used in adult sheep (Henry *et al.* 1999) results in a blunting of the normal pre-parturient rise in mean ACTH and cortisol concentrations. This is a consequence of a reduction in pulse amplitude rather than a decrease in pulse frequency. The effect is likely to be centrally mediated since the pituitary response to challenge with AVP and CRH did not differ between treated and control fetuses.

The observation that exogenous leptin inhibits the fetal HPA axis is consistent with findings in rodents where leptin blocks the adrenocortical response to fasting (Ahima *et al.* 1996) and stress activation of HPA function (Heiman *et al.* 1997). The effects of leptin on the neuroendocrine axis in larger animals have been questioned. In one study in ovariectomised adult ewes, recombinant human leptin infused intracerebroventricularly at a rate of 20 μg/h for 72 h reduced food intake, but did not alter pulsatile luteinising hormone (LH) or growth hormone (GH) secretion (Henry *et al.* 1999). Pooled plasma cortisol, follicle-stimulating hormone (FSH) and prolactin (PRL) also remained unchanged. Control animals did not have their dietary intake reduced to match that of the treated group, so that it is impossible to assess if leptin inhibited neuroendocrine responses to energy restriction. In another study of oestrogen-treated castrated males, subcutaneous recombinant human leptin (150 μg/kg per day) achieving plasma levels of 18 ng/ml (in comparison with endogenous plasma levels of 1–2 ng/ml) prevented the decline in LH pulse frequency during a 78-h fast, and at the same time increased the amplitude of GH pulses (Nagatani *et al.* 2000). In long-term feed-restricted adult sheep, intracerebroventricular infusion of leptin increases GH concentrations (Henry *et al.* 2001, Morrison *et al.* 2001). The reported effects on the reproductive axis conflict with studies finding either no change (Morrison *et al.* 2001) or an increase in LH pulse frequency (Henry *et al.* 2001). There is a paucity of literature on the HPA axis and leptin under such conditions.

The fetus differs from the adult in a number of ways and might not be expected to respond to leptin in the same way as an adult. Nutrient intake cannot be increased by stimulation of feeding behaviour. Fetal growth is metabolically constrained and provision of extra calories either by direct intrafetal infusion of glucose, by maternal overnutrition or insulin-like growth factor-I (IGF-I) treatment promotes fetal somatic growth rather than accumulation of fat stores (Charlton & Johengen 1987, Stevens *et al.* 1990, Stephenson *et al.* 2001). The fetus is relatively lean and there seem to be endocrine mechanisms inhibiting energy accumulation as adipose tissue (Hay 1995). Leptin is present both in fetal adipose tissue and the placenta in the sheep (Thomas *et al.* 2001) as in other species (Hoggard *et al.* 1997, Henson *et al.* 1998, 1999, Chen *et al.* 2000). The expression of leptin in perirenal adipocytes in the fetal sheep increases with gestation (Yuen *et al.* 1999). However, the proportion of perirenal fat in comparison with fetal body weight declines in late gestation (Hay 1995, Stephenson *et al.* 2001) and the relative contributions of placental and adipose tissue to circulating leptin concentrations are unknown. It is unlikely, however, that plasma leptin concentrations convey information solely about the state of fetal adipose stores.

In adults, the disproportionate change in leptin in proportion to fat mass following weight reduction or acute overfeeding has led to the suggestion that leptin signals caloric intake rather than fat mass *per se* (Considine *et al.* 1996, Kolaczynski *et al.* 1996, Barb *et al.* 1999). Regardless of whether fetal plasma leptin levels are determined by calorie intake or by some more complex interaction of placental and adipose tissue growth, we show that increases in circulating leptin have the potential to inhibit the fetal HPA axis in late gestation. We speculate that the functional consequences of this may be to prevent the accelerated fetal HPA activation that leads to parturition and to promote continued fetal growth. Infusion of cortisol

into adrenalectomised fetuses to mimic the normal pre-parturient increase in cortisol inhibits fetal growth (Fowden *et al*. 1996). A fetus receiving adequate metabolic substrate via the placenta might be expected to maintain relatively higher plasma leptin concentrations, that in turn suppress the fetal HPA axis, thereby allowing continued growth and postponing birth. Removal of that leptin-imposed inhibition in late pregnancy might then contribute to activation of fetal HPA function and parturition.

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