PROGRAMMING OF ADULT CARDIOVASCULAR FUNCTION AFTER EARLY
MATERNAL UNDERNUTRITION IN SHEEP

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Running title: Early diet and adult cardiovascular function

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The prenatal nutritional environment influences the subsequent risk of hypertension in adulthood. Animal studies have used, generally, the rat as a model species to illustrate the association between maternal nutrient intake and blood pressure in the resulting adult offspring. No study to date has shown programming of adult cardiovascular function in the sheep through maternal dietary intervention. We therefore fed pregnant sheep to either 100% recommended intake from day 0 of gestation to term (~147 dGA; controls n=8) or to 50% recommended intake from day 0-95 dGA and thereafter to 100% intake (NR; n=9). Sheep lambed naturally, offspring were weaned at 16 weeks and the male offspring were reared on pasture until 3 years of age. At this time, cardiovascular catheters were inserted under halothane anaesthesia and sheep allowed 2-4 days recovery. Resting cardiovascular status and pressor responses to infusion of noradrenaline, angiotensin II and captopril were then assessed alongside resting plasma concentrations of glucose, cortisol and leptin. NR sheep were of similar birth weight to controls but at three years of age had higher blood pressure prior to, but not after, feeding. Peripheral sensitivity to vasoconstrictor infusion was similar between dietary groups, although a reflex bradycardia was not apparent in NR sheep during noradrenaline infusion. Circulating leptin correlated well with fat mass and increased more after vasoconstrictor infusion in NR sheep. In conclusion, early NR has been shown to programme aspects of cardiovascular control and adipocyte function in adult sheep. 

Keywords: Programming, Nutrition, Noradrenaline, SNS, Blood pressure, Leptin
INTRODUCTION

Hypertension is a major risk factor for ischaemic heart disease (IHD). An age-specific increase of 20(10) mmHg systolic(diastolic) blood pressure above average more than doubles the risk of IHD (42). Hypertension is therefore a common risk factor for death in the population over the age of 50 (40; 52). Hypertension and IHD are both multifactorial in their etiology and amongst other predisposing factors (e.g. high fat and salt intake, smoking and obesity) a sub-optimal intrauterine environment has been shown to be of particular importance in determining potential risk status (4). While poor in utero development is often marked by low birth weight, the association with elevated adult blood pressure is not necessarily dependent upon a reduction in birth weight per se, but is perhaps due to complex interactions between the pre- and postnatal environment (20). The methodologies and inferences implied by the ‘developmental origins of adult disease hypothesis’ have been criticised (33; 43), but appear robust when subjected to meta-analyses (32; 40). Furthermore, through the use of animal models, that are largely free from methodological bias and socio-economic confounding, there is sufficient evidence to suggest the hypothesis is biologically plausible and of major importance in terms of public health (6; 31; 39).

To date, however, physiological programming of adult cardiovascular risk has only been demonstrated repeatedly in small animal models such as the rat (38; 51). Rats, being a litter-bearing species, are particularly nutritionally sensitive to either specific (protein; Langley & Jackson 1994) or global undernutrition (51) or high dose glucocorticoid exposure (5) during pregnancy, owing to the high rate of fetal growth and thus protein accretion relative to maternal size (47). In contrast, the
sheep has a similar rate of pre- and postnatal growth to the human, and usually produces only one or two offspring weighing between 3-6 kg – not unlike humans. However, in this species any observed programmed changes in cardiovascular control following nutritional restriction have been limited to the late gestation fetus or young lamb (18; 26). Programming of adult cardiovascular function in the sheep has only ever been observed following high dose glucocorticoid therapy for two days in early gestation (14-16); however, this study does not address the potential for undernutrition during this period to programme adult cardiovascular function, as suggested by retrospective human data (56; 58).

Based upon the aforementioned rodent studies, the elevation in resting blood pressure of the adult, after maternal undernutrition, appears related in part to programmed elements of the renin-angiotensin-system (49; 59; 60), sympathoadrenal function (41) and a tendency to deposit more fat as an adult (65). Interrelationships between these factors exist: for example, increased circulating and local angiotensin II action has been shown to stimulate leptin release from adipocytes (10) and prenatal nutrient restriction reduces intrarenal renin (71) which, combined, are predicted to result in reduced circulating angiotensin II concentrations and thus up-regulation of tissue angiotensin II receptor densities. Indeed, this has been shown, although the response may be species and tissue-specific, and time-dependent i.e. type I angiotensin II receptor densities are increased in the adrenal, kidney and liver of neonatal sheep (67) but decreased in the kidney of adult rats (49) after maternal undernutrition. In addition, circulating leptin – which reflects body adiposity – has clear effects on
autonomic, in particular, sympathetic function (28). Early undernutrition of pregnant sheep has been shown to result in fatter term fetuses (7).

In the present study we have extended these findings into adulthood in the sheep. In addition to investigating the effects of global nutrient restriction during early-mid gestation on body composition of the resultant male offspring at three years of age, we have undertaken an *in vivo* analysis of basal and stimulated cardiovascular and endocrine function. As an index of cardiovascular function we have measured resting systolic, diastolic and mean arterial blood pressure, heart rate and pressor responses to exogenous infusion of the potent vasopressor hormones angiotensin II and noradrenaline – given the observed programming of neonatal angiotensin II receptor densities using this model (67) and of sympathoadrenal activity in others (41), and to the physiological pressor stimulus of feeding in sheep. During the short-term pressor challenges, we have also made an assessment of baroreflex function through the parallel changes in blood pressure and heart rate. Operation of the cardiovascular baroreflex is key to maintaining central pressure during ambulatory changes in blood pressure: if inadequate, then risk of later hypertension is increased (17; 24; 50). Alongside these cardiovascular indices, the current study assesses the resting plasma concentration of leptin – as an indicator of body fat mass – and the change in leptin concentration before and after angiotensin II or noradrenaline infusion – to indicate the endocrine sensitivity of adipose tissue (9; 10; 30). In addition, the resting plasma concentration of glucose and cortisol were measured to provide indices of resting metabolic and stress status, respectively.
MATERIALS AND METHODS

Animals. All procedures were performed under the UK Animals (Scientific Procedures) Act, 1986. Seventeen mature Scottish Blackface male-bearing ewes of similar age, live weight and body condition score were mated during a synchronized oestrus and randomly allocated to receive either a control (C; n=8) or nutrient restricted (NR; n=9) diet from day 1 of pregnancy. Ewes were group housed under natural day length conditions with unlimited access to water. During the first 95 days of gestation, control ewes were fed 100% metabolisable energy (ME) requirements for live weight maintenance (8.0 MJ/day) as defined by the AFRC (1) while NR sheep were fed to 50% of that amount (4.0 MJ/day). Thereafter rations were increased according to litter size and the changing requirements associated with the increase in conceptus weight, so that at the time of lambing they had sufficient tissue reserves to sustain a normal lactation (1). The basal diet consisted of 250 g hay per day (1.0 MJ/day) and 650 g dried grass pellets per day (7 MJ/day; Green Keil, North Eastern Farmers Ltd., Aberdeen, UK). The nutrient restricted diet consisted of a similar proportion of hay (250 g; 1.0 MJ/day) but reduced pellet intake (280 g; 3 MJ/day). The diets contained an adequate amount of vitamins (Vit A, 8121mg/kg; D, 2005mg/kg & and E, 50mg/kg diet) with minerals provided as blocks (“Baby Rockies”, Tithebarn Ltd, Winsford, Cheshire; containing magnesium, 1g/kg; iron 200mg/kg; manganese, 100mg/kg; iodine 50mg/kg; zinc 120mg/kg; cobalt 100mg/kg; selenium 20mg/kg and sodium 38%). All ewes were weighed and body condition scored at 21-day intervals. At term, lambs were delivered naturally with no intervention and birth weights recorded. The offspring were ewe reared until weaning at 16 weeks of age and thereafter grass-fed at the Macaulay Institute Glensaugh Research Station, Aberdeen until three years of age.
**Experimental protocols.** At approximately one month prior to surgery all uncastrated male sheep were transported to the Sutton Bonington Campus, University of Nottingham and group housed indoors. 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⁻¹ I.V. Intraval Sodium; Rhone Mérieux, Dublin, Ireland) and maintained with 1-2% halothane in 50:50 O₂/N₂O. Right carotid and jugular catheters were inserted into each sheep, secured and the neck incision closed. Catheters emerging from the neck were coiled and protected within a 10” bandage. All sheep received a dose of long-acting antibiotic (15 mg.kg⁻¹ I.M. amoxycillin, ‘Duphamox‘; Fort Dodge Animal Health Ltd, Southampton, UK) and analgesia (1 mg.kg⁻¹ flunixin meglumine; ‘Finadyne‘; Shering-Plough, Kenilworth, UK) post-operatively. Catheter patency was maintained by daily flushing with heparinized saline (50 I.U. heparin.ml⁻¹). All sheep were housed in individual pens but together in a room with controlled lighting (12h on 12h off; 8.00am-8.00pm), had established normal feeding patterns within 1-2 hrs after surgery, and showed no visible signs of discomfort for the duration of the experimental period. A period of 2-3 days post-operative recovery was allowed prior to any experiment being performed and the investigator was blinded to the dietary origin of the sheep. For experimental protocols, catheters were connected to pre-calibrated pressure transducers (SensorNor 840; S 4925), attached at heart level and linked to a data acquisition system (Po-Ne-Mah; Version 3, Gould Instrument Systems Inc). Over a 7-10 day period, cardiovascular variables were recorded from all sheep on 4 separate occasions and days, with *ad libitum* hay and water available. Each experiment was begun between 9.00-10.00am. After a baseline period of one hour, analogue signals for real-time systolic, diastolic, mean arterial pressure and heart
rate were recorded sec-by-sec during a further 30 min period, were digitized and stored on an Excel spreadsheet for further analysis. From these basal data, pulse pressure (systolic-diastolic) and the rate pressure product ([mean arterial blood pressure (mmHg) x heart rate (beats min\(^{-1}\)])/10^3), an index of myocardial work and thus oxygen consumption, were derived. This 30 min recording period was considered the baseline for daily cardiovascular variables. The following protocols were conducted in a random order on separate days and for angiotensin II, noradrenaline and captopril in all sheep. For the cardiovascular response to feeding, data for n=5 controls and n=8 NR only are available.

**Feeding:** Sheep were not fed concentrate for a period of 24 hr. The following morning after a baseline period of 10 min the sheep were fed hay and concentrate with *ad libitum* access to water. Cardiovascular variables were recorded for a 10 min period during which all concentrate feed was consumed and for a further 10 min post-prandial period.

**Angiotensin II:** After a baseline period of 10 min, step-wise I.V. increases in angiotensin II (0, 1, 2, 4, 8, 16 & 32 ng.kg\(^{-1}\).min\(^{-1}\)) were administered every 10 min, followed by a 30 min recovery period in which cardiovascular variables returned to baseline.

**Noradrenaline:** After a baseline period of 10 min, step-wise I.V. increases in noradrenaline (0, 2, 4, 8, 16, 32 & 48 ng.kg\(^{-1}\).min\(^{-1}\)) were administered every 10 min, followed by a 10 min recovery period in which cardiovascular variables returned to baseline.

**Captopril:** After a baseline period of 30 min the potent angiotensin converting enzyme (ACE) inhibitor, captopril, was infused for 30 min at a dose of 0.12 mg.kg\(^{-1}\).hr\(^{-1}\). This dose has been previously validated to be the lowest effective dose to
completely block the pressor effect of 0.5µg angiotensin I (61). Indeed, in our own hands, doubling the dose rate of captopril had no greater hypotensive effect (23). After infusion, cardiovascular variables were recorded for a further 20 min recovery period or until blood pressure had returned to baseline.

Blood samples: On each day of an experiment a blood sample (5 ml) was taken prior to the experiment and subsequently immediately after infusion of the highest dose given. In addition, on a separate day after animals had previously been fasted for 12 h, blood samples (2 ml) were taken during baseline (30 min prior to feeding) and every 30 min thereafter for a total period of 6 hrs. The blood was drawn into heparinised syringes, placed in chilled blood tubes and centrifuged at 3500rpm (800g), 4°C for 5 min and the resultant plasma stored at -20°C for later analysis of glucose, cortisol and leptin concentration. After all experimental protocols sheep were euthanased with a lethal dose of sodium pentobarbitone (Euthatal; 100mg/kg) and tissue weights recorded.

Hormone analysis: Plasma concentrations of glucose were measured enzymatically (Trinder; glucose oxidase) as described by Symonds et al (64). Plasma concentrations of leptin were assayed using a double antibody RIA, validated for use with ovine plasma as previously described in detail (13). Samples were assayed in duplicate (200µl) using a rabbit anti-ovine leptin primary antibody, iodinated ovine leptin and sheep antirabbit secondary antibody. The leptin assay has a sensitivity of 0.10 ng/ml with intra- and interassay coefficients of variation of 4 and 11% (n=5), respectively. Total cortisol was measured using a commercially available coated-tube RIA kit (Coat-a-Count cortisol, Diagnostic Products Corp, Ltd,
Caernarfon, UK) validated for use with ovine plasma (7). The minimum detection limit for the assay was 0.5 ng/ml and the intra- and interassay (n=5) coefficients of variation were 4 and 11%, respectively.

Statistical analyses. All data are expressed as Means ± S.E.M. unless otherwise stated. The data were first tested for normality of distribution and appropriate parametric or non-parametric tests used. The data for birth weight and current weight were continuous and analysed by one-way ANOVA. Cardiovascular variables (blood pressures, heart rate, rate pressure product) were analysed by two-way ANOVA with repeated measures for effects of group e.g. control vs. NR, time e.g. prior to, during and after feeding/pressor challenges and any interaction between group*time. Post hoc statistics were run where indicated using Tukeys’ t-test. Hormone data were analysed by two-way ANOVA with repeated measures. Area under the curve (AUC) for heart rate was calculated using a custom designed Excel spreadsheet according to Equation 1.

Equation 1: Analysis of area under the curve $\frac{0.5(a+z)}{2} + \left(\sum_{b}^{y} y \right)$.

Where a is the first data point, z is the last data point and b-y are the data points enclosed by the curve. For example, during the 60 minutes of noradrenaline infusion; a is the AUC for the trapezium describing minute 1 of infusion, b is the AUC for minute 60 and b-y represent minutes 2-59. Average values were then compared by one-way analysis of variance. For a comparison between the slopes of linear regression curves for mean arterial blood pressure and heart rate obtained
during noradrenaline infusion the analyses was conducted using ANCOVA according to Armitage & Berry (3). All statistical comparisons were conducted using SPSS 11.1 (SPSS Inc, Chicago, USA). For all comparisons, statistical significance was accepted when P<0.05.
RESULTS

**Offspring weights:** Lamb weights were similar between maternal dietary groups (Control, 4.4 ± 0.2; NR, 4.0 ± 0.3 kg) and were appropriate given the size of the ewe and plane of nutrition during pregnancy, according to recommended guidelines for the nutrition of housed, pregnant ewes (1). At three years of age there was no difference in body weight between the two groups of sheep (control, 75.6 ± 2.8; NR, 75.0 ± 2.6 kg).

**Basal cardiovascular status:** Baseline cardiovascular variables were assessed prior to, during and after feeding and/or each experimental protocol. In the morning, prior to feeding of concentrate, values for systolic (Control, 99±2 vs. NR, 110±2 mmHg), diastolic (65±3 vs. 74±2 mmHg) and mean arterial pressure (79±2 vs. 89±3 mmHg), heart rate (83±5 vs. 102±4 beats.min⁻¹) and the rate pressure product (6.54±0.37 vs. 9.11±0.41 beats.min⁻¹.mmHg⁻¹/10³) were significantly elevated (all \( P<0.05 \)) in NR sheep. However, after feeding, these values in NR sheep were not significantly different to controls (Figure 1). On subsequent days, cardiovascular variables were recorded after the morning feed and averaged values for individual sheep over 2-3 baseline recording periods were not significantly different between dietary groups (systolic, 109±3 vs. 113±2 mmHg; diastolic, 74±3 vs. 76±2 mmHg; mean arterial pressure, 87±3 vs. 91±2 mmHg; heart rate, 87±5 vs. 92±5 beats.min⁻¹), in control and NR sheep, respectively.

**Cardiovascular responses to angiotensin II infusion:** In both control and NR sheep, angiotensin II infusion resulted in dose-dependent increments in arterial
blood pressure and decrements in heart rate (Figure 2A & 2B). The magnitude of
the increase in pressure and decrement in heart rate were not significantly different
between dietary groups (Figure 2).

Cardiovascular responses to noradrenaline infusion: In both control and NR
sheep, noradrenaline infusion resulted in dose-dependent increments in arterial
blood pressure (Figure 3A & B). In NR sheep, the increase in arterial blood pressure
was similar but heart rate failed to decline significantly (Figure 3D). Consequently,
the area under the curve for delta heart rate during the challenge was significantly
lower in NR relative to control sheep (98 ± 58 vs. –163 ± 46 AUC units
respectively; P=0.004). Furthermore, when plotted as a linear relationship between
individual data points for mean arterial blood pressure and heart rate (baroreflex
sensitivity) the intercept, but not the slope, was significantly elevated in NR (y=-
0.23x + 116) sheep relative to controls (y=-0.35x + 114, t= 4.92; P<0.001), as
illustrated in Figure 4.

Cardiovascular responses to captopril infusion: In control and NR sheep,
captopril infusion resulted in a decrease in systolic, diastolic and mean arterial blood
pressure by an average of 4.6±1.6 mmHg and 6.2±1.7 mmHg (systolic pressure),
respectively – without change in heart rate. There were no significant differences
between groups in the response to captopril infusion.

Basal glucose, cortisol and leptin status: Plasma concentrations for glucose,
cortisol and leptin did not vary over the course of the study period (Figure 5) and
mean values were not significantly different in control and NR sheep (Glucose, 5.38±0.30 vs. 5.06±0.72 mmoles.L\(^{-1}\); cortisol, 29.0±5.3 vs. 23.6±1.6 nmoles.L\(^{-1}\); leptin, 3.14±1.03 vs. 4.37±1.15 ng.ml\(^{-1}\), for control and NR sheep, respectively). However, during both noradrenaline and angiotensin II infusion the delta change in plasma leptin was significant (P<0.05) in NR but not in control offspring (Controls, before infusion 3.22±1.9 and 2.73±0.96, after infusion 3.16±1.7 and 3.13±1.10 ng/ml for before and after noradrenaline and angiotensin II infusion, respectively; NR, before infusion 3.47±0.9 and 3.50±1.46, after infusion 4.5±1.3 and 5.30±2.27 ng/ml, respectively). Captopril infusion had no effect on plasma leptin in either dietary group (Controls, 3.75±2.65 & 4.16±2.44 ng/ml; NR, 3.81±2.45 & 3.55±2.45 ng/ml before & after infusion, respectively). The delta change in plasma leptin during each pressor challenge is illustrated in Figure 6B.

**Sheep biometry at three years of age:** There were no differences between the two dietary groups in the weights of any organ measured with the exception of the liver, which was significantly smaller in NR relative to control sheep, when expressed in absolute (Table 1) or relative (NR; 15.0±0.5 vs. Control; 16.8±0.6 g/kg; P=0.01) terms. While the lower weight of the gonads in NR approached significance in absolute terms (Table 1), this effect was weakened when expressed relative to body weight (P=0.09). The degree of fatness of the sheep regardless of dietary group (g fat per kg empty carcass) correlated well with plasma leptin (r\(^2\)= 0.47, P= 0.007, Figure 6A).
DISCUSSION

We have shown for the first time in adult sheep that maternal nutritional restriction from conception to the end of the period of maximal placental growth results in programmed alterations to cardiovascular function in the three-year-old offspring. Specifically, irrespective of weight at birth, nutrient restricted (NR) sheep had higher blood pressure prior to, but not after, feeding and altered baroreflex responses to step-wise noradrenaline infusion. These cardiovascular changes were not associated with altered glucose or glucocorticoid profiles. As adults, NR sheep were of similar body weight to controls, but this masked a significant reduction in hepatic weight. Plasma leptin correlated significantly with the ratio of fat/fat-free mass in both groups, and increased after noradrenaline and angiotensin II infusion in NR, but not control, sheep.

The developmental origins of adult disease hypothesis has been subject to methodological and interpretive criticism (32; 43), but stands up when meta-analyses of the human data (33; 40) and evidence from animal models are considered (6; 31; 34; 39). From very early on in the studies of the nutritional regulation of fetal growth, it was clear that the ‘timing’, severity and duration of nutrient restriction with respect to later postnatal outcome was important (47; 53; 68; 69). These ‘programming’ influences may be reconciled with the nature of the growth process. When the episode of nutrient restriction coincided with, or persisted beyond, a period of hyperplastic vs. hypertrophic organ development its structure and/or function can be permanently and irretrievably altered (47; 68; 69). In the context of the developmental origins of adult disease hypothesis, such
alterations lead to a profound limitation in organ function such that the maximal functional capacity is achieved earlier and more readily, creating premature pathophysiological sequelae (34). Perhaps one of the most demonstrated examples in animal models is a permanent reduction in nephron number, as a result of an early insult, and increased incidence of hypertension (66; 70; 71).

Epidemiological data from the ‘Dutch Hunger Winter Famine’ of World War II highlighted early gestation as a particularly sensitive period for the programming of subsequent cardiovascular development (57). This is not surprising given the early priority for establishing systems necessary to support adequate nutrient delivery for tissue growth, e.g. the placenta and fetal cardiovascular system. In the human, a heart beat is detectable from as early as 22 days of a ~276 day gestation period while in the sheep, placental growth is most rapid during days 30-80 of a ~150 day gestation (19). For this reason we chose to restrict maternal nutrient intake over a period encompassing both embryonic and maximal placental growth. To date many different studies in rats have shown nutritional programming of hypertension (for review see (6; 39). The rat, however, may be particularly vulnerable to any nutritional imbalance during gestation given the exceptional rate of protein accretion during prenatal development (estimated at 23-fold that of the human fetus (48)) and the sum weight of the products of conception relative to maternal weight (25-35% vs. 6-10% in the sheep and 3-5% in humans). The programming of any outcome measure, such as blood pressure for example, may therefore be amplified in the rat; hence the 20-40 mmHg difference in systolic blood pressure after prenatal protein restriction (39). To date the one group to have shown
prenatal programming of hypertension in sheep, by targeted high-dose glucocorticoid therapy, observed group differences of only 5-10 mmHg (16).

The present study is the first to show that a physiological challenge (undernutrition) during gestation can programme resting blood pressure (~7-10mmHg higher in NR prior to feeding) and influence cardiovascular control (altered baroreflex responses during noradrenaline infusion) in the adult offspring of a species with similar pre- and postnatal growth rates to humans, which were followed longitudinally from birth. Clearly, however, despite the degree of undernutrition (50%) reflecting the difference between upper and lower quartiles for energy intake in a representative human population (8), and being sufficient to significantly alter maternal metabolism (12); it had no effect on birth weight or postnatal growth rate and only resulted in a moderate increase in offspring blood pressure as the adult sheep ages. Nevertheless, cardiovascular programming is evident in these sheep. The lack of any difference in peripheral responses to vasopressor infusion coupled with an effect on baroreflex function, may suggest a centrally-orientated, rather than peripheral origin, for the programmed change in physiological function. On the other hand, greater increases in plasma leptin concentration after noradrenaline/angiotensin II infusion in NR may indicate persistent programming of the adipocyte, since angiotensin II stimulates leptin release from fat cells (10). However, long-term changes in adipocyte function and angiotensin II activity in NR may well also reflect programming at the level of the brain, given the effects centrally acting leptin and angiotensin II can have on autonomic function (10; 21; 28). Furthermore, the data suggest that the increase in tissue-specific angiotensin II receptor densities evident at birth (67) persists into later life: however, this has
yet to be clarified with further molecular studies in adipose tissue and other
important sites for angiotensin II action such as the kidney, brain and peripheral
vascular tissue.

It has long been known that baroreflex function attenuates during an angiotensin
II/noradrenaline mediated increase in blood pressure (for review see (55). The
mechanism is thought to reflect a combination of decreased sympathetic withdrawal
together with less parasympathetic outflow from the area postrema, mediated by
angiotensin II stimulation of catecholaminergic neurons, rather than a direct effect
on the heart or baroreceptors themselves (55). With respect to the current study,
the attenuated fall in heart rate during noradrenaline infusion in NR sheep suggests
a weaker vagal dominance during increases in arterial pressure. Further in vivo
studies using atropine to antagonise cardiac vagal tone during noradrenaline
infusion together with molecular characterisation of cardiac adrenergic receptor
densities may well clarify this suggestion. A similar alteration to baroreflex function
has recently been observed in NR sheep at one year of age although, at this time,
no change in basal blood pressure was observed (22). Thus, given that early
gestational undernutrition of sheep lowers the resting blood pressure of the fetus
(27), has only a marginal effect by one year of age, but increases pre-feeding
systolic pressure at 3 years of age suggests that programming of cardiovascular
dysfunction in sheep develops gradually with age, reflecting the ontogenesis of
essential hypertension in humans (62); in contrast to the overt hypertension
observed in the offspring of rats soon after weaning (38).
Taken together, past and present data suggest that the adipocyte is sensitive to the programming influence of maternal undernutrition in terms of size and/or number and function (7; 54; 63; 67). Greater relative fat mass and, in particular, leptin production is thought to influence central blood pressure control and autonomic outflow and is often associated with increased blood pressure (25; 44; 45). In particular, excess weight (fat) gain appears to specifically increase renal sympathetic nerve activity, as renal denervation attenuates obesity-related hypertension (35). Leptin correlates well with relative fat:fat-free mass, as in the current study; however, it is becoming increasingly clear that leptin is more than the signalling molecule thought to mediate ‘lipostatic’ control of body weight (36). Rather leptin, in part, regulates overall energy balance by reducing food intake but increasing sympathetic activity, and thus energy expenditure (45; 46). Leptin infusion has been shown to increase sympathetic, in particular, renal sympathetic nerve activity (28) and blood pressure (11). Indeed transgenic mice over-expressing leptin have increased blood pressure despite being lean and of lower body weight, whereas leptin deficient, obese ob/ob mice have lower arterial blood pressure that is restored to levels observed in wild-type controls when leptin is replaced (2). Ganglionic blockade ameliorates the hypertensive state in mice over-expressing leptin, suggesting mediation by sympathetic nerve activity (2). Thus, functional hypothalamic leptin receptors and an intact sympathetic reflex arc appear a prerequisite for expression of obesity-related hypertension.

One putative hypothalamic target site is the melanocortin-type 4 receptor (MC4-R) as intracerebroventricular administration of leptin had been shown to activate the melanocortin system, resulting in increased renal sympathetic nerve activity (29).
Chronic blockade of the MC4-R produces gross obesity but has no effect on blood pressure (37). Thus in the context of the present study in which NR sheep show differing leptin responses to ‘stress-hormones’ (Fig 6B), elevated heart rate at any given mean arterial pressure (perhaps indicating increased sympathetic activity (Fig 4)) and increased pre-feeding blood pressure (Fig 1) it seems reasonable to presume that over a number of years a tendency to store more fat in NR offspring will lead to chronic, yet mild, increases in plasma leptin and influences central, perhaps renal, neural pathways associated with blood pressure regulation. Clearly to establish proof of principle we need to determine, in future studies, whether NR sheep are more susceptible (with respect to blood pressure regulation) to a drive for increased weight gain, either by restricting physical activity and/or increasing postnatal food intake. Any in vivo physiological measures can then be correlated with changes to putative hypothalamic receptors such as leptin and MC4-R.

In conclusion, the present study has shown for the first time that global undernutrition, during the periods of embryonic and placental development can programme adult physiology in the sheep. Increased pre-feeding blood pressure, elevated heart rate at a given mean arterial pressure and increased leptin responses to catecholamines and angiotensin II were all observed, and all are key mediators of the body’s stress response. Further detailed analysis of the hypothalamic expression of leptin and MC4-R and of angiotensin II receptor densities in key tissues such as adipose and kidney are suggested to substantiate the in vivo physiological data of the present study.
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LEGENDS

Figure 1. Cardiovascular response to feeding in control and nutrient restricted sheep.

Values are 5 min means ± S.E.M. for control (-○-, n = 5) and nutrient restricted (NR; -●-, n = 8) sheep for systolic (SBP), diastolic (DBP), mean arterial (MABP) blood pressure and heart rate (HR) before, during and after feeding a concentrate diet following a 24h restriction. Statistical differences are: *, P<0.05 control vs. NR. Box indicates time to consume all concentrate food. For dietary details see Materials and Methods.

Figure 2. Mean arterial blood pressure and heart rate response to incremental step-wise infusion of angiotensin II in control and nutrient restricted sheep.

Values are 5-min means ± S.E.M. for control (left panel, n = 8) and nutrient restricted (NR; right panel, n = 9) sheep for a baseline period (10 min), 1 h of angiotensin II infusion (stepwise dose increments of 0, 1, 2, 4, 8, 16 & 32 ng.kg⁻¹.min⁻¹ every 10 min) and 30 min of recovery. Box indicates the period of infusion.

Figure 3. Mean arterial blood pressure and heart rate response to incremental step-wise infusion of noradrenaline in control and nutrient restricted sheep.

Values are 5-min means ± S.E.M. for control (left panel, n = 8) and nutrient restricted (NR; right panel, n = 9) sheep for a baseline period (10 min), 1 h of
noradrenaline infusion (stepwise dose increments of 0, 2, 4, 8, 16, 32 & 48 ng.kg\(^{-1}\).min\(^{-1}\) every 10 min) and 30 min of recovery. Box indicates the period of infusion.

Figure 4. Relationship of mean arterial blood pressure with heart rate during noradrenaline infusion.

Values are the 5-min group means ± S.E.M. for control (-○-, n = 8) and nutrient restricted (NR; -●-, n = 9) sheep for a baseline period (10 min), 1 h of noradrenaline infusion (stepwise dose increments of 0, 2, 4, 8, 16 & 48 ng.kg\(^{-1}\).min\(^{-1}\) every 10 min) and 30 min of recovery. The intercept of the mean curve, but not the slope, of the relationship for individual data points between mean arterial blood pressure and heart rate was significantly elevated in NR \((y=-0.23x + 116)\) sheep relative to controls \((y=-0.35x + 114, t= 4.92; P<0.001)\).

Figure 5. The plasma concentrations of glucose (A), cortisol (B) and leptin (C) in control and nutrient restricted sheep. Values are means ± S.E.M. for control (-○-) and nutrient restricted (NR; -●-) sheep for a 6 h period with blood sampling every 30 min. Data for glucose are Control, n=8 and NR, n=9; cortisol, n= and NR, n=7; leptin, n=7 and NR, n=8. Arrow indicates the time of feeding of concentrate to the sheep.

Figure 6. Correlation of plasma leptin with relative fat mass in control and NR sheep (A), and the delta change in leptin with noradrenaline and angiotensin II infusion (B). Values are means ± S.E.M. for control and nutrient
restricted sheep: A, for baseline leptin concentration prior to feeding and g fat per kg carcass weight obtained at post mortem. Leptin correlated significantly with g/kg fat mass ($y = 0.1668x + 0.6315, r^2 = 0.4718; P<0.01$). B, for plasma leptin before and after infusion of angiotensin II or noradrenaline, *$P<0.05$; see Materials and Methods for details of infusion protocol.

Table 1. Biometrical measurements in control and nutrient restricted sheep at three years of age.

Values are means ± SEM.
### TABLE 1.

**Sheep biometry at three years of age**

<table>
<thead>
<tr>
<th></th>
<th>CONTROLS</th>
<th>NR</th>
<th><strong>P</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (kg)</strong></td>
<td>75.6 ± 2.8</td>
<td>75.0 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Heart weight (g)</strong></td>
<td>360 ± 29</td>
<td>325 ± 19</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Liver weight (g)</strong></td>
<td>1262 ± 40</td>
<td>1118 ± 35</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Kidney weight (g)</strong></td>
<td>211 ± 6</td>
<td>205 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Adrenal weight (g)</strong></td>
<td>5.9 ± 0.6</td>
<td>4.6 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Spleen weight (g)</strong></td>
<td>331 ± 41</td>
<td>328 ± 26</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Lung weight (g)</strong></td>
<td>724 ± 26</td>
<td>702 ± 33</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Pancreas weight (g)</strong></td>
<td>73.9 ± 9.0</td>
<td>73.0 ± 9.0</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Gonad weight (g)</strong></td>
<td>533 ± 29</td>
<td>469 ± 17</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Total adipose tissue weight (g)</strong></td>
<td>1283 ± 242</td>
<td>1451 ± 284</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Adipose:carcass weight (g/kg)</strong></td>
<td>16.8 ± 2.6</td>
<td>19.4 ± 3.4</td>
<td>NS</td>
</tr>
</tbody>
</table>
Fig 1

SBP (mmHg)  DBP (mmHg)  MABP (mmHg)  HR (beats.min⁻¹)

-10  -5  0  5  10  15  20

Time (min)

NR (n=8)  Control (n=5)
Fig 2

A
Mean arterial blood pressure (mmHg)

B
- NR (n=9)
- Control (n=8)

C
Heart rate (beats.min⁻¹)

D
Fig 3

A
Mean arterial blood pressure (mmHg)

B

C
Heart rate (beats.min⁻¹)

D

NR (n=9)
Control (n=8)
Fig 4

Mean arterial blood pressure (mmHg) vs. Heart rate (beats.min⁻¹)

- NR (n=9)
- Control (n=8)
A  Plasma [glucose] (mmoles.L\(^{-1}\))

B  Plasma [cortisol] (nmoles.L\(^{-1}\))

C  Plasma [leptin] (ng.ml\(^{-1}\))

Time of day
Fig 6

A

Plasma [leptin] (ng.ml\(^{-1}\)) vs g/kg fat mass

- Controls, n=7
- NR, n=7

B

∆ leptin (ng.ml\(^{-1}\))

- Noradrenaline infusion
- AII infusion

* indicates significance compared to controls.