Effect of maternal cold exposure and nutrient restriction on insulin-like growth factor sensitivity in adipose tissue of newborn sheep

E. Butt, M.A. Hyatt, H. Budge, M. E. Symonds and T. Stephenson

Centre for Reproduction and Early Life, Institute of Clinical Research, Queen's

Medical Centre, University Hospital, Nottingham, NG7 2UH

Running title: Insulin-like growth factors and adipose tissue in the newborn

Key words: Adipose tissue, insulin-like growth factor, nutrition

Correspondence to:

Professor Michael E Symonds Academic Division of Child Health, Queen's Medical Centre, University Hospital, Nottingham, NG7 2UH

Tel: +44 (0) 115 823 0611 Fax: +44 (0) 115 823 0626

E-mail: michael.symonds@nottingham.ac.uk

ABSTRACT

Adipose tissue mass in the newborn is determined in part by insulin-like growth factor (IGF)s, which are dependent on the maternal nutritional and metabolic environment during late gestation. The present study was designed to determine whether maternal cold exposure (CE) commencing in mid gestation could modulate some of the adaptive effects of nutrient restriction in late gestation on adipose tissue endocrine sensitivity in the resulting offspring. Twenty eight pregnant sheep were entered into the study and were either shorn, i.e. cold exposed, from 70 days gestation (term = 147 days), or remained unshorn, and were fed either their total calculated metabolisable energy (ME) requirements for body weight and pregnancy from 110 days gestation or 50% of this amount (n=7 per group). Adipose tissue was sampled from the offspring at one day of age and the mRNA abundance for IGF-I, II their receptors (R) and GH secretagogue receptor-1a (GHSR-1a) were determined. CE mothers produced larger offspring with more perirenal adipose tissue, an adaptation prevented by maternal nutrient restriction. Nutrient restriction in unshorn mothers increased IGF-I and IIR mRNA abundance. The mRNA abundances for IGF-I, II and IIR in adipose tissue were reduced by CE, adaptations independent of maternal food intake, whereas CE plus nutrient restriction increased GHSR-1a mRNA. In conclusion, maternal nutrient restriction with or without CE has very different effects on IGF sensitivity of adipose tissue and may act to ensure adequate fat stores are present in the newborn in the face of very different maternal endocrine and metabolic environments.

INTRODUCTION

In large mammals, including sheep and humans, the maternal metabolic and hormonal environment has a large influence on fetal adipose tissue development, and can determine adaptation at birth as well as longer term fat deposition (Symonds et al., 2003; McMillen et al., 2004). Changes in maternal food intake through gestation can, therefore, have a large impact both on fetal fat mass and its endocrine sensitivity, particularly that of the insulin-like growth factor (IGF) axis (Symonds et al., 2003). For example, during late gestation maternal nutrient restriction reduces prolactin sensitivity in adipose tissue of the newborn as a consequence of a decrease in the abundance of long, but not short, form of the prolactin receptor (Pearce et al., 2005). This adaptation is not affected by maternal cold exposure induced by winter shearing although the latter can promote fetal adipose tissue growth (Symonds et al., 1992; Symonds et al., 1995; Gate et al., 2000). The extent to which the maternal and fetal endocrine adaptations to chronic cold exposure (Thompson et al., 1982; Symonds et al., 1989) can impact on its endocrine sensitivity to the IGF-growth hormone (GH) axis has not previously been investigated and was the primary aim of the present study.

The amount of adipose tissue laid down by the fetus is primarily determined by the rate of glucose supply (Stevens *et al.*, 1990) which also regulates fetal plasma concentration of IGF-I (Owens *et al.*, 1994). A reduction in maternal nutrition between early to mid gestation increases mRNA abundance for both the IGF-I and -II receptor (R) in conjunction with enhanced adipose tissue deposition (Bispham *et al.*, 2003) but whether nutrient restriction in late gestation may have the same effect has not been examined. Fetuses sampled from mothers nutrient restricted in late gestation

have reduced fat mass near to term (Symonds et al., 1998) but, as young adults, they exhibit increased fat deposition in conjunction with raised insulin receptor (IR) β subunit abundance which, itself, may contribute to the accompanying insulin resistance (Gardner et al., 2005). The extent to which IRβ abundance may already be up regulated at birth, as opposed to accompanying the greater fat mass with age, is unknown. Another potentially important hormone in regulating adipose tissue growth is ghrelin. It is a gut derived endogenous ligand of the growth hormone (GH) secretagogue receptor (GHSR) (Kojima et al., 1999) that in the adult stimulates appetite and regulates GH release (Sugino et al., 2002). In rats, ghrelin synthesised by the placenta (Gualillo et al., 2001) or mother, has an important role in fetal development in late gestation when it can regulate growth of the fetus (Nakahara et al., 2006). Messenger RNA expression for both ghrelin and its novel G proteincoupled receptor (GHSR-1a) have been found in a wide variety of fetal and adult organs including adipose tissue (Gnanapavan et al., 2002; Nakahara et al., 2006) but, to date, the extent to which it may be nutritionally regulated during early development has not been investigated.

The aim of the following study was to determine whether maternal cold exposure induced from mid gestation could overcome the effects of later nutrient restriction on fetal whole body and fat growth and its endocrine sensitivity. Our analysis was conducted on perirenal adipose tissue as this constitutes up to 80% of total fat stores in the newborn sheep (Clarke *et al.*, 1997b). Tissue analysis was focussed on gene expression as this critical stage of development is coincident with their maximal expression that itself acts to ensure tissue function meets the pronounced metabolic

and endocrine changes occurring around the time of birth (Symonds *et al.*, 2003; Gnanalingham *et al.*, 2005).

Materials and Methods

Animals and diet

Twenty eight multiparous Bluefaced Leicester cross Swaledale female sheep of known mating date were entered into the study. All mothers were of similar body weight (Control 79 \pm 4; Nutrient restricted 81 \pm 3; Cold exposed 78 \pm 4; Cold exposed and nutrient restricted $79 \pm 4 \text{ kg}$ (n = 7 per group)) and fat distribution as assessed indirectly using body condition score measurements. In December they were all housed indoors, under conditions of natural day length, from 68 days of gestation and 14 randomly assigned mothers were shorn two days later. All animals were fed a diet of straw ad libitum plus a fixed amount of concentrate that was sufficient to fully meet their total metabolisable energy requirements with respect to fetal number and stage of gestation (Mostyn et al., 2003). One month before predicted lambing date, 7 shorn and 7 unshorn mothers were nutrient restricted by providing a diet sufficient to meet only 50% of their energy requirements for maintenance and pregnancy. After giving birth normally at term, the offspring were humanely euthanased at one day of age with an overdose of barbiturate (100 mg kg⁻¹ pentobarbital sodium: Euthatal: RMB Animal Health, UK) administered through the jugular vein. All perirenal adipose tissue was rapidly dissected, weighed, snap frozen in liquid nitrogen and stored at -80°C until analysed. These operative procedures and experimental protocols had the required Home Office and institutional approval as designated by the Animals (Scientific Procedures) Act (1986).

Messenger RNA detection

Total RNA was isolated using Tri-Reagent (Sigma, Poole, UK). In order to maximise sensitivity, a two-tube approach to reverse transcription (RT) was adopted as

previously described (Bispham *et al.*, 2003). The conditions used to generate first strand cDNA RT were: 70°C (5 min), 4°C (5 min), 25°C (5 min), 25°C (10 min), 42°C (1 hour), 72°C (10 min), 4°C (5 min). The RT reaction (final volume 20 μl) contained: buffer (250 mM Tris-HCl, 40 mM MgCl₂, 150 mM KCl, 5 mM dithioerythritol pH 8.5), 2 mM dNTPs, 1 x hexanucleotide mix, 10 units RNase inhibitor, 10 units M-MLV reverse transcriptase and 1 μg total RNA. All these commercially available products were purchased from Roche Diagnostics Ltd (Lewes, UK).

The expression of each gene was determined by RT-polymerase chain reaction (RT-PCR) (Bispham *et al.*, 2003). The analysis used oligonucleotide cDNA primers for each gene under test by generating specific exon-intron spanning products (see Table 1 for details of previously unpublished sequences). Briefly, the PCR programme consisted of an initial denaturation (95 °C (15 min)), amplification (stage I, 94 °C (30 s); stage II, annealing temperature (30 s); stage III, 72 °C (60 s)) and final extension (72 °C (7 min); 8 °C 'hold'). The PCR mixture (final volume 20 ul) contained 7 ul DEPC H₂O, 10 ul Thermo-Start PCR Master Mix® (50 ul contains 1.25 units Thermo-Start® DNA Polymerase, 1 x Thermo-Start® reaction buffer, 1.5 mM MgCl₂ and 0.2 mM each of dATP, dCTP, dGTP and dTTP, catalogue number AB-0938-DC-15 ABgene®), 1 uM Forward Primer, 1 uM Reverse Primer and 1 ul RT (cDNA) product. The annealing temperature and cycle numbers of all primers were optimised so as to be in the linear range.

Agarose gel electrophoresis (2.0 – 2.5%) and ethidium bromide staining confirmed the presence of both the product and 18S at the expected sizes. Densitometric analysis was performed on each gel by image detection using a Fujifilm LAS-1000 cooled charge-coupled device camera to determine mRNA abundance for each gene.

Consistency of lane loading for each sample was verified from the measurement of 18S ribosomal RNA. All results were then expressed as a ratio of a reference sample ran on all gels. Each analysis was conducted in duplicate with appropriate positive and negative controls and a range of molecular weight markers. In addition, the resultant PCR product was extracted (QIAquick gel extraction kit, catalogue number 28704), sequenced and results cross-referenced against the Genebank website (Website) to determine specificity of the target gene.

Statistical Analysis

As Kolmogorov-Smirnov normality tests (SPSS 11.0.1) confirmed that the data was normally distributed, parametric statistical analyses were performed. Statistical analyses with respect to significant differences (P<0.05) between mean values obtained from offspring of control and nutritionally manipulated mothers were carried out using a two way analysis of variance for the effects of cold exposure and nutrient restriction.

RESULTS

Weight at birth and perirenal adipose tissue mass

Maternal cold exposure resulted in an increase in birth weight, an adaptation that was dependent on maternal food intake and was thus not observed in cold exposed mothers that were nutrient restricted (Table 2). The greater birth weight in well fed cold exposed mothers was accompanied by a proportional increase in total perirenal fat mass. Interestingly offspring born to mothers that were cold exposed and nutrient restricted were the smallest and thus possessed the least amount of adipose tissue although this was not statistically significant.

IGF-I, -II, receptor mRNA abundance in perirenal adipose tissue

Maternal cold exposure resulted in a pronounced decrease in IGF-I mRNA abundance, whereas nutrient restriction in unshorn sheep significantly increased IGF-I abundance (Figure 1) in adipose tissue. These changes in IGF-I were not, however, accompanied by any significant difference in IGF-IR mRNA. A similar pattern of changes in mRNA abundance for IGF-II and its R were observed with the modification that in offspring born to unshorn mothers IGF-IIR rather than IGF-II were up regulated in adipose tissue by nutrient restriction (Figure 2).

GHSR-1a and IRβ mRNA abundance in perirenal adipose tissue

In offspring born to cold exposed, nutrient restricted mothers there was a marked increase in GHSR-1a mRNA abundance that was not seen in offspring of well fed

mothers (Figure 3). There was no effect of either maternal cold exposure or nutrient restriction on IR β mRNA abundance (Control 218 \pm 65; Nutrient restricted 178 \pm 14; Cold exposed 316 \pm 13; Cold exposed and nutrient restricted 206 \pm 33 (n = 7 per group) arbitrary units as a ratio of 18S rRNA).

DISCUSSION

The major finding of our study is that the increase in both body and adipose tissue weight in offspring born to cold exposed mothers was not accompanied by an increase in IGFR mRNA abundance within their fat. These findings extend previous studies of cold exposure and nutrient restriction when pregnant sheep were only shorn four weeks before term but also produce larger offspring with enhanced fat stores. They are, however, in contrast with the effect of maternal nutrient restriction between early to mid gestation followed by restoration of the maternal diet in late gestation when the increase in fetal fat deposition is accompanied by an increase in mRNA abundance for both the IGF-I and -IIR (Bispham et al., 2003). Under these nutritional conditions it has been suggested that it is actually the level of feed that is consumed following the period of nutrient restriction that may be critical in determining fetal fat deposition up to birth. In the present study fat mass in the newborn was in proportion to body weight and as such these indices of fetal growth were only increased in those offspring born to cold exposed and well fed mothers. Taken together these results indicate that long term changes in the maternal diet from mid gestation can influence fat mass in the newborn in the absence of an increase in IGF-R. Interestingly it is only nutrient restriction in late and not earlier in gestation that causes an increase in fat deposition in later life (Gopalakrishnan et al., 2004; Gardner et al., 2005; Gopalakrishnan et al., 2005).

Despite fat mass being proportional to body weight a striking molecular difference in the adipose tissue of offspring born to mothers that were cold exposed and nutrient restricted is the increase in GHSR-1a that may be related to an increase in plasma ghrelin and subsequent changes in cell signalling (Murata *et al.*, 2002). The extent to

which such an adaptation may be mediated by changes in both the acylated and desacyl forms of ghrelin (Hosoda *et al.*, 2000; Nakahara *et al.*, 2006) remain unknown as this analysis has yet to be undertaken in sheep. Increased circulating ghrelin and/or tissue sensitivity would be predicted to effect both insulin action (Murata *et al.*, 2002) and sympathetic innervation (Yasuda *et al.*, 2003). Indeed, studies in adult mice have shown that chronic administration of ghrelin decreases UCP1 expression as well as increasing fat mass (Tsubone *et al.*, 2005). The increase in GHSR-1a mRNA in adipose tissue from offspring born to cold exposed and nutrient restricted mothers does not appear to impair UCP1 abundance (Pearce *et al.*, 2005). It may act to prevent any further depletion of adipose tissue that accompanies undernutrition in unshorn mothers (Symonds *et al.*, 1998). Interestingly this adaptation is only found in the offspring of cold exposed mothers thus emphasising their very different maternal metabolic and endocrine environments compared to unshorn sheep that is maintained, irrespective of food intake (Thompson *et al.*, 1982; Symonds *et al.*, 1988; Symonds *et al.*, 1989).

Although there was no difference in adipose tissue mass between offspring born to normally fed and nutrient restricted unshorn mothers there was a significant difference in the response between the IGF-I and -II axis. Notably the change in IGF-I and -II mRNA differed with regard to ligand and R responses in that although IGF-I mRNA was increased it was only accompanied by enhanced IGF-IIR mRNA abundance. As already discussed above these are different responses to that seen with nutrient restriction in earlier gestation. At the same time there was no effect of nutrient restriction on mRNA abundance for the IRβ that is found in these offspring at one year of age when their fat mass is greater than controls (Gardner *et al.*, 2005).

Taken together it may be that these early changes in both IGF-I and IGF-IIR act to increase fat growth after birth when it is one of the fastest growing organs in the body (Clarke *et al.*, 1997b). The lack of a significant effect on the IGF-IR may be explained by the expected decrease in plasma IGF-I, with undernutrition (Bauer *et al.*, 1995) which then only has the potential for a limited effect on its R within the adipocyte because of the observed enhanced capacity for an increase in paracrine secretion. With regard to the potential impact of increased IGF-IIR mRNA on adipose tissue growth this normally determines the bioavailability of IGF-II by acting as a negative regulator of its anabolic effects by sequestering plasma IGF-II into the cell for degradation (O'Dell & Day, 1998). It remains to be established whether the same function occurs in fetal adipose tissue when an increase in both IGF-I and IIR can be accompanied by an increased fat mass in the newborn (Bispham *et al.*, 2003).

An important point to note with respect to the findings in the present study is that in the ovine fetus adipose tissue deposition is minimal (Symonds *et al.*, 2004) because of the much higher metabolic demands for lipid deposition compared with that required for carbohydrate and protein deposition (i.e. 39 c.f. 15-25 MJ/kg). The primary function of fat in the fetus is to provide an endogenous energy store and facilitate the rapid initiation of nonshivering thermogenesis at birth following cold exposure to the extrauterine environment (Power, 1989; Symonds *et al.*, 1995). This may mean that any adaptation in endocrine sensitivity within fetal or newborn adipose tissue will have very limited impact on total fat mass at birth but may contribute to its mitochondrial composition and function (Clarke *et al.*, 1997a). It is obviously important that sufficient fat stores are present in the newborn as these are then rapidly mobilised after birth before being replaced through the lactational period (Clarke *et*

al., 1997b). It is therefore possible that the very different molecular profiles within the IGF-GH axis between offspring born to cold exposed and nutrient restricted mothers act to ensure that at the very least fat mass is proportionate to body weight and that fat deposition is then adequately maintained in the postnatal period (Pearce et al., 2005). Then after weaning as the animal attains its mature body weight persistent adaptations within the adipocyte can contribute to enhanced fat mass found at one year of age (Gardner et al., 2005).

In conclusion, we have shown for the first time that there are pronounced differences in the endocrine sensitivity of adipose tissue between offspring born to cold exposed and nutrient restricted mothers. This particularly impacts on the IGFs which may adapt to ensure that sufficient adipose tissue is deposited to enable the newborn to effectively adapt to cold exposure of the extrauterine environment.

ACKNOWLEDGEMENTS

E. Butt was supported by a Biotechnology and Biological Sciences Research Council Studentship.

REFERENCES

- Bauer MK, Breier BH, Harding J, Veldhuis JD & Gluckman PD (1995) The fetal somatotrophic axis during long term maternal undernutrition in sheep; evidence of nutritional regulation in utero. *Endocrinology* **136**, 1250-1257.
- Bispham J, Gopalakrishnan GS, Dandrea J, Wilson V, Budge H, Keisler DH, Broughton Pipkin F, Stephenson T & Symonds ME (2003) Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. *Endocrinology* **144**, 3575-3585.
- Clarke L, Bryant MJ, Lomax MA & Symonds ME (1997a) Maternal manipulation of brown adipose tissue and liver development in the ovine fetus during late gestation. *British Journal of Nutrition* **77**, 871-883.
- Clarke L, Buss DS, Juniper DS, Lomax MA & Symonds ME (1997b) Adipose tissue development during early postnatal life in ewe-reared lambs. *Experimental Physiology* **82**, 1015-1017.
- Gardner DS, Tingey K, van Bon BWM, Ozanne SE, Wilson V, Dandrea J, Keisler DH, Stephenson T & Symonds ME (2005) Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *American Journal of Physiology* **289**, R947 R954.
- Gate JJ, Clarke L, Lomax MA & Symonds ME (2000) Chronic cold exposure has no effect on brown adipose tissue in newborn lambs born to wellfed ewes. *Reproduction, Fertility and Development* **11**, 415-418.
- Gnanalingham MG, Mostyn A, Gardner DS, Stephenson T & Symonds ME (2005)

 Developmental regulation of adipose tissue: Nutritional manipulation of local glucocorticoid action and uncoupling protein 2. *Adipocytes* 1, 221-228.
- Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB & Korbonits M (2002) The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *Journal of Clinical Endocrinology and Metabolism* **87**, 2988.
- Gopalakrishnan G, Gardner DS, Dandrea J, Langley-Evans SC, Pearce S, Kurlak LO, Walker RM, Sweetho I, Keisler DH, Ramsay MM, Stephenson T & Symonds ME (2005) Influence of maternal pre-pregnancy body composition and diet during early-mid pregnancy on cardiovascular function and nephron number in juvenile sheep. *British Journal of Nutrition* **94**, 938-947.
- Gopalakrishnan G, Gardner DS, Rhind SM, Rae MT, Kyle CE, Brooks AN, Walker RM, Ramsay MM, Keisler DH, Stephenson T & Symonds ME (2004) Programming of adult cardiovascular function after early maternal undernutrition in sheep. *American Journal of Physiology* **287**, R12-20.
- Gualillo O, Caminos J, Blanco M, Garcia-Caballero T, Kojima M, Kangawa K, Dieguez C & Casanueva F (2001) Ghrelin, a novel placental-derived hormone. *Endocrinology* **142**, 788-794.
- Hosoda H, Kojima M, Matsuo H & Kangawa K (2000) Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochemical and Biophysical Research Communications* **279**, 909-913.
- Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**, 656-660.

- McMillen IC, Muhlhausler BS, Duffield JA & Yuen BS (2004) Prenatal programming of postnatal obesity: Fetal nutrition and the regulation of leptin synthesis and secretion before birth. *Proceedings of Nutrition Society* **63**, 405-412.
- Mostyn A, Wilson V, Dandrea J, Yakubu DP, Budge H, Alves-Guerra MC, Pecqueur C, Miroux B, Symonds ME & Stephenson T (2003) Ontogeny and nutritional manipulation of mitochondrial protein abundance in adipose tissue and the lungs of postnatal sheep. *British Journal of Nutrition* **90**, 323-328.
- Murata M, Okimura Y, Iida K, Matsumoto M, Sowa H, Kaji H, Kojima M, Kangawa K & Chihara K (2002) Ghrelin modulates the downstream molecules of insulin signaling in hepatoma cells. *Journal of Biological Chemistry* **277**, 5667-5674.
- Nakahara K, Nakagawa M, Baba Y, Sato M, Toshinai K, Date Y, Nakazato M, Kojima M, Miyazato M, Kaiya H, Hosoda H, Kangawa K & Murakami N (2006) Maternal ghrelin plays an important role in rat fetal development during pregnancy. *Endocrinology* **147**, 1333-1342.
- O'Dell SD & Day INM (1998) Molecules in focus Insulin-like growth factor II (IGF-II). *The International Journal of Biochemistry and Cell Biology* **30**, 767-771.
- Owens JA, Kind KL, Carbone F, Robinson JS & Owens PC (1994) Circulating insulin-like growth factors-I and -II and substrates in fetal sheep following restriction of placental growth. *Journal of Endocrinology* **140**, 5-13.
- Pearce S, Budge H, Mostyn A, Korur N, Wang J, Ingleton PM, Symonds ME & Stephenson T (2005) Differential effects of maternal cold exposure and nutrient restriction on prolactin receptor and uncoupling protein 1 abundance in adipose tissue during development in young sheep. *Adipocytes* **1**, 57-64.
- Power G (1989) Biology of temperature: the mammalian fetus. *Journal of Developmental Physiology* **12**, 295-304.
- Stevens D, Alexander G & Bell AW (1990) Effects of prolonged glucose infusion into fetal sheep on body growth, fat deposition and gestation length. *Journal of Developmental Physiology* **13**, 277-281.
- Sugino T, Hasegawa Y, Kikkawa Y, Yamaura J, Yamagishi M, Kurose Y, Kojima M, Kangawa K & Terashima Y (2002) A transient ghrelin surge occurs just before feeding in a scheduled meal-fed sheep. *Biochemical and Biophysical Research Communications* **295**, 255-260.
- Symonds ME, Bird JA, Clarke L, Gate JJ & Lomax MA (1995) Nutrition, temperature and homeostasis during perinatal development. *Experimental Physiology* **80**, 907-940.
- Symonds ME, Bryant MJ, Clarke L, Darby CJ & Lomax MA (1992) Effect of maternal cold exposure on brown adipose tissue and thermogenesis in the neonatal lamb. *Journal of Physiology, London* **455**, 487-502.
- Symonds ME, Bryant MJ & Lomax MA (1988) Metabolic adaptation during pregnancy in winter-shorn sheep. *Journal of Agricultural Science, Cambridge* **111**, 137-145.
- Symonds ME, Bryant MJ & Lomax MA (1989) Lipid metabolism in shorn and unshorn pregnant sheep. *British Journal of Nutrition* **62**, 35-49.
- Symonds ME, Mostyn A, Pearce S, Budge H & Stephenson T (2003) Endocrine and nutritional regulation of fetal adipose tissue development. *Journal of Endocrinology* **179**, 293-299.
- Symonds ME, Pearce S, Bispham J, Gardner DS & Stephenson T (2004) Timing of nutrient restriction and programming of fetal adipose tissue development. *Proceedings of the Nutrition Society* **63**, 397-403.

- Symonds ME, Phillips ID, Anthony RV, Owens JA & McMillen IC (1998) Prolactin receptor gene expression and foetal adipose tissue. *Journal of Neuroendocrinology* **10**, 885-890.
- Thompson GE, Bassett JM, Samson DE & Slee J (1982) The effect of cold exposure of pregnant sheep on fetal plasma nutrients, hormones and birth weight. *British Journal of Nutrition* **48**, 59-64.
- Tsubone T, Masaki T, Katsuragi I, Tanaka K, Kakuma T & Yoshimatsu H (2005) Ghrelin regulates adiposity in white adipose tissue and UCP1 mRNA expression in brown adipose tissue in mice. *Regulatory Peptides* **130**, 97-103.
- Yasuda T, Masaki T, Kakuma T & Yoshimatsu H (2003) Centrally administered ghrelin suppresses sympathetic nerve activity in brown adipose tissue of rats. *Neuroscience Letters* **349**, 75-78.

Table 1. Primer sequences for genes used in RT-PCR.

Primer Set	Primer Sequence	Product size (bp)
IRβ	F 5'-CTGCACCATCAACGGAA-3' R 5'-CGTAACTTCCGGAAGAAGGA-3'	150
GHSR-1a	F 5'-CTACTTCGCCATCTGCTTCC-3' R 5'-GAGGGTCGGTACCATTCTCA-3'	155

GHSR- 1a: growth hormone secretagogue receptor-1a

 $IR\beta \ insulin \ receptor \ \beta \ subunit$

Table 2. Effect of chronic maternal cold exposure (CE) and nutrient restriction (NR) on whole body and perirenal adipose tissue (PAT) weights of the resulting offspring at one day of age. Values are means with their standard errors and n=7 per group. Significant differences between the same nutritional groups (i.e. effect of cold exposure) represented by * p < 0.05 and significant differences between control and nutrient restricted groups that were both shorn group represented by different superscripts a vs b P < 0.01. For full details of maternal feeding regime see Materials and Methods.

	Unshorn		Shorn	
	Control	NR	Control	NR
Bodyweight (kg)	4.42 ± 0.34	4.36 ± 0.21	5.45 ± 0.45 *	3.96 ± 0.25
Total PAT (g)	15.21 ± 1.8	17.86 ± 1.8	22.34 ± 3.4^{a}	12.98 ± 1.0^{b}
Relative PAT (g:kg)	3.45 ± 0.37	4.16 ± 0.43	4.14 ± 0.59	3.31 ± 0.20

FIGURE TITLES

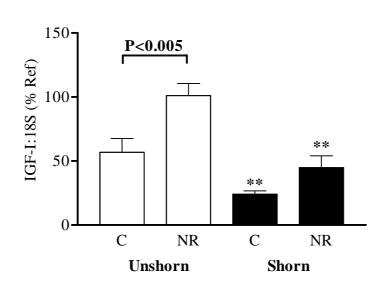
Figure 1. Effect of chronic maternal cold exposure and nutrient restriction on mRNA abundances for a) insulin-like growth factor (IGF)-I and b) its receptor (R) in perirenal adipose tissue in the resulting offspring at one day of age (n=7 per group). Values are means with their standard errors. Controls (unshorn), open bars; cold exposed (shorn), closed bars; nutrient restriction (NR). Significant differences between nutritional groups: ** p < 0.01. For full details of maternal feeding regime see Materials and Methods.

Figure 2. Effect of chronic maternal cold exposure and nutrient restriction on mRNA abundances for a) insulin-like growth factor (IGF)-II and its b) receptor (R) in perirenal adipose tissue in the resulting offspring at one day of age (n=7 per group). Values are means with their standard errors. Controls (unshorn), open bars; cold exposed (shorn), closed bars; nutrient restriction (NR). Significant differences between nutritional groups: * p < 0.05; ** p < 0.01. For full details of maternal feeding regime see Materials and Methods.

Figure 3. Effect of chronic maternal cold exposure and nutrient restriction on mRNA abundance for growth hormone secretagogue receptor (GHSR-1a) in perirenal adipose tissue in the resulting offspring at one day of age (n=7 per group). Values are means with their standard errors. Controls (unshorn), open bars; cold exposed (shorn), closed bars; nutrient restriction (NR). Significant differences between nutritional groups: ** p < 0.01. For full details of maternal feeding regime see Materials and Methods.

Figure 1





(b)

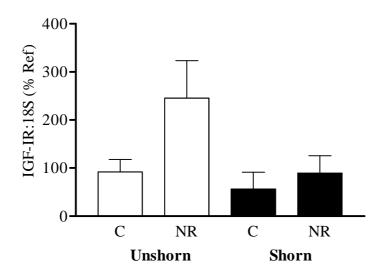
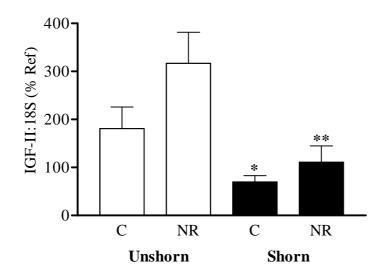


Figure 2

(a)



(b)

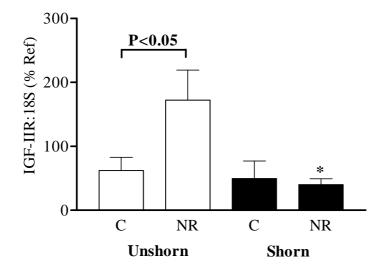


Figure 3

