Maternal parity and its effect on adipose tissue deposition and endocrine sensitivity in the postnatal sheep

M A Hyatt1, D H Keisler3, H Budge1,2 and M E Symonds1,2

1Early Life Nutrition Research Unit, Division of Human Development, Academic Child Health and 2Respiratory Biomedical Research Unit, School of Clinical Sciences, University Hospital, University of Nottingham, E Floor East Block, Derby Road, Nottingham NG7 2UH, UK
3Department of Animal Sciences, University of Missouri, Columbia, Missouri 65201, USA

Abstract

Maternal parity influences size at birth, postnatal growth and body composition with firstborn infants being more likely to be smaller with increased fat mass, suggesting that adiposity is set in early life. The precise effect of parity on fat mass and its endocrine sensitivity remains unclear and was, therefore, investigated in the present study. We utilised an established sheep model in which perirenal–abdominal fat mass (the major fat depot in the neonatal sheep) increases ~10-fold over the first month of life and focussed on the impact of parity on glucocorticoid sensitivity and adipokine expression in the adipocyte. Twin-bearing sheep of similar body weight and adiposity that consumed identical diets were utilised, and maternal blood samples were taken at 130 days of gestation. One offspring from each twin pair was sampled at 1 day of age, coincident with the time of maximal recruitment of uncoupling protein 1 (UCP1), whilst its sibling was sampled at 1 month, when UCP1 had disappeared. Plasma leptin was lower in nulliparous mothers than in multiparous mothers, and offspring of nulliparous mothers possessed more adipose tissue with increased mRNA abundance of leptin, glucocorticoid receptor and UCP2, adaptations that persisted up to 1 month of age when gene expression for interleukin-6 and adiponectin was also raised. The increase in fat mass associated with firstborn status is therefore accompanied by a resetting of the leptin and glucocorticoid axis within the adipocyte. Our findings emphasise the importance of parity in determining adipose tissue development and that firstborn offspring have an increased capacity for adipogenesis which may be critical in determining later adiposity.


Introduction

Worldwide childhood obesity is increasing, an occurrence that is of great concern as it often tracks through to adulthood, suggesting that adiposity is set in early life (Field et al. 2005). The cause of obesity is multifactorial with environmental, biological and genetic factors all having an influence (Rosenbaum et al. 1997), although the relative contribution of each remains uncertain. There is, however, evidence that reduced foetal growth can promote later adiposity, particularly if accompanied by accelerated postnatal growth (Ong et al. 2000, Stettler et al. 2003). Maternal parity can determine birth weight (Lumey & Stein 1997, Gardner et al. 2007), postnatal growth and the longer term health of the offspring (Bai et al. 2002, Ong et al. 2002), resulting in increased fat mass in both school children (Wilkinson et al. 1977) and adolescents (Celi et al. 2003, Wang et al. 2007). Surprisingly, no study to date has looked at the cellular mechanisms mediating this response. We have previously reported the effect of maternal parity on foetal and postnatal development using a sheep model (Hyatt et al. 2007), and found that, like humans, being firstborn is associated with being smaller at birth. These offspring also have pronounced differences in their hepatic GH–insulin–like growth factor (IGF) axis that may impact upon their postnatal growth (Hyatt et al. 2007).

In addition to providing an endogenous energy storage, adipose tissue secretes a number of cytokines and peptides, termed adipokines, which are involved in regulating insulin sensitivity, appetite, energy balance, inflammation and lipid metabolism (see Trayhurn & Wood 2004). Excess fat mass that accompanies obesity appears to be established in early life and is associated with a state of chronic low-grade inflammation where pro-inflammatory cytokines such as interleukin 6 (IL6) are raised, whilst anti-inflammatory markers including adiponectin are decreased in proportion to fat mass (Das 2001). Other important endocrine factors that are susceptible to in utero programming and critical in determining adipose tissue function and later adiposity include the glucocorticoid receptor (GR or NR3C1 as listed in the HUGO Database) and the enzymes 11β-hydroxysteroid dehydrogenase (HSD11B) types 1 and 2 as well as uncoupling protein 2 (UCP2) and peroxisome proliferator-activated receptor γ (PPARγ; Whorwood et al. 2001, Bispham et al. 2005, Gnanalingham et al. 2005b, Berthiaume et al. 2007, De Sousa Peixoto et al. 2008).
Gene expression and function of these proteins are dependent in part on the maternal diet through pregnancy (Whorwood et al. 2001, Bispham et al. 2005, Gnaulattingham et al. 2005b) that can also determine later adipose tissue function. Using our previously established animal model of maternal parity (Hyatt et al. 2007), we have now investigated the impact of maternal parity on adipose tissue development with regard to glucocorticoid sensitivity and adipokine gene expression. Our analysis was focused on the perirenal–abdominal depot as this is the largest fat depot in the newborn sheep and undergoes rapid growth after birth (Clarke et al. 1997a) that is accompanied by adaptations in its inflammatory and related responses (Sharkey et al. 2009b). We hypothesised that gene expression of key regulators of adipose tissue function and composition would be increased in firstborn offspring during early postnatal development as fat deposition is enhanced over this period.

Materials and Methods

Animals and experimental design

Fifteen twin-bearing (six nulliparous (N) and nine multiparous (M)) Border Leicester X Swaledale sheep that were all reproductively mature adults were entered into the study. Nulliparous sheep were 2 years old and had never been previously mated, whilst the multiparous sheep were aged 3–4 years and had all experienced two previous successful pregnancies, which is important because there is little increase in birth weight after a second pregnancy (Gardner et al. 2007). There were no differences in maternal weight gain or body condition score (BCS) throughout pregnancy as determined by repeated weighing and body condition scoring at fortnightly intervals (e.g. 110 days of gestation – N 80.2 ± 2.8; M 76.5 ± 3.0 kg; BCS – N 2.5 ± 0.2; M 2.6 ± 0.3 and 140 days of gestation – N 82.2 ± 2.8; M 79.5 ± 3.5 kg; BCS – N 2.2 ± 0.2; M 2.4 ± 0.4). Following conception, animals were group-housed, and they consumed 100% of their total metabolisable energy requirements for maternal body weight and stage of twin pregnancy. The diet comprised chopped hay and concentrate, and it was provided in a 3:1 weight ratio with all animals having access to a mineral block and fresh water. Offspring were delivered naturally at term (i.e. 145 ± 2 days), and birth weights were recorded. Within 6 h of birth, one twin was randomly selected from each mother to be tissue sampled following humane euthanasia using an i.v. injection of barbiturate (100 mg/kg pentobarbital sodium: Euthatal: RMB Animal Health, Dagenham, UK). The perirenal–abdominal adipose tissue (PAT) depots were completely dissected out and weighed, and a representative sample was stored at −80°C until further analysis (Hyatt et al. 2007). Mothers were housed with their remaining offspring and were fed a diet of hay ad libitum, together with a fixed amount of concentrate that was sufficient to fully meet their own energy requirements plus that required for lactation. Thus, the remaining twin was reared as a singleton with its mother until tissue sampling at 30 days postnatal age as described above. The resultant groups with regard to gender were 1 day: N, three males: three females; M, four males: five females and 30 days: N, one male: five females; M, six males: three females. There was no effect of gender, either within or between twin sets, on birth weight (<15% difference) irrespective of maternal parity. It should be noted that the aim of this study was not to examine any differential effect of gender on adipose tissue development.

Laboratory measurements

Blood sampling and plasma measurements All mothers had a jugular vein catheter inserted under local anaesthesia to enable fasted maternal blood sampling (0800 h; 5 ml) to be carried out at 130 days of gestation. A 5–ml venous blood sample from the offspring was the same and collected into heparinised syringes prior to dissection at 1 and 30 days of age. Blood samples were centrifuged at 800 g at 4°C for 15 min, and plasma supernatant was transferred to a sterile 1.5-ml Eppendorf tube (within 10 min of collection) and stored at −20°C. Plasma leptin and cortisol (Coat-a-Count; Euro DPC, Caernarfon, UK) were measured by RIA (Delavaud et al. 2000, Gardner et al. 2006), and plasma IGF1 was assessed by ELISA (OCTEIA IGF1; IDS Ltd, Tyne and Wear, UK). Plasma glucose (Randox GPO-PAP; Randox, Crumlin, UK) and non-esterified fatty acid (NEFA; NEFA-c Kit; Wako Chemicals GmbH, Neuss, Germany) concentrations were assessed spectrophotometrically (Sebert et al. 2009).

Total RNA isolation, reverse transcription and standard curve generation Total RNA was extracted from 1 g of frozen PAT using Tri-Reagent (Sigma). Total RNA samples were treated for potential genomic DNA contamination with DNase 1 (Promega Ltd), and their A260/A280 ratio was assessed to confirm purity and concentration. cDNA was synthesised from 3 μg RNA using 200 U Superscript II (Invitrogen Ltd) by reverse transcription in accordance with the manufacturer’s protocol. For standard curve generation, 1–10^{-8} ng/μl of primer-specific gel-purified amplicon was used to ensure PCR amplification efficiency (1.95–2.0) as described previously (Williams et al. 2007). 18S rRNA was used as a housekeeping gene, and results were calculated using the 2^{-ΔΔC_t} method (Livak & Schmittgen 2001). Gene expression data are normalised to the 1-day-old group born to multiparous mothers and are presented as a fold change.

Quantitative real-time PCR analysis The relative abundance of GR, HSD11B-1/2, adipsin, leptin, IGF1, adiponectin, IL6, tumour necrosis factor α (TNFα), insulin receptor, PPARA/G, UCP1/2, IGF1 receptor (R), IGF2R, IGF binding protein (BP) and 18S mRNA transcripts were determined by qRT-PCR amplification using a real-time
thermocycler (Quantica, Techno Incorporated, Barloworld Scientific Ltd, Stone, UK) and Quantitect SYBR green PCR kit (Qiagen Ltd) as described previously (Williams et al. 2007). Primer sequences have been published previously (Bispham et al. 2005, Hyatt et al. 2007, Mulhhausler et al. 2007, Williams et al. 2007, Sebert et al. 2009) with the exception of HSD11B1 (F: GTG CCA GAT CCC TGT CTG AT, R: AGC GGG ATA CCA CCT TCT TT (60 °C)), HSD11B2 (F: AGC AGG AGA CAT GCC GTT TC, R: AGC GGG ATA CCA CCT TCT TT (60 °C)) and IL6 (F: TGG AGG AAA AAG ATG GAT GC, R: GAC ATG CTG GAG AAG ATG CA (60 °C)). Real-time PCR conditions were set at 95 °C (15 min); 45 cycles of 94 °C (30 s), annealing temperature (30 s) followed by 72 °C (8 min). Melt curves were generated to confirm reaction specificity. Real-time PCRs were performed in duplicate with appropriate positive and negative controls as described previously (Williams et al. 2007).

Protein detection Mitochondria were prepared from ~1 g of PAT, and protein content was determined using the Lowry method (Lowry et al. 1951). Western blotting was utilised to measure UCP1 abundance in 10 μg of mitochondrial protein as described previously (Mostyn et al. 2003).

Statistical analysis
All data were explored for normality of their distribution using the Kolmogorov–Smirnov test and were log transformed where necessary (SPSS version 16.0, Chicago, IL, USA). Plasma concentrations and gene abundance were compared by general linear model analysis. The terms fitted to the model were maternal parity, age and parity × age interactions. The present study was neither designed nor powered to analyse the effect of gender, therefore offspring gender was added to the model as a covariate. Where parity × age interactions were present, further independent sample t-tests were performed to identify within which postnatal age group parity differences lay. Data are expressed as mean values with their standard errors. For all comparisons, statistical significance was accepted when a probability of 5% was observed (P<0.05).

Adipokine gene abundance
Leptin mRNA abundance was persistently higher in offspring born to nulliparous mothers irrespective of sampling age (Table 2; P<0.05). Maternal parity had no effect upon adiponectin or IL6 mRNA abundance at birth, but both were raised in offspring born to nulliparous mothers by 1 month of age. Neither maternal parity nor postnatal age had any effect upon TNFα mRNA abundance.

GR and HSD11B 1/2 mRNA abundance
Being born to a first-time mother resulted in significantly higher GR and HSD11B2 mRNA abundance in adipose tissue, a difference that only persisted up to

### Results

**Offspring body weight and adiposity**

Offspring born to nulliparous mothers were lighter at birth and had significantly more PAT (Table 1). At 30 days after birth, despite achieving a similar body weight, firstborn offspring still possessed greater fat mass.

**Maternal and neonatal plasma metabolites**

Maternal plasma leptin concentration, as measured in late gestation, was substantially higher in multiparous mothers than in nulliparous mothers (N: 1·1±0·3, M: 5·2±0·9 ng/ml, P<0·005). A similar difference but of much smaller magnitude was also seen in their offspring at birth (N: 0·6±0·2, M: 1·9±0·6 ng/ml, P<0·05). In contrast, parity had no effect upon offspring plasma IGF1 or cortisol concentrations at birth (Table 1). However, plasma IGF1 was lower in 30-day-old offspring born to nulliparous mothers despite increasing with postnatal age in both groups, whilst plasma cortisol decreased with age in all offspring. Neither plasma glucose nor NEFAs were affected by parity (data not shown).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>1 day of age</th>
<th>30 days of age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nulliparous</td>
<td>Multiparous</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>3·9±0·2*</td>
<td>4·6±0·2†</td>
</tr>
<tr>
<td>PAT mass (g)</td>
<td>17·0±1·6</td>
<td>11·7±1·3</td>
</tr>
<tr>
<td>Relative PAT (g/kg)</td>
<td>4·2±0·2*</td>
<td>2·6±0·3†</td>
</tr>
<tr>
<td>Plasma IGF1 (nmol/l)</td>
<td>8·3±1·3</td>
<td>11·3±1·6</td>
</tr>
<tr>
<td>Plasma cortisol (nmol/l)</td>
<td>173±18</td>
<td>179±21</td>
</tr>
</tbody>
</table>

* Different superscripts within an age group denote statistically significant effect of parity (P<0·05). †P<0·05. ‡P<0·005 mean values are significantly different from those of the respective 1-day-old group. PAT, perirenal–abdominal adipose tissue.
1 day of age & 30 days of age

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nulliparous (a.u)</th>
<th>Multiparous (a.u)</th>
<th>Nulliparous (a.u)</th>
<th>Multiparous (a.u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP1 mRNA</td>
<td>1.0 ± 0.13</td>
<td>0.01 ± 0.11</td>
<td>1.9 ± 0.14</td>
<td>1.4 ± 0.01</td>
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<tr>
<td>UCP2 mRNA</td>
<td>1.9 ± 0.28*</td>
<td>1.0 ± 0.12</td>
<td>1.1 ± 0.06</td>
<td>1.3 ± 0.03</td>
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<tr>
<td>UCP1 (% ref)</td>
<td>1.18 ± 3.8*</td>
<td>1.00 ± 2.6*</td>
<td>ND*</td>
<td>1.04 ± 2.6*</td>
</tr>
<tr>
<td>Leptin mRNA</td>
<td>1.9 ± 0.2*</td>
<td>1.0 ± 0.2*</td>
<td>1.6 ± 0.3*</td>
<td>0.5 ± 0.04*</td>
</tr>
<tr>
<td>Adiponectin mRNA</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.23</td>
<td>3.1 ± 0.6*</td>
<td>1.0 ± 0.13</td>
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<td>HSD11B1 mRNA</td>
<td>1.38 ± 0.4</td>
<td>1.0 ± 0.23</td>
<td>10.48 ± 2.2*</td>
<td>1.38 ± 1.0*</td>
</tr>
<tr>
<td>TNFα mRNA</td>
<td>0.7 ± 0.2</td>
<td>1.0 ± 0.4</td>
<td>1.8 ± 0.8*</td>
<td>7.7 ± 6.7*</td>
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<tr>
<td>GR mRNA</td>
<td>1.9 ± 0.4*</td>
<td>1.0 ± 0.3*</td>
<td>36.4 ± 9.5*</td>
<td>1.0 ± 0.4*</td>
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<tr>
<td>HSD11B2 mRNA</td>
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<td>1.0 ± 0.3</td>
<td>0.3 ± 0.1</td>
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<tr>
<td>IGF2R mRNA</td>
<td>2.7 ± 0.5*</td>
<td>1.0 ± 0.1*</td>
<td>0.8 ± 0.2*</td>
<td>1.0 ± 0.6</td>
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<tr>
<td>IGF1R mRNA</td>
<td>1.7 ± 0.2*</td>
<td>1.0 ± 0.3*</td>
<td>0.5 ± 0.1*</td>
<td>1.0 ± 0.2*</td>
</tr>
<tr>
<td>IGF2 mRNA</td>
<td>0.0 ± 0.18</td>
<td>1.0 ± 0.09</td>
<td>0.4 ± 0.1</td>
<td>1.0 ± 0.2*</td>
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<tr>
<td>PPARG mRNA</td>
<td>1.6 ± 0.12*</td>
<td>1.0 ± 0.08*</td>
<td>0.54 ± 0.17*</td>
<td>0.34 ± 0.12*</td>
</tr>
<tr>
<td>PPARG mRNA</td>
<td>1.4 ± 0.4</td>
<td>1.0 ± 0.2</td>
<td>0.1 ± 0.05*</td>
<td>0.1 ± 0.02*</td>
</tr>
</tbody>
</table>

*Different superscripts within an age group denote statistically significant effect of parity (P<0.05). †P<0.01. §P<0.001. ‡P<0.0005 denote statistically significant effect of postnatal age within parity group. PAT, perirenal–abdominal adipose tissue; ND, not detected; a.u, arbitrary units.

1 month for GR. Consequently, there was an age-related increase in GR together with decreased HSD11B2 gene expression. In contrast, there was no effect of maternal parity or postnatal age on mRNA abundance for HSD11B1.

**Insulin receptor and IGF-binding protein 3 mRNA abundance**

Insulin receptor and IGF1R mRNAs were significantly higher in adipose tissue sampled from 1-day-old offspring born to nulliparous mothers (Table 2), an adaptation reversed by 1 month of age. Gene expression for both IGF1R and IGF2R was significantly reduced with postnatal age. In contrast, there was no effect of postnatal age or maternal parity on IGF-binding protein 3.

**UCP1 and UCP2**

There was no effect of maternal parity on UCP1 mRNA, but protein expression was significantly higher in offspring of nulliparous mothers at 1 day of age, a difference that was reversed by 1 month of age as UCP1 content decreased markedly in all offspring over this period (Table 2). In contrast, UCP2 mRNA abundance was significantly higher in adipose tissue sampled from offspring born to nulliparous mothers at 1 day of age, a difference that was no longer apparent by 1 month of age due to a pronounced decrease in its expression in offspring born to nulliparous mothers.

**Discussion**

We have shown that the increase in fat mass associated with firstborn status is accompanied by a potential resetting of the leptin and glucocorticoid axis within the adipocyte. The enhanced fat mass seen at birth is thus associated with endocrine changes which are likely to have contributed to increased rates of adipogenesis both during late gestation and continuing after birth. These findings further emphasise the importance of the foetal and early postnatal environment in determining both adipose tissue development and later adiposity.

**The influence of maternal parity on placental and foetal development and neonatal leptin and adiposity**

Our observation that firstborn offspring are lighter at birth is in agreement with findings from other species (Ong et al. 2002, Gardner et al. 2007), including humans, and occurs even when there is no change in maternal body weight and the pregnancy is twin-bearing (Symonds et al. 2004). As size...
Offspring of nulliparous mothers possessed more UCP1, which is likely to reflect increased translation of the UCP1 gene to protein following rapid activation at birth (Clarke et al. 1997b). Interestingly, this was accompanied by enhanced UCP2 gene expression and is in accordance with the effect of foetal growth restriction induced by umbilical cord occlusion (Gnanalingham et al. 2005a). It may be that the comparatively restricted in utero environment that accompanies a first pregnancy (Bai et al. 2002) acts to promote both maturation and growth of adipocytes in the foetus. Raised UCP1 would increase its effectiveness in producing heat after birth, an adaptation previously shown to be accompanied by increased fat mass at 1 month after birth in sheep born to chronically cold exposed mothers (Symonds et al. 1992).

Maternal parity influences glucocorticoid and insulin sensitivity of adipose tissue

Local adipose tissue glucocorticoid sensitivity, set during the neonatal period, is nutritionally regulated in utero and can determine later adiposity (Gnanalingham et al. 2005b). In the present study, being born to a nulliparous mother resulted in an increased mRNA abundance of GR and HSD11B2 mRNAs at birth, indicating increased local adipose tissue glucocorticoid sensitivity in the absence of any change in plasma cortisol. Interestingly, this adaptation was no longer apparent by 1 month of age when there is an ~40-fold increase in GR in conjunction with an age-related decrease in HSD11B2, which are responses predicted to increase risk of later obesity (Watts et al. 2005). Adipocyte differentiation is a complex process that requires coordinated communication between hormones, growth factors and transcription factors. Glucocorticoids, for example, are major stimulators of adipose tissue development and fat accumulation especially in combination with insulin (Brindley 1992) and IGF1R (Mur et al. 2003). These hormones act together to induce expression of metabolic genes (Teruel et al. 1996). However, the precise signalling mechanism and transcription factors involved in GR-, insulin- and IGF1R-regulated adipogenesis and differentiation are still being elucidated. Recent studies have suggested a role for GR-dependent lipin-1 in adipogenesis in both mouse (Zhang et al. 2008) and human adipocytes. Moreover, expression of lipin-1 in differentiating preadipocytes is essential for normal expression of adipogenic transcription factors and for synthesis of triglycerides. In the present study, we observed higher GR and IGF1R mRNA abundance in firstborn offspring at birth that was accompanied by a transient upregulation of the insulin receptor that would promote both differentiation and adipogenesis (Chapman et al. 1985, Rosen & Spiegelman 2000). At 1 month of age, adiponectin mRNA abundance was also increased in perirenal adipose tissue of firstborn offspring, suggesting increased insulin sensitivity (Tsai et al. 2004) despite reduced IR at this stage. In contrast, the rate of loss of both PPARG and PPARA over the first month of life was similar between groups, and confirms that PPARs are not

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involved in promoting adipose tissue growth after birth (Lomax et al. 2007). Taken together, such adaptations would be predicted to promote fat mobilisation if food supply became limited in later life.

In conclusion, the increase in fat mass of firstborn offspring and the accompanying alterations in adipose tissue endocrine sensitivity may be significant risk factors for obesity in early childhood as well as for the onset of the metabolic syndrome and its associated disorders in later life. Whether this is due to a difference in maternal body composition or pregnancy interval is unclear. Nonetheless, the role of maternal parity in determining offspring adiposity and later metabolic disease is especially important, given that contemporary women in the western world are limiting the size of their families and that this may result in a generation with a greater proportion of firstborn children, thereby exacerbating the current obesity epidemic.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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