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Impact of birth weight and gender on early postnatal hypothalamic energy balance regulatory gene expression in the young lamb

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ABSTRACT

Intra-uterine growth restriction (IUGR) is involved in developmental metabolic programming and here we test the hypothesis that IUGR affects the developing hypothalamic energy balance regulatory pathways in a sex-specific manner. This experiment investigated early postnatal hypothalamic gene expression for six primary leptin- and insulin-sensitive neuropeptides and receptors in male and female IUGR ($n = 8$ and 9, respectively) and normal (N) birth weight lambs ($n = 8$ per gender) gestated and suckled by overnourished mothers. IUGR lambs were smaller at birth, had increased fractional growth rates (FGR), lower final body weight (11 weeks) and similar body fat content compared with N lambs, while males had higher final body weight and insulinemia but lower body fat and leptinemia than females. In situ hybridization revealed greater gene expression in the hypothalamic arcuate nucleus at 11 weeks for anorexigenic genes in females and orexigenic genes in males, with no effect of IUGR. Leptinemia correlated with gene expression for neuropeptide Y (NPY, negatively) in both sexes and pro-opiomelanocortin (POMC, positively) in females but with leptin receptor (negatively) only in males. Current FGR for girth correlated negatively with gene expression for NPY in males and POMC in females. Neither IUGR nor gender affected suckling activity (proxy for appetite) assessed at 3 weeks, but final NPY gene expression correlated with suckling weight gain in males. This study has revealed no effect of IUGR on early postnatal hypothalamic energy balance gene expression but a major effect of gender associated with major sex differences in adiposity and leptinemia.

1. Introduction

The basic model of energy homeostasis in the mature animal includes peripheral metabolic feedback hormones leptin and insulin regulating the activities of opposing orexigenic and anorexigenic circuits in the hypothalamus (Schwartz et al., 2000). These neuronal circuits develop in the fetal brain, they are well established by birth in precocial species such as sheep and primates, and their development is influenced by the prenatal nutritional environment (Grayson et al., 2010). Changes in the developing hypothalamic circuitry may underlie the apparent programming of a predisposition to obesity and altered metabolic phenotype by intra-uterine growth restriction (IUGR), especially when such offspring are born into an unrestricted nutritional environment (Gluckman and Hanson, 2008). However, existing data largely come from studies of rodents in which hypothalamic neuroendocrine maturation occurs after birth (Breton, 2013); it is now appropriate to increase our understanding of prenatal nutritional programming of the hypothalamus in larger, precocial mammalian species.

Previously we have demonstrated the presence of key anorexigenic and orexigenic gene expression in the hypothalamic arcuate nucleus of the ovine fetus at mid as well as late gestation (Adam et al., 2008; Mühlhäusler et al., 2004). A consistent finding was the sensitivity of anorexigenic neuropeptides in the arcuate nucleus to fetal nutrient (glucose) supply, thus pro-opiomelanocortin (POMC) gene expression correlated with fetal glycemia at 81 days (term = 147 days; Adam et al., 2008), intra-fetal glucose infusion increased POMC gene expression at 140 days (Mühlhäusler et al., 2005), and cocaine- and amphetamine-regulated transcript (CART) gene expression correlated with fetal liver glycogen and was decreased in IUGR compared with normally growing fetuses at 130 days (Adam et al., 2011a). It remains to be determined to what extent prenatal changes in hypothalamic neuropeptides persist in postnatal life when they may affect appetite and body weight regulation in the free-living sheep. Findings from rodent offspring indicate early postnatal down-regulation of anorexigenic and up-regulation of orexigenic hypothalamic neuropeptides following prenatal fetal undernutrition caused by maternal undernutrition or placental insufficiency (Cripps et al., 2009; Desai et al., 2007;

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Huizinga et al., 2001). Earlier we reported in sheep that the presumed increase in fetal arcuate POMC gene expression induced in late gestation by maternal overnutrition and elevated glycemia was sustained in lambs at postnatal day 30 (Mühlhäusler et al., 2006). Now it is pertinent to investigate whether the presumed changes in anorexigenic hypothalamic gene expression in IUGR ovine fetuses are sustained postnatally.

Although insulin and leptin are present in the fetal circulation, there is little evidence for either hormone playing an adult-like nutritional signalling role in the hypothalamus. Precocial species like sheep lay down adipose tissue in the latter stages of gestation, which secretes leptin in proportional concentrations (Mühlhäusler et al., 2002), and the ovine fetal pancreas secretes insulin from mid gestation onwards (Aldoretta et al., 1998). Gene expression for receptors for both hormones is detected in the arcuate nucleus from mid gestation, but no correlation was found between circulating concentrations of either hormone and expression of key appetite regulatory genes in the fetal arcuate nucleus (Adam et al., 2008, 2011a,b; Mühlhäusler et al., 2004). Circulating fetal insulin concentrations correlated negatively with arcuate insulin receptor (Ins-R) gene expression in an apparently adult-like ligand–receptor relationship, but circulating leptin concentrations were found to correlate positively with arcuate leptin receptor (OB-Rb) gene expression in late gestation (Adam et al., 2011a,b). These latter findings were consistent with the substantial evidence for leptin playing a neurotrophic role in the neonate (Bouret, 2010). However by 5–6 months of age intracerebroventricular (ICV) leptin suppresses appetite in both male and female sheep indicating that a functional role for leptin signalling in the hypothalamus has developed by this age (Adam et al., 2011b). Conversely, ICV-administered insulin had no effect on appetite in 5- to 6-month-old sheep (Adam et al., 2011b). In the present study, we examine relationships between hypothalamic arcuate neuropeptide and receptor gene expression, leptinemia and insulinemia at 3 months of age.

In addition to the effects of IUGR, we examine the influence of gender since there is evidence for sex differences in hypothalamic programming with respect to the hypothalamo-pituitary-adrenal 'stress' axis (Gardner et al., 2006; Wallace et al., 2011), and yet there is a lack of equivalent data with respect to the hypothalamic appetite regulatory axis. Furthermore, in the cohort of lambs used for the present study, whilst IUGR impacted postnatal fractional growth rates and glucose metabolism, gender had the overriding influence on body composition and metabolic hormone status (Wallace et al., 2013). Thus, in these lambs from our overnourished adolescent dam model of utero-placental insufficiency, IUGR led to increased fractional growth rates to 11 weeks of age and impaired glucose handling at 7 weeks compared with normal birth weight lambs whereas females had increased adiposity and leptinemia compared with males (Wallace et al., 2013). Importantly, IUGR and normal birth weight lambs in this cohort were both born to dams that had high dietary intakes throughout pregnancy and lactation, thus allowing us to examine their early postnatal phenotype without the confounding effect of differences in maternal nutrition. In order to estimate appetite drive and voluntary food intake in the lambs, suckling activity was assessed at 3 weeks of age.

This experiment therefore investigated the influences of IUGR and gender on the early postnatal phenotype with respect to the developing hypothalamic appetite regulatory pathways. Specifically, we examined gene expression for six primary leptin- and insulin-sensitive hypothalamic neuropeptides and receptors at 11 weeks of age in low and normal birth weight male and female lambs born to overnourished adolescent mothers. We test the hypothesis that IUGR affects the developing hypothalamic energy balance regulatory pathways in a sex-specific manner.

2. Materials and methods

2.1. Animals

All procedures were licensed under the UK Animals (Scientific Procedures) Act 1986 and approved by local Ethical Review Committee. The derivation of the lambs is described in detail by Wallace et al. (2013). Briefly, growing adolescent recipient ewes (Dorset Horn × Mule) had been implanted with singleton embryos, derived from superovulated donors (Border Leicester × Scottish Blackface) and a single sire (Dorset Horn), and given a high quality complete diet ad libitum throughout pregnancy and lactation. The complete diet contained 12 MJ metabolizable energy and 140 g crude protein per kg dry matter and ad libitum intakes were calculated to promote rapid maternal growth during pregnancy leading to restricted placental growth, and hence restricted fetal growth in ~50% cases, followed by maximal milk yields during the 11-week lactation. In addition, lambs had access to their mothers' feed at all times. There was a continuous distribution of birth weights from which lambs were categorized as intra-uterine growth restricted (IUGR) or normal birth weight (N) (Wallace et al., 2013). The present study comprised 17 IUGR ($n = 8$ male, $n = 9$ female) and 16 N lambs ($n = 8$ per gender), with most of the individual embryo donors represented in both categories.

The lambs were weighed, measured and blood sampled mid-morning at 5-day intervals up to ~68 days of age and just before euthanasia at 77 days (11 weeks). Plasma leptin and insulin were determined by in-house radioimmunoassays (Marie et al., 2001; MacRae et al., 1991, respectively) with all inter and intra-assay coefficients of variation less than 10% (as reported in Wallace et al., 2013). They were euthanized by lethal injection of sodium pentobarbitone (10–15 ml Euthesate; 200 mg pentobarbitone/ml; Willows Francis Veterinary, Crawley, UK). Whole brains were removed, immediately frozen in isopentane over dry ice and stored at −80 °C. Perirenal and visceral (omental and mesenteric) fat depots were dissected out and weighed.

2.2. Suckling activity assessment

Suckling activity was determined at 23 ± 0.9 days of age, coincident with the presumed peak lactation and prior to lambs showing any significant interest in eating their mothers' food. Ewes were milked by hand following intravenous oxytocin injection (Oxytocin-S® 10 i.u. per ewe; Intervet Ltd, Cambridge, UK) in order to empty the udder, the lamb was weighed and access to the udder was prevented using an udder cover. After 3 h, the lamb was reweighed to determine fasting weight loss, the udder cover was removed and the number and duration of suckling bouts were determined for a period of 60 min. Lambs were weighed at 15-min intervals throughout this observation period and again at 90 min.

2.3. Hypothalamic gene expression

The frozen brains were trimmed down to a mid-ventral block, mounted and sectioned by cryostat coronally through the hypothalamus from the mammillary body (caudal) to the optic chiasm (rostral). Sections (20 μm) were thaw-mounted onto poly-L-lysine-coated slides and stored at −80 °C. Gene expression for neuropeptide Y (NPY), agouti-related peptide (AGRP), POMC, CART, OB-Rb and Ins-R was measured by in situ hybridization, using techniques described in detail elsewhere (Adam et al., 1997). The NPY riboprobe was generated from a rat cDNA (Adam et al., 1997), the CART probe from a cloned sheep cDNA (Barrett et al., 2001), and AGRP and POMC probes were generated from cloned Siberian hamster cDNAs (Mercer et al., 2000). A riboprobe complementary to fragments of the intracellular domain of OB-Rb was generated from a cloned sheep cDNA (Mercer et al., 1998), and the Ins-R riboprobe was generated from a partial ovine cDNA (Archer et al., 2005). All probes have previously been validated in sheep brain (Adam et al., 2002; Archer et al., 2005) and corresponding sense probes showed no hybridization. Briefly, sections were fixed, acetylated, and hybridized overnight at 58 °C using ^{35}S-labelled cRNA probes ($1–1.5 \times 10^7$ cpm/ml). They were then treated with RNase A, desalted, with a final high stringency wash (30 min) in 0.5× SSC at 60 °C (Ins-R at 75 °C), dried and apposed for 7–10 days to Hyperfilm β-max (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK). Intensity and total area of hybridization were quantified in the hypothalamic arcuate nucleus on each autoradiographic image, using the Image-Pro Plus system (Media Cybernetics, Silver Spring, MD, USA). Example images are shown in Fig. 1. The integrated intensity of the hybridization signal (i.e. the optical density integrated over the total hybridization area) was computed using standard curves generated from ^{14}C autoradiographic micro-scales (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK). For each probe, up to 6 sections spanning the medial hypothalamus (i.e. in the region midway between the mammillary body and optic chiasm, further identified by third ventricle morphology) were examined from each brain. All reagents were obtained from Sigma (Sigma UK, Poole, Dorset, UK) unless otherwise stated.

2.4. Statistical analyses

Effects of birth weight category, gender and their interaction were examined by analysis of variance (General Linear Model; Minitab 16, Minitab Inc., State College, PA), and data are presented as means ± standard errors. Pearson product-moment