Hypothalamic alterations in fetuses of high fat diet-fed obese female rats

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Abstract

The offspring of high fat (HF) diet-fed rats display increased body weight during adulthood. However, it is not known whether the changes in appetite regulation in these animals occur *in utero* or postnatally. We investigated the effects of maternal obesity induced by a HF diet prior to and during pregnancy on leptin and insulin signaling and the expression of orexigenic and anorexigenic peptides in term fetal hypothalami. The consumption of a HF diet prior to and during pregnancy resulted in obesity in HF female rats; additionally, HF female rats exhibited hyperinsulinemia and hyperleptinemia which were exaggerated in late gestation compared with control female rats that were fed a standard rodent laboratory chow (LC). Term fetuses of HF female rats (FHF) also had significantly higher serum leptin and insulin levels compared with control fetuses (FLC) while there was no difference in average fetal weight between the two groups.

FHF hypothalami showed elevated levels of mRNA and proteins for leptin long receptor and insulin receptor β -subunit. However, the protein levels of signal transducers and activators of transcription-3 and insulin receptor substrate-2, the downstream signaling components of leptin and insulin signaling respectively were decreased. Also, FHF hypothalami had increased mRNA levels of neuropeptide Y and agouti-related polypeptide indicating that orexigenic neuropeptides in HF progeny are already upregulated by term fetal stage. Additionally, the mRNA levels of pro-opiomelanocortin and melanocortin receptor-4 were also increased in the HF fetal hypothalami. These findings indicate potential programming effects of an altered intrauterine environment induced by HF diet consumption on appetite-regulating neuropeptides and leptin and insulin signaling in the late fetal period.

Journal of Endocrinology (2009) **200**, 293–300

Introduction

Recent studies published by the NHANES III survey point to an alarming increase in the prevalence of obesity, metabolic syndrome and type 2 diabetes in adults as well as in children (Flegal *et al.* 2002). In 1999–2000, within the US, >15% of 6–19 year-old children, 10% of children between the ages of 2 and 5 years-old and more than 12% of 6–23 month-old infants were classified as obese (>95% body mass index (BMI); Flegal *et al.* 2002). This demographic shift in phenotype of obesity towards a younger age is not explained by genetic changes over the last 100 years. There is increasing evidence to indicate that genes interact with the intrauterine, perinatal and early postnatal environments of an organism leading to adaptations for immediate survival. However, over the long-term, these adaptations can predispose individuals to the development of the obese phenotype (Plagemann 2005). In the context of the phenomenon of developmental programming, obesity during pregnancy is implicated in playing a pathogenic role in the development of the obese phenotype in offspring. Children who are born to obese mothers are twice as likely to be obese by 2 years

of age (Whitaker 2004). Abnormal maternal pregravid weight along with diabetes is an additional risk factor for adolescent obesity (Yogev & Langer 2008). Adolescents born to obese mothers are also twice as likely to have metabolic syndrome if the mother is both diabetic and obese (Boney *et al.* 2005). A strong correlation exists between maternal BMI and the BMI of offspring in adulthood. Also, studies show an increased risk of large gestational age for babies if the mother has abnormal weight gain between successive pregnancies (Parsons *et al.* 2001, Villamor & Cnattingius 2006). Fetal development in a diabetic–intrauterine environment resulted in a diabetogenic tendency in the offspring (Van Assche *et al.* 2001). Among Pima Indians high birth weight resulting from abundant supply of glucose due to maternal diabetes during pregnancy predisposed the offspring to the development of diabetes later in life (Pettitt 1996). These epidemiological studies strongly indicate that the origins of obesity and metabolic syndrome lie *in utero*.

Animal studies in various models including gestational under-nutrition, infants of diabetic mothers, neonatal over-nutrition, and glucocorticoid exposure provide valuable supportive evidence confirming the role of intrauterine

environment in developmental metabolic programming of offspring. Studies in these diverse animal models of altered environment have shown a programming effect on growth, adipose tissue and the appetite regulating pathways in the brain in adult offspring resulting in an increased risk of type 2 diabetes, hypertension and cardiovascular dysfunction (Whitaker 2004, Plagemann 2005). Maternal overnutrition during pregnancy has been similarly demonstrated to lead to increased appetite, adiposity, impaired glucose tolerance and cardiovascular dysfunction in the offspring (Khan *et al.* 2005, Muhlhausler *et al.* 2006, Taylor & Poston 2007).

The hypothalamus plays a central role in energy homeostasis by regulating both appetite and energy expenditure. The arcuate nucleus in the hypothalamus is the prime area involved in the control of food intake (Schwartz *et al.* 2000, Valassi *et al.* 2008). It contains two interconnected groups of 'first order' neurons which release the orexigenic neuropeptides (neuropeptide Y (NPY) and agouti-related peptide (AgRP)) and the anorexiogenic substance pro-opiomelanocortin (POMC; Schwartz *et al.* 2000, Valassi *et al.* 2008). The axons from these neurons project to second order neurons in the paraventricular nucleus and the lateral hypothalamic area where appropriate anabolic or catabolic pathways are activated in response to the peripheral signals received (Schwartz *et al.* 2000, Valassi *et al.* 2008).

The central nervous system maturation including that of the hypothalamus begins *in utero* and continues into early postnatal life. Therefore, changes in nutrient availability in the intrauterine and neonatal periods can cause permanent changes in expression of key appetite-regulating neuropeptides and their receptors. Neonatal overnutrition studies in suckling rats have demonstrated changes in expression of NPY and AgRP in the hypothalamus in association with hyperphagia and increased adiposity (Plagemann 2005). The mRNA levels of long form of leptin receptor (OB-Rb), NPY and AgRP were inversely related to total fat mass in lambs of over-nourished ewes pointing to a state of leptin resistance in the adult offspring (Muhlhausler *et al.* 2006). Adult offspring of prenatally overfed rats had an exaggerated feeding response to central NPY injection compared with control animals (Kozak *et al.* 2005). Studies from our laboratory have shown that artificial rearing of newborn rat pups on a high carbohydrate milk formula resulted in hypothalamic adaptations predisposing them to hyperphagia in the post-weaning period (Srinivasan *et al.* 2008).

Most studies on programming effects have been done in the neonatal period. However, considering that the hypothalamus starts developing *in utero*, it becomes imperative to segregate the effects of prenatal overnutrition on the offspring from effects of lactation and postnatal overnutrition. This is important for two reasons. First, unlike models that induce fetal hyperglycemia, maternal overfeeding models are more closely related to the current obesity epidemic. Secondly, if these effects are established during the fetal period, it will provide important supportive information to epidemiological studies for designing intervention strategies during the prenatal period to prevent permanent establishment of adverse programming effects in the offspring.

The present study evaluated the maternal and fetal environments during pregnancy complicated by obesity in rats fed a high-fat (HF) diet. We investigated the signaling pathways of insulin and leptin in the fetal hypothalamus to investigate putative alterations in downstream signaling as well as expression levels of neuropeptides involved in energy homeostasis. Knowledge of these changes will further our understanding of the role of leptin and insulin in the brain in the developing fetus.

Materials and Methods

Animal experimentation

All animal protocols were approved by the Institutional Animal Care and Use Committee. Newly weaned (postnatal day 23) Sprague-Dawley female rats were procured from ZivicMiller Laboratories (Zelienople, PA, USA) and housed under controlled conditions of light, temperature, and humidity. They were randomly assigned either to a standard rodent laboratory chow diet (LC; Harlan Teklad, Madison, WI, USA) or a high-fat diet (Product # F3282; Bio-Serv; Frenchtown, NJ, USA) with access to water and their specific diets *ad libitum*. Two rats receiving the same diet were housed per cage from the time of weaning until initiation of breeding. The caloric composition of the HF diet was 24.4% carbohydrate, 59.5% fat and 16.2% protein compared with 70% carbohydrate, 10.9% fat and 19.2% protein in the LC diet (Srinivasan *et al.* 2006). The energy densities of the diets were 5.3 kcal/g for HF and 3.2 kcal/g for LC.

The female rats were weighed weekly from postnatal day 25. Blood (0.2 ml) was obtained from the tail site every 2 weeks for plasma analyses outlined below. At 100 days of age, the females were housed individually and mated with normal adult males fed a standard LC diet. Expulsion of vaginal plug was considered day one of gestation. The female rats were housed individually during pregnancy. The respective diets were continued during pregnancy for the females. Pregnant females were killed on day 21 of gestation and blood samples were obtained. For optimal blood sample amounts, term fetuses from each female were considered as a single unit and specimens were pooled from each litter. Plasma from female rats and sera from term fetuses were analyzed for insulin and leptin levels using rat RIA kits (Linco Research, St Louis, MO, USA).

Isolation of hypothalami

The skull was opened and the hypothalamus was dissected from the ventral side of the brain using the anatomical margins of the optic chiasm anteriorly and the mammillary bodies posteriorly. All the samples were snap frozen in liquid nitrogen and stored at -80°C until subsequent analyses by PCR and western blot.

Quantitative RT-PCR

Total RNA was extracted from the hypothalami of rat fetuses using the TRIZOL reagent phenol–chloroform procedure. (Gibco). One microgram of RNA was subsequently reverse transcribed into cDNA by using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions and analyzed further using SYBR green quantitative PCR technique. Primer sequences were designed to span at least one exon–exon junction of the target mRNA to prevent amplification of contaminating genomic DNA using Primer 3 software and NLM sequence database. Primer sequences are described in Table 1. The mRNA levels detected by SYBR Green (Bio-Rad) analysis were normalized using 18S mRNA levels as endogenous reference standard. (Quantum RNA Classic II 18S Internal Standard, 324 bp; Ambion, Austin, TX, USA). Relative mRNA levels were calculated according to the comparative $\Delta\Delta C_t$ method.

Western blot analysis

Total protein was extracted from pooled hypothalami of term rat fetuses. Briefly, 2 hypothalami were resuspended in 300 μ l solubilization buffer (50 mM HEPES, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM $NaVO_4$, 10 mM NaP_2O_7 , 10 mM NaF, 2 mM EDTA, 1% Igepal, 10% glycerol, 2 μ g/ml aprotinin, antipain, leupeptin, and pepstatin, 1.5 μ g/ml benzamide, 34 μ g/ml phenylmethylsulphonyl fluoride), homogenized by sonication and centrifuged. Equal amounts of protein (20–50 μ g) for both groups were separated by SDS–PAGE for determination of levels of the different proteins examined. Protein was transferred to a nitrocellulose membrane and incubated with primary and secondary antibodies as described earlier (Srinivasan *et al.* 2006). Protein bands were visualized using chemiluminescence (PerkinElmer, Wellesley, MA, USA), and densitometric analysis was performed using the Quantity One program. Antibodies were obtained from sources as indicated: OB-Rb (Linco Research), β -subunit of the insulin receptor (IR- β) and insulin receptor substrate-2 (IRS-2; Upstate Cell

Signaling, Chicago, IL, USA), signal transducers and activators of transcription-3 (STAT-3; Cell Signaling Solutions, Lake Placid, NY, USA) and suppressors of cytokine signaling-3 (SOCS-3; Affinity BioReagents, Golden, CO, USA).

Statistical analysis

All data are expressed as means $\pm$ S.E.M. (n as indicated). Litter from one female was considered as a single observation. One-way ANOVA followed by *post hoc* analysis using the Student–Newman–Keuls test was used to determine significance of the difference of the means of data for multiple time points. For analysis of data for a single time point Student unpaired *t*-test was performed. *P* value ≤ 0.05 was considered as significant.

Results

Body weight gain and food intake

Our earlier studies showed that female rats fed a HF diet exhibited significant increases in weight gain by postnatal day 65 which was amplified over time (Srinivasan *et al.* 2006). The present study also showed that female rats weaned onto a high-fat diet were significantly heavier than age-matched LC female rats in the post-weaning period (Fig. 1A). During pregnancy HF female rats were markedly heavier than age-matched LC pregnant rats at the end of the first, second and third weeks of gestation (Fig. 1B). The HF diet is more calorie dense compared with the LC diet (5.3 kcal/g for HF diet and 3.2 kcal/g for LC diet). HF female rats consumed a significantly increased number of calories compared with age-matched LC female rats in the pre-pregnancy period (Fig. 1C) although in terms of weight of diet consumed there were no marked differences between the two groups of rats per each data point (data not shown). During gestation HF female rats continued to demonstrate hyperphagia as indicated by the increased caloric consumption during the third week of pregnancy (Fig. 1D).

Table 1 Primer sequences for quantitative RT-PCR

OB-Rb	370 bp	Sense: 5'-CCTGAAACATTTGAGCATCTTT-3' Antisense: 5'-CGATGCACTGGCTGACAGAA-3'
AgRP	114 bp	Sense: 5'-AGCAGACCGAGCAGAAGATG-3' Antisense: 5'-GACTCGTGACGCTTACACA-3'
NPY	236 bp	Sense: 5'-AGAGATCCAGCCCTGAGACA-3' Antisense: 5'-AACGACAACAAGGGAAATGG-3'
POMC	173 bp	Sense: 5'-CGACGGAGGAGAAAAGAGGT-3' Antisense: 5'-CTATGGAGGTCTGAAGCAGGAG-3'
IR- β	331 bp	Sense: 5'-ATCTGGATCCCCCTGATACTGTC-3' Antisense: 5'-ATGTGGGTGTAGGGGATGTGTTC-3'
STAT-3	104 bp	Sense: 5'-TGATGCGCTCTTATGTGAGG-3' Antisense: 5'-GGCGGACAGAACATAGGTGT-3'
SOCS-3	133 bp	Sense: 5'-CCTTTGAGGTTACAGGACAG-3' Antisense: 5'-GTAGCCACGTTGGAGGAGAG-3'

Figure 1 Body weights and food intake for HF and age-matched LC female rats in the pre-pregnancy and pregnancy periods. Body weights in the pre-pregnancy period from postnatal days 24 to 105 (A) and during pregnancy (B) and food intake in the pre-pregnancy period (C) and during pregnancy (D) for HF and age-matched LC female rats. The results are expressed as means $\pm$ S.E.M. ($n=6-8$). * $P \leq 0.05$ compared with LC.

Insulin, leptin and glucose levels in the pre-pregnancy and pregnancy periods

Within 2 weeks of consumption of the high-fat diet, plasma leptin levels were significantly higher (1.98-fold increase; $P \leq 0.05$) in HF female rats (Fig. 2A). While plasma leptin levels continued to increase in both groups in the post-weaning period, HF females had significantly higher plasma leptin levels compared with LC females at all the time points in the pre-pregnancy period (1.90-fold; $P \leq 0.05$, 1.99-fold; $P \leq 0.05$, and 2.61-fold $P \leq 0.05$ on postnatal days 60, 73, and 87 respectively; Fig. 2A). On gestational day 21 there was a further heightened disparity (2.75-fold increase; $P \leq 0.05$) in plasma leptin levels between the two groups of rats (Fig. 2A). Although, plasma insulin levels of HF and LC females were not different up to postnatal day 73, they became significantly higher (1.48-fold; $P \leq 0.05$) in HF females by postnatal day 87 and continued to be increased to 2.34-fold ($P \leq 0.05$) during late gestation (Fig. 2B). Both groups had similar glucose levels suggesting a state of insulin resistance (78 ± 8 mg/dl for LC and 74 ± 10 mg/dl for HF in the pre-pregnancy period on postnatal day 87; 83 ± 13 mg/dl for LC and 78 ± 12 mg/dl for HF during pregnancy on gestational day 21).

Characterization of fetal weights, leptin and insulin levels

There is evidence that maternal leptin can cross the placenta and is a significant source of fetal leptin (Smith & Waddell 2003) but there is little transplacental transfer of insulin (Boskovic *et al.* 2003). In our previous study, we showed that HF fetuses were hyperinsulinemic due to enhanced pancreatic insulin secretion (Srinivasan *et al.* 2006). Since, serum levels of leptin and insulin

Figure 2 Plasma levels of leptin (A) and insulin (B) in HF and age-matched LC female rats in the post-weaning period (during the pre-pregnancy and pregnancy periods). The results are expressed as means $\pm$ S.E.M. ($n=6-12$). * $P \leq 0.05$ compared with LC. G21: gestational day 21.

may affect fetal development, we evaluated their levels in the fetuses of LC mothers (FLC) and fetuses of HF mothers (FHF). The average term fetal weights (5.00 ± 0.3 g for FLC and 5.20 ± 0.2 g for FHF) and the average term placental weights ($FLC = 0.50 \pm 0.02$ g and $FHF = 0.47 \pm 0.02$ g) were similar for both the groups. The average number of term fetuses in HF group was similar to that of the LC group (average litter size for LC = 15.1 ± 1.8 vs 14.1 ± 2.2 for HF females). Fetal weight/placental weight ratio was not significantly different between the two groups of fetuses (data not shown). Serum insulin levels of FHF were 1.62-fold higher ($P \leq 0.05$) than that of FLC (Fig. 3A). The serum leptin levels of FHF were also significantly higher ($P \leq 0.05$) than that of FLC (Fig. 3B). In an earlier study we showed that fetal glucose levels were not different between the two groups of fetuses (Srinivasan *et al.* 2006).

Effect of HF diet on levels of appetite-regulating neuropeptides and components of leptin and insulin signaling

Quantitative RT-PCR Although it has been shown that leptin and insulin signaling pathways in the hypothalamus play

Figure 3 Serum levels of insulin (A) and leptin (B) in term fetuses of LC and HF female rats. The results are expressed as means $\pm$ S.E.M. ($n=6-8$). * $P \leq 0.05$ compared with LC.

a pivotal role in the regulation of food intake in adults, it is not known if alterations in these signaling pathways are initiated during fetal development due to an adverse maternal intrauterine environment. In order to decipher the role of the obese/hyperinsulinemic maternal environment of the HF female rat on the development of programming effects at the level of insulin and leptin signaling in fetal hypothalamus, the mRNA levels of the receptors and some of the proximal components were determined by real-time PCR. We observed a 3.6-fold ($P \leq 0.05$) increase in mRNA levels of OB-Rb in FHF hypothalamus compared with FLC hypothalamus (Fig. 4). This was accompanied by a 1.72-fold ($P \leq 0.05$) increase in levels of STAT-3 mRNA in FHF hypothalamus (Fig. 4), the primary mediator of leptin's activity on the downstream neurons that express AgRP and NPY. There was no significant change in level of SOCS-3 mRNA. There was a 2.24-fold ($P \leq 0.05$) increase in levels of the IR- β in FHF hypothalamus (Fig. 4). mRNA levels of AgRP and NPY were increased by 1.77-fold ($P \leq 0.05$) and 3.41-fold ($P \leq 0.05$) respectively (Fig. 4) and POMC and melanocortin receptor 4 (MC4R) levels were elevated by 4.29-fold ($P \leq 0.05$) and 3.14-fold ($P \leq 0.05$) respectively in the FHF hypothalamus compared with FLC hypothalamus (Fig. 4).

Western blot analysis An increased OB-Rb mRNA level was associated with an increased level of OBRb protein by 2.08-fold in the FHF hypothalamus (Fig. 5). However, total

Figure 4 mRNA levels of AgRP, NPY, POMC, MC4R, OB-Rb, STAT-3, SOCS-3 and IR- β in the hypothalamus of term fetuses of high-fat-diet fed (FHF) and laboratory chow fed (FLC) female rats. mRNA levels are normalized relative to 18S as endogenous reference standard using the $\Delta\Delta C_t$ comparative method. Results are expressed as means $\pm$ S.E.M. ($n=5-7$). * $P \leq 0.05$ compared with LC.

STAT-3 protein level in the FHF hypothalamus was only 85% of the FLC hypothalamus ($P \leq 0.05$; Fig. 5). No significant difference was observed in levels of SOCS-3 protein between the two groups.

Protein levels of IR- β were also increased (Fig. 5) corresponding to the increased mRNA levels of IR- β in FHF compared with FLC. However, IRS-2 protein amount in the FHF group was only 65% of the FLC group ($P \leq 0.05$; Fig. 5).

Discussion

The present study confirms our previous report about the consequences of maternal high-fat diet consumption leading to fetal programming (Srinivasan *et al.* 2006). It is possible that

Figure 5 Western blot analysis of leptin (OB-Rb, STAT-3, SOCS-3) and insulin (IR- β , IRS-2) signal transduction components in the hypothalamus of term FHF and FLC female rats. Results are expressed as means $\pm$ S.E.M. ($n=5-7$). * $P \leq 0.05$ compared with LC.

the observed alterations in the maternal intrauterine environment and the resultant fetal programming effects could also be due to the reduced availability of carbohydrate-derived calories in the HF diet (due to increases in the fat-derived calories). It extends the findings from fetal pancreatic insulin hypersecretion in the previous study to altered signaling in the hypothalamus of term fetuses of female rats fed a high-fat diet. This demonstrates that altered hormonal and metabolic milieu in obese mothers may play a significant role in establishing altered metabolic programming in the fetal period compounding postnatal effects. The study shows that HF fetuses are hyperleptinemic and hyperinsulinemic and points to the presence of abnormalities in the signal transduction pathways of leptin and insulin in the hypothalamus of FHF-diet fed female rats. This is associated with increased levels of NPY and AgRP mRNAs in the hypothalamus which are key appetite-regulating neuropeptides in the postnatal period.

Our findings of hyperinsulinemia in the previous and current studies in fetuses exposed to a hyperinsulinemic/obese intrauterine environment in the HF female rats are closely related to findings of other investigators in various models of overnutrition during early periods of life. It has been shown that offspring of women with gestational diabetes have high insulin levels perinatally which continue into adult life (Kohlhoff & Dorner 1990, Dorner & Plagemann 1994). Similarly, rats reared in smaller litters also display hyperinsulinemia in early postnatal life (Kohlhoff & Dorner 1990, Dorner & Plagemann 1994, Plagemann *et al.* 1999b). Our earlier studies showed that HF fetal islets displayed a hypersecretory capacity which probably contributed to the observed fetal hyperinsulinemia (Srinivasan *et al.* 2006). It has been reported that NPY and AgRP neurons stimulate pancreatic insulin secretion (Szabo & Szabo 1983). A plausible reason for the observed fetal hyperinsulinemia in term HF fetuses could be the observed increases in the mRNA levels of these neuropeptides in term fetal HF hypothalami. Rats reared in small litters and the offspring of diabetic women have been observed to be hyperleptinemic while those raised in large litters or exposed to maternal undernutrition have hypoleptinemia (Davidowa & Plagemann 2000). Since rat fetuses do not possess much s.c. fat at birth (Kawai *et al.* 1997, Amico *et al.* 1998), increased adipogenesis is not likely to be the mechanism of hyperleptinemia. However, it has been shown that maternal leptin can be transported across the placenta and is a significant source of fetal leptin (Smith & Waddell 2003). Therefore, we postulate that enhanced transplacental transport of leptin from mother to fetus may have contributed to the fetal hyperleptinemia observed in our study.

Leptin and insulin have important actions in the appetite-regulating neurons in the hypothalamus (Niswender & Schwartz 2003). Binding of leptin and insulin to OBRb and IR- β respectively stimulates downstream activity in their respective signaling pathways. This involves activation of non-receptor Janus kinases followed by autophosphorylation of the

receptor and binding of STATs. Specifically, STAT-3 isoform dimerizes and translocates to the nucleus to bind to the promoter region of the genes that regulate transcription (Fruhbeck 2006). It stimulates expression of POMC and MC4R genes (Bates & Myers 2004) while suppressing expression of AgRP/NPY (Kitamura *et al.* 2006). Similarly, activation of tyrosine kinase activity of IR- β leads to phosphorylation of IRS-2 followed by PI3K activation. This results in stimulation or repression of POMC and AgRP/NPY respectively (Plum *et al.* 2006). Also, SOCS-3 is involved in feedback inhibition of leptin receptor in response to leptin (Bjorbak *et al.* 2000) as well as targeting insulin receptor and IRS-2 for degradation (Ueki *et al.* 2004).

It has been previously reported that adult rats fed a hyperlipidemic diet display hyperleptinemia and hyperinsulinemia with resistance to their actions at the level of the hypothalamus. Specifically, there is reduced tyrosine phosphorylation of insulin receptor and IRS-2 with no change in their protein levels in the hypothalamus of adult high-fat-diet fed rats (De Souza *et al.* 2005). Also, there have been reports of decreased STAT-3 signaling in response to leptin in mice fed a high-fat diet pointing to abnormal transport as well as signal transduction (El-Haschimi *et al.* 2000). In our study, high levels of leptin and insulin in the HF fetuses in response to intrauterine environment were associated with enhanced mRNA and protein levels of their respective membrane receptors in the hypothalamus of HF fetuses. However, there was a significant decrease in the level of STAT-3 protein although its mRNA level was increased in the FHF hypothalamus. Transcriptional regulation has been considered the major point of regulation of protein production in eukaryotic cells but recent evidence brings the role of post-transcriptional processes to light. In this context, regulation of mRNA stability at cellular level is postulated to be a major control point of cellular mRNA levels (Staton *et al.* 2000). Hormones are postulated to play a major role in this process (Staton *et al.* 2000). Therefore, we suggest that altered hormonal milieu in the hypothalamus of HF fetuses may be the potential reason for observed discrepancy between mRNA and protein levels of STAT-3. Also, the protein level of IRS-2 in FHF hypothalamus was significantly decreased. This indicates impaired post-receptor signaling at the level of STAT-3 and IRS-2. SOCS-3 is a major endogenous feedback inhibitor of STAT-3 (Bjorbak *et al.* 2000) and IRS-2 (Ueki *et al.* 2004) but we did not find an increase in SOCS-3 mRNA or protein levels in our study.

The findings of increased NPY/AgRP in the HF rat fetus are in parallel with observations of other investigators in adult offspring exposed to maternal overnutrition (Muhlhausler *et al.* 2006). However, POMC and MC4R levels were increased. This is consistent with observations from another study where acute and chronic leptin treatment increased or had no effect on POMC level respectively (Proulx *et al.* 2002). Leptin and insulin normally act via STAT-3 and IRS-2 respectively to inhibit AgRP/NPY neurons while stimulating POMC neurons (Porte *et al.* 2002, Morton *et al.* 2005, Xu

et al. 2005). Our findings of increased mRNA levels of AgRP and NPY with decreased levels of STAT-3 and IRS-2 proteins are suggestive of impairment of leptin and insulin signaling. In adult rats exposed to maternal overnutrition or overfed postnatally, resistance to actions of insulin and leptin results in abnormal appetite regulation, overweight and glucose intolerance.

Although leptin and insulin do not affect appetite in the fetal period, hyperleptinemia and hyperinsulinemia with decreased levels of STAT-3 and IRS-2 may have different implications for the developing fetus. Leptin has been shown to promote growth of arcuate neurons secreting NPY and AgRP preferentially in the neonatal period (Bouret *et al.* 2004, Horvath & Bruning 2006). Also, it has been shown that perinatal hyperinsulinism in subcutaneously insulin-treated neonatal rats and offspring of gestational diabetic rats show hypoplasia and hypotrophy of the ventromedial nucleus, which is an important hypothalamic nucleus involved in inhibition of appetite and pancreatic insulin secretion (Dorner & Plagemann 1994, Plagemann *et al.* 1999a). Thus, it has been suggested that perinatal hyperinsulinism and hyperleptinism may be neuroendocrine teratogenic risk factors. However, substrates involved in mediating these effects are still unknown. It has been recently shown that brain IRS-2 null mice exhibited abnormal neuron development and brain growth (Schubert *et al.* 2003) and it is predominantly distributed in brain areas regulating energy homeostasis (Pardini *et al.* 2006). Also, STAT-3 is known to maintain neural precursor cells in the mouse embryonic neocortex (Yoshimatsu *et al.* 2006). Therefore, it is likely that abnormal signaling of leptin and insulin via STAT-3 and IRS-2 respectively in term rat fetuses may lead to abnormal development of neurons and their projections leading to altered levels of appetite-regulating neuropeptides thus predisposing them to abnormal appetite regulation in the postnatal period. The possibility that altered post-transcriptional processes may be operational in decreased STAT-3 production will need to be explored since SOCS-3 is traditionally considered to be involved in impaired signaling was not increased here.

In summary, this study shows that abnormalities of leptin and insulin signaling are present in the critical period of fetal development at the levels of STAT-3 and IRS-2. This is associated with altered regulation of levels of appetite-regulating neuropeptides and may have a role in abnormal development of hypothalamic energy regulating neurons since these proteins are involved in neuronal development. This may provide an explanation to the observed phenotype of obesity and metabolic syndrome in the adult offspring of obese females. Therefore, this study points to a potential intrauterine programming effect of maternal high-fat diet with implications for abnormal appetite regulation in offspring in the postnatal period.

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.

Funding

This work was supported by National Institute of Health grant (DK-061518).

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Received in final form 5 December 2008

Accepted 9 December 2008

Made available online as an Accepted Preprint

11 December 2008