A high-carbohydrate diet in the immediate postnatal life of rats induces adaptations predisposing to adult-onset obesity

Malathi Srinivasan*, Paul Mitrani*, Gigani Sadhanandan, Catherine Dodds, Suhad Shbeir-ElDika, Shanthie Thamotharan1, Hussam Ghanim2, Paresh Dandona2,3, Sherin U Devaskar1 and Mulchand S Patel

Department of Biochemistry, School of Medicine and Biomedical Sciences, University at Buffalo, State University of New York at Buffalo, 140 Farber Hall, 3435 Main Street, Buffalo, New York 14214, USA
1Department of Pediatrics, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California 90095, USA
2Department of Medicine, School of Medicine and Biomedical Sciences, University at Buffalo, State University of New York, Buffalo, New York 14214, USA
3Diabetes and Endocrinology Center of Western New York, Kaleida Health, Buffalo, New York 14209, USA
Note: Correspondence should be addressed to M S Patel; Email: mspatel@buffalo.edu

*MSrinivasan and P Mitrani contributed equally to this work

Abstract

Newborn rat pups artificially raised on a high-carbohydrate (HC) milk formula are chronically hyperinsulinemic and develop adult-onset obesity. As HC rats display aberrations in body weight regulation, hypothalamic adaptations predisposing to obesity have been investigated in this study. The artificial rearing of neonatal rat pups on the HC milk formula resulted in significant increases in the mRNA levels of neuropeptide Y, agouti-related polypeptide, and galanin in the hypothalamus of 12-day-old HC rats. Simultaneously, decreases in the mRNA levels of POMC, melanocortin receptor-4, cocaine- and amphetamine-regulated transcript, and corticotrophin-releasing factor were observed in the hypothalamus of these rats. These changes persisted in 100-day-old HC rats despite weaning onto a rodent diet on postnatal day 24. Marked hyperphagia and increased body weight gain were observed in the post-weaning period. The mRNA levels and protein content of insulin receptor β (IR-β) and leptin receptor (long form) showed significant decreases in the hypothalamus of both 12- and 100-day-old HC rats. Further investigation of insulin signaling in the hypothalamus of HC rats indicated significant decreases in the proximal signaling components (insulin receptor substrate proteins 1 and 2 and phosphotyrosine-linositol 3-kinase) in 100-day-old HC rats. These results suggest that hypothalamic neuropeptides respond to the increased carbohydrate availability with associated hormonal alterations during the period of dietary modulation and that these adaptations by persisting in the post-weaning period predispose the HC rats for adult-onset obesity. Journal of Endocrinology (2008) 197, 565–574

Introduction

Overweight now prevalent in epidemic proportions especially in Western societies is a source of concern because it is a risk factor for several adult-onset metabolic diseases, the most important amongst them being type 2 diabetes and cardiovascular diseases (Mokdad et al. 2001, Zimmet et al. 2001). In addition to genetics, dietary habits, and sedentary lifestyle, altered nutritional experiences in early life via malprogramming effects in target organs also contribute to the etiology of obesity. Metabolic malprogramming effects occur due to the overlap of the critical window of developmental plasticity of target organs with the period of nutritional modification (Lucas 1991). In the long term, these malprogramming effects have deleterious consequences. Several retrospective epidemiological studies and investigations in laboratory animal models support this concept (Barker 1995, Godfrey & Barker 2000, Van Assche et al. 2001, Bertram & Hanson 2001, Petry et al. 2001, Holemans et al. 2003).

Many previous studies focusing on the long-term consequences of perinatal nutritional interventions were limited to fetal development in response to altered maternal nutritional status. Examples of animal models include total calorie restriction (Garofano et al. 1998, Thamotharan et al. 2005), protein restriction (Berney et al. 1997, Latorraca et al. 1998, Bennis-Taleb et al. 1999, Oznane & Hales 1999), and gestational diabetes (Van Assche et al. 2001). These studies linked aberrant fetal development and long-term malprogrammed consequences in various target organs such as pancreatic islets, hypothalamus, liver, muscle, and adipose tissue (Berney et al. 1997, Garofano et al. 1998, Latorraca et al. 1998, Bennis-Taleb et al. 1999, Oznane & Hales 1999, Van Assche et al. 2001). The long-term consequences of an altered nutritional experience in the immediate postnatal life have...
been demonstrated by adjusting the litter size in rats (McCance 1962, Li et al. 2002).


The hypothalamus plays a critical role in the regulation of energy homeostasis by receiving and processing afferent information and orchestrating energy homeostasis (Horvath 2005). The central regulation of appetite and energy expenditure is dependent on interactions between specific neuropeptides. Neuropeptide Y (NPY), agouti-related protein (AgRP), and galanin (GAL) are important orexigenic neuropeptides that promote food intake and reduce energy expenditure (Kalra et al. 1999, Leibowitz & Wortley 2004). The anorexigenic neuropeptides α-melanocortin-stimulating hormone (α-MSH), one of the products of the pro-opiomelanocortin (POMC) gene, is the primary neuropeptide responsible for the regulation of food intake by the central melanocortin system and functions by binding to the melanocortin receptors (MCR), the important one being MC4-R (Schwartz et al. 2000, Cowley et al. 2001). AgRP functions as the endogenous competitive antagonist of the MC4-R. Cocaine- and amphetamine-regulated transcript (CART) and corticotropin-releasing factor (CRF) are other important anorexigenic signals that inhibit food intake and stimulate energy expenditure via activation of the sympathetic nervous system (Horvath et al. 2004). The two important afferent signals involved in the long-term regulation of food intake and energy balance are insulin and leptin and they transduce their effects by binding to their cognate receptors in the hypothalamus (Schwartz et al. 2000, Cowley et al. 2001). Hypothalamic neuronal development initiated during fetal life extends into the immediate postnatal period (Pozzo-Miller & Aoki 1992). The timing of artificial rearing of newborn rats on the HC milk formula (starting on postnatal day 4) overlaps with the postnatal development of hypothalamic neurons. Based on the above observation, we hypothesize that hypothalamic malprogramming predisposing to later onset of obesity occurs immediately in neonatal HC rats in response to the HC dietary modification as observed in the case of pancreatic islets and that these early adaptations persist into adulthood causing obesity in these rats.

Materials and Methods

The antibodies for insulin receptor β-subunit (IR-β) (Cell Signaling Solutions, Lake Placid, NY, USA), insulin receptor substrate-1 (IRS-1), IRS-2 and phosphatidylinositol 3-kinase (PI3K) (Upstate Cell Signaling, Chicago, IL, USA), and leptin receptor long form (OB-Rb; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were obtained from vendors as indicated. The secondary antibodies bovine anti-goat horseradish peroxidase (HRP) conjugate and goat anti-rabbit HRP conjugate were obtained from Santa Cruz Biotechnology and Bio–Rad Laboratories respectively. The chemiluminescence reagent was obtained from Perkin–Elmer (Boston, MA, USA). All chemicals used were of analytical grade.

Artificial rearing of rat pups

The Institutional Animal Care and Use Committee approved all animal protocols. Pregnant Sprague–Dawley rats purchased from Zivic Miller Laboratories (Zellenope, PA, USA) were housed individually with access to standard rodent diet (16% Protein Rodent Diet, Harlan Teklad, Madison, WI, USA; % weight distribution: carbohydrate 61%, protein 16%, fat 4%, ash 6%, fiber 4%, and moisture 10%) and water made available ad libitum under controlled conditions of temperature (25 ± 2 °C) and a 12 h light (0600–1800 h):12 h darkness cycles. The artificial rearing technique has been described in detail previously (Hiremagalur et al. 1992, 1993). Briefly, intragastric cannulas were introduced into 4-day-old neonatal rat pups under mild anesthesia and these pups were artificially reared on the HC or HF milk formula. The milk formulae were delivered at a rate of 0.45 kcal/g body weight/day for 20 min every 2 h (one-twelfth of daily allotment). The calorie distribution of macronutrients in HC milk formula was 56% carbohydrate, 24% protein, and 20% fat and in both rat milk and HF milk formula was 8% carbohydrate, 24% protein, and 68% fat. Rat pups reared by their dams (mother-fed; MF) served as the control group. In order to establish that the artificial rearing protocol per se did not influence the development of the HC phenotype, rat pups were artificially raised on the HF milk formula, the macronutrient composition of which was similar to that of rat milk. In addition to the MF control group, the HF group was used as the experimental control group for most of the investigations reported in this study. For one set of experiments, HC and age-matched MF and HF rats (both male and female pups were used) were killed on postnatal day 12. For studies on 100-day-old rats only male pups from the three groups were used. For these studies, the HC and HF rat pups continued to receive their respective milk formula until postnatal day 24 when they were weaned onto a standard rodent diet and water ad libitum and killed on postnatal day 100. MF pups were also weaned onto a standard rodent diet and water ad libitum and killed on postnatal day 100.
diet on postnatal day 24. Trunk blood was collected from both 12- and 100-day-old rats, plasma separated, and stored frozen until analysis for insulin and leptin concentrations. Food intake and body weights were recorded periodically in the post-weaning period.

After death, the brain was dissected out from the skulls of 12- and 100-day-old rats. A region defined dorsally by the thalamus, rostrally by the optic chiasm, and caudally by the mamillary bodies was excised and the hypothalami were snap-frozen in liquid nitrogen and stored at −80°C as described previously (Mantzoros et al. 1998). Plasma insulin and leptin concentrations were measured by RIA using commercially available kits as per the manufacturer’s instructions (Linco Research, St Louis, MO, USA). Hypothalamic NPY content was measured by RIA using rabbit anti-rat NPY antibody as described previously (Singh et al. 1997).

Real-time PCR
RNA was isolated from the hypothalamus of 12- and 100-day-old MF and HC rats using the TRIzol reagent–phenol–chloroform procedure (Gibco BRL). Total RNA was quantified and mRNA samples were reverse-transcribed into cDNAs by using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions. mRNA levels of NPY, AgRP, GAL, POMC, MC4-R, CART, CRF, IR-β, and OB-Rb were measured via real-time RT-PCR using the iCycler system (Bio-Rad). Primer sequences, which were designed to span at least one exon–exon junction of the target mRNA in order to prevent amplification of contaminating genomic DNA, are described in Table 1. The mRNA levels detected by SYBR Green (Bio-Rad) according to the manufacturer’s instructions. The mRNA levels were normalized to 18S mRNA levels (QuantumRNA Classic II 18S Internal Standard, 324 bp; Ambion, Inc., Austin, TX, USA). PCR efficiency was examined by serially diluting the template cDNA, and melting curve data were collected to assess PCR specificity. Each cDNA sample was run in triplicate and a corresponding mRNA sample that had not been subjected to reverse transcription was included as a negative control in each run. The relative mRNA levels were calculated according to the comparative ΔΔCt method.

Western blot analysis
Hypothalami obtained from 100-day-old MF and HC rats were homogenized in a solubilization buffer (50 mM HEPES, 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM NaVO4, 10 mM Na2P2O7, 10 mM NaF, 2 mM EDTA, 1% Igepal, 10% glycerol, 2 μg/ml aprotime, antipain, leupeptin, and pepstatin, 1-5 μg/ml benzamidine, and 34 μg/ml phenylmethylsulphonyl fluoride). The homogenates were centrifuged and the protein content in the supernatant was measured by the BCA method (Bio-Rad). Equal amounts of protein for each preparation was added to an equal volume of 2× Laemmli sample buffer and separated by sodium dodecyl sulfate-PAGE. The protein was transferred to a nitrocellulose membrane and blocked with 5% skimmed milk in 0·1% Tween-tris-buffered saline for 2 h at 4°C, followed by incubation with the specific antibodies (dilutions: OB-Rb, 1:100; IR-β, 1:1000; IRS-1and IRS-2, 1:2000; PI3K, 1:2000) in 3% dry milk in 0·1% Tween-tris-buffered saline overnight at 4°C. The membranes were then washed and incubated with goat-anti-rabbit or bovine anti-goat IgG-HRP in Tween-tris-buffered saline with 3% dry milk for 2 h at room temperature. Protein bands were visualized using chemiluminescence (Perkin–Elmer, Wellesley, MA, USA), and densitometry analysis was performed using the Quantity One program.

Statistical analysis
Results are expressed as means ± s.e.m. of six to eight animals in each experiment. One-way ANOVA was used for comparisons

Table 1 Primers for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY</td>
<td>5’-AGA GAT CCA GCC CTG AGA CA-3’</td>
<td>3’-CGA TGC ACT GGC TGA CAG AA-5’</td>
</tr>
<tr>
<td>AgRP</td>
<td>5’-GAG AGA CAC AAG GGA AAT GG-3’</td>
<td>3’-CGA TGC ACT GGC TGA CAG AA-5’</td>
</tr>
<tr>
<td>GAL</td>
<td>5’-CGA CGG AGG AGA AAA GAG GT-3’</td>
<td>3’-CGA TGC ACT GGC TGA CAG AA-5’</td>
</tr>
<tr>
<td>POMC</td>
<td>5’-CGA CGG AGG AGA AAA GAG GT-3’</td>
<td>3’-CGA TGC ACT GGC TGA CAG AA-5’</td>
</tr>
<tr>
<td>MC4-R</td>
<td>5’-GAG ACT TGG CCG TAC TTC TCT-3’</td>
<td>3’-GCA AGG AGG AGA AAA GAG GT-3’</td>
</tr>
<tr>
<td>CART</td>
<td>5’-GAT AGC TGG TGC CTG AGT GA-3’</td>
<td>3’-GCA AGG AGG AGA AAA GAG GT-3’</td>
</tr>
<tr>
<td>CRF</td>
<td>5’-GAG ACT TGG CCG TAC TTC TCT-3’</td>
<td>3’-GCA AGG AGG AGA AAA GAG GT-3’</td>
</tr>
<tr>
<td>IR-β</td>
<td>5’-ATT TGG GTG TAG GGG ATG TGT TCA-3’</td>
<td>3’-GCA AGG AGG AGA AAA GAG GT-3’</td>
</tr>
<tr>
<td>Ob-Rb</td>
<td>5’-CCT GAA ACA TTT GAG CAT TTT-3’</td>
<td>3’-GCA AGG AGG AGA AAA GAG GT-3’</td>
</tr>
</tbody>
</table>
between the difference of the means of MF, HF, and HC groups of rats followed by post hoc analysis using the Student–Newman–Keul’s test. Statistical analysis of the differences between the means of only MF and HC groups was done using Student’s t-test. \( P<0.05 \) was considered significant.

**Results**

Artificial rearing of 4-day-old rat pups on the HC milk formula (56% of total calories derived from carbohydrate in comparison with 8% of calories from carbohydrate in the HF milk formula or rat milk) had no effect on the body weight of 24-day-old HC rat pups (day of weaning) compared with age-matched HF and MF rats (Fig. 1A). MF, HF, and HC male rats were weaned onto a standard rodent laboratory chow on postnatal day 24. The body weights of HC male rats were significantly increased from postnatal day 40 onwards compared with age-matched HF or MF male rats (Fig. 1A). On postnatal day 100, HC male rats were approximately 25% heavier than the age-matched HF and MF rats (Fig. 1A). The food intake was measured on a weekly basis from postnatal day 30 onwards. HC rats consumed significantly more feed compared with age-matched HF and MF rats at all the time points investigated (Fig. 1B). In order to estimate the correlation between the body weight and food intake data, the ratio of the feed efficiency (body weight gain per week/g feed consumed per week) for HC/MF and HF/MF assuming feed efficiency as 1 for MF was determined. It was observed that there was a significant increase in the mean feed efficiency for the entire period from postnatal day 30 to postnatal day 99 (approximately 50%; \( P<0.05 \)) for the HC group (HC/MF, 1.52±0.24) compared with the control group (HF/MF, 0.993±0.03). A significant increase in plasma insulin levels was observed in 12-day-old HC rats compared with age-matched HF and MF rats (Fig. 2A). Hyperinsulinemia persisted in 100-day-old HC rats compared with age-matched HF and MF rats despite weaning HC rats onto laboratory chow on postnatal day 24 (Fig. 2A). The results

![Figure 1](A) Body weights of HC and age-matched MF and HF male rats from postnatal days 30–100. (B) The mean weekly food intake of HC and age-matched MF and HF male rats in the post-weaning period. Results are means ± S.E.M. of eight animals/group. *\( P<0.05 \) compared with HF and MF (ANOVA/Student–Newman–Keul’s test).

![Figure 2](A) Plasma insulin levels in 12- and 100-day-old HC and age-matched HF and MF rats. (B) Plasma leptin levels in 12- and 100-day-old HC and age-matched HF and MF rats. Results are means ± S.E.M. of six animals/group. *\( P<0.05 \) compared with HF and MF (ANOVA/Student–Newman–Keul’s test).
obtained on body weights and plasma insulin levels in this study are in agreement with those published earlier from this laboratory (Hiremagalur et al. 1993, Vadlamudi et al. 1993, 1995). The plasma leptin levels were significantly lower in 12-day-old HC rats and significantly increased in adult HC rats compared with age-matched HF and MF rats (Fig. 2B). Although plasma leptin levels were reduced in 12-day-old HC rats, our earlier results showed that there were no significant differences in the body weights of MF, HF, and HC rats during the suckling period (Hiremagalur et al. 1993).

**Early effects of the HC dietary modification in the immediate postnatal period**

In order to determine whether the HC dietary modification in the immediate postnatal life of newborn rats induced alterations at the level of signals involved in appetite regulation, mRNA levels of relevant neuropeptides/receptor genes were determined by real-time PCR analysis in total RNA preparations obtained from whole hypothalami of 12-day-old HC, HF, and MF rats. The mRNA levels of NPY and AgRP genes were significantly increased (more than twofold) in whole hypothalamic extracts of 12-day-old HC rats compared with their levels in the hypothalami of age-matched HF and MF rats (Fig. 3A). The mRNA levels of anorexigenic neuropeptide genes such as POMC (precursor of α-MSH), MC4-R, CART, and CRF were significantly reduced in whole hypothalami extracts obtained from 12-day-old HC rats compared with their levels in the hypothalami extracts obtained from age-matched MF rats (Fig. 4A). Additionally, there was a significant decrease in the mRNA levels of both the IR-β and OB-Rb genes (approximately 50 and 60% respectively) in 12-day-old HC rats compared with the corresponding mRNA level in age-matched MF rats (Fig. 4A). NPY concentrations in hypothalamic extracts obtained from 12-day-old HC and age-matched MF pups were quantified by RIA. NPY content was significantly increased in the hypothalamic extract of 12-day-old HC rats compared with age-matched MF rats (Fig. 5).

**Long-term effects in adult animals**

To determine whether the changes observed in the hypothalami of 12-day-old HC rats persisted into adulthood, mRNA levels of neuropeptide/receptor genes were determined by real-time PCR analysis in total RNA prepared from whole hypothalami of 100-day-old HC, HF, and MF male rats. A marked increase in the mRNA levels of NPY and AgRP genes (approximately a more than twofold change) was observed in the hypothalami of adult HC rats compared with their levels in the hypothalami of age-matched HF and MF rats (Fig. 3B). As observed for 12-day-old rats, significant decreases in the mRNA levels of POMC, MC4-R, CART, and CRF genes were observed in the hypothalami of 100-day-old HC rats compared with age-matched HF and MF rats (Fig. 3B). When compared with age-matched MF rats, hypothalamic mRNA levels of GAL was significantly higher in adult HC rats (Fig. 4B). Additionally, significant decreases in the mRNA levels of IR-β and OB-Rb genes (approximately 20 and 25% respectively) compared with the mRNA levels in age-matched MF rats (Fig. 4B) were observed. The total NPY content in hypothalamic extracts as determined by ELISA showed a significant increase in its content in 100-day-old HC rats (Fig. 5).

Protein levels of the insulin and leptin receptors were determined by western blot analysis of hypothalamic extracts of 100-day-old MF and HC rats. A significant decrease (approximately 40%) in the protein content of IR-β was observed in hypothalamic extracts of 100-day-old HC rats.
compared with age-matched MF rats (Fig. 6). Additionally, the OB-Rb protein content was also markedly reduced (approximately 50%) in hypothalamic extracts of 100-day-old HC rats. In order to determine whether insulin signaling was reduced in the hypothalamus of 100-day-old HC rats, protein content of IRS-1, IRS-2, and PI3K, the proximal components of the insulin signaling cascade were determined by western blot analysis in hypothalamic extracts of adult HC rats. As seen in Fig. 6, the protein content of IRS-1, IRS-2, and PI3K were reduced by approximately 25% compared with their levels in the hypothalamus of age-matched MF rats.

Discussion

The results from the present study indicate that the hypothalamus is vulnerable to metabolic programming effects of the high carbohydrate content of the milk formula received in the immediate postnatal life (suckling period) due to the overlap of the postnatal neuronal developmental period with the HC dietary modification. The observed alterations in 12-day-old HC rats in the expression of the components of the hypothalamic mechanism regulating body weight homeostasis suggest a predisposition to hyperphagia. This is supported by the observed increases in food intake of the HC rats beginning immediately in the post-weaning period. Additionally, changes similar to those observed in the hypothalamus of 12-day-old rats were observed in the hypothalamus of adult rats resulting in adult-onset obesity. Our earlier findings showed that metabolic programming effects occurred in pancreatic islets due to the HC dietary modification and that these early malprogrammed effects persist beyond the period of dietary modification (Vadlamudi et al. 1993, Aalinkeel et al. 1999, 2001). Taken together, these results indicate that malprogramming of both islets and hypothalamus occurring during the period of dietary modification predisposes the HC rats to the HC phenotype of chronic hyperinsulinemia and adult-onset obesity.

One plausible reason for the observed increases in the mRNA levels of the orexigenic neuropeptides (NPY, AgRP, and GAL) and concomitant reduction in the mRNA levels of the anorexigenic neuropeptides and receptors (POMC, MC4-R, CART, CRF, IR-β, and OB-Rb) in the hypothalamus of 12-day-old HC rats could due to the switch in the major source of calories (from fat in rat milk to carbohydrate in the HC milk formula). The glucose-oxidizing pathway is not completely developed in the rat brain during the early suckling period due to low levels of the pyruvate dehydrogenase complex activity (Cremer & Teal 1974, Land et al. 1977) and the developing rat brain depends at least in part, on ketone bodies as a source of energy (Hawkins et al. 1971). The HC milk formula has a considerably reduced fat content (only 20% of total calories) compared with rat milk (68% of total calories). Furthermore, the immediate onset of hyperinsulinemia (within 24 h) compared with age-matched MF rats (Fig. 6). Additionally, the OB-Rb protein content was also markedly reduced (approximately 50%) in hypothalamic extracts of 100-day-old HC rats. In order to determine whether insulin signaling was reduced in the hypothalamus of 100-day-old HC rats, protein content of IRS-1, IRS-2, and PI3K, the proximal components of the insulin signaling cascade were determined by western blot analysis in hypothalamic extracts of adult HC rats. As seen in Fig. 6, the protein content of IRS-1, IRS-2, and PI3K were reduced by approximately 25% compared with their levels in the hypothalamus of age-matched MF rats.

Figure 4 Quantitative real-time PCR analysis of GAL, IR-β, and OB-Rb mRNAs in hypothalami obtained from (A) 12-day-old and (B) 100-day-old HC and age-matched MF rats. The threshold cycle values were normalized using 18S as an internal standard and fold-difference was calculated using the comparative Ct method (ΔΔCt). Individual samples were run in triplicate and values represent the means ± S.E.M. of six independent experiments. *P<0.05 compared with MF (Student’s t-test).

Figure 5 Hypothalamic NPY concentrations in 12- and 100-day-old MF and HC rats as determined by RIA. The results are means ± S.E.M. For the 12-day-old rats, n≥15 and for 100-day-old rats, n≥8. *P<0.05 compared with MF (Student’s t-test).
markedly reduces the ability of liver to convert fatty acids to ketone bodies resulting in a marked reduction in the availability of ketone bodies in blood for brain energy production (Haney & Patel 1985, Haney et al. 1986). Until the glucose metabolizing system is fully developed in the brain, the deficit in the availability of ketone bodies as a source of energy for the brain in the HC rat pups may be interpreted as a sign of energy deficit by specific neurons involved in energy homeostasis thereby promoting adaptations favoring increased food intake.

HC rats begin to consume significantly increased amounts of feed beginning from the immediate post-weaning period. Although the observed changes in the mRNA levels of neuropeptides and receptor genes in the hypothalami of HC rats are not very large (but statistically significant), these results are consistent with the observed increases in food intake and body weight in HC rats compared with age-matched MF and HF control rats in the post-weaning period. Both NPY and AgRP are potent stimulators of food intake and decrease energy expenditure during periods of negative energy balance (Horvath et al. 2004). AgRP functions as an endogenous antagonist of α-MSH by binding to MC4-R (Horvath et al. 2004). The dominant effect of AgRP in the HC rats is indicated by the observed decrease in the mRNA levels of MC4-R as well as POMC in HC rats. In the face of reduced availability of receptors for which both α-MSH and AgRP compete, the increased levels of AgRP (along with decreased levels of POMC) may confer a distinct advantage for the effects propagated by this neuropeptide. The anorexigenic neuropeptides POMC, CART, and CRF are components of the central melanocortin system that reduce food intake and promote energy expenditure by stimulating the sympathetic nervous system (Horvath et al. 2004). The observed reduction in the mRNA levels of these factors in HC rats suggests aberrations in appetite regulation leading to hyperphagia and increased body weight gain.

It is of interest to note that both central and peripheral systems may be responding to the HC dietary modulation and hence the observed phenotype could be the result of cross-talk between these systems. Due to the complexity of the whole-body physiological response it is a very difficult task to identify a cause–effect relationship for the observed phenomenon. However, the altered hormonal environment in neonatal HC rats could be an important cue for the development of the predisposition for adult-onset obesity. Although in adulthood increased levels of insulin and leptin are signs of satiety, during early periods in life both these hormones function as trophic factors. Insulin has been shown to be a potent modulator of hypothalamic neuronal differentiation and maturation (Dorner & Plagemann 1994). When rats were intrahypothalamically treated with insulin on their second or eighth day of postnatal life, beginning by the age of 3 weeks these rats became overweight which persisted throughout life accompanied by impaired glucose tolerance and chronic hyperinsulinemia (Plagemann et al. 1992a,b). In the overnourished (small litter size) suckling rats characterized by hyperinsulinemia (and hyperleptinemia), increases in the number of NPY neurons in the arcuate nucleus (ARC) and increased concentrations of NPY in the paraventricular medial nucleus (PVN) were observed (Plagemann et al. 1999b). The administration of insulin to the third ventricle of the brain resulted in the downregulation of NPY in the ARC (Schwartz et al. 1992, Sipols et al. 1995) and increased expression of POMC (Benoit et al. 2002). Studies on the brain-specific IR−−deficient mice that displayed obesity and impaired fertility assigned an important role for brain IR signaling in the central regulation of body weight homeostasis and reproduction (Bruning et al. 2000).

The neonatal leptin surge observed in rodents has been implicated to function as a signal for neuronal development (Ahima et al. 1999, Stepan & Swick 1999, Ahima & Hileman 2000). Bouret et al. (2004a,b) showed that leptin is essential for the development of neuronal projections from the ARC to the paraventricular nucleus in the immediate postnatal period. Due to the absence of leptin during this critical period in the ob/ob mice, the neuronal projections from the ARC were compromised and this defect was indicated as being the cause for the development of obesity in these mice (Bouret et al. 2004b). Several abnormalities in the brain such as reduced...
brain DNA content, disruption of neuronal projections, and reduction in cortical brain volume have been reported in the ob/ob mice that lack endogenous leptin (Bereiter & Jeanrenaud 1979, Steppan & Swick 1999, Bouret et al. 2004b). In the ob/ob mouse hypothalamus, the mRNA levels of NPY were increased and those of POMC were decreased and leptin treatment of these mice normalized the amounts of these mRNAs (Friedman & Halaas 1998, Mistry et al. 1999). In the leptin receptor-deficient Zucker rats, NPY mRNA levels were significantly increased as early as postnatal day 2 (Kowalski et al. 1999).

In the context of hormonal environment, neonatal HC rats share similarities with both the small-litter rat model (hyperinsulinemia) and the ob/ob mouse (hyperleptinemia). Plasma insulin levels are markedly increased within 24 h of the pups receiving the HC milk formula and plasma leptin levels are significantly reduced in 12-day-old HC rats. As critical periods of neuronal differentiation and maturation continue into the immediate postnatal life in rats (Pozzo-Miller & Aoki 1992, Grove et al. 2003), abnormal levels of plasma insulin and leptin in HC rats during this period may underlie an altered development of the hypothalamic melanocortin system predisposing for hyperphagia and increased body weight gain in these rats. The observed increases in the mRNA levels of NPY, AgRP, and GAL and decreases in the mRNA levels of POMC, MC4-R, CART, CRF, IR-β, and OB-Rb in the hypothalamus of 12-day-old HC rats suggest such a possibility. The obese phenotype of adult HC rats is possibly due to the persistence of the early responses to the HC milk formula (as observed in 12-day-old HC rats) although the contribution of other factors to adult-onset obesity cannot be excluded.

IRs are particularly highly expressed in the ARC where they are co-expressed with NPY, AgRP, and POMC. Hormone binding to the IR results in rapid autophosphorylation of the receptor followed by tyrosine phosphorylation of IR substrate proteins, inducing an activation of the downstream pathways such as the MAPK and PI3K cascades (White 2003). The signaling cascade involved in leptin action in the hypothalamus has been documented (Munzberg & Myers 2005). Insulin and leptin resistance in the brain are commonly observed in obese conditions (Schwartz & Porte 2005). In the hypothalamus of 100-day-old HC rats, the observed significant decreases in the protein content of IR-β, IRS-1, IRS-2, and PI3K suggest a state of insulin resistance in the hypothalamus. Leptin resistance is indicated by reduced levels of the leptin receptor. De Souza et al. (2005) showed that a local proinflammatory response induced by a HF diet in the hypothalamus resulted in impaired anorexigenic insulin signaling, which predisposed these rats for hyperphagia and overweight. The observed insulin and leptin resistance in the hypothalamus of 100-day-old HC rats may be important for the onset of obesity in these rats.

The only other model that explores the consequences of an altered dietary experience in the sucking period only is overfeeding during this period achieved by reducing the litter size to three pups per dam (the small litter model). Pups raised in the small litter were heavier and hyperinsulinemic and hyperleptinemic during the sucking period in response to overfeeding and continued to be obese in adulthood (Faust et al. 1980, Plagemann et al. 1992b, 1999b). Hypothalamic neurons in these adult rats responded differentially to various stimuli and these effects were postulated to be in response to overfeeding that occurred during the sucking period (Daviddowa & Plagemann 2001, 2004, Davidowa et al. 2003). Schmidt et al. (2001) showed that prophylactic leptin treatment did not prevent hyperinsulinemia and increased the fat deposition in small litter pups and that selective hyperleptinemia in normal rat pups did not trigger leptin resistance and obesity. Based on these observations, the authors have suggested that rather than hyperleptinemia per se other factors associated with over nourishment in the small litter pups (for example, hyperinsulinemia) may play a role in the development of obesity in the small litter rats (Schmidt et al. 2001). A comparison between the small litter and the HC rat models indicates that increased plasma concentrations of insulin during the period of dietary treatment (suckling period) were observed in both the models. In contrast to the small litter pups, plasma leptin levels were significantly reduced in HC rats during the suckling period and there were no changes in their body weight gains during this period (Hiremagalur et al. 1993). Although Schmidt et al. (2001) have suggested that hyperleptinemia per se does not play a role in the development of obesity in small litter rat model, in HC rats the reduced levels of leptin along with hyperinsulinemia may play a role in the malprogramming of the hypothalamic melanocortin system (as indicated by the studies of Bouret et al. 2004b)). The observation of a similar metabolic phenotype in adult HC and small litter rats (hyperinsulinemic, hyperleptinemic, obesity) underscores the long-term effects of altered hormonal environments during critical periods of development. In the present study, we have shown that hypothalamic neuronal changes occurred in the sucking period (postnatal day 12) and persisted onto adulthood in the HC rats. In the small litter model, changes in the hypothalamus were reported at the time of weaning (postnatal days 21–24) (Plagemann et al. 1999a,b, Lopez et al. 2005).

Our earlier studies showed that rats artificially reared on a HF milk formula, the macronutrient composition of which was similar to that of rat milk, did not develop the HC phenotype of chronic hyperinsulinemia and adult-onset obesity (Hiremagalur et al. 1993, Vadlamudi et al. 1995). This study further extends the earlier observation by showing that several orexigenic and anorexigenic factors involved in energy homeostasis are not altered in the hypothalami of HF rats. This observation therefore indicates that the artificial rearing technique per se does not play a role in the malprogramming of the hypothalamus in the context of predisposition to adult-onset obesity and that the observed malprogramming effects are primarily due to the high carbohydrate content of the HC milk formula and the concomitant alterations in the hormonal profile of these rats. As discussed above, potent adaptations that strongly favor increased food intake and possibly reduced energy...
expenditure may be integrated in the brains of HC pups due to neuronal developmental plasticity. These early neuronal adaptations then become permanent and are most likely responsible for adult-onset obesity.

In the context of the increasing incidence of obesity in humans, the role of alterations in feeding practices for infants is being increasingly considered. It has been suggested that decreases in breastfeeding rates combined with early introduction of baby supplemental foods (carbohydrate-enriched) may contribute to this phenomenon. The HC rat model is a useful tool to investigate the long-term consequences of increased carbohydrate intake in the immediate postnatal life. The results presented in this study show that increased carbohydrate intake in rats during the suckling period can cause altered expression of hypothalamic neuropeptides involved in the regulation of body weight homeostasis.

Acknowledgements

This work was supported in part by the National Institutes of Health Grants DK 61518 (M S P) and HD 25024 (S U D). We would like to thank Drs Gail Willsky and Malcolm Slaughter at the School of Medicine and Biomedical Sciences, State University of New York at Buffalo for their critical reading of this manuscript. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References


Bertram CE & Hanson MA 2001 Animal models and programming of the metabolic syndrome. British Medical Bulletin 60 103–121.


Davidska H & Plagemann A 2001 Inhibition by insulin of hypothalamic VMN neurons in rats overweight due to postnatal overfeeding. Neuropeptides 34 3201–3207.


Journal of Endocrinology (2008) 197, 565–574

Downloaded from Bioscientifica.com at 07/31/2019 08:43:04PM via free access


Received in final form 17 March 2008
Accepted 3 April 2008
Made available online as an Accepted Preprint 3 April 2008