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ACTIVATION OF HYPOTHALAMIC NPY, AgRP, MC4R, AND IL-6 mRNA LEVELS IN YOUNG LEWIS RATS WITH EARLY-LIFE DIET-INDUCED OBESITY.

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Objective. Obesity represents a low-grade inflammatory disease and appears a risk factor for insulin resistance, but little is known on whether this may contribute to the development of autoimmune inflammatory diseases. The aim of this work was to study the early-life diet-induced obesity in Lewis rats which are known to be highly susceptible to autoimmunity.

Methods. Obesity was induced by reduced litter size (4 pups per litter) followed by high-fat diet (SHF rats). Control rats (8 pups per litter) were fed with standard diet (CN rats). Oral glucose tolerance test (3 g glucose per kg b.w.) was performed by intra-gastric tube in conscious rats after 12 h fast. Adipocyte size was assessed by light microscope after collagenase digestion. Hypothalamic arcuate (ARC) and paraventricular nuclei (PVN) were isolated by the punching technique. Target mRNAs were quantified by real-time PCR with the use of TaqMan probes and primers. Serum hormones (leptin, ghrelin, adiponectin, visfatin and insulin) were assayed by specific RIAs .

Results. During the experimental period SHF rats had the same body weight gain and caloric intake as CN rats. At the age of 8 weeks SHF rats showed increased epididymal fat mass and adipocyte volume, impaired glucose tolerance, normal basal fasting insulin, visfatin, and ghrelin level, but decreased adiponectin and high leptin level. In the ARC, the SHF rats showed increased expression of mRNA for orexigenic neuropeptide Y (NPY), agouti-related protein (AgRP) and anorexigenic pro-inflammatory cytokine IL-6. In the PVN, the SHF rats showed increased expression of mRNA for anorexigenic melanocortin 4 receptor (MC4R) and IL-6.

Conclusion. Overexpression of orexigenic NPY and AgRP in the ARC indicates leptin resistance in SHF rats. The increased expression of MC4R in PVN points to the activation of melanocortin anorexigenic system which, along with increased hypothalamic IL-6, might prevent the animals from overfeeding. Higher adiposity in these rats results from the high fat-diet composition and not from increased caloric intake. Furthermore, enhanced leptin production appears the main factor indicating the predisposition to autoimmunity in these overfed rats.

Keywords: Overnutrition – Glucose intolerance – Adiponectin – Leptin – Visfatin – Ghrelin – Hypothalamus – Orexigens

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Feeding behavior is regulated by the interaction of peripheral orexigenic and anorexigenic inputs with central hypothalamic regulatory neuronal pathways. The orexigenic neuropeptide Y (NPY), agouti-related protein (AgRP) as well as anorexigenic pro-opiomelanocortin (POMC – precursor of α-MSH) and cocaine- and amphetamine-regulated transcript (CART) are expressed and released from the hypothalamic arcuate nucleus (ARC) which appears the major site integrating the signals about energy status. Among them NPY/AgRP neurons are activated by ghrelin, but inhibited by leptin and insulin. In contrast, POMC/CART neurons are activated by leptin and insulin, but inhibited by ghrelin (Valassi et al. 2008). The signals generated by activated ARC neurons are transmitted to other hypothalamic areas among which the projections to the paraventricular nucleus (PVN) play a key role in maintaining energy homeostasis (Kalra et al. 1999).

In neonatal rodents, ARC projections as well as central anorexigenic activity of leptin are not yet fully functional (Proulx et al. 2002), since the hypothalamic appetite regulating circuitry mostly develops during the first three postnatal weeks (Bouret and Simerly 2004; Grove et al. 2005). Therefore, early postnatal overnutrition induced by raising the rats in small litters (SL), i.e. 2 to 4 pups with a nursing female, offers a suitable opportunity to study the development of energy dysbalance. The pups and adolescent SL rats were found leptin resistant (Dawidova and Plagemann 2000; Schmidt et al. 2001). Young Sprague-Dawley post weaning rats from SL have shown decreased hypothalamic expression of mRNA for biologically active long isoform of leptin receptor, while NPY and AgRP showed an overexpression in the arcuate nucleus as a response to endogenous hyperleptinemia (Lopez et al. 2005). Wistar rats from SL have shown a decrease of adiponectin in adipose tissue which was further intensified by high-fat diet leading consequently to insulin resistance and glucose intolerance (Boullu-Ciocca et al. 2008). On the other hand, high-fat diet obese mice or obese humans had higher circulating levels of visfatin which is known by its insulin-mimetic properties (Fukuhara et al. 2005; Berndt et al. 2005; Haider et al. 2006).

In general, obesity is considered a low-grade inflammatory condition. Nevertheless, it has not been established so far whether the overnutrition in early life may contribute to the severity of inflammatory diseases. It was reported that the inbred Lewis rat strain with high vulnerability to develop experimental autoimmune adjuvant arthritis appeared a suitable model to study this condition (Sternberg et al. 1989; Oitzl et al. 1995).

The objective of this study was to investigate the effect of overnutrition induced by the reduction of litter size followed by high-fat diet on the mechanisms involved in feeding behavior in young post-pubertal Lewis rats. We have chosen this feeding paradigm to mimic the situation of young population who is being overfed during the nursing period and subjected to continuous overfeeding by high fat consumption thereafter. We focused our attention at the parameters particularly involved in the obese state and autoimmunity such as the glucose tolerance and plasma level of insulin, ghrelin, leptin, adiponectin and visfatin. At the same time we examined also the expression of feeding behavior related neuropeptides in the ARC such as NPY, AgRP, IL-6, and in the PVN IL-6, and α-MSH receptor (MC4R) as well.

Materials and Methods

Experimental design. Male Lewis rats were adjusted to litter size of 4 (small litter) and 8 (normal litter) after birth. At the age of 21 days the pups along with their nursing females were obtained from Charles River farm (Germany) and housed in an animal room of the Department of Normal, Pathological and Clinical Physiology, Third Medical Faculty of Charles University, Prague. After weaning (day 23) the rats from the small litter (SHF, n = 12) were fed with high-fat diet D 12451 (Ssniff, Germany) with energy density of 22.7 MJ/kg (45 % calories as fat), and rats from the normal litter (CN, n = 12) were fed with standard laboratory diet ST1 (Velaz, CZ) with energy density of 14.5 MJ/kg (10 % calories as fat) for five following weeks. The animals were housed four per cage under 12/12 h light/dark cycle having free access to the respective diet and tap water; food intake and body weight was measured daily. They were treated in accordance with the national law of the Czech Republic on the use of laboratory animals, No. 167/1993 (fully compatible with European Community Council directives 86/609/EEC).

At the age of 56-days oral glucose tolerance test (OGTT) was performed in all animals after the administration of glucose by intragastric tube (3 g glucose/kg b.w. as 30 % water solution after 12 h fasting). Blood glucose was determined by Glucocard II GT-1640 glucometer (Arkray Inc., Kyoto, Japan) from tail-bled samples at 0, 30, 60, and 120 min after glucose administration.

The animals were decapitated after overnight fast on day 58 of their life. Trunk blood was collected into tubes containing EDTA, centrifuged and plasma was

stored at –30 °C. Brains were dissected, chilled in 2-methylbutane, snap frozen in dry-ice and stored at –80 °C. Left and right part of epididymal fat were dissected and weighed. Left epididymal fat was kept in plastic tubes with PBS containing 4 % glucose (Sigma-Aldrich Inc., St. Louis, MO) and the adipocyte diameter was determined within 24 hours after dissection. Right epididymal fat was snap-frozen in liquid nitrogen and stored at –80 °C until homogenized according to Wu et al. (2001). Separated fat homogenates were stored in aliquots at –80 °C until analyzed.

Diameter of adipocytes determination. Adipocyte size was assessed microscopically after their isolation by collagenase digestion (Pinterova et al. 2001). The cells were photographed using the camera (Canon Digital Power Shot S40) attached to the microscope and resulting cell diameter was calculated as the average value of at least 100 cells from each adipocyte suspension.

Analyses of hormones plasma. Leptin, ghrelin, adiponectin, visfatin, and insulin levels were determined by RIA using kits by Linco Research (St. Charles, MO). Total protein concentrations in adipose tissue were analyzed according to Bradford protein assay method (Bradford, 1976). The level of glucose in plasma was measured with the use of autoanalyser Hitachi 911 (Hitachi, Japan).

Isolation of hypothalamic nuclei. The arcuate and paraventricular nuclei were isolated from the frozen brains by a punching technique (Palkovits and Brownstein 1988). The brains were cut horizontally up to the beginning of the PVN. The PVN was isolated by 1 mm deep punch, using a needle with 0.6 mm of inner diameter, bilaterally between the fornices and the lumen of the third ventricle. The ARC (including the median eminence) was isolated by 1 mm deep punch, using a needle with 0.6 mm of inner diameter. The isolated frozen tissues were collected in 1.5 ml Eppendorf tubes and kept under -80 °C until used.

Total mRNA isolation and quantitative TaqMan PCR procedure. Poly(A)RNA was isolated from both hypothalamic nuclei using Chemagic mRNA Direct Kit (Chemagen Biopolymer-Technologie AG, Beasweiler, Germany). The signal was reversely transcribed to cDNA by a reaction containing commercial Omniscript RT Kit (Quiagen Inc., Valencia, CA) components, RNase inhibitor (Takara Holdings Inc., Shiga, Japan) and $pd(N)_6$ random hexamer primers (Amersham Biosciences, Piscataway, NJ). The expressions of NPY, AgRP, IL-6, MC4R in individual nuclei were quantitated using ABI Prism 7000 Sequence Detector (Applied Biosystems, Foster City, CA). The reactions were performed with TaqMan gene expression products (Applied Biosystems, Foster City, CA). Multiplex PCR reaction mix contained cDNA, TaqMan Universal PCR Master Mix, TaqMan primers and fluorogenic probes for endogenous control (labeled with the VIC™ reporter dye) and target (labeled with the FAM™ reporter dye) genes. TaqMan eukaryotic rat 18S RNA reagents were used as endogenous control. Samples were run in triplicate. Thermal cycling proceeded according to manufacturer's protocol with two initial setup steps: 2 min 50 °C, 10 min 95 °C steps and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analyzed using ABI Prism 7000 Sequence Detection System Software. Input RNA amounts were calculated with a multiple comparative method for the mRNAs of interest and 18S RNA.

Statistical evaluation. Total area under the curve was calculated from the time when glucose was administered (time 0) up to 120 minutes by using GraphPad Prism 3.0 Software. Statistical significance of all differences between two groups was evaluated by Student's t-test. Repeated measurements of food consumption, body weight and OGTT values were analyzed by one-way ANOVA followed by Bonferroni test. Values of $p<0.05$ were considered as statistically significant. Results were expressed as mean ± SEM for each group.

Results

Biometric parameters. Reduction of litter size followed by high fat diet after weaning did not affect body weight and caloric intake in the rats fed high-fat diet (SHF rats) as compared to these fed standard diet (CN rats) (Fig. 1A). However, SHF rats had significantly more epididymal fat (Fig. 1B), and larger adipocyte size (Fig. 1C) than these found in CN rats.

Glucose tolerance and insulin plasma levels. Oral glucose tolerance test was performed in 56-days old animals, two days before decapitation. In both SHF and CN rats, the initial glucose level in tail-bled samples (0 min) was not different (Fig. 2A). However, impaired glucose tolerance became manifest by reduced utilization, and higher glucose levels at 120 minutes in SHF compared to CN (6.48 ± 0.57 mmol/l vs. 4.77 ± 0.62 mmol/l, $p<0.01$). Furthermore, the total area under the curve was significantly larger in the SHF than in the CN group (875.1 ± 16.8 vs. 759.6 ± 30.6 mmol/l · 0-120 min, $p<0.01$). Plasma glucose level from trunk blood samples was found slightly, but significantly increased in the same SHF rats compared to CN rats two days

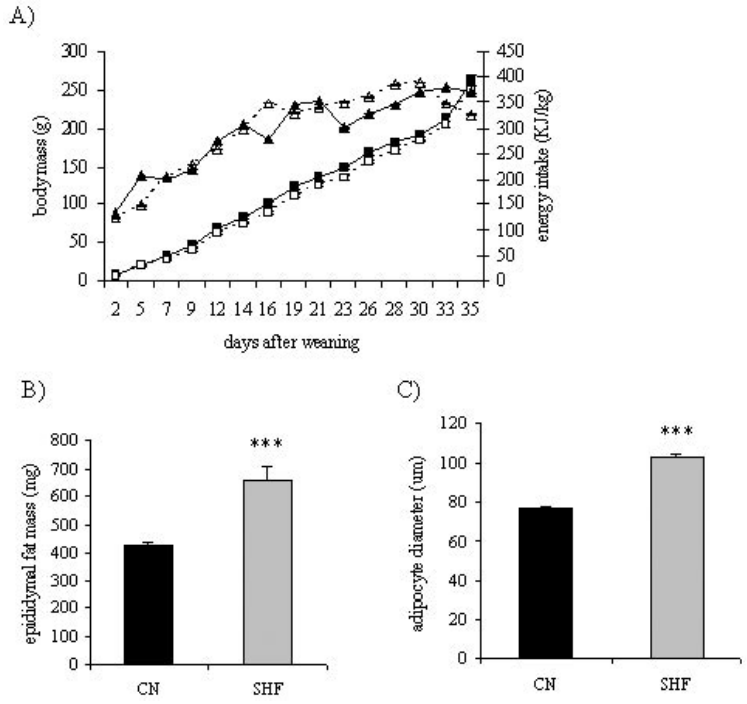

Figure 1: Biometric parameters. (A) Changes of body mass (square lines) and energy intake (triangle lines) in CN (filled squares or triangles) and SHF rats (opened squares or triangles) in selected days of the study. (B) Epididymal fat mass and (C) diameter of adipocytes in 58-days old CN and SHF rats. Abbreviations: CN – control rats from normal-size litter on standard diet, SHF – rats from small-size litter on high-fat diet. ***p<0.001 CN vs. SHF

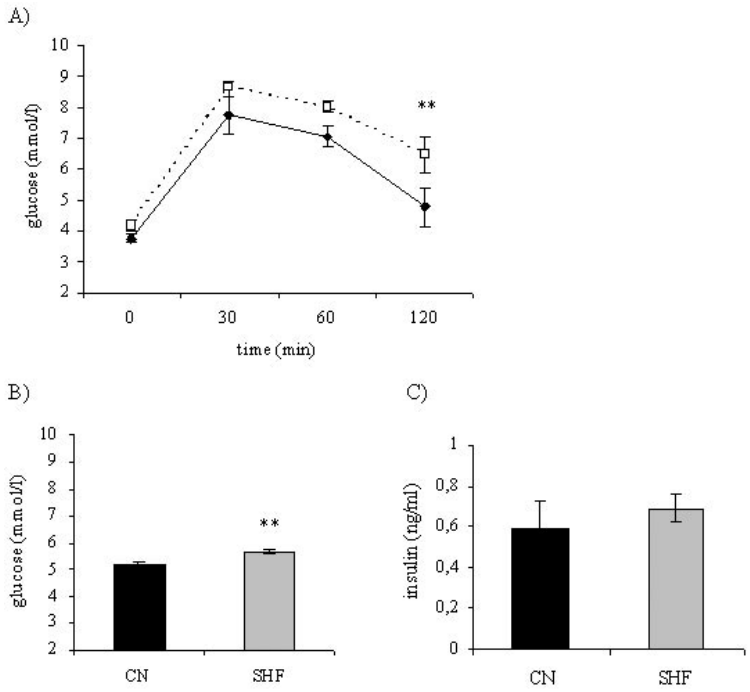

Figure 2: Glucose tolerance and insulin plasma levels. (A) Effects of small-size litter and high-fat diet consumption on OGTT values in 56-days old SHF rats (opened squares) in comparison with CN rats (filled squares). (B) Glucose and (C) insulin plasma levels in 58-days old CN and SHF rats. For abbreviations see Fig. 1. **p<0.01 CN vs. SHF

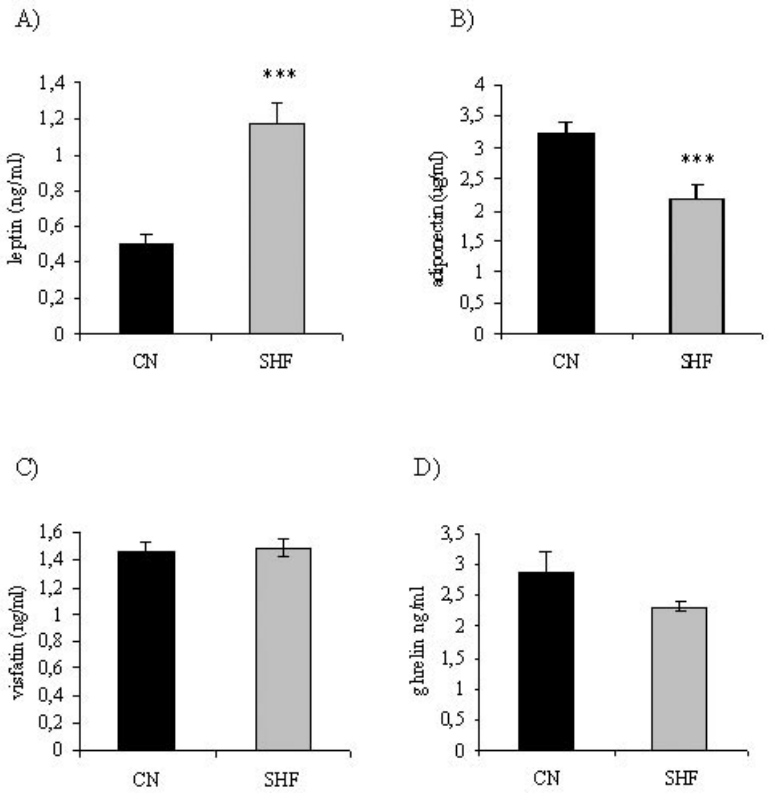

Figure 3: Hormone levels. Differences between 58-days old CN and SHF rats in plasma levels of (A) leptin, (B) adiponectin, (C) visfatin, and (D) ghrelin. For abbreviations see Fig. 1. ***p<0.001 CN vs. SHF

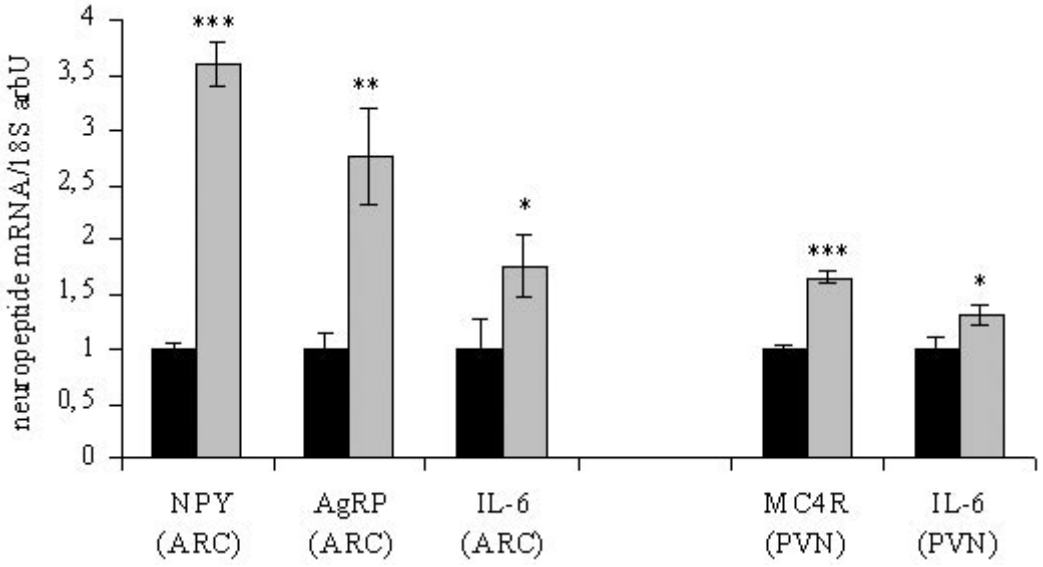

Figure 4: Neuropeptide mRNA expressions in the hypothalamic nuclei. Changes in mRNA expressions for NPY, AgRP and IL-6 in the ARC, and MC4R and IL-6 in the PVN in 58-days old CN and SHF rats. For abbreviations see Fig. 1. * p<0.05, **p<0.01, ***p<0.001 CN vs. SHF

later after decapitation (58-days old animals), while basal insulin plasma levels were similar in both groups (Fig. 2B, 2C).

Hormone levels. In SHF rats significantly higher level of leptin was found in plasma ($p<0.001$; Fig. 3A), and in epididymal fat as well (271.94 ± 13.5 pg/100 μg total protein vs. 174.8 ± 10.05 pg/100 μg total protein, $p<0.01$). Plasma adiponectin was significantly lower in SHF rats compared to controls ($p<0.001$; Fig. 3B), while no difference was found between the two groups of rats for visfatin (Fig. 3C) and ghrelin (Fig. 3D) plasma level.

Neuropeptide mRNA expression in hypothalamic nuclei, ARC or PVN (Fig.4). In arcuate nucleus of 58-days aged SHF rats increased mRNA expression for orexigenic peptides (NPY – $p< 0.001$; AgRP – $p<0.01$) was found as well as of that for IL-6 ($p<0.05$). In addition, in the PVN of the same group also increased mRNA expression was found for MC4R ($p<0.001$) and IL-6 ($p<0.05$).

Discussion

This study demonstrates that the overnutrition induced by a small liter size followed by high-fat diet in Lewis rats resulted in increased epididymal fat mass, impaired glucose tolerance, decreased adiponectin and unchanged visfatin plasma levels in adulthood. In addition these rats showed enhanced leptin production, normal circulating ghrelin levels and overexpression of the hypothalamic orexigenic neuropeptides NPY and AgRP, as well as of anorexigenic IL-6 and MC4R.

In contrast to other studies (Plagemann et al. 1999; Lopez et al. 2005; Boullu-Ciocca et al. 2005), our SHF rats did not increase caloric intake and body mass compared to CN rats. This discrepancy could be related to different diets and strains of rats used. In our experimental diet 45 % of caloric intake was covered by fat which resulted in the expansion of adipose tissue with all related after-effects. Similarly to our findings Pedersen et al. (1991) showed that high-fat feeding induced insulin resistance independently of body mass enhancement in Sprague-Dawley rats. Moreover, their rats had even lower body weight than controls, but heavier epididymal fat pads and larger adipocytes which was crucial for the development of insulin resistance state.

In our study, early overfeeding followed by high-fat diet consumption resulted in the impaired glucose tolerance which was associated with slightly enhanced basal plasma glucose but not with the alteration in basal insulin levels. The unchanged insulin levels along with impaired glucose uptake have been shown by Rodrigues et al. (2007) in early overnutrition rat model. We assume that impaired glucose tolerance in our SHF rats may be related to a decreased plasma adiponectin level, since adiponectin acts as a positive modulator of glucose uptake in insulin sensitive tissues without being an insulin secretagogue (Chandran et al. 2003; Fu et al. 2005). This hypothesis was supported by several human and animal studies showing the link between insulin resistance and decreased adiponectin production (Weyer et al. 2001; Yamauchi et al. 2001; Tajtakova et al. 2006).

Visfatin is another beneficial adipokine which functions as an enzyme essential in the NAD biosynthetic pathway critical for glucose-stimulated insulin secretion from the β-cell (Revollo et al. 2007). Increased circulating visfatin levels have been reported in most human and mice studies of obesity (Fukuhara et al. 2005; Berndt et al. 2005; Haider et al. 2006). However, our SHF rats did not show any changes in plasma visfatin levels which appears consistent with the study of Mercader et al. (2008), who found unaffected visfatin levels in the cafeteria diet-induced model of obesity in Wistar rats and in obese Fa/Fa rats. Thus, unlike in humans and mice, here we showed that, in rat model of early overnutrition followed by high-fat diet consumption, visfatin does not seem to interfere with glucose intolerance to ameliorate insulin resistance.

The activation of orexigenic neuropeptides (NPY and AgRP) mRNA levels in arcuate nucleus of SHF rats is in line with previous results by Lopez et al. (2005) in 24-days old SL rats. Moreover, our findings infer that the overfeeding from early life followed by high-fat diet consumption can prolong these changes up to the adulthood. Regarding the enhanced leptin levels and normal ghrelin levels that we found in SHF rats, the activated NPY and AgRP in the ARC obviously result from the lack of leptin inhibition rather than from the activation of ghrelin, since the levels of the latter were unaltered.

Increased expression of mRNA for MC4R in paraventricular nucleus is suggesting the activation of anorexigenic melanocortin system in SHF rats. Since AgRP is the natural melanocortin antagonist (Yang et al. 1999), we expected the attenuation of MC4R mRNA expression by AgRP enhancement. However, under our conditions AgRP does not seem to have this effect. Although the increased expression of IL-6 in the hypothalamus of obese rats has been reported previously (De Souza et al. 2005), its physiological relevance as related to obesity is currently unclear. Considering

the anorectic effects of pro-inflammatory cytokines (Buchanan and Johnson 2007), IL-6 might be a part of the mechanisms of the anorexigenic system preventing the animals from overfeeding. Taken together, increased leptin production could lead to desensitization of the central orexigenic system (NPY, AgRP), while the sensitivity of the anorexigenic system (MC4R, IL-6) remained preserved.

In conclusion, this study showed that in Lewis rats, the early postnatal overnutrition followed by high-fat diet until adulthood resulted in enhanced adipose tissue accumulation, increased leptin levels, impaired glucose tolerance, normal fasting insulin, visfatin, ghrelin, and reduced adiponectin levels. The decreased adiponectin is the candidate to explain the glucose intolerance in this study. Overexpression of the orexigenic neuropeptides in the ARC could be explained by leptin-resistant state based on enhanced leptin production. Up-regulation of the mRNAs for MC4R in the PVN along with IL-6 in the ARC and PVN indicates the activation of the anorexigenic systems which consequently resulted in unaltered food intake of SHF animals. Furthermore, enhanced leptin production appears the main factor indicating the predisposition to autoimmunity in these overfed rats.

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