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Early Neuroendocrine Alterations in Female Rats Following a Diet Moderately Enriched in Fat

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SUMMARY

1. High-fat diets disrupt metabolic equilibrium and hypothalamic-pituitary-adrenal axis function and may lead to the development of metabolic and endocrine dysfunctions. The early neuroendocrine responses elicited by a combination of short-term metabolic and emotional stressors is not fully elucidated.

2. The purpose of the present study was to determine the impact on female rats, of a short-term enriched in fat diet, combined with an acute stressor.

3. Adult female Wistar rats were fed a fat diet for 7 days and subsequently exposed to 5 min swimming stress. Plasma leptin, insulin, glucose, luteinizing hormone (LH) and corticosterone, along with brain corticosteroid receptors' mRNAs were measured at 1 h post stress.

4. Diet, compared to chow, reduced food intake and body weight gain, increased leptin and LH, and decreased glucose in the periphery. The diet increased plasma corticosterone and reduced GR mRNA in the hippocampus, similarly to swim stress.

5. The diet significantly modified the animals' response to the subsequent swim stress, by blocking further corticosterone rise and GR mRNA reduction. In addition, exposure of diet-fed rats to stress, altered their endocrine response, in terms of leptin and LH.

6. These observations suggest that even short, moderately unbalanced diets can affect peripheral and central components of energy balance, reproduction and stress response.

KEY WORDS: hippocampus; corticosteroid receptors; leptin; insulin; stress; dietary fat.

INTRODUCTION

The macronutrient composition of a diet is a major regulator of energy homeostasis and consequently affects an organism's development, physiology and reproduction. High-fat diets alter circulating leptin and insulin levels (Havel *et al.*, 2001), impair glucose metabolism (Kraegen *et al.*, 1991), and affect stress response (Buwalda *et al.*, 2001; Kamara *et al.*, 1998; Pascoe *et al.*, 1991; Tannenbaum *et al.*, 1997) and reproduction (Frisch *et al.*, 1975).

Glucocorticoids are key components of the organism's stress response system. In rodent brain, corticosterone control on basal and stress-induced function of the

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limbic-hypothalamic-pituitary-adrenal (LHPA) axis is exerted through its mineralocorticoid (MR) and glucocorticoid (GR) receptors, respectively (de Kloet, 2003). Following stress, activated GRs mediate the negative feedback on the release of hypothalamic corticotropin releasing hormone (CRH), leading to the termination of the stress response (Makino *et al.*, 2002). The rat brain corticosterone receptors system appears considerably plastic in response to various stressors (Herman *et al.*, 1995; Karandrea *et al.*, 2002; Kitraki *et al.*, 1999). However, deregulation in LHPA axis negative control can result in excessive levels of glucocorticoids and is implicated in the development of severe pathologies (de Kloet, 2003).

Glucocorticoids, through their receptors, also participate in the central control of food intake and energy balance. There are mutual interactions between the hormonal regulators of food intake and those of the stress response. In the periphery, leptin levels change upon exposure to metabolic stress (Ahima *et al.*, 1996) or exercise (Pagano *et al.*, 1999) and these changes have been implicated in the efficacy of the stress response (Kamara *et al.*, 1998). Leptin inhibits glucocorticoid production by the adrenal gland (Spinedi *et al.*, 1998) and exerts an inhibitory effect on HPA axis response to various stress stimuli (Heiman *et al.*, 1997; Giovambattista *et al.*, 2000). Inactivation of glucocorticoid receptor gene in the nervous system, alters leptin levels and energy accumulation (Kellendonk *et al.*, 2002). In the hypothalamus, GRs influence the expression of orexigenic peptides, counteracting the anorexigenic actions of leptin and insulin in the central nervous system (Kalra *et al.*, 1999). In peripheral tissues, glucocorticoids act as catabolic signals and through their receptors enhance hepatic enzyme-mediated glucose availability (Pilkis *et al.*, 1992). High levels of corticosterone increase body fat and affect plasma insulin (Strack *et al.*, 1995) and leptin (Slieker *et al.*, 1996) concentrations. Diets that disturb endocrine balance, can possibly exacerbate the impact of the LHPA response to a stressful event, further increasing the risk for endocrine and metabolic diseases (Chan *et al.*, 2003; Kamara *et al.*, 1998). Glucocorticoids and stress can exert either inhibitory or facilitatory effects on reproduction, through inhibition or stimulation of gonadotropin secretion (Brann *et al.*, 1991), the final outcome depending on the length of stress exposure and the milieu of gonadal steroids. Normal reproductive function is intimately related to food intake, diet composition and adiposity, both in animals and humans (Cheung *et al.*, 2000; Frisch *et al.*, 1975; Kiess *et al.*, 2000). The adipocyte-derived hormone leptin acts as a metabolic signal that under low energy reserves can inhibit the activity of neuroendocrine reproductive axis in both sexes. Leptin, along with insulin, control luteinizing hormone (LH) secretion and pulsatility by regulating gonadotropin releasing hormone (GnRH) release in the hypothalamus (Burcelin *et al.*, 2003; Nagatani *et al.*, 1998). Leptin administration in ob^{-}/ob^{-} mice of both sexes restores body weight, food intake and fertility (Chehab *et al.*, 1996).

Most of the studies using a rather extended dietary period, have shown that the LHPA axis function of male rats is significantly influenced by dietary fat (Buwalda *et al.*, 2001; Kamara *et al.*, 1998; Pascoe *et al.*, 1991; Tannenbaum *et al.*, 1997). We have recently shown that even a moderately fat-enriched diet can in a short time disrupt the metabolic neuroendocrine balance and the stress response in male rats, potentially rendering the organism more vulnerable to stressful insults (Kitraki *et al.*, 2004). However, in particular in females, little is known about the nature of the

interactions between the regulators of stress response and those of energy balance, at the initial steps of an homeostatic disruption, that are of major importance for permanent metabolic and endocrine dysregulations (Unger and Orci, 2001).

The present study was undertaken to determine the impact of a short term, specific, moderately fat-enriched diet applied alone or in combination with a subsequent acute stress, on the early events affecting major regulators of energy homeostasis, reproduction and HPA axis, in female rats.

METHODS

Animals and Experimental Protocol

In total, forty-eight adult female Wistar rats, 80 days old at the beginning of the experiment were used. The animals were kept three per cage under a 12-h light/dark cycle at 24°C, having free access to tap water and standard chow food in pellets. One week prior to the experimental procedure, all animals were fed with powdered chow (fat, 2% per weight, carbohydrates, 40%, protein, 16.5%) ad libitum. Rats were randomly divided into two equal groups and were fed for the next 7 days either with the powdered chow or with the fat-enriched regime (diet). The diet mixture was made fresh each day and was composed by adding 18 g of corn oil to every 82 g of powdered chow, in order to obtain a fat enriched (20% in corn oil), low protein (13,5%), low carbohydrate (33%) mixture. The energy content of standard chow was 2440 kcal/kg and that of experimental diet 3660 kcal/kg. Food consumption in each cage was determined daily for the 7-day period. Body weights were measured in the morning prior to the beginning of the experiment (day 0) and on day 7. In the morning of day 8, food was removed from the cages 3 h prior to sacrifice and animals in each group were randomly assigned into two subgroups. One subgroup was left undisturbed and the other was subjected to acute stress consisted of 5 min forced swimming in a glass cylinder 20 cm in diameter, filled with water of 24°C. All animals were euthanized by decapitation upon instant ether anesthesia. Stressed rats were decapitated 1 h following the stress procedure, alternating with non-stressed animals, always between 10:00 AM and 12:00 PM. Trunk blood was collected for hormonal determinations. The brains were immediately removed and individual hypothalami and hippocampi were collected for corticosterone receptors' mRNA analysis. Samples of twelve animals per group were used for hormonal determinations and of six animals per group for glucose and corticosteroid receptors measurements. Vaginal smears were taken from all animals at sacrifice, to determine the estrous phase. All animal treatments were carried out in accordance with the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Determination of Plasma Hormones and Glucose Levels

Plasma corticosterone levels were determined by radioimmunoassay (RIA) using Amersham's Biotrak$^{(TM)}$ rat corticosterone [^{125}I] assay system, following

corticosterone displacement from corticosterone-binding globulin (CBG) by heating to 60°C for 30 min, in a water bath. The assay sensitivity was 2.55 ± 0.25 and 11.0 ± 0.59 ng/mL at 80 and 50% respectively. The coefficients of variation within and between assays were 5 and 4.0–5.9%, respectively. The non-specific binding defined as the proportion of tracer bound in the absence of antibody was <3%.

Plasma leptin levels were determined by a two-step RIA, using Linco's rat leptin [^{125}I] assay system. The assay sensitivity was 0.44 ± 0.01 and 1.32 ± 0.04 ng/mL at 80 and 50% respectively. The coefficients of variation within and between assays were 2-4.6 and 3.0–5.7%, respectively. The non-specific binding was <2%.

Plasma insulin was measured by a sensitive two-step RIA (18 h preincubation, in the absence of the tracer, followed by 1 h and 30 min of incubation; for details see Kitraki *et al.*, 2004), using commercially available reagents (DiaSorin, Sallugia, Italy). The assay sensitivity was 1.78 ± 0.03 and 5.57 ± 0.07 μU/mL at 80% and 50% respectively. The coefficients of variation within and between assays were 5–10 and 6–10%, respectively. The non-specific binding was <6%.

Plasma LH levels were determined by Amersham's Biotrak$^{(TM)}$ rat LH [^{125}I] RIA assay system. The cross-reactivity towards rat TSH was 0.56% and for rat FSH, GH, PRL or ACTH <0.1%. The assay sensitivity was 0.5 ng/mL at 80% and 0.13 ng/mL at 50%. The coefficients of variation within and between assays were 6–7% and 6.5–10.9%, respectively. The non-specific binding was <5%.

For the enzymatic determination of plasma glucose by the glucose oxidase/peroxidase technique, the bioMerieux (Lyon, France) Glucose RTU assay system and a Perkin Elmer spectrophotometer were used.

RNA Isolation and Northern Blot Analysis

Total RNA from the hypothalamus and hippocampus of each rat separately was isolated by using the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Northern blot analysis was performed as described previously (Kitraki *et al.*, 1999, 2004). For either GR or MR riboprobes, the hybridization solution (Kitraki *et al.*, 1999) contained 3×10^6 cpm of the probe per milliliter of hybridization buffer. Autoradiograms were quantified by using the densitometric program Image Pro Plus (Media Cybernetics). The densitometric reading for the major GR and MR bands was divided by the reading for 28S rRNA in each sample, to normalize possible variations in the amount of loaded RNA samples.

Statistics

Univariate analysis of variance for the effects of diet, of stress and of their interaction on peripheral hormone levels, on blood glucose and on brain corticosterone receptors was performed by using the General Linear Model of SPSS software (version 11.0) followed, when appropriate, by the *LSD* post hoc test for multiple comparisons. The means ±SEM of body weight gain during the 7-day period and of daily food intake between experimental diet-fed and chow-fed groups were compared by using Student's *t-test*. Significance was accepted for $p < 0.05$.

RESULTS

Effects of Diet on Food Intake and Body Weight Gain

The experimental diet used, significantly affected both food intake and body weight gain of rats. Rats fed the fat diet consumed daily a smaller amount of food (48.3 g/cage), corresponding to 76% of the amount of chow taken by the controls, and gained less body weight during the one week of diet, compared to their chow-fed counterparts (Table I).

Effects on Peripheral Hormone Levels

Fat-enriched diet, acute swimming stress, as well as their combination significantly affected plasma leptin levels (Fig. 1(A)). The between groups multiple comparisons showed a significant increase of leptin levels in the diet-fed non-stressed group, as compared to all other groups. The diet-induced increase in leptin levels was abolished following stress (compare the diet-fed control, DC, with the diet-fed stressed, DS, group in Fig. 1(A)).

Diet, acute stress, as well as their combination significantly affected plasma corticosterone levels (Fig. 1(B)). Post hoc analysis showed that the 7-day diet alone significantly increased plasma corticosterone levels, as also did the acute stress and the combination of diet and acute stress treatment, compared to CC group.

Stress, but not diet, significantly affected plasma insulin levels. Insulin levels were decreased at 1 h following acute swimming stress in stressed animals fed either the normal chow or the diet regimen, as compared to non-stressed animals (Fig. 1(C)).

Morning plasma LH levels were also affected by the diet, the acute stress, as well as by their combination (Fig. 1(D)). The between groups multiple comparisons showed a significant increase of LH levels in the diet-fed non-stressed group, as compared to all other groups. Notably, the diet-induced increase in LH levels was attenuated at 1 h following acute stress (compare DC with DS group in Fig. 1(D)). Vaginal smears taken at sacrifice revealed that in all the four groups 90% of animals were either in proestrous or estrous. No correlation between morning LH levels and the estrous cycle was observed.

Effects on Plasma Glucose Levels

Plasma glucose levels were significantly lower in diet-fed rats compared to chow-fed (Fig. 2). No significant effect of stress was observed.

Table I. Effects of Fat-Enriched Diet on Body Weight Gain and Food Intake

Experimental groups	Body weight gain (g) per animal in 7 days (Means ± SEM, $n = 24$ per group)	Food intake (g) per cage (Means ± SEM, 3 animals per cage)
Normal chow diet	14.3 ± 0.64	63.83 ± 1.89
Fat-enriched diet	10.9 ± 0.67*	48.30 ± 1.33*

*Statistically significant from the normal chow diet group, Student's t-test ($p < 0.05$).

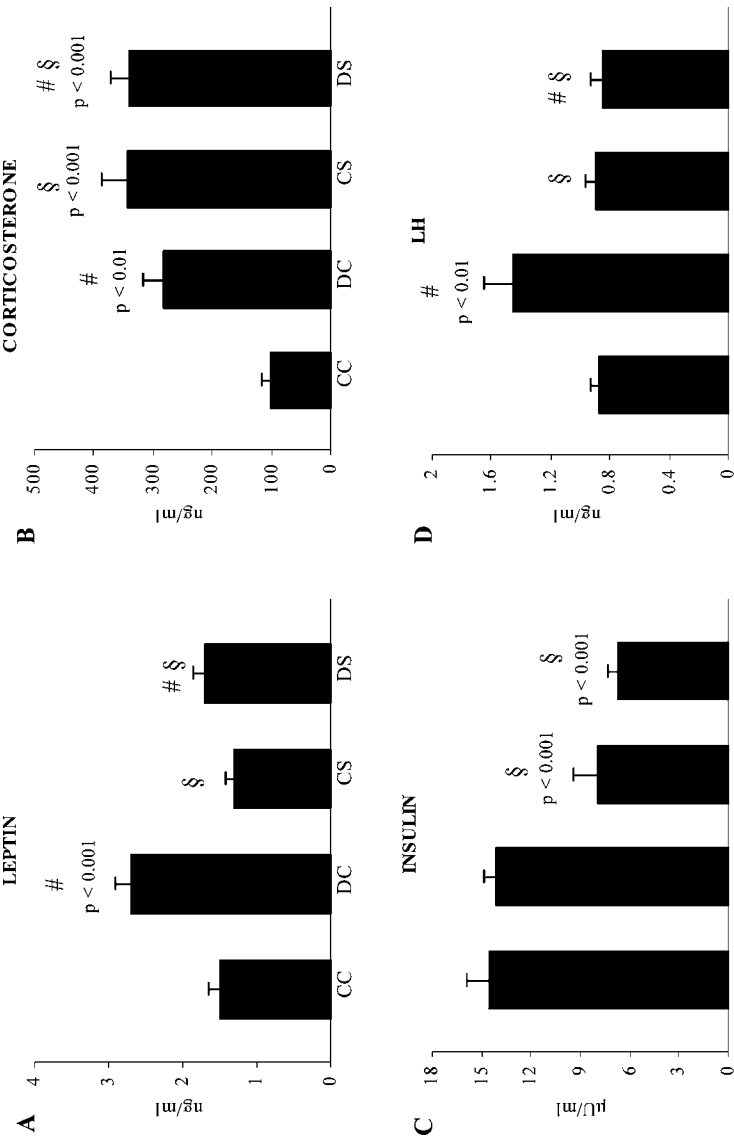

Fig. 1. Plasma leptin (A), corticosterone (B), insulin (C) and LH (D) levels of: chow-fed control (CC), diet-fed control (DC), chow-fed stressed (CS) and diet-fed stressed (DS) female rats. Values in bars represent means ±SEM ($n = 12$ per group). # indicates the significant effect of diet ($F_{(1,45)} = 23.82$, $P < 0.001$ for leptin, $F_{(1,40)} = 6.24$, $P = 0.017$, for corticosterone and $F_{(1,47)} = 5.15$, $P = 0.028$ for LH) § the significant effect of stress ($F_{(1,45)} = 12.58$, $P = 0.001$ for leptin, $F_{(1,40)} = 18.10$, $P < 0.001$ for corticosterone, $F_{(1,47)} = 41.35$, $P < 0.001$ for insulin and $F_{(1,47)} = 5.99$, $P = 0.018$ for LH) and #§ the significant effect of *diet x stress* interaction ($F_{(1,45)} = 4.52$, $P = 0.039$ for leptin, $F_{(1,40)} = 6.82$, $P = 0.013$ for corticosterone, and $F_{(1,47)} = 7.38$, $P = 0.009$ for LH). Significance for group comparisons, when appropriate, is given on the graph.

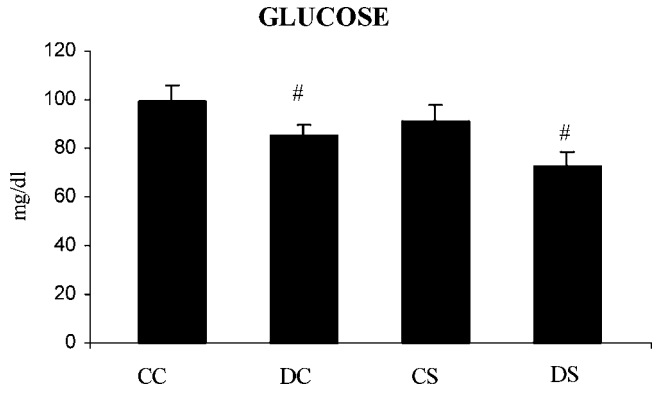

Fig. 2. Plasma glucose levels of: chow-fed control (CC), diet-fed control (DC), chow-fed stressed (CS) and diet-fed stressed (DS) female rats. Values in bars represent means ±SEM ($n = 6$ per group). # indicates the significant effect of diet ($F_{(1,22)} = 7.03$, $P = 0.016$).

Effects on Brain Corticosteroid Receptors

The expression of corticosterone receptors was affected in an area and receptor type-specific manner. Statistical evaluation of Northern blot results revealed a significant effect of diet and of its combination with stress on the expression of GR gene in the hippocampus (Fig. 3), but not in the hypothalamus (data not shown).

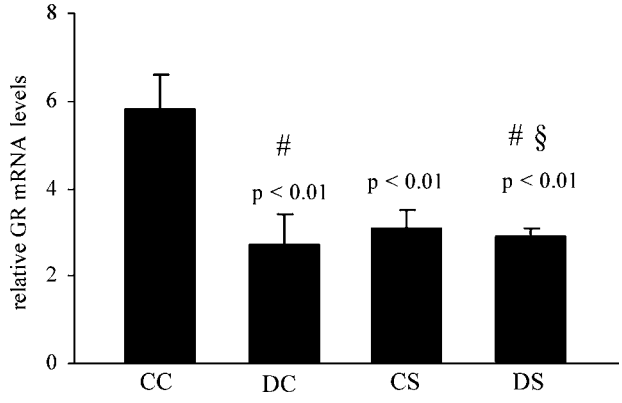

Fig. 3. Quantification of Northern blot results for glucocorticoid receptor (GR) mRNA levels in the hippocampus of: chow-fed control (CC), diet-fed control (DC), chow-fed stressed (CS) and diet-fed stressed (DS) female rats, ($n = 6$ per group). Each bar represents the means ±SEM of the densitometric GR mRNA reading divided by the respective 28S rRNA reading (relative mRNA levels). # indicates the significant effect of diet ($F_{(1,20)} = 6.44$, $P = 0.021$), and #§ the significant effect of *diet x stress* interaction ($F_{(1,20)} = 4.96$, $P = 0.040$). Significance for group comparisons, when appropriate, is given on the graph.

Hippocampal GR mRNA levels were significantly decreased as a result of the 7-day diet, compared to CC group. A similar reduction in GR mRNA levels was also observed in the diet-fed rats exposed to acute stress, compared to CC group. Exposure to acute swimming stress also led to down-regulation of GR mRNA in the hippocampus. In contrast to GR, MR mRNA levels in the hippocampus were not affected by the experimental conditions (data not shown).

DISCUSSION

The present study designed to test whether a short-term fat-enriched diet can significantly modify important endocrine factors of the adult female rat linked to energy homeostasis, reproduction and stress response, gave a positive answer. One week exposure to the diet increased circulating levels of leptin, LH and corticosterone, while it decreased glucose levels in the periphery and GR mRNA in the hippocampus. The diet significantly modified the animals' response to a subsequent acute stress, by blocking both further corticosterone rise and GR mRNA down-regulation. In addition, the exposure of diet-fed rats to stress, altered the animals' prior endocrine response to the diet, in terms of leptin and LH levels.

The fat-enriched diet we used contained 20% corn oil, commonly used as a lipid source in rodent diets (Ackroff *et al.*, 1990). The diet-fed rats ingested daily a reduced amount of food and gained less body weight than the chow-fed controls. This could be attributed to the increased plasma leptin levels observed in these animals and is in agreement with our observations in male rats treated under the same protocol (Kitraki *et al.*, 2004). In this study, increased leptin levels were detected as an early response to the fat-enriched diet. When longer fat diet protocols were applied in female (Ainslie *et al.*, 2000) or male (Tannenbaum *et al.*, 1997) rats, they have resulted to decreased leptin levels and increased body weights. These treatments can potentially lead to reduced leptin sensitivity or leptin resistance in analogy with insulin sensitivity or resistance and ultimately to metabolic and endocrine dysfunction, in particular in obese prone animals (Wang *et al.*, 2001).

Acute swimming stress did not affect leptin levels measured at 1 h post stress in normally-fed female rats, similarly with our observations in male rats (Kitraki *et al.*, 2004) and in agreement with previous studies on the effects of acute stress in leptin levels (Pagano *et al.*, 1999). However, when the acute stress was applied in diet-fed females it blocked the previously seen diet-induced increase in leptin levels. This reduction in leptin levels at 1h post stress in diet-fed stressed animals could not be attributed to the actions of high corticosterone levels, also present in the diet-fed non-stressed group, but rather to the negative feedback exerted by the sympathoadrenal system on leptin secretion. Sympathetic nervous system activation regulates leptin production and secretion by adipocytes and it has been proposed that following stress, the 'fight or flight' system operates to reduce leptin levels in order to increase appetite and conserve energy (Rayner and Trayhurn, 2001).

One week exposure to the diet did not alter basal insulin levels in female rats, in accordance with the previous observations (Havel *et al.*, 1999; Pascoe *et al.*, 1991) and our own in males, fed the same short term diet (Kitraki *et al.*, 2004).

The above observations are not inconsistent with a potential glucose induced insulin hypersecretion observed in male rats (Cruciani-Guglielmacci *et al.*, 2004). However, in our study the animals were at the fasted state when sampled and the glycemia was low, particularly in diet fed rats. In addition, the present study was not designed to unmask either a potential induced insulin hypersecretion or a reduced insulin sensitivity. It is known that following acute stress catecholaminergic system activation leads to low insulin levels and hyperglycemia (Gotoh *et al.*, 1998; Pascoe *et al.*, 1991; Smythe *et al.*, 1989). However in this study, plasma glucose levels were significantly reduced on day 7 of the diet. The fall in glucose levels we observed in the diet-fed female rats could be a consequence of leptin increase following fat diet. Leptin alone or in combination with insulin can reduce hepatic glucose production by decreasing the synthesis of phosphoenolpyrouvate carboxykinase (Anderwald *et al.*, 2002). Sandoval and Davis have proposed that a decrease of leptin during stress may serve as a signal to the brain, to switch fuel utilization to carbohydrates, a quicker energy source (Sandoval *et al.*, 2003). Our data suggest that leptin could serve the above purpose in fat-fed stressed female rats, although the concomitant increase in glucose levels could not be detected at 1h post stress in these animals, probably due to the stress-induced increase in glucose utilization.

The fat diet used was experienced as stress by female rats. This is evidenced by the similarly increased circulating corticosterone levels in diet-fed non-stressed and chow-fed stressed rats, as well as by the parallel down-regulation of hippocampal GR mRNA in these two groups. Notably, when the swimming stress was applied in sequence to the 'metabolic stress', the neuroendocrine response was abolished, as to both corticosterone and its receptors, implying an overloading of the response system that do not further respond to a novel stimulus. As transcription remains the main level of control of GR gene (Dong *et al.*, 1998; Webster *et al.*, 1994), one could suggest that the reduced GR mRNA levels observed at 1 h post stress could lead to reduced receptor levels and subsequent impairment of stress response. However, since no protein levels were determined in this study, the potential implications of this reduction on stress responsivity are speculative. By using the same protocol in male rats, we have detected increased corticosterone release in acutely stressed animals of either diet and significant reduction of GR mRNA in the hypothalamus of fat-fed stressed animals (Kitraki *et al.*, 2004). These findings show the existence of sex differences in the impact of a metabolic and a subsequent physical stressor on neuroendocrine stress response system: the metabolic insult elicits significant responses in female LHPA axis, while the subsequent swimming stress affects glucocorticoid receptors' expression in both sexes, though at different levels of the axis response.

An interaction between the neuroendocrine circuits controlling stress response and energy balance in female rats was also seen in the case of LH levels. In our study, the pattern of diet- and stress-induced changes in LH levels coincided that of leptin: we observed the same increase in plasma LH levels due to the diet, which was abolished following stress. This reduction of LH levels in the diet-fed females following stress could be attributed to the concerted action of low leptin, insulin and glucose levels detected in these stressed animals. Leptin is more than a metabolic signal for the brain, as it conveys information about environmental conditions and energy reserves and can influence reproductive function (Watanobe, 2002). Leptin,

together with insulin, regulate LH secretion and pulsatility in females (Burcelin *et al.*, 2003; Nagatani *et al.*, 1998). On the other hand, stress-induced hypoglycemia and sympathetic system activation that are potent inhibitors of LH secretion (Cagampang *et al.*, 1997) could also have contributed to the final outcome in plasma LH levels.

The present study aimed to clarify some aspects of the complex early neuroendocrine responses elicited in the adult female rat by the combination of a diet, simulating the diets often consumed in western societies and an acute stress of short duration. The combined action of the 7-day fat-enriched diet and stress affected peripheral and central components of energy balance, reproduction and stress response systems, suggested that even short term, moderate dietary alterations can significantly alter the metabolic equilibrium and the neuroendocrine response to an acute stress. The observed changes have pointed out the importance of leptin in these early neuroendocrine responses in female rats, which, though characteristic of the gender and the short duration, are in keeping with those witnessed in the potentially harmful longer fat-diet protocols that promote metabolic and endocrine dysfunctions.

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