Chronic stress reduces body fat content in both obesity-prone and obesity-resistant strains of mice

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Abstract

Unpredictable stressors have been used to assess the effect of stress on energy metabolism in obesity-prone (C57BL6J) and obesity-resistant (AJ) mice. Mice were exposed for 25 days to a stress protocol. Both strains of mice were divided into groups of control and stressed mice, which had access to either a high-fat or a high-carbohydrate diet. Twenty-four hours after the last session of stress, mice were sacrificed for blood and brain collections. Insulin, corticosterone, and glucose concentrations in plasma were measured, and expressions of corticotropin-releasing factor (CRF) in the paraventricular hypothalamic nucleus (PVH) and the central amygdala (CeA) were determined by in situ hybridization. Stressed mice in all groups had lower body fat contents than control mice, and all mice fed with the high-fat diet had heavier retroperitoneal and inguinal fat pads than mice fed with carbohydrate. CRF mRNA level in the CeA was lower in B6 mice than in AJ mice. Stressed mice had a lower expression of CRF in the CeA than control mice. In conclusion, chronic stress reduces body fat content in obesity-prone as well as in obesity-resistant mice.

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Introduction

There is strong evidence from investigations carried out in laboratory rodents that stress induces catabolic effects (Armarrio et al., 1985; Harris et al., 1998; Krahn et al., 1990; Marti et al., 1994; Michel and Cabanac, 1999a,b; Rybkin et al., 1997; Smagin et al., 1999; Yahata et al., 1987). Acutely, stress activates the corticotropin-releasing factor (CRF) system, which orchestrates the hormonal and behavioral response to stress (Sutton et al., 1982; Weninger et al., 1999). CRF has been recognized for years as a major anorectic agent (Cole et al., 1990; Koob and Thatcher-Britton, 1985; Murakami et al., 1989; Vale et al., 1981). Infusion of CRF into the brain decreases food intake, body weight gain, as well as the body weight set point (Britton et al., 1982; Cabanac and Richard, 1995; Morley and Levine, 1982; Rivest and Richard, 1990). CRF mediates stress-induced anorectic effects (Koob and Heinrichs, 1999; Menzaghi et al., 1993) that generally persist for quite a few hours after CRF administration. Not too surprisingly, day-to-day repetition of stress generally leads to a negative energy balance (Favre and Vermorel, 1975; Harris et al., 2002a,b; Michel and Cabanac, 1999b; Rybkin et al., 1997). Data from Harris (Harris et al., 1998) have even demonstrated that a 3-day exposure to stress can lead to a long-term retardation in weight growth in rats.

In addition to the demonstration for an association between stress and negative energy balance, there is also evidence from human studies to suggest that stress is positively associated with the development of obesity. Recently, Michel et al. (2003) demonstrated that stress leads to either body weight gain or body weight loss depending on whether stress is applied with a high-energy diet, or with a chow diet to obesity-prone animals. We indeed reported that 20 min of restraint stress in obesity-prone rats fed an energy-dense diet, led to an excessive body weight gain over the
subsequent 9 days. It remains to be demonstrated whether the chronic exposure to stress would also promote positive energy balance in obesity-prone animals.

The present study was aimed at investigating fat deposition and food intake in obesity-prone (C57BL/6J) and obesity-resistant (AJ) mice, which were chronically exposed to stress. C57BL/6J (B6) mice are known to become obese when placed on a high-fat diet (Kraegen et al., 1986; Storlien et al., 1986; Surwit et al., 1988). This dietary effect is sustained as long as the energy-dense diet is provided. If the animals are returned to a standard chow diet, their body weights drop to a normal non-obese state (Parekh et al., 1998). On the other hand, AJ mice remain lean even when offered a high-fat diet. For this study, we designed an original stress protocol made of five stressors known to initiate hormonal stress responses. The stressors were selected from the literature (Barr and Phillips, 1998; Briese and Cabanac, 1991; Michel and Cabanac, 1999b; Monnikes et al., 1993). As B6 are obesity-prone when subjected to a high-fat diet, we hypothesized that a mild chronic stress would increase their food intake and facilitate their body weight gain. Finally, CRF mRNA contents in the paraventricular hypothalamic nucleus (PVH) and central nucleus of amygdala (CeA) were measured under the various conditions of the study. PVH is the site of the hypophysiotropic CRF and the CeA is known to be responsible for the behavioral response to stress and the fear conditioning response (Makino et al., 1999; Roozendaal et al., 1991; Roozendaal et al., 1990), and thus were the main areas of interest.

Materials and methods

Animals and diets

Male B6 and AJ mice weighing 23.2–27.8 g, were pair-housed at 23°C in a humidity-controlled room. Lights were on at 0900 and off at 2100. Each strain was divided into 4 groups of eight individuals: high-carbohydrate control (CHO-Ctl), high-carbohydrate stressed (CHO-Str), high-fat control (FAT-Ctl), and high-fat stressed (FAT-Str). They were adapted to their ambient conditions for at least 7 days and to their specific diets for 3 days before the beginning of the stress protocol. Animals were offered purified diets. The high-carbohydrate diet consisted of starch (63%), corn oil (15%), casein (20%), methionine (0.3%), vitamins (1%), and minerals (0.6%). The high-fat diet was 65% lipid (half from corn oil and half from pure lard), 10% starch, 3.2% dextrose, 20% casein, 0.3% methionine, 1% vitamins, and 0.6% minerals.

Stress protocol

The animals were subjected to the following stressors: (i) conspecific exposure (care was taken to never pair the same mice) (Barr and Phillips, 1998); (ii) water avoidance (a mouse is placed on a wood cube surrounded by water) (Monnikes et al., 1993); (iii) cage inclination at 40° (Barr and Phillips, 1998); (iv) damp bedding (addition of water to the litter) (Barr and Phillips, 1998); (v) colonic temperature taking every 30 min (Michel and Cabanac, 1999b). The 5 stressors (one different stressor each day) were randomly assigned to each group so that each mouse had the five stressors over a period of 5 consecutive days in an unpredicted manner. In order to do so, random numbers were assigned to both stressors and animals, which were randomly picked up by a blind experimenter and then stressors were assigned to each group of animal in a random and unpredictable way. The exposure to each stressor lasted 2 h per day and took place at the beginning of the light cycle. The block of 5 stressors was repeated 5 times over 25 days.

Body weight, food intake, and tissue weight

Every morning, mice were weighed. The day-to-day food consumption was assessed by subtracting the food left uneaten from the amount of food provided to the animals. Twenty-four hours after the end of the treatment period, mice were anesthetized as quick as possible with a mixture of ketamine/xylazine for mice (0.1 ml/10 g). Then, they were decapitated and the inguinal (IWAT) and retroperitoneal (RWAT) white adipose tissue pads as well as the interscapular brown adipose tissue (IBAT) depot were removed and weighed. The soleus and vastus lateralis muscles were also removed and weighed.

Plasma determination

An intracardiac blood sample was taken in anesthetized mice, just before decapitation, for the determination of plasma corticosterone, insulin, and glucose. Blood was sampled without delay, usually 1 to 2 min after the injection of the anesthetics. Plasma glucose was determined (glucose oxidase method) using a glucose analyzer (Beckman, Palo Alto, Calif., USA). Serum corticosterone was determined by radioimmunoassay (Medicorp, Royalmount, Mtl., Canada) (sensitivity, 0.05 nmol/l; inter-assay coefficient of variation, 6.9%, intra-assay coefficient of variation, 7.3%). Insulin was determined by RIA with a reagent kit from Linco Research (St. Charles, MO, USA) (inter-assay coefficient of variation, 9%, intra-assay coefficient of variation, 2.9%) using rat insulin as a standard.

Brain preparation

Mice were anesthetized and without delay were perfused intracardially with 200 ml of ice-cold isotonic saline at first followed by 500 ml of paraformaldehyde (4%). Brain was removed at the end of perfusion and kept in paraformaldehyde (4%) pending its slicing. It was transferred to a
solution containing paraformaldehyde and sucrose (10%) before being cut 24 h later using a sliding microtome (Histoslide 2000, Reichert-Jung). Twenty-five-μm-thick sections were collected and stored at −30°C in a cold sterile cryoprotecting solution containing sodium phosphate buffer (50 mM), ethylene glycol (30%), and glycerol (20%).

**In situ hybridization histochemistry**

In situ hybridization histochemistry was used to assess CRF mRNA on tissue sections taken from the PVH and CeA. The protocol used was largely adapted from the technique described by Simmons et al. (1989). Briefly, brain sections were mounted onto poly-L-lysine-coated slides and allowed to desiccate overnight under vacuum. The sections were then successively fixed for 20 min in paraformaldehyde (4%), digested for 30 min at 37°C with proteinase K (10 μg/ml in 100 mM Tris–HCl containing 50 mM EDTA, pH 8.0), acetylated with acetic anhydride (0.25% in 0.1 M triethanolamine, pH 8.0) and dehydrated through graded concentrations (50, 70, 95, and 100%) of alcohol. After vacuum drying for at least 2 h, 90 μl of the hybridization mixture, with an antisense 35S-labeled cRNA probe (10⁷ cpm/ml), were spotted on each slide. Each slide was sealed under a coverslip and incubated overnight at 60°C in a slide warmer. The next day, the coverslips were removed and the slides rinsed four times with 4× SSC (0.6 M NaCl, 60 mM sodium citrate buffer, pH 7.0), digested for 30 min at 37°C with RNase-A (20 μg/ml in 10 mM Tris–500 mM NaCl containing 1 mM EDTA), washed in descending concentrations (50, 70, 95, and 100%) of alcohol. After a 2-h period of vacuum drying, the slides were exposed on an X-ray film (Eastman Kodak, Rochester, N.Y., USA) for a period of 48 h. Once removed from the autoradiography cassettes, the slides were exposed for 14 days before being developed in D19 developer (Eastman Kodak) for 3.5 min at 14–15°C and fixed in rapid fixer (Eastman Kodak) for 5 min. Finally, tissues were rinsed in running distilled water for 1 to 2 h, counterstained with thionine (0.25%), dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX.

**Antisense 35S-labeled cRNA probes**

The CRF cRNA probe was generated from the 1.2-kb EcoRI fragment of rat CRF cDNA (Dr. K. Mayo, Northwestern University, Evanston, Ill., USA) subcloned into a pGEM4 vector (Stratagene, La Jolla, Calif., USA) and linearized with HindIII and EcoRI (Pharmacia Biotech Canada Inc., Baie d’Urfé, Qué., Canada) for antisense and sense probes, respectively. The specificity of the probe was confirmed by the absence of a positive signal in sections hybridized with sense probe. Radioactive riboprobes were synthetized by incubation of 250 ng linearized plasmid in 10 mM NaCl, ATP/GTP/CTP, α-35S-UTP, 40 U Rnasin (Promega, Madison, Wisc., USA) and 20 U SP6 RNA polymerase for CRF antisense probe, for 60 min at 37°C. The DNA templates were treated with 100 μl of DNase solution (1 μl DNase, 5 μl of 5 mg/ml tRNA, 94 μl of 10 mM Tris/10 mM MgCl₂). The preparation of the riboprobe was completed through a phenol-chloroform extraction and an ammonium acetate precipitation.

**Quantitative analysis of the hybridization signals**

The hybridization signals revealed on NTB2 dipped nuclear emulsion slides were analyzed and quantified under a light microscope (Olympus, BX50) equipped with a black and white video camera (Sony, XC-77) coupled to a Macintosh computer (Power PC 7100/66) using Image software (version 1.55 non-FPU, Wayne Rasband, NIH, Bethesda, MD, USA). The optical density of the mRNA hybridization signal was determined from coronal brain sections under dark-field illumination at a magnification of 25×. The optical density determination was performed on each side of the brain and the two optical density values obtained were averaged. This average was included in the statistical analyses at the individual score of a mouse. The sections quantified for in situ hybridization were those where the areas of interest were clearly identified through anatomical cues. Then, the two highest scores in intensity were used and averaged again for the statistical analysis. While the experimenter was quantifying sections, he was unaware of which group each section belongs to. Only the mice number, with no description, was given to this experimenter. A section with no signal could be drawn according to anatomical tips, and was measured in order to be consistent for the statistical analysis. When no hybridization signal was visible under dark-field illumination, the brain structures were outlined under bright-field illumination and then subjected to densitometric analysis under dark-field illumination. The optical density for each specific region was corrected for the average background signal, which was determined by sampling unlabeled areas outside of the areas of interest. Four mice per group were used for the in situ hybridization.

**Statistical analyses**

Analysis of variance (ANOVA) was used to detect significant (P < 0.05) interaction effects of strain (B6 vs. AJ), treatment (stress vs. control), and diet (CHO vs. FAT) on body weight and food intake, on insulin, glucose, and corticosterone, tissue weights (muscle and fat), and CRF mRNA levels in the PVH and CeA. Each group included 8 animals, and 4 per groups were used for the optical density analyses. When a significant interaction was found, Fisher post hoc tests were performed to make individual comparisons.
Results

After 5 days of treatment (1 block of stressors), there were already significant main effects of stress (stress vs. control, $P = 0.002$) and strain (AJ vs. B6, $P = 0.0003$) on body weight gain. The main effect of diet appeared on day 8 (Fig. 1A). After day 8, mice that exhibited weight loss began to regain their weight, except for stressed AJ mice group fed a carbohydrate diet, which continued to lose weight until day 15. At the end of the protocol, stressed mice did not weigh significantly less than their respective controls except for the B6 mice fed high fat which gained less weight than controls (Fig. 1B; $P = 0.048$).

Stressed mice had lower WAT depots than their corresponding control mice (Fig. 2, panels A, B, C). IWAT, RWAT, and IBAT were, respectively, 33%, 38%, and 17% lighter in stressed than in control mice. Mice fed a high-fat diet had higher retroperitoneal and inguinal fat mass than mice on a high-carbohydrate regimen (Figs. 2B, C). With no regard to diet or stress, B6 mice had more IBAT and more RWAT (26%) but less IWAT tissue (21%) than AJ mice (Figs. 2A–C). There was no significant difference among groups with regard to food intake [$F(1,56) = 1.529$, $P = 0.22$].

Fig. 1. The time course of the $\Delta$ body weight (A) and the total weight gain (B) for each group (AGC = AJ glucides control; AGS = AJ glucides stress; AFC = AJ fat control; AFS = AJ fat stress; BGC = B6 glucides control; BGS = B6 glucides stress; BFC = B6 fat control; BFS = B6 fat stress). Stress began at day one. Bars are means ± SE, $n = 8$, Fisher post hoc test.

Fig. 2. The mean fat mass (g) for the brown adipose tissue [BAT; upper panel (A)], for the white retroperitoneal fat [WR; lower left panel (B)], and the white inguinal fat [WI; lower right panel (C)], 25 days after the beginning of the stress protocol. Stress rats are represented by scattered bars and control rats by solid bars. Bars are means ± SE, $n = 8$. 

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Significant differences (ANOV A for multiple factors, $P = 0.22$). No change was observed regarding the soleus and vastus lateralis muscle weights (data not shown).

There was no significant difference between groups in the plasma corticosterone and insulin concentrations. Plasma glucose levels were however higher in the high-fat groups than in the high-carbohydrate groups. There was no significant difference in plasma glucose between stressed and control animals (Table 1).

There was no significant difference between groups in CRF mRNA expression in the PVH (data not shown). However, stress significantly decreased CRF mRNA expression in the CeA [Fig. 3; $F(1,18) = 6.309, P = 0.02$]. In the same brain area, CRF mRNA expression was also lower in B6 mice [$F(1,18) = 10.305, P = 0.05$] than in AJ mice. Diet had no effect on the CRF mRNA expression in the CeA (see Fig. 4 for examples of photomicrographs).

**Discussion**

The present results are largely consistent with those obtained in previous studies done primarily in laboratory rats (Armario et al., 1985; Harris et al., 1998; Krahn et al., 1990; Marti et al., 1994; Michel and Cabanac, 1999a,b; Rybkin et al., 1997; Smagin et al., 1999; Yahata et al., 1987), which showed that chronic exposure to daily stress led to catabolic effects that translate into a reduction in body weight and body fat gains. In this study, stressed mice were unable to compensate (through an increase in food intake) the increase in energy expenditure associated with stress, which is consistent with a CRF-like effect. Stress strongly stimulates the CRF system, which comprises peptides with strong catabolic attributes (Britton et al., 1982; Cabanac and Richard, 1995; Cole et al., 1990; Koob and Heinrichs, 1999; Koob and Thatcher-Britton, 1985; Menzaghi et al., 1993; Morley and Levine, 1982; Murakami et al., 1989; Rivest and Richard, 1990; Vale et al., 1981). CRF and urocortins 1, 2, and 3 all reduce food intake when injected into the brains of obesity-prone rats fed an energy-dense diet indicating that obesity somewhat contrasts with the recent demonstration that a single session of stress could lead to an increase in body weight during the 9 days that follow the stress session in obesity-prone rats fed an energy-dense diet (Michel et al., 2003). The reasons for the difference in the metabolic effect of stress between the two studies are not known but can certainly relate to species differences. Also, the duration and the frequency of stress are unquestionably important factors in determining whether stress will promote or block fat accretion. Infrequent short-lasting periods (a few minutes) of stress probably have less catabolic effects than long-lasting exposures such as the ones used in the present study. Long-lasting exposures to stress activate the CRF system and are likely to cause stable catabolic actions if they are frequent. Duration and frequency of stress could however not be the sole factors to determine whether the effects of stress would be anabolic or catabolic. In fact, a single session of stress would favor fat deposition solely in obesity-prone rats fed an energy-dense diet indicating that

Fig. 3. The expression of CRF mRNA expression in the CeA between after the 25 days of protocol. Stress rats are represented by scattered bars and control rats by solid bars. Bars are means ± SE, ($n = 4$).

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Corticosterone (mmol/l)</th>
<th>Insulin (mmol/l)</th>
<th>Glucose (mmol/l)</th>
</tr>
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<tbody>
<tr>
<td><strong>AJ carbohydrates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>88.2 ± 22.6</td>
<td>0.066 ± 0.027</td>
<td>8.15 ± 0.72</td>
</tr>
<tr>
<td>Stress</td>
<td>114.9 ± 24.5</td>
<td>0.054 ± 0.012</td>
<td>8.20 ± 0.86</td>
</tr>
<tr>
<td><strong>AJ fat</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>91.7 ± 19.9</td>
<td>0.047 ± 0.007</td>
<td>8.44 ± 0.50</td>
</tr>
<tr>
<td>Stress</td>
<td>86.6 ± 21.2</td>
<td>0.031 ± 0.017</td>
<td>7.63 ± 0.64</td>
</tr>
<tr>
<td><strong>B6 carbohydrates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>62.0 ± 14.7</td>
<td>0.189 ± 0.117</td>
<td>9.06 ± 0.66</td>
</tr>
<tr>
<td>Stress</td>
<td>78.4 ± 26.5</td>
<td>0.072 ± 0.032</td>
<td>6.67 ± 0.50</td>
</tr>
<tr>
<td><strong>B6 fat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>79.8 ± 29.2</td>
<td>0.082 ± 0.020</td>
<td>10.15 ± 0.46</td>
</tr>
<tr>
<td>Stress</td>
<td>64.1 ± 23.4</td>
<td>0.061 ± 0.012</td>
<td>9.89 ± 0.33</td>
</tr>
</tbody>
</table>

Significant differences (ANOVA for multiple factors, $P ≤ 0.05$) are expressed in the lower cell of each variable.
genes and environment need to be propitious for a fat gain response. It is noteworthy that a single session of stress, such as that enhancing fat gain in obesity-prone rats, slows down fat deposition in obesity-resistant rats (Michel et al., 2003), suggesting that stress does not obligatory need to be long-lasting and frequent to be catabolic, which is in agreement with the work of Harris et al. (1998). Altogether, the present results and others from the literature on stress and obesity suggest that stress would be either catabolic especially if it is long lasting and frequent or anabolic in the presence of obesity genes in an obesogenic environment if it is short lasting and infrequent. However, the effects of chronic stress in obesity-prone rats fed energy-dense and palatable diets needs to be further investigated. From the results obtained in the present study, one would predict that obesity-prone rats exposed to stress would also deposit less fat than controls but perhaps to a lesser extent (Levin et al., 2000).

Another factor liable of influencing the metabolic response to stressors is the stress-stimulated activation of the HPA axis. Each of the stressful conditions used in the present study has been shown to acutely stimulate the secretion of corticosterone (Barr and Phillips, 1998; Monnikes et al., 1993), which has been reported to favor visceral fat deposition, inhibit thermogenesis, and enhance the ingestion of high-fat/high-sugar diet (referred to as comfort food by Dallman et al., 2003; Pecoraro et al., 2004). Despite all those reported effects, none of the anabolic effects of corticosterone was however apparent in the present study. Energy expenditure tended to be higher in stressed rats, the lowering effect of stress on fat depots was not preferentially seen in RWAT (a visceral depot) and stress did not induce the intake of the fat diet. The possibility that stressed mice have not been sufficiently exposed to elevated corticosterone levels cannot be excluded. Indeed, corticosterone levels measured 24 h following stress were similar among groups, which is not inconsistent with previously published results, which have shown that glucocorticoid levels often return readily to normal values after stress (Armario et al., 1985; Natelson et al., 1987). The present

![Fig. 4. Represents example of photomicrograph of the CRF mRNA in situ hybridization in the PVH and the CeA for each group. Solid bar = 100 μm with a 20× objective.](image-url)
study does not therefore invalidate the recent observation that stress can promote the ingestion of comfort food and the finding that such food can reduce the activity of the HPA axis (Dallman et al., 2003; Pecoraro et al., 2004) and attenuate the catabolic action to stress.

The lack of increase in expression of CRF mRNA in both the PVH and CeA 24 h after the last session of stress rules out the possibility that the CRF system could have been chronically activated by our stressful conditions. In contrast, stressed B6 and AJ mice had lowered expression of CRF mRNA in the CeA, which further suggests that mice were not chronically exposed to corticosterone in the present study as glucocorticoids increase the expression of CRF mRNA in the CeA (Dallman et al., 2003; Pecoraro et al., 2004). Reductions in CRF expression in the CeA have also been reported in anxious rats, which is consistent with the possibility that a reduced expression could be a compensatory effect to the acute overstimulation of the CRF system (Hwang et al., 2004). This reduced CeA expression of CRF could also be the result of a reduction in the fat gain and the leptin production (Costa et al., 1997). Leptin has been shown to enhance the expression of CRF mRNA in the CeA (Arvaniti et al., 2001).

In conclusion, this study demonstrates that the long-term exposure to daily stress does not facilitate or exacerbate obesity even in obesity-prone laboratory rodents. In fact, daily exposure to a 2-h stress session during 25 days led to a reduction in body fat in both obesity-prone and obesity-resistant mice. They confirmed that long-lasting chronic stress is more catabolic than anabolic in mice even when genes and environment are propitious to obesity.

References


