Stress facilitates body weight gain in genetically predisposed rats on medium-fat diet

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Submitted 6 February 2003; accepted in final form 17 June 2003

Michel, Chantal, Barry E. Levin, and Ambrose A. Dunn-Meynell. Stress facilitates body weight gain in genetically predisposed rats on medium-fat diet. Am J Physiol Regul Integr Comp Physiol 285: R791–R799, 2003. First published June 19, 2003; 10.1152/ajpregu.00072.2003.—To assess the interaction between stress and energy homeostasis, we immobilized male Sprague-Dawley rats prone to diet-induced obesity (DIO) or diet resistance (DR) once for 20 min and then fed them either low-fat (4.5%) chow or a medium-fat (31%), high-energy (HE) diet for 9 days. Stressed, chow-fed DIO rats gained less, while stressed DIO rats on HE diet gained more body weight and had higher feed efficiency and plasma leptin levels than unstressed controls. Neither stress nor diet affected DR body weight gain. While stress-induced plasma corticosterone levels did not differ between phenotypes, DIO rats were initially more active in an open field and had higher hippocampal dentate gyrus and CA1 glucocorticoid receptor (GR) mRNA than DR rats, regardless of prior stress or diet. HE diet intake was associated with raised dentate gyrus and CA1 GR and amygdalar central nucleus (CeA) corticotropin-releasing hormone (CRH) mRNA expression, while stress was associated with reduced hypothalamic dorsomedial nucleus Ob-R mRNA and CeA CRH specifically in DIO rats fed HE diet. Thus a single stress triggers a complex interaction among weight gain phenotype, diet, and stress responsivity, which determines the body weight and adiposity of a given individual.

BODY WEIGHT AND FOOD INTAKE are maintained within a relatively narrow range throughout life (19, 20, 29, 31, 56). Despite this fact, there is now an obesity epidemic in many developed countries of the world (55). The stresses of life in modern society may contribute to this increased incidence of obesity. In humans, psychological stress can facilitate abdominal fat accumulation (22, 58). However, most rodent studies suggest that severe stress induces anorexia and body weight loss (28, 50, 54). This difference between humans and rodents is largely unexplained. Most human obesity is inherited as a polygenic trait (9). In rats, diet-induced obesity (DIO) also appears to be inherited as a polygenic trait (40). Outbred Sprague-Dawley rats gain weight homogeneously when fed a low-fat chow diet from weaning. However, when fed a diet of moderate fat (31%) content [high-energy (HE) diet], one-half the rats develop DIO while the rest are diet resistant (DR). These weight gain phenotypes can be isolated by selectively breeding for the DIO and DR traits with an inheritance pattern that suggests a polygenic trait (40). Before the onset of their obesity, chow-fed DIO-prone rats show many abnormalities, such as reduced leptin sensitivity, altered sympathetic activity, and increased expression of hypothalamic neuropeptide Y mRNA (35, 36, 38, 39, 43). When such rats are selectively bred for the DIO and DR traits through many generations and fed either HE or a palatable diet, DIO rats are largely unresponsive to chronic, mild unpredictable stress, while DR rats lose weight and have elevated plasma corticosterone and paraventricular nucleus (PVN) corticotropin-releasing hormone (CRH) mRNA expression (42). This suggests that, compared with DR rats, selectively bred DIO rats are either hyporesponsive or quickly habituate, i.e., have reduced responses to the effects of chronic stress on energy homeostasis and the central and peripheral pathways mediating the stress response.

The hypothalamo-pituitary-adrenal (HPA) axis plays a pivotal role in the response to stress and the control of body weight and fat distribution. Stress increases PVN CRH release (45). This increases pituitary release of ACTH with subsequent increase in adrenal cortical release of glucocorticoids (49). Glucocorticoids then feed back to inhibit CRH and ACTH release (16) by acting primarily on glucocorticoid (GR) and mineralocorticoid (MR) receptors (10, 44). GR are most responsive to feedback inhibition by high levels of corticosterone in rats (2) and are expressed prominently in the hippocampus, a brain area that plays a role in learning and memory (30, 57), as well as the adaptive response to stress (51). Repeated stress and chronic elevation of glucocorticoids are associated with remodeling of dendrites of hippocampal pyramidal neurons (51), and this may contribute to habituation, possibly by reducing GR expression or function (49). On the other hand, acute stress may sensitize animals such that they have an increased response to subsequent stressors. This might occur at the level of hippocampal metabolism; hypothalamo-pituitary-adrenal axis; corticotropin-releasing hormone; food intake; glucocorticoid receptor; corticosterone; norepinephrine; leptin receptor

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GR and other stress-related neural systems to induce long-term alterations in body weight, neurohumoral function, and behavioral responses to a subsequent stressful event.

In light of our prior studies showing hyperresponsiveness and/or habituation to the effects of chronic repeated stress on energy homeostasis in selectively bred DIO rats (42), we postulated that outbred DIO-prone rats would have an inherent difference in their central pathways mediating acute stress responsivity, which would antedate their development of obesity. Because acute stress can have dramatic effects on subsequent body weight gain (28, 59), we also postulated that the response to a single acute stressor might lead to differential long-term effects on body weight gain between obesity-prone and -resistant rats. To test this hypothesis, we assessed the effect of a single stress in chow-fed outbred DIO- and DR-prone rats on their subsequent regulation of energy homeostasis and stress responsivity when fed either chow or an HE diet. In this case, outbred DIO-prone and DR rats were used since their propensity to be obesity prone or resistant can be predicted in adulthood, before the introduction of HE diet and the development of their weight gain phenotypes (34). Thus any differences in stress responsivity would reflect a preexisting trait, unaffected by diet or the presence of obesity.

METHODS

Animals. Male 3- to 4-month-old Sprague-Dawley rats (Charles River Labs, Kingston, NY) were used for this experiment. Animal usage was in compliance with the Animal Care Committee of the East Orange Veterans Affairs Medical Center (East Orange, NJ) and the American Physiological Society guidelines (3). Animals were fed Purina rat chow (no. 5001) ad libitum from their arrival and were housed on a 12:12-h light-dark schedule with lights on at 0800 and lights out at 2000. Purina rat chow contains 3.30 kcal/g with 23.4% as protein, 4.5% as fat, and 72.1% as carbohydrate, which is primarily in the form of complex polysaccharide (41). After a minimum of 1 wk adaptation, rats were placed in metabolic cages for collection of 24-h urine norepinephrine (NE) levels to separate DIO-prone from DR-prone rats (34). Initially, 84 rats were screened; the 32 with the highest NE levels (2.61 ± 0.20 ng·ml⁻¹·h⁻¹) were taken as DIO prone, and the 32 with the lowest NE levels (0.85 ± 0.14 ng·ml⁻¹·h⁻¹) were taken as DR prone (34).

Experimental protocol. At 1300, 16 DIO- and 16 DR-prone chow-fed rats were placed in a Plexiglas restrainer, and blood was quickly collected by tail nip for assay of basal plasma corticosterone (Basal). Restraint stress was performed in the presence of obesity.

After 9 days, the previously stressed rats were again stressed (Stress2) at 1300 with blood drawn 18 min into the restraint. We did not draw basal corticosterone levels before Stress2 because of the concern that too much stress would have an additive effect on the terminal GR and CRH mRNA measures.

On the 10th day, previously stressed rats had their openfield activity measured, in a randomized order, over a 15-min period between 1000 and 1400, in a 40 × 40 × 30.5-cm 16-beam Digiscan open-field apparatus (Omnitech). This was done to evaluate their anxiety level since stress has been shown to reduce the activity level (23, 53). Immediately after open-field testing, rats were decapitated, and brains were removed and quickly frozen on dry ice. Control rats were also killed on the 10th day with time of death randomized across all eight experimental groups.

Antisense 35S-labeled cRNA probes. The CRH cRNA probe was generated by high-performance liquid chromatography with electrochemical detection as previously described (34). Tail blood samples were collected into heparinized tubes, and the plasma was removed for corticosterone assay using a double-antibody radioimmunoassay kit (ICN Biomedicals).

In situ hybridization for CRH, GR, and leptin receptor (Ob-R) mRNA. Fresh frozen brains were cut in five sequential sets of 15-μm sections. Brain sections were mounted onto gel-coated slides that were then fixed for 20 min in paraformaldehyde (4%) and kept frozen at −80°C until they were assayed. In situ hybridization was used to localize CRH, GR, and Ob-R mRNA expression using modifications of previously described methods (33, 60). Briefly, brain sections were thawed for 15 min and then were placed in paraformaldehyde (4%) for 20 min, 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 hybridization mixture containing an antisense 35S-labeled cRNA probe (~10⁶ cpm/ml) were spotted on each slide. The slides were sealed under a coverslip and incubated overnight at 60°C in a water bath. The next day, the coverslips were removed, and slides were 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 of SSC (2×, 10 min; 1×, 5 min; 0.5×, 5 min; 0.1×, 10 min at 60°C), and dehydrated through graded concentrations of alcohol. After a 24-h period of vacuum drying, the slides were apposed on an X-ray film (Eastman Kodak, Rochester, NY) for a period of 2–21 days depending on the probe.

Antisense 35S-labeled cRNA probes. The CRH cRNA probe was generated from the 1.2-kb EcoRI fragment of rat CRH cDNA (D. Richard, Université Laval, Quebec, Canada) subcloned into a pGEM4 vector (Stratagene, La Jolla, CA) and linearized with HindIII and EcoRI for antisense and sense probes, respectively. The GR cRNA probe was generated from EcoRI fragment of rat GR cDNA (D. Richard) subcloned into a pGEM4 vector (Stratagene) and linearized with BamHI and XbaI for antisense and sense probes, respectively. The probe for all splice variants of the leptin receptor (Ob-R) was generated from EcoRI fragment of rat Ob-R cDNA (J. Elmquist, Harvard Medical School, Boston, MA) subcloned
into a pGEM-IIZF vector (Stratagene) and linearized with SacI and HindIII for antisense and sense probes, respectively. The specificity of the probes was confirmed by the absence of a positive signal in sections hybridized with sense probe. Radioactive riboprobes were synthesized by incubation of 250 ng linearized plasmid in 10 mM NaCl, ATP/GTP/CTP (0.55–0.64 mM), α-35S-UTP (1,000 Ci/mmol; New England Nuclear, Beverly, MA), 40 U Rnasin (Promega, Madison, WI) and 20 U SP6, T7, or T3 RNA polymerase for CRH antisense probes, 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 resulting autoradiograms were read using computer-assisted densitometry (Drexel) by an observer blinded to the experimental groups (38). 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. Sections (n = 2/rat) for hypothalamic PVN and central nucleus of the amygdala (CeA) CRH mRNA expression and hippocampal Gr mRNA expression were read at the rostral-caudal level of the midportion of the PVN. Sections for hypothalamic arcuate (ARC) and dorsomedial (DMN) nuclei Ob-R expression (n = 2/rat) were read through the rostral-caudal extent of the compact portion of the DMN.

Statistics. Body weights, food intake, feed efficiency, plasma leptin, and trunk blood corticosterone levels and mRNA expression data were analyzed using three-way ANOVA (phenotype × stress/no stress × diet). Corticosterone levels at the beginning and end of stress and from Stress1 to Stress2 were compared by two-way ANOVA (genotype × diet) with repeated measures. Open-field data for stressed rats were compared by two-way ANOVA (genotype × diet). If significant differences for a given parameter were found by ANOVA (P ≤ 0.05), intergroup comparisons were made by post hoc Bonferroni test. Because of the complexity of potential interactions, if marginal significant differences among factors were found by three-way ANOVA, significant difference for each factor alone was assessed separately by one-way ANOVA with post hoc Bonferroni comparisons.
alone. Indeed, stressed rats had an overall reduction in Ob-R mRNA expression in the DMN (Fig. 5) compared with unstressed groups [F(1,42) = 4.137, P = 0.048]. There were no significant phenotype-diet-stress main effects for Ob-R in the ARC, nor was there any significant correlation between plasma leptin levels and Ob-R expression in the DMN or ARC.

Open-field activity. Only rats previously subjected to immobilization stress were tested in the open-field apparatus. This test was performed to compare the anxiety levels of the stressed rats (22). There was a main effect of phenotype on open-field activity. Stressed DIO rats, regardless of their diet, had more horizontal activity during 7 min in an open field [F(1,28) = 4.23, P = 0.049; Fig. 6]. Overall, stressed DIO rats had more vertical activity than stressed DR rats [F(1,28) = 11.53; P = 0.002]. However, this was due to a main effect of diet [F(1,28) = 8.91; P = 0.001] because DIO rats on HE diet had more vertical activity than the other groups (Fig. 6).

Plasma corticosterone and brain CRH and GR mRNA expression. Basal concentrations of plasma corticosterone did not differ among the experimental groups (Table 1). As expected, corticosterone levels rose significantly after 20 min of restraint compared with basal levels [F(2,40) = 24.03, P = 0.001], but there were no phenotype-specific differences in this corticosterone response. Although no basal corticosterone levels were drawn on the 9th day after the first stress (Stress1), there were no intergroup differences in corticosterone levels among the four stressed groups after Stress2 (Table 1). Because no basal levels were drawn before Stress2, it was not possible to assess what effect prior Stress1 might have had on the relative increase in corticosterone levels after the second Stress2.

In the brain, there were no differences among the groups for PVN CRH mRNA expression, regardless of phenotype, stress, or diet (Fig. 7). Whereas PVN CRH expression tends to be highly correlated with plasma corticosterone levels, CeA CRH expression appears to be independent of plasma corticosterone levels (48). In keeping with this, CeA CRH mRNA expression showed a significant main effect of both diet and stress [F(1,38) = 18.52, P = 0.001]. In DIO rats on HE diet, CeA CRH mRNA expression was 244% higher in unstressed than stressed DIO rats (P = 0.006) and 209% higher than unstressed DIO rats on chow (P = 0.025; Fig. 7). GR mRNA expression in the CA2 and CA3 areas of the hippocampus was very low or not detect-
able, and there were no significant intergroup differences. However, there were significant main effects of phenotype and diet \( F(1,46) = 5.32, P = 0.026 \) on GR mRNA expression in the CA1 area and dentate gyrus of the hippocampus (Fig. 8). In the CA1 area, GR expression was 31% higher in DIO than DR rats and was 32% higher in rats fed HE diet than chow \( F(1,46) = 4.11, P = 0.048 \). There was also a tendency to 20% lower CA1 GR expression in stressed vs. control rats \( F(1,46) = 3.84, P = 0.056 \). There were, however, no significant differences by post hoc testing between all groups. Dentate gyrus GR mRNA also tended to be 125% higher in DIO than DR rats \( F(1,46) = 3.59, P = 0.064 \) and in rats fed HE diet than those fed chow \( F(1,46) = 3.43, P = 0.071 \), while stress had no effect. Again, there were no specific intergroup differences for dentate GR mRNA expression by post hoc testing. Finally, there were no significant intergroup differences in GR mRNA expression in the PVN by ANOVA (data not shown).

**DISCUSSION**

The main finding of this study is that outbred, obesity-prone DIO rats appear to be more responsive than DR rats to the diet-dependent effects of a single stress upon body weight gain. The prolonged response to a
Thus prior stress led to decreased feed efficiency, reduction in either food intake or apparent adiposity.

On the other hand, the same stress in chow-fed DIO rats was associated with reduced body weight gain without a change in food intake per kcal food intake) in DIO rats fed HE diet. On the other hand, the same stress in chow-fed DIO rats was associated with reduced body weight gain without a change in either food intake or apparent adiposity. Thus prior stress led to increased feed efficiency at the expense of food intake when DIO rats were fed a low-fat diet. This suggests that energy expenditure was reduced by prior stress when stressed DIO rats were fed HE diet and increased when they were fed chow.

In addition, DIO rats, as a phenotype, had elevated expression of GR mRNA in the hippocampal CA1 area and a stress- and diet-dependent reduction in CeA CRH mRNA expression. Finally, stressed DIO rats fed HE diet also showed increased open-field activity, suggesting that they were less anxious than other groups of stressed rats (22). Unlike the DIO rats, neither stress nor diet affected DR weight gain, feed efficiency, apparent adiposity, open-field activity, CRH, or GR mRNA expression. On the other hand, a single bout of restraint stress was associated with reduced DMN Ob-R mRNA and a tendency toward lower CA1 GR mRNA expression in both DIO and DR rats, regardless of dietary exposure. These data suggest that a single stress may have a long-lasting effect on neural systems involved in stress and energy homeostasis in both DIO and DR rats but that energy homeostasis is altered by the stress-diet interaction only in DIO rats.

This specific effect of a single stressor predominantly on DIO body weight gain is in distinct contrast to our previous study in selectively bred DIO rats (42). Those DIO rats were first made obese and then subjected to chronic unpredictable stress while they were fed sequentially on HE diet and then a highly palatable liquid diet. As opposed to selectively bred DR rats that lost weight and had chronic HPA activation, obese DIO rats had little response to chronic stress (42). The difference in outcome of these two studies demonstrates the complex interactions between the control of energy homeostasis and stress (42). There are several reasons why obese DIO rats on an HE diet might be more responsive to the physiological and metabolic effects of chronic unpredictable stress, while obesity-prone DIO rats appear to be more readily sensitized to the diet-dependent effects of a single stressor on energy homeostasis. First, chronic stress is likely to alter the diurnal rhythm of animals, and the time at which stress is administered during the day can be a major determinant of the long-term effects of stress on body weight (1, 59). The chronically stressed DIO and DR rats in our prior study were stressed twice a day during the light phase (42), while the rats in the current study were stressed once at midnight cycle. Second, dietary factors (macronutrient content, caloric density, length of exposure) and/or the presence of obesity can have major effects on stress responsivity (61, 62). Finally, animals can habituate to chronic stress, but such habituation is very dependent on the strength, duration, frequency, pattern, prior exposure and age of subject (25). The methods and parameters used to assess the physiological response to habituation are also critical (52). While sensitization leads to increased responsivity to subsequent stress, it too is dependent on many of the same factors that modulate habituation and on the particular outcome measure assessed (7, 13, 26). Thus the common thread between our prior (42) and present studies is that DIO rats differ markedly from DR rats in their overall responsivity to stress. While DIO rats might be less responsive or habituate more readily to the weight-reducing effects of chronic stress, they are clearly more responsive to the sensitizing effects of a single stress on weight gain in the context of differing dietary composition.

Both the present and previous studies (42) demonstrate the important interactions between central pathways mediating stress responsivity and those regulating energy homeostasis. Brain areas such as the ARC and DMN contain neurons that play critical roles...
in the regulation of energy homeostasis (5, 17, 37). They also contain leptin receptors that allow the brain to monitor and control adipose stores (4, 21). Lesions that disconnect the ARC from the PVN alter both stress responsivity and energy homeostasis (5). It is well described that adrenalectomy reduces adiposity in various rodent models of obesity (12, 63). One potential explanation is that adrenalectomy leads to corticosterone-dependent, constitutive activation of the long form (signaling) of the leptin receptor (Ob-Rb) in the hypothalamus (46). In the current study, we found that prior stress was associated with reduced expression of mRNA coding for all splice variants of the leptin receptor in the DMN but not ARC. Thus the elevated levels of corticosterone that occurred during the first stress might be implicated in a long-term depression of DMN Ob-R expression. Importantly, the DMN, like the ARC, has also been implicated in both energy homeostasis (6, 8, 27) and stress responsivity (18). These findings must be interpreted with caution, however, as we did not directly assess the expression of the signaling form (Ob-Rb) of the leptin receptor and because mRNA expression does not necessarily reflect receptor function. In addition, rats were stressed a second time with both restraint and open-field testing before terminal assessment of Ob-R expression.

The interaction of stress with genotype and diet was also apparent in the central pathways involved in stress responsivity. DIO rats had a phenotype-dependent increase in hippocampal CA1 and a tendency for elevated dentate GR expression, which were independent of stress and diet. This was selective for the hippocampus as there were no intergroup differences in PVN GR expression. Thus increased hippocampal GR expression appears to be a phenotypic trait of obesity-prone DIO rats. Given the complexity of the feedback systems in which hippocampal GR systems are involved and the lack of difference in DIO and DR corticosterone responses to acute stress, it is difficult to predict how this underlying GR increase might alter DIO energy homeostasis in response to acute or chronic stress.

As with hippocampal GR expression, CRH mRNA expression showed site-specific differences among groups. There was a selective reduction in CeA CRH expression in stressed vs. nonstressed DIO rats on HE but not chow diet. As with PVN GR expression, PVN CRH expression was unaffected by genotype, stress, or diet. This differential regulation of CRH in the CeA vs. the PVN is well described. The CeA is associated with the psychological component of stress (46), and the behavioral changes resulting from the stimulation of the CeA appear to be independent of stress-induced HPA axis activation (11, 48). Psychological stress increases CeA but not PVN CRH mRNA (46), and CeA CRH mRNA expression can be increased by corticosterone, while PVN CRH is decreased (47). Behaviorally, stress is generally associated with reduced exploratory activity as a function of anxiety (23). Interestingly, stressed DIO rats on HE diet actually had lower CeA CRH expression than stressed DIO rats on chow. Because psychological stress would be expected to increase CeA CRH, this reduction might be due to the salutary effects of a relatively high-fat diet on the physiological and behavioral responses to stress (14), interacting with the genotype-specific neural circuitry of the DIO rats. However, it is uncertain whether the diet- and stress-dependent differences in CeA CRH expression played any role in the overall increase in open-field activity in DIO rats. Whatever the relationship, their increased open-field activity suggests reduced anxiety that may lead to a reduced stress responsiveness such as we previously documented in selectively bred obese DIO rats (42).

In summary, there is a complex interaction between the effects of stress and diet on energy homeostasis in DIO vs. DR rats. On the one hand, DIO rats appear to be less sensitive or to quickly habituate to the effects of chronic unpredictable stress on energy homeostasis (42). On the other hand, DIO rats have a diet-dependent sensitization to the effects of a single stressor on energy homeostasis. It is unlikely that corticosterone was a critical determinant of their differences in energy homeostasis after a single stress. However, their differences in hippocampal GR expression suggest that DIO rats have a preexisting alteration in central pathways mediating stress responses. This finding may have important implications for the hyporesponsiveness of DIO rats to chronic stress (42). Of course it is important to keep in mind that mRNA expression does not necessarily predict the amount of neuropeptide released at a synapse or the function of a given receptor. Clearly, our studies leave a number of questions unanswered as to how these various findings fit together to explain why stress and diet have such critical effects on energy homeostasis in DIO and DR rats. Nevertheless, they do demonstrate that the DIO model is a fruitful one for the further exploration of the ways in which stress alters the regulation of energy homeostasis in obesity.

We thank C. Salter, E. Dziopa, O. Gordon, and A. Moraischvili for expert technical assistance.

DISCLOSURES

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1-30066, the Research Service of the Department of Veterans Affairs, and a postdoctoral fellowship award from Merck Research Laboratories.

REFERENCES


