Glucocorticoid-dependency of increased adiposity in a model of hypothalamic obesity

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Abstract
It is known that rats treated, at neonatal age, with monosodium L-glutamate (MSG) develop neuroendocrine and metabolic abnormalities, resulting in a phenotype of hypothalamic obesity, characterized by increased adiposity, corticosteronemia and leptinemia.

OBJECTIVE: We explored whether adrenal manipulations could result in the reversion of this phenotype of hypothalamic obesity.

EXPERIMENTAL DESIGNS: Newborn male rats, treated with MSG or vehicle (CTR), were submitted to sham operation, bilateral adrenalectomy (ADX) or bilateral adrenal enucleation (AE) on day 120 of age. Animals were examined 21 days after ADX, combined or not with corticosterone (B) substitution (ADX+B), and on days 21 and 35 after AE. Food intake, body weight and body fat mass were monitored; additionally circulating levels of insulin, leptin, ACTH and B were measured.

RESULTS: Our data indicate that: a) normalization of basal B circulating levels in, 21 day-ADX and -AE, MSG rats fully reversed hyperinsulinemia, hyperleptinemia and significantly decreased body fat mass; and b) recovery of hypercorticosteronemia in, 35 day-AE, MSG rats fully restored this phenotype of hypothalamic obesity.

CONCLUSION: Our study strongly supports that high glucocorticoid production is the main factor responsible for the development of enhanced adiposity in MSG rats and, importantly, that this abnormality could be reversed by an appropriate therapy.
Introduction

Bi-directional interplay between adipose tissue and neuroendocrine functions is now widely accepted. In particular, hypothalamic-corticotrope-adrenal (HCA) axis activity is strongly suggested as a main regulator of leptin expression and secretion [1, 2]; in an opposite way, the adipocyte product is known to inhibit ACTH-elicited glucocorticoid secretion [3]. Hypothalamic obesity could be the result of site-dependent hypothalamic malfunctions. In fact, paraventricular nuclei (PVN) loss results in a phenotype of obesity characterized by increased food intake, while ventromedial nuclei (VMN) lesion develops obesity independently of increased food intake [4, 5].

Neonatal administration of rats with monosodium L-glutamate (MSG), a treatment able to induce arcuate nuclei (ARC) ablation [6–8], generates adult hypophagic obese animals bearing reduced both orexigenic and anorexigenic, NPY and POMC respectively, signals [8–10]. It is known that the ARC is a pivotal target involved in the regulatory process of the organism’s energy storage/expenditure [11], whose mechanisms include adipose tissue leptin secretion and the leptin signaling system acting at the hypothalamic level. Regarding the effects of MSG treatment on the hypothalamo-adipocyte axis function, it has been reported that MSG-treated rats develop increased adiposity and hyperleptinemia by 90 days of age [12]; and that adult MSG animals treated with exogenous leptin, as it happens with other model of hypothalamic obesity [13], are partly refractory to the leptin inhibitory effect on food intake and body weight gain [14]. In addition, MSG-injected animals develop at adult age non-insulin dependent diabetes by a mechanism due to insulin resistance. Regarding the effects of neonatal MSG treatment on HCA axis activity, up today, it is well known that adult obese rats present, in vitro [15] and in vivo [16], over-activity of this neuroendocrine axis and that, recent data from our laboratory, a transient decrease in glucocorticoid circulating levels, in MSG-treated male rats, restored adrenocortical sensitivity to the leptin inhibitory effect on ACTH-elicited corticosterone secretion [17].

The aim of the present study was to test the hypothesis that enhanced glucocorticoid production is the key element characterizing increased adiposity in the ARC-damaged male rat model of obesity. Adrenal manipulations, in normal and hypothalamic obese rats, are proposed as the main tool to demonstrate that this phenotype of obesity is developed indirectly after the neonatal lesion of the central nervous system.

Materials and Methods

Animals and treatment. Adult male (300–330 g BW) and female (240–280 g BW) Sprague-Dawley rats were allowed to mate in colony cages in a light (lights on from 07:00 to 19:00 h)- and temperature (22 °C)-controlled room. Rat chow and water were available ad libitum. Pregnant rats were transferred to individual cages. Beginning on day 2 after parturition, newborn pups were injected i.p. with either 4 mg/g BW MSG (Sigma Chemical CO, St. Louis, MO) dissolved in sterile 0.9% NaCl or 10% NaCl (litter-mate controls; CTR) once every two days and up to day 10 of age [18]. Rats were weaned and sexed at 21 days of age; daily body weight and food intake of individual male rats were recorded up to the experimental day. CTR and MSG rats were used for experimentation on, approximately, day 150 of age. MSG-injected animals were screened for effectiveness of treatment by macroscopic observation of degeneration of the optic nerves at the time of sacrifice.

Animals were killed by decapitation according to protocols for animal use, in agreement with NIH Guidelines for care and use of experimental animals. All experimentation received approval from our Institutional Animal Care Committees.

Experimental designs.

CTR and MSG, 120 day-old, animals were submitted, after light ketamine anesthesia, to bilateral adrenalectomy (ADX) or adrenal enucleation (AE), by the dorsal approach. ADX rats received a subcutaneous pellet containing either placebo (ADX) or 75 mg corticosterone (Innovative Research of America, Toledo, Ohio, USA) (ADX+B) for 21 days [19], they drank 0.9% NaCl up to the experimental day. AE was performed as previously described [20]. Briefly, each adrenal was exposed and a puncture was made in the capsule, squeezing the gland gently with forceps, leaving the capsule intact, and then extruded the parenchyma. AE rats drank 0.9% NaCl for the first 4 days after sur-
Surgery. Sham-operation was performed by exposing both adrenals without touching the glands or their pedicles. Twenty one (adrenalectomized rats) or 35 (enucleated rats) days post surgery (approximate age of 140–150 days) animals were killed by decapitation and trunk blood, collected into plastic tubes containing 0.2 ml EDTA 10 g%, was immediately centrifuged for further determination of plasma concentrations of ACTH, total corticosterone (B), free corticosterone (FB), corticosteroid binding globulin (CBG), insulin and leptin. Thereafter, total fat (TF; the sum of omental, epididymal, and retroperitoneal) pads from animals of both experimental groups were dissected and weighted.

Assays. Circulating ACTH concentrations were measured by a previously described immunoradiometric assay [21] with a standard curve between 15 and 3,000 pg/ml and with intra- and inter-assay coefficients of variation (CVs) of, respectively, 2–3 and 6–8%. Plasma concentrations of B were evaluated by a specific radioimmunoassay (RIA) earlier reported [22] with a standard curve between 1 and 250 µg/dl and intra- and inter-assay CVs of respectively 4–6 and 8–10%. Plasma corticosteroid binding globulin (CBG) concentrations were measured as earlier reported [23]. Briefly, 50 µl steroid-stripped serum sample, appropriately diluted in assay buffer, was incubated overnight (4 C) with graded concentrations of cold B (200 µl) in the presence of 100,000 cpm in 50 µl of assay buffer; the separation of bound and free hormone fractions was achieved by the addition of 0.1 ml charcoal (1 g%)-dextran T 70 (0.1 g%). Bound radioactivity was determined in a Tracor Analytic Scintillation System. Analysis of binding data for further determination of the concentration and affinity of circulating CBG and calculation of plasma free corticosterone (FB) levels were performed as previously reported [24].

Insulin circulating levels were determined by a specific rat RIA previously developed in our laboratory [25]; the standard curve ranged from 0.1 to 10 mg/ml, CVs intra- and inter-assay were of 3–7 and 8–11%, respectively. Finally, plasma leptin concentrations were determined by a specific RIA from our laboratory [26], the standard curve ranged between 0.4–50 ng/ml, CVs intra- and inter-assay of 5–8 and 10–13%, respectively.

Statistics. Results were expressed as mean ± SEM. Means were analyzed by ANOVA, with multiple comparisons, followed by the Fisher’s test [27]. Values of p < 0.05 were considered statistically significant.

**Results**

**Body weight values and food intake in normal and hypothalamic-damaged rats of different ages**

Table 1 shows body weights values and daily food intake in normal (CTR) and MSG-lesioned rats of different ages (60, 90 and 120 days). While CTR rats displayed normal increases in body weights with age, MSG rats were significantly (p < 0.05) lighter than their normal counterparts (CTR) at all ages examined. As described in this animal model, MSG rats were hypophagic after comparison with the amount of food eaten by their age-matched counterparts; in fact the grams of food consumed during 24 hours was significantly (p < 0.05) lower in MSG than in CTR rats, regardless of the day examined. Even more, MSG rats were hypophagic from weaning up to the experimental day (data not shown).

Important to denote is that MSG rats were also characterized by enhanced adiposity (see below), reduced weight of the anterior pituitary, decreased nasal-anal length, self-mutilation and typical degeneration of the optic nerves, when observed at the time of sacrifice.

**Effects of adrenalectomy and corticosterone replacement therapy on HPA and adipo-insular axes functions in normal and hypothalamic obese rats**

Figure 1 and Table 2 show the results of circulating levels of ACTH (Fig. 1A) and corticosterone (in its total and free forms, Fig. 1B and Table 2, respectively) in different groups of experimental animals. As depicted, ADX for 21 days similarly increased several fold, over the respective baseline values, plasma ACTH concentrations in CTR and MSG rats; this effect was fully abrogated by B replacement therapy (ADX+B) in both groups of rats. MSG rats were hyper-corticosteronemic in basal condition (MSG SHAM group) and, while ADX alone removed all detectable amount of B in circulation, corticosterone replacement therapy restored B plasma levels to values similar to those of observed in CTR SHAM rats, regardless of whether the hypothalamic lesion was performed or not. Figure 1 shows also the results of circulating insulin (panel C) and leptin (panel D) levels in different groups of experimental animals. ADX alone or combined with corticosterone replacement therapy did not modify circulating insulin concentrations in CTR rats. Although MSG SHAM rats were hyperinsulinemic, vs. CTR SHAM rats, ADX blunted hyperinsulinemia, even to values lower than those of CTR ADX rats; restoration of low circulating B levels in MSG ADX rats (ADX+B) completely reversed the effect of ADX alone, although values attained were still lower (p < 0.05) than those of MSG SHAM rats. Important to denote is that no group differences were noticed in basal plasma glucose levels, regardless of whether ADX ADX+B were performed (data not shown). Circulating leptin levels in CTR rats

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Group</th>
<th>Body Weight (g)</th>
<th>Food intake (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>CTR</td>
<td>251.4 ± 6.9</td>
<td>20.74 ± 0.78</td>
</tr>
<tr>
<td></td>
<td>MSG</td>
<td>200.9 ± 5.1</td>
<td>16.41 ± 1.11</td>
</tr>
<tr>
<td>90</td>
<td>CTR</td>
<td>316.4 ± 12.3</td>
<td>23.61 ± 0.89</td>
</tr>
<tr>
<td></td>
<td>MSG</td>
<td>269.7 ± 6.1</td>
<td>14.96 ± 1.27</td>
</tr>
<tr>
<td>120</td>
<td>CTR</td>
<td>333.2 ± 9.3</td>
<td>18.17 ± 0.97</td>
</tr>
<tr>
<td></td>
<td>MSG</td>
<td>269.7 ± 6.7</td>
<td>14.82 ± 1.37</td>
</tr>
</tbody>
</table>

*: p < 0.05 vs. CTR values at same age.

Table 1. Body weight values and food intake in, control (CTR) and MSG-damaged, male rats of different ages. Values are the mean ± SEM (n = 9–13 rats per group).
were significantly (p < 0.05) decreased by ADX alone, an effect fully reversed by B therapy (ADX+B rats). As expected, MSG SHAM rats were hyperleptinemic, vs. CTR SHAM animals, and ADX significantly (p < 0.05) reduced plasma leptin levels, although with values still significantly (p < 0.05) higher than CTR rats in similar condition; B replacement therapy in ADX rats, although partially prevented the effect induced by ADX alone, was not able to fully restore circulating leptin concentrations to values obtained in MSG SHAM rats, but these values were undistinguishable from those in CTR ADX+B rats.

Changes in BW values induced by ADX and ADX+B in CTR and MSG rats are also depicted in Table 2. As shown, MSG were lighter (p < 0.05) than CTR rats, regardless of the experimental condition. ADX for 21 days decreased (p < 0.05) BW values in both groups of rats, an effect fully reversed by B therapy in both groups.

**Effects of adrenal enucleation for different periods on HPA and adipo-insular axes functions in CTR and MSG rats.**

Table 2. Basal circulating levels of corticosteroid binding globulin (CBG), and their respective Kd values, and of free corticosterone (Free B) concentrations in different groups of control (CTR) and MSG-damaged male rats. SHAM values included in this table are those combined from SHAM animals used in both experimental designs (sham-adrenalectomy and -enucleation). Values are the mean ± SEM (n = 6-12 rats per group).

<table>
<thead>
<tr>
<th>Surgery/Treatment</th>
<th>Group</th>
<th>CBG (nM)</th>
<th>Kd (nM)</th>
<th>Free B (ng/dl)</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>CTR</td>
<td>156 ± 28</td>
<td>4.63 ± 0.91</td>
<td>114 ± 17</td>
<td>331.2 ± 8.5</td>
</tr>
<tr>
<td></td>
<td>MSG</td>
<td>309 ± 41</td>
<td>4.59 ± 0.88</td>
<td>238 ± 19</td>
<td>271.7 ± 5.9</td>
</tr>
<tr>
<td>ADX</td>
<td>CTR</td>
<td>628 ± 57</td>
<td>5.37 ± 1.01</td>
<td>ND</td>
<td>298.8 ± 8.4</td>
</tr>
<tr>
<td></td>
<td>MSG</td>
<td>587 ± 69</td>
<td>6.53 ± 1.19</td>
<td>ND</td>
<td>238.6 ± 11.1</td>
</tr>
<tr>
<td>ADX + B</td>
<td>CTR</td>
<td>213 ± 16</td>
<td>3.27 ± 0.83</td>
<td>129 ± 18</td>
<td>311.9 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>MSG</td>
<td>228 ± 19</td>
<td>3.16 ± 0.66</td>
<td>111 ± 13</td>
<td>249.7 ± 7.9</td>
</tr>
<tr>
<td>21 day-AE</td>
<td>CTR</td>
<td>142 ± 18</td>
<td>5.23 ± 0.98</td>
<td>167 ± 39</td>
<td>338.5 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>MSG</td>
<td>295 ± 37</td>
<td>4.67 ± 0.86</td>
<td>105 ± 16</td>
<td>263.6 ± 8.1</td>
</tr>
<tr>
<td>35 day-AE</td>
<td>CTR</td>
<td>162 ± 21</td>
<td>4.29 ± 0.78</td>
<td>94 ± 11</td>
<td>343.2 ± 10.3</td>
</tr>
<tr>
<td></td>
<td>MSG</td>
<td>364 ± 31</td>
<td>5.11 ± 0.95</td>
<td>261 ± 61</td>
<td>275.7 ± 6.9</td>
</tr>
</tbody>
</table>

*, p < 0.05 vs. CTR values in similar condition.
+, p < 0.05 vs. SHAM values of the same group.
#, p < 0.05 vs. 21 day-AE MSG values.
ND, non detectable.

Effects of adrenal manipulations on total body fat content and on the relation of leptin secretion and adiposity in normal and hypothalamic obese rats

Figure 3 shows total body fat (TF) contents in different groups of rats. TF was significantly (p < 0.05) higher in MSG than in CTR rats 21 days after SHAM operation (Fig. 3A). While 21 days after ADX TF was slightly, although significantly (p < 0.05), reduced in CTR rats (Fig. 3A), a robust effect of ADX has taken place in MSG on this period; in fact TF was significantly (p < 0.05) reduced vs. SHAM levels, leptinemia in MSG rats at 21 days post-surgery; interestingly, hyperleptinemia was fully restored on day 35 post-AE.

The effects of AE, for different periods, in BW values in CTR and MSG animals are shown in Table 2. Again, MSG were lighter (p < 0.05) than CTR animals, regardless of the experimental condition, and AE did not modify BW values in both groups.
Fig. 1 (right). Basal circulating levels of ACTH (A), corticosterone (B), insulin (C) and leptin (D) in control (CTR, white bars) and hypothalamic obese (MSG, black bars) rats on day 21 after either sham operation (SHAM ADX), bilateral adrenalectomy (ADX) or ADX combined with corticosterone therapy (ADX+B). Bars are the mean ± SEM (n = 8-11 rats per group).

*, p < 0.05 vs. values of the respective SHAM group.
+, p < 0.05 vs. values of the respective ADX group.
#, p < 0.05 vs. CTR values in similar condition.

Fig. 2 (right). Basal circulating levels of ACTH (A), corticosterone (B), insulin (C) and leptin (D) in control (CTR, white bars) and hypothalamic obese (MSG, black bars) rats either sham operated (SHAM ENUC) or bilateral adrenal enucleation for 21 (21 d-AE) or 35 (35 d-AE) days. Bars are the mean ± SEM (n = 8-11 rats per group).

*, p < 0.05 vs. values of the respective SHAM group.
#, p < 0.05 vs. CTR values in similar condition.
ϕ, p < 0.05 vs. values of the respective 21 d-AE group.

Fig. 3 (above). Total fat (TF) content in different groups of control (CTR, white bars) and hypothalamic obese (MSG, black bars) rats after either ADX alone or combined with corticosterone therapy (A) or submitted to bilateral adrenal enucleation (B) for 21 or 35 days. Basal circulating leptin concentrations, expressed in ng/ml of plasma per grams of TF, after adrenalectomy (C) or bilateral adrenal enucleation (D) in CTR and MSG animals are also depicted. Bars are the mean ± SEM (n = 8-11 rats per group).

#, p < 0.05 vs. CTR values in similar condition.
*, p < 0.05 vs. values of the respective SHAM group.
+, p < 0.05 vs. values of the respective ADX group.
ϕ, p < 0.05 vs. values of the respective 21 d-AE group.
It has been previously reported [28], in MSG damaged mice, that the major cause of this phenotype of hypothalamic obesity is dependent on constant thermoregulation at a low body temperature. Moreover, these authors described that ADX, in MSG-treated mice, is able to enhance body temperature thus allowing to increase thermogenic activity and, as a consequence, to prevent the development of enhanced adiposity [28]. However, in the present study we were able not only to reverse fat deposition by ADX but also to demonstrate that the development of adiposity takes place by a mechanism due to a glucocorticoid molecular basis; in fact, we found that the spontaneous establishment of high, but not normal, circulating levels of this steroid is able to enhance TF content. Moreover, in our experimental designs, by blunting B production, after ADX, hypothalamic-corticotrope response remained normal (by comparing to that developed by CTR rats) and, interestingly, the ablation of adrenal glands in MSG rats also was able to decrease both hyperinsulinemia and hyperleptinemia. Importantly, restitution of low constant circulating B concentrations in ADX animals, a treatment similarly effective for normalizing circulating ACTH levels in both non-obese and obese rats, significantly enhanced circulating insulin concentrations (vs. ADX rats) in MSG animals, although not reproducing the hyperinsulinemic state characterizing MSG SHAM rats. It was earlier reported that only fasting MSG rats, for 24 hours, could result in the lowering of high circulating levels of insulin in this model [29]. We found that the effect of ADX has taken place not only on insulin but also on leptin plasma levels; in fact, ADX rats from both groups displayed very low leptin levels (although MSG remained with higher adipokine concentrations in circulation than CTR rats). The effect of ADX on lowering circulating leptin levels has been already shown by a pioneering report from our team [2], in which ADX-induced high ACTH output seems to be the main responsible molecular effect to decrease adipocyte ob production; moreover, this effect has been recently supported by in vitro adipose tissue experiments [30].

Additional experiments, performed after bilateral adrenal enucleation, not only did reproduce the glucocorticoid dependency of adiposity after the transient lowering in glucocorticoid concentrations in circulation, but also further support that allowing adrenals to regenerate in MSG animals, rendering in either moderate or high circulating B concentrations in plasma, depending on the time elapsed after AE; as a consequence, normalization of insulin and leptin concentrations in plasma occurred on day 21 after AE and, on day 35 post-AE, when almost full adrenal regeneration takes place in AE MSG rats [31], high basal levels of circulating glucocorticoids are re-established, coinciding with elevated circulating concentrations of both insulin and leptin, being similar to those observed in SHAM MSG rats.

Regarding to insulin production in MSG animals, it is known that low glucose concentration-stimulated insulin output by isolated pancreatic islets from MSG animals is significantly increased [31], probably resembling the hyperinsulinemia found in the circulation of MSG rats in non-fasting condition. This over production of insulin has been attributed to a compensatory enhancement of the parasympathetic tonus in MSG rats [31, 32]. In addition, some other effects could be related, besides to the hyperinsulinemic state, it is known that MSG rats are insulin-resistant [33]. This effect can be explained by an altered balance of the parasympathetic and sympathetic nervous activity [34]; in fact, besides the enhanced parasympathetic tone developed by MSG rats [31, 32], these animals, as observed in human obesity, also develops diminished adrenomedullar epinephrine secretion [35].

Results and interpretations from our study could be strongly differentiated from those previously reported by using, hyperphagic, leptin-deficient mice (ob/ob) [36, 37] and spontaneously leptin-resistant [38] (fa/fa) rats. In fact, and conversely to our hypophagic MSG rats, the above mentioned hyper-corticosteronemic animals did develop an obese phenotype directly related to increased daily food intake in the absence of either leptin synthesis or functionality, respectively. MSG rats did synthesize leptin and, depending on the metabolic status, their Ob-Rb could be functionally full active. Recent data from our laboratory [17] demonstrated that adrenal glands from AE MSG rats, characterized by low leptin circulating levels, are fully responsive to the in vitro inhibitory effect of exogenous leptin on ACTH-elicited glucocorticoid production. We now show that MSG rats also decrease the adipogenic process under two clear circumstances: first, in the absence of circulating B levels (ADX) and, second, in the transient state of low circulating levels of glucocorticoid (21 d-AE animals). However, when full regeneration of adrenal glands is reached, now characterized by hypercorticosteronemia, adipogenesis is fully allowed
to run up to reach TF mass similar to that observed in SHAM MSG rats.

Finally, and by analyzing TF contents and the particular expression of circulating leptin concentrations (concentrations expressed by grams of individual TF), it results fully clear that: a) the development of increased adiposity is directly related to the rate of glucocorticoid circulating levels, and b) the concentration of relative, to TF, leptin in circulation is fully dependent on changes in TF mass induced by adrenal manipulations.

In summary, our data strongly support that neonatal damage of the ARC is able to induce in vivo HCA hyperactivity, namely enhanced glucocorticoid production, thus resulting in an obese phenotype characterized by hyperadiposity, hyperinsulinemia and hyperleptinemia; this phenotype, although permanently installed after hypothalamic damage, could be however reversed by an appropriate therapy related to the use of central [39] and/or peripheral [38] antagonists of HCA axis function.

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