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Prolonged but Not Short Negative Energy Condition Restored Corticoadrenal Leptin Sensitivity in the Hypothalamic Obese Rat

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Key Words
Leptin · Hypothalamo-pituitary-adrenal axis · *ob*-Rb gene expression · Food restriction

Abstract
Background/Aim: We have reported that neonatal treatment with monosodium L-glutamate (MSG), which causes damage to the arcuate nucleus, leads to severe hyperleptinemia and reduced adrenal leptin receptor (*ob*-Rb) expression in adulthood. As a result, rats given MSG neonatally display corticoadrenal leptin-resistance, a defect that is overridden by normalization of corticoadrenal hyperfunction. The aim of the present study was to determine whether negative energy conditions could correct corticoadrenal cell dysfunction in rats given MSG neonatally. ***Methods:*** Normal (CTR) and MSG-treated female rats were subjected to food removal for 1–5 days, or prolonged (24–61 days) food restriction (FR). Plasma levels of several biomarkers and in vitro corticoadrenal function were evaluated following starvation or FR. ***Results:*** Fasting for 1–5 days reduced plasma leptin levels in CTR and MSG rats, compared to levels in the respective groups fed ad libitum ($p < 0.05$), but adrenal leptin-resistance was unchanged. With prolonged FR, isolated adrenal cells from MSG rats became sensitive to leptin, which lowered ACTH-induced glucocorticoid release. This restoration of leptin response was associated with normalization of adrenal *ob*-Rb gene expression. ***Conclusion:*** Dietary restriction in some leptin-resistant obese phenotypes may normalize adrenocortical function.

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Introduction

Neonatal administration of monosodium L-glutamate (MSG) damages neurons in the hypothalamic arcuate nucleus (ARC) [1–3], leading to behavioral, morphological, neuroendocrine and metabolic changes which persist in adulthood [2–7]. Thus, neonatal MSG treatment leads to self-mutilation [1], hypogonadism [1], enhanced hypothalamo-pituitary-adrenal (HPA) axis activity [4, 8–12], growth disturbances [1], hypophagia [13] and a paradoxical hyperadiposity [14]. The HPA axis is important in the maintenance of homeostasis, and the changes caused in this axis by neonatal MSG treatment have been attributed to dysfunctions of several hypothalamic systems [15–17], which control pituitary and adrenal function [11]. Leptin is a satiety factor [18], but also inhibits ACTH-dependent adrenal glucocorticoid production [19]. Circulating leptin

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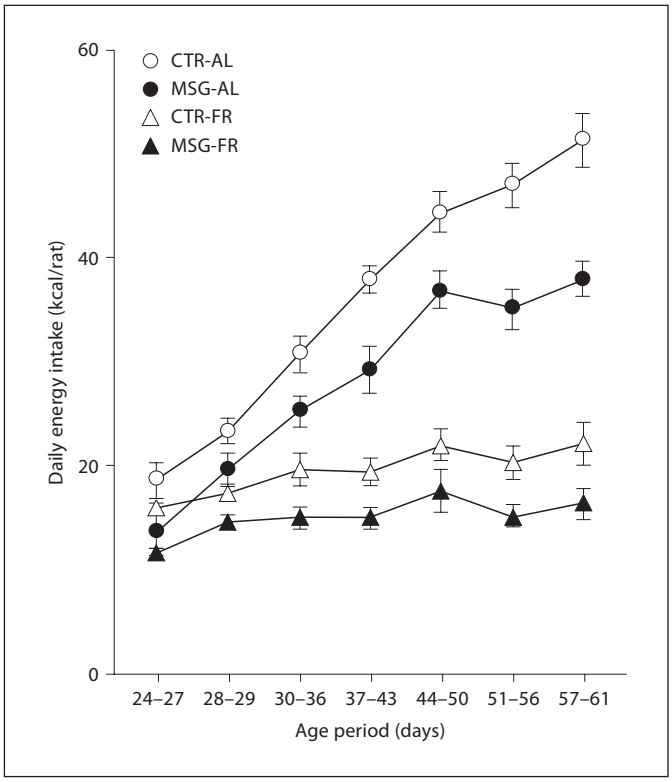


Fig. 1. Energy intake (in kcal) in fed ad libitum (AL) and food-restricted (FR) conditions in CTR and MSG rats at different age periods. Values are the means ± SEM (a representative experiment, with n = 7–9 rats per group/age/condition).

levels are increased in rats treated with MSG neonatally [4] and this could be the cause of up-regulation of adrenal function in these animals. The expression of *ob*-Rb is reduced in the adrenal gland of female rats receiving neonatal MSG treatment [20] and this could be a cause of enhanced corticoadrenal activity. In other words, elevated circulating levels of free corticosterone [20] may be due, at least in part, to leptin-resistance of the adrenal gland [20]. Recent work from our laboratories described adrenal leptin-resistance in adult rats treated with MSG neonatally, which can be overridden after normalization of circulating glucocorticoid levels [4]. Our working hypothesis was that chronic hyperleptinemia in the female rat treated neonatally with MSG [21] is a key factor for the development of leptin resistance in the adrenal gland. The aim of the present study was to determine whether reduction in leptin levels by either acute or chronic negative energy balance could correct adrenal dysfunction in rats treated with MSG neonatally.

Materials and Methods

Animals and Experimental Designs

Adult male Sprague-Dawley rats with body weight (BW) 300–330 g and female ones with BW 240–280 g were mated in a light (lights on from 07:00 to 19:00 h) and temperature (22°C) controlled room, with rat chow and water available ad libitum. Pregnant rats were transferred to individual plastic cages. On ages day 2, 4, 6, 8 and 10, female pups were injected i.p. with either 4 mg/g BW MSG (Sigma Chemical Co., St. Louis, Mo., USA) in sterile 0.9% NaCl or 10% NaCl (littermate controls; CTR) [22]. Rats were weaned and housed in individual cages on age day 21. MSG-injected animals were screened for effectiveness of treatment by macroscopic observation of degeneration of the optic nerves when euthanized. All experimentation was approved by our Institutional Animal Care Committee. We complied with international regulations concerning the ethical use of animals throughout this research.

Experiment 1: Food Withdrawal (FW). On age day 55, food was removed from cages; water was provided ad libitum to both groups of animals (CTR and MSG). Animals were sacrificed on age days 56, 57, 58, 59 and 60 (1, 2, 3, 4 and 5 days after FW, respectively). Additional rats from both groups were fed ad libitum (CTR-AL and MSG-AL) and sacrificed on age day 61 (experimental day zero). Experiments were run between 08:00 and 09:00 h. Trunk blood was collected and immediately centrifuged for further determination of plasma concentrations of leptin, ACTH, and corticosterone (B). Following sacrifice, freshly dissected visceral fat (VF; the sum of retroperitoneal, omental and parametrial fat) pads were weighed. Adrenal glands from rats on experimental days 0, 1, 3 and 5 after FW were dissected and processed as indicated below.

Experiment 2: Food Restriction (FR). CTR-AL and MSG-AL animals were fed and supplied water ad libitum between age days 21 and 61 (AL groups). In parallel, a previously described FR protocol was set up for CTR and MSG animals [23]. Briefly, daily food intake (calculated from the daily amount of food eaten by CTR and MSG animals in ad libitum condition) at different age-periods were approximately: 85, 74, 61, 51, 48 and 43% on days 24–27, 28–29, 30–36, 37–43, 44–50 and 51–61, respectively.

Figure 1 depicts the energy intake of animals from the different groups in different age periods. On age day 61, between 08:00 and 09:00 h, CTR-AL, CTR-FR, MSG-AL and MSG-FR rats were euthanized; trunk blood was collected and immediately centrifuged for determination of plasma concentrations of leptin, ACTH, and B. Following sacrifice, freshly dissected VF pads were weighed. Adrenal glands were also dissected and processed as indicated below.

Determination of Peripheral Biomarkers

Plasma leptin levels were determined by a specific radioimmunoassay (RIA) developed in our laboratory [24], the standard curve ranging between 0.04 and 12.5 ng/ml and intra- and interassay coefficients of variation (CVs) of 5–8 and 10–13%. Circulating ACTH concentrations were measured by a previously described immunoradiometric assay [25] with a standard curve between 15 and 3,000 pg/ml and intra- and interassay CVs of 2–3 and 6–8%, respectively. Plasma and medium concentrations of B were evaluated by a specific RIA as previously described [26], with standard curves between 1 and 250 μg/dl and, intra- and interassay CVs of 4–6 and 8–10%, plasma total protein and triglycerides (TG) concentrations were determined using a commercially available enzymatic-colorimetric kit from Weiner Lab. (Rosario, Argentina).

Evaluation of Adrenocortical Function in vitro

This method has been extensively described in previous studies [4]. Dissected adrenal glands (free of adipose tissue) were cut with a thin dissecting knife and digested in Earle's balanced salt solution (EBSS) containing 0.3% collagenase (type 1, Sigma; 1 ml of solution per gland) and gently shaken at 37°C in a 95% air-5% CO_2 atmosphere. Cells were washed with 10 ml of incubation medium (IM): EBSS containing 0.2% bovine serum albumin (BSA), 20 mg/l ascorbic acid, 100 U/ml aprotinin and antibiotics (pH 7.4). Then, cells were resuspended to a density of 100,000 cells per 0.9 ml of IM. This volume was distributed into 12 × 75 mm polystyrene test tubes and 0.1 ml of IM, either alone or containing ACTH (final concentrations 0.01–1 ng/ml), was added in the absence or presence of leptin (PrePro Tech Inc., Rocky Hill, N.J., USA; 0.1–1 nM final concentrations). Cells were then incubated for 2 h at 37°C. At least 6–8 tubes per condition were used in each experiment. At the end of incubation, tubes were centrifuged at 100 *g* for 10 min at room temperature and supernatants were separated and kept frozen (–20°C) until measurement of media B concentrations.

Adipose Tissue RNA Isolation and Real-Time PCR

Total RNA was isolated from adrenal glands by a modification of the single step, acid guanidinium isothiocyanate-phenol-chloroform extraction method [27] (Trizol; Invitrogen, Life Tech., Carlsbad, Calif., USA; catalog number 15596-026). The yield and quality of extracted RNA were assessed by 260/280 nm optical density ratio and electrophoresis in denaturing conditions on 2% agarose gel. 1 μg of total RNA was reverse transcribed using random primers (250 ng) and Superscript III RNase H-Reverse Transcriptase (200 U/μl; Invitrogen, Life Tech; catalog number 18989-093). For quantitative real-time PCR the following primers were applied: β-actin (R): 5′-ACCCTCATAGATGGGCACAG-3′, (F): 5′-AGCCATGTACGTAGCCATCC-3′ (115 bp) (GenBank accession number: NM 031144); *ob*-Rb (R): 5′-TGTGGAATCTGGAGTGGTCA-3′; (F): 5′-TCTGGAGCCTGAACCAGTTT-3′ (115 bp) (GenBank accession number: NM-012596). 2 μl of the reverse transcription mix were amplified with QuantiTect Syber Green PCR kit (Qiagen, Düsseldorf, Germany; catalog number 204143) containing 0.5 μM of each specific primer, using A Light-Cycler Detection System (MJMini Opticon, Biorad, Hercules, Calif., USA). PCR efficiency was ~1. The threshold cycles (Ct) were measured in separate tubes by duplicate. The identity and purity of the amplified product were checked by electrophoresis on agarose mini-gel and the melting curve was analyzed at the end of amplification. The ΔCt values were calculated in every sample for each gene of interest as follows: Ct gene of interest – Ct reporter gene with β-actin, whose mRNA levels did not differ between control and test groups, as the reporter gene. Relative changes in the expression level of one specific gene (ΔΔCt) were calculated as ΔCt of the test group minus ΔCt of the control group, then presented as $2^{-\Delta\Delta Ct}$.

Data Analysis

Data (peripheral biomarkers and in vitro experiments) expressed as means (± SEM) were analyzed by either one (treatment) or two (treatment and metabolic condition) factor ANOVA, followed by Student-Newman-Keul's test for comparison of different mean values [28]. The nonparametric Mann-Whitney test was used for analysis of data from adrenal gland mRNA expressions [28].

Results

FW induced changes in BW, adipose tissue mass and circulating levels of several biomarkers in CTR and MSG rats.

FW-induced changes in BW and VF mass are depicted in figure 2: BWs (fig. 2, left) were significantly ($p < 0.05$) lower in MSG compared with CTR rats, regardless of the day examined and decreased in a similar, time-related, fashion in both groups. CTR rats (day 2 and beyond of food deprivation) showed an earlier VF mass reduction as compared with MSG (day 5 of food deprivation) rats. However, a significantly ($p < 0.05$) higher VF mass was observed in MSG compared with CTR rats, regardless of the day examined (fig. 2, right). TG circulating levels were similar in non-fasting rats from both groups (0.79 ± 0.09 and 0.83 ± 0.11 g/l in CTR and MSG rats, respectively). While fasting transiently decreased circulating TG levels (on days 2, 3 and 4), this occurred in CTR rats only, in contrast MSG animals displayed similar circulating TG levels, in both fed and fasting states. Moreover, on fasting days 2, 3 and 4 circulating TG values were higher ($p < 0.05$) in MSG than in CTR rats (not shown). However, on day 5 of fasting, CTR rats recovered day zero values. Total protein concentrations in plasma were similar in both CTR and MSG rats on day 0 and did not alter by 1–3 days of FW (data not shown). However, on days 4 and 5 of the FW protocol, CTR rats displayed decreased proteinemia (vs. day 0 values) with levels significantly ($p < 0.05$) lower than MSG animals (5.21 ± 0.33 and 3.62 ± 0.54 g/dl vs. 7.49 ± 0.64 and 6.82 ± 0.28 g/dl, on days 4 and 5 after FW and in CTR and MSG rats). MSG rats on day 0 showed significantly ($p < 0.05$) higher circulating leptin levels compared with CTR animals in similar condition (fig. 3, upper left). Although leptinemia was significantly ($p < 0.05$) decreased on day 1 of fasting in both groups, it remained significantly higher ($p < 0.05$) in MSG compared with CTR rats up to 4 days after food deprivation. Circulating levels of ACTH (fig. 3, upper right) and corticosterone (fig. 3, lower) were similar in both experimental groups of fed animals (day 0), and were not influenced on day 1 after FW. Conversely, 2 days of food deprivation induced peak values in plasma ACTH and B levels of a similar magnitude in CTR and MSG rats. Thereafter (day 3 or more), plasma concentrations of both hormones declined toward the respective day zero values. However, full recovery of initial (day 0) values occurred 1 day later in MSG (day 5) compared with CTR (day 4) rats (fig. 3, lower).

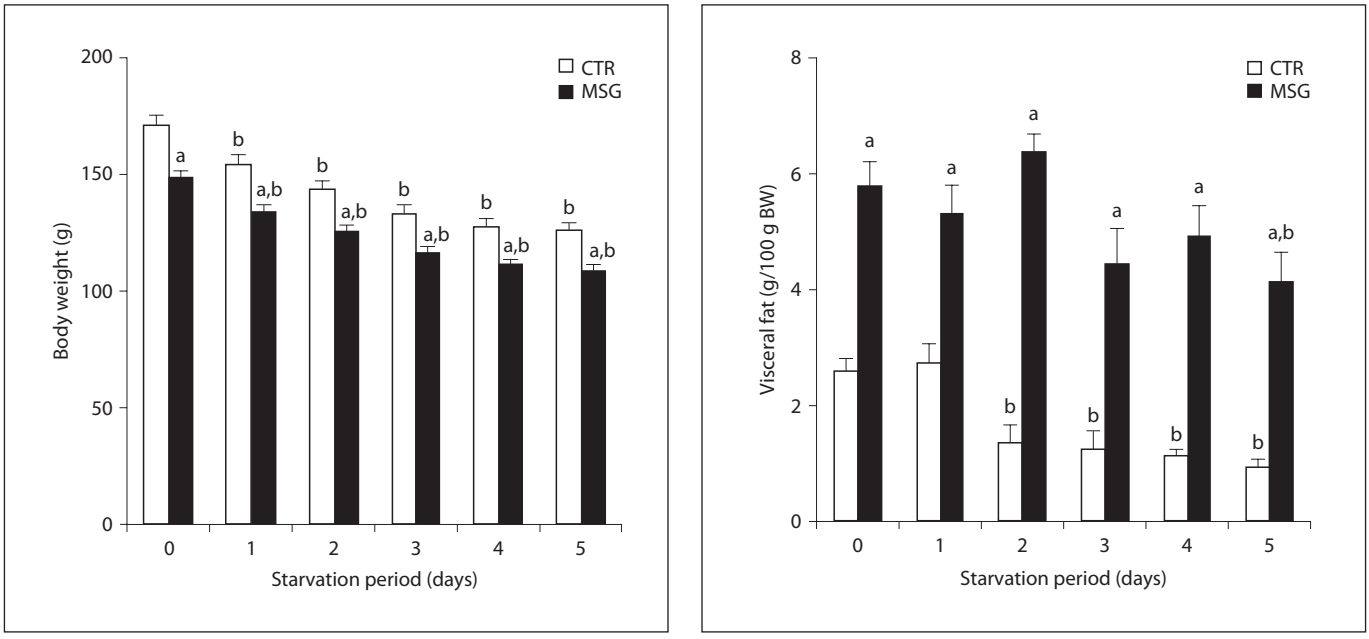


Fig. 2. Effect of food withdrawal for several days on body weight and visceral fat mass in CTR and MSG rats. Values are the means ± SEM (n = 7–9 rats per group/condition). [a] $p < 0.05$ vs. CTR values on the same day. [b] $p < 0.05$ vs. day 0 values of the same group.

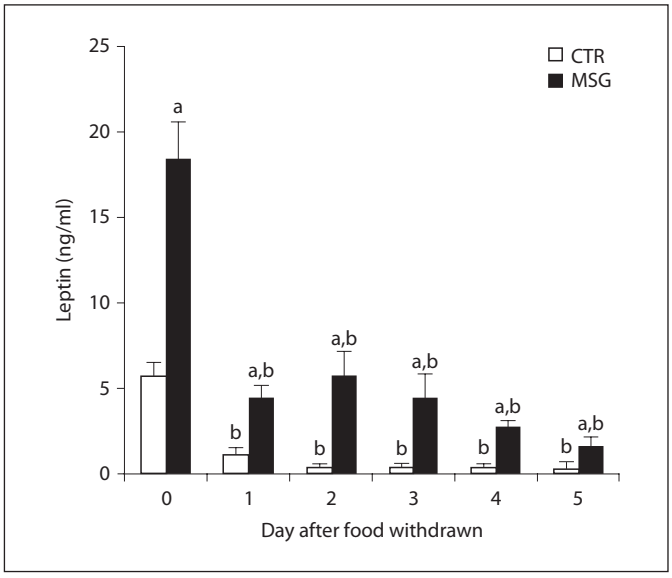
Food Restriction-Dependent Changes in Body Weight, Adipose Tissue Mass and Circulating Levels of Several Biomarkers in CTR and MSG Rats

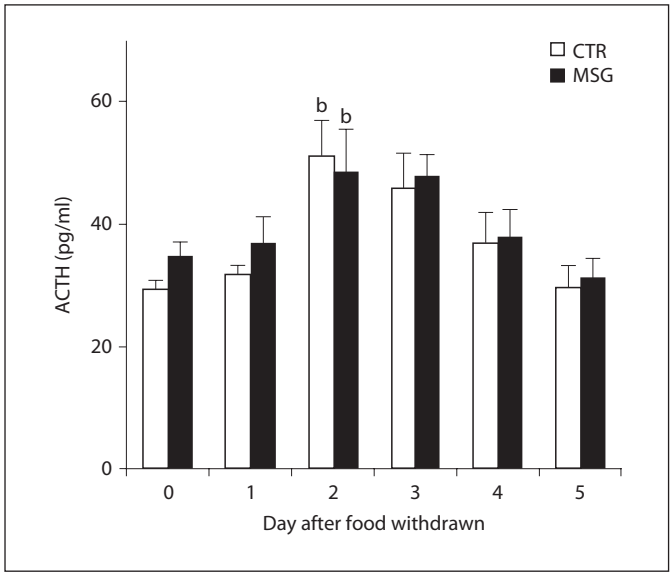
FR significantly ($p < 0.05$) reduced BW values in both experimental groups (table 1). As expected, fresh VF pad mass was significantly ($p < 0.05$ vs. CTR-AL rats) enlarged in MSG-AL animals (table 1). VF pad mass decreased after FR ($p < 0.05$ vs. respective AL group values) in both groups but revealed higher VF pad mass absolute values in CTR-FR rats compared with MSG-FR rats (table 1). Correcting for BW revealed no significant differences between both groups (0.51 ± 0.09 and 0.44 ± 0.12 g of VF/100 g BW in CTR-FR and MSG-FR rats). Circulating TG levels were significantly ($p < 0.05$ vs. respective AL values) reduced after FR in both groups of rats (0.56 ± 0.04 and 0.59 ± 0.08 g/l in CTR-FR and MSG-FR rats, respectively). As previously described [23], the present FR protocol did not modify (vs. respective AL values) plasma total protein concentrations in either group studied (values being 6.95 ± 0.23 and 7.49 ± 0.64 g/dl in CTR-FR and MSG-FR rats).

Circulating leptin (fig. 4, upper left) levels were significantly ($p < 0.05$) higher in MSG-AL compared with CTR-AL rats, and FR produced a significant ($p < 0.05$) decrease in leptinemia in both groups ($p < 0.05$ vs. respective AL group values). However, peripheral leptin concentrations remained significantly higher in MSG-FR than in CTR-FR rats (fig. 4, upper left), whether expressed by grams of VF (4.49 ± 0.44 vs. 17.34 ± 1.82 ng/ml/g VF in MSG- and CTR-FR rats, $p < 0.05$) or by 100 g BW (3.98 ± 0.24 vs. 2.32 ± 0.14 ng/ml/100 g BW in MSG-FR and CTR-FR rats, $p < 0.05$). HPA axis hormone plasma concentrations indicated that circulating ACTH and B levels were similar in CTR-AL and MSG-AL rats (fig. 4, upper right and lower panels). While CTR-FR and MSG-FR rats displayed similar circulating ACTH levels (fig. 4, upper right), conversely, significantly ($p < 0.05$ vs. respective AL group values) higher plasma corticosterone values were found in both FR groups (fig. 4, lower). Circulating B concentrations were significantly ($p < 0.05$) greater in CTR-FR than in MSG-FR rats (fig. 4, lower).

Effects of Food Manipulation on in vitro Adrenocortical Functions

Adrenocortical functions were evaluated in vitro by using isolated adrenal cells from CTR (fig. 5a) and MSG (fig. 5b) rats either fed ad libitum (day 0) or at time points after FW (1, 3 and 5 days). The results indicate that spontaneous glucocorticoid secretion was similar in both cell groups on day 0 and that adrenal responses to ACTH





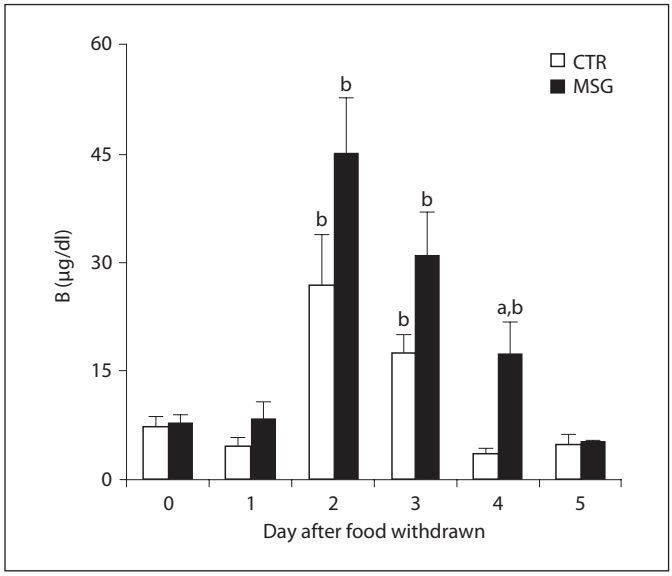


Fig. 3. Leptin (upper left), ACTH (upper right) and corticosterone (lower) circulating levels before (day zero) and several days after food removal in normal (CTR) and MSG rats. Values are the means ± SEM (n = 7–9 rats per group/day). [a] $p < 0.05$ vs. CTR values on the same day. [b] $p < 0.05$ vs. day zero values in the same group.

(0.01–1 ng/ml) stimulation were concentration-dependent in both cell groups. In CTR cells, responses to ACTH (0.1 and 1 nM) were significantly ($p < 0.05$) higher after FW (days 1, 3 and 5) than that observed on day 0 (fig. 5a). Adrenal cells from non-fasting (day 0) MSG rats were hyper-responsive to ACTH stimulation in comparison to the day zero CTR adrenal cell response. Food deprivation in MSG animals did not modify adrenal cell responses to ACTH, regardless of the day examined (fig. 5b). Adrenocortical functions were also evaluated using isolated adrenal cells from 61-day-old CTR (fig. 5c) and MSG (fig. 5d) rats, in AL and FR conditions. The results indicated that spontaneous glucocorticoid secretion was similar in both cell groups from AL rats, and that adrenal responses to ACTH (0.01–1 ng/ml) stimulation were concentration-dependent regardless of cell group. We compared the patterns of ACTH-stimulated B output among CTR-AL and MSG-AL groups and found that cells from MSG-AL did release a significantly ($p < 0.05$) higher amount of B than that released by CTR-AL cells in response to 0.1 and 1 ng/ml ACTH. In addition, FR significantly ($p < 0.05$) enhanced adrenal cell responses to 0.1 and 1 ng/ml ACTH in CTR-FR and MSG-FR cell groups. Moreover, the adrenal hyper-response to ACTH charac-

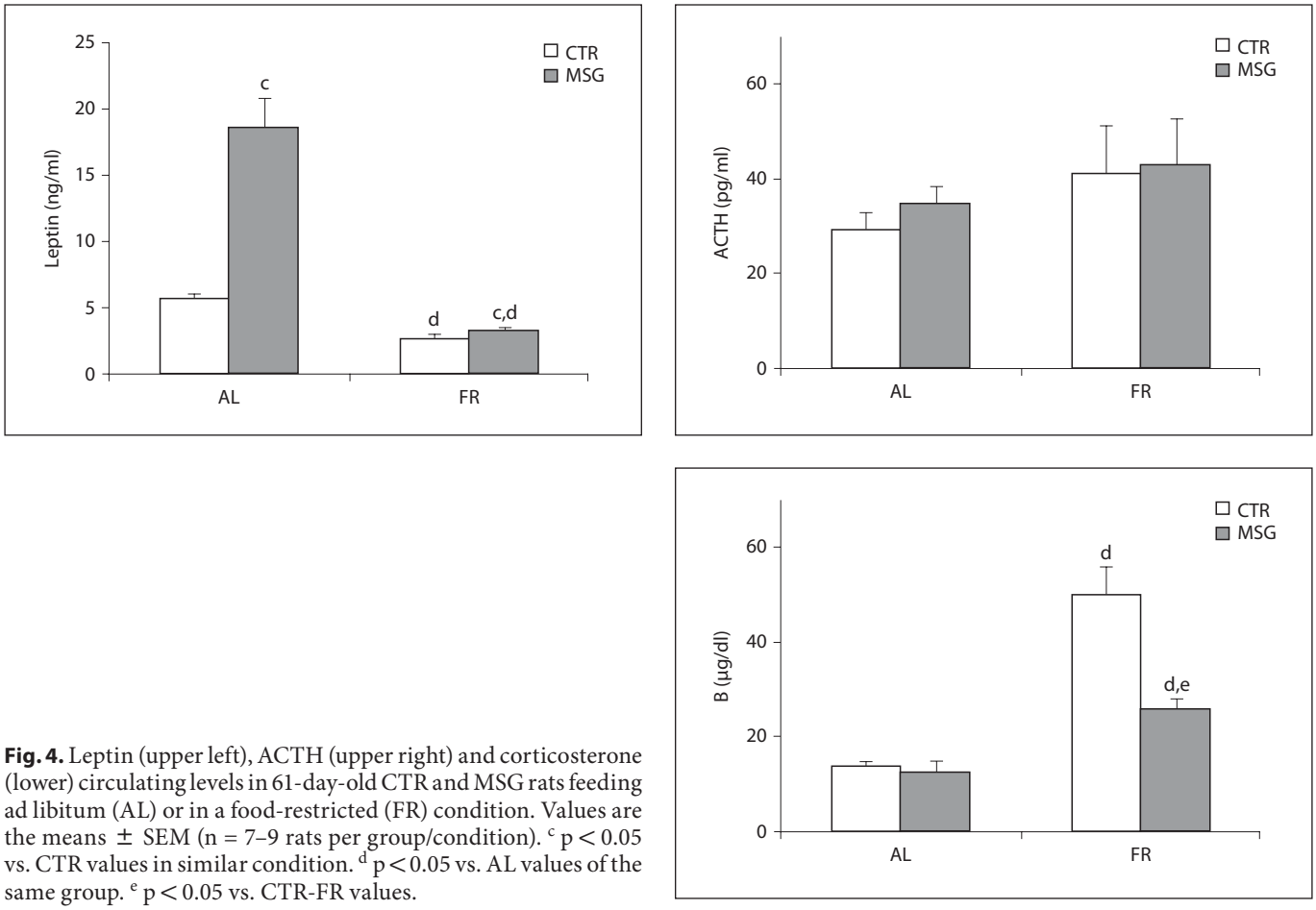


Fig. 4. Leptin (upper left), ACTH (upper right) and corticosterone (lower) circulating levels in 61-day-old CTR and MSG rats feeding ad libitum (AL) or in a food-restricted (FR) condition. Values are the means ± SEM (n = 7–9 rats per group/condition). [c] $p < 0.05$ vs. CTR values in similar condition. [d] $p < 0.05$ vs. AL values of the same group. [e] $p < 0.05$ vs. CTR-FR values.

terizing MSG-AL cells (vs. CTR-AL cells) was also observed in the FR condition (CTR-FR vs. MSG-FR).

In vitro Effects of Leptin on ACTH-Stimulated Glucocorticoid Secretion by Isolated Adrenal Cells from CTR and MSG Rats Submitted to Food Manipulation

These data are expressed as percent of inhibition in relation to values obtained in the absence of leptin, conventionally considered zero percent inhibition in adrenal cells from rats of the FW protocol (experiment 1). Although the addition of exogenous leptin 0.1 (fig. 6a) or 1 nM (fig. 6b) into the incubation medium did not modify spontaneous glucocorticoid secretion in all cell groups (data not shown), it was able to significantly ($p < 0.05$ vs. values, in similar condition, in the absence of leptin) inhibit 0.1 ng/ml ACTH-stimulated B output in CTR adrenal cells regardless of energy condition. Conversely, exogenous leptin (0.1 or 1 nM) failed to inhibit ACTH (0.1 ng/ml)-elicited B output by MSG adrenal cells, whether rats were fed or not (see also fig. 6a, b). Exogenous leptin (0.1 or 1 nM) did inhibit 0.1 ng/ml ACTH-stimulated B output by CTR cells from AL and FR rats (fig. 6c). Conversely, adrenal cells from MSG-AL rats were refractory to any inhibitory effect of leptin on 0.1 ng/ml ACTH-stimulated B secretion (fig. 6d). Interestingly, adrenal cells from MSG rats subjected to FR did develop leptin sensitivity. In fact, leptin (0.1 and 1 nM) significantly ($p < 0.05$) inhibited ACTH-elicited B release (fig. 6d).

Effect of Food Restriction on Adrenal ob*-Rb mRNA Expression*

We have observed in vitro that isolated adrenal cells from energy replete MSG rats (MSG-AL) were resistant to leptin inhibition of ACTH-driven B secretion. Also, food-restricted but not starved MSG rats became sensitive to leptin inhibitory action on ACTH-induced glucocorticoid output. Therefore, we investigated whether this

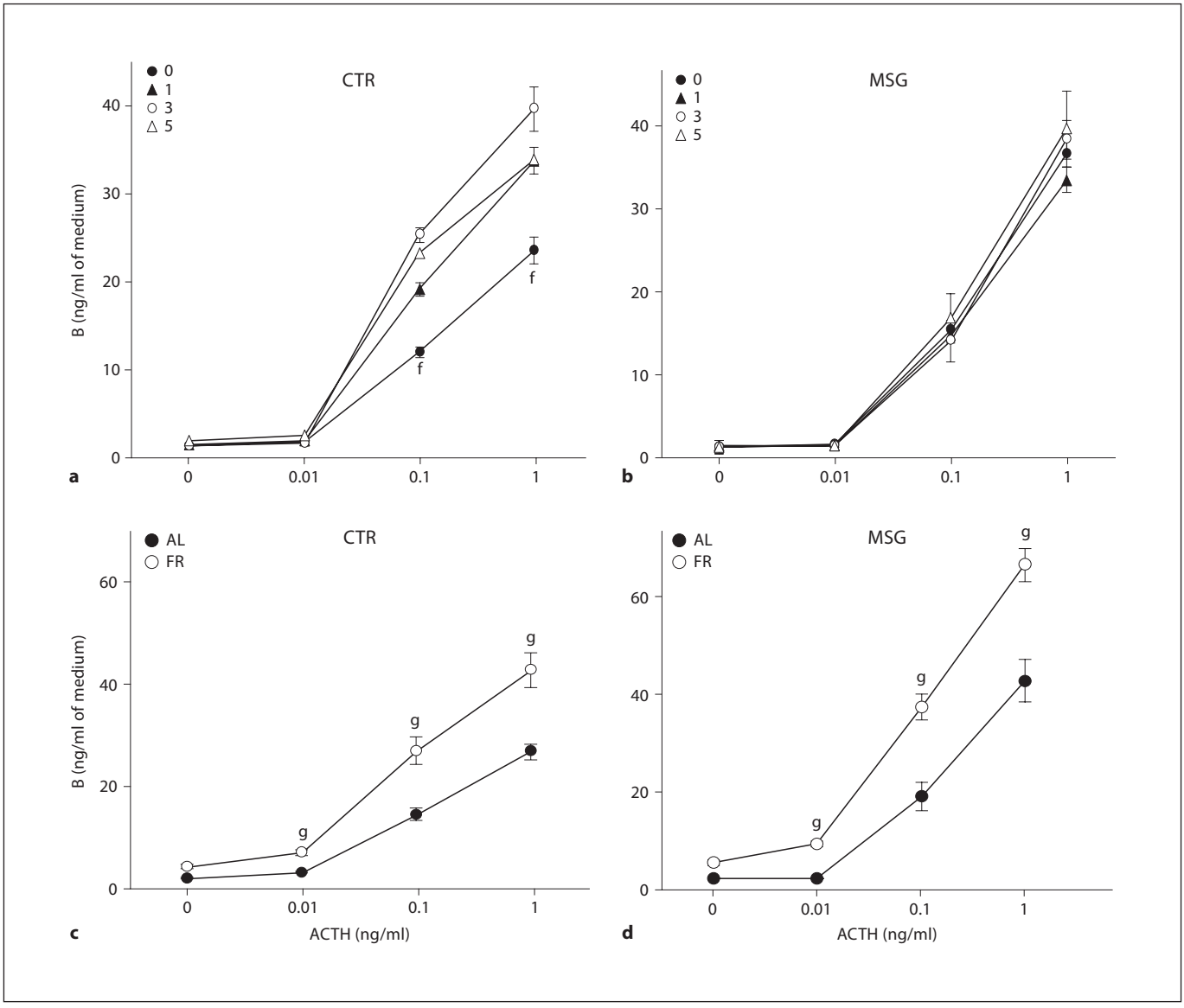


Fig. 5. In vitro spontaneous (ACTH concentration zero) and ACTH-induced corticosterone secretion by isolated adrenal cells from nonfasting (day 0) and fasting (for 1–5 days) CTR (**a**) and MSG (**b**) rats; and, from fed ad libitum (AL) and food restricted (FR), CTR (**c**) and MSG (**d**) rats. Values are the means ± SEM (n = 3–4 different experiments, with 5 replicates per experiment). [f] $p < 0.05$ vs. values obtained with equal ACTH concentrations in both the CTR group on remaining days, and the MSG group, regardless of the day. [g] $p < 0.05$ vs. AL values in similar conditions.

change could be explained by any modification in adrenal *ob*-Rb gene expression. We found, in relation to the CTR-AL group, that adrenal *ob*-Rb mRNA expression was not modified by FR in CTR rats (fig. 7). Conversely, this expression was significantly ($p < 0.05$) reduced in hyperleptinemic obese rats fed ad libitum (MSG-AL) (fig. 7). Interestingly, adrenal glands from FR hypothalamic obese rats (MSG-FR) showed upregulation in adrenal of *ob*-Rb mRNA expression when compared ($p < 0.05$) to MSG-AL rats; these values were also similar to those observed in the reference group (CTR-AL rats) (fig. 7).

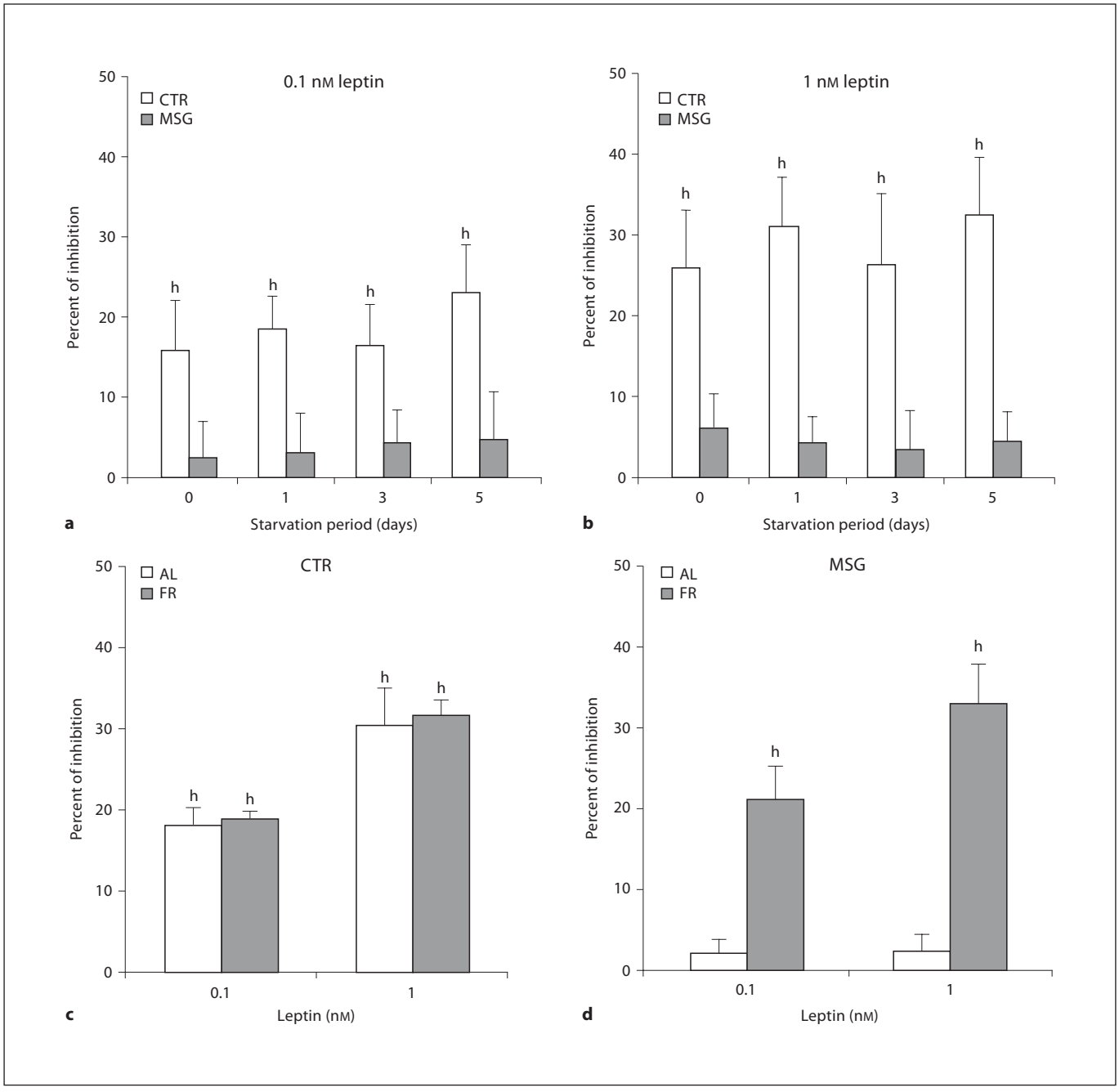


Fig. 6. In vitro effects of different concentrations of leptin (**a**, **b**) on 0.1 ng/ml ACTH-induced glucocorticoid output by isolated adrenal cells from CTR and MSG rats, in non-fasting (day zero) and after different days (1–5) of fasting. Similarly, the in vitro effects of leptin (0.1 and 1 nM) on ACTH (0.1 ng/ml)-stimulated corticoadrenal function are shown in fed ad libitum (AL) and food-restricted (FR), CTR (**c**) and MSG (**d**) rats. For full details on the expression of these data, see 'Results'. Values are the means ± SEM (n = 3–4 different experiments, with 5 replicates per experiment). ^{h} $p < 0.05$ vs. values in the absence of leptin.

Discussion

In the present study, we found that decreasing leptinemia by prolonged FR, but not a short fasting period of time reverses adrenal leptin-insensitivity in severe hyperleptinemic (MSG) female rats, and that this change involved normalization of the adrenal expression of the functional leptin receptor gene.

Our data indicate that after five days of food deprivation, hypothalamic obese rats restored leptinemia to values found in normal rats. However, even in the presence of an endogenous environment with a very low circulating leptin concentration, adrenal cells from starved MSG rats remained refractory to any leptin inhibitory effect on ACTH-dependent glucocorticoid production. This observation could be attributable to the short time period during which adrenal cells were exposed to low (although they may not be low enough, vs. fasting normal animals) circulating levels of leptin.

Moreover, the fact that starvation failed to reduce circulating triglyceridemia in MSG rats could indicate that this signal might be contributing to the leptin-resistant state of hypothalamic obese animals [29]. Conversely, prolonged negative energy condition due to FR not only induced low circulating leptin levels but also reversed MSG adrenal cell insensitivity to leptin inhibitory effect on ACTH-driven glucocorticoid secretion. This correction was also observed when the downregulation in adrenal *ob*-Rb mRNA expression was overridden. Thus, our study strongly supports that prolonged hyperleptinemia could be a crucial factor for the development of adrenal leptin insensitivity in this model of hypothalamic obesity. Regarding changes in BW, fat mass and other metabolic parameters induced by FW, our data tally with those already reported by other researchers in both normal [30, 31] and MSG-damaged [30] male rats. In one of these studies [31] the authors have addressed similar changes induced by fasting (up to 10 days) on circulating TG levels in both normal and MSG rats. Moreover, they reported that early (24 h) starved MSG rats displayed (as in the fed state) hyperinsulinemia, whereas similar insulinemia was noticed in normal and MSG rats between days 3 and 10 of fasting. Although we do not show these data, we found a similar effect in food removal-induced changes in insulinemia in our female MSG rats. Indeed, we found that VF fat stores decreased more rapidly in CTR (day 2 of fasting, approximately 49% loss) than in MSG (day 5 of fasting, approximately 30% loss). Similarly, Ribeiro et al. [30] found in starved male rats that while 83% of fat stores were consumed after 10 days of FW in normal animals, only 58% of initial fat stores were reduced 14 days after food removal in MSG rats. Whether these differences can be accounted for by enhanced sympathetic outflow on adiposity cannot be answered by our study. Starvation is accepted to decrease sympathetic activity [32], and because the impairment of both sympathetic activity [33] and adrenal medulla catecholamine output [34] are characteristics of the MSG rat, it is unlikely that these events in the starved MSG rat could significantly contribute to reduction of VF mass. Although TG levels transiently decreased (between days 2–4) in fasting CTR rats, this decline could be representing a metabolic adaptive mechanism preceding a crucial factor for the organism's survival such as protein consumption, as found on days 4–5 after food removal. Regarding data on a starvation-induced rapid reduction in leptinemia in normal rats, our results agree with those previously reported [30] after 24 h of fasting, although we now have extended data up to five days after food removal. On the other hand, in hyperleptinemic (MSG) rats, only five days of fasting were effective to induce peripheral leptin levels with a similar

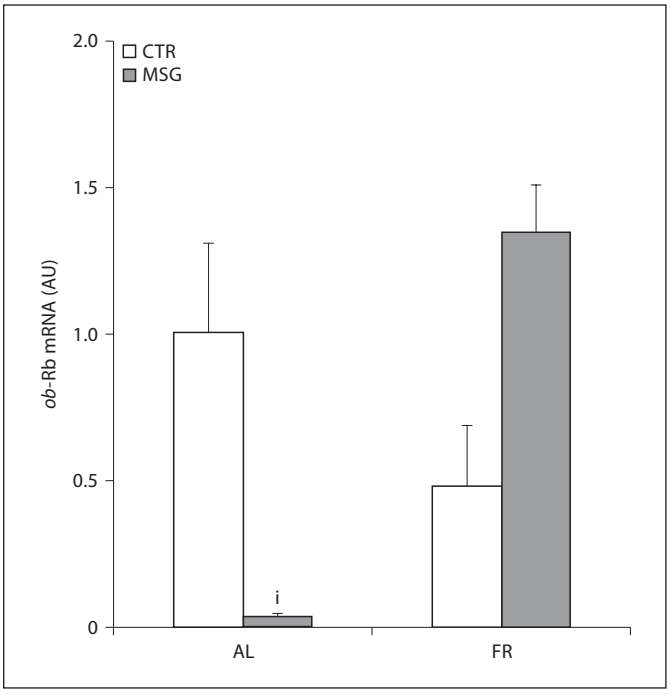


Fig. 7. *ob*-Rb mRNA levels measured in the adrenal glands from fed ad libitum (AL) and food-restricted (FR), CTR and MSG rats by q-PCR, normalized to the levels of β-actin, then presented as expression relative to values obtained in glands from CTRAL rats. Values are the means ± SEM (n = 4–5 experiments). [i] $p < 0.05$ vs. CTR-AL and MSG-FR values.

Table 1. Effect of prolonged food restriction (FR) or not (AL) on body weight and visceral fat (VF) mass, in 61-day-old CTR and MSG rats

	AL		FR	
	CTR	MSG	CTR	MSG
Body weight, g	168.8 ± 6.6	134.9 ± 8.6[a]	112.1 ± 2.2[b]	80.4 ± 3.1[a, b]
VF mass, g/100 g BW	2.35 ± 0.18	5.21 ± 0.62[a]	0.49 ± 0.08[b]	0.27 ± 0.05[a, b]

Values are the means ± SEM (n = 7–9 rats per group/condition).
[a] $p < 0.05$ vs. CTR values in similar condition. [b] $p < 0.05$ vs. AL values of the same group.

order of magnitude to those for 24 h fasting normal rats. Interestingly, changes in HPA axis hormones after fasting developed in a similar fashion in CTR and MSG rats, although the resiliency of the corticoadrenal response was, as previously described [20], delayed in MSG rats. Moreover, the area under the curve of circulating glucocorticoid concentrations throughout the whole fasting period was significantly ($p < 0.05$) higher in MSG (68.18 ± 11.84 μg/dl/5 h) than in normal (30.79 ± 7.05 μg/dl/5 h) rats. These observations fully agree with our present in vitro observations and with earlier data from our laboratory [4, 20, 21, 35, 36] and others [8, 9, 11] indicating HPA axis hyperactivity in MSG rats. Moreover, enhanced glucocorticoid levels could also be accounted for by decreased metabolic clearance rate of this hormone in this animal model [9, 10].

In point of fact, we used this model of hypothalamic obese female rat previously and demonstrated that exogenous leptin failed to inhibit ACTH-stimulated glucocorticoid secretion by isolated fasciculata reticularis-enriched adrenal cells [20]. In that study we also found that adrenal glands from MSG rats displayed (although semiquantitatively) low *ob*-Rb mRNA expression [20]. Thus, the present data fully agree with these observations. In fact, we have now determined by quantitative RT-PCR significantly decreased adrenal *ob*-Rb mRNA expression in the MSG rat fed AL and a lack of leptin effect on ACTH-induced glucocorticoid output by adrenal cells.

Interestingly, we found that decreasing circulating leptin levels by a short period of time (fasting) was not effective to change adrenal sensitivity to leptin.

Regarding the long FR protocol, as previously reported [23], we found that this experimental design did not vary total protein levels in either group of rats. Conversely, it induced reduced plasma TG levels in both experimental groups. Thus, the decreased triglyceridemia, namely in MSG-FR rats, could be favoring the restoration of adrenal leptin sensitivity [29]. It should be noted that the differences in BW values comparing individual AL rats remained the same after FR. However, paradoxically, the decrease in VF mass was higher in MSG-FR than in CTR-FR animals. This finding could indicate that the neonatal MSG treatment did not delay FR-induced VF mass reduction [37]. However, while MSG-FR rats attained significantly lower VF mass than CTR-FR rats, circulating leptin concentrations remained higher in MSG-FR vs. CTR-FR rats (table 1; fig. 4), although these values are not representative of hyperleptinemia. Among endogenous factors inducing leptin secretion, glucocorticoid [38, 39] and insulin [40, 41] are the most important. With respect to the latter signal, although the data are not shown, we observed similar circulating insulin levels in both groups of FR rats. In addition, peripheral glucocorticoid levels in both groups of FR rats were higher than those observed in AL rats; however, circulating corticosterone levels were lower in MSG-FR than in CTR-FR animals. This suggests that this signal does not significantly contribute to enhance leptinemia in MSG-FR rats. Dietary protein deficiency [42] but not FR [23, 42] enhances ACTH synthesis and secretion; thus the increase in circulating glucocorticoid levels in both groups of FR animals could be related to the decrease in leptinemia and full adrenal *ob*-Rb functionality [19]. In addition, the discrepancy between VF mass and leptinemia in MSG-FR rats could be related to the morphological characteristics of MSG adipocytes. In fact, we previously reported that adipocytes from obese hypothalamic female rats are larger than those from their normal counterparts [21]. It is accepted that there is a direct relationship between adipocyte size and leptin production [43]; thus enlarged adipocytes of MSG rats could contribute to their enhanced circulating leptin levels even after prolonged FR. However, because FR modifies adipocyte size in normal rats [44], possible modifications in this process in MSG-FR

rats should not be discarded. Decreased leptin concentrations in circulation induced by FR fully restored both normal adrenal *ob*-Rb mRNA expression and a leptin inhibitory effect on ACTH-driven glucocorticoid release. These data further indicate that when adrenal *ob*-Rb is expressed normally, it is fully operative in MSG rat, thus supporting that post *ob*-Rb events, if any, are not permanently impaired in MSG damaged rats. Similarly, we previously found that transient correction of enhanced corticoadrenal function in the MSG rat resulted in the recovery of functionally active adrenal *ob*-Rb [4] and other metabolic dysfunctions [21]. However, when the adrenal cortex of enucleated MSG animals reached full regeneration, adrenal insensitivity to leptin inhibition reappeared [4]. Therefore, the present data, combined with our previous findings, strongly suggest that downstream leptin receptor events are not seriously damaged in MSG rats. Common forms of human obesity and diet-induced obesity (DIO) in rodents, as in this rat model of hypothalamic obesity, are associated with development of leptin resistance [45]. The means by which the downstream receptor mechanisms become impaired are not yet fully understood. However, our data suggest that MSG obese rats, unlike DIO rats [46], did develop peripheral (adrenal) leptin resistance because of a substantial decrease in adrenal *ob*-Rb gene expression. However, through our study, possible transient changes in post-*ob*-Rb events [46] cannot be excluded in MSG rats.

In summary, our study supports that appropriate corrections in the diet in some peripheral leptin-resistant obese phenotypes could account for their decreases in both body and fat masses and leptinemia. Hyperleptinemia is a common characteristic of normal aged [47] and MSG rats [4, 20, 21]. Several studies suggest that prolonged FR in aged normal rats decreased circulating levels of leptin, improved central leptin sensitivity, and restored adipocyte insulin sensitivity [48, 49]. We have presently found that a decrease in leptinemia after a prolonged (FR) but not short (fasting) period of negative energy intake fully restored adrenal leptin sensitivity in hyperleptinemic, MSG rats. Thus, ACTH-driven adrenal glucocorticoid secretion [4, 19] and probably other leptin-dependent functions [50–53] were normalized. As a consequence, normal adrenal glucocorticoid production could be beneficial for curtailing adipogenesis [39] and for preserving the organism against the undesirable effects of excessive endogenous glucocorticoid [54–56].

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