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Modulatory Effects of Leptin on Leydig Cell Function of Normal and Hyperleptinemic Rats

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Key Words
Leptin · Testis · Steroidogenesis · Hypogonadism · Gonadal steroids · Leydig cells · Monosodium glutamate

Abstract
Neonatal *L*-monosodium glutamate (MSG) administration in rats induces several neuroendocrine and metabolic disruptions. Leptin, the adipocyte product, modulates several neuroendocrine systems including the hypothalamic-pituitary-gonadal (HPG) axis in mammals. The aim of the present study was to determine whether MSG-induced chronic hyperleptinemia could play any relevant role in the hypogonadism developed by male rats when examined in adulthood. We found that 120-day-old MSG male rats displayed significant hyperleptinemia, hypogonadism, and undisturbed basic testis structure and spermatogenesis. In vitro studies in purified Leydig cells from normal (CTR) and MSG-damaged rats revealed that basal and human chorionic gonadotropin (hCG)-stimulated 17-hydroxy-progesterone (17-HO-P_4), Δ_4-androstenedione (Δ_4A) and testosterone (T) secretions were significantly lower in MSG than in CTR cells. Exposure to murine leptin (mleptin, 10^{-8} *M*) significantly inhibited hCG-elicited T secretion by CTR cells after 180 min incubation. While mleptin significantly inhibited hCG-stimulated Δ_4A output and the Δ_4A:17-OH-P_4 ratio of secretion, conversely, it failed to modify the ratio T:Δ_4A release by CTR Leydig cells. Interestingly, the effects of mleptin found on CTR Leydig cells were absent in MSG Leydig cells. Finally, endogenous hyperleptinemia was associated with a significant decrease in Leydig cell expression of Ob-Rb mRNA in MSG rats. In summary, this study demonstrates that: (1) mleptin inhibited testicular steroidogenesis in CTR rats; (2) MSG-treated rats showed lower in vitro 17-OH-P_4, Δ_4A and T production under basal and post-hCG stimulation conditions; (3) purified Leydig cells from MSG-treated rats displayed resistance to the inhibitory action of mleptin on T release, and (4) endogenous leptin exerts a modulatory effect on Leydig cell Ob-Rb mRNA expression. The inhibitory effect of leptin on testicular function is thus abrogated in MSG-damaged rats. The testicular leptin-resistance developed by MSG rats seems to be due to early chronic exposure of Leydig cells to high leptin circulating levels, which in turn down-regulate testicular Ob-Rb expression. It remains to be determined whether the testicular dysfunction of MSG rats can be reversed after correction of hyperleptinemia or whether it is an irreversible effect of the hypothalamic lesion.

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Introduction

The adipocyte product, leptin, is a representative parameter of energy homeostasis and body fat. It was also described in recent years as a metabolic and neuroendocrine hormone [1]. Leptin modulates several endocrine systems including the hypothalamo-pituitary-gonadal (HPG) axis function in both sexes [2].

Specifically, in relation to the HPG axis, several reports described leptin as being involved in normal sexual maturation and reproductive process [2, 3]. Some evidence suggests, however, that the regulatory effect of leptin on the HPG axis depends on the threshold of circulating leptin levels [4]. Compelling evidence indicates that the main sites of action of leptin in modulating the HPG axis seem to be the hypothalamus [5], pituitary [6] and gonads [7].

Effects of leptin on different endocrine targets, including the HPG axis, appear to be mediated by its specific binding to the leptin receptor, which includes different splicing variants, thus resulting in several isoforms of the receptor. Leptin receptors are present in male gonadal tissue, indicating that leptin could also exert a direct endocrine action on the gonads. Up to now, six leptin receptor isoforms have been recognized [8]. The Ob-Rb is the most widely distributed in mammals, and it is also expressed at the testicular level [7]. The local effect of leptin on the testis seems to be inhibitory [2]. Nevertheless, leptin administration elevated circulating levels of follicle-stimulating hormone (FSH) in male *ob/*ob mice and stimulated gonadal development [2]. In addition to these findings, leptin was shown to exert a concentration-dependent inhibition on luteinizing hormone (LH)-stimulated testosterone (T) production by either rat Leydig cells in culture or adult testis tissue [9, 10]. More recently, expression of leptin receptor has also been shown to take place during germ cell development of mouse testis [11]. This set of data indicates that leptin acts at different levels of the HPG axis, and its effects on reproductive functions could be related to different hormone thresholds [12].

Different phenotypes of obesity are characterized by hyperleptinemia, combined or not with hyperinsulinemia, and associated with hypogonadism. The association between hypogonadism and obesity is already recognized; thus endogenous hyperleptinemia is a potential afferent signal to influence testicular function. The aim of the present study was thus to develop a model of chronic hyperleptinemia combined with hypogonadism, by application of a neurotoxic treatment with *L*-monosodium glutamate (MSG) in the male newborn rat. Administration of this neurotoxin, which damages the arcuate nucleus of the hypothalamus, results in a syndrome characterized by stunted growth, pseudo-obesity and hypogonadism during adulthood [13]. This model allows to explore whether hypogonadism of adult MSG-treated male rats is dependent on endogenous hyperleptinemia or whether it results from testicular leptin resistance. To evaluate these issues, HPG function during endogenous hyperleptinemia and in vitro effects of exogenous leptin on testicular function was studied in adulthood in MSG-treated and control (CTR) rats. In addition, the impact of MSG-induced hyperleptinemia on testicular Ob-Rb expression was explored.

Materials and Methods

Animals and Experimental Designs

Adult male (350–450 g) and female (240–280 g) Sprague-Dawley rats were allowed to mate in colony cages, in a light- (12 h light cycle, lights on at 07:00 h) and temperature-controlled (20–22 °C) room, and were fed standard food pellets and water ad libitum. Pregnant female rats were placed in clear individual plastic cages. From 2 days after birth, male pups were injected i.p. with 4 mg/g BW MSG (Merck, Darmstadt, Germany) dissolved in 0.9% NaCl or 10% NaCl (CTR) on every 2 days up to day 10 of age [14], weaned at 21 days of age, and used for experimentation on day 120 of age.

Groups of 16 rats (CTR and MSG) were sacrificed and trunk blood was collected for further measurement of different hormones (see below); immediately after sacrifice their testes, epididymides and seminal vesicles were quickly dissected and weighted. Testes were then used for either testicular androgen determination, semi-quantitation of Ob-Rb mRNA expression or histological studies. A separate set of experimental animals (10–12 per group) was sacrificed and their testes were used for isolation of purified Leydig cells to be submitted for either in vitro experimentation on the effects of the neurotoxic damage and exogenous mleptin on spontaneous/hCG-stimulated androgens output or determination of Ob-Rb mRNA expression.

Animals were killed by decapitation following protocols for animal use from the NIH Guidelines for care and use of experimental animals.

Hormone Measurements

Plasma leptin concentrations were determined by a radioimmunoassay (RIA) previously described in detail [15]. The detection range of the standard curve was 0.4–15 ng/ml. The within-assay and interassay coefficients of variation (CVs) were 5–8 and 10–13%, respectively. Serum levels of LH and FSH were determined by double antibody RIA as previously described [16]. Results were expressed in terms of the reference preparations (rat LH-RP3 and FSH-RP-2), supplied by the NIDKK, Bethesda, Md., USA. Sensitivity for the LH assay was 0.024 ng/tube and for FSH was 0.10 ng/tube. The within- and interassay CVs were less than 8 and 13%, respectively, for both RIAs [17]. Testosterone (T) levels in serum, tissue and incubation medium were measured by RIA using testosterone [1,2–^{3}H (N), 60 Ci/mmol] from New England Nuclear (Boston, Mass., USA) and a specific antibody from Immunotech Diagnostic (Montreal, Canada), as previously de-

scribed and validated [18]. Androstandiol was evaluated by RIA using 5α-androstane-3α,17β-diol-1,2–^{3}H (SA 40.9 Ci/mmol) and a highly specific antibody (5α-androstane-3α,17β-diol-15-CMO-HAS) from Immunotech Diagnostic. Intratesticular T and androstandiol concentration was determined by RIA in samples processed as previously described and validated [19]. Briefly, pieces of testicular tissue were homogenized in acetone and centrifuged, and supernatants were evaporated to dryness. After resuspension in water, samples underwent a sequence of solvent partitions: water-dithylether (1:10, v/v), 70% methanol-hexane (1:1, v/v), and methanol-dichloromethane (1:3, v/v). Samples were evaporated, and the residues were resuspended in buffer and assayed for T and androstandiol by RIA as described above. Recovery of the initial tritiated standard was 83.80 ± 1.78% (mean standard ± error (SE)). Results were expressed in terms of ng/g tissue. The sensitivity of the T and androstandiol assays was 12.5 pg/ml. The within- and inter-assay CVs were less than 10%. The concentrations of 17-hydroxy-progesterone (17-HO-P_4) and Δ_4-androstenedione (Δ_4-A) in the incubation medium were measured by commercially available kits from Immunotech Diagnostic. The within- and inter-assay CVs were 5 and 4.4%, and 5.5 and 8.6%, respectively. Free thyroxin (FT4) levels in serum were also assayed by RIA using a kit from Diagnostic Product Corp. (■■■■, ■■■■, USA), and validated in our laboratory for rat plasma samples. The within- and inter-assay CVs were 4.5 and 8%, respectively.

Testicular Histological Studies

Tissue Preparation. 15 min before the initiation of perfusion for tissue fixation, rats were injected intraperitoneally with heparin at a dose of 130 IU/kg of body weight. The anesthetized (ketamine + xylazine) rats were perfused through the left ventricle with 0.9% saline for approximately 5 min to clear blood vessels. After clearance of vessels, a two-way valve apparatus was used to introduce 4% glutaraldehyde in phosphate buffer 0.05 *M*, pH 7.4 without removal of the needle [20] and animals were perfused for 25–30 min. To perform light microscopic investigations testis fragments were routinely processed and embedded in plastic (glycol methacrylate). Subsequently, sections of 4 μm in thickness were obtained and stained with toluidine blue to perform standard histological analysis.

Testis Histology. Qualitative histological analysis of the testis was performed carefully in order to analyze the testis structure in rats, and to verify to most advanced germ cell type present in the seminiferous epithelium of each animal, according to the criteria previously described [20]. To characterize rat germ cell association through the acrosomic system method.

Isolation of Leydig Cells

Purified Leydig cell preparations were obtained from, MSG and CTR, rats killed at 120 days of age. Fractions were prepared from 8–12 testes per group per experiment. Purified Leydig cell preparations were obtained as previously described [17]. Briefly, testes were removed, decapsulated and digested with 0.25 mg/ml collagenase (Worthington Biochemical Corporation, Freehold, N.J., USA) in M199 medium plus 0.1% BSA fraction V (Sigma Chemical Co., St. Louis, Mo., USA), at 34°C for 12–15 min. The digestion procedure was stopped by dilution with fresh medium. Two successive washes and sedimentations were then performed. The supernatants were pooled and interstitial cells collected by centrifugation at 220 *g*, for 10 min. Crude cell preparations were purified on a discontinuous five-layer Percoll density gradient (21, 26, 34, 40, and 60%) (Pharmacia, Uppsala, Sweden). The gradient was centrifuged at 800 *g*, for 30 min, and the interphase between 40 and 60% was collected and washed with medium to remove Percoll [21]. Cell viability, assessed by the trypan blue exclusion method, was approximately 95%. Purity of cell preparations, determined by a positive cytochemical reaction for 3β-hydroxysteroid dehydrogenase (3β-HSD) [22], was 90%.

Effect of Murine Leptin on Leydig Cell Testoterone Production

To evaluate a possible direct effect of leptin on in vitro secretion of 17-OH-P_4, Δ_4-A and T, purified Leydig cells (10^5 cells/tube, approximately) were obtained from CTR and MSG rats and incubated in M199, containing 0.1% BSA, in the presence or absence of mleptin (10^{-9}–10^{-7} *M*). Incubations were performed in a Dubnoff shaking incubator (88 cycles/min) in the absence (basal) or presence of various concentrations of hCG (0.05–100 ng/ml) (Ayerst Laboratory, Montreal, Canada), for 90 and 180 min, at 34°C, in an atmosphere of 95% O_2-5% CO_2. At the end of the first 90 min of incubation, 100 μl aliquots of media were taken and, after 180 min, tubes were centrifuged and all media samples were kept frozen (–20°C) until determination of medium concentrations of different steroids.

RNA Isolation and RT-PCR Analysis

Total RNA was isolated from testicular samples and purified Leydig cells by using the single-step, acid guanidinium isothiocyanate-phenol-chloroform extraction method described by Chomzynski et al. [23]. The yield and quality of extracted RNA were assessed by 260/280 nm optical density ratio and electrophoresis, under denaturating conditions, on 2% agarose gel. One microgram of total RNA was incubated with 0.2 m*M* dNTPs, 1 m*M* $MgSO_4$, 1 *M* Ob-Rb primers, 0.25 *M* β-actin primers, 0.1 U/μl AMV reverse transcriptase (5 U/μl), 0.1 U/μl Tfl DNA polymerase (5 U/μl). Final volume was 25 μl. Amplifications were done in a thermal cycler (Perkin-Elmer) in the following conditions: 48°C – 45 min for reverse transcription step (1 cycle); 94°C – 2 min for AMV RT inactivation and RNA/cDNA/primer denaturation (1 cycle); 94°C – 30 s for denaturation; 54°C – 1 min for annealing; 68°C – 2 min for extension (40 cycles); 68°C – 7 min for final extension (1 cycle), and 4°C for soak (Promega Access RT-PCR System No. A1250). Because we determined in preliminary experiments that co-linearity exists (data not shown) in β-actin (r^2 = 0.982) and Ob-Rb (r^2 = 0.968) cDNA amplification between 30 and 45 cycles when processed, then 40 cycles was used. Primers were designed for a high homology region of the long form leptin-receptor (Ob-Rb) (encoded with intracellular domain which is essential for signal transduction) (F) 5′-TGG CCC ATG AGT AAA GTG AAT-3′, and (R) 5′-CCA GAA GAA GAG GAC CAA ATA -3′. In this semiquantitave technique, the second set of primers was specific for the β-actin gene, having the following sequences: (F) 5′-CGG AAC CGC TCA TTG CC-3′, and (R) 5′-ACC CAC ACT GCC CAT CTA-3′ (289 bp). Controls without reverse transcriptase were systematically performed to detect cDNA contamination. The amplified products were analyzed on 2% agarose gel and visualized by ethidium bromide UV transillumination in a Digital Imaging System (Kodak Digital Science, Electrophoresis Documentation and Analysis 120 System).

Statistical Analysis

Data of circulating and medium hormone concentrations were analyzed by ANOVA, followed by Fisher's test for comparison of different mean values. The nonparametric Mann-Whitney test was used for analysis of data from testicular Ob-Rb mRNA expression [24].

Table 1. Body weight (BW), relative organ weight (g of organ per 100 g BW) and intratesticular T and androstandiol (ng per g of tissue), in 120-day-old CTR and MSG rats

Parameter	MSG	CTR
BW	323.91 ± 4.91	292.4 ± 9.43*
Testis	4.83 ± 0.11	4.12 ± 0.09*
Seminal vesicles	2.06 ± 0.23	1.59 ± 0.19*
Epididymides	1.98 ± 0.07	1.51 ± 0.11*
Intratesticular testosterone	64.24 ± 6.08	50.36 ± 5.36
Intratesticular androstandiol	20.06 ± 2.06	23.30 ± 1.74

* $p < 0.05$ or less vs. CTR values.
Values are the mean ± SEM (n = 16 rats per group).

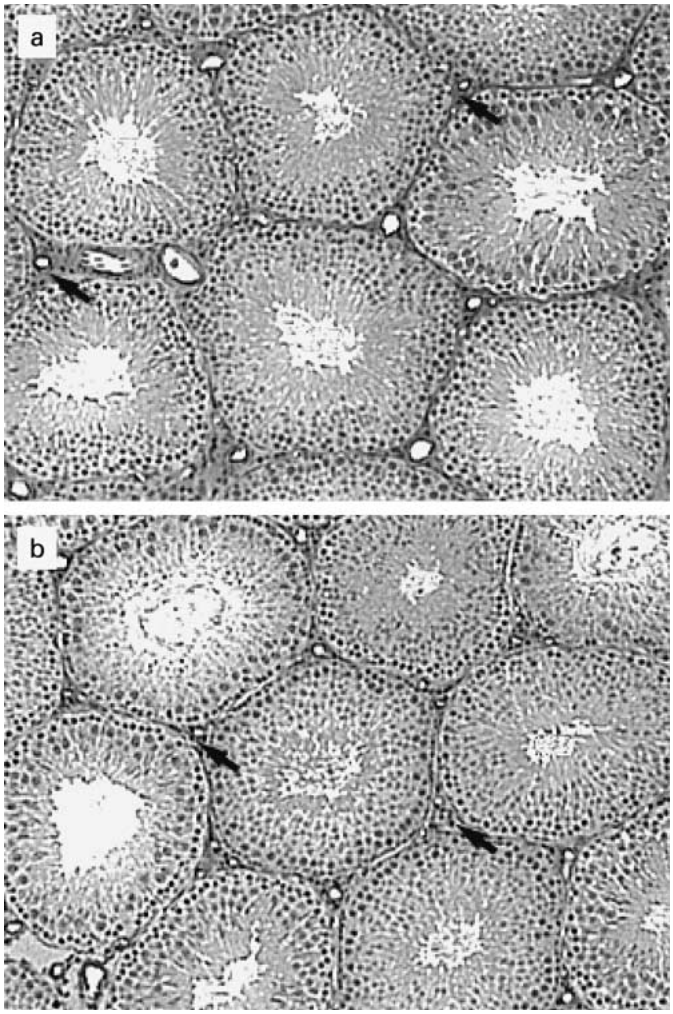

Fig. 1. Lower magnification of the testis in 120-day-old, CTR (**a**) and MSG-treated rats (**b**). It can be observed that, apparently, MSG treatment did not cause any alteration in the testis structure as evidenced by the seminiferous tubules (ST) cross-sections at different stages of the cycle and the interstitial compartment (arrows). The testis tissue was stained with toluidine blue (a representative animal per group, magnification × 100).

Results

Body and Organ Weights and Testicular Morphology

Exposure of newborn rats to MSG resulted in the expected involution of the reproductive system. Body weight (BW) was lower ($p < 0.05$) than in CTR age-matched rats (table 1). MSG rats also presented a significant ($p < 0.05$) regression of testes, seminal vesicles and epididymis when compared to CTR rats (table 1). These differences, when expressed as relative organ weights (g/100 g BW) persisted (table 1).

Finally, when compared to age-matched CTR, testis structure was apparently normal in MSG-treated rats (fig. 1). In fact, no qualitative alterations were observed in testis structure and in spermatogenesis in MSG-treated rats at 120 days of age (fig. 1).

Tissue and Peripheral and Hormone Levels

Results of hormone profiles are shown in table 1 and figure 2. Intratesticular concentrations of T and androstanediol were similar in both experimental groups when expressed by mass of tissue (table 1). However, when androgen tissue concentrations were expressed per organ we detected significant ($p < 0.005$) differences between groups, regardless of the androgen, concentrations being higher in CTR than in MSG rats (data not shown). Nevertheless, it should be stressed that BW and testis weight values in MSG animals were significantly ($p < 0.05$) lower than those found in their normal counterparts (table 1).

Circulating LH (fig. 2a) and FSH (fig. 2b) levels were significantly ($p < 0.05$) reduced by MSG treatment as compared to the CTR day-matched group. However, rats exposed to MSG displayed plasma T levels within the CTR range (fig. 2c). When plasma leptin levels were measured, a significant increase ($p < 0.05$) in MSG-treated rats in comparison to CTR animals was observed (fig. 2d). In fact, plasma leptin levels were 3-fold higher in the MSG group, and persisted in relation to individual BW (table 2). Finally, and because of the stunted growth development of MSG rats, we explored whether this characteristic could be related to changes in thyroid function as a consequence of MSG treatment. Results indicated that 120-day-old MSG-treated rats showed plasma FT_4 levels similar to those found in age-matched CTR rats (table 2).

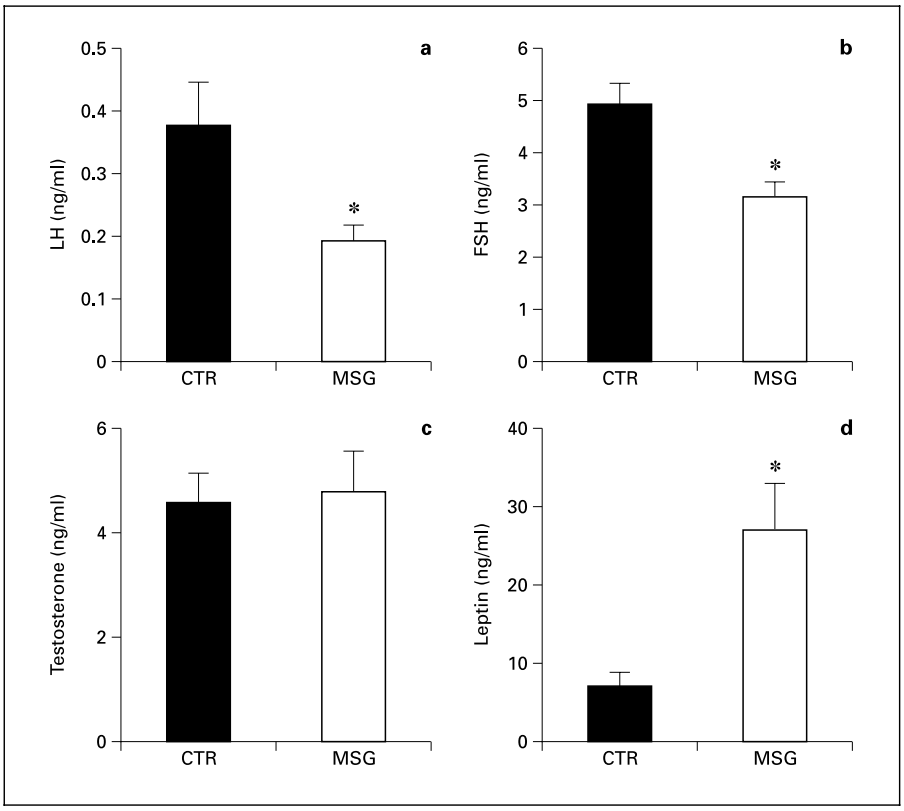

Fig. 2. Basal circulating levels of LH (**a**), FSH (**b**), total testosterone (**c**) and leptin (**d**) in 120-day-old, CTR and MSG-treated male rats. Values are the mean ± SEM (n = 16 animals per group). * $p < 0.05$ or less vs. CTR values.

Table 2. Circulating plasma concentrations of leptin, expressed in relation to individual BW, and free thyroxin (FT_4) in 120-day-old CTR and MSG rats

Group	CTR	MSG
Leptin, ng/ml/100 g BW	2.91 ± 0.46	10.39 ± 2.41*
FT_4, ng/dl	1.21 ± 0.09	1.11 ± 0.11

* $p < 0.05$ or less vs. CTR values.
Values are the mean ± SEM (n = 16 rats per group).

Effect of Endogenous Hyperleptinemia on in vitro T Production by Leydig Cells in the Presence or Absence of Mleptin

Figure 3 shows the results of basal and hCG-stimulated (0.05–100 ng/ml) T production by purified isolated Leydig cells from CTR and MSG rats after either 90 (fig. 3a) or 180 min (fig. 3b) incubation in the absence or presence of mleptin (10^{-9} and 10^{-8} *M*). In the absence of mleptin, the results indicated that, as a consequence of MSG treatment, basal and hCG-stimulated T production by Leydig cells was significantly ($p < 0.01$ or less) lower at both incubation times than the respective responses developed by a similar number of CTR cells. For both groups, the minimal hCG concentrations able to enhance basal T release into the medium were 0.5 and 0.1 ng/ml, after 90 and 180 min incubation, respectively.

It is important to remark that, after 90 min incubation, the relative response (hCG-stimulated:basal ratio) of Leydig cells from MSG rats was significantly higher than in CTR cells although MSG cells released significantly ($p < 0.05$) lower amounts of T than CTR cells. On the other hand, hCG-stimulated:basal ratio of T released into the medium was significantly ($p < 0.05$) lower in the MSG than in the CTR cell-group after 180 min incubation (data not shown).

Basal and hCG-stimulated T production in vitro by CTR Leydig cells was unmodified by the lower mleptin (10^{-9} *M*) concentration. Conversely, although mleptin (10^{-8} *M*) did not modify basal T production, it significantly ($p < 0.05$) increased the response to 0.1 ng/ml hCG in CTR cells at both incubation times (fig. 3a, b). Interestingly, the effect of 10^{-8} *M* mleptin on 0.1 ng/ml hCG-induced T secretion became significantly ($p < 0.05$) higher

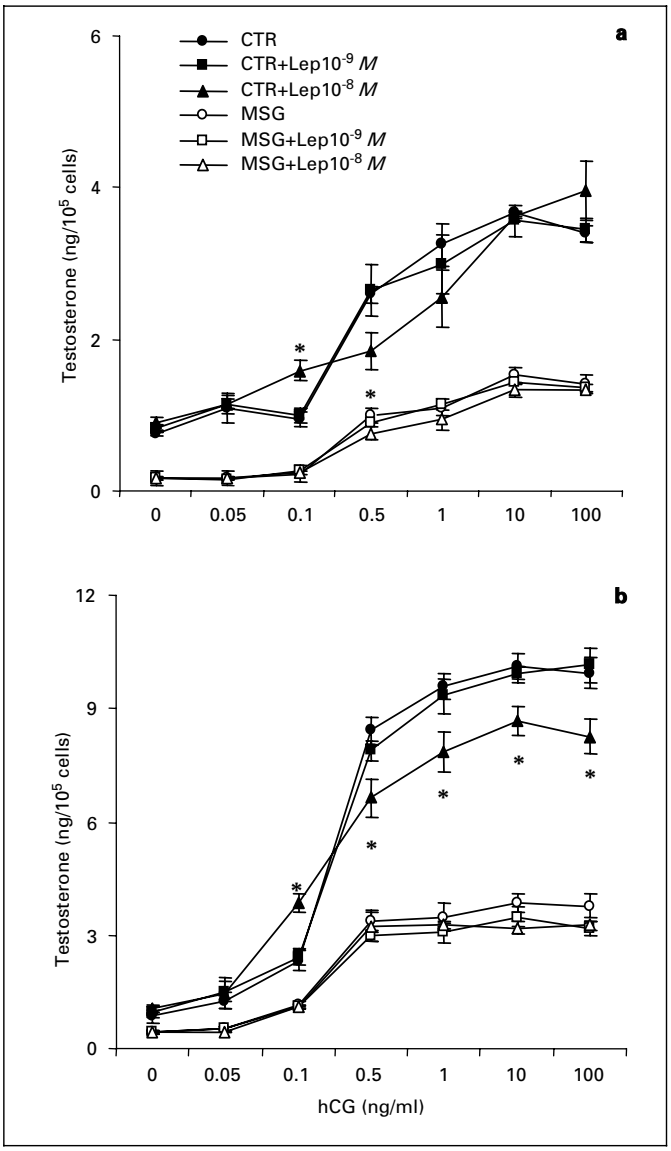

Fig. 3. Basal and hCG-stimulated testosterone release by isolated Leydig cells from 120-day-old CTR and MSG rats incubated in vitro for 90 (**a**) and 180 (**b**) min under metabolic conditions. Incubations were done in the absence or presence of mleptin (Lep; 10^{-9} and 10^{-8} *M*). Values are the mean ± SEM (n = 5 experiments, with 5–6 tubes per group-condition in each experiment). All MSG values are significantly ($p < 0.01$ or less) different vs. CTR values in similar conditions. * $p < 0.05$ or less vs. CTR values without leptin under similar conditions.

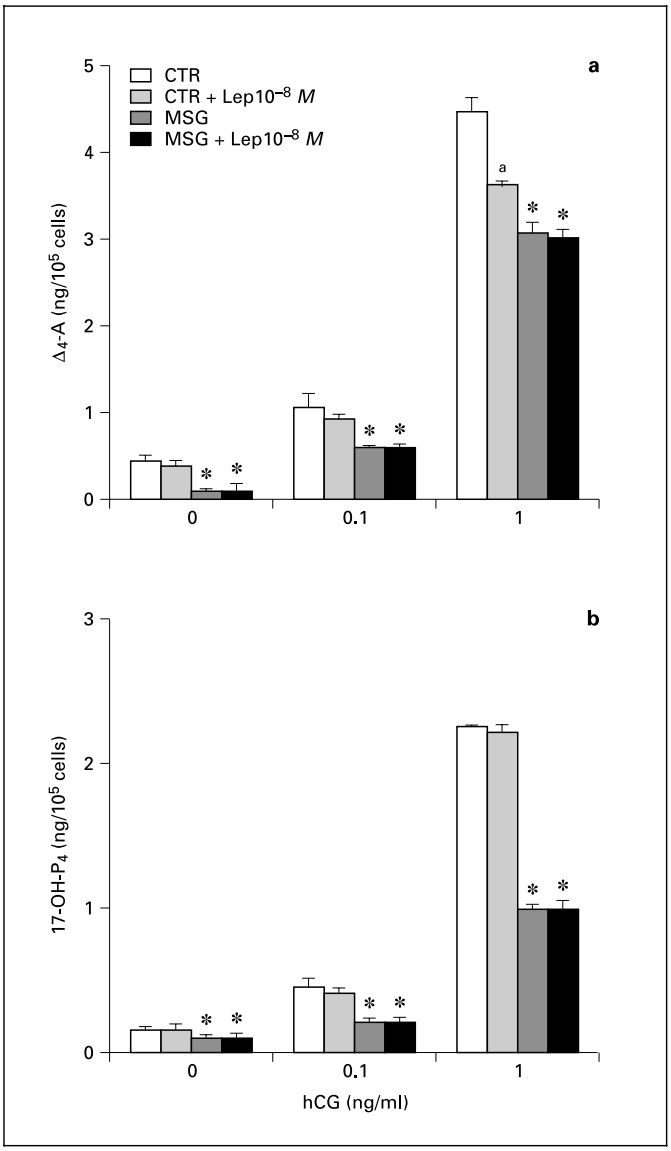

Fig. 4. Basal and hCG-stimulated Δ_4-A (**a**) and 17-HO-P_4 (**b**) release by isolated Leydig cells from 120-day-old CTR and MSG rats incubated in vitro for 180 min under metabolic conditions. Incubations were done in the absence or presence of 10^{-8} *M* leptin. Values are the mean ± SEM (n = 5 experiments, with 5–6 tubes per group-condition in each experiment). * $p < 0.05$ or less vs. CTR values in similar conditions. [a] $p < 0.05$ vs. CTR values without leptin in similar conditions.

than basal T release (fig. 3a). On the other hand, 10^{-8} *M* mleptin significantly ($p < 0.05$) inhibited 0.5 ng/ml hCG-induced T output at 90 min (fig. 2a), while at 120 min incubation (fig. 3b) the inhibitory effect of mleptin was found with concentrations of 0.5, 1, 10 and 100 ng/ml hCG. Conversely to results for CTR cells, the presence of mleptin (at either concentration) in the incubation medium had no effect on basal and hCG-stimulated T release by incubated MSG Leydig cells, regardless of incubation time (fig. 3a, b).

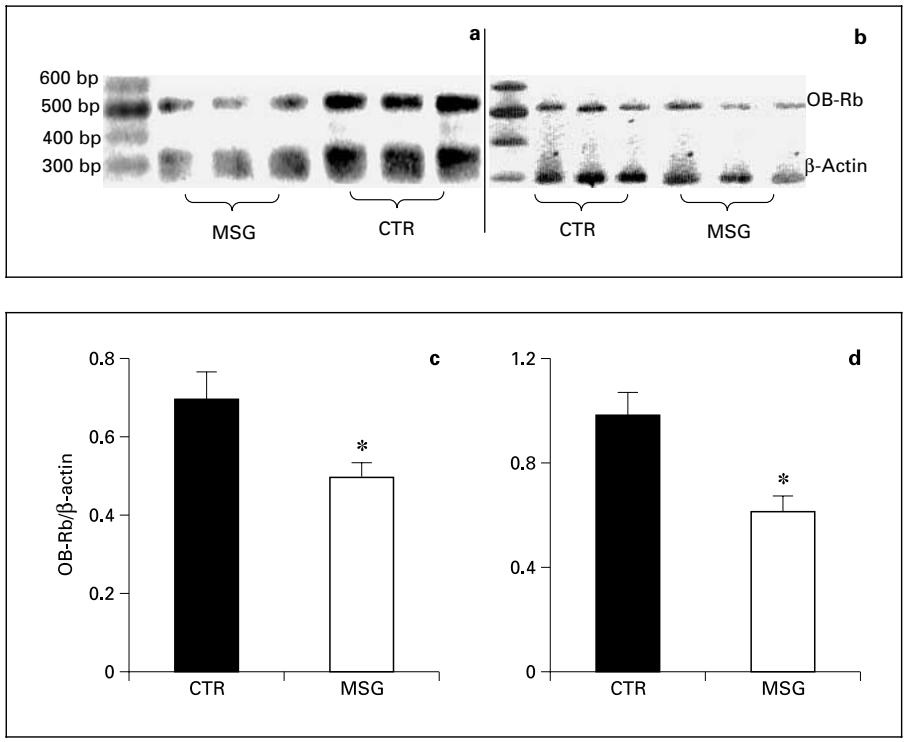

Fig. 5. RT-PCR expression of Ob-Rb mRNA (3 representative animals per group) in testes (**a**) and isolated Leydig cells (**b**) from control and MSG rats of 120 days of age (M = molecular marker, 500-bp ladder), co-expressed with β-actin. Ob-Rb expression, relative to β-actin, testes (**c**) and isolated Leydig cells (**d**) from CTR and MSG 120-day-old animals (**b**). Values are the mean ± SEM (n = 9 testis and 200,000 Leydig cells per group, respectively). * $p < 0.05$ vs. CTR values.

When a higher mleptin (10^{-7} *M*) concentration was assayed in our model, it was found to mimic the pattern on both Leydig cell groups (data not shown).

Effect of Endogenous Hyperleptinemia on in vitro Testosterone Precursor Production by Leydig Cells in the Presence or Absence of Mleptin

To explore potential effects of leptin-induced inhibition of CTR Leydig cell steroidogenesis, basal and hCG-stimulated 17-HO-P_4 and Δ_4A secretions were measured in the media after 180 min incubation. The results of Δ_4-A and 17-HO-P_4 secretion, in basal and hCG (0.1 and 1 ng/ml)-stimulated conditions, were significantly ($p < 0.05$) lower in Leydig cells from MSG than CTR rats (fig. 4a, b, respectively). Although mleptin failed to modify basal steroidogenesis in CTR cells, interestingly, mleptin (10^{-8} *M*) was able to significantly ($p < 0.05$, vs. similar condition in the absence of leptin) inhibit 1 ng/ml hCG-stimulated Δ_4-A, but not 17-HO-P_4, secretion. Moreover, 10^{-8} *M* mleptin significantly ($p < 0.05$) reduced the normal ratio (obtained in the absence of mleptin) of Δ_4-A:17-HO-P_4 secretion from 2.12 ± 0.28 to 1.63 ± 0.36 in CTR cells stimulated with 1 ng/ml hCG and, conversely, mleptin failed to modify the ratio of T:Δ_4-A secretion (2.15 ± 0.21 and 2.18 ± 0.16) when calculated in the absence and presence of mleptin, respectively, in CTR Leydig cells stimulated with the similar hCG concentration (1 ng/ml). Similar to findings for T secretion, mleptin had no effect on hCG-elicited release of either of the T precursors by MSG cells, regardless of the incubation period (fig. 4a, b).

Effect of Endogenous Hyperleptinemia on Testicular Expression of Ob-Rb mRNA

In order to explore whether the lack of effect of leptin on testicular steroidogenesis depended on any change in testicular Ob-Rb expression after MSG treatment, the expression of leptin receptor Ob-Rb mRNA in testicular sections from CTR and MSG rats was evaluated. Figure 5a shows a representative ethidium bromide gel electrophoresis of Ob-Rb mRNA amplified by RT-PCR from total testicular RNA samples in both groups. Semiquantitative analysis of relative (to β-actin co-expressed) Ob-Rb expression in testicular samples indicated that a significantly ($p < 0.05$) lower receptor expression was found in MSG than in CTR tissues (fig. 5b). Interestingly, the reduced expression (vs. CTR rats) of testicular Ob-Rb in MSG rats was even present when examined in isolated Leydig cells (fig. 5c, d).

Discussion

Leptin, the adipocyte hormone, is important not only for the regulation of food intake and energy balance but is also a significant signal modulating metabolic and neuroendocrine functions.

As already mentioned, the main sites of action of leptin in modulating HPG axis function are the hypothalamus, pituitary and gonads. Leptin regulation of individual reproductive capacity in adulthood seems, however, to depend on the threshold of circulating leptin; the regulation of neuroendocrine or gonadal functions [12] is very sensitive to hyper- or hypoleptinemia. The present study demonstrates that neonatal MSG-induced hyperleptinemia induces significant changes in male gonads and accessory sex organs in adulthood. In fact, our experimental model confirmed earlier reports on neurotoxin-induced hypothalamic denervation, a model characterized by stunted growth, pseudo-obesity and hypogonadism in adulthood [13]. To our knowledge, this is the first demonstration that this neuronal lesion also causes a 3-fold increase in plasma leptin levels that persists irrespective of the individual BW [25].

In addition, plasma LH and FSH levels correlate well with the hypogonadism already described [13, 14]. Unexpectedly, circulating T levels in the adult MSG rat were similar to the CTR group. Nevertheless, this parameter does not always parallel the androgen tissue concentrations. Incidentally, we have detected significantly lower plasma T levels at 30 days of age [26]. It is then likely that during the critical period of sexual accessory organ growth, these tissues are exposed to low circulating androgens and also low GH [27].

Among several other factors, LH and thyroid hormone are crucial for testicular cell proliferation and differentiation during postnatal development [28]. In this regard, studies in progress in our laboratory [29] indicate that free tetraiodotyronine (T4), LH and FSH circulating levels are significantly lower in MSG than in CTR rats at 30 days of age, along with a slightly reduced Leydig cell volume. When rats reached adulthood, however, the two main testis compartments were not altered, thus indicating that alterations in young MSG-treated rats are probably due to decreased free T4 and LH circulating levels characteristic of these animals. Restoration of normal thyroid hormone levels at adult age could thus contribute to normalize testicular structure.

The pathway for T metabolization is an enzymatic process which takes place in microsomes from rat lung, liver, kidneys, testis, prostate and brain, and the cytochorme P-450 isozymes are responsible for the process [30]. The effects of neonatal MSG administration to rodents, in addition to the well-defined syndrome developed, induces a depression of hepatic T metabolizing enzymes. Within these, the sex-dependent activity and the expression of cytochrome P-450 isoforms (e.g. CYP2C11) are altered in MSG-treated rats [31]. Moreover, when MSG is administered to male rats, in the same dose and schedule to the one used in the present study, both GH circulating levels and liver expression of CYP2C11 isoform, are not detected at adult life [32]. Additionally, it was explored, in an in vitro study, whether isolated corticoadrenal cells from MSG rats could be a significant source of T production, either in basal or ACTH-stimulated conditions. The results did not shown a significant release of T into the incubation medium by incubated adrenal cells from both experimental groups examined (data not shown). Thus, the adrenal does not seem to contribute to the overall concentration of circulating T levels in MSG adult rats.

More recently, leptin has been presented as a local regulator of gonadal activity in the male. The *ob* gene product was reported to directly inhibit T secretion in rats [33]. Testicular function is impaired, however, in anorexic and hypoleptinemic men [34], and endogenous leptin was shown to reverse hypogonadism and to accelerate the onset of puberty in both sexes [35, 36]. As a consequence of MSG treatment, basal and hCG-stimulated T production by Leydig cells was significantly lower at both incubation times studied than that of the same number of CTR cells. Conversely, although mleptin (10^{-8} *M*) failed to modify basal T production, it significantly increased the response to 0.1 ng/ml hCG in CTR cells. Interestingly, the effect of 10^{-8} *M* mleptin on 0.1 ng/ml hCG-induced T secretion became significantly higher than basal release. Within this context, exogenous mleptin administration in mice with genetically determined leptin deficiency is able to restore the delayed puberty characterizing this strain [36]. Additionally, increased nocturnal levels of leptin (among others) have been shown to precede the onset of puberty in agonadal primates [37].

On the other hand, in adult CTR Leydig cells, 10^{-8} *M* mleptin significantly inhibited 0.5 ng/ml hCG-induced T release at 90 min; while at 120 min, the inhibitory effect of leptin was clearly found with 0.5, 1, 10 and 100 ng/ml hCG concentrations.

Conversely to that observed in CTR cells, the presence of mleptin in the incubation medium had no effect on either basal or hCG-stimulated T release by incubated MSG Leydig cells regardless of incubation time. When a higher mleptin (10^{-7} *M*) concentration was assayed in our

model, leptin was found to mimic the patterns in both Leydig cell groups.

To explore the potential effects of leptin-induced inhibition of CTR Leydig cell steroidogenesis, basal and hCG-stimulated 17-HO-P_4 and Δ_4-A secretion were measured in the media after 180 min incubation. The results of Δ_4-A and 17-HO-P_4 release in basal and hCG (0.1 and 1 ng/ml)-stimulated conditions indicated that they were both significantly lower in Leydig cells from MSG than from CTR rats. These results confirm that hyperleptinemia is able to inhibit testicular steroidogenesis. Interestingly, and although mleptin failed to modify basal steroidogenesis in CTR cells, mleptin (10^{-8} *M*) inhibited 1 ng/ml hCG-stimulated Δ_4-A and the Δ_4-A:17-HO-P_4 ratio of secretion, but not the T:Δ_4-A ratio. mleptin was also ineffective on the release of both T precursors by MSG cells regardless of the incubation time. On this point, it has been suggested that leptin is able to affect 17,20-lyase activity, the enzyme that converts 17α-hydroxylated intermediates to Δ_4-A [9]. A previous report has also shown that in vitro recombinant leptin (10^{-9}–10^{-7} *M*) is able to decrease the hCG-stimulated expression levels of steroidogenic factor 1, StAR and $P450_{sc}$ mRNAs after 180 min [7] in a concentration-dependent manner without significantly altering 17β-HSD type III expression. Another report [33] also indicated that in vivo hCG-induced down-regulation of 17β-HSD expression is only detected 12 h after treatment. In our study, the leptin effect could be related to sustained endogenous hyperleptinemia although we did not measure directly expression levels of the enzyme.

Leptin is already accepted as an important factor directly modulating Leydig cell activity [7]. Effects of leptin on different endocrine target tissues, including the HPG axis are mediated by specific binding to the leptin receptor, which exhibits several splicing variants and several isoforms. Expression of the Ob-Rb receptor in different tissues is down-regulated by high circulating leptin levels [15, 38]. In the case of testis, leptin receptor isoforms are present [7], suggesting that leptin also exerts a direct endocrine action on the gonads. The local effect on the testis seems to be inhibitory [2]. In order to explore whether the lack of leptin effect on testicular steroidogenesis depended on changes in testicular Ob-Rb receptors after MSG treatment, we evaluated the expression of leptin receptor Ob-Rb mRNA in testicular sections from CTR and MSG rats. Ob-Rb expression in both testicular samples and purified Leydig cells was significantly ($p < 0.05$) lower in MSG than in CTR tissues. These results agree with the concept of homologous regulation of the expression of Ob-Rb in the rat hypothalamus [15], adrenal [38] and testis [7] shown by in vivo and in vitro experiments.

In summary, our data strongly support that: (1) MSG-induced hyperleptinemia induces in vitro Leydig cell dysfunction in terms of basal 17-HO-P4, Δ_4A and basal and post-hCG T secretion; (2) mleptin in CTR Leydig cells stimulates T secretion over basal release at the lowest hCG concentration tested; however, the same concentration of mleptin (10^{-8} *M*), significantly inhibits hCG-induced T release (in the range of 0.5, 1, 10 and 100 ng/ml hCG) in CTR Leydig cells; (3) the presence of mleptin in the incubation medium has no effect on basal and hCG-stimulated T release by incubated Leydig cells in rats treated with MSG, and (4) Ob-Rb mRNA expression is significantly reduced in testes and isolated Leydig cells in MSG-treated rats at 120 days of age with regard to CTR.

Adult hypogonadism characteristic of the MSG model is partly accounted for by hyperleptinemia at an early stage of development and by the inhibitory role of leptin on reproductive functions.

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