Molecular mechanisms of leptin action in adult rat testis: potential targets for leptin-induced inhibition of steroidogenesis and pattern of leptin receptor messenger ribonucleic acid expression

M Tena-Sempere, P R Manna[2], F-P Zhang[2], L Pinilla, L C González, C Diéguez[1], I Huhtaniemi[2] and E Aguilar

Department of Physiology, University of Córdoba, 14004 Córdoba, Spain

[1]Department of Physiology, University of Santiago de Compostela, 15705 Santiago de Compostela, Spain

[2]Department of Physiology, University of Turku, 20520 Turku, Finland

(Requests for offprints should be addressed to M Tena-Sempere, Department of Physiology, Faculty of Medicine, University of Córdoba, Avda Menéndez Pidal s/n, 14004 Córdoba, Spain; Email: fi1tesem@lucano.uco.es)

Abstract

Leptin, the product of the *ob* gene, is a pivotal signal in the regulation of neuroendocrine function and fertility. Although much of the action of leptin in the control of the reproductive axis is exerted at the hypothalamic level, some direct effects of leptin on male and female gonads have also been reported. Indeed, recent evidence demonstrated that leptin is able to inhibit testosterone secretion at the testicular level. However, the molecular mechanisms behind this effect remain unclear. The focus of this study was twofold: (1) to identify potential targets for leptin-induced inhibition of steroidogenesis, and (2) to characterize in detail the pattern of expression and cellular distribution of leptin receptor (Ob-R) mRNA in adult rat testis. In pursuit of the first goal, slices of testicular tissue from adult rats were incubated with increasing concentrations of recombinant leptin (10^{-9}–10^{-7} M) in the presence of human chorionic gonadotropin (hCG; 10 IU/ml). In this setting, testosterone secretion *in vitro* was monitored, and expression levels of mRNAs encoding steroidogenic factor 1 (SF-1), steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side-chain cleavage enzyme (P450 scc) and 17β-hydroxysteroid dehydrogenase type III (17β-HSD) were assessed by Northern hybridization. In pursuit of the second goal, the pattern of cellular expression of the Ob-R gene in adult rat testis was evaluated by *in situ* hybridization using a riboprobe complementary to all Ob-R isoforms. In addition, testicular expression levels of the different Ob-R isoforms, previously identified in the hypothalamus, were analyzed by means of semi-quantitative RT-PCR. In keeping with our previous data, recombinant leptin significantly inhibited hCG-stimulated testosterone secretion. In this context, leptin, in a dose-dependent manner, was able to co-ordinately decrease the hCG-stimulated expression levels of SF-1, StAR and P450 scc mRNAs, but it did not affect those of 17β-HSD type III. *In situ* hybridization analysis showed a scattered pattern of cellular expression of the Ob-R gene within the adult rat testis, including Leydig and Sertoli cells. In addition, assessment of the pattern of expression of Ob-R subtypes revealed that the long Ob-Rb isoform was abundantly expressed in adult rat testis. However, variable levels of expression of Ob-Ra, Ob-Re, and Ob-Rf mRNAs were also detected, whereas those of the Ob-Rc variant were nearly negligible. In conclusion, our results indicate that decreased expression of mRNAs encoding several up-stream elements in the steroidogenic pathway may contribute, at least partially, to leptin-induced inhibition of testicular steroidogenesis. In addition, our data on the pattern of testicular expression of Ob-R isoforms and cellular distribution of Ob-R mRNA may help to further elucidate the molecular mechanisms of leptin action in rat testis.

Journal of Endocrinology (2001) **170,** 413–423

Introduction

The obese (*ob*) gene product, leptin, is an adipocyte-secreted plasma hormone that plays a key role in the regulation of food intake, energy expenditure and body weight homeostasis (Friedman & Halaas 1998). In addition, leptin has recently emerged as a pivotal metabolic signal in the regulation of reproductive function (Barash *et al*. 1996, Friedman & Halaas 1998, Rosenbaum & Liebel 1998, Casanueva & Dieguez 1999). The absence of biological actions of leptin, due to mutations in the *ob* gene (*ob/ob* mice) or leptin receptor gene (*db/db* mice), leads to infertility whereas leptin administration advances the onset of puberty, maintains reproductive cyclicity despite acute

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fasting, and prevents sterility in *ob/ob* mice (Ahima *et al.* 1996, 1997, Chehab *et al.* 1997, Mounzih *et al.* 1997). In addition, a stimulatory role of leptin in the control of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion has been documented (Barash *et al.* 1996, Nagatani *et al.* 1998). Compelling evidence indicates that the hypothalamus is the primary target for most of the action of leptin on the reproductive axis (Schwartz *et al.* 1996). However, based on the characterization of leptin receptor distribution and leptin effects on *in vitro* systems, additional sites for leptin action have been suggested, including the pituitary, testis and ovary (Zamorano *et al.* 1997, Spicer & Francisco 1997, Caprio *et al.* 1999, Tena-Sempere *et al.* 1999*a*, Jin *et al.* 2000). Interestingly, evaluation of the direct testicular effects of leptin revealed its ability to inhibit testosterone secretion (Caprio *et al.* 1999, Tena-Sempere *et al.* 1999*a*, 2000*a*), a response analogous to that observed in terms of ovarian steroidogenesis after leptin stimulation *in vitro* (Spicer & Francisco 1997, Zachow *et al.* 1999). Thus, it is tempting to propose a complex mode of action of leptin at multiple sites of the hypothalamic–pituitary–gonadal axis that involves both stimulatory and inhibitory responses.

The biological actions of leptin are carried out through interaction with its specific surface receptor. The leptin receptor (Ob-R) belongs to the cytokine receptor superfamily, containing a single membrane-spanning domain (Tartaglia 1997). Worthy to note, expression of the Ob-R gene results in an array of alternatively spliced isoforms (Ob-Ra to Ob-Rf), that share the extracellular domain but differ in the transmembrane/cytoplasmic regions (Tartaglia *et al.* 1995, Tartaglia 1997, Lee *et al.* 1996, Takaya *et al.* 1996). Among them, the Ob-Rb variant, with the longest cytoplasmic domain, appears as the functional, signal-transducing isoform in the hypothalamus (Tartaglia 1997, Casanueva & Dieguez 1999). The functional role of the shorter Ob-R subtypes remains to be fully elucidated. However, the ability of the Ob-Ra isoform to perform signal transduction has been reported (Murakami *et al.* 1997), and this variant has been shown to be involved in leptin transport across the blood–brain barrier (Tartaglia 1997, Bjorbæk *et al.* 1998). In addition, a role for the Ob-Re isoform as a soluble leptin-binding protein has been proposed (Tartaglia 1997). Moreover, modulation of Ob-Rb function by co-expression of shorter Ob-R isoforms has been reported (White *et al.* 1997, White & Tartaglia 1999). In this scenario, it is likely that regulation of leptin action upon target tissues could depend, at least partially, on the balance of expression of the different Ob-R isoforms.

The aim of the present study was to further explore the molecular mechanisms of leptin action upon adult rat testis. For this purpose, two experimental approaches were undertaken. First, we evaluated the ability of leptin to modulate mRNA expression levels of several key elements in the testicular steroidogenic pathway. Second, we assessed the pattern of testicular Ob-R gene expression. To this end, cellular distribution of Ob-R mRNA within adult rat testis was studied by means of *in situ* hybridization, and semi-quantitative RT-PCR was used to evaluate relative expression levels of mRNAs encoding different Ob-R isoforms.

Materials and Methods

Animals and drugs

Adult (75-day-old) male Wistar rats were purchased from Charles River (Criffa, Barcelona, Spain). On arrival, the animals were housed under constant conditions of light (14 h of light; lights on at 0700 h) and temperature (22 °C), with free access to standard laboratory animal food and tap water. All experimental procedures were approved by the Córdoba University Ethical Committee for Animal Experimentation and were conducted in accordance with the European Union standards for care and use of experimental animals.

Human recombinant leptin was produced in *Saccharomyces cervisae* as described elsewhere (Considine *et al.* 1996), and kindly donated by Eli Lilly (Indianapolis, IN, USA). Highly purified human chorionic gonadotropin (hCG; Profasi HP500) was purchased from Serono (Madrid, Spain).

Tissue incubations

For the analysis of the direct effects of leptin on steroidogenic-related genes, static incubation of testicular tissue was carried out as described elsewhere (Tena-Sempere *et al.* 1999*a*, 2000*a*). This setting allows up to 4 h of incubation of slices of testicular tissue of approximately 20·0 mm thickness with preserved cell viability, as indirectly evidenced by appropriate secretory responses to known stimuli and absence of significant RNA breakdown in incubated samples (Tena-Sempere *et al.* 1999*a*, 2000*a*, and present results). Upon decapitation of experimental animals, testes were removed immediately, decapsulated, and cut into pieces of approximately equal size (mean weight/piece: $383 \pm 8·5$ mg; 4 slices per testis). Testicular slices (2 slices/well) were incubated in 2 ml DMEM–F12 medium (1:1; Life Technologies, Grand Island, NY, USA) supplemented with 0·1 g/l gentamicin (Biological Industries, Bet-Haemek, Israel) in a Dubnoff shaker (60 cycles/min) at 32 °C under an atmosphere of 5% CO_2–95% O_2. After preincubation for 1 h, the media were replaced either by fresh medium or medium containing increasing doses of human recombinant leptin (10^{-9}–10^{-7} M) in the presence of hCG (10 IU/ml). An additional group of testicular samples was challenged with hCG alone. After 180 min incubation, 100 µl aliquots from the incubation media were taken for testosterone measurement and samples of testicular tissue were frozen

in liquid nitrogen and stored at $-70\,^{\circ}\text{C}$ until used for RNA analysis. On the latter, four major targets were selected: steroidogenic factor 1 (SF-1), steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side-chain cleavage enzyme (P450 scc) and 17β-hydroxy steroid dehydrogenase type III (17β-HSD).

Testosterone measurements

Testosterone was measured from diethyl ether extracts of tissue incubation media by RIA using ^{3}H-labeled testosterone as tracer, as described elsewhere (Rodriguez-Padilla *et al.* 1987). The levels of testosterone in the media were expressed as normalized values per gram of incubated tissue.

RNA isolation and Northern blot analysis

Total RNA was isolated from testicular samples using the single-step acid guanidinium thiocyanate–phenol–chloroform extraction method, as described previously (Chomczynski & Sacchi 1987). The quantity of extracted RNA was assessed by spectrophotometry.

For Northern hybridization analyses, RNA samples (20 µg/lane) were resolved on 1·2% denaturing agarose gels and transferred onto Hybond-N$^+$ nylon membranes (Amersham International, Aylesbury, UK) using the capillary method. The membranes were cross-linked by short-wave UV irradiation and prehybridized for 4–6 h at $64\,^{\circ}\text{C}$ in a solution containing 50% deionized formamide, $3 \times$ SSC, $5 \times$ Denhardt's solution, 0·1 g/l heat-denatured calf thymus DNA, 1% SDS, and 0·1 g/l yeast transfer RNA. For hybridization, ^{32}P-labeled complementary RNA (cRNA) and DNA (cDNA) probes specific for the target genes were generated using the Riboprobe system II kit and the Prime-a-Gene labeling kit (Promega, Madison, WI, USA), respectively. For generation of cRNA probes, *Not*I linearized pGEM-T vector (Promega) containing the full-length mouse StAR cDNA, and *Eco*RI linearized pBS plasmid (Promega) containing a 298 bp fragment of rat P450 scc cDNA were used as templates. *In vitro* transcription was carried out using T7 RNA polymerase, and [^{32}P]UTP (Amersham), following the instructions of the manufacturer. For generation of cDNA probes, *Eco*RI–*Pst*I 780 bp fragment of rat SF-1 cDNA, and *Eco*RI excised 367 bp fragment of mouse 17β-HSD type III (Mustonen *et al.* 1997) were used as templates. Random priming was carried out using DNA polymerase I (Klenow fragment) and [^{32}P]CTP (Amersham), following instructions of the manufacturer.

Hybridizations were carried out at $66\,^{\circ}\text{C}$ (cRNA probes) or $42\,^{\circ}\text{C}$ (cDNA probes) for 20 h in the same prehybridization solution after addition of the corresponding radiolabeled probe. After hybridization, the membranes were washed in $2 \times$ SSC–0·1%SDS at room temperature for 20 min, $0\cdot5 \times$ SSC–0·1%SDS for 20 min at $65\,^{\circ}\text{C}$, and three times in 0·1%SSC–0·1%SDS for 1 h at $65\,^{\circ}\text{C}$. The filters were exposed to Kodak X-ray films (Kodak XAR-5 and XLS 5, Eastman Kodak, Rochester, NY, USA) at $-70\,^{\circ}\text{C}$ for 24–120 h. Relative mRNA levels were obtained by densitometric scanning of the autoradiograms (1-D Manager software, TDI Ltd, Madrid, Spain), and the values were normalized by the amount of 18S ribosomal RNA transferred per lane, as estimated under ethidium bromide staining. The molecular sizes of the mRNA species were estimated by comparison with mobility of the 18S and 28S ribosomal RNAs. Reagents for RNA analysis were obtained from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise stated.

In situ hybridization

Five micrometre sections of adult testis tissue were used for *in situ* hybridization. To assess cellular distribution of overall Ob-R gene expression, a specific riboprobe recognizing all Ob-R isoforms was generated. An antisense ^{35}S-labeled RNA probe complementary to an area of the extracellular domain of Ob-R was synthesized using the Riboprobe system II kit (Promega), SP6 RNA polymerase, [^{35}S]CTP, and *Nco*I-linearized template, constructed by subcloning a 418 bp fragment of Ob-R cDNA into pGEM-T vector. This cDNA was generated by RT-PCR, using the primer pair *Ob-Rsense* (5′-CTC CGC ACT CAC AGG CAA CA-3′) and *Ob-Ras* (5′-TGG ATC GGG CTT CAC AAC AA-3′). As control, adjacent sections were hybridized using a sense radiolabeled RNA probe generated as described above except for the use of *Spe*I-linearized template and T7 RNA polymerase. Pretreatment of sections was performed as described previously (Tena-Sempere *et al.* 1994). The hybridization of pre-treated slides with antisense or sense probes was carried out overnight at $50\,^{\circ}\text{C}$, and thereafter the samples were washed first in $2 \times$ SSC, 50% formamide, 10 nM dithiothreitol (DTT; Boehringer Mannheim, Ingelheim, Germany) at $50\,^{\circ}\text{C}$ for 30 min, and then in $0\cdot2 \times$ SSC, 50% formamide, 10 nM DTT at $50\,^{\circ}\text{C}$ for 10 min. The slides were rinsed with $1 \times$ PBS and treated with 10 µg/ml ribonuclease A (Boehringer Mannheim) in Tris–EDTA buffer ($T_{10}E_5$), 0·5 M NaCl (pH 8·0) at $37\,^{\circ}\text{C}$ for 30 min. After digestion, washing with $2 \times$ SSC, 50% formamide, 10 nM DTT was repeated. Finally, the slides were rinsed with $2 \times$ SSC, dehydrated in ethanol, and processed for liquid emulsion autoradiography using NTB-3 emulsion solution (Eastman Kodak). The slides were exposed at $4\,^{\circ}\text{C}$ for 2–3 weeks, and developed at $12\,^{\circ}\text{C}$ by treatment with D-19 solution (Eastman Kodak) for 2·5 min. After developing, the slides were counterstained with hematoxylin (BDH Ltd, Poole, UK).

RNA analysis by semi-quantitative RT-PCR

The pattern of mRNA expression of Ob-R isoforms in adult rat testis was assessed by semi-quantitative

RT–PCR, using isoform-specific oligo-primer pairs. Total RNA was isolated from adult testicular samples as described above (Chomczynski & Sacchi 1987). For amplification of Ob-R variants, the following primer pairs were used: *Ob-Ra sense* (5′-CCT ATC GAG AAA TAT CAG TTT A-3′) and *Ob-Ra as* (5′-TCA AAG AGT GTC CGC TCT CT-3′), for amplification of a 285 bp fragment of rat Ob-Ra isoform cDNA; *Ob-Rb sense* (5′-TGG CCC ATG AGT AAA GTG AAT-3′) and *Ob-Rb as* (5′-CCA GAA GAA GAG GAC CAA ATA -3′), for amplification of a 386 bp fragment of rat Ob-Rb isoform cDNA; *Ob-Rc sense* (5′-GTG TCC TGC TGC TCG GAA CAC TGT-3′) and *Ob-Rc as* (5′-AAA GAT CTC TAT GTA ATA GAG TAT-3′), for amplification of a 188 bp fragment of rat Ob-Rc isoform cDNA; *Ob-Re sense* (5′-GCA GAA TCA GCA CAC ACT GTT-3′) and *Ob-Re as* (5′-GTA AAA GCA CAG TAC ACA TAC C-3′), for amplification of a 301-bp fragment of rat Ob-Re isoform cDNA; and *Ob-Rf sense* (5′-AGA GGA TAT ATA GTG GAT GCC G-3′) and *Ob-Rf as* (5′-CAC AAA TGA GCC ATC TTC AAA CC-3′), for amplification of a 411 bp fragment of rat Ob-Rf isoform cDNA. The reported sets of primers were selected based on previous references (Garcia *et al.* 2000) with minor modifications, and synthesized according to the published cDNA sequences of the cloned Ob-R isoforms (Tartaglia *et al.* 1995, Lee *et al.* 1996, Takaya *et al.* 1996, Tartaglia 1997). In addition, to provide an appropriate internal control, co-amplification of a 290 bp fragment of the L19 ribosomal protein mRNA was carried out in each sample using the primer pair: *L19-sense* (5′-GAA ATC GCC AAT GCC AAC TC-3′) and *L19-as* (5′-ACC TTC AGG TAC AGG CTG TG-3′), generated according to the rat L19 ribosomal protein cDNA (Chang *et al.* 1987).

For amplification of the targets, RT and PCR were run in two separate steps. Furthermore, to enable appropriate amplification in the exponential phase for each target, PCR amplification of Ob-Ra, -Rb, -Rc, -Rf, -Re and L19 ribosomal protein transcripts was carried out in separate reactions with different number of cycles (see below), but using similar amounts of the corresponding cDNA templates, generated in single RT reactions, as described elsewhere (Santana *et al.* 1996, Tena-Sempere *et al.* 2000b). Briefly, equal amounts of total testicular RNA (4 µg) were heat denatured and reverse transcribed by incubation at 42 °C for 90 min with 12·5 U AMV RT (Promega), 20 U ribonuclease inhibitor RNasin (Promega), 200 nM deoxy-NTP mixture, and 1 nM isoform-specific and L19 antisense primers, in a final volume of 30 µl of 1 × AMV-RT buffer. The reactions were terminated by heating at 97 °C for 5 min and cooling on ice, followed by dilution of the RT cDNA samples with nuclease-free H_2O (final volume 60 µl). For semi-quantitative PCR, 10 µl aliquots of the cDNA samples (equivalent to 650 ng total RNA input) were amplified in 50 µl of 1 × PCR buffer in the presence of 2·5 U

Taq-DNA polymerase (Promega), 200 nM deoxy-NTP mixture, and the appropriate primer pairs (1 nM of each primer; see above). PCR reactions consisted of a first denaturing cycle at 97 °C for 5 min, followed by a variable number of cycles of amplification defined by denaturation at 96 °C for 1·5 min, annealing at 55 °C for 1·5 min, and extension at 72 °C for 3 min. A final extension cycle of 72 °C for 15 min was included. The number of cycles was optimized to ensure amplification in the exponential phase of PCR. Different numbers of cycles were tested for each Ob-R isoform (ranging between 25 and 45) and L19 ribosomal protein (ranging between 14 and 30). Based on the analysis of cycle dependency of the intensity of the generated PCR signals, 33 and 20 cycles were chosen for further analysis of Ob-R variants and L19 ribosomal protein transcript, respectively.

The cDNA fragments generated were resolved in 1·5% agarose gels containing ethidium bromide (0·1 µg/ml), and visualized using a digital imaging system (1-D Manager software, TDI Ltd). The molecular sizes of the transcripts were determined by comparison with size markers run together with the cDNA products (PCR 50 bp Step Ladder, Promega). Specificity of the PCR products was confirmed by Southern hybridization, using radiolabeled nested oligo-primers and/or digestion with specific restriction enzymes (data not shown), as described elsewhere (Tena-Sempere *et al.* 1999b, García *et al.* 2000). To ensure that equal inputs of RNA were added to RT-PCR reactions, only samples yielding roughly similar optical density (OD) values for L19 bands were considered for further analysis. In addition, to minimize potential RT-PCR artefacts due to inherent reaction variability, all data points were repeated, for each target, at least 3 times using independent RNA samples. Finally, in all assays, liquid controls and reactions without RT were included, yielding negative amplification (data not shown).

Statistics

Hormonal results from testicular incubations are presented as means ± S.E.M. from 8–12 independent determinations. When appropriate, semiquantitative data from RNA analyses were expressed as means ± S.E.M. from at least three independent determinations. Significant differences between groups were determined by one-way ANOVA, followed by Tukey's test. $P<0·05$ was considered significant.

Results

Expression of several steroidogenic-related genes is regulated by leptin in adult rat testis

To identify potential targets for leptin-induced inhibition of testosterone secretion, secretory responses were

Table 1 Effects of human recombinant (hr) leptin on *in vitro* hCG-stimulated testosterone (T) secretion by adult rat testicular slices. The testicular samples were challenged with increasing concentrations of leptin (10^{-9}–10^{-7} M) in the presence of 10 IU/ml hCG, and testosterone release to the incubation medium was assessed after 180 min (see Materials and Methods). Testes incubated with 10 IU/ml hCG served as controls. In addition, testosterone secretion by testicular samples incubated in the presence of medium (DMEM) alone is presented. Values are given as means ± S.E.M. (*n* = 10–12 samples/group)

	T secretion ((ng/ml) · g tissue)
DMEM	13.46 ± 1.34
hCG 10 IU	210.5 ± 22.5^{a}
hCG+hr leptin 10^{-9} M	$164.2 \pm 18.7^{a,*}$
hCG+hr leptin 10^{-8} M	$140.7 \pm 17.5^{a,**}$
hCG+hr leptin 10^{-7} M	$147.1 \pm 10.2^{a,**}$

[a] $P \leq 0.01$ vs values from DMEM group; [*] $P \leq 0.05$; [**] $P \leq 0.01$ vs values from the corresponding hCG-stimulated group (ANOVA followed by Tukey's test).
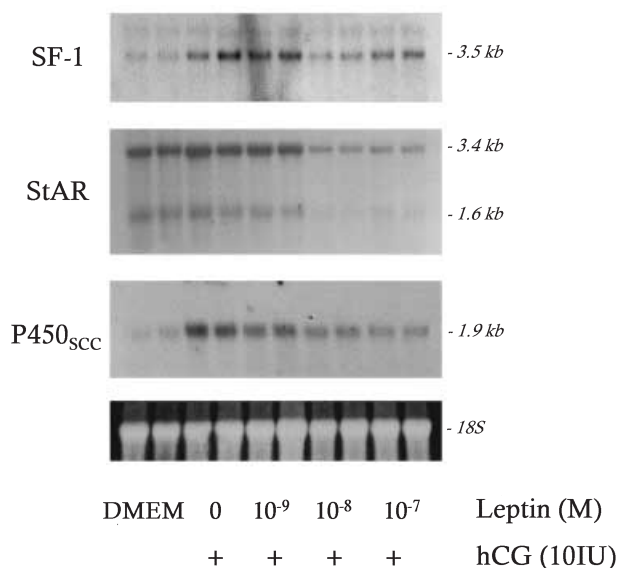

Figure 1 Representative Northern hybridization analyses of steroidogenic factor-1 (SF-1), steroidogenic acute regulatory protein (StAR) and cytochrome P450 cholesterol side-chain cleavage enzyme (P450 scc) mRNA expression levels in individual testicular samples incubated in the presence of increasing doses of recombinant leptin (10^{-9}–10^{-7} M) plus hCG (10 IU/ml). Samples incubated in the presence of hCG served as controls. In addition, data from samples incubated with medium (DMEM) alone are presented. Two samples per treatment group are shown. Northern hybridizations were carried out using specific complementary RNA and DNA probes, as described in Materials and Methods. For each sample, the amount of 18S ribosomal RNA transferred per lane was used as control for even loading and transfer efficiency. The molecular sizes of the expected mRNA species are indicated on the right.

correlated to mRNA expression levels of several steroidogenic key factors in hCG-stimulated testicular samples after exposure to increasing doses of human recombinant leptin (10^{-9}–10^{-7} M). The mRNAs to be analyzed were selected based on previous references on the effects of leptin upon other steroidogenic tissues (Kruse *et al.* 1998, Zachow *et al.* 1999), as well as on their pivotal role in testosterone biosynthesis (Stocco & Clark 1996, Parker & Schimmer 1997). RNA analyses were carried out by Northern hybridization using specific cRNA and cDNA probes. Hybridization signals of 3·5 kb, 3·4 and 1·6 kb, 1·9 kb, and 1·3 kb were obtained for SF-1, StAR, P450 scc and 17β-HSD type III, respectively, in agreement with previous reports (Miller 1988, Mustonen *et al.* 1997, Clark *et al.* 1995, Manna *et al.* 1999b, Tsai-Morris *et al.* 1999). In keeping with our previous data (Tena-Sempere *et al.* 1999a), 180 min incubation of testicular tissue in the presence of 10 IU/ml hCG elicited a significant increase in testosterone secretion. This response was significantly inhibited by co-incubation with leptin, at all doses tested (10^{-9}–10^{-7} M; Table 1). In this experimental setting, hCG significantly increased the steady-state mRNA levels of SF-1, StAR and P450 scc, as estimated by Northern hybridization after 180 min stimulation, whereas the expression level of 17β-HSD type III mRNA remained unchanged. Exposure to recombinant leptin induced a co-ordinate decrease in the hCG-stimulated expression levels of SF-1, StAR and P450 scc mRNAs. However, subtle differences in the dose dependency of this effect were noted: 10^{-9} M leptin moderately inhibited hCG-elicited P450 scc mRNA levels without altering those of SF-1 and StAR, while 10^{-8}–10^{-7} M doses significantly decreased mRNA levels of the three targets. On the latter, it has to be noted that no significant difference in the magnitude of such an inhibitory response was detected between 10^{-8} and 10^{-7} M leptin concentrations for any of the messages under evaluation. On the contrary, recombinant leptin, at all doses tested, failed to modify the 17β-HSD type III mRNA levels in hCG-stimulated testicular samples (Figs 1–3).

Pattern of cellular distribution and isoform expression of Ob-R mRNA in adult rat testis

To provide further insight into the mode of action of leptin in rat testis, the pattern of cellular expression of leptin receptor (Ob-R) mRNA was explored in testicular sections from adult (75-day-old) rats by means of *in situ* hybridization (ISH), using a specific antisense RNA probe complementary to all Ob-R isoforms. Distribution analysis revealed a scattered pattern of Ob-R gene expression within rat testis, hybridization signals being detected in the seminiferous tubules and in the interstitial space (Fig. 4A). At higher magnification, specific signals were clearly located in Leydig and Sertoli cells (Fig. 4B), with possible deposition of silver grains in germ cells also. Specificity of
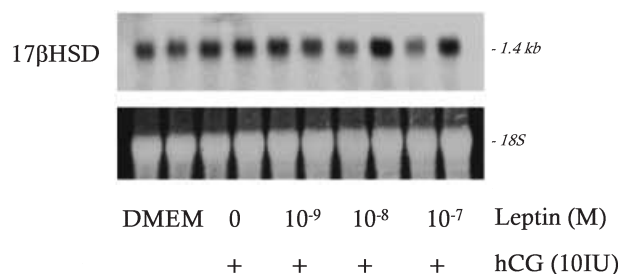
Figure 2 Representative Northern hybridization analysis of 17β-hydroxy steroid dehydrogenase type III (17β-HSD) mRNA expression levels in individual testicular samples incubated in the presence of increasing doses of recombinant leptin (10^{-9}–10^{-7} M) plus hCG (10 IU/ml). Samples incubated in the presence of hCG served as controls. In addition, data from samples incubated with medium (DMEM) alone are presented. Two samples per treatment group are shown. Northern hybridizations were carried out using a specific complementary DNA probe, as described in Materials and Methods. For each sample, the amount of 18S ribosomal RNA transferred per lane is presented, and the molecular size of the expected mRNA species is indicated on the right.

the ISH results was confirmed by hybridization of adjacent testicular sections with the corresponding sense cRNA probe, that yielded negative results (Fig. 4C).

In addition, to characterize further the pattern of Ob-R gene expression in adult rat testis, the relative mRNA levels of the different Ob-R subtypes were assayed by means of semi-quantitative RT-PCR. Such an analysis, using isoform-specific primer pairs, revealed that Ob-Rb mRNA was abundantly expressed in the adult (75-day-old) rat testis. In addition, variable expression levels of mRNAs encoding Ob-Ra, Ob-Re, and Ob-Rf isoforms were detected, whereas almost negligible signals for Ob-Rc were amplified (Fig. 5).

Discussion

Testicular steroid hormone biosynthesis is a hormonally regulated multi-step process that involves the sequential conversion of cholesterol into testosterone by the co-ordinated actions of a group of cytochrome P450 hydroxy-lase and hydroxysteroid dehydrogenase enzymes (Miller 1988). The first and rate-limiting key steps of this cascade are the translocation of cholesterol to the inner mitochondrial membrane and its subsequent conversion into pregnenolone; events that are mediated by steroidogenic acute regulatory (StAR) protein and cytochrome P450 cholesterol side-chain cleavage (P450 scc) enzyme, respectively (Miller 1988, Stocco & Clark 1996). In rodent testis, the predominant Δ^4 steroidogenic pathway ends with the conversion of androstenedione to testosterone by the specific testicular type III isoform of 17β-hydroxysteroid dehydrogenase (17β-HSD) (Mustonen *et al.* 1997,
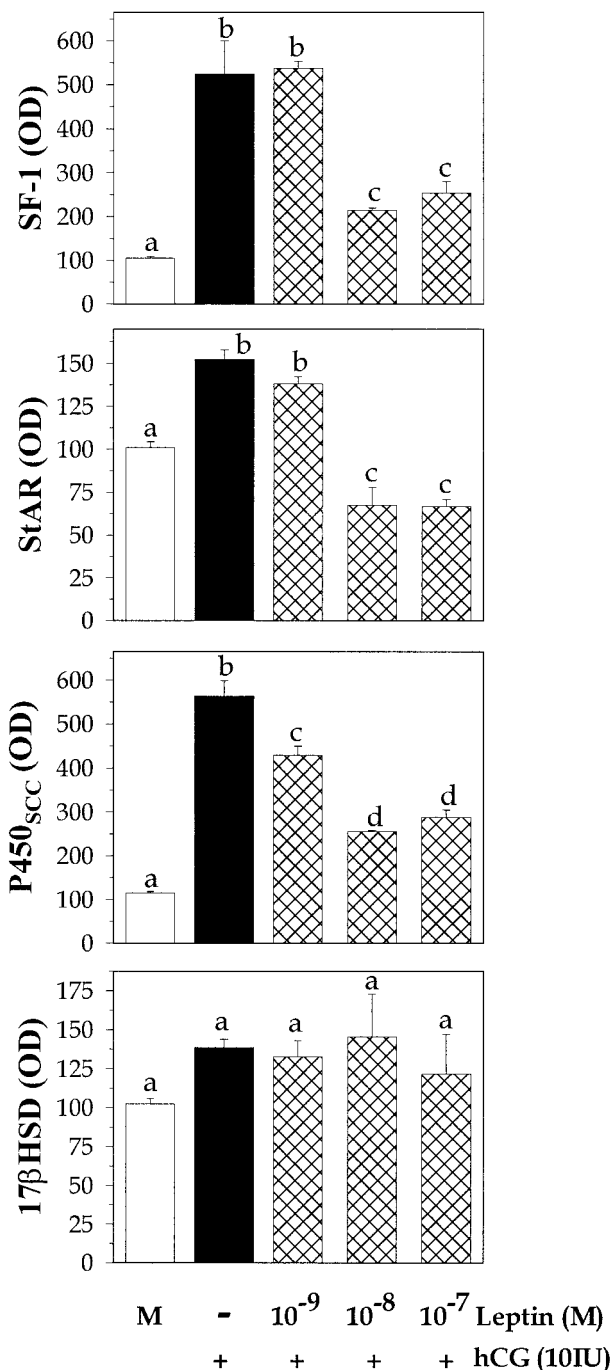

Figure 3 Compilation of quantitative data on the steady-state levels of SF-1, StAR, P450 scc and 17β-HSD mRNAs in hCG-stimulated testicular samples challenged with increasing concentrations of recombinant leptin (10^{-9}–10^{-7} M). Relative expression levels were obtained, in each sample, by normalization of absolute optical densities (OD) of each target to that of 18S ribosomal RNA transferred per lane. Values are given as means ± S.E.M. of at least three independent determinations. Groups with different superscript letters are statistically different (ANOVA followed by Tukey's test).
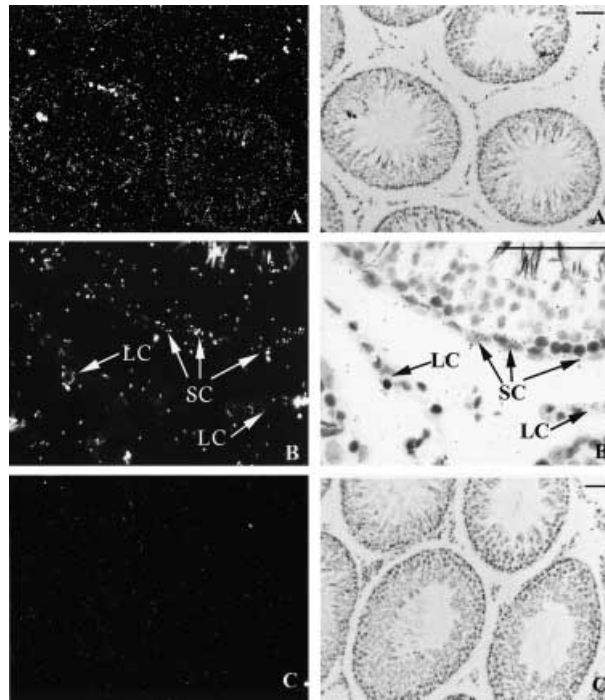
Figure 4 Representative photomicrographs of Ob-R mRNA *in situ* hybridization of adult rat testis. Testicular sections from 75-day-old rats were hybridized with a specific antisense RNA probe complementary to all Ob-R isoforms, as described in Materials and Methods. Darkfield (A, B, C) and brightfield (A′, B′, C′) photomicrographs are presented. The arrows in B and B′ indicate Leydig cells (LC) and Sertoli cells (SC). An adjacent section hybridized with the corresponding sense cRNA probe is shown in C. The emulsion-coated slides were exposed in the dark for 2–3 weeks at 4 °C. Scale bars in A′, B′ and C′=50 μm.

Tsai-Morris *et al.* 1999). Tissue-specific and hormone-regulated expression of the pivotal elements in the steroidogenic pathway is likely depend on the concerted action of several transcription factors. Among them, a crucial role for steroidogenic factor 1 (SF-1) has recently emerged not only as a transcriptional regulator of various steroidogenic enzyme genes, but also as a pivotal element in gonadal and adrenal development and function (Parker & Schimmer 1997, Hammer & Ingraham 1999).

Compelling evidence demonstrates direct inhibitory actions of leptin upon steroid hormone secretion. Such effects have been independently reported by different groups in the three major steroidogenic tissues, namely the adrenal gland, the ovary and the testis (Spicer & Francisco 1997, Pralong *et al.* 1998, Kruse *et al.* 1998, Caprio *et al.* 1999, Tena-Sempere *et al.* 1999a, 2000a, Zachow *et al.* 1999). However, the mechanisms for such an inhibitory action are only partially characterized, and little attention has been paid to the molecular events involved in leptin-induced inhibition of testicular testosterone secretion. To gain insight into the mechanisms whereby leptin

suppresses testosterone secretion *in vitro*, we correlated the secretory responses to mRNA expression levels of SF-1, StAR, P450 scc and 17β-HSD in hCG-stimulated testicular samples after exposure to increasing doses of human recombinant leptin (10^{-9}–10^{-7} M). Results from our Northern hybridization analyses suggest that decreased expression of mRNAs encoding several up-stream elements in the steroidogenic route may contribute, at least partially, to leptin-induced inhibition of testicular steroidogenesis.

The pivotal role of cytochrome P450 scc in steroid hormone biosynthesis makes it a target for the regulatory actions of tropic hormones in steroidogenic tissues (Miller 1988). In addition, since leptin was recently reported to significantly reduce ACTH-stimulated P450 scc mRNA levels in cultured bovine adrenocortical cells (Kruse *et al.* 1998), we aimed at evaluating whether an analogous response is detected in adult rat testis. Indeed, our Northern analyses demonstrated a dose-dependent decrease in P450 scc mRNA expression levels in hCG-stimulated rat testicular samples exposed for 180 min to
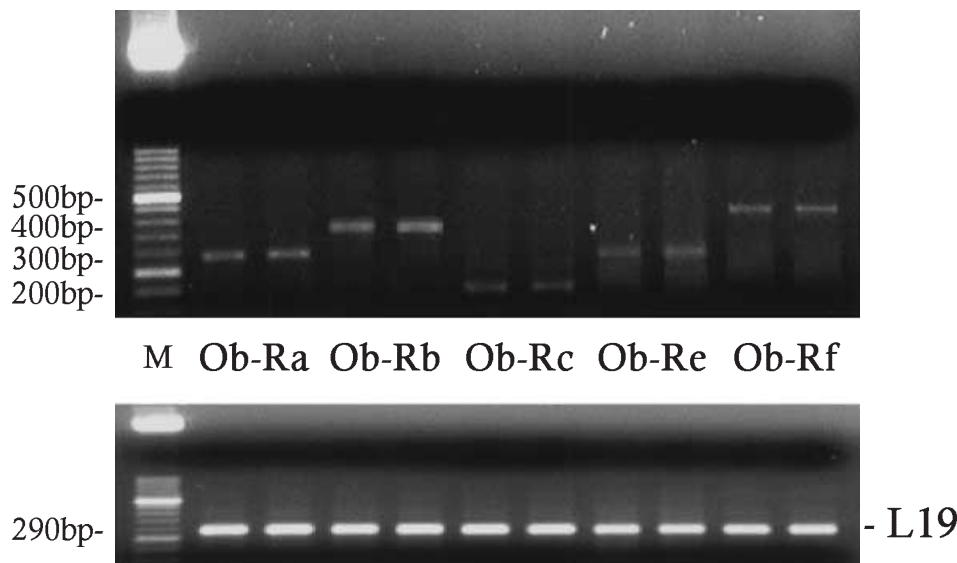
Ob-R isoforms

Figure 5 Assessment of expression levels of the mRNAs encoding Ob-Ra, Ob-Rb, Ob-Rc, Ob-Re and Ob-Rf subtypes in adult (75-day-old) rat testis. A representative ethidium bromide-stained gel electrophoresis of isoform specific Ob-R cDNA fragments, amplified by semi-quantitative RT-PCR, is presented. As internal control, amplification of L19 ribosomal protein transcript was carried out in each sample as described in Materials and Methods, yielding an even amplification pattern. The sizes of the generated products were calculated by comparison with the mobility of 50 bp DNA step ladder (M); the base pair length of representative markers being indicated on the left.

increasing concentrations of recombinant leptin. These data suggest that such a step in steroid hormone biosynthesis may be under leptin regulation in different steroidogenic tissues (i.e. adrenal and testis). Moreover, they open up the possibility that the mechanism by which leptin inhibits testosterone secretion may involve, at least partially, modulation of early events in the steroidogenic pathway. This is further supported by the dose–dependent leptin–induced decrease in StAR mRNA levels observed in the same samples. To our knowledge, this is the first study to report the inhibitory effect of leptin on the expression levels of StAR mRNA. Given that regulation of steroidogenesis by various hormonal signals is tightly correlated with concomitant changes in StAR gene expression in different experimental settings (Clark *et al.* 1995, Manna *et al.* 1999*a*,*b*), our present results suggest that leptin–induced decrease in StAR mRNA expression may contribute, at least partially, to the reported inhibition of testosterone secretion *in vitro*, and open up the possibility that StAR mRNA expression may be under leptin regulation in other steroidogenic tissues. However, further experimental work, including analysis of expression at the protein level, is needed to fully demonstrate the involvement of P450 scc and StAR in the reported inhibitory effect of leptin upon rat testicular steroidogenesis.

Recently, SF–1 has emerged as a pivotal regulator of development and function of the hypothalamic–pituitary–gonadal axis. Concerning steroid hormone production, SF–1 has a key role in tissue–specific transcriptional regulation of several steroid hydroxylases, including cytochrome P450 scc (Parker & Schimmer 1997, Hammer & Ingraham 1999). Moreover, expression of the StAR gene, as well as those encoding several non-cytochrome P450 enzymatic components of the steroidogenic pathway, are under SF–1 regulation (Parker & Schimmer 1997, Hammer & Ingraham 1999). Our initial observation on the ability of leptin to inhibit hCG-stimulated StAR and P450 scc mRNA levels in rat testicular tissue prompted us to evaluate whether SF–1 mRNA expression itself can be regulated by leptin. In fact, little is known about the hormonal regulation of SF–1 gene expression, and conflicting results on the ability of hCG and other stimuli of mouse Leydig cell steroidogenesis to modulate SF–1 mRNA levels have been reported (Chau *et al.* 1997, Manna *et al.* 1999*a*,*b*). In keeping with a previous reference (Manna *et al.* 1999*b*), our analysis demonstrated increased expression of testicular SF–1 mRNA after hCG stimulation *in vitro*. In this setting, incubation with increasing concentrations of recombinant leptin induced a significant, dose–dependent decrease in the expression levels of SF–1 mRNA, i.e. a response roughly similar to that observed in terms of StAR and P450 scc mRNAs. Considering that expression of these targets is activated by SF–1, it is possible, although yet to be proven, that

leptin-induced suppression of SF-1 expression levels may contribute, at least partially, to the decrease in StAR and P450 scc mRNA expression after exposure to leptin. Nevertheless, our results suggest that leptin is a novel regulator of SF-1 mRNA expression in rat testis. It remains to be solved whether a similar role is carried out in other tissues, such as the adrenal, the ovary, the ventromedial hypothalamus and the pituitary, where co-expression of functional Ob-Rs (Schwartz *et al.* 1996, Casanueva & Dieguez 1999, Jin *et al.* 2000) and the SF-1 gene (Parker & Schimmer 1997, Hammer & Ingraham 1999) has been demonstrated.

Analysis of the molecular mechanisms whereby leptin inhibits estradiol production in the ovary demonstrated that leptin impairs stimulated P450 aromatase activity and mRNA expression, i.e. the final steroidogenic step in rat granulosa cells (Zachow *et al.* 1999). In order to explore the possibility that an analogous mechanism could operate in rat testis, leptin effects on mRNA expression levels of 17β-HSD type III were evaluated. This was considered relevant given the recent cloning of the enzyme isoform in the rat and the limited knowledge available on hormonal regulation of expression of this target gene (Tsai-Morris *et al.* 1999). Our analyses demonstrated the lack of effect of 180 min exposure to hCG *in vitro* in terms of 17β-HSD type III mRNA levels. This is in keeping with a previous report showing that hCG-induced down-regulation of 17β-HSD expression *in vivo* becomes evident only after 12 h treatment (Tsai-Morris *et al.* 1999). In addition, our results indicate that leptin is unable to acutely regulate 17β-HSD type III mRNA expression levels in hCG-stimulated testicular tissue. Thus, although the possibility of a regulatory action of leptin at this level cannot be completely ruled out on the basis of the present data, our results are suggestive of a possible sexual dimorphism in the overall mechanism whereby leptin inhibits gonadal sex steroid secretion.

Clearly, our current data do not exclude other possible sites of inhibitory action of leptin in testosterone biosynthesis. In fact, it was recently reported that leptin is able to decrease the 17,20 lyase activity in primary cultures of rat Leydig cells, thus decreasing the rate of conversion of 17-OH-progesterone into androstenedione (Caprio *et al.* 1999). However, since no acute changes in mRNA expression levels of cytochrome P450c17 after leptin stimulation were observed (Caprio *et al.* 1999), we did not analyze the potential regulation of this message by recombinant leptin in our experimental setting.

To expand our knowledge on the mode of action of leptin on adult rat testis, the pattern of cellular expression of leptin receptor (Ob-R) gene was evaluated by means of *in situ* hybridization (ISH) using a specific riboprobe complementary to all cloned Ob-R isoforms. Worthy to note, most of the information on the location of Ob-R in rodent testis comes from studies using the mouse, and species differences may exist. Our distribution analysis

revealed a scattered pattern of Ob-R gene expression within rat testis, with specific hybridization signals detected in the seminiferous tubules and in the interstitial space. Indeed, at higher magnification, signals were located in apparent Leydig and Sertoli cells, with possible expression in germ cells also. These results are in line with previous evidence on the expression of the Ob-R gene in murine Leydig cells, as detected by ISH (Hoggard *et al.* 1997) and RT-PCR (Caprio *et al.* 1999). In addition, the expression of Ob-R in mouse germ cells has been reported very recently (El-Hefnawy *et al.* 2000). Overall, although data on testicular location of Ob-R protein are not provided, our current results on the pattern of cellular distribution of Ob-R mRNA in adult rat testis may pave the way for further elucidation of the molecular basis for leptin action upon the steroidogenic machinery, either through direct or indirect actions on Leydig cells.

Finally, analysis of Ob-R isoform expression was carried out in adult rat testis. RT-PCR assays using isoform-specific primer pairs revealed that the Ob-Rb isoform is apparently the most abundant mRNA variant at this age, in line with previous references (Caprio *et al.* 1999). However, other Ob-R subtype messages were detected, with moderate to high expression levels for Ob-Ra, Ob-Re, and Ob-Rf isoforms, and nearly negligible signals for Ob-Rc; a pattern of expression that is roughly analogous to that of the prepubertal rat testis (Tena-Sempere *et al.* 2001). As a next step, we will aim at identifying the pattern of cellular distribution of Ob-R isoforms within rat testis. Nevertheless, novel data on expression levels of the mRNAs encoding different Ob-R variants in adult rat testis are relevant considering the different functional capacities of the Ob-R subtypes (Tartaglia *et al.* 1995, Lee *et al.* 1996, Takaya *et al.* 1996, Tartaglia 1997, Murakami *et al.* 1997, White *et al.* 1997, White & Tartaglia 1999). Thus, the complex pattern of processing of Ob-R mRNA in rat testis may result in the generation of different receptor isoforms with variable signaling ability, ranging from complete (Ob-Rb) to partial (Ob-Ra) or absent (Ob-Re and others). Moreover, the specific functional features of testis-expressed Ob-R isoforms such as Ob-Ra, involved in leptin transport across biological barriers (Tartaglia 1997, Bjorbæk *et al.* 1998), and Ob-Re, as soluble leptin-binding protein (Tartaglia 1997), as well as the proposed interaction between Ob-R isoforms in leptin signaling (White *et al.* 1997, White & Tartaglia 1999) should be taken into account when evaluating the mechanisms for testicular actions of leptin. In this context, identification of the specific roles of Ob-R variants in rat testis merits further investigation.

In conclusion, our results demonstrate that 180 min exposure to leptin is able to down-regulate hCG-stimulated SF-1, StAR and cytochrome P450 scc mRNA expression levels in adult rat testis without altering significantly those of 17β-HSD type III. In addition, evidence for a scattered pattern of cellular expression of

Ob-R gene within rat testis is presented that includes Ob-Rb as well as other shorter isoforms. Overall, decreased expression of mRNAs encoding several up-stream elements in the steroidogenic pathway may represent a novel contributing factor to leptin-induced inhibition of rat testicular steroidogenesis.

Acknowledgements

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