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Expression of Functional Leptin Receptors in Rodent Leydig Cells*

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ABSTRACT

Several studies indicate that the size of body fat stores and the circulating levels of the adipocyte-derived hormone leptin are able to influence the activity of the hypothalamic-pituitary-gonadal axis. The leptin-hypothalamic-pituitary-gonadal interactions have been mainly studied at the level of the central nervous system. In this study, we investigated the possibility that leptin may have direct effects on the rodent Leydig cell function. To probe this hypothesis, we first analyzed the expression of leptin receptors (OB-R) in rodent Leydig cells in culture. RT-PCR studies showed that rat Leydig cells express both the long (OB-Rb) and short isoform (OB-Ra) of leptin receptor, whereas MLTC-1 cells (a murine Leydig tumor cell line) express only the long isoform. Short-term (30–90 min) incubation of rat Leydig cells with increasing concentrations of leptin (2–500 ng/ml) led to a significant and dose-dependent inhibition of human (h)CG-stimulated testosterone (T) production (~60% reduction, IC_{50} = 20 ng/ml) but no change in basal androgen release. Also, leptin (150 ng/ml) amplified hCG-induced intracellular cAMP formation (1- to 2-fold) without modifying basal cAMP levels. Subsequent experiments showed that leptin inhibited 8Br-cAMP-stimulated T production, indicating that leptin's effect is exerted beyond cAMP. The inhibitory effect of leptin on hCG-induced T secretion was accompanied by a significant reduction of androstenedione and a concomitant rise of the precursor metabolites pregnenolone, progesterone, and 17-OH-progesterone, conceivable with a leptin-induced lesion of 17,20 lyase activity. Separate experiments performed with the MLTC-1 cells (not expressing cytochrome P450–17α) showed that leptin, though amplifying hCG-stimulated cAMP production, did not modify hCG-stimulated pregnenolone and progesterone release. These results further indicate that leptin action on steroidogenesis occurs downstream of progesterone synthesis. Northern Blot experiments showed no acute effect of leptin on cytochrome P450–17α messenger RNA accumulation in rat Leydig cells in basal and hCG-stimulated conditions, excluding that the rapid changes observed were caused by messenger RNA degradation. In conclusion, these findings, for the first time, show that leptin has direct, receptor-mediated actions on rodent Leydig cells in culture, at concentrations within the range of obese men. (*Endocrinology* **140:** 4939–4947, 1999)

LEPTIN, the recently identified product of the *ob* gene, is a 16-kDa protein produced by white adipose tissue. Leptin circulates in the blood at concentrations that parallel the amount of fat reserves, and was initially found to act at the hypothalamic level as a satiety factor (1, 2). The mutation in the *ob* gene causes severe obesity in the mouse, which is caused by a defect in energy expenditure, increased food intake, and altered nutrient partitioning (3). Several more recent observations suggest that leptin is also an important biochemical message between fat stores and the reproductive axis. Peripheral injection of recombinant leptin restores fertility in male (4) and female (5) *ob/ob* mouse models that show a severe sterility caused by an insufficient hypothalamic-pituitary drive. Leptin treatment counteracts the fasting-induced inhibition of gonadotropin secretion in rodents (6) and monkeys (7), whereas the administration of leptin antiserum into the lateral ventricles of rats causes a decrease in LH pulsatility and cessation of estrous ciclicity (8). Also, leptin has been implicated in triggering the onset of puberty in rodents (9, 10) and humans (11, 12). The interactions between leptin and the hypothalamic-pituitary-gonadal (HPG) axis have been mainly investigated at the level of the central nervous system and shown to involve a direct/indirect leptin regulation of GnRH release (13).

The long and short isoforms of leptin receptor are widely expressed in peripheral tissues (14–16), and recent reports suggest that leptin could have important peripheral actions, including effects on the endocrine pancreas (17, 18), the hematopoietic stem cells (19, 20), and steroidogenic tissues such as the ovary (21–24) and the adrenals (25–27). In particular, leptin has been found to suppress insulin-induced progesterone and 17β-estradiol production by isolated bovine granulosa cells (21), prevent insulin-induced progesterone and androstenedione secretion in bovine ovarian thecal cells (22), and impair hormonally-stimulated release of 17β-estradiol by rat granulosa cells in culture (23). Furthermore, leptin has been shown to inhibit glucocorticoid secretion by cultured bovine adrenocortical cells (25) and to significantly reduce the ACTH-stimulated cortisol

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release from normal human and rat adrenal gland (26), as well as the ACTH-stimulated expression of cytochrome P450–17α messenger RNA (mRNA) accumulation in human and bovine adrenal cells in culture (25, 27).

These data suggest that leptin may have relevant peripheral actions on target steroidogenic cells. In the present study, we have tested the hypothesis that, in addition to its central nervous system effects on the reproductive axis, leptin may exert direct effects in the control of steroid production from isolated rodent Leydig cells in culture. To test this hypothesis, we first studied leptin receptor expression in isolated adult rat Leydig cells and in a murine Leydig tumor cell line (MLTC-1), then we investigated the effects of leptin on basal and human (h)CG-stimulated steroid production by rat Leydig cells and MLTC-1 cells in culture.

Materials and Methods

Isolation of Leydig cells

Adult male Sprague Dawley rats (Charles River Laboratories, Inc. Breeding Laboratories, Wilmington, MA) were killed by oxygen deprivation, and testes were rapidly removed and placed in ice-cold PBS, pH 7.4. Interstitial cells were obtained by collagenase digestion of decapsulated testes, as previously described (28). Crude cell suspension was washed and then pelleted at 200 $\times$ *g* for 10 min. The cell pellet was resuspended with elutriation buffer consisting of regular Medium 199 (Whittaker M.A. Bioproduct, Walkerville, MD) with Hanks' salts and L-glutamine containing 1.4 g/l NaHCO3, 0.5% BSA, 1 mM EDTA, 50 U/ml heparin, 12.5 mg/liter deoxyribonuclease, and 50 mg/liter gentamicin, pH 7.4. The purified cells were obtained by centrifugal elutriation (29). Nitro-blue-tetrazolium staining showed a purity of approximately 95%. Cells were centrifuged and resuspended in Medium 199 containing 0.1% BSA, 50 mg/liter gentamicin, and 0.125 mM 3-methylisobutyl xanthine (Aldrich Chemical Co., Inc., Milwaukee, WI).

MLTC-1 cells propagation and culture

Stock cultures of MLTC-1 cells were grown and maintained in RPMI-1640 medium (Whittaker M.A. Bioproduct) containing 25 mM HEPES, 300 mg L-glutamine/liter, 2000 mg glucose/liter, and supplemented with 10% FCS and 50 mg/liter gentamicin. The cells were subcultured by trypsinization [0.05% (wt/vol) trypsin], and experimental cultures were plated in 6-well tissue culture dishes and grown to 70–80% confluency before initiation of the experiments. The medium was then removed, and the cells were washed three times with PBS and returned to fresh RPMI medium supplemented with 0.1% BSA, 50 mg/liter gentamicin, and 0.125 mM 3-methylisobutyl xanthine. Cell viability was determined by the trypan blue dye-exclusion test (30).

Incubation and assays

The purified rat Leydig cells and MLTC-1 cells were plated in 6-well tissue culture dishes (1.5×10^6 cells/well·2 ml) and then incubated at 37 C under 95% [O_2] and 5% [CO_2] for the designed time period (see figure legends), in the presence or absence of saturating doses of hCG (1 ng/ml for rat Leydig cells, 20 ng/ml for MLTC-1 cells; CR 121, preparation kindly provided by the Center for Population Research, National Institutes of Child Health and Development, Bethesda, MD), or 8Br-cAMP (final concentration 1 mM) (Sigma Chemical Co., St. Louis, MO) with or without recombinant murine leptin (R&D Systems, Minneapolis, MN) at different concentrations, for the designed time period (see figure legends). At the end of incubation, media were removed and centrifuged at 250 *g* for 12 min, and the supernatants were saved for the assay of extracellular pregnenolone, progesterone, (from both rat Leydig cells and MLTC-1 cells), 17-OH-progesterone, androstenedione, and testosterone (T) (only from rat Leydig cells). All plated cells were also processed for the analysis of intracellular cAMP, as described elsewhere (31). The measurements of steroids and cAMP were performed by RIA, as previously described (32).

All the experiments were performed at least three times, in triplicate; results are the mean ± SE unless otherwise specified. Statistical significance was determined by ANOVA test.

Cytochrome P450–17α and c-fos expression

In other experiments, purified rat Leydig cells were plated in 100 $\times$ 20-mm tissue-culture dishes (7.5×10^6 cells/dish·10 ml) and incubated in the same conditions as above, for 30 min, in the presence or absence of hCG (1 ng/ml), with or without recombinant murine leptin (150 ng/ml). Cells were then washed three times with ice-cold PBS, scraped, and centrifuged at 250 $\times$ *g* for 12 min. Total RNA was isolated from cell pellets by using TRIzol RNA reagent (Life Technologies, Inc., Gaithersburg, MD) followed by deoxyribonuclease I (Life Technologies, Inc.) treatment to remove DNA contamination. The total RNA samples (15 μg for each lane) were resolved in 1.5% agarose gel containing 10 $\times$ 3-[*N*-morpholino]propanesulfonic acid and formaldehyde, transferred onto a Gene screen membrane (Biotechnology System, NEN Life Science Products, Boston, MA). The fixed membrane was prehybridized with a buffer containing 6 $\times$ SSC, 5 $\times$ Denhardt's and 0.5% SDS (Quality Biological Inc., Gaithersburg, MD) and hybridized overnight with a ^{32}P-labeled rat P450–17α complementary DNA (cDNA) probe (33). Thereafter, the membrane was washed twice in 2 $\times$ SSC-0.1% SDS at 65 C for 15 min each and twice in 0.5 $\times$ SSC-0.1% SDS for 10 min each. Hybridization was evaluated by autoradiography. The same membrane was stripped by two washes of 10 min each in 1% SDS at 85 C and one wash of 2 min in 0.2 $\times$ SSC. After confirmation of the removal of radioactivity, by exposing the membrane to x-ray film overnight, the membrane was hybridized again with a ^{32}P-labeled rat c-*fos* cDNA probe, as described elsewhere (34), and hybridization was evaluated by autoradiography. Quantification of P450–17α and c-*fos* mRNA was evaluated by Phosphorimage Scanner (Molecular Dynamics, Inc., Sunnyvale, CA) after normalization with β-actin, as previously reported (33).

Leptin receptor expression

Total RNA from primary rat Leydig cells, MLTC-1 cells, and rat and mouse, full-brain (positive) control was obtained as previously described. RNA was then subjected to RT-PCR using a kit from Perkin-Elmer Corp. Europe B. V. (Cretkrevz, Switzerland) (Gene Amp RNA PCR kit). First-strand cDNA was synthesized from 1 μg of total RNA using oligo dT primers and an annealing temperature of 65 C, and then amplified by PCR in 50 μl of reaction. Four sets of primers, based on rat and mouse leptin receptor sequences, were chosen (Fig. 1). Set 1 primers [5′-ATGCTGTGCAGTCACTCAGTG-3′ (sense, nucleotides 2284–2303) and 5′-CAACTCCTTCCATAAATACTGGG-3′ (antisense, nucleotides 2526–2503), rat sequence, GenBank accession no. U52966; 5′-GTGCTGTGGAGTCACTCAGTG-3′ (sense, nucleotides 2210–2230) and 5′-CAACTCCTTCCATAAATACTGGG-3′ (antisense, nucleotides 2452–2430), murine sequence, GenBank accession no. U49110] generate a 242-bp PCR product corresponding to the extracellular domain common to all Ob-R isoforms. Set 2 primers [5′-GATATTTGGTCCTCTTCTTCTGG-3′ (sense, nucleotides 2786–2809) and 5′-AGTTGTGGTGAAATCACATTGG-3′ (antisense, nucleotides 3223–3201), rat sequence, GenBank accession no. U52966; (5′-GATATTTGGTCCTCTTCTTCTGG-3′ (sense, nucleotides 2712–2735) and 5′-AGTTGTGGTGAAATCATGGTGGG-3′ (antisense, nucleotides 3149–3127), murine sequence, gene bank accession no. U49110] generate a 437-bp PCR product, corresponding to the intracellular domain of the receptor, specific of the Ob-Rb isoform. Set 3 primers [5′-ATGCTGTGCAGTCACTCAGTG-3′ (sense, nucleotides 2284–2303) and 5′-ACTTCAAAGAGTGTCCGCTCT-3′ (antisense, nucleotides 2668–2689), rat sequence, GenBank accession no. D84125; 5′-GTGCTGTGGAGTCACTCAGTG-3′ (sense, nucleotides 2210–2230) and 5′-AGTCATTCAAACCATTAGTTTAGG-5′ (antisense, 2805–2782), murine sequence, GenBank accession no. U49106] were chosen to generate, respectively, a fragment of 479 (rat) and 595 bp (mouse), specific only of the Ob-Ra isoform, as the downstream primers corresponded to the cDNA of the intracytoplasmic domain of the short isoform (Fig. 1). Finally, another set of primers (set 4) was used, combining the upstream primer of set 1 and the downstream primer of set 2, both from rat and murine OB-Rb sequence, to generate longer PCR products (940 bp), spanning different exons in the extra- and intracytoplasmic regions of rat and murine OB-Rb.

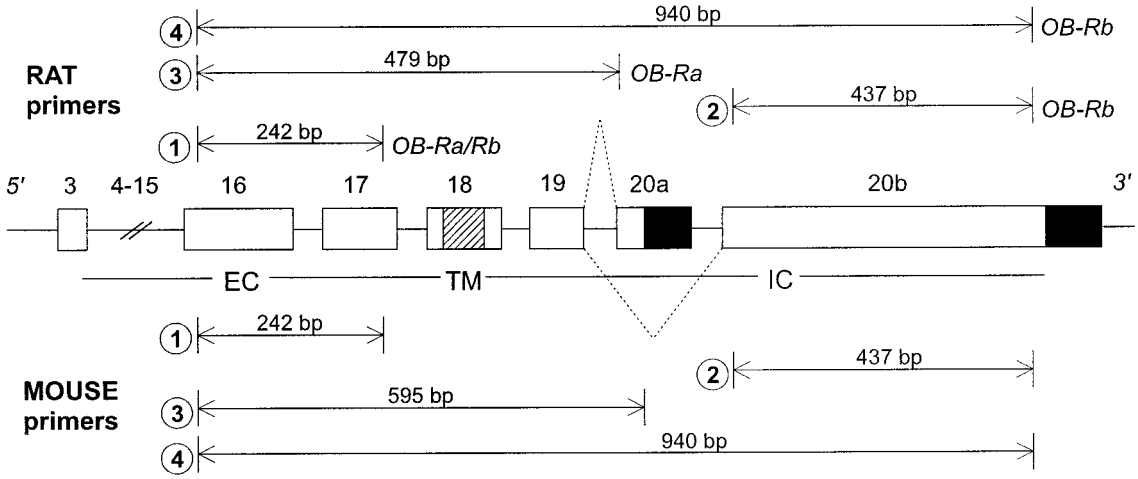

FIG. 1. Schematic illustration of the gene encoding leptin receptor and of the location of the sets of primers (1–4) used in RT-PCR experiments. Exons are represented as *white* or *black boxes*. *White boxes* correspond to exon sequences that are translated; *black boxes* correspond to 3′-untranslated sequences. Alternative splicing can result in transcripts that encode either the short (*dashed line above exons*) or the long (*dashed lines below exons*) intracellular domain. EC, Extracellular; TN, transmembrane; IC, intracellular.

Thermocycling conditions were: 30 sec of denaturation at 94 C, 30 sec of annealing at 55 C, and 60 sec of extension at 72 C for 34 cycles. An additional 2-min extension step at 72 C was added after the 34 cycles. The PCR products were then subjected to electrophoresis in 1% agarose gel and visualized by ethidium bromide staining. No PCR product was obtained with any of the set of primers used in the absence of reverse transcriptase (negative control, data not shown). The identity of the generated PCR products was confirmed by cycle sequencing (Thermo Sequenase Radiolabeled Terminator Cycle sequencing kit, Amersham Pharmacia Biotech Life Science), and the size was compared with molecular weight markers (100-bp ladder, Promega Corp., Madison, WI). In addition, the PCR products obtained by using set 4 primers were gel-purified (QIAquick gel extraction kit, QIAGEN), denatured and, after labeling with random exonucleotide method (RadPrime labeling kit, Life Technologies), used as a probe in a Northern Blot analysis of total RNA and mRNA from primary rat Leydig cells and MLTC-1 cells. Fifteen micrograms of total RNA and 1 μg of mRNA were used, following the same procedures previously described (prehybridization, hybridization, and washing temperatures this time were 50 C).

Results

Figure 2 (A and B) is the photograph of two 1% agarose gels with ethidium bromide staining. A shows the presence of PCR products of the expected size from rat full-brain total RNA (positive control, lanes 1–3) and primary rat Leydig cells total RNA (lanes 4–6). The generated PCR products corresponded to a 242-bp cDNA fragment of the extracytoplasmic domain of OB-R common to all isoforms (lanes 1 and 4), and 437-bp (lanes 2 and 5) and 479-bp (lanes 3 and 6) cDNA fragments specific of the intracytoplasmic domain of OB-Rb and OB-Ra, respectively. B shows the same experiment as A, performed on mouse full-brain total RNA (positive controls, lanes 1–3) and on MLTC-1 cells total RNA (lanes 4–6), using the respective sets of primers chosen from the murine OB-R sequence (see *Materials and Methods* and Fig. 1). All expected PCR products could be detected, with the exception of the fragment specific for OB-Ra that could not be amplified from MLTC-1 cells (B, lane 6), suggesting that the short isoform of leptin receptor is not expressed in MLTC-1 cells. Another set of primers (set 4) was used and generated 940-bp cDNA fragments specific of the OB-Rb, both from rat Leydig cells (A, lane 8) and from MLTC-1 cells (B, lane 8). Lane 7 shows the presence of the 940-bp fragment from rat and mouse full-brain total RNA (A and B, respectively). Tentative confirmations of these data, by Northern Blot analysis of rat Leydig cells and MLTC-1 cells mRNA, were negative (data not

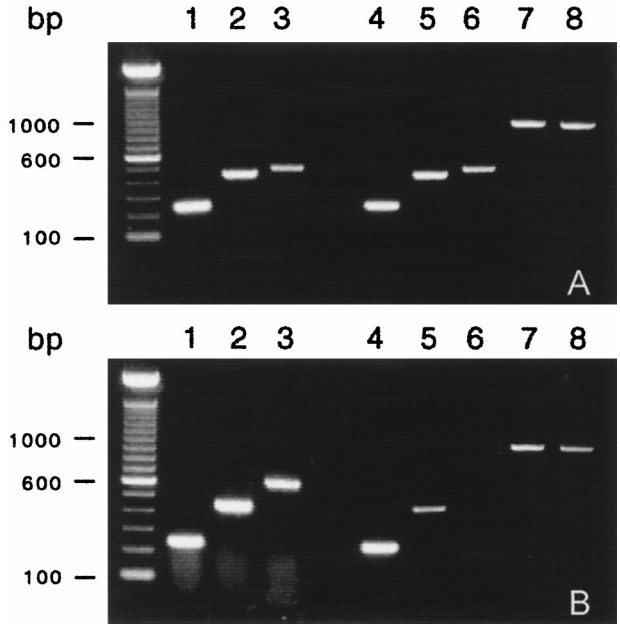

FIG. 2. A, RT-PCR expression analysis of OB-Ra and OB-Rb isoforms in rat brain (lanes 1, 2, 3, and 7, positive controls) and rat Leydig cells (lanes 4, 5, 6, and 8); B, expression analysis of leptin receptor in mouse brain (lanes 1, 2, 3, and 7, positive controls) and in MLTC-1 cells (lanes 4, 5, 6, and 8), by using the murine specific primers sets, equivalent to those used in experiment of A (rat specific). Lanes 1 and 4 show a 242-bp PCR product specific for the extracytoplasmic domain of OB-R obtained by using set 1 primers. Lanes 2 and 5 show a 437-bp band specific for the intracytoplasmic domain of OB-Rb, obtained by using set 2 primers. Lanes 3 and 6 (A) show a 479-bp DNA fragment specific for OB-Ra. In B, this fragment could be amplified only from mouse brain (lane 3, 595 bp), not from MLTC cells (lane 6). Lanes 7 and 8 show a 940-bp DNA fragment obtained combining the upstream primer of set 1 and the downstream primer of set 2 (set 4), spanning both the extracellular and the intracellular domains of OB-Rb.

shown), suggesting that, in these cells, the level of expression of leptin receptor is not abundant.

Studies were then performed to establish whether leptin receptor has functional roles in Leydig cells. Figure 3A shows basal and hCG-stimulated (1 ng/ml) T secretion by rat cultured Leydig cells, after 30 and 90 min of incubation with leptin (150 ng/ml). Leptin significantly inhibited hCG-stimulated T production after 30 min (13.1 ± 0.3 ng/ml *vs.* 27.3 ± 1.0 ng/ml, 50–60% reduction, $P < 0.01$); such inhibitory effect was maintained, even if at a lesser extent (~35% reduction) after 90 min (56.5 ± 2.2 ng/ml *vs.* 87.3 ± 5.4 ng/ml, $P < 0.01$). Leptin alone did not modify T production at any of the examined times. B shows basal and hCG-stimulated intracellular cAMP production from the same experiment as A. Again, leptin did not alter intracellular cAMP levels in the absence of hCG stimulus, whereas it tended to increase hCG-induced cAMP production after 30 min and significantly enhanced it after 90 min (56.2 ± 3.2 fmol/ml *vs.* 37.3 ± 1.4 fmol/ml, $P < 0.05$). Interestingly, in other experiments, leptin significantly reduced 8Br-cAMP-stimulated (1 mM) T production by 40–50% (19.4 ± 1.3 ng/ml *vs.* 34.7 ± 2.1 ng/ml, $P < 0.01$), suggesting that its action on steroidogenesis is exerted beyond cAMP formation. Because leptin effects on steroidogenesis were maximal after 30 min, subsequent experiments all were performed by using this incubation time.

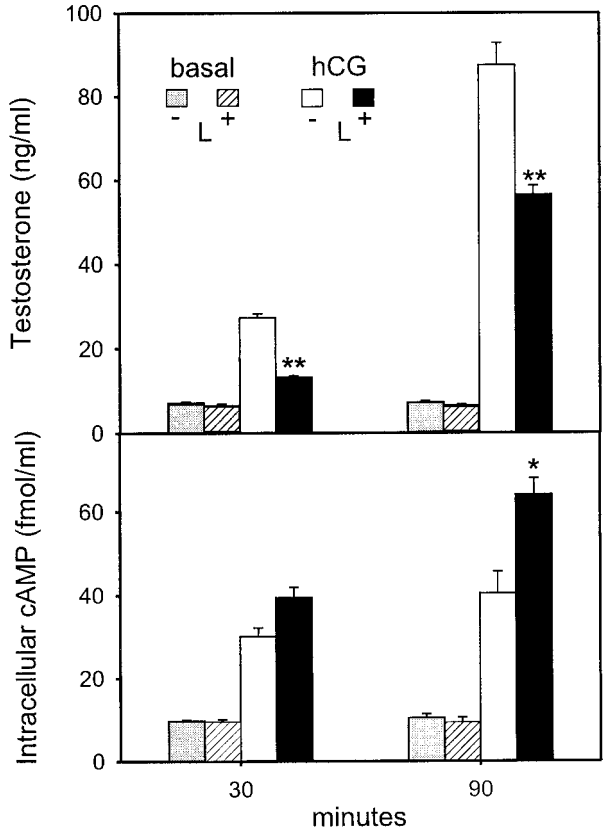

FIG. 3. Effects of leptin (150 ng/ml) on basal and hCG-stimulated (1 ng/ml) T (A) and intracellular cAMP (B) production by rat Leydig cells in culture after 30 and 90 min incubation. ▨, −hCG −leptin; ▨, −hCG +leptin; □, +hCG −leptin; ■, +hCG +leptin; *, $P < 0.05$ level of significance; **, $P < 0.01$ level of significance.

Dose-response studies showed that leptin inhibition of hCG-stimulated T production was present at concentrations ≥ 10 ng/ml leptin, with an IC_{50} of 20 ng/ml and maximal inhibition at 150 ng/ml, at which a 50–60% reduction was observed (Fig. 4). Leptin alone did not alter T secretion at any of the concentrations tested (data not shown).

The effects of maximal inhibitory concentrations of leptin on dose-dependent hCG stimulation of T production by cultured Leydig cells were also evaluated (Fig. 5). As expected, hCG stimulated T production in a dose-dependent manner (ED_{50} = 0.52 ng/ml). Leptin did not modify basal T production but markedly reduced the steroidogenic response to hCG with no significant change in hCG sensitivity (hCG ED_{50}, in the presence of leptin, = 0.51 ng/ml). These results were suggestive of the presence of a leptin-induced lesion of the steroidogenic pathway stimulated by hCG, with possible accumulation of T precursors. Indeed, subsequent experiments showed that leptin addition to cell cultures caused lower hCG-induced increments in the production of androstenedione (259.3 ± 3.2 Δ percent change from baseline, hCG + leptin, *vs.* 392.9 ± 26.1%, hCG alone) and T (202.9 ± 9.3% *vs.* 382.2 ± 13.9%) ($P < 0.01$), which were accompanied by higher hCG-induced changes in the accumulation of the precursor metabolites pregnenolone (336.7 ± 36.6% *vs.* 165.5 ± 32.1%) ($P < 0.05$), progesterone (290.7 ± 6.7% *vs.* 176.6 ± 7.8%), and 17-OH-progesterone (302.3 ± 20.9% *vs.* 160.1 ± 21.2%) ($P < 0.01$) (Fig. 6). These results indicate that leptin inhibition was likely caused by a leptin-induced defect in 17,20 lyase conversion of 17-OH-progesterone to androstenedione and T, as also shown by the augmented percentage changes in precursor (17-OH-progesterone) to product (androstenedione) molar ratios (848 ± 12.6% *vs.* 405.6 ± 17.4%) ($P < 0.05$).

Figure 7 represents basal and hCG-stimulated (20 ng/ml) pregnenolone (A), progesterone (B), and cAMP (C) produc-

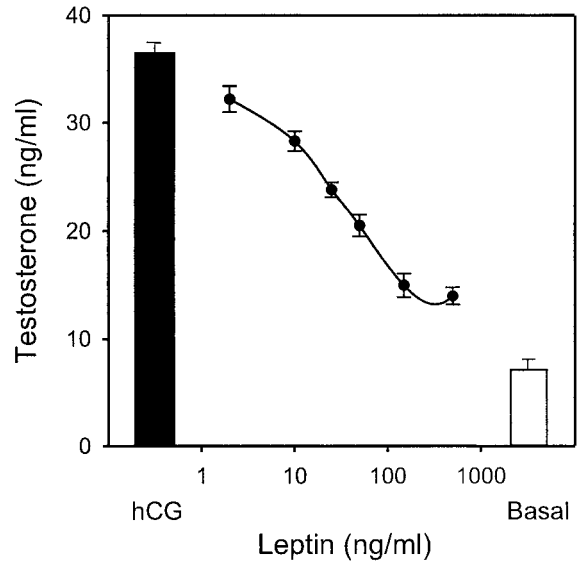

FIG. 4. Effect of increasing concentrations of leptin on hCG-stimulated T production by rat Leydig cells in culture after 30 min of incubation. Leptin IC_{50} was approximately 20 ng/ml, and level of significance of leptin + hCG *vs.* leptin alone ranged from $P < 0.05$ to $P < 0.01$ at leptin concentrations ≥ 10 ng/ml.

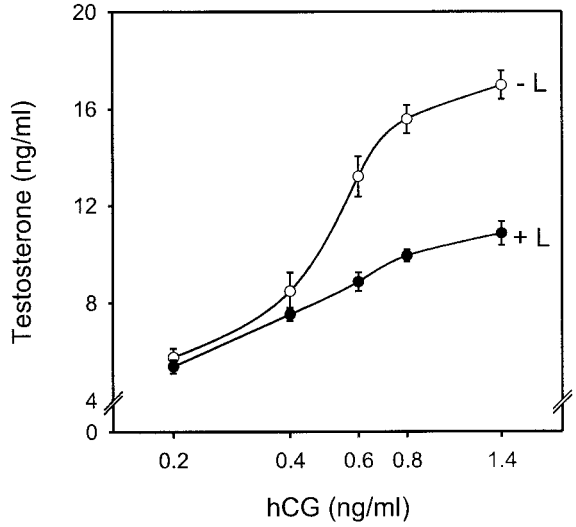

FIG. 5. Effect of increasing concentrations of hCG on T production by rat Leydig cells in culture in the absence (○) or presence (●) of leptin (150 ng/ml).

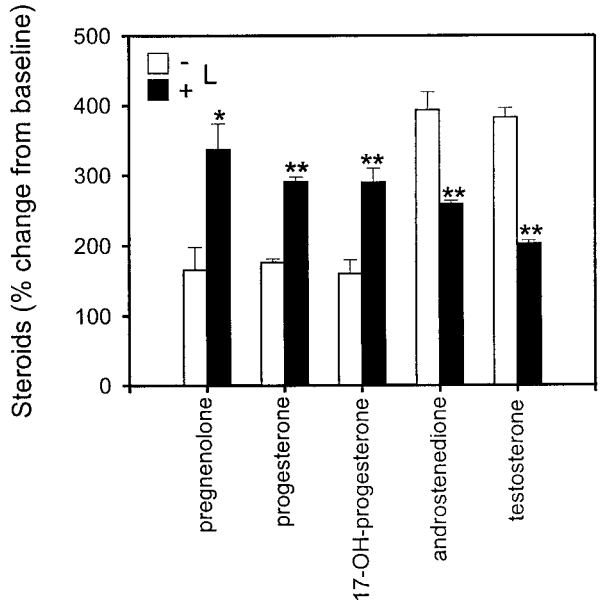

FIG. 6. Percentage changes from baseline (−hCG) in steroid production by primary rat Leydig cells in culture incubated for 30 min with hCG (1 ng/ml) in the presence ■ or absence □ of leptin (150 ng/ml); *, $P < 0.05$ level of significance; **, $P < 0.01$ level of significance.

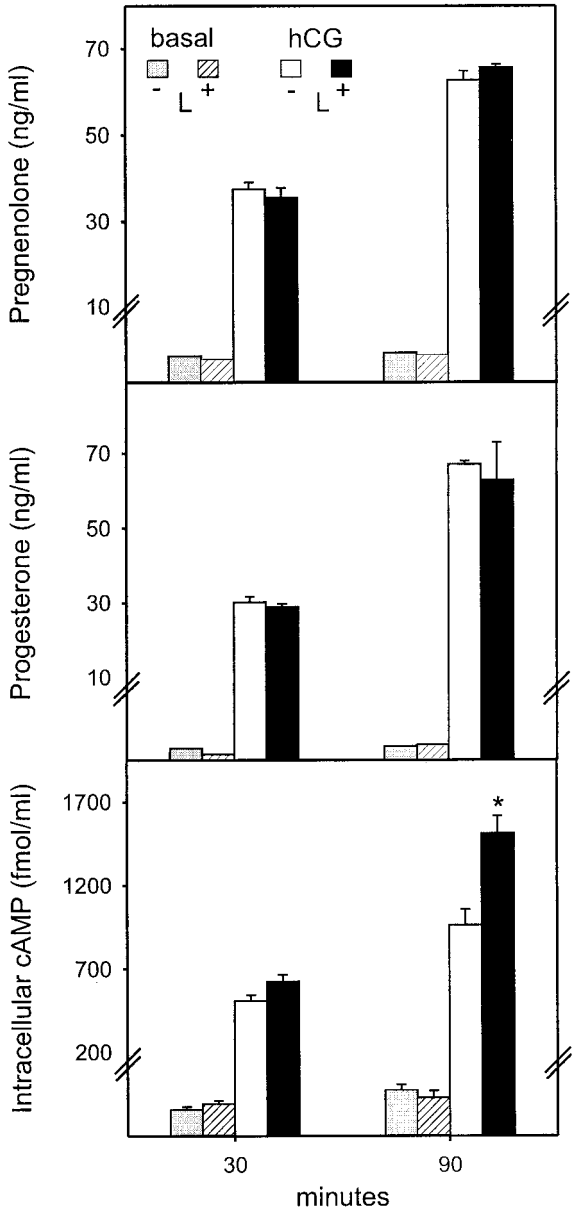

FIG. 7. Effects of leptin (150 ng/ml) on basal and hCG-stimulated (20 ng/ml) pregnenolone (A), progesterone (B), and intracellular cAMP (C) production by MLTC-1 cells after 30 and 90 min of incubation. ▨, −hCG −leptin; ▨, −hCG +leptin; □, +hCG −leptin; ■ +hCG +leptin; *, $P < 0.05$ level of significance.

tion by MLTC-1 cells in the presence or absence of leptin (150 ng/ml). For these cells, a 20-times-higher concentration of hCG was chosen because MLTC-1 cells are known to require approximately 70% occupancy of the LH receptor for maximal steroid stimulation (35). In MLTC-1 cells, leptin did not modify steroid output, either in the absence or presence of hCG stimulus, at any of the examined times. Interestingly, and in analogy to what was observed in rat Leydig cells, leptin treatment amplified hCG-induced intracellular cAMP production by 60% ($P < 0.05$) at 90 min (C), indicating that also MLTC-1 cells express a functional leptin receptor. Again, leptin alone did not alter intracellular cAMP levels.

In Northern blot studies (Fig. 8), leptin did not alter the accumulation of P450–17α mRNA from primary cultured rat Leydig cells, both in basal (lanes 1–2) and hCG-stimulated (lanes 3–4) conditions, excluding that the rapid changes observed in hCG-induced steroidogenesis were caused by mRNA degradation. The same membrane was stripped and hybridized with a c-*fos*-specific probe to study whether leptin was able to activate an early gene expression. In contrast to hCG that caused a quick increase in c-*fos* mRNA accumulation by approximately 3-fold, as already reported by others (36, 37), leptin did not modify c-*fos* expression in basal and hCG-stimulated conditions.

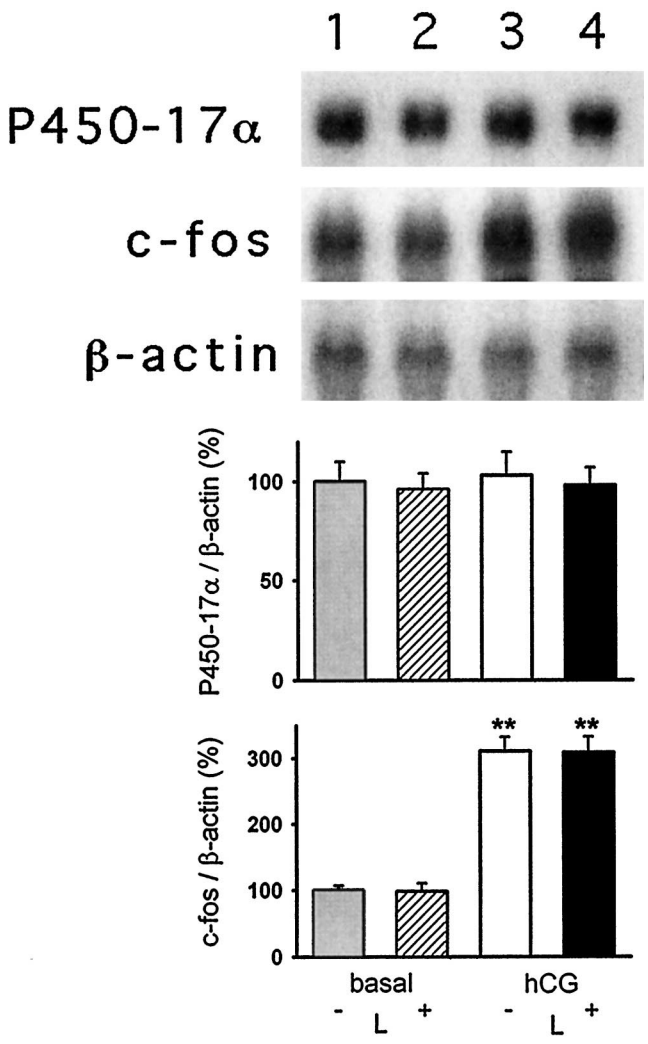

FIG. 8. Nothern blot analysis of P450–17α and c-*fos* expression in rat Leydig cells after 30 min incubation with hCG (1 ng/ml) and leptin (150 ng/ml) alone or in combination. Results were recorded by autoradiography for visual display (*upper*) and quantified from the membranes by phosphorimager (*lower*). Values are the mean ± SE of three separate experiments. A representative experiment is provided in the *upper part* of the figure. Lane 1, −hCG −leptin; lane 2, −hCG +leptin; lane 3, +hCG −leptin; lane 4, +hCG +leptin; ▨, −hCG −leptin; ▨, −hCG +leptin; □, +hCG −leptin; ■, +hCG +leptin; **, $P < 0.01$ level of significance.

Discussion

In this study, we have demonstrated that a functional leptin receptor is expressed in rat Leydig cells and a mouse tumoral Leydig cell line, and have shown that leptin can exert biological effects on rodent Leydig cells at concentrations within the range of obese men. These findings are consistent with an endocrine action of leptin on the testis.

Positional cloning of the leptin receptor gene showed that it encodes several alternate spliced forms (a–e, as well as others), all of which, except Ob-Re (soluble form), contain a single transmembrane domain (38). All isoforms share identical extracellular ligand binding domain with homology to the class I cytokine receptor family, but they differ at the C-terminus. Only Ob-Rb, which has the longest (303 amino acids) cytoplasmic domain, encodes all protein motifs capable of activating the JAK-STAT signal transduction pathway (39). In mice, Ob-Rb is expressed at high levels in the hypothalamus, the primary site where leptin is thought to be acting, with lower-level expression in brain, testes, and adipose tissue (40, 41). In general, in extra-brain tissues, expression levels of Ob-Rb account for only a small part of the total Ob-R expression, which is mostly represented by the expression of the short forms of the leptin receptor. These forms are products of the same gene; have no (OB-Re) or short (less than 50 amino acids) cytoplasmic domains, which contain only one (box 1) out of the two JAK binding domains; are apparently incapable of signaling via the JAK-STAT pathway; and play a role that remains to be defined (38). Besides Ob-Rb, probably only Ob-Ra, which is expressed ubiquitously and is abundant in the choroid plexus, may play important physiological roles, including leptin uptake and/or efflux from the cerebrospinal fluid, and possible increase of the local leptin concentration in a given target tissue, thereby presenting the ligand to the signaling form of the leptin receptor (41). Recent reports have shown that the Ob-Ra has distinct signal capacities and is able to transmit signals through the MAP kinase pathway in transiently transfected cells (42) and in CHO cell lines stably expressing Ob-Ra (43); the significance of these activities for leptin biology *in vivo* is not known. The Ob-R has been found at all points along the HPG axis, and several groups have documented evidence of Ob-R mRNA in the gonads of mice, rats, and humans (15, 16, 24). In particular, in adult mice, *in situ* hybridization studies performed with [^{35}S]-labeled antisense riboprobes to the common extracellular domain of the leptin receptor have shown that Ob-R mRNA is present in the stratified epithelium of spermatic cells and in Leydig cells (15); however, the analysis used detected all isoforms of the leptin receptor and does not give information on its/their eventual functional properties. In this study, we were able to demonstrate that Leydig cells isolated from adult rat testis or derived from a mouse tumoral cell line express the long isoform of leptin receptor; rat Leydig cells, but not MLTC cells, coexpressed also the short Ob-Ra isoform. Because the leptin receptor was only detected by RT-PCR, which is a very sensitive method for mRNA detection, it is conceivable that the level of expression of leptin receptor in Leydig cells was low; however, the receptor was highly efficient and functional because, upon activation, it led to a rapid and dose-dependent inhibition of LH/hCG-stimulated T production. Interestingly, also in human ovarian cells, the signaling isoform of leptin receptor is expressed at levels detectable only by RT-PCR (24); in these cells, in analogy to results in rat Leydig cells, leptin has been found to inhibit LH-stimulated estradiol production, with no effects on basal steroid release. These results, together with other reports that showed that leptin has inhibitory actions on hormonally-stimulated ovarian steroidogenesis *in vitro* in different animal models (21–23), indicate that leptin, at proper levels, may signal metabolic information directly to the gonads via functional peripheral receptors besides the hypothalamic-pituitary (GnRH-FSH/LH) unit. Furthermore, the availability of a Leydig tumor cell line, *i.e.* MLTC-1 cells, expressing the func-

tional leptin receptor is of great importance for future studies on leptin signal transduction pathway/s.

Several reports have addressed the association between leptin and circulating steroids in human males. It has been shown that leptin levels are lower in males, compared with females (44–47); that hypogonadal men have higher circulating leptin, compared with hypogonadal patients under effective androgen substitution therapy (48); and that long-term exposure of human fat cells to T inhibits leptin expression *in vitro* (49). These results lead to the accepted knowledge that T is an important contributor to the gender difference in serum leptin levels. In our study, the inhibitory effect of leptin on hCG-stimulated T production was dose dependent and appeared at concentrations within the range of circulating levels in obese men. Obese subjects, as a group, have elevated levels of leptin in the blood and reduced androgen concentrations (50). It has been known for years that the entity of androgen reduction is related to the amount of fat mass (51) and, recently, that it is also related to leptin levels (52); in addition, we found that the androgen response to hCG stimulation is impaired in obese man, and multivariate analysis showed that leptin was the best hormonal predictor of the obesity-related reduction in androgen response (53). These observations, together with the present results, indicate that leptin excess may have an important role in the development of reduced androgens in male obesity. It is possible that leptin has a dualistic effect on male reproduction and that the major site of action may differ, depending on the concentration of leptin in the blood. The direct inhibitory action of leptin on the testis may be of importance under certain conditions, such as obesity (54, 55), with elevated plasma leptin/cerebrospinal fluid ratio and exposure of the peripheral tissues to very high leptin concentrations. Conversely, the positive leptin action at the hypothalamic level may be of relatively greater importance during conditions with low concentrations of leptin in the blood, as in subjects with low body mass index (6) and in ob/ob mice (5, 56, 57). It is also likely that leptin's *in vivo* actions on Leydig cell steroidogenesis are more complex than those observed *in vitro* and could be influenced by various factors, including events that modify leptin concentrations (*i.e.* food restriction) and/or changes in hypotalamic peptides known to be modified by leptin and to regulate the HPG axis (*i.e.* POMC products, neuropeptide Y, galanin, and CRH) (13, 58).

The mechanism of leptin-induced inhibition of hCG-stimulated T production from rat Leydig cells was also investigated. The inhibitory actions of leptin were exerted after a short-time incubation (minutes) enough to reveal the action of other substances previously found to regulate Leydig cell function in a stimulatory (59) or inhibitory (60, 61) manner. Leptin markedly inhibited the androgen response, with no change in hCG sensitivity, suggesting a not-competitive mechanism of interaction between leptin and hCG; also, the observation that leptin inhibited 8-Br-cAMP-stimulated T production indicates that leptin action was exerted beyond cAMP. Interestingly, leptin significantly amplified hCG-induced cAMP formation both in MLTC-1 and rat Leydig cells, with no change in basal cAMP levels. These results were unexpected. The leptin receptor is known to stimulate JAK phosphorylation and in-sequence STAT protein phosphorylation, dimerization, translocation to the nucleus, and stimulation of transcription (38). It is believed that STAT3 and, maybe, STAT5 are activated by the triggering of Ob-R (39), and Stat5b protein has been recently found to be present and phosphorylated in rat Leydig cells and MA10 mouse Leydig tumor cell line after GH treatment (62). Thus, it is likely that, in Leydig cells, leptin stimulation of the Ob-R leads to the activation of the JAK/STAT pathway, which should be distinct from the adenylate cyclase-cAMP-PKA pathway. Explanations of leptin amplification of hCG stimulation of cAMP should exclude leptin-induced changes in phosphodiesterase activity, which has been shown to occur in other cells responsive to leptin (63), because all experiments were performed in the presence of adequate concentrations of MIX, a universal inhibitor of phosphodiesterases. Interestingly, we previously demonstrated that, in rat Leydig cells, CRF acutely inhibits hCG-stimulated T production (60), and that this is attributable to a rapid cross-talk between two distinct transducing signal pathways, *i.e.* the CRF-PKC and the hCG-adenylate cyclase pathways (61). It is possible that, in rodent cells, a positive interaction occurred between the JAK-STAT pathway and adenylate cyclase activity stimulated concomitantly by leptin and hCG. This possibility will be investigated in future studies.

The leptin-induced inhibition of hCG-stimulated androstenedione and T production was explained by a lesion of 17,20-lyase activity, the enzyme that converts 17α-hydroxylated intermediates to androstenedione (64), as shown by the increase in precursor metabolites (pregnenolone, progesterone, and 17-OH-progesterone) and the augmented percentage changes in precursor-to-product molar ratios (17-OH-progesterone/androstenedione). Data obtained from MLTC-1 cells, a tumor cell line not expressing cytochrome P450–17α (65), showed that leptin, though causing a significant increase in hCG-stimulated cAMP production, did not modify steroid release, further indicating that leptin action occurs downstream of progesterone synthesis. In other studies, long-term leptin incubation (1 day) has been found to inhibit glucocorticoid release from bovine adrenocortical cells and to significantly reduce ACTH-stimulated human and bovine adrenal steroidogenesis through a transcriptional inhibition of cytochrome P450–17α (25, 27). In our experiments, acute leptin treatment did not cause any reduction in P450–17α mRNA accumulation, by Northern blot analysis, indicating that its rapid action was not caused by mRNA degradation but possibly by an inhibitory modulation of 17,20-lyase activity. Along this possibility, we have previously shown that rapid modifiers of hCG-stimulated Leydig cell function act via specific alterations in the activity of cell enzymatic machinery (60, 61). Further studies performed with prolonged treatment periods (24 h and longer) to account for the half-life of the P450–17α message will be needed to assess the effect of leptin on new P450–17α mRNA synthesis. However, adult primary rat Leydig cells seem unsuitable for this purpose, because these cells usually lose their LH/hCG receptor after 1–3 days in culture along with a parallel decline of 17-α-hydroxylase/17,20-lyase activities (66).

The mechanism/s of action of leptin on 17,20-lyase activity is presently unknown. In Leydig cells, the production of

steroids in response to hormone stimulation requires *de novo* protein synthesis (67), but changes in phosphorylation of steroidogenic enzymes also play an important role (68). In particular, serine phosphorylation seems to be required exclusively for 17,20-lyase activity, as in human adrenocortical cells, dephosphorylation of microsomal proteins with alkaline phosphatase causes a complete loss of 17,20-lyase but no change in 17α-hydroxylase activity (69). Because a rapid increase in phosphatase activity occurs after Ob-R activation (70), a leptin-induced change in hormonally-stimulated phosphorylation of specific P450–17α amino acids cannot be excluded. Finally, recent reports showed that *in vivo* and *in vitro* treatment with leptin activates c-*fos* gene expression in specific hypothalamic nuclei (71, 72) and in stable transfected cells (73), respectively. In Leydig cells, in contrast to hCG, which induced a clear stimulation of c-*fos* expression, we were unable to show any increase in c-*fos* mRNA levels by leptin, indicating the lack of involvement of this early gene in leptin response.

In conclusion, the results of our study demonstrate that functional leptin receptors are expressed in rodent Leydig cells and that leptin has a novel direct negative action on LH/hCG-stimulated androgen production from Leydig cells in culture. These results are consistent with an endocrine action of leptin on the rat testis and have possible implications in some clinical aspects of male reproduction, such as the reduced testicular function in obese males.

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