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Leptin Modulates the Glucocorticoid-Induced Ovarian Steroidogenesis*

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ABSTRACT

Leptin regulates food intake and other activities through its hypothalamic receptor. Leptin receptors are also found in other organs, including the ovary. Direct effects of leptin in ovarian steroid production were studied in primary rat granulosa cells and in rat and human granulosa cell lines. Leptin (0.6–18 nM) suppressed ovarian steroid synthesis costimulated by FSH and dexamethasone. Production of pregnenolone, progesterone, and 20α-hydroxy-4-pregnen-3-one was inhibited by leptin. This inhibition was due at least in part to reduced expression of adrenodoxin, a component of the P450scc system enzyme. Costimulation of progesterone production by forskolin and dexamethasone was also inhibited by leptin, whereas the forskolin-induced cAMP production was not affected. We find that leptin induces c-Jun expression and attenuates the transcriptional activity of the glucocorticoid receptor (GR) in granulosa cells. Elevation of c-Jun expression by other means, *e.g.* 12-*O* tetradecanoylphorbol-13-acetate or transfecting with a c-Jun expression vector, abolished the transcriptional activity of the GR. A leptin-induced elevation of c-Jun modulates the transcriptional activity of the GR, possibly leading to the observed attenuation of steroidogenesis. It was recently shown that glucocorticoids stimulate leptin expression *in vivo*, which in turn, inhibits cortisol synthesis. A direct action of leptin on the ovary is an additional element of a regulatory network that maintains the homeostasis of steroid production. (*Endocrinology* **140:** 1731–1738, 1999)

LEPTIN, a product of the *obese* (ob) gene, is a cytokine secreted by adipocytes, which in mammals plays a major role in controlling body fat mass through coordinated regulation of feeding behavior and metabolic energy balance (1). Leptin's activities are mediated through the leptin receptor, (OB-R), a member of the class I cytokine receptor superfamily, expressed in several forms, which differ in the length of their cytoplasmic domain (2, 3). Furthermore, a different repertoire of these leptin receptor-splice variants is expressed in a tissue-specific manner (2–4).

OB-Rb, the long form of OB-R, is expressed predominately in the hypothalamus, where it mediates many leptin's activities by regulating metabolic and behavioral activities. Ligand binding to OB-Rb activates Janus kinase 2 and its substrates STAT-1, STAT-3, and STAT-5 (5, 6). COS cells transfected with either the shorter variant OB-Ra or OB-Rb express the immediate early genes such as c-Jun, c-Fos, and Jun B upon treatment with leptin (7). OB-Ra is expressed in various tissues, and therefore leptin may exert other physiological functions by acting directly on peripheral cells and tissues. Indeed, it has been shown that leptin exerts various activities on peripheral targets such as liver cells, pancreatic β-cells, and muscle cells (8–10).

Several studies have shown that leptin also plays a major role in reproduction. For example, leptin treatment rescued the sterility of genetically obese *ob/ob* mice (11, 12). In addition, leptin accelerated the onset of puberty in female mice (13). Because leptin levels are correlated with body mass index, it appears that leptin acts as a message, informing the brain that maternal energy stores are sufficient to support the high-energy demands of reproduction. Recently, it has been reported that the messenger RNA of the long and short forms of leptin receptors are present in granulosa and cumulus oophorus cells (14), suggesting that leptin may have a direct effect on ovarian function.

Granulosa cells, which differentiate during each estrous cycle and nurse the mammalian egg, are the main site of progesterone production (15). The induction of steroidogenesis in granulosa cells is initiated by the gonadotrophic hormones FSH and LH, which interact with specific cell membrane receptors (16). The interaction of these gonadotropins with their receptors leads to the activation of adenylate cyclase and subsequently to the induction of steroidogenic enzymes such as cytochrome P450 side chain cleavage enzyme (P450scc) and its ancillary electron transport proteins adrenodoxin (ADX) and ADX reductase (17, 18). P450scc catalyses the first step in the biosynthesis of progesterone by converting cholesterol to pregnenolone. (15).

The mechanism by which steroidogenesis is induced cannot be solely attributed to the action of the gonadotropins. Additional substances, including growth factors and corticosteroids are known to modulate gonadotropin's activity (19–21). For example, it was shown that glucocorticoids aug-

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ment the FSH-induced production of progesterone in rat preantral primary granulosa cells (19). Glucocorticoids also stimulate leptin expression in adipocytes, whereas leptin inhibits glucocorticoid synthesis in the adrenal cortex (22, 23). This regulatory feedback prompted us to study the direct effect of leptin on the glucocorticoid response in granulosa cells. We report here that leptin inhibits the glucocorticoid augmentation of steroid synthesis in granulosa cells. A leptin-induced c-Jun expression is possibly involved in this process.

Materials and Methods

Cells and treatments

Primary granulosa cells of preovulatory follicles were obtained from 25-day-old female rats treated with PMSG (25 U), as previously described (24). Cells were plated in DMEM/F12 medium (1:1) containing 10% FBS. After 18 h, the cells were washed and the medium replaced by serum-free medium containing the desired stimulants. The steroidogenic rat FSHR-17 cell line expressing the FSH receptor was previously described (25). Cells (2×10^4) were cultured in 24-well plates in DMEM/F12 medium containing 10% FBS. After 18 h, the medium was replaced by a serum-free medium containing the desired stimulants.

Human granulosa cells (clone HO23) were immortalized by cotransfection with SV40, Ha-ras, and a p53 temperature-sensitive mutant (p53 val 135), (26). Cells were cultured and stimulated as described for FSHR-17 cells.

Plasmids

Plasmid pΔG46TCO contains two copies of a synthetic glucocorticoid receptor response element (GRE) linked to the herpes virus thymidine kinase (TK) promoter, followed by a chloramphenicol acetyl transferase (CAT) gene (27). Plasmid pRSVc-Jun for mammalian expression of c-Jun and the control plasmid pRSV-β Gal were previously described (28, 29).

DNA transfection

FSHR-17 cells (1×10^5 cells) in 30-mm plates were transfected with various plasmids with the aid of the FuGENE transfection reagent (Boehringer Mannheim, Mannheim, Germany). Eighteen hours after transfection, the medium was replaced with fresh DMEM/F12 medium containing 10% FBS, and the transfected cells were cultured for an additional 24 h in the presence or absence of various stimulants.

CAT assay

CAT activity was determined as described (29). After transfection and various treatments, sonicates of FSHR-17 cells were heated for 10 min at 65 C before analysis. CAT activity in various cells was adjusted to the same amount of protein. The percentage of chloramphenicol acetylation was calculated by scanning the TLC plates with a phospho-imager. The value of CAT activity in cells treated with dexamethasone (DEX) only was used to normalize all other results.

cAMP assay

Intracellular cAMP levels were determined in extracts of cells that were cultured with [^{3}H]-adenine. Preformed monolayers of FSHR-17 cells in 24-well plates (2×10^4 cells) were incubated for 16 h in a serum-free medium containing [2-^{3}H]adenine (10 μCi/ml). The medium was then replaced by a serum-free medium (0.5 ml) containing the appropriate stimulant. After 15 min the medium was discarded and ice-cold perchloric acid (2.5%) containing 0.1 mM cAMP (1 ml/well) was added. The perchloric acid extract was neutralized with 1 M Tris/4.2 M KOH, [2-^{3}H]-cAMP was isolated by sequential chromatography on a Dowex-50 cation exchanger and neutral alumina. Intracellular accumulation of [2-^{3}H]-cAMP was calculated as the percentage of total [2-^{3}H]-adenine uptake per well (30).

Progesterone assay

FSHR-17 or primary rat preovulatory granulosa cells were cultured in 24-well plates in DMEM/F12 medium containing 10% FBS. After 18 h the medium was replaced by a serum-free medium containing the desired stimulant. Progesterone accumulation in the culture medium was determined after 24 h by RIA (31).

Immunoblot assay of ADX

FSHR-17 cells were plated in 60-mm dishes (4×10^5 cells/dish) in DMEM/F12 medium containing 10% FBS. After 18 h the medium was replaced by a serum-free medium containing the desired stimulant. After 24 h, the cells were washed and pelleted (1500 $\times$ *g*, 10 min). The pellets were lysed for 15 min at 4 C in Lysis Buffer (0.1 ml of 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM Na orthovanadate, 5 μg/ml aprotinin, 1 nM phenylmethanesulfonyl fluoride, 20 mM NaF). The clarified extracts (30 μg protein) were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane and probed with a polyclonal antibody against ADX (1:1000), followed by goat antirabbit antibody conjugated to horseradish peroxidase (Jackson Laboratories, Bar Harbor, ME). The complexes were visualized by the enhanced chemiluminescence (ECL, Amersham) and quantitated by densitometry.

Immunoblot analysis of AP1

FSHR-17 cells were plated in 60-mm dishes (4×10^5 cells/dish) in DMEM/F12 medium containing 10% FBS and incubated for 18 h. The medium was then replaced by a serum-free medium and the cultures incubated for additional 24 h. The medium was then replaced by a serum-free medium containing the desired stimulant and nuclear extracts were prepared after 2 h by the following procedure: washed cell pellets were resuspended for 15 min at 4 C in 0.4 ml hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM p-amino benzoic acid, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 50 μg/ml aprotinin). Triton X-100 (10%, 25 μl) was then added and the suspension was mixed vigorously for 10 sec. The nuclei were separated from the cytoplasmic fraction by centrifugation (15,000 $\times$ *g*, 30 sec, 4 C). The nuclear fraction was then extracted by resuspending it in 0.2 ml ice cold nuclear extraction buffer (20 mM HEPES, pH 9.7, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM p-amino benzoic acid, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μg/ml aprotinin, and 1 mM PMSF). After rotating the tubes for 15 min at 4 C the nuclear extract was cleared by centrifugation. Nuclear extracts (30 μg protein) were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane and probed with a rabbit anti c-Jun/AP1 antibody, followed by goat antirabbit antibody conjugated to horseradish peroxidase. The bands were visualized by the enhancer chemiluminescence kit (Amersham) and quantitated by densitometry.

Antibodies and other reagents

Antibodies against pregnenolone, progesterone and 20α,hydroxy-4-pregnen-3-one were a kind gifts from Dr. F. Kohen, Weizmann Institute of Science (Rehovot, Israel). Polyclonal antibody against adrenodoxin was kindly provided by Dr. W. L. Miller, University of California (San Francisco, CA). Rabbit c-Jun/AP1 polyclonal antibody was from Santa Cruz Biotechnology, Inc.; DEX, 2-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and forskolin were from Sigma Chemical Co. (St. Louis, MO). Human recombinant FSH was from the National Institute of Health (Bethesda, MD). Murine leptin was from PeproTech Inc. (Rocky Hill, NJ). Human leptin was the kind gift of R. Devos and Y. Guisez, Roche Gehnt.

Statistical significance

All experiments were repeated at least three times with different batches of cells. Values are the mean ± SE of these experiments where noted. Significance between experimental values was determined by unpaired Student's *t* test and are significant if *P* value were <0.05 when data from all experiments were considered.

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Results

Leptin inhibits progesterone production in granulosa cells

We studied the effect of leptin on progesterone production in granulosa cells using both rat primary granulosa cells and steroidogenic rat granulosa cell lines (25). In rat primary granulosa cells, FSH induced the production of progesterone, whereas dexamethasone (DEX) augmented progesterone production by about 5-fold. Leptin by itself did not induce the production of progesterone, nor did DEX. Leptin had no significant effect on the production of progesterone induced by FSH alone. However, leptin significantly reduced the DEX-augmented production of progesterone (46% inhibition, $P < 0.05$, Fig. 1). Inhibition was dose-dependent with the maximal effect obtained at 18 nM leptin (Fig. 2A). This inhibitory effect of leptin was obtained also in the rat granulosa cell line FSHR-17, where leptin lowered the levels of progesterone in a dose-dependent manner, with a maximal effect of murine leptin obtained at 0.6 nM ($P < 0.05$, Fig. 2B).

Forskolin, a potent activator of adenylate cyclase, mimics the action of FSH in granulosa cells. Primary granulosa cells and two steroidogenic granulosa cell lines, rat FSHR-17 and human HO23 were induced by a combination of forskolin (20 μM) and DEX. We found that in all three cell cultures, both human and murine leptin significantly reduced the levels of progesterone in a dose-dependent manner. Specifically, murine leptin (60 nM) reduced the level of progesterone by 50% ($P < 0.05$) in FSHR-17 cells induced by forskolin and DEX (Fig. 3A). Similar results were obtained with rat primary granulosa cells (30% inhibition, $P < 0.05$; Fig. 3B) and with human HO23 cells (34% inhibition, $P < 0.05$; Fig. 3C). The higher concentration of leptin needed for attenuating the forskolin-induced cells as compared with FSH-induced cells may be attributed to the observation that FSH exerts only a transient increase of cAMP in the rat granulosa cells, whereas forskolin was shown to induce a sustained and potent rise of cAMP in these cells (24).

The dose-response curve of leptin's effect on the DEX + FSH induction of progesterone assumed an inverse bell shape (Fig. 2). Leptin at a physiological concentration range (32) suppressed the level of progesterone induced by FSH and DEX. In contrast, at higher concentrations, leptin elevated the level of progesterone, rather than further suppressing it. However, leptin alone did not induce any change in the progesterone level, even when added at high concentrations (>60 nM) (Figs. 1 and 2B).

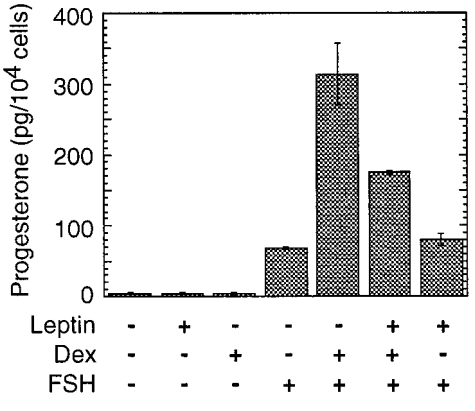

FIG. 1. Leptin lowers progesterone levels in rat primary preovulatory granulosa cells. Rat primary preovulatory granulosa cells were stimulated for 24 h with FSH (2.5 nM), DEX (10 nM) or both, in the absence or presence of murine leptin (18 nM). Progesterone levels in the culture media were then determined. Data are mean ± SE (n = 3).

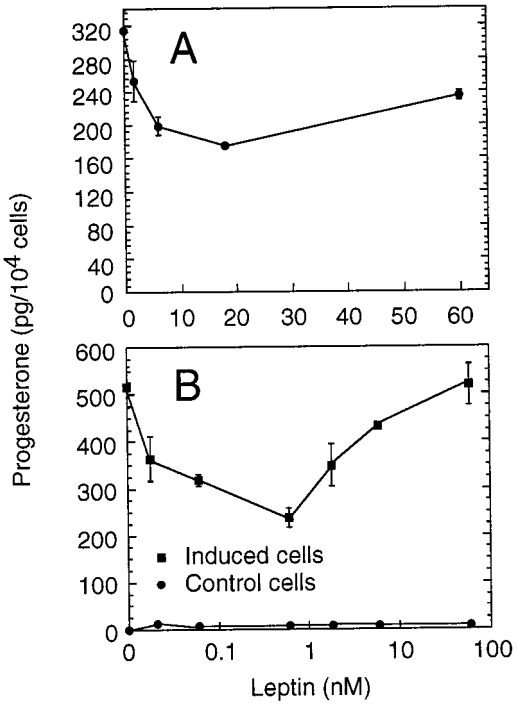

FIG. 2. Leptin lowers the level of progesterone in a dose-dependent manner. A, Rat primary preovulatory granulosa cells were induced for 24 h with FSH (2.5 nM) and DEX (10 nM) in the presence of increasing doses of murine leptin. Progesterone levels in the culture media were then determined. Data are mean ± SE (n = 3). B, Rat granulosa FSHR-17 cells were similarly induced with FSH (1 nM) and DEX (3 nM) in the presence of increasing doses of murine leptin. Progesterone levels in the culture media were then determined. Data are mean ± SE (n = 3).

Leptin may reduce progesterone levels in granulosa cells either by inhibition of its synthesis or by enhancing its metabolism (33). To distinguish between these two possibilities, we determined the effects of leptin on the levels of the progesterone precursor pregnenolone, as well as the progesterone metabolite 20α hydroxy-4-pregnen-3-one. The levels of both the precursor and the metabolite were reduced by 32% and 40%, respectively ($P < 0.05$ in both cases) when leptin (2 nM) was added to cultures of FSHR-17 cells costimulated by FSH and DEX (Fig. 4A). These results suggest that in rat granulosa cells leptin inhibits progesterone synthesis rather than enhancing its further metabolism.

P450scc is the rate limiting enzyme of the steroidogenic pathway in granulosa cells (18). P450scc, and its ancillary electron transport protein, ADX and ADX reductase are induced by gonadotropins/cAMP in primary rat granulosa cells and cell lines (17, 34). Therefore, we measured ADX expression as a marker for cytochrome P450scc activity. We checked if DEX can augment the forskolin-induced expression of ADX and whether leptin may attenuate this induction. FSHR-17 cells were treated for 24 h with forskolin, or forskolin together with DEX, in the presence or absence of

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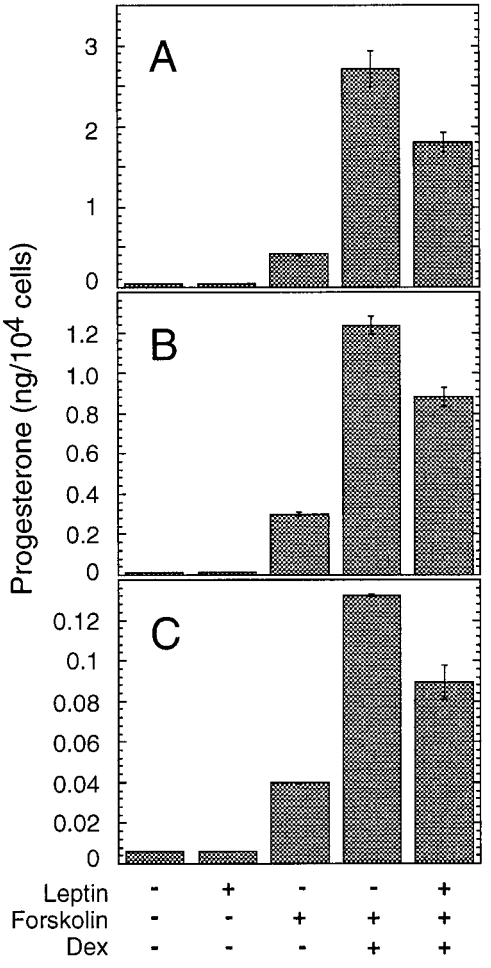

FIG. 3. Leptin modulates progesterone levels in ovarian granulosa cells treated with forskolin and DEX. Three different granulosa cell cultures were induced for 24 h with forskolin (20 μM) alone or forskolin and (10 nM) DEX in the absence or presence of leptin (60 nM). Progesterone levels in the culture media were then determined. Data are mean ± SE (*panel A*) rat granulosa FSHR-17 cells (n = 4). B, Rat primary preovulatory granulosa cells (n = 3). C, Human granulosa HO23 cells (n = 3).

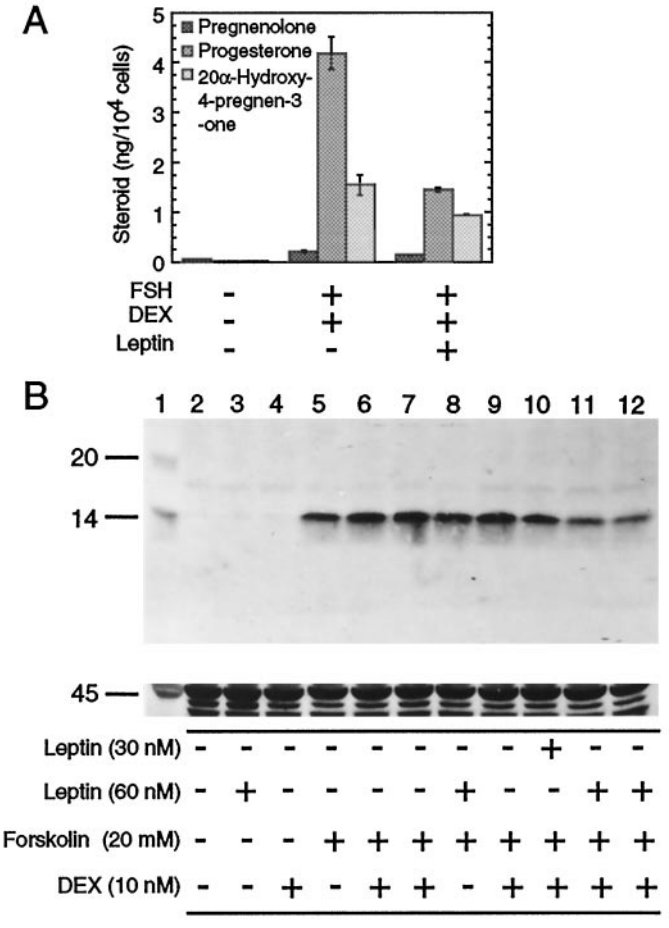

FIG. 4. Leptin inhibits steroidogenesis and lowers the induction of adrenodoxin. A, FSHR-17 cells were induced for 24 h with FSH (1 nM) and DEX (3 nM) in the presence or absence of murine leptin (2 nM). The levels of pregnenolone, progesterone and 20α-hydroxy-4-pregnen-3-one in the culture media were then determined. Data are mean ± SE (n = 3). B, FSHR-17 cells were incubated for 24 h with the indicated combinations of forskolin (20 μM), murine leptin and DEX (10 nM). Cell extracts were immunoblotted with antibody to ADX (migrating as an 14-kDa protein). The lanes were normalized by reprobing the membrane with an antibody to α-actin (*lower panel*). Molecular mass markers (lane 1; in kDa) are indicated on the *left side*. The blot is a representative of three independent experiments.

murine leptin. Immunoblotting with antibodies to ADX revealed that DEX up-regulated the forskolin-induced expression of ADX by 2.4 ± 0.38-fold ($P < 0.05$, n = 3, Fig. 4B, compare lane 5 with lanes 6, 7, and 9), whereas leptin (60 nM) down-regulated by 2.1 ± 0.31-fold the forskolin + DEX-induced expression of ADX ($P < 0.05$, n = 3, compare lane 9 with lanes 11 and 12). Neither leptin alone nor DEX alone affected ADX expression (lanes 3 and 4). Furthermore, leptin had no significant effect on the forskolin-induced level of ADX (n = 3, compare lane 5 with lane 8).

Leptin attenuates the transcriptional activity of the glucocorticoid receptor

Both P450scc system enzyme and its product progesterone may be induced by FSH or forskolin in the absence of DEX. Therefore, it is possible to determine if leptin modulates the activity of either FSH/forskolin or that of glucocorticoids. We found that leptin did not affect the level of forskolin-induced ADX in FSHR-17 cells (Fig. 4B, compare lane 5 with lane 8). Similarly, we found that leptin had no effect on the basal and forskolin-induced progesterone production in FSHR-17 cells ($P > 0.05$, Fig. 5A), nor did it affect ($P > 0.05$) the basal and forskolin-induced production of cAMP in these cells (Fig. 5B). We therefore concluded that leptin does not affect the induction of cAMP by FSH or forskolin and their subsequent effect on steroidogenesis. Rather, it is likely that leptin inhibits the DEX-induced pathway.

The glucocorticoid receptor (GR) is a ligand-dependent transcription factor, activated by glucocorticoids such as DEX. Activated GR induces gene expression by binding to the glucocorticoid response element (GRE) (35, 36). Therefore, we examined whether leptin affected the transcriptional activity of GR in granulosa cells. FSHR-17 cells were transiently transfected with the reporter plasmid pΔG46TCO,

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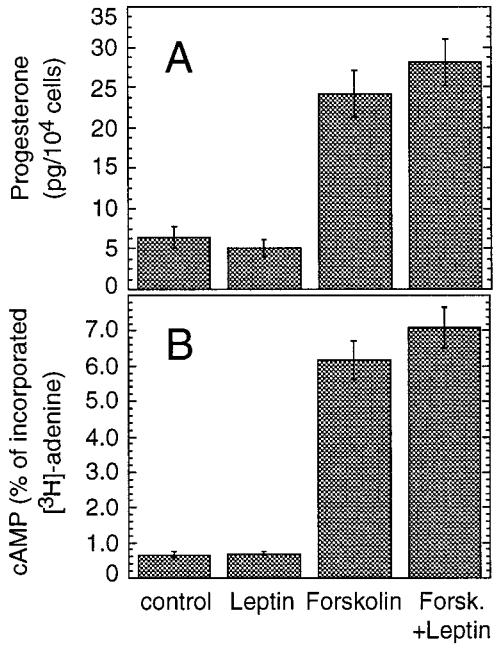

FIG. 5. Leptin does not affect forskolin-induced progesterone and cAMP levels in FSHR-17 cells. A, Rat FSHR-17 cells were induced with forskolin (50 μM, 24 h) in the presence or absence of murine leptin (60 nM). Progesterone levels in the culture media were then determined. Data are mean ± SE (n = 3). B, Rat FSHR-17 cells were induced with forskolin (10 μM, 15 min) in the presence or absence of murine leptin (60 nM). cAMP accumulation in the cells was then determined. Data are mean ± SE (n = 3).

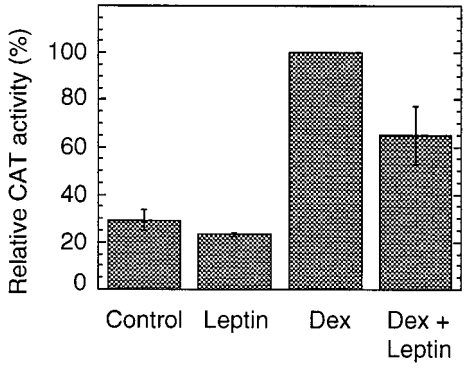

FIG. 6. The transcriptional activity of the glucocorticoid receptor is repressed by leptin. FSHR-17 cells were transiently transfected with the glucocorticoid-inducible CAT construct pΔG46TCO (2 μg/10^5 cells). Transfected cells were induced with DEX (100 nM, 24 h) in the presence or absence of murine leptin (60 nM). Cell extracts were subjected to CAT assay. CAT activity in cells treated with DEX only was used to normalize all other results. The data are mean ± SE (n = 6).

containing a GR response element, the TK promoter, and the CAT gene (27). Transfected cells were treated for 24 h with DEX in the presence or absence of leptin and the level of CAT was then determined. Leptin (60 nM) was found to significantly inhibit CAT activity induced by DEX (35% inhibition, $P < 0.05$), whereas leptin alone had no effect on CAT activity (Fig. 6). Thus leptin probably lowers the expression of the ADX gene by attenuating the transcriptional activity of GR.

Modulation of the transcriptional activity of GR can occur by different mechanisms, including reduction of its expression level or interaction of GR with other factors. We found by immunoblot analysis that leptin had no effect on the steady-state level of GR in FSHR-17 cells (data not shown). It was previously shown that c-Jun directly modulates the transcriptional activity of GR in various cell types. However, elevated c-Jun may either up-regulate or down-regulate the transcriptional activity of GR in a cell-specific manner (27, 37). We found that treatment of FSHR-17 cells for 2 h with murine leptin (6 and 60 nM) or with the c-Jun inducer TPA (50 nM) elevated the steady-state level of c-Jun by 1.4, 2.65 and 1.97-fold, respectively (Fig. 7A). c-Jun levels remained significantly higher than untreated cells 6 and 24 h post treatment (data not shown).

The effect of overexpressing c-Jun on the transcriptional activity of GR was then studied. FSHR-17 cells were cotransfected with the GRE-CAT reporter pΔG46TCO, and the expression vector pRSVc-Jun. As a control, cells were cotransfected with pRSV-βGal and pΔG46TCO. Transfected cells were cultured with or without DEX for 24 h and then assayed for CAT activity. In addition, the steady-state level of c-Jun was elevated by treatment of GRE-CAT-transfected cells with TPA (100 nM, 24 h) followed by DEX. Both overexpression of c-Jun or elevation of c-Jun by TPA abolished the DEX-induced CAT activity, whereas transfection of FSHR-17 cells with the control plasmid pRSV-βGal had no inhibitory effect on GR activity (Fig. 7B). Furthermore, the c-Jun inducer TPA also inhibited progesterone synthesis in FSHR-17 cells (Fig. 7C). Induction of progesterone synthesis by forskolin alone was inhibited by >50% ($P < 0.05$), whereas the DEX-augmented induction was inhibited by 93% ($P < 0.01$). Similarly, TPA (50 nM) inhibited the forskolin induced expression of ADX by 1.6 ± 0.15-fold ($P < 0.001$, n = 3, Fig. 7D, compare lane 3 with lane 4), whereas the forskolin + DEX-augmented induction of ADX was reduced by 3 ± 0.65 fold ($P < 0.05$, n = 3, compare lane 5 with lane 6).

Discussion

The present study demonstrates a novel role of leptin as a modulator of steroid biosynthesis in granulosa cells. Steroid biosynthesis is induced by FSH in primary preovulatory or cultured granulosa cells and is augmented by growth factors (20, 21). Glucocorticoids were previously found to increase the progesterone production induced by FSH in rat preantral primary granulosa cells and in the rat granulosa cell line FSHR-17 (19, 25). We have extended these findings by showing that glucocorticoids also increase the FSH/cAMP-induced steroidogenesis in rat preovulatory primary granulosa cells and in a human granulosa cell line. We have found that activated glucocorticoid receptor (GR) up-regulates the cAMP induction of the electron carrier ADX, an intrinsic part of the P450scc system enzyme (17, 18).

A minimal level of leptin is required for activating the hypothalamic-pituitary axis, which triggers gonadotropin secretion. Indeed, lack of leptin due to starvation or due to a genetic defect (*e.g.* in *ob/ob* mice), or lack of the hypothalamic leptin receptor (as in *db/db* mice), all lead to gonadotropin deficiency and unovulation (11). The local effects of leptin on ovarian steroidogenesis are in opposite direction to

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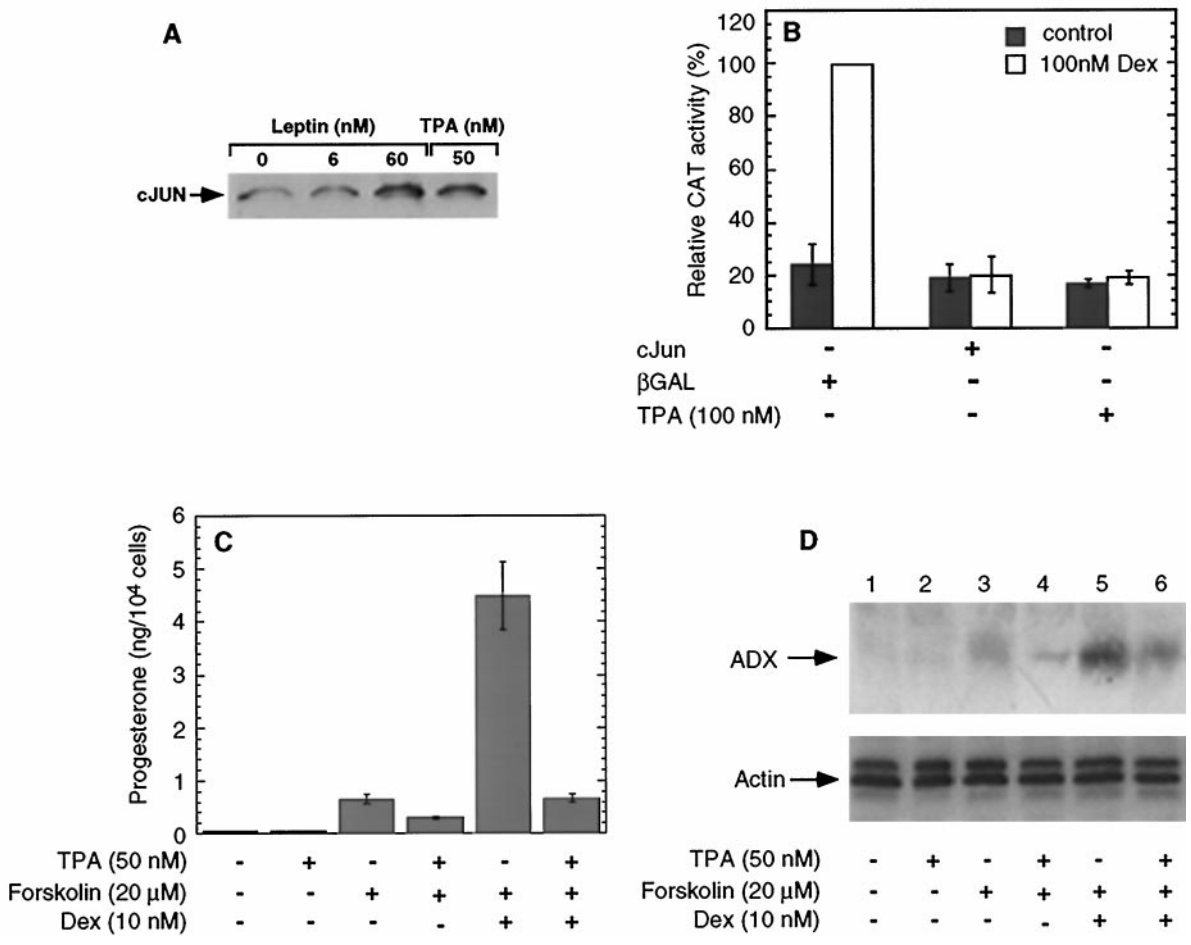

FIG. 7. Leptin up-regulate the expression of c-Jun in FSHR-17 cells, thereby modulating the transcriptional activity of the glucocorticoid receptor and steroidogenesis. A, Nuclear extracts (30 μg protein/lane) of FSHR-17 cells induced for 2 h with medium alone (lane 1), murine leptin (6 and 60 nM, lanes 2 and 3, respectively) or TPA (50 nM, lane 4) were subjected to SDS-PAGE and immunoblotting with polyclonal antibody to c-Jun/AP1. This blot is a representative result of three independent experiments. B, FSHR-17 cells were transiently transfected with the glucocorticoid-inducible CAT vector pΔG46TCO (2 μg/10^5 cells) together with either a c-Jun expression vector (RSVc-Jun) or with a control vector RSV-βGal (0.6 μg/10^5 cells each). Transfected cells were then kept unstimulated or induced with DEX (100 nM, 24 h). Similarly, cells transfected with the glucocorticoid-inducible CAT vector pΔG46TCO were induced with DEX or DEX and TPA (100 nM, 24 h). Both overexpression of c-Jun or c-Jun induction by TPA abolished the transcriptional activity of the glucocorticoid receptor. Data are mean ± SE (n = 5). C, FSHR-17 cells were stimulated for 24 h with forskolin (20 μM), DEX (10 nM), TPA (50 nM), either alone or in various combinations. Progesterone levels in the culture media were then determined. Data are mean ± SE (n = 4). D, Rat FSHR-17 cells were incubated with forskolin (20 μM, 24 h) in the presence or absence of TPA (50 nM). Cells were also incubated with forskolin (20 μM, 24 h) and DEX (10 nM) in the presence or absence of TPA (50 nM). Cell extracts were immunoblotted with anti-ADX. The lanes were normalized by reprobing the membrane with an antibody to α-actin (*lower panel*).

its central effects. Thus, leptin was previously reported to inhibit steroid synthesis in granulosa cells when induced by FSH in combination with growth factors such as IGF-I and insulin (38, 39). It was also found recently that leptin inhibits the LH-induced estradiol production in rat granulosa cells (40). We show here for the first time that DEX-stimulated production of progesterone in FSH-treated primary rat granulosa cells is inhibited in a dose-dependent manner by leptin at physiological concentrations (0–20 nM). At high, nonphysiological levels, the inhibition was attenuated, indicating that leptin may act by multiple mechanisms. Thus, at physiological concentrations, leptin seems to attenuate locally the steroidogenic activity of various signals that enhance FSH activity. Hence, the homeostasis of steroid production in granulosa cells is maintained by the opposite local and central effects of leptin. These regulatory mechanisms are part of a complex network, which also includes the induction of leptin expression in adipocytes by glucocorticoids (22) and the inhibition of glucocorticoid synthesis in the adrenal cortex by leptin (23).

The molecular events leading to attenuation of steroidogenesis by leptin were studied in FSHR-17 cells. Our findings that leptin inhibits the production of pregnenolone, the precursor of progesterone, as well as the progesterone metabolite 20α-hydroxy-4-pregnen-3-one, indicates that leptin may act as an inhibitor of ovarian steroidogenesis. Becuase leptin had no effect on the basal and the forskolin-induced progesterone and cAMP production, we tested if leptin affects the cellular response to glucocorticoids. Glucocorticoids were reported to augment the gonadotropin-induced transcription of P450scc in a Leydig tumor cell line (41). Therefore, we studied the effect of leptin on the transcriptional

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activity of GR. We found that glucocorticoids augment the cAMP induced transcription of ADX in FSHR-17 cells and leptin inhibits this activity. Thus, our finding that leptin attenuates the transcriptional activity of GR is a possible mechanism by which leptin modulates the expression of ADX, thereby lowering ovarian steroid synthesis. Perhaps leptin can also modulate the expression of other steroidogenic enzymes such as P450scc, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 Isomerase or 20 α-hydroxysteroid dehydrogenase. Alternatively, leptin may affect the expression of the steroidogenic acute regulatory protein (StAR) (42), steroidogenic transcription factor SF-1/Ad4BP (43) or Sterol Carrier Protein 2 (SCP_2) (44).

The mechanism by which activated GR elevates ADX remains to be elucidated. DEX effectively induces ADX in FSHR-17 cells but the promoter region of the rat ADX gene was not cloned. The activated GR may induce the transcription of ADX gene by directly binding to a classical or variable GRE (45, 46) in the ADX promoter. Alternatively, DEX may induce the transcription of ADX indirectly, as was reported for P450scc induction in a Leydig tumor cell line (41).

The AP1 family of transcription factors consists of homodimers and heterodimers of Jun (v-Jun, c-Jun, Jun B, Jun D), Fos (v-Fos, c-Fos, Fos B, Fra1, Fra2) or activating transcription factors (ATF2, ATF3/LRF1, B-ATF) bZIP (basic region leucine zipper) proteins (47). The ability of c-Jun to interact with GR and alter the program of gene expression was shown previously (35, 36). Overexpression of c-Jun has already been shown to repress GR activity in other systems (27). Therefore, our finding that leptin induces the expression of c-Jun in FSHR-17 cells and that the overexpression of c-Jun in these cells abolishes the transcriptional activity of the GR provide one possible explanation how leptin attenuates GR activity, thus reducing the steroid production. Both TPA and leptin induce the expression of c-Jun and attenuate the induction of ADX expression. However, TPA is more potent than leptin as an inhibitor of GR transcriptional activity, as well as an inhibitor of DEX-augmented progesterone production. The lower inhibitory action of leptin suggests that it may transduce in parallel other signaling cascades that could attenuate c-Jun activity. For example, leptin may induce other members of the AP1 family such as Jun D, a potential inhibitor of c-Jun (27).

The GRE-CAT reporter vector used here for determination of the transcriptional activity of GR (pΔG46TCO) does not contain an AP1 binding site. Hence, the overexpressed c-Jun must have abolished the transcriptional activity of the GR by interacting directly with it. This mode of interaction was previously shown to occur between the DNA-binding domain of GR and the leucine zipper region of c-Jun (35, 36). The resulting complex could not bind to any one of the cognate DNA elements *in vitro*. Alternatively, it is possible that a GR/c-Jun complex can still bind to a DNA element, but such binding represses transcription by blocking transactivation (48). We have excluded another possible mode of GR inhibition, namely, down-regulation of its expression by leptin (data not shown). Therefore, the induction of c-Jun in granulosa cells by leptin inhibits the transcriptional activity of GR, which may lead to a reduced steroid hormone synthesis.

The signaling mechanism by which leptin up-regulates the steady-state level of c-Jun in granulosa cells remains to be elucidated. c-Jun is frequently subject to transcription-regulation (47). However, new findings have suggested that c-Jun activity can also be regulated by modulating its rate of degradation. Thus, phosphorylation of serine and threonine residues at its amino-terminal activation domain by mitogen-activated protein kinase (MAPK) reduces the ubiquitinylation of this protein and hence its rate of degradation (49). Recent reports have suggested that leptin activates MAPK (50). Thus, induction of MAPK by leptin may account for at least part of its signaling cascade, leading to the activation of c-Jun.

In conclusion, the present data demonstrates that leptin exerts opposite central and local effects on ovarian steroid synthesis. It would be of great interest to find how general is this effect of leptin on the GR, as it may impinge upon the transcription of many other genes, thus giving new insights to the endocrine role of leptin.

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