BIOLOGY OF REPRODUCTION **68,** 789–796 (2003)
Published online before print 23 October 2002.
DOI 10.1095/biolreprod.102.010702

Biphasic Effects of Leptin in Porcine Granulosa Cells[1]

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ABSTRACT

The direct effects of recombinant porcine leptin on porcine granulosa cells were studied to test the hypothesis that leptin, acting through the nuclear transcription factor signal transducer and activator of transcription 3 (STAT-3), modulates sterol regulatory element-binding protein 1 (SREBP1) thereby increasing steroidogenesis. In porcine granulosa cells in culture over 48 h, leptin at 10 ng/ml increased progesterone accumulation 3-fold while it was reduced by leptin at 1000 ng/ml. Leptin had no effect on progression of granulosa cells through the cell cycle nor on the frequency of cell death. Leptin treatment at 24 or 48 h of culture resulted in dose-dependent 2- to 4-fold increases in tyrosine phosphorylation of STAT-3. Leptin had a biphasic effect on the abundance of membrane-bound and transcriptionally active forms of SREBP1. In transient transfection of primary porcine granulosa cells, the plasmid expressing the transcriptionally active form of SREPB-1 induced transcription of the key regulator of steroidogenesis, the steroidogenic acute regulatory protein (StAR). StAR transcription was also increased by the low dose of leptin and was further upregulated in the presence of the SREBP plasmid. Leptin at 1000 ng/ml inhibited SREBP1-induced StAR expression. Thus, leptin, acting through STAT-3, modulates steroidogenesis in a biphasic and dose-dependent manner, and SREBP1 induction of StAR expression may be in the cascade of regulatory events.

corpus luteum, leptin, leptin receptor, progesterone, steroid hormones

INTRODUCTION

The peptide leptin is produced primarily by adipocytes and achieves hormonal status by virtue of its secretion into the bloodstream [1]. Its role in reproduction includes important actions on the hypothalamus to bring about release of LH-releasing hormone, thereby triggering gonadotropin release and leading to development of the reproductive tract and induction of puberty [1]. Administration of leptin to obese leptin-deficient mutant mice caused decreased food intake, body weight loss, increased ovarian weight, increase in ovarian follicles, and restoration of fertility [2–4]. A direct involvement of leptin in ovarian function has been postulated. The expression of leptin receptors has been demonstrated in human, mouse, rat, and pig ovaries [4–7]. Given its positive effects on gonadotropin secretion and fertility, leptin is expected to have either a positive local effect or no effect at all. The majority of researchers have suggested that the direct effects of leptin on ovarian cells are inhibitory and can be attributed to attenuation of gonadotropin, insulin, insulin-like growth factor 1 (IGF-I), and/or glucocorticoid-mediated steroidogenesis [6, 8–14]. Contrasting studies have revealed direct stimulatory effects of leptin in rat and human ovaries in the form of induction of cytochrome P450aromatase and consequent estrogen synthesis [15].

Alternative splicing of the leptin receptor results in the production of multiple isoforms that contain a common extracellular domain [16, 17] and cytoplasmic domains of differing lengths. The longest form of the leptin receptor, form b, is a member of the interleukin (IL) 6 receptor family of class I cytokine receptors [18, 19] and is the only isoform that contains intracellular tyrosine residues that can serve as a target for phosphorylation [18]. This receptor isoform is highly expressed in the porcine ovary, and its cytoplasmic domain contains the potential Janus kinase (JAK) binding domains and the potential consensus sequence for binding of signal transducers and activators of transcription (STAT) [7, 19].

Steroidogenesis depends on the supply of its precursor, cholesterol, derived from intracellular and extracellular sources. Intracellular levels are tightly controlled by regulation of the uptake, storage, and synthesis by a unique family of transcription factors known as the sterol regulatory element-binding proteins (SREBP) [20]. These transcription factors are localized to the endoplasmic reticulum in an approximately 125-kDa precursor form under conditions of replete intracellular sterol/cholesterol stores. Upon depletion of cholesterol, the membrane-bound proteins are cleaved by proteases, releasing a 68-kDa transcription regulator. The mature SREBPs enter the nucleus, where they bind sterol regulatory sites located in the promoter regions of genes involved in cholesterol homeostasis and transport [21]. Circulating leptin has an effect on the gene expression profile and phenotype of white adipose tissue. In this context, leptin modulates SREBP1 activity by decreasing the amount of mRNA and of cleaved (transcriptionally active) SREBP1 protein [22].

Little is known about the genes targeted by leptin in the ovary, other than the apparent activation of c-jun and the inhibition of serum glucocorticoid kinase [23]. Given the known influences of leptin on steroid synthesis, the steroidogenic acute regulatory protein (StAR) is an interesting candidate for leptin regulation, especially since SREBP1

[1]This work was supported by grant STR0202133 from the Natural Sciences and Engineering Research Council of Canada. Z.T.R.-C. is on leave from Universidad de Antioquia, Medellín, Colombia.
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Received: 27 August 2002.
First decision: 3 September 2002.
Accepted: 10 September 2002.

ISSN: 0006-3363. http://www.biolreprod.org

regulates StAR [24]. To gain further insight into the mechanisms of action of leptin in the ovary, we investigated the effects of leptin on porcine granulosa cells in vitro. The data indicate that leptin, acting through STAT-3, modulates steroidogenesis in a biphasic, dose-dependent manner and that SREBP1 induction of StAR expression may be in the cascade of regulatory events.

MATERIALS AND METHODS

Cell Culture

Granulosa cells were aspirated from medium-sized (3–5 mm) follicles of ovaries from prepubertal gilts and cultured as previously described [25]. Viable cells (7–9 $\times$ 10^6 cells/ml) were pooled in minimum essential medium (MEM; Gibco BRL, Burlington, ON, Canada) containing 1 mg/L insulin (Sigma, Oakville, ON, Canada), 0.1 mM nonessential amino acids (Gibco BRL), 5 $\times$ 10^4 IU/L penicillin (Gibco BRL), 50 μg/L streptomycin (Gibco BRL), 0.5 mg/L fungizone (Gibco BRL), and 10% fetal calf serum (Gibco BRL). Cells were incubated at 37°C. To evaluate the effects of leptin on progesterone production and abundance of the signaling molecule SREBP1, cells were incubated with 0, 10, 100, or 1000 ng/ml recombinant porcine leptin for 12, 24, and 48 h. Phosphorylated STAT-3 abundance was evaluated after treatment of porcine granulosa cells with the same leptin doses for 5, 15, or 30 min at various times (12, 24, and 48 h) after initiation of cultures. To characterize the interactions among leptin, FSH, and IGF-I, and progesterone, cells were incubated with porcine pituitary FSH (100 ng/ml; Sigma) alone or in combination with human recombinant IGF-I (100 ng/ml; Sigma) and/or leptin (10 or 1000 ng/ml). To provide sufficient protein for Western blot analysis, cells from three wells/treatment at 12, 24, and 48 h were pooled. Similarly, pooled samples of medium were collected for RIA of progesterone. There were three independent replicates of each experiment in which the cell populations were derived from ovaries collected at the abattoir on different days.

Determination of Granulosa Cell Viability

To evaluate the effect of leptin on granulosa cell proliferation and/or viability, cells were cultured as described above for 12, 24, and 48 h with various doses of leptin. Cells from three independent granulosa cell cultures (i.e., derived from ovaries collected on three separate occasions) were subjected to flow cytometric analysis. Cultures were dispersed by treatment with 1% trypsin (Sigma) and collected by centrifugation at 1000 rpm for 10 min. The cell pellets were resuspended in 200 μl PBS, and 5 ml of ice-cold 70% ethanol was added, dropwise under agitation, to the cell suspension, which was maintained at 4°C. The cells were collected by centrifugation (1000 rpm for 5 min), and the cell pellets were washed twice with PBS and resuspended in 500 μl PBS. Propidium iodide (100 μl) (1 mg/ml; Sigma) was added to label nuclei, followed by 10–20 μl of deoxyribonuclease-free RNase A (10 mg/ml; Sigma). The cell suspension was protected against light, incubated for 1 h on ice, and then analyzed with a FACScan flow cytometer (Beckman Coulter, Mississauga, ON, Canada). The relative percentage of cells in each stage of the cell cycle was then analyzed using the ModFitLT version 2.0 DNA analysis program (Becton Dickinson, San Jose, CA), and mean percentage values for each stage were calculated. Total proteins were extracted from porcine granulosa cells by addition of 400 μl of 1 M NaOH per well (7.5-mm plates) for 2 h at room temperature and then 400 μl of 1 M HCl for 1 h at 37°C. Total protein extracted from three independent granulosa cell experiments was assessed by the Bradford (Bio-Rad, Mississauga, ON, Canada) protein assay.

Recombinant Porcine Leptin Production and Purification

In preliminary experiments, recombinant human leptin (BIOMOL, Plymouth Meeting, PA) altered steroidogenesis in porcine granulosa cells. On the basis of these positive results, we undertook the production of recombinant porcine leptin. The cDNA encoding the mature porcine leptin protein (amino acids 22–167) was amplified by polymerase chain reaction (PCR) from the complete leptin coding sequence that was previously subcloned in plasmid pBluescript KSII+ [26] (GenBank accession AF026976). The forward primer, 5′-GGTGGCATATGGTGCCCA-TCTGGAGAGTCC-3′, contains an *Nde*I site (underlined), and the reverse primer, 5′-CCGGAATTCAGCAGCCAGGGCTGAGG-3′, contains an *Eco*RI site (underlined). The PCR mixture (50 μl) contained 1× Expand HF buffer (Roche Diagnostics, Indianapolis, IN), 1 ng plasmid DNA, 0.2 mM of each dNTP, 2.6 units of Expand HF PCR System enzyme mix (Roche), and 30 pmol of each primer. The PCR amplification profile comprised 2 min at 94°C followed by 10 cycles of 15 sec at 94°C, 30 sec at 62°C, and 2 min at 68°C, followed by 28 cycles of 15 sec at 94°C, 30 sec at 62°C, and 2 min at 68°C with a 5-sec time increment/cycle, and a final 5-min extension at 72°C. The amplified PCR products were then double digested with *Nde*I and *Eco*RI and were directly ligated to *Nde*I-*Eco*RI double-digested pTYB12 plasmid (IMPACT-CN system; New England Biolab, Beverly, MA) to yield the pTYB12lep plasmid. Restriction digestion and DNA sequencing verified insertion of leptin cDNA.

The pTYB12lep plasmid was used to transform *Escherichia coli* strain ER2566 and was incubated at 37°C in Luria broth supplemented with 100 μg/ml ampicillin. Cultures were grown to mid log (optical density $[27]_{600}$ of 0.6), induced with 0.5 mM isopropyl-D-thiogalactoside, and then incubated at 15°C for 16 h. Cells were harvested by centrifugation at 5000 $\times$ *g* for 10 min, and the pellet was resuspended in lysis buffer (20 mM Hepes, pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100). Cells were then disrupted by two 2-min sequences of pulse sonication. The supernatant was separated from cell debris by centrifugation at 12 000 $\times$ *g* for 30 min and passed through a chitin bead column (5 $\times$ 2 cm) previously equilibrated with buffer A (20 mM Hepes, pH 8.0, 1 M NaCl, 1 mM EDTA, 1% Triton X-100). The chitin column was washed with 6 $\times$ the bed volume of buffer A followed by a second wash with 6 $\times$ the bed volume of buffer A with NaCl concentration adjusted to 1.5 M. Two $\times$ the bed volume of cleavage buffer (20 mM H, pH 8.0, 1.5 M NaCl, 1 mM EDTA, 1% Triton X-100, 100 mM dithiothreitol [DTT]) was then passed through the column to distribute the DTT evenly throughout the resin, and the flow was arrested. The column was incubated at 16°C for 48 h. Fractions (3 ml) containing the recombinant leptin were obtained by eluting the column with 3 $\times$ the bed volume of elution buffer (20 mM Hepes, pH 8.0, 2 M NaCl, 1 mM EDTA, 1% Triton X-100). All fractions were analyzed on a 12% SDS-polyacrylamide gel, and a protein with mobility of authentic leptin was eluted in the first 10 fractions. Visualization of the proteins was made possible with Coomassie blue staining. Protein concentrations were estimated using the D_c Protein Assay (Bio-Rad), and fractions containing leptin were pooled and glycerol was added to a final concentration of 20% (v/v). The purified recombinant protein was used to generate polyclonal antibodies in rabbits against the porcine leptin.

Progesterone RIA

Medium collected at termination of cultures was assayed for progesterone according to procedures previously described [28] employing antibody provided by Dr. G.D. Niswender (Colorado State University, Ft. Collins, CO). The intraassay coefficient of variation, calculated between duplicates, ranged from 3% to 9%. The interface value, based on a sample of 0.1 ng that displaced approximately 50% of the tracer, was 9.8%. The interassay value, calculated from four samples present in all assays, ranged from 5% to 11%. In early experiments, individual culture wells were assayed for progesterone to estimate variation within individual experiments. Interwell coefficients of variation ranged from 0.1% to 16.9%, with an overall average of 6.8%.

Immunoblotting

Three wells per treatment were pooled to provide sufficient protein for detection by Western blot. Solubilized granulosa cell were homogenized on ice in TED buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1 mM diethyldithiocarbamic acid [DEDTC]) containing 2 mM octyl glucoside (Sigma) and centrifuged at 30 000 $\times$ *g* for 1 h at 4°C. The crude pellets (membranes, nuclei, and mitochondria) were sonicated (8 sec/cycle, three cycles) in TED sonication buffer (20 mM Tris, pH 8.0, 50 mM EDTA, 0.1 mM DEDTC) containing 32 mM octyl glucoside. The sonicates were centrifuged at 16 000 $\times$ *g* for 15 min at 4°C. The supernatants (solubilized cell extract) were stored at −70°C until electrophoresis was performed. The protein concentration was determined by the Bradford (Bio-Rad) protein assay. Nuclear protein extracted from immortalized human ovarian granulosa cells (provided by Dr. N. Auersperg, University of British Columbia, Vancouver, BC, Canada) that had been transiently transfected with a plasmid constitutively expressing the transcriptionally active form of SREBP1 (pSVSPORT1-ADD1-403; provided by K. Schoonjens, University Louis Pasteur) was used as a positive control for SREBP1. For STAT-3, the positive control was a nuclear extract from epithelial growth factor-stimulated A431 human carcinoma cells (Upstate Biotechnology, Lake Placid, NY). Proteins (50 μg) in cell extracts were resolved by one-dimensional 5% SDS-PAGE minigel for 45 min at 200 V and then electro-

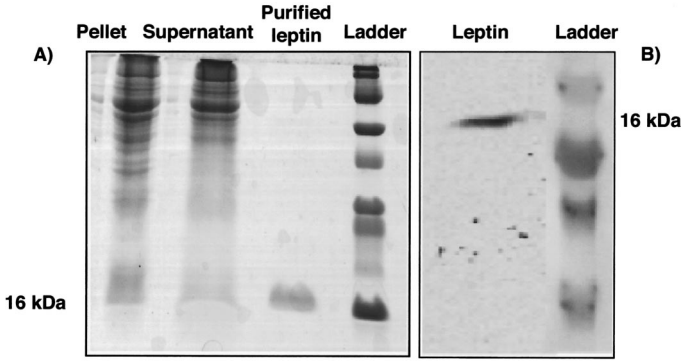

FIG. 1. Analysis of the expression and purification of recombinant porcine leptin. **A**) SDS-PAGE results showing purified leptin protein. Coomassie blue. Lane 1: pellet fraction after sonication and centrifugation; lane 2: soluble fraction containing the leptin-intein fusion protein before passage through the chitin column; lane 3: purified recombinant leptin after DTT cleavage and elution from chitin column; lane 4: molecular mass markers. **B**) Western blot analysis of purified recombinant leptin. Lane 1: purified leptin; lane 2: molecular mass marker.

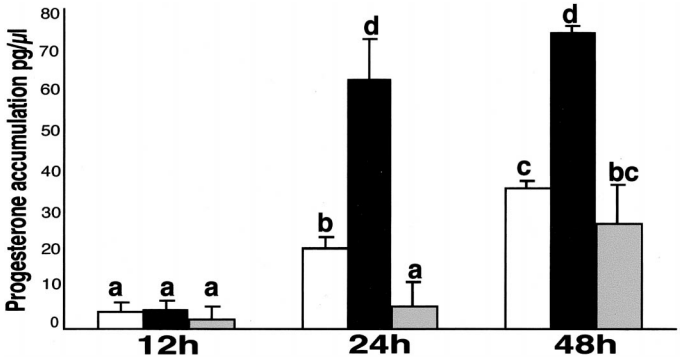

FIG. 2. Progesterone accumulation in porcine granulosa cells in culture over 12, 24, and 48 h after incubation with medium alone. Treatments were control (open bars), 10 ng/ml (solid bars), or 1000 ng/ml (shaded bars) leptin. Means are derived from three independent experiments, and means with different letters differ significantly ($P < 0.05$).

phoretically transblotted to polyvinylidene fluoride membranes (Amersham Pharmacia Biotech, Arlington Heights, IL). The membranes were washed in 0.1% (v/v) Tween 20 in Tris-buffered saline (TTBS: 100 mM Tris, 0.9% sodium chloride, pH 7.5) and incubated with primary antibodies raised in mouse against the phosphorylated tyrosine-704 of the 92-kDa STAT-3 protein (Upstate Biotechnology), SREBP1 rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 41–200 mapping near the amino terminus (Santa Cruz Biotechnology, Santa Cruz, CA), and porcine leptin polyclonal antibody raised against the recombinant leptin protein purified as described previously. The second antibody, horseradish peroxidase anti-rabbit/mouse IgG, was added in the same buffer. The signal was detected by adding the peroxidase substrate (ECL⊕ kit; Amersham Pharmacia Biotechnolgy, Piscataway, NJ), which produces luminescence at the site of second antibody binding. Blots were then exposed to Kodak (Rochester, NY) photographic films. The optical density of protein bands was quantified by scanning with a densitometer (Storm; Amersham), and the data were analyzed with the NIH imaging program.

Transient Transfection Assays

The porcine StAR promoter-luciferase construct (pStAR1423Luc; 1553 base pairs) provided by Dr. H. Lavoie (University of South Carolina, Columbia, SC) and the plasmid expressing the active form of SREBP1 were employed in transfection experiments. Porcine granulosa cells (8×10^6 cells/well) were plated as described above. After attachment, cells were rinsed once with MEM with fetal bovine serum and antibiotics for 30 min. Transfection medium (710 μl/well) was prepared in MEM with 400 ng of plasmid DNA and 4 μl of Effectene (Qiagen, Mississauga, ON, Canada). Cells were incubated with transfection medium for 24 h, after which the medium was replaced with new MEM containing treatments or vehicle for 12–24 h. At the end of the treatment period, cells were rinsed once at room temperature with PBS, lysed in 200 μl of 1× passive lysis buffer (Luciferase Assay System; Promega, Madison, WI), and stored at −70°C until assayed. Cotransfection of the plasmid containing the *Renilla* luciferase gene under control of the SV40 early enhancer/promoter region was used to correct for differences in transfection efficiency. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase substrate (70 μl) was added to 10 μl lysate, and luciferase activity was measured using a 20/20 luminometer (Lumat, LB-9507, version 5.03; Berthold Technologies, Bad Wildbad, Germany). Luciferase data were expressed as the mean ± SEM. Each luciferase assay experiment was performed in duplicate within an experiment, and experiments were repeated in three cell cultures from slaughterhouse ovaries collected on different days.

Statistical Analyses

Hormone assay, optical density, and promoter activity data were subjected to Shapiro test to determine normality of distribution and to Bartlett test for homogeneity of variances. In the absence of homogenous variance, the analysis was performed on square root-transformed data. Each experiment was repeated three times. When pooling of samples within an experiment was necessary to provide sufficient material for analysis, a randomized complete block ANOVA was employed to determine effects of treatment and time. Transfection experiments were subjected to a nested ANOVA. In the presence of a significant F value in the ANOVA, individual means were compared using the Student-Newman-Keuls procedure. The level of probability selected to determine significance was $P < 0.05$.

RESULTS

Expression and Purification of Recombinant Porcine Leptin

Recombinant human leptin was first shown to have direct effects on porcine granulosa cells. To ensure that these effects were pertinent to the pig, recombinant pig leptin was produced and purified (Fig. 1). The apparent molecular mass of 16 kDa of the eluted leptin was consistent with the predicted molecular mass of leptin (Fig. 1A), and Western analysis with anti-porcine leptin antibodies confirmed the authenticity of the recombinant porcine leptin (Fig. 1B). The porcine antibody also recognized recombinant human leptin, which migrated identically in Western blot (data not shown). The biological activity of recombinant porcine leptin was confirmed by comparing its effects to those of the human recombinant leptin on granulosa cell steroidogenesis in vitro. Because the levels of progesterone were similar after treatments with both human and porcine recombinant leptin, all further experiments were performed with recombinant porcine hormone.

Leptin Effects on Progesterone Accumulation in Porcine Granulosa Cells

Recombinant porcine leptin, at doses of 10, 100, and 1000 ng/ml, was added to porcine granulosa cells that were then cultured over 12, 24, and 48 h, and the progesterone accumulation in the medium was measured by RIA. An ANOVA revealed highly significant effects of treatment ($P < 0.002$) and time ($P < 0.001$), with no significant interaction. Individual comparisons demonstrated the presence of progressive increases of progesterone in untreated control cultures from 12 through 48 h (Fig. 2, $P < 0.01$). The 100-ng/ml dose of recombinant leptin produced neither stimulation nor inhibition compared with control cultures (data not shown), and the high and low doses were therefore selected for further study. There was a biphasic variation in progesterone accumulation in the culture medium

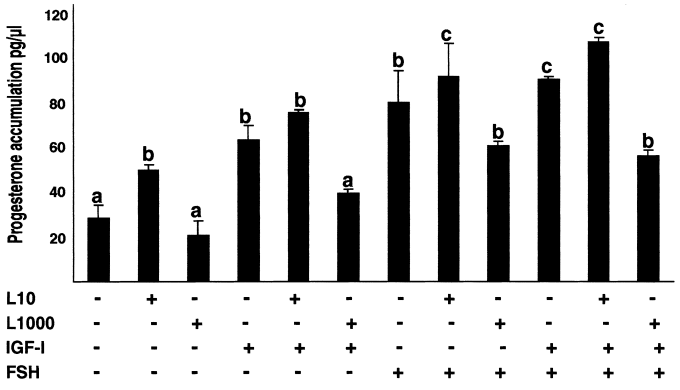

FIG. 3. Progesterone accumulation in porcine granulosa cell cultures terminated at 48 h after incubation with medium alone (control), leptin (10 and 1000 ng/ml), IGF-I (100 ng/ml), and FSH (100 ng/ml) or with combinations of high and low leptin doses with FSH, IGF-I, and FSH + IGF-I. Results are presented as mean ± SEM resulting from three independent experiments. Means with different letters differ significantly ($P < 0.05$).

at 24 and 48 h in cultures treated with 10 or 1000 ng/ml recombinant leptin. The 10-ng/ml dose consistently increased progesterone accumulation, whereas 1000 ng/ml was inhibitory at 24 h ($P < 0.03$) and tended toward being inhibitory at 48 h ($P < 0.08$).

An ANOVA revealed a significant treatment effect ($P < 0.001$) in granulosa cells cultured with leptin, FSH, IGF-I, or their combinations. Individual comparison of means (Fig. 3) demonstrated that leptin 10 ng/ml increased ($P < 0.05$) the accumulation of progesterone. IGF-I and FSH, alone or in combination, increased the progesterone concentrations in culture medium relative to controls ($P < 0.01$). Leptin at 10 ng/ml in the presence of FSH resulted in stimulation in excess of that produced by either reagent alone ($P < 0.01$), with a mean similar to that for the combination of FSH and IGF-I. Leptin at 10 ng/ml did not further stimulate progesterone in the presence of FSH and IGF-I relative to the combination of FSH + IGF-I alone. The higher dose of leptin (1000 ng/ml) prevented the IGF-I-induced stimulation of progesterone accumulation and interfered with FSH + IGF-I-induced increases in progesterone concentration in culture medium (Fig. 3, $P < 0.05$).

Leptin Effects on Granulosa Cell Cycle and Total Protein in Porcine Granulosa Cells

We also investigated whether the doses of leptin employed affected the numbers of granulosa cells in culture, either by induction of mitosis or by increasing the frequency of cell death by apoptosis. Figure 4 is a representative flow cytometric evaluation of populations of granulosa cells treated with 10 and 1000 ng/ml recombinant leptin over 48 h in culture. There was no significant variation in the pattern of distribution of the cell population across the cell cycle. The majority of granulosa cells were in G0–G1 phase in both control (mean ± SEM = 93.5% ± 2.7%) and treated (91.2% ± 1.3% and 95.2% ± 1.6% for leptin at 10 and 1000 ng/ml, respectively) cultures. The proportion of the cell populations that had reduced total DNA, indicative of apoptosis, did not differ among controls (1.1% ± 0.2%) and treatments. (1.8% ± 0.2% and 1.7% ± 0.9% for leptin at 10 and 1000 ng/ml, respectively). In addition, total protein analysis indicated that after 12, 24, and 48 h in culture granulosa cells had virtually identical levels of total protein, and there were no significant differences among control cultures and those treated with leptin at 10 or 1000 ng/ml (data not shown). These results demonstrate that there was no effect of leptin on cell populations.

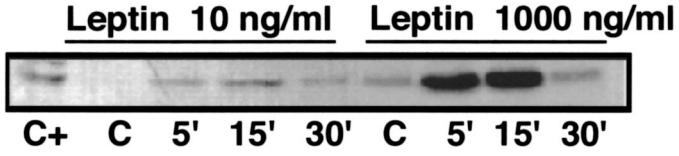

FIG. 5. Immunoblot showing the time course of expression of phosphorylated STAT-3 protein in primary cultures of porcine granulosa cells treated with 10 or 1000 ng/ml recombinant porcine leptin for 5–30 min beginning at 48 h after initiation of culture. Antibodies raised in mice against the phosphorylated tyrosine-704 of the STAT-3 protein were employed, and bands migrating at approximately 92 kDa are shown. Untreated cells (C) are followed by cells derived from cultures terminated at 5, 15, and 30 min after treatment with leptin. The positive control (C+) represents phosphorylated STAT-3 from cell lysate of the epidermal growth factor-stimulated A432 human carcinoma cell line.

Leptin Effects on STAT-3 Phosphorylation in Porcine Granulosa Cells

To examine the effect of recombinant porcine leptin on granulosa cell signaling pathways, we first evaluated the ability of leptin to induce STAT-3 phosphorylation. In preliminary trials, leptin treatment elevated phosphorylation of STAT-3, with maxima at 15 min and in a profile that was dose dependent (Fig. 5). We then undertook short-term treatments (15 min) with the two doses of leptin in cells previously cultured for 12, 24, or 48 h. As before, the ANOVA revealed significant effects of treatment ($P < 0.03$) and time ($P < 0.008$), with a significant interaction ($P < 0.01$). Individual comparisons of means (Fig. 6) indicated that both doses of leptin increased STAT-3 phosphorylation

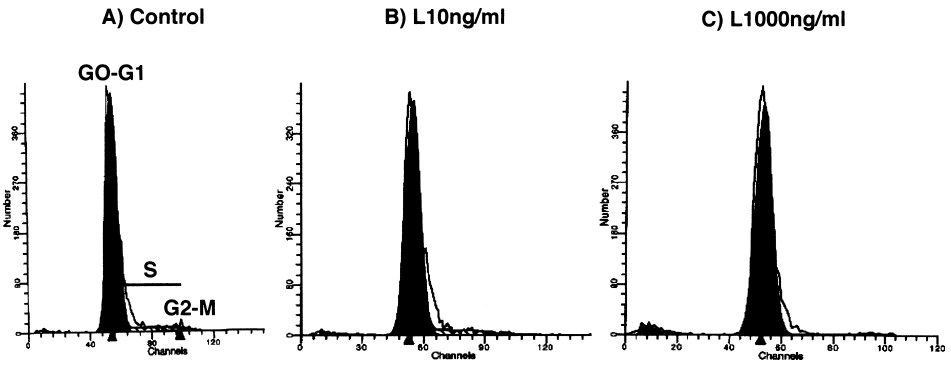

FIG. 4. Analysis of the porcine granulosa cell cycle using flow cytometry. Granulosa cell cultures were terminated at 48 h after incubation with medium alone (control, **A**), medium + 10 ng/ml leptin (**B**), or medium + 1000 ng/ml leptin (**C**). The cell cycle phases (G0–G1, S, and G2–M) are indicated in **A**. The G0–G1 phase is shaded and thus includes the majority of granulosa cells. The figure is representative of three independent experiments.

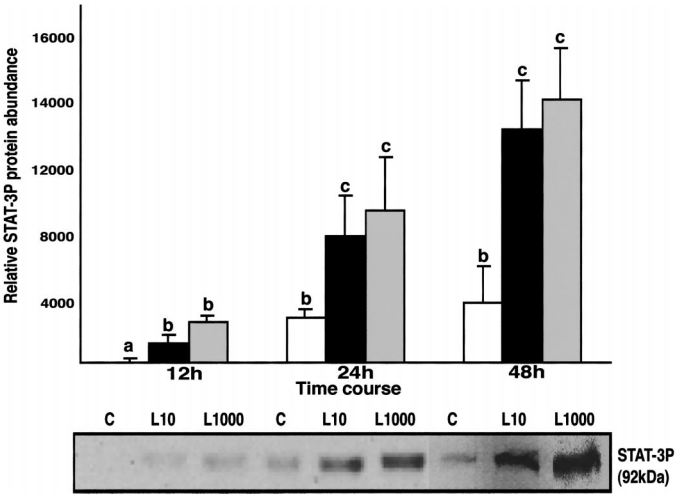

FIG. 6. Western analysis demonstrating the relative protein abundance of phosphorylated STAT-3 in porcine granulosa cells cultured for 12, 24, or 48 h and then treated for 15 min with 0 (open bars), 10 (solid bars), or 1000 (shaded bars) ng/ml leptin. The upper panel depicts mean (±SEM) of densitometric scans of the band migrating at approximately 92 kDa from three independent granulosa cell cultures. Means with different letters are significantly different ($P < 0.05$).

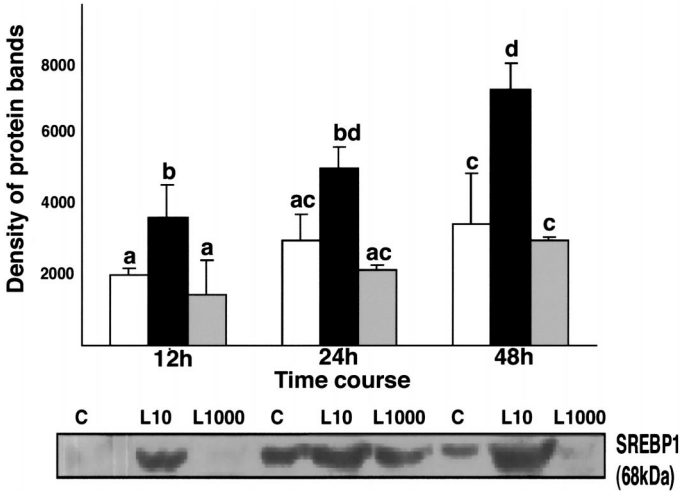

FIG. 7. Immunoblot of proteins from porcine granulosa cells over 12, 24, and 48 h in culture treated with 10 or 1000 ng/ml of porcine recombinant leptin. A rabbit polyclonal antibody raised against amino acids 41–200 mapping near the amino terminus of SREBP1 was used. The upper panel is the mean (±SEM) of three replicate experiments and comprises the nondimensional densitometric scans of the band migrating at approximately 68 kDa (mature or transcriptionally active form of SREBP1). Controls are designated by open bars, 10 ng/ml leptin by solid bars, and 1000 ng/ml leptin by shaded bars. Means with different letters are significantly different ($P < 0.05$). This figure is representative of three independent analyses.

at all times tested and that the abundance of phosphorylated STAT-3 over the 15-min window was greater at 24 and 48 h than at 12 h ($P < 0.05$).

SREBP1 Abundance after Leptin Treatment of Porcine Granulosa Cells

To confirm the proteolytic activation of SREBP1 after leptin treatment, we measured the concentration of the 68-kDa transcriptionally active form of SREBP in granulosa cells by immunoblot. An ANOVA revealed significant effects of treatment ($P < 0.05$) but not time. Individual comparisons (Fig. 7) indicated that mature SREBP1 increased with time in control cultures such that the 48-h control value was greater than the corresponding 12-h control ($P < 0.05$). At all time points studied, there was an increase in 68-kDa SREBP in cultures treated with 10 ng/ml leptin ($P < 0.001$). In contrast, in cultures treated with a 1000 ng/ml dose of leptin, the concentration of the transcriptionally active form of SREBP1 did not differ from that of controls but was consistently lower than that in cultures treated with 10 ng/ml leptin ($P < 0.03$).

The immunoblots were performed with the SREBP1 rabbit polyclonal antibody raised against amino acids 41–200, mapping near the amino terminus. This antibody also interacts with the band corresponding to the precursor form of the SREBP1, which migrates at approximately 125 kDa. The pattern of expression of the precursor was directly proportional to that of the active form, i.e., the low dose of leptin increased the 125-kDa form, whereas the high dose reduced it (data not shown). This finding suggests that the effect of leptin was on both de novo synthesis of SREBP1 and its cleavage to the mature form.

Leptin and SREBP1 Regulate the StAR Promoter

Given the effects of leptin on SREPB1 and steroidogenesis, we sought to identify target genes that might be involved, with the focus on StAR, the rate-limiting protein in steroid synthesis. Transfection of the StAR promoter-reporter plasmid resulted in constitutive promoter activity in excess of that resulting from the empty plasmid control ($P < 0.05$) (Fig. 8). Leptin alone at 10 ng/ml increased StAR promoter luciferase activity by 1.5- to 2-fold over constitutive luciferase expression ($P < 0.05$), but the 1000-ng/ml dose had no effect. Cotransfection of the plasmid constitutively expressing the transcriptionally active form of SREBP1 elevated promoter activity 2- to 3-fold over basal levels ($P < 0.05$), and addition of leptin at 10 ng/ml significantly increased the signal. When higher doses of recombinant leptin (1000 ng/ml) were used, the luciferase signal was reduced to constitutive levels (Fig. 8, $P < 0.05$).

DISCUSSION

We generated and purified recombinant porcine leptin and confirmed its authenticity by Western blot using antibodies that interact with human leptin. We previously dem-

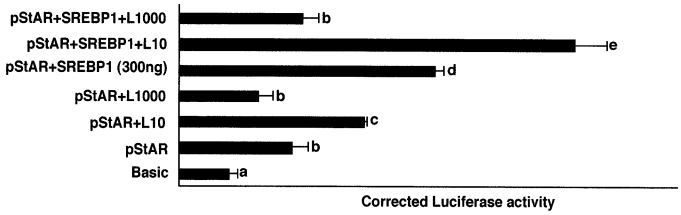

FIG. 8. Mean (±SEM) corrected luciferase activity in primary porcine granulosa cells transiently transfected with the porcine StAR promoter-luciferase construct (pStAR1423Luc, 1553 base pairs), the active form of SREBP1 (pSVSPORT1-ADD1-403), or the promoterless luciferase plasmid, pGL3-basic. Cells were cotransfected with simian virus 40 *Renilla*-luciferase control vector to correct for transfection efficiency. Cells were treated for 24 h after transfection with 10 and 1000 ng/ml porcine recombinant leptin. Means of three independent experiments are depicted, and those with different letters are significantly different ($P < 0.05$).

onstrated the presence of the long form of the leptin receptor in the same cell model that we employed in the present investigation, i.e., primary cultures of pig granulosa cells [7]. Here, we provide new information to indicate that recombinant porcine leptin can stimulate the JAK-STAT pathway in this model, resulting in time-dependent phosphorylation of STAT-3.

The importance of leptin to successful reproduction is indicated by infertility in mice bearing mutations that prevent synthesis of biologically active leptin [6] or the leptin receptor [29]. Fertility can be restored in female mice lacking in leptin by treatment with leptin [3], and exogenous leptin accelerates the onset of puberty in immature rats [30]. Although the general view has been that the principal effects of leptin are on the neuroendocrine component of reproduction (see [31] for review), evidence has emerged to indicate the presence of direct effects on the ovary. Functional leptin receptors are expressed in the ovary of numerous species, including humans [5], mice [4], rats [6, 13], and pigs [7]. Further, in the pig ovary, transcripts and protein consistent with the full length leptin receptor increase with progesterone synthetic capacity, both in vitro and in vivo [7]. Leptin receptor abundance in the hypothalamus is believed to govern the biological actions of leptin [32]. It seems unlikely that leptin, a hormone that has positive effects on gonadotropin secretion and ovarian function in vivo, should have direct negative effects on the ovary.

Nevertheless, conflicting observations among species and among experimental paradigms have prevented the development of a consistent view of the effect of leptin on steroidogenesis. The occurrence of direct stimulatory effects on progesterone production, as demonstrated for 10-ng/ml leptin, are in contrast to the results of many of the studies of granulosa cells in vitro. Equally rare is evidence for the direct inhibitory effects on progesterone synthesis that we observed for the higher dose; most studies show leptin inhibits some combination of gonadotropin and growth factor stimulation of steroidogenesis and, more specifically, estrogen synthesis [33]. Spicer and associates [8, 9] first demonstrated that leptin impairs insulin, or insulin or IGF-I in combination with FSH stimulation of progesterone and estradiol accumulation in cultures of bovine granulosa cells in serum-free medium. This finding was confirmed in rat [11, 13] and human [14] granulosa cell cultures. However, other investigators [15] demonstrated that leptin at 1 ng/ml increased P450aromatase expression and estradiol-17β accumulation in luteinized human granulosa cells, with no effect on progesterone. In that study, there was positive interaction between leptin, IGF-I, and FSH in induction of P450aromatase expression. In light of the inconsistency among these results, we repeated these experiments using as a model porcine granulosa cells cultured with serum. As previously shown [34], FSH and IGF-I increased progesterone accumulation, as did the low dose of leptin, consistent with the results of other experiments. Further, our higher dose, in the 10^{-7} M range, clearly inhibited progesterone accumulation stimulated by FSH, IGF-I, or their combination. These results support the hypothesis that the inhibitory effects of leptin include interference with ligand-induced steroidogenesis. At the doses we employed (10^{-10}–10^{-7} M), the effects of leptin were biphasic with respect to stimulation and inhibition of progesterone synthesis.

Findings of stimulation by low doses (10^{-10}, 10^{-9} M) and inhibition by higher doses (10^{-8}, 10^{-7} M) of leptin in ovarian cells in vitro concur with observations of the effects of leptin on release of GnRH from median eminence-arcuate nucleus explants in the rat [35]. Leptin triggers release of LH and FSH from pituitary cultures in a pattern in which low doses are stimulatory and higher doses are ineffective [35, 36], again consistent with the idea that low doses of leptin have stimulatory effects.

Granulosa cells in their luteinized state in vivo, i.e., in highly vascularized porcine corpus luteum [37], are subject to circulating leptin concentrations ranging from 1.5 to 5 ng/ml ([38] and unpublished results). The 10-ng/ml dose employed in vitro in the present study is of the same order of magnitude as that found in circulation, suggesting that the results are of physiologic significance. In the process of luteinization in the pig, there is a reduction in P450aromatase expression by the granulosa cells [34]. Based on the results of other studies that have demonstrated that leptin inhibits P450aromatase expression and activity, and on our observation that physiological doses of leptin increase progesterone synthesis, we postulate that leptin plays a role in the process of luteinization. This hypothesis is supported by observations that leptin receptor abundance in porcine granulosa cells increases with luteinization in vitro and in vivo [7], and leptin binding increases with time in culture (and presumably luteinization) of bovine granulosa cells [9]. This hypothesis merits further investigation.

The present investigation provides new insight into the mechanism for the direct effects of leptin on the ovary through modulation of SREBP1 cleavage and consequent effects on the expression of StAR. Leptin directly affects the abundance of SREBP transcripts in adipose tissue [22] and hepatic and pancreatic tissues [39]. In these studies, administration of high levels of leptin in vivo [22] or overexpression of leptin in vitro [39] diminished SREBP1 mRNA. This finding is consistent with the our observation that the precursor form of SREBP1 (125 kDa) is less abundant in cells treated with 1000 ng/ml leptin. There is no precedent for either upregulation of the transcriptionally active form of SREBP1 by low doses of leptin or for downregulation of this form by the high dose. Cleavage to the mature form is regulated by intracellular sterol concentrations [21, 40]; thus, the up- and downmodulation of the mature form of SREBP1 in the present study may be secondary to alteration of intracellular cholesterol concentrations and may reflect changes in steroid synthesis.

We demonstrated that the low dose of leptin alone caused modest but significant increase in porcine StAR promoter activity in a homologous cell system. Transfection of a plasmid constitutively expressing SREBP1 increases porcine StAR gene promoter activity in primary cultures of porcine granulosa cells. The role of SREBP as a positive regulator of the StAR gene has been demonstrated in other species [24, 41], but the present investigation is the first to demonstrate that leptin interacts with SREBP in StAR gene transcription. The pattern of activity recapitulates the effects of leptin, both in progesterone accumulation and in expression of the precursor and mature forms of SREBP1. The low dose of leptin is clearly stimulatory, whereas the high dose is inhibitory. The mechanism of these interactions is not currently known. Tena-Sempere et al. [42] recently demonstrated that high doses of leptin (10^{-8} and 10^{-7} M) reduced the expression of both steroidogenic factor 1 (SF-1) and StAR in response to gonadotropin treatment of rat Leydig cells. SF-1 is a transcription factor essential for StAR expression [43]. Thus, the negative effects of the high dose of leptin in the present study may have resulted

from downregulation of SF-1. Whether the converse is true for low doses awaits further experimentation.

The experiments in this investigation demonstrate that two doses of porcine leptin, 10 ng/ml and 1000 ng/ml, stimulate phosphorylation of STAT-3 in porcine granulosa cells in vitro. The low dose of leptin increased progesterone accumulation by luteinized porcine granulosa cells in vitro, whereas the high dose was inhibitory. The low dose of porcine leptin increased both the precursor and mature forms of SREBP1, but the high doses decreased both. Low doses of leptin interacted with SREBP1 to increase the transcription of a StAR promoter-luciferase construct, and again the high dose had an inhibitory effect. The present study provides evidence that physiologic doses of leptin are stimulatory to steroidogenesis and may play a role in the process of luteinization.

ACKNOWLEDGMENTS

The authors thank Mira Dobias for her high quality technical work in aid of this investigation, Dr. H. Lavoie for generously sharing her protocols for transfection of primary porcine granulosa cells, and Dr. Alan K. Goff for statistical advice.

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