Different levels of leptin regulate different target enzymes involved in progesterone synthesis

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Objective: To study the effects of different doses of leptin on the expression of proteins involved in P synthesis, such as steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (P450scc), and 3β-hydroxysteroid dehydrogenase (3βHSD).
Design: Experimental studies.
Setting: Research laboratory.
Animal(s): Immature rats primed with gonadotropins to induce ovulation.
Intervention(s): In vivo studies: rats received either an acute or daily treatment with leptin. In vitro studies: ovarian explants were cultured in the absence or presence of leptin (0.3–500 ng/mL).
Main Outcome Measure(s): The expression of both messenger RNA and protein of StAR, P450scc, and 3βHSD were measured by reverse transcription-polymerase chain reaction (PCR) and Western blot, respectively.
Result(s): The acute treatment with leptin, which inhibits the ovulatory process, caused a significant reduction in the ovarian expression of P450scc without changes in StAR or 3βHSD. In contrast, the daily treatment, which induces the ovulatory process, showed an increased expression of the ovarian 3βHSD protein, without differences in the other proteins measured. We also found that leptin increased the protein of both P450scc and 3βHSD at physiological levels and inhibited both messenger RNA and protein of 3βHSD at higher concentrations.
Conclusion(s): The results indicate that 1) leptin is able to regulate the expression of the 3βHSD protein in a dose-dependent manner; and 2) leptin seems to exert its dual effects on P synthesis on different targets in a dose-dependent manner. (Fertil Steril® 2013;99:1460–6. ©2013 by American Society for Reproductive Medicine.)
Key Words: Leptin, StAR, P450scc, 3βHSD, ovary

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Leptin, the obese gene product, is produced mainly by adipocytes, is secreted into the bloodstream, and is involved in neuroendocrine signaling and reproduction, including ovarian function (1). Leptin mediates its effects by binding and activating specific leptin receptors that are widely distributed in different systems, including the hypothalamus-pituitary-ovarian axis (2–5). Both leptin receptors and leptin are expressed in granulosa (GC) and theca, interstitial and cumulus cells of humans (3, 6, 7), rats (8, 9), and mice (10, 11). Likewise, leptin protein has been found in follicular fluid (FF) at levels similar to those found in plasma (6, 7). In vivo and in vitro studies have demonstrated that leptin causes stimulating (12–14) and inhibitory (15–23) effects on P production in a dose-, tissue-, and species-dependent manner. Previously, we found that an acute treatment with leptin inhibits (24), whereas a daily administration of low doses of leptin increases (25) plasma P during the ovulatory process.

The steroidogenic acute regulatory protein (StAR) and the cytochrome P450 side chain cleavage (P450scc) enzyme mediate the rate-limiting steps of steroidogenesis in gonadal cells. The StAR is involved in the acute regulation of steroidogenesis by faciliting the access of cholesterol to the P450scc action, and P450scc is the enzyme responsible for conversion of cholesterol to pregnenolone, the immediate precursor of P. Once pregnenolone is produced, it may be converted to P by the action of a single

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microsomal enzyme, 3β-hydroxysteroid dehydrogenase (3βHSD) (26, 27). Thus, the aim of this work was to examine whether the alteration in P levels caused by leptin observed in our previous studies involves changes in the expression of ovarian StAR, P450scc, and/or 3βHSD.

MATERIALS AND METHODS

Animals

Immature female Sprague Dawley rats aged 21 days were purchased from the School of Veterinarian Sciences (Buenos Aires University). Animals were kept under controlled conditions of light (14 hours light:10 hours darkness), temperature (22°C) and humidity, with free access to food and water. At 26–27 days of age, rats were intraperitoneally (IP) injected with 15 IU of equine chorionic gonadotropin (eCG) and 48 hours later, with 15 IU of hCG to induce the first generation of preovulatory follicles and ovulation. Animals were handled according to the Guiding Principles for the Care and Use of Research Animals, and all the protocols were approved by the Institutional Committee of both the Centro de Estudios Farmacológicos y Botánicos and the School of Medicine of the Buenos Aires University.

In Vivo Studies

Rats received one of the two following treatments: 1) acute treatment (24), in which the rats received five IP injections of either recombinant rat leptin (5 μg/0.15 mL of phosphate buffer saline-bovine serum albumin [PBS–BSA]) or PBS–BSA alone (control) 1 hour before hCG administration and at 150-minute intervals until killing; and 2) daily treatment (25), at 22 days of age, in which the rats received an IP injection of either recombinant rat leptin (3 μg/0.15 mL PBS–BSA) or PBS–BSA alone (control) per day until the day the animals were killed. After 10 days of treatment, these animals were injected with gonadotropin as indicated previously. In total, these animals received 12 injections of leptin. All the rats (8–10 per group) were killed by decapitation 10 hours after hCG administration and both ovaries were immediately dissected out, frozen on dry ice, and stored at −78°C until use. Only one ovary from each animal was used in these studies.

In Vitro Studies

Animals were killed by decapitation 4 hours after hCG administration and the ovarian tissue was incubated as described previously (24). Ovarian slices (four slices per ovary per well) were randomly distributed in 24-well plates containing 500 μL/well of Dulbecco's minimum essential medium (DMEM)/F12 (1:1) medium (Bio-Rad Laboratories) with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5 μg/mL fungizone, and 2 mM L-glutamine, and different leptin concentrations (0.3–500 ng/mL) (24, 28, 29) at 37°C in a humidified atmosphere (5% CO_2:95% O_2). After a 4-hour incubation, ovarian tissues were recovered and frozen on dry ice and stored at −75°C until use. Each experiment was repeated at least six times.

RNA Isolation and Semiquantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from all frozen tissues using TRI Reagent (Molecular Research Center) according to the manufacturer's instructions. The organic phase of each sample was saved for protein extraction. Total RNA quantitation and purity determination was assessed by spectral absorption (A260/A280) before reverse transcription-polymerase chain reaction (PCR) reactions. Complementary DNA was synthesized from 4 μg RNA in 25 μL of reaction mixture containing 200 U Moloney murine leukemia virus, oligonucleotides (Random Primers; Invitrogen), and deoxyribonucleotides (Promega). Complementary DNA was amplified by PCR in a total volume of 25 μL using the primers and the cycles detailed in Table 1. Each reaction also contained 1 U *Taq*-DNA polymerase (Invitrogen), 0.2 mM of each primer (Invitrogen), 0.2 mM of each deoxyribonucleotide, and 1.5 mM $MgCl_2$. The PCR profiles consisted of an initial denaturing step at 94°C for 5 minutes and an appropriate number of denaturing cycles at 94°C for 40 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 1 minute, and a final extension step at 72°C for 5 minutes. The primer sequences used to amplify the messenger RNA (mRNA) of P450scc, 3βHSD, and β-actin were designed by Primer3-BLAST software, as described previously (30), whereas that to amplify StAR mRNA was that described by Moreau et al. (31) (Table 1). In preliminary experiments, the optimum cycle number was determined for each target, therefore signals were always in the exponential portion of the amplification curve. An aliquot of each sample of the PCR reaction was electrophoresed in 2% (wt/vol) agarose gel

TABLE 1

Primers used for polymerase chain reaction (PCR) for detection of StAR, P450scc, 3βHSD, and actin.

Gene	Oligonucleotide sequences	Annealing temperature (°C)	Cycles	Product size	GenBank
StAR	Sense 5′-CATCCAGCAAGGAGAGGAAG-3′ Antisense 5′-CGTGAGTTTGGTCTTTGAGG-3′	55	35	496 bp	NM031558.2
P450scc	Sense 5′-TTGTGCCCCCCTGGG-3′ Antisense 5′-TTTGATGAAGTCCTGAGCTACACC-3′	54	30	176 bp	NM017286.2
3βHSD	Sense 5′-AGTCCTGATCTGAGGGCTGA-3′ Antisense 5′-GCAGTTCTTTCTCCTGCACC-3′	53	20	218 bp	NM001042619.1
β-actin	Sense 5′-AGCCATGTACGTAGCCATCC-3′ Antisense 5′-CTCTCAGCTGTGGTGGTGAA-3′	57	35	228 bp	NM031144.2

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with subsequent ethidium bromide (10 mg/mL) staining. The mRNA bands were visualized and quantified using Image Quant RT ECL (General Electric, Amersham Bioscience Argentina SA) and ImageJ, respectively. Data were normalized to β-actin mRNA in each sample. Negative controls were performed without reverse transcriptase or RNA.

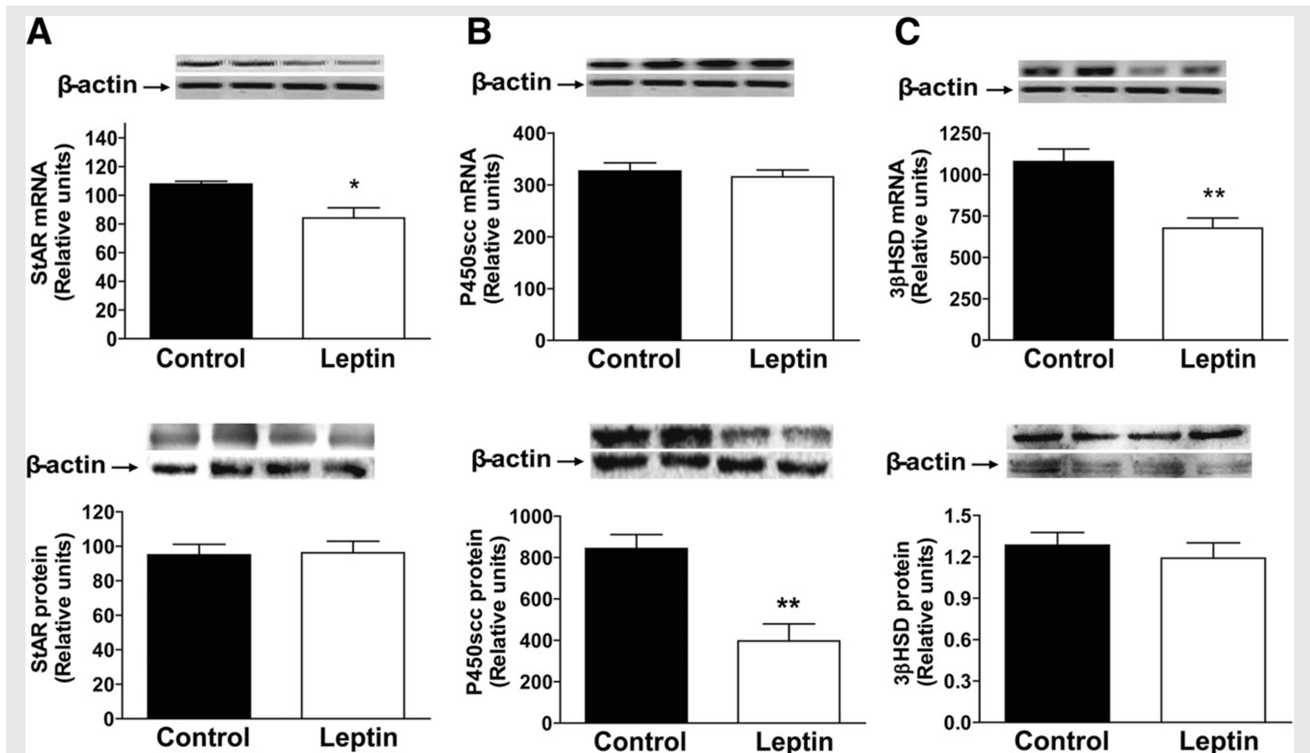
Protein Isolation by Western Blot

Proteins were isolated from the organic phase of the RNA isolation according to the manufacturer's instructions. Equal amounts of protein (30 μg for StAR, 100 μg for P450scc, 100 μg for 3βHSD) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%). Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) for 60 minutes in a cold chamber using a Bio-Rad transblot apparatus. Membranes were first blocked at 4°C overnight in Tris-HCl:saline (50 mM Tris-HCl: 150 mM NaCl at pH 7.5) containing 5% (wt/vol) of nonfat milk powder (for rabbit antibodies) or normal goat serum (for goat antibody), and then incubated at 4°C for 48 hours with the primary antibodies (rabbit anti-StAR and goat anti P450scc; Santa Cruz Biotechnology), rabbit anti-3βHSD II (Novus Biologicals), and rabbit anti-actin (Sigma–Aldrich), diluted 1:200. After washing, membranes were treated with goat anti-rabbit IgG for StAR and 3βHSD II (Santa Cruz Biotechnology) or bovine anti-goat IgG for P450scc (Jackson ImmunoResearch Laboratories Inc.) as secondary antibodies, diluted 1:2,000 at room temperature for 1 hour. Immunoreactive bands were visualized using chemiluminescence detection reagents (Sigma-Aldrich) and Image Quant RT ECL (General Electric), and quantified using ImageJ. Before reuse, membranes were stripped, blocked, and reprobed according to the manufacturer's instructions. Negative controls were carried out by omitting the incubation with the primary antibody and no bands were detected. Molecular weight standards (Bio-Rad Laboratories) were run under the same conditions to identify the protein bands. The data were normalized to β-actin protein levels in each sample to avoid procedural variability.

Statistical Analysis

All data are expressed as means $\pm$ SEM. The difference between two groups was analyzed using Student's t test. Comparisons between more than two groups were performed using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test. Differences between groups were considered significant when $P<.05$.

FIGURE 1



In vivo effect of the acute treatment with leptin on the ovarian expression of messenger RNA (*top*) and protein (*bottom*) of StAR (A), P450scc (B), and 3βHSD (C). Immature rats were primed with equine chorionic gonadotropin/hCG and treated with vehicle (Control) or 5 μg leptin at 1 hour before hCG and at 150-minute intervals. Animals were killed 10 hours after hCG administration. Quantitative analysis of both messenger RNA and protein bands were normalized to β-actin as control. The results are expressed as the mean $\pm$ SEM for 8–10 samples per group. Each sample represents one ovary from each animal with the same treatment (n = 8–10). *$P<.05$, **$P<.01$ versus controls (Student's test).

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RESULTS

In Vivo Studies

After the acute treatment with leptin, we observed a significant reduction in the expression of mRNA of both StAR (19%, $P<.05$) and 3βHSD (37%, $P<.01$) compared with controls, without changes in their proteins (Fig. 1A and C). In contrast, this treatment reduced the expression of P450scc protein by 53% ($P<.01$), without differences in the mRNA (Fig. 1B).

After the daily treatment with leptin, we found that neither StAR nor P450scc showed significant differences in the expression of the transcript or protein (Fig. 2A and B). However, this treatment caused a significant increase in the ovarian expression of 3βHSD protein (44%, $P<.05$) compared with controls, without changes in the mRNA (Fig. 2C).

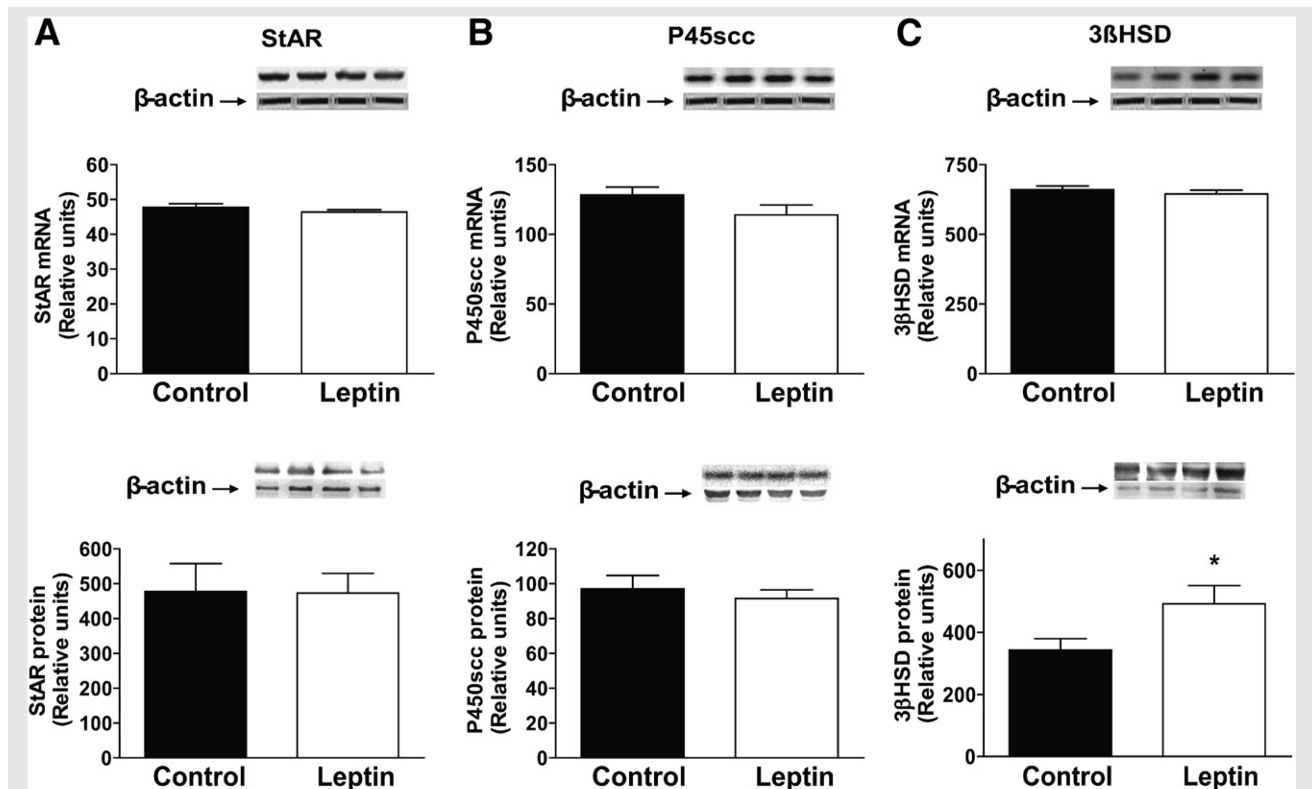
In Vitro Studies

To study the direct effect of a narrow range of leptin concentrations, the same proteins studied in the in vivo experiments were measured in ovarian explant cultures after a 4-hour incubation. Neither the transcript nor the protein of StAR was modified by the presence of different leptin concentrations compared with controls (Fig. 3A). However, the ovarian expression of P450scc protein was increased in the presence of leptin (Fig. 3B), but only significantly at 3 ng/mL ($P<.01$). This increase represented 75% compared with controls. No differences were found in the P450scc mRNA expression. In the case of 3βHSD, the ovarian tissues exposed to leptin clearly exhibited lower expressions of mRNA than the control tissues, although these reductions were only significant at leptin concentrations of 100–300 ng/mL (47%–60%). However, the protein shows a dose-dependent biphasic effect, as its expression was significantly increased (96%–38%) at 1–10 ng/mL and significantly decreased (54%) at 500 ng/mL of leptin when compared with controls (Fig. 3C).

DISCUSSION

The fact that P450scc protein was reduced by the acute treatment with leptin, without changes in StAR and 3βHSD proteins, means that this treatment induced a lower availability of the ovarian P450scc enzyme, the first rate-limiting step in P synthesis. The original purpose of this treatment design was to maintain high levels of leptin after hCG administration

FIGURE 2



In vivo effect of the daily administration of leptin (3 μg/d) on the ovarian expression of messenger RNA (*top*) and protein (*bottom*) of StAR (A), P450scc (B), and 3βHSD (C). On day 22 of age, rats received injections of either vehicle (Control) or 3 μg of leptin per day. After 10 days of treatment, animals received the equine chorionic gonadotropin/hCG stimulation as indicated in Materials and Methods. Animals were killed 10 hours after hCG administration. Quantitative analysis of both messenger RNA and protein bands were normalized to β-actin as control. The results are expressed as the mean $\pm$ SEM for 8–10 samples per group. Each sample represents one ovary from each animal with the same treatment (n = 8–10). *$P<.05$ versus controls (Student's test).

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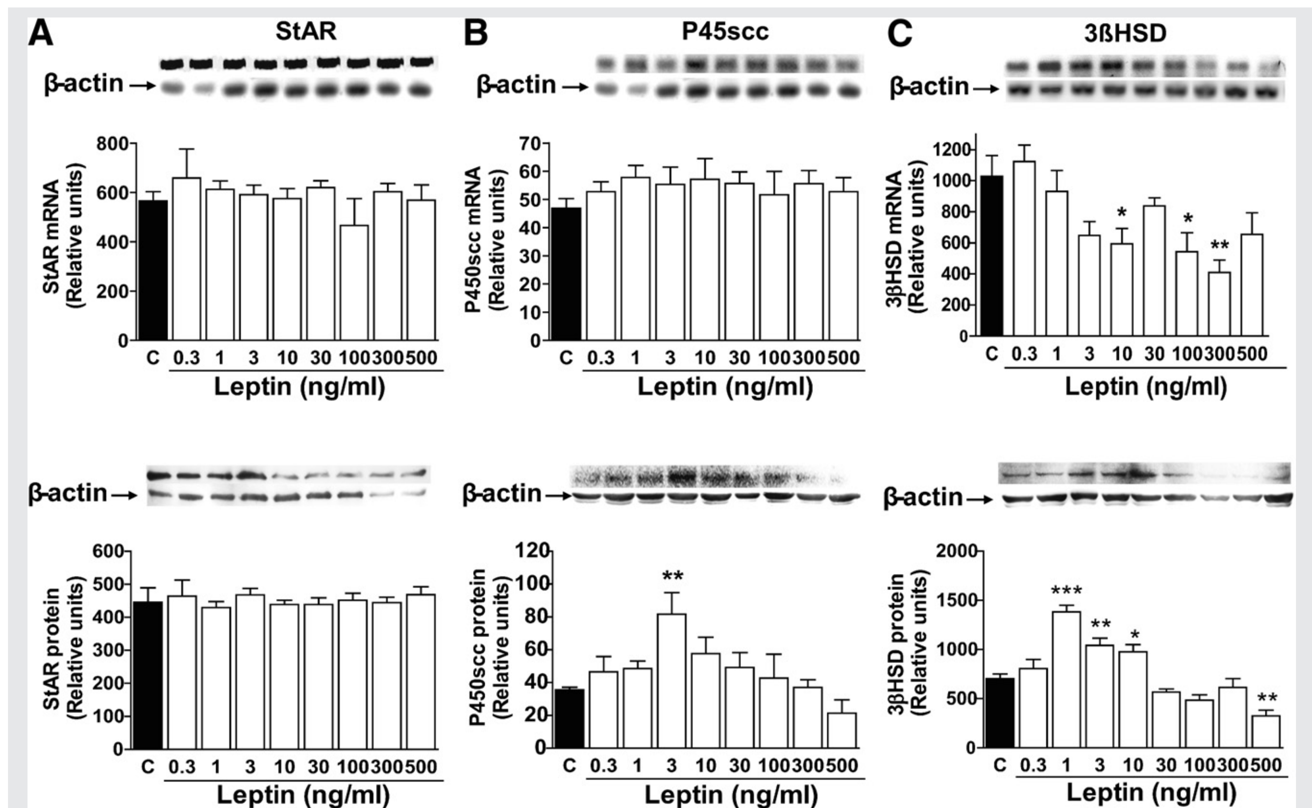
FIGURE 3



In vitro effect of leptin on the ovarian expression of messenger RNA (*top*) and protein (*bottom*) of StAR (A), P450scc (B), and 3βHSD (C). Ovarian explants were obtained 4 hours after hCG administration from immature rats primed with equine chorionic gonadotropin/hCG and incubated for 4 hours either in the presence or in the absence (Control) of different concentrations of leptin (0.3–500 ng/mL). Quantitative analysis of messenger RNA and protein bands were normalized to β-actin as control. The results are expressed as the mean $\pm$ SEM of six independent experiments (n = 6). *$P<.05$, **$P<.01$, ***$P<.001$ versus controls (one-way analysis of variance [ANOVA] and Dunnett's multiple comparison test).

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to avoid the decrease in the levels of this protein observed in circulation before ovulation, as described previously (24, 32). Therefore, this result is consistent with findings showing that this treatment is able to inhibit plasma P levels (24) and ovulation (24, 28, 29). Unlike the acute treatment, the ovarian expression of the 3βHSD protein, a microsomal enzyme that converts pregnenolone to P, was increased when the rats received the daily treatment with low doses of leptin and no changes were found in the other proteins studied. This increase is consistent with that found in other studies, where a daily treatment with leptin, as well as other chronic treatments, was found to have an effective and positive action on the ovarian function, to accelerate the onset of puberty in rodents (12, 33) and humans (34, 35), to induce the ovulatory rate, the plasma P levels, and recover the sexual function in malnourished female rats (25, 36). To our knowledge, this work is the first to demonstrate that leptin is able to regulate the expression of the 3βHSD protein in a dose-dependent manner. A direct inhibitory action of leptin in P secretion has been demonstrated by several groups of investigators in different in vitro systems (15–20). However, few studies have reported the in vivo action of leptin on the P levels in the rat. No differences have been found in the P levels between rats treated with high doses of leptin during the ovulatory process and control animals (29). However, an increase in the ovarian expression of both StAR and adrenodoxin, which is an intrinsic component of the cytochrome P450scc enzymatic system, has been found after a daily treatment with leptin in immature rats for 13 days, consistent with high serum P levels and early puberty exhibited by these animals (12). Furthermore, daily injections with physiological doses of leptin to immature rats increased P levels (37). Thus, the opposite response of the ovarian tissue to leptin could be due to different mechanisms of leptin action, as these results suggest that leptin regulates different targets throughout P synthesis in a dose-dependent manner.

The StAR expression was not altered by the presence of leptin in our in vitro experiments, whereas P450scc protein was significantly increased at 3 ng/mL, without differences in its transcript. In addition, 3βHSD protein exhibited a concentration-dependent biphasic effect. These results are consistent with previous findings where 1–10 ng/mL of leptin increased the P secretion in the culture medium of ovarian explants after a 4-hour incubation (38). It has been shown that leptin influences the basal (21) or the gonadotropin- (16, 17),

or insulin- (15, 39), or insulin-like growth factor I (IGF-I)- (7, 22), or cyclic adenosine 3′:5′ monophosphate (cAMP)- (20) induced ovarian P secretion in many different biological systems. Without considering the biological differences between all of these systems, these effects were obtained with concentrations higher than those used in our in vitro studies. It is important to point out that the 3–10 ng/mL concentrations used in our in vitro studies are of the same order of magnitude as that found in circulation of normally fed rats, and that they have stimulatory effects on the ovarian function, as described previously (12, 40). It is well known that the expression of P450scc is induced by cAMP in steroidogenic tissues such as the ovary. Some studies have shown that leptin is able to inhibit the 8-cAMP-stimulated P secretion in culture of different cell systems, probably through a cAMP degradation mechanism (20, 41). Neither the cAMP levels nor cAMP-specific phosphodiesterase were measured in the present study, but it is probable that leptin acts on the production of P through a mechanism involving cAMP signaling to regulate the key enzymes involved in its synthesis.

Although it is difficult to compare an in vivo effect caused by a repeated or daily systemic exposure, as in our in vivo studies, with an effect caused by a tissue directly exposed to effective and known doses, it is important to point out that our in vitro assays confirmed the results obtained in our in vivo studies from this and previous works (24, 25), where physiological levels of leptin have a stimulatory effect and high levels of leptin have an inhibitory effect on the P production by regulating the expression of P450scc and/or 3βHSD.

Finally, and considering that steroidogenesis is mainly responsive to two regulatory systems that determine the steroid levels, our results suggest that leptin appears to be acting as a chronic regulation factor of steroidogenesis during the ovulatory process as it modulates P levels by altering the expression of P450scc and/or 3βHSD without altering the StAR content as an acute regulation factor.

In conclusion, this work provides evidences that 1) leptin is able to regulate the expression of the 3βHSD protein in a dose-dependent manner; and that 2) the dual and opposite response of the ovarian tissue to leptin could be due to its action on different targets, as different enzymes involved in P synthesis are modified by this protein in a dose-dependent manner.

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REFERENCES

1. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994; 372:425–32.
2. Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Trayhurn P. Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. FEBS Lett 1996;387:113–6.
3. Karlsson C, Lindell K, Svensson E, Bergh C, Lind P, Billig H, et al. Expression of functional leptin receptors in the human ovary. J Clin Endocrinol Metab 1997;82:4144–8.
4. Zamorano PL, Mahesh VB, De Sevilla LM, Chorich LP, Bhat GK, Rann DW. Expression and localization of the leptin receptor in endocrine and neuroendocrine tissues of the rat. Neuroendocrinology 1997;65:223–8.
5. Sone M, Nagata H, Takekoshi S, Osamura RY. Expression and localization of leptin receptor in the normal rat pituitary gland. Cell Tissue Res 2001;305: 351–6.
6. Cioffi JA, van Blerkom J, Antczak M, Shafer A, Wittmer S, Snodgrass HR. The expression of leptin and its receptors in pre-ovulatory human follicles. Mol Hum Reprod 1997;3:467–72.
7. Agarwal SK, Vogel K, Weitsman SR, Magoffin DA. Leptin antagonizes the insulin-like growth factor-1 augmentation of steroidogenesis in granulosa and theca cells of the human ovary. J Clin Endocrinol Metab 1999;84: 1072–6.
8. Zachow RJ, Weitsman SR, Magoffin DA. Leptin impairs the synergistic stimulation by transforming growth factor-β of follicle-stimulating hormone-dependent aromatase activity and messenger ribonucleic acid expression in rat ovarian granulosa cells. Biol Reprod 1999;61:1104–9.
9. Archanco M, Muruzabal FJ, Llopiz D, Garayoa M, Gomez-Ambrosi J, Fruhbeck G, et al. Leptin expression in the rat ovary depends on estrous cycle. J Histochem Cytochem 2003;51:1269–77.
10. Antczak M, van Blerkom J. Oocyte influences on early development: the regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. Mol Hum Reprod 1997;3:1067–86.
11. Ryan NK, Woodhouse CM, van der Hoek KH, Gilchrist RB, Armstrong DT, Norman RJ. Expression of leptin and its receptor in the murine ovary: possible role in the regulation of oocyte maturation. Biol Reprod 2002;66: 1548–54.
12. Almog B, Gold R, Tajima K, Dantes A, Salim K, Rubinstein M, et al. Leptin attenuates follicular apoptosis and accelerates the onset of puberty in immature rats. Mol Cell Endocrinol 2001;183:179–91.
13. Kendall NR, Gutierrez CG, Scaramuzzi RJ, Baird DT, Webb R, Campbell BK. Direct *in vivo* effects of leptin on ovarian steroidogenesis in sheep. Reproduction 2004;128:757–65.
14. Swain JE, Dunn RL, McConnell D, Gonzalez-Martinez J, Smith GD. Direct effects of leptin on mouse reproductive function: regulation of follicular, oocyte, and embryo development. Biol Reprod 2004;71:1446–52.
15. Spicer LJ, Francisco CC. Adipose obese gene product, leptin, inhibits bovine ovarian theca cell steroidogenesis. Biol Reprod 1998;58:207–12.
16. Brannian JD, Zhao Y, McElroy M. Leptin inhibits gonadotrophin-stimulated granulosa cell progesterone production by antagonizing insulin action. Hum Reprod 1999;14:1445–8.
17. Barkan D, Jia H, Dantes A, Vardimon L, Amsterdam A, Rubinstein M. Leptin modulates the glucocorticoid-induced ovarian steroidogenesis. Endocrinology 1999;140:1731–8.
18. Spicer LJ, Chamberlain CS, Francisco CC. Ovarian action of leptin. Endocrine 2000;12:53–9.
19. Zerani M, Boiti C, Zampini D, Brecchia G, Dall'Aglio C, Ceccarelli P, et al. Ob receptor in rabbit ovary and leptin *in vitro* regulation of corpora lutea. J Endocrinol 2004;183:279–88.
20. Lin Q, Poon SL, Chen J, Cheng L, HoYuen B, Leung PC. Leptin interferes with 3',5'-cyclic adenosine monophosphate (cAMP) signaling to inhibit steroidogenesis in human granulosa cells. Reprod Biol Endocrinol 2009;7:115.
21. Ruiz-Cortés ZT, Martel-Kennes Y, Gévry NY, Downey BR, Palin MF, Murphy BD. Biphasic effects of leptin in porcine granulosa cells. Biol Reprod 2003;68:789–96.
22. Karamouti M, Kollia P, Kallitsaris A, Vamvakopoulos N, Kollios G, Messinis IE. Growth hormone, insulin-like growth factor I, and leptin interaction in human cultured lutein granulosa cells steroidogenesis. Fertil Steril 2008;90: 1444–50.
23. Karamouti M, Kollia P, Kallitsaris A, Vamvakopoulos N, Kollios G, Messinis IE. Modulating effect of leptin on basal and follicle stimulating hormone stimulated steroidogenesis in cultured human lutein granulosa cells. J Endocrinol Invest 2009;32:415–9.

24. Ricci AG, Di Yorio MP, Faletti AG. Inhibitory effect of leptin on the rat ovary during the ovulatory process. Reproduction 2006;132:771–80.
25. Roman EA, Ricci AG, Faletti AG. Leptin enhances ovulation and attenuates the effects produced by food restriction. Mol Cell Endocrinol 2005;242:33–41.
26. Stocco DM, Clark BJ. Regulation of the acute production of steroids in steroidogenic cells. Endocr Rev 1996;17:221–44.
27. Miller WL. Role of mitochondria in steroidogenesis. Endocrinol Dev 2011;20: 1–19.
28. Duggal PS, Van der Hoek KH, Milner CR, Ryan NK, Armstrong DT, Magoffin DA, et al. The *in vivo* and *in vitro* effects of exogenous leptin on ovulation in the rat. Endocrinology 2000;141:1971–6.
29. Duggal PS, Ryan NK, van der Hoek KH, Ritter LJ, Armstrong DT, Magoffin DA, et al. Effects of leptin administration and feed restriction on thecal leukocytes in the preovulatory rat ovary and the effects of leptin on meiotic maturation, granulosa cell proliferation, steroid hormone and PGE_2 release in cultured rat ovarian follicles. Reproduction 2002;123:891–9.
30. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365–86.
31. Moreau C, Froment P, Tosca L, Moreau V, Dupont J. Expression and regulation of the SCD2 desaturase in the rat ovary. Biol Reprod 2006;74:75–87.
32. Ryan NK, Van der Hoek KH, Robertson SA, Norman RJ. Leptin and leptin receptor expression in the rat ovary. Endocrinology 2003;144:5006–13.
33. Ahima RS, Dushay J, Flier SN, Prabakaran D, Flier JS. Leptin accelerates the onset of puberty in normal female mice. J Clin Invest 1997;99:391–5.
34. Strobel A, Issad T, Camoin L, Ozata M, Strosberg AD. A leptin missense mutation associated with hypogonadism and morbid obesity. Nat Genet 1998; 18:213–5.
35. García-Mayor RV, Andrade MA, Rios M, Lage M, Dieguez C, Casanueva FF. Serum leptin levels in normal children: relationship to age, gender, body mass index, pituitary-gonadal hormones, and pubertal stage. J Clin Endocrinol Metab 1997;82:2849–55.
36. Gruaz NM, Lalaoui M, Pierroz DD, Englaro P, Sizonenko PC, Blum WF, et al. Chronic administration of leptin into the lateral ventricle induces sexual maturation in severely food-restricted female rats. J Neuroendocrinol 1998;10: 627–33.
37. Cannady WE, Brann DW, Mahesh VB. The potential role of periovarian fat and leptin in initiation of puberty in the immature rat. Int J Obes Relat Metab Disord 2000;24:S146–7.
38. Di Yorio MP, Bilbao MG, Pustovrh MC, Prestifilippo JP, Faletti AG. Leptin modulates the expression of its receptors in the hypothalamic-pituitary-ovarian axis in a differential way. J Endocrinol 2008;198: 355–66.
39. Spicer LJ, Francisco CC. The adipose obese gene product, leptin: evidence of a direct inhibitory role in ovarian function. Endocrinology 1997;138: 3374–9.
40. Watanobe H, Suda T. A detailed study on the role of sex steroid milieu in determining plasma leptin concentrations in adult male and female rats. Biochem Biophys Res Commun 1999;259:56–9.
41. Hsu HT, Chang YC, Chiu YN, Liu CL, Chang KJ, Guo IC. Leptin interferes with adrenocorticotropin/3',5'-cyclic adenosine monophosphate (cAMP) signaling, possibly through a Janus kinase 2-phosphatidylinositol 3-kinase/Akt-phosphodiesterase 3-cAMP pathway, to down-regulate cholesterol side-chain cleavage cytochrome P450 enzyme in human adrenocortical NCI-H295 cell line. J Clin Endocrinol Metab 2006;91:2761–9.