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In Vivo Regulation of the Steroidogenic Activity of Rat Luteal Cells by Insulin

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The aim of the present study was to determine the long-term effects of insulin treatment on luteal cell function. For this purpose, superovulated prepubertal rats were treated with insulin (group I) or vehicle (group C) for 9 days. Serum progesterone (P_4) levels were increased in the insulin-treated group (55 ± 10 vs 134 ± 31 ng/ml, $P < 0.05$). Isolated luteal cells were incubated 3 h, and P_4 and 20α-hydroxy-progesterone (20α-OH-P) were measured in the incubation media. A decrease in P_4 levels and an increase in 20α-OH-P values [P_4 (ng/ml): $C = 26.6 \pm 0.3$; $I = 20 \pm 2$; 20α-OH-P (ng/ml): $C = 62 \pm 2$; I: 120 ± 7; $P < 0.01$] were observed in group I. In addition, progestagen (P_4 + 20α-OH-P) levels were higher in group I ($C = 88 \pm 2$; $I = 140 \pm 9$ ng/ml; $P < 0.001$). When cytochrome $P450_{SCC}$ contents were measured by immunoblotting, a marked increase was observed in luteal cells obtained from group I. LH receptor numbers were decreased in luteal cells isolated from group I ($C = 388{,}834 \pm 14{,}146$; $I = 303{,}057 \pm 13{,}392$ sites/cell; $P < 0.001$) with a concomitantly diminished LH responsiveness. It is concluded that *in vivo* treatment of superovulated rats with insulin increases luteal progestagen production by increasing the content of cytochrome $P450_{SCC}$.

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INTRODUCTION

Hyperinsulinemia is frequently associated with clinical manifestations of hyperandrogenism in patients with polycystic ovarian syndrome, insulin resistance syndromes and acanthosis nigricans [1–4]. In view of the fact that the ovary is at present considered a target organ for insulin [5, 6], a correlation between high circulating levels of insulin and some ovarian disorders has been postulated [1, 3, 5, 7].

In several experimental models, the *in vitro* modulatory action of insulin and insulin-like growth factors on granulosa cell steroidogenesis [8–14] and LH receptor induction [15–18] has been demonstrated. Previous results in our laboratory indicated the presence of insulin receptors in freshly isolated rat luteal cells and a stimulatory action of this hormone on progesterone (P_4) production by these cells [19]. In addition, insulin was found to stimulate both the enzymes responsible for cholesterol side-chain cleavage as well as aromatase, in cultured rat luteal cells [20]. However, there have been few reports on the *in vivo* modulation of ovarian function by insulin. Poretsky *et al.* [21] demonstrated that experimental hyperinsulinemia in rats stimulates aromatase activity, tends to down-regulate ovarian insulin receptors and up-regulates ovarian insulin-like growth factor I (IGF-I) receptors.

Ovarian steroidogenesis is regulated by the rate of cholesterol transport across the mitochondrial membranes to the site of its conversion to pregnenolone, and by the amount and/or activity of steroidogenic enzymes. The mitochondrial cholesterol side-chain cleavage enzyme system is comprised of three main components that participate in electron transport: cholesterol side-chain cleavage cytochrome $P450$ ($P450_{SCC}$); an iron–sulphur protein known as adrenodoxin; and a flavoprotein, NADPH-adrenodoxin reductase. Several reports indicate that both gonadotropin and IGF-I increase the protein content of these enzymes as well as their specific mRNAs [22–25]. However, little is known about the *in vivo* regulation by insulin of $P450_{SCC}$.

The aim of the present work was to study the long-term effects of insulin treatment on luteal cell function. For this purpose, a hyperinsulinemic state was generated by administering insulin to super-

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Trivial name for 20α-hydroxypregn-4-ene-3-one: 20α-hydroxy-progesterone.
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ovulated rats for 9 days. The production of steroids, responsiveness to LH, number of LH/hCG receptors and content of cholesterol side-chain cleavage cytochrome $P450$ were measured and compared to those from control animals.

MATERIALS AND METHODS

Materials

Pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) used for superovulation were from König and Elea Laboratories (Buenos Aires, Argentina), respectively. Bovine protamine-zinc insulin was from Beta Laboratories (Buenos Aires, Argentina). [1,2,6,7-^{3}H(N)]progesterone (98.3 Ci/mmol), [1,2-^{3}H(N)]estradiol (100.5 Ci/mmol) and carrier-free [^{125}I]Na (100 mCi/ml) were purchased from New England Nuclear (Boston, MA). Unlabeled progesterone, 20α-hydroxypregn-4-ene-3-one, estradiol, HEPES, deoxyribonuclease I (DNase I), bovine serum albumin fraction V (BSA), acrylamide, bis-acrylamide, sodium dodecyl sulfate (SDS), glycine, ammonium persulfate, *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), horseradish peroxidase-labeled goat anti-rabbit IgG and 3,3′-diaminobenzidine were from Sigma Chemical Co. (St Louis, MO). Dulbecco's Modified Eagle Medium with 4.5 g glucose/liter (DMEM) and Ham's F-12 nutrient mixture (F-12) were obtained from Gibco Laboratories (Grand Island, NY). Collagenase type CLS II (125 IU/mg) was from Worthington Biochemical Co. (Freehold, NJ). Human LH (hLH: 7.2 IU/μg) was kindly supplied by the National Pituitary Agency (NIH, Bethesda, MD). Rabbit antibody against bovine cytochrome $P450_{SCC}$ was a generous gift from Dr Anita H. Payne (Ann Arbor, MI).

In vivo insulin treatment and superovulation

Female Sprague–Dawley rats, 23–25 days old, allowed food and water *ad libitum* and kept in an air-conditioned atmosphere, were injected subcutaneously with PMSG (25 IU/rat) followed 48 h later by hCG (25 IU/rat). Rats were treated with 0.5 IU insulin-Zn-protamine (s.c.) on the day of PMSG administration and 1 IU/rat/day on the following days. The last injection took place 1 h before sacrifice (Group I). The same schedule was performed with control rats (Group C), employing vehicle. Animals in group I had free access to a 2.5% glucose solution. Experiments were carried out 8 days after PMSG administration. Blood glucose was measured in both groups of animals with reflexion photometer equipment (Glucometer, Miles Laboratories, Buenos Aires). One hour after insulin injection, animals in group I were found to be hypoglycemic: blood glucose (mg/dl) $C = 144 \pm 8$; $I = 51 \pm 6$ ($P < 0.001$). Euglycemic values were reached 6 h after insulin injection.

Preparation of collagenase-dispersed luteal cells from superovulated rats

Luteal cells from luteinized ovaries were isolated basically as described previously [20, 26]. Briefly, ovaries were finely minced in DMEM:F12 (1:1 v/v), containing 10 mM HEPES (DMEM:F12-HEPES) and 0.1% BSA, and enzymatic digestion was performed using 1 ml per ovary of DMEM:F12-HEPES plus 0.5% BSA, 0.1% collagenase and 0.008% deoxyribonuclease I (DNase I). Tubes were shaken for 40 min at 37°C, the suspension was passed through nylon mesh (Nytex 50) and centrifuged for 10 min at 250 *g*. Pellets were washed twice in DMEM:F12-HEPES 0.1% BSA, and resuspended in the same medium supplemented with 0.5% BSA. Cells were counted in a hemocytometer. Viability, determined by trypan blue staining, was in the 90% range.

Incubation of luteal cells

Isolated luteal cells (200,000 cells/0.5 ml) from superovulated C and I rats were incubated 3 h in DMEM:F12-HEPES, 0.5% BSA, under constant shaking at 37°C with or without increasing doses of hLH (0.1–100 ng/ml).

Incubations were terminated by centrifuging the vials for 15 min at 800 *g* at 4°C and supernatants were frozen for steroid determination by radioimmunoassay (RIA; see below).

Preparation of iodinated hCG

[^{125}I]hCG was prepared using the chloramine-T technique [27]. The iodinated hormone was purified in a Sephadex G-50 column and stored in aliquots at −20°C. Biological activity of the hormone was tested in radioligand assays using testis membrane preparations, as described elsewhere [28]. Maximal binding capacity and precise specific activity calculations were performed by binding in the presence of excess receptors and by self-displacement assays, respectively [29, 30]. Specific activity and maximal binding capacity of typical preparations of [^{125}I]hCG averaged 65 μCi/μg and 35%, respectively.

LH/hCG receptor measurement

Luteinizing hormone receptors in rat luteal cells were measured by incubating the cells for 2 h in DMEM:F12-HEPES 0.5% BSA at 37°C (40,000 cells/0.25 ml). Scatchard analysis was performed using increasing concentrations of [^{125}I]hCG. Nonspecific binding was assessed by parallel incubations containing 5 IU of unlabeled hormone. After the addition of cold phosphate-buffered saline (PBS), containing 2 mg/ml BSA and 1 mg/ml celite, tubes were centrifuged for 20 min at 1500 *g* and bound [^{125}I]hCG was measured in the pellets. Results are expressed in terms of number of binding sites per cell.

Western blot analysis of luteal cytochrome $P450_{SCC}$

Isolated luteal cells obtained from both experimental groups (C and I), were homogenized in 0.25 M sucrose, 50 mM KCl, 1 mM $CaCl_2$ and 2 mM $MgCl_2$ with the addition of protease inhibitors. Luteal homogenates were centrifuged for 10 min at 800 *g* and supernatants further centrifuged for 15 min at 10,000 *g*. Pellets (mitochondrial fractions) were resuspended in Laemmli electrophoresis sample buffer [31], denatured by 5 min in a boiling water bath and equal amounts of protein (10 μg) were applied to each lane of the gel. Proteins separated by 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis were then transferred onto nitrocellulose membranes in transfer buffer containing 20% methanol (v/v), 0.192 M glycine, 0.025 M Tris–base (pH 8.3) 16 h at 4°C. Blots were blocked 1 h in PBS containing low-fat powdered milk (2%) and Tween 20 (0.2%) at room temperature and then exposed 1 h to an anti-$P450_{SCC}$ polyclonal antibody (1:10,000 dilution). After three washes, the nitrocellulose sheets were exposed to horseradish peroxidase-labeled goat anti-rabbit IgG (1:1000) and antigen–antibody complexes visualized by using 3,3′-diaminobenzidine as substrate. The amounts of immunoreactive $P450_{SCC}$ protein were compared in mitochondrial fractions from groups C and I. The quantification of $P450_{SCC}$ was validated by subjecting different quantities (0.2–20 μg) of luteal cell protein to Western blot.

Protein determination was performed by the method of Lowry *et al.* [32], after dissolving the samples for 16 h in 0.5 N NaOH at 20°C. Bovine serum albumin was employed as standard.

Other methods

Progesterone, 20α-hydroxy-progesterone (20α-OH-P) and estradiol (E_2) production by luteal cells was evaluated by RIA in suitable dilutions of incubation media. Serum P_4 levels were measured by RIA, following a modification of the method of Abraham *et al.* [33, 34].

RIAs were performed as described previously [20]. Under our conditions, the within-assay and between-assay variations were 8.0 and 14.2%, respectively for P_4; 7.2 and 12.5%, respectively for E_2; and 7.5 and 13.2%, respectively, for 20α-OH-P.

All incubations were carried out in triplicate. Results are presented as means ± SE. Statistical analysis was done using the Student's *t*-test for paired data or the ANOVA test for comparisons between multiple data. Differences between treatment groups were considered to be significant at $P < 0.05$. Experiments were repeated at least three times using 8–10 animals per experiment.

RESULTS

Progesterone concentration in serum was measured in both control (C) and insulin-treated (I) groups following ether extraction. A significant increase in circulating levels of P_4 in group I was observed (C: 55 ± 10, I: 134 ± 31 ng/ml, $n = 10$, $P < 0.05$).

Basal levels of P_4 and 20α-OH-P were measured in the incubation media of luteal cells obtained from groups C and I (Fig. 1, left panel). Luteal P_4 accumulation decreased significantly in group I, while 20α-OH-P increased. In addition, luteal progestagen production, defined as the sum P_4 + 20α-OH-P, showed increase in group I (Fig. 1, right panel).

The stimulatory effect of increasing doses of hLH on P_4 accumulation by rat luteal cells obtained from control or insulin-treated rats is shown in Fig. 2. Progesterone was significantly higher in group C than in group I at all LH concentrations tested. On the contrary, 20α-OH-P production in response to hLH was diminished in control luteal cells (Fig. 3). How-

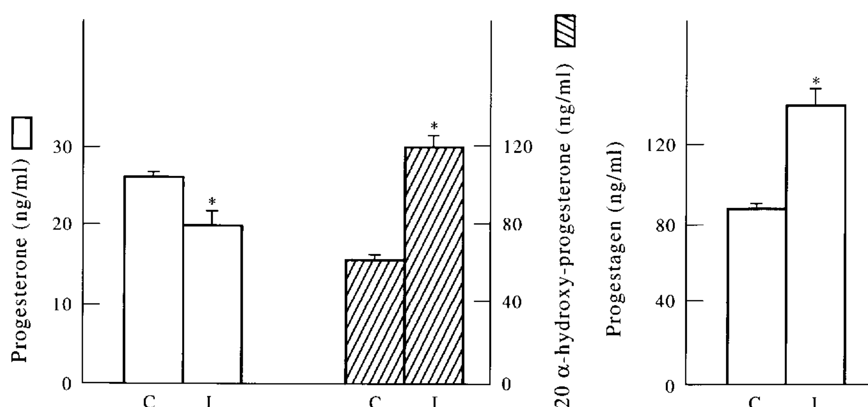

Fig. 1. Progestagen production by isolated luteal cells from control (C) or insulin-treated (I) rats. Cells (200,000/0.5 ml) were incubated for 3 h in DMEM:F12, 10 mM HEPES, 0.5% BSA at 37°C. Progesterone and 20α-OH-P released by the cells were measured by radioimmunoassay, using specific antibodies. Left panel: P_4 and 20α-OH-P levels in the media of luteal cell incubations. Right panel shows progestagen release by the cells, defined as: P_4 + 20α-OH-P. Results are given as means ± SE. Asterisks indicate significant differences with respect to controls ($P < 0.01$).

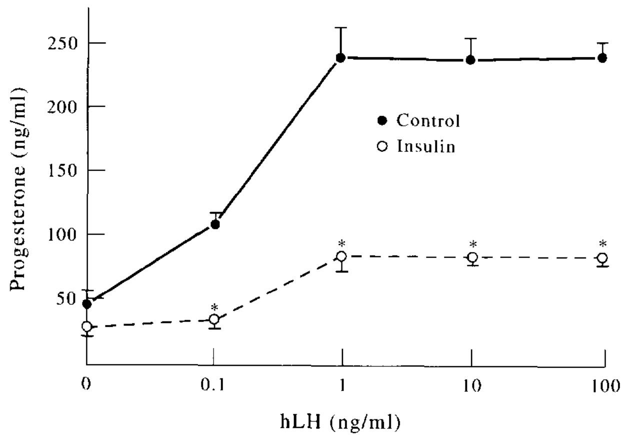

Fig. 2. Effect of increasing doses of hLH on P_4 accumulation by luteal cells. Cells (200,000/0.5 ml), obtained from control or insulin-treated rats, were incubated for 3 h with the indicated amounts of hLH at 37°C. At the end of the incubation, tubes were centrifuged for 15 min at 800 *g* at 4°C and supernatants kept frozen until P_4 determination by radioimmunoassay. Data are means ± SE of triplicate incubations. Asterisks indicate significant differences with respect to corresponding controls ($P < 0.05$).

ever, LH stimulation was greater for both steroids in control cells (P_4: 5-fold vs 2.7-fold stimulation for groups C and I, respectively; 20α-OH-P: 6.3-fold vs 2.3-fold stimulation for groups C and I, respectively).

Scatchard plotting of binding data derived from luteal cells revealed the presence of a single class of high affinity binding sites for [^{125}I]hCG (Fig. 4). In the control group, the apparent dissociation constant (K_d) was 0.059 ± 0.007 nM, while the number of hCG binding sites (Q) was 388,834 ± 14,146 sites/cell. The K_d was not modified in luteal cells from insulin-treated rats but the number of binding sites was reduced (K_d: 0.076 ± 0.016 nM, Q: 303,057 ± 13,392 sites/cell, $P < 0.001$). The decrease in the number of LH binding sites could be responsible for the diminished responsiveness of the cells to hormonal stimulation *in vitro*.

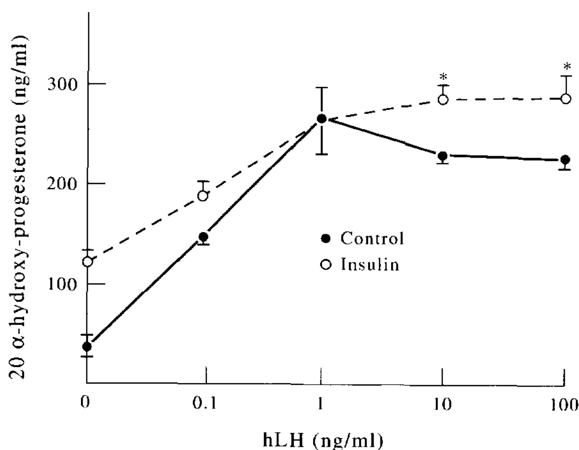

Fig. 3. Effect of increasing doses of hLH on 20α-OH-P accumulation by luteal cells. Cells (200,000/0.5 ml), obtained from control or insulin-treated rats, were incubated for 3 h with the indicated amounts of hLH at 37°C. At the end of the incubation, tubes were centrifuged for 15 min at 800 *g* at 4°C and supernatants kept frozen until 20α-OH-P determination by radioimmunoassay. Data are means ± SE of triplicate incubations. Asterisks indicate significant differences with respect to corresponding controls ($P < 0.05$).

Aromatase activity was assessed by adding androstenedione (0.25 μM) to the incubation media. Neither basal nor hLH-stimulated luteal estradiol production were modified by the *in vivo* insulin treatment (data not shown).

The specific amounts of immunoreactive cytochrome $P450_{SCC}$ were studied in luteal cells obtained from control and insulin-treated rats. Mitochondrial fractions of luteal cells were subjected to SDS–polyacrylamide gel electrophoresis and Western blotting with a specific antibody (see Fig. 5). The effect of *in vivo* insulin treatment was to induce synthesis (or decrease the degradation rate) of immunoisolated proteins which migrated on SDS–polyacrylamide gels in regions corresponding to cytochrome $P450_{SCC}$. The molecular weight of the cytochrome $P450_{SCC}$, as estimated from the position of standards, was 56,000.

DISCUSSION

The purpose of this study was to evaluate the effect of insulin administration on the luteal cell steroidogenesis of superovulated rats. Results indicate that insulin treatment increases the steroidogenic activity of rat luteinized ovaries. This action of insulin is reflected in an increase in circulating blood levels of P_4 and in the progestagen production by isolated luteal cells obtained from treated rats.

Although the *in vitro* luteal P_4 accumulation is diminished in the cells recovered from treated rats, this phenomenon is accompanied by an important increase in the level of 20α-OH-P, suggesting activation of the conversion of P_4 to its main metabolite 20α-OH-P, probably due to an increase in the amount of available substrate. However, while basal progestagen production is higher in luteal cells from insulin-treated rats, LH responsiveness is diminished, possibly as a result of the decreased number of LH binding sites in these cells.

Even though *in vitro* insulin and IGF-I action on progesterone biosynthesis has been exhaustively studied in rat granulosa [8, 9, 35] or luteal cells [19, 20, 36, 37], this study is the first to demonstrate an *in vivo* effect of insulin on luteal progesterone synthesis and LH responsiveness. We have previously shown [20] that low concentrations of insulin (0.2 nM) stimulate progesterone production in luteal cell cultures, a result consistent with the high affinity component detected by Scatchard analysis of luteal cells [19]. These findings suggest that, as postulated in other models [1, 7], under *in vitro* conditions insulin acts through its homologous luteal receptor and not by interaction with IGF-I receptors. The present results do not allow the elucidation of which receptors are

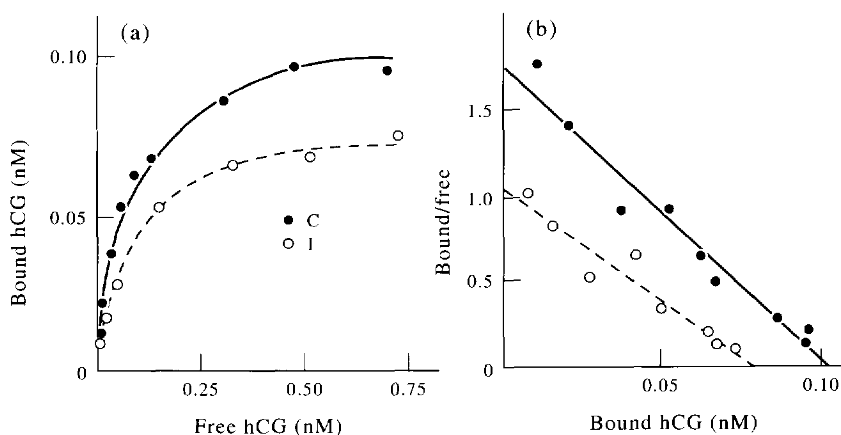

Fig. 4. (a) Saturation plot of [^{125}I]hCG specifically bound to rat luteal cells from control (C) or insulin-treated (I) rats. Aliquots of cell suspensions (40,000 cells/0.25 ml) were incubated for 3 h at 37°C with increasing concentrations of [^{125}I]hCG (6×10^{-11}–3×10^{-9} M). Parallel incubations were carried out in the presence of 5 μg nonradiactive hCG in order to determine nonspecific binding. Tubes were then centrifuged for 15 min at 1500 *g* at 4°C and supernatants, containing unbound hormone, removed. Specific binding of [^{125}I]hCG was calculated by substracting the nonspecific binding observed in the presence of excess nonradiactive hCG from the binding obtained with [^{125}I]hCG alone. (b) Scatchard plot of the same data, showing that insulin treatment down-regulated a single class of high affinity binding sites.

involved in the *in vivo* insulin stimulation, since it is difficult to determine the amount of hormone that actually reaches the corpus luteum.

On the other hand, the possibility that other hormones whose secretion is sensitive to the hypoglycemic state reached after insulin administration have an effect on the parameters investigated cannot be discarded. However, the *in vitro* insulin action mentioned above supports a direct effect of the hormone on luteal function.

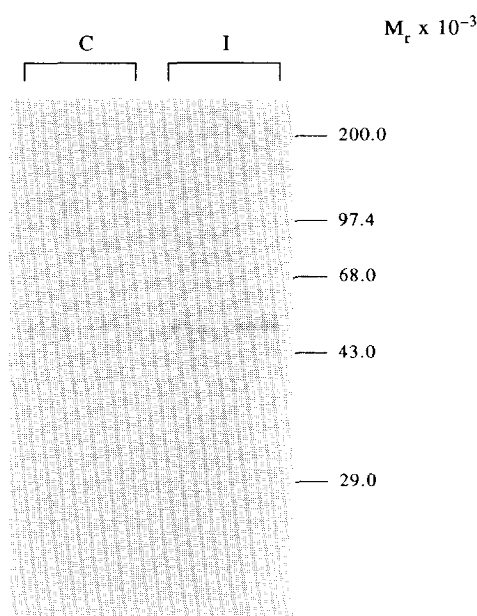

Fig. 5. Effect of *in vivo* insulin treatment on the contents of cytochrome $P450_{SCC}$ in luteal cells. Mitochondrial fractions from control (C) or insulin-treated (I) rats were subjected to SDS–polyacrylamide gel electrophoresis and Western blot with a specific antibody. Positions of prestained molecular weight markers ($\times 10^{-3}$) are indicated on the left.

Surprisingly, insulin treatment caused a down-regulation of LH receptors in isolated luteal cells. Homologous down-regulation of LH receptors has been extensively described in ovarian and Leydig cells [38–42], but there are few reports concerning the *in vivo* regulation by insulin of LH receptors. Chronic diabetic states in rats produce a decrease in LH receptors of Leydig or luteal cells [28, 34]. Several studies demonstrated that both insulin and growth factors can enhance FSH induction of LH receptors in cultured rat granulosa cells [15, 16]. However, other reports have shown that insulin inhibits LH receptor formation [17, 18]. Furthermore, heterologous down-modulation of LH receptors by prolactin has been described in cultured porcine granulosa cells [43, 44]. Using differential labeling and flow cytometry techniques, Lane *et al.* demonstrated that prolactin and LH can be cointernalized and entrapped in identical endosomes. These authors suggest that elevated prolactin levels induce a cointernalization of prolactin receptors and unoccupied LH receptors and this would provide a cellular basis for the ovarian unresponsiveness associated with hyperprolactinemia. A similar mechanism might be taking place in our experimental model, where a down-regulation of luteal LH receptors is associated with hyperinsulinemia.

It has been suggested that the synthesis of side-chain cleavage cytochrome $P450$ could be one of the rate-limiting steps of steroid biosynthesis in steroidogenic tissues [45–47]. Trophic hormones, namely ACTH or gonadotropins, which act via the classic cyclic AMP pathway, enhance protein and mRNA contents of cytochrome $P450_{SCC}$ and adrenodoxin in adrenal and ovarian cells [23, 48, 49]. By means of an immunoblotting technique, we have observed an increase in the content of mitochondrial $P450_{SCC}$ in luteal cells from

insulin-treated rats. This could be one of the mechanisms responsible for the increase in serum progesterone and *in vitro* progestagen production in the insulin-treated group. In agreement with our results, Veldhuis *et al.* [22] demonstrated that IGF-I increases the synthesis of cytochrome $P450_{SCC}$ and adrenodoxin in porcine granulosa cell cultures. Since IGF-I and insulin act through a cyclic AMP-independent pathway (beginning with the tyrosine phosphorylation of their plasma membrane receptors), an alternate mechanism has been suggested whereby steroidogenic cells respond to hormonal stimulation by increasing the synthesis of cholesterol side-chain cleavage components [22].

In summary, we conclude that *in vivo* insulin treatment of superovulated rats produces an increase in the content of cholesterol side-chain cleavage cytochrome $P450$, with a concomitant rise in serum progesterone levels and *in vitro* luteal progestagen production. On the other hand, hyperinsulinemia produces a down-regulation of luteal LH receptors accompanied by a decrease in LH responsiveness. These results could contribute to the understanding of some ovarian dysfunctions frequently observed in insulin-resistant syndromes.

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