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Inhibition of Steroidogenesis by Luteal Cells of Early Pregnancy in the Rat in Response to *In Vitro* Administration of a Gonadotropin-releasing Hormone Agonist

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Previous studies from this laboratory have demonstrated that the administration of a gonadotropin-releasing hormone agonist (GnRH-Ag) *in vivo* in early or mid-pregnancy to rats induces antifertility effects by suppressing the luteal production of progesterone (P_4) within 24 h with a concomitant increase in luteal lipid droplets and decreases in the luteal cytochrome *P*450 side chain cleavage (*P*450scc) enzyme and its mRNA content. These observations suggest a direct inhibitory effect of GnRH-Ag on the corpus luteum. Here we demonstrate a suppressive effect of GnRH-Ag *in vitro* on the basal P_4, pregnenolone (P_5) and 20α-dihydroprogesterone (20α-DHP) production by luteal cells obtained during early pregnancy in rats. We further studied its effect on two key enzymes, namely *P*450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD), which participate in the conversion of cholesterol to P_5 and conversion of P_5 to P_4, respectively. We observed that two doses of GnRH-Ag, 10^{-4} and 10^{-7} M, suppress the basal P_4 production *in vitro* after 12 h of incubation by luteal cells; P_4 remained suppressed after 48 h of incubation. Basal P_5 production was also suppressed after luteal cells were incubated for 12 h with 10^{-4} and 10^{-7} M GnRH-Ag, but incubation for 48 h with GnRH-Ag failed to alter P_5 production by these cells. 20α-DHP production was suppressed after incubating the luteal cells with both doses of GnRH-Ag for 12 h. GnRH-Ag inhibited *P*450scc activity after 12 h of incubation and 3β-HSD protein content at all time periods measured. These results suggest that GnRH exerts a direct inhibitory effect on luteal steroidogenesis. This inhibition is due to its suppressive effect on *P*450scc and/or 3β-HSD and not due to an increase in P_4 metabolites.

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INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is indispensable for normal reproduction as it regulates the secretion of gonadotropins by the pituitary. However, chronic administration of GnRH or its highly potent agonists induces a paradoxical antireproductive effect in both sexes of numerous mammalian species [1–4]. The luteolytic effect of GnRH is well documented in several pregnant and pseudopregnant rodent models [5, 6] and is due to the diminished production of progesterone (P_4). Earlier studies from this laboratory have demonstrated that *in vivo* administration of a GnRH agonist (GnRH-Ag) suppresses luteal P_4 synthesis with a concomitant increase in lipid droplets and a decrease in the number of tubular cristae within the mitochondria of the corpus luteum [7]. Furthermore, the GnRH-Ag treatment causes a decrease in the luteal cytochrome *P*450 side chain cleavage (*P*450scc) enzyme and its mRNA content [7]. These observations suggest a direct inhibitory effect of GnRH-Ag on luteal steroidogenesis. There is much evidence in the literature that implicates extrapituitary, direct inhibitory effects of GnRH or its agonists on ovarian steroidogenesis [8–10]. However, these studies have primarily

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focused on LH- or hCG-stimulated P_4 production by luteal cells obtained from the luteinized ovary of immature rats. In the present study we have examined the effect of various doses of a GnRH-Ag on basal steroid production by luteal cells obtained from pregnant rats, viz. P_4 and pregnenolone (P_5), a precursor of P_4, as well as 20α-dihydroprogesterone (20α-DHP), which is a metabolite of P_4. Further, we estimated the activity of *P*450scc, which is a rate limiting enzyme involved in the synthesis of P_5 from cholesterol, and 3β-hydroxysteroid dehydrogenase (3β-HSD), which catalyzes the oxidation of P_5 to P_4 to examine the possible locus/loci of GnRH-Ag action on the steroidogenic pathway.

EXPERIMENTAL

Reagents

Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}, 0.4% solution of trypan blue, 0.02% solution of ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA; endotoxin tested), HEPES buffer, collagenase type IV, penicillin, streptomycin and gentamicin were purchased from Sigma Chemical Co. (St Louis, MO). Medium 199, 0.25% trypsin–EDTA and fetal bovine serum were obtained from GIBCO (Grand Island, NY). DNAse I and dispase (neutral protease) were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). GnRH-Ag ([Pyro]-Glu-His-Trp-Ser-Tyr-D-TRP-NMeLEU-Arg-Pro-Ethylamide-LHRH; Wyeth-40972) was provided by Dr A. Corbin, Wyeth-Ayerst Lab. (Philadelphia, PA); [^{3}H]P_4 and [^{3}H]20α-DHP were purchased from NEN Research Products (Boston, MA). P_4 antiserum (GDN-337) was provided by Dr G. D. Niswender (Fort Collins, CO) and antiserum to 20α-DHP was a gift from Dr K. Quadri (Manhattan, KS). [^{3}H]P_5, standards and antiserum for P_5 were purchased from ICN Biomedicals (Costa Mesa, CA).

Animals

Timed-pregnant Sprague–Dawley rats were purchased from Holtzman Co. (Madison, WI) and maintained at 23 to 25°C with a daily 14 L:10 D photoperiod. Purina rat chow and water were available *ad libitum*. The day of insemination was designated as day 1 of pregnancy.

Tissue collection and enzymatic dissociation

On day 8 of pregnancy (with day 1 as day of insemination), ovaries were dissected with rats under ether anesthesia. Corpora lutea were removed carefully from the adhering follicles and placed into Medium 199 containing 2.2 g/l of sodium bicarbonate previously gassed with 95% O_2:5% CO_2. The methodology used for enzymatic dissociation of corpora lutea has been described previously [11]. After dissociation, the cells were counted with a hemocytometer and viability was assessed after brief incubation of the cells with 0.4% trypan blue solution. Approximately 4–5 × 10^5 cells per ml of complete Medium 199 (containing 25 mM Hepes buffer, 100 IU of penicillin and 100 μg of streptomycin/ml, 50 μg of gentamicin/ml, and 10% fetal bovine serum) were plated in 24-well Falcon tissue culture plates and incubated for approx. 90 min in an atmosphere of 95% air:5% CO_2 at 37°C for the attachment of the cells. After this period of preincubation, medium was replaced with fresh medium, and then the attached cells were treated with GnRH-Ag 10^{-4} or 10^{-7} M, dissolved in 0.9% saline in a total volume of 10 μl/ml of medium/well. The cells were then incubated for 4, 12 or 48 h in an atmosphere of 95% air:5% CO_2 at 37°C. Control groups of cells received 10 μl of saline/ml of medium/well. At the end of the designated incubation period, the medium was aspirated, snap frozen and stored at −20°C until assayed for P_4, P_5 and 20α-DHP. Cells were detached using 0.25% trypsin–EDTA solution, and one set of cells was processed for the estimation of *P*450scc activity and another set was snap frozen and stored at −70°C until subjected to immunoblot analysis of 3β-HSD.

RIAs

P_4 was measured in the medium as described previously [12]. This assay uses a specific antibody (GDN-337) prepared against P_4. The specificity of antiserum was very high and the sensitivity of the assay was 0.10 ng/assay tube. Inter- and intra-assay coefficients of variation were <14.2 and <3.4%, respectively.

P_5 was measured in the medium using a specific antibody. This has shown <0.01% cross-reactivity with other steroids. Sensitivity of the assay was 0.25 ng/assay tube. The coefficients of variation between and within assays were <11.4 and 4.2%, respectively.

20α-DHP was assayed in the medium by employing a specific antiserum. Sensitivity of the assay was 0.01 ng/assay tube. Inter- and intra-assay coefficients of variation were <10.2 and 3.8%, respectively.

3β-HSD protein immunoblot analysis of luteal cells

Luteal proteins were fractionated by SDS–PAGE and electroblotted onto nitrocellulose filters [13]. Purified human placental 3β-HSD and BRL protein weight markers were used for a positive control and estimation of molecular size, respectively. The blots were washed three times for 30 min with the solution containing 5% fat-free milk (Carnation)/0.15 Nonidet P-40 in phosphate buffered saline (PBS) and then incubated for 18 h at 4°C with a 1:2000 dilution of rabbit antiserum raised against purified human placental 3β-HSD. The blots were washed three times (30 min/wash) and incubated for an additional 4 h at 4°C in a 1:1000 dilution of ^{125}I-labeled goat antirabbit immunoglobulin G. After three more washes in the same solution, the filters were radioautographed at

−80°C using an intensifying screen and XAR-5 film. The intensity of the signal emitted by ^{125}I-antibodies recognizing the 42 kDa protein was quantified using an Amersham RAS Image Analyzer System.

Estimation of P450scc activity

*P*450scc activity was estimated using a fluorogenic probe (22-phenoxazonoxy-5-cholene-3β-ol), the synthesis and characterization of which has been reported elsewhere [14, 15]. This fluorescent probe was used for the measurement of the acute effects of GnRH-Ag on cholesterol conversion to P_5. The fluorogenic probe, a cholene-based steroid with a fluorogenic moiety (resorufin) strategically placed at the site of side chain cleavage, when exposed to *P*450scc enzyme, yields P_5 and the highly fluorescent resorufin. This sensitive fluorescent signal represents the enzyme activity. A stock solution (100 μM) of the *P*450scc probe was made in 95% ethanol and filtered with a 0.2 μm filter before use. The probe was added in a 1:100 dilution to the cell suspension. Luteal cells suspended in Medium 199 without phenol red were incubated with this fluorogenic probe for 2 h in a water bath at 37°C. Fluorescence emission measurements were obtained on a Perkin–Elmer 650-40 fluorescence spectrophotometer fitted with a thermostatted cuvette holder. Resorufin fluorescence was measured from 550–700 nm (8 nm bandpass) with 530 nm excitation (10 nm bandpass). Maximum fluorescence intensity was observed at the emission wavelength of 586 nm. Spectra were background-corrected by spectral subtraction of the initial (zero) time point.

Statistics

There were 3–7 replicates in each experiment and 3 independent experiments were done. Data from independent experiments were pooled for presentation except when immunoblot analysis of luteal content of 3β-HSD was presented. The experiments for immunoblot analysis of luteal content of 3β-HSD were performed twice. Hormone concentrations were normalized to 5×10^5 cells.

The data were analyzed by one-way analysis of variance followed by Scheffe's test when differences were significant. $P < 0.05$ was considered significant.

RESULTS

Basal levels of steroid production by luteal cells (Fig. 1)

Basal levels of P_5, P_4 and 20α-DHP in control groups at various periods of incubation are shown in Fig. 1. These data indicate that the maximum production of P_5 occurred at 4 h and that of P_4 occurred at 48 h of incubation, respectively. The levels of 20α-DHP increased gradually with the progression of incubation.

Effect of GnRH-Ag on the luteal 3β-HSD protein content (Fig. 2)

The data from the first experiment is given in Fig. 2. Immunoblot analysis of luteal cells in the control group shows a progressive increment in the luteal content of

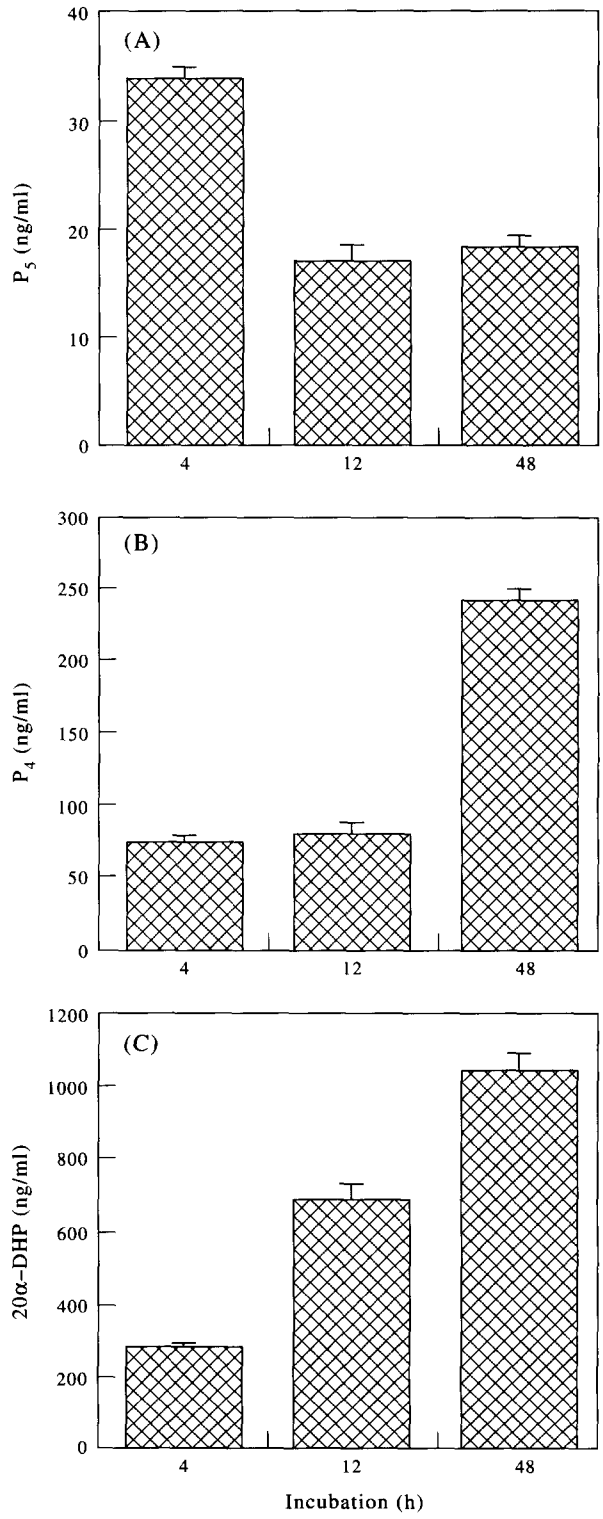

Fig. 1. Production of P_5 (A), P_4 (B) and 20α-DHP (C) by the luteal cells in control groups incubated for different time periods. Values are mean ± SEM.

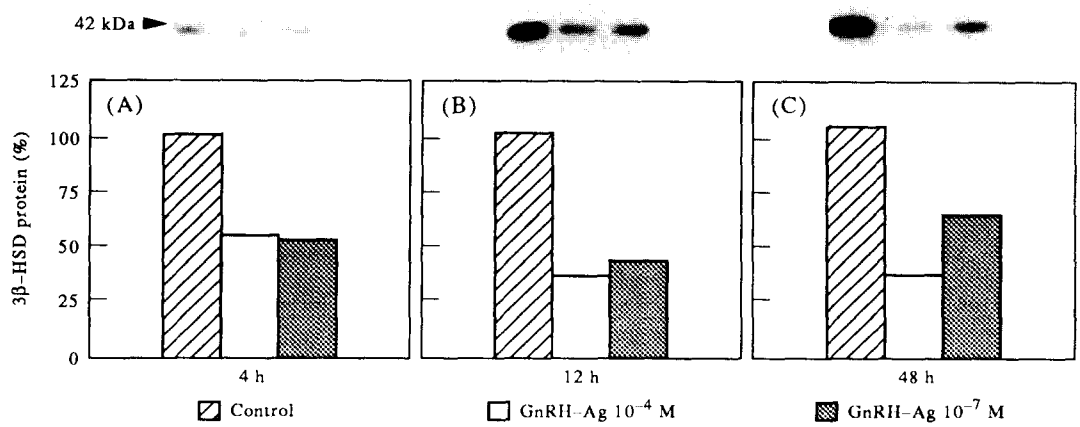

Fig. 2. Effect of GnRH-Ag treatment on the luteal 3β-HSD content as revealed by immunoblot analysis (upper panels). Histogram in the lower panel corresponding to each blot represents the densitometric units expressed in % compared to its own control values at each time period.

3β-HSD with the highest levels at 48 h of incubation (arbitrary densitometric units: *first experiment*: 4 h:9.5; 12 h:15.2 and 48 h:18.5; *repeat experiment*: 4 h:4.37; 12 h:8.1 and 48 h:11.8). This is consistent with the P_5 and P_4 levels shown in Fig. 1.

Even as early as 4 h after GnRH-Ag treatment, 3β-HSD protein content was decreased in the luteal cells by 45 and 47% (*repeat experiment*: 42 and 40%) with two doses of GnRH-Ag, 10^{-4} and 10^{-7} M, respectively. There was a considerable decrease in the luteal 3β-HSD protein content after 12 h of incubation, with values being 67 and 58% (*repeat experiment*: 74 and 71%) lower than control values with the higher and lower doses of GnRH-Ag, respectively. While the attenuation of luteal 3β-HSD content at 48 h was similar to that at 12 h with higher dose of GnRH-Ag, the attenuation was not as steep with the lower dose when compared to levels at 12 h of incubation.

Effect of GnRH-Ag on basal steroid production by luteal cells (Fig. 3)

GnRH-Ag at doses 10^{-4} and 10^{-7} M decreased the P_4 production by luteal cells within 12 h after the commencement of treatment and production remained suppressed after 48 h of incubation. Neither dose of GnRH-Ag suppressed the P_4 production when incubated for 4 h with luteal cells.

Incubation of luteal cells with two different doses of GnRH-Ag for 4 h was ineffective in altering the P_5 production. Nonetheless, both doses of GnRH-Ag inhibited P_5 production by luteal cells within 12 h. An extended period of incubation (48 h) with both doses of GnRH-Ag failed to alter P_5 production by luteal cells.

Incubation of luteal cells with two different doses of GnRH-Ag for 4 h was ineffective in altering 20α-DHP production as compared to control. However, both doses of GnRH-Ag suppressed 20α-DHP production by the luteal cells after 12 h of incubation. Incubation for 48 h with either dose of GnRH-Ag failed to alter the 20α-DHP production by the luteal cells.

Effect of GnRH-Ag treatment on the P450scc activity of the luteal cells (Fig. 4)

Luteal cells incubated for 12 h with two doses of GnRH-Ag showed a marked decrease in the maximum fluorescence intensity after incubating them with *P*450scc probe. Incubating the luteal cells for 4 or 48 h with both doses of GnRH-Ag was ineffective in altering the *P*450scc activity as measured by fluorescence intensity. These results demonstrate that *P*450scc activity was maximally suppressed after 12 h of incubation.

DISCUSSION

The results of these experiments have clearly demonstrated, for the first time, the direct suppressive effect of GnRH-Ag on basal P_4 production by luteal cells obtained during early pregnancy prior to any stimulation with LH or hCG. In this study, we observed that both doses of GnRH-Ag suppressed P_5 production by luteal cells after 12 h of incubation. This suggests a probable lesion at the luteal *P*450scc following treatment with GnRH-Ag. We observed a suppression in the activity of *P*450scc after incubating the luteal cells for 12 h with both doses of GnRH-Ag. This corroborates an earlier study in this laboratory wherein *in vivo* treatment with GnRH-Ag during early pregnancy suppressed the luteal *P*450scc enzyme and its mRNA expression within 24 h [7]. In the present study, we also observed that both doses of GnRH-Ag suppressed P_4 production by the luteal cells after 12 and 48 h of incubation. This is suggestive of an inhibitory action of GnRH-Ag on the luteal 3β-HSD. We did notice a decrease in the 3β-HSD content of luteal cells after incubating them with two doses of GnRH-Ag at 4, 12 or 48 h, although we could not observe an attenuation in P_4 production of luteal cells incubated for 4 h with GnRH-Ag treatment. Therefore, the data presented here support the hypothesis that the two key enzymes of steroidogenic pathway, *P*450scc and 3β-HSD, are involved in the suppression of luteal steroidogenesis by

GnRH-Ag, probably due to its direct effect on these enzymes. Our findings are consistent with the results of the earlier studies in which GnRH is shown to suppress P_4 synthesis by decreasing P_5 production by cultured rat granulosa cells [16] or the conversion of P_5 to P_4 by inhibiting the 3β-HSD [17]. However, contrary to the report that GnRH further reduces the production of P_4 by cultured rat granulosa cells by stimulating 20α-HSD dehydrogenase activity [18], which results in the enhanced levels of 20α-DHP, we observed, using luteal cells of pregnant rats, that both doses of GnRH-Ag inhibited the production of this metabolite after 12 h of incubation. Therefore, it seems unlikely that the inhibition in P_4 production is due to a stimulation in 20α-hydroxysteroid dehydrogenase, although we did not estimate the activity of this enzyme. On the basis of our post-incubation trypan blue dye exclusion test, the reduced luteal production of P_4 or P_5 in GnRH-Ag treated rats cannot be associated with cell death.

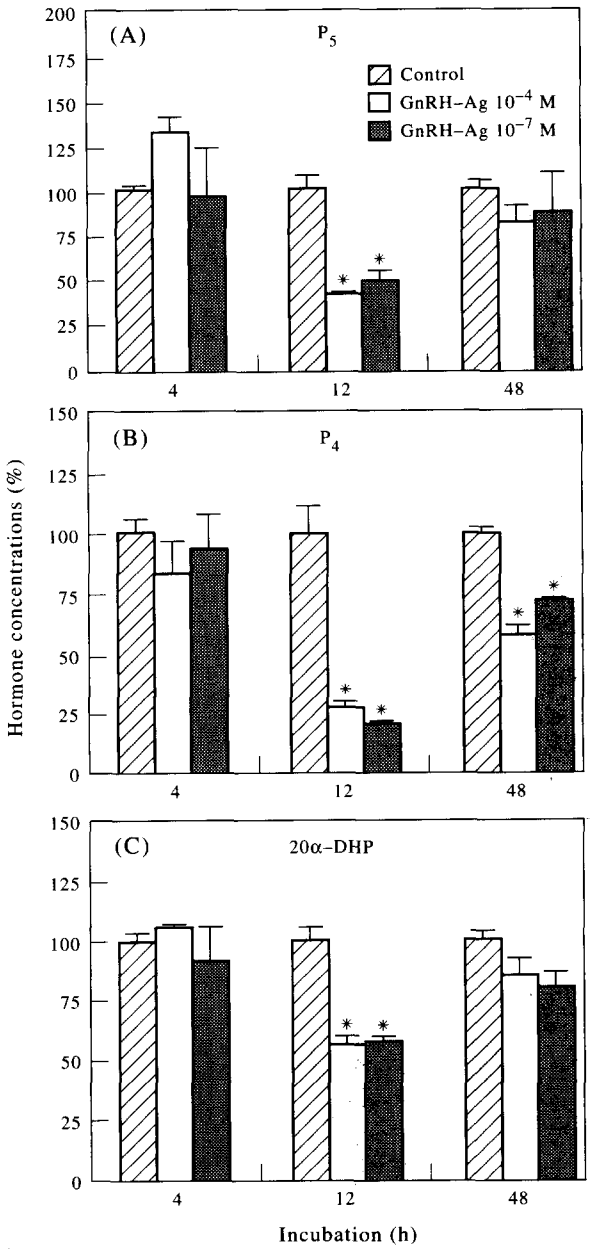

Fig. 3. Effect of GnRH-Ag treatment on steroid hormone production by luteal cells incubated for different time periods. Values are mean ± SEM expressed in % as compared to its control value at each time period. *$P < 0.05$ compared to its control.

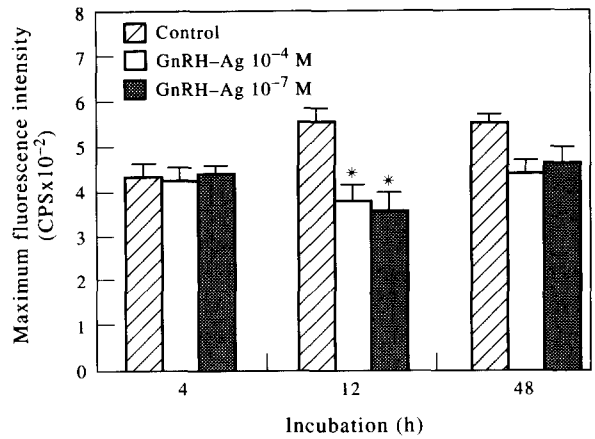

Fig. 4. Effect of GnRH-Ag treatment on the maximum fluorescence intensity of the luteal cells incubated with a specific fluorogenic probe for *P*450scc (as described in Experimental). CPS, counts per second. Values are mean ± SEM. *$P < 0.05$ compared to its control.

There are several reports available wherein the P_4 production by corpora lutea that were stimulated by LH (not basal levels of P_4) was suppressed by GnRH or its highly potent agonists [19–22]. Our findings in this report corroborate the earlier work [19] which demonstrated that GnRH-Ag suppressed the P_4 production by luteal cells obtained from the 25-day-old immature rats, previously treated with eCG and hCG. However, the results are contrary to the report wherein basal P_4 secretion from luteal cells appears to be unaffected in an immature rat model, previously treated with eCG and hCG, by different doses of LHRH or (D-Trp6) LHRH [23]. The possible mechanism of GnRH suppression of LH-stimulated P_4 secretion appears to be the interference of the coupling of LH–receptor complex to adenylate cyclase, thus impairing the cAMP generation and hence, P_4 production [23]. *In vivo* GnRH-Ag treatment failed to alter the luteal cAMP production during pregnancy (Sridaran *et al.*, unpublished observations). Furthermore, earlier studies from this laboratory have clearly shown that GnRH-Ag treatment *in vivo* has considerably reduced the ability of corpus luteum to synthesize P_4 without any significant changes in its ability to synthesize testosterone or estradiol during early [24] or mid [25] pregnancy. Luteal testosterone production during early pregnancy in the rat is LH-mediated [26]. Therefore, in the present study, it seems that suppression of P_4 after *in vitro* treatment cannot be associated with the LH-mediated event, but is due to an effect that is beyond the gonadotropic hormone–receptor inter-

action, probably mediated by specific receptor sites for GnRH in the luteal cells.

Furthermore, demonstration of specific receptor sites for GnRH or its analog in isolated luteal cells or particulate membrane-rich fractions prepared from luteinized ovaries [19] suggest a direct action of these compounds on the luteal cells by binding to these specific high affinity receptors. GnRH-Ag treatment has no effect on the number of GnRH receptors present in the luteal membranes during early pregnancy (Sridaran *et al.*, unpublished observations). Although these effects of GnRH-Ag on luteal steroidogenesis could merely reflect a pharmacological phenomenon, a physiological role for GnRH on luteal function could not be eliminated. Recent demonstration of GnRH mRNA expression in granulosa cells by RT-PCR [27] and the expression of the GnRH gene [28] in different components of the rat ovary by *in situ* hybridization histochemistry [29] strongly implies a paracrine or autocrine function for GnRH-like molecule, which may be actively participating under normal physiological conditions in modulating ovarian steroidogenesis.

Earlier observation of the steroidogenic response of two different populations of luteal cells with regard to various substrates by our laboratory [11], and demonstration of two to three times more cytochrome *P*450scc in the large luteal cells [30], asserts that large luteal cells are the primary source of P_4 production. Therefore, it will be of interest to elucidate the suppressive effect of GnRH-Ag on these two different populations of cells, and studies are in progress in this laboratory in this regard.

GnRH has been shown to increase the calcium influx into the pituitary cells [31, 32]. The intracellular signalling biochemistry of GnRH in the corpus luteum appears to be identical to that in the pituitary [33]. On the basis of earlier observations [23], it is suggested that the cellular mediator for such antigonadotropic action could be calcium. We have observed that there is a rapid and transient increase in the Ca^{2+} levels in the large luteal cells after treating them with GnRH-Ag (Sridaran *et al.*, unpublished observations). In rat granulosa cells, the binding of GnRH to its membrane receptor activates the rapid breakdown of polyphosphoinositides into inositol phosphates and diacylglycerol. Inositol phosphates, especially 1,4,5, triphosphates, are known to mobilize the calcium from its intracellular stores, whereas diacylglycerol is known to activate protein kinase C [34]. GnRH-induced changes in intracellular calcium concentrations have been shown to modify second messenger generation in rat granulosa cells [35, 36]. However, the existence of such a system in the rat luteal cells of pregnancy remains to be elucidated.

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