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Levonorgestrel Inhibits Luteinizing Hormone-Stimulated Progesterone Production in Rat Luteal Cells

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The effects of the synthetic progestin levonorgestrel (LNG) on basal and LH-stimulated progesterone production were studied in collagenase-dispersed luteal cells obtained from 9-day pregnant rats. Luteal cells responded to ovine LH (oLH) with an increase in progesterone output which was maximal at a dose of 100 ng/ml. No effect of LNG was observed at 0.1–10 μM, but at 100 μM, it inhibited basal progesterone production. On the other hand, a dose of 10 μM LNG suppressed the stimulation of progesterone secretion induced by oLH, dibutyryl-cAMP and pregnenolone. It is suggested that the possible mechanism of action of the progestin involves a post-cAMP site and, in some way, may lead to an interference with 3β-hydroxysteroid dehydrogenase activity, which catalyzes the formation of progesterone from pregnenolone, the last step of progesterone biosynthesis. This study provides a different point of view supporting an autocrine control mechanism by which progesterone, the principal steroidogenic product of luteal cells, may exert a negative ultra-short loop regulation of its own biosynthesis.

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INTRODUCTION

Rat ovarian steroidogenesis is primarily controlled by pituitary and placental hormones [1]. However, the possibility of autocrine and paracrine controls of steroid production in the rat ovary has been debated. There is a growing body of empirical evidence that supports the hypothesis originally proposed by Rothchild [2], that progesterone would modulate its own secretion at the ovarian level via a feedback mechanism. The synthetic progestin R5020 augments gonadotropin stimulated progesterone production in rat granulosa cells [3]. The relevance of these findings is supported by the observation of progesterone receptors in the nuclei of rat granulosa cells [4, 5] as well as messenger ribonucleic acid encoding for progesterone receptors [6]. Rat corpora lutea incubated with the synthetic anti-progesterone RU-486 (17β-hydroxy-11β-[4-dimethyl-aminophenyl]-17α-[1-propynyl]-estra-4,9-diene-3-one) which has no agonist effect [7], respond with an increase in progesterone production [8]. These results suggest the existence of luteal progesterone receptors in this species.

Rat luteal cells produce progesterone in the absence of LH, but respond to an acute LH stimulus with increased progesterone synthesis [9, 10]. LH exerts its acute effects on steroidogenesis mainly through the activation of adenylyl cyclase, with the resultant increase in intracellular cAMP and activation of cAMP-dependent protein kinases [11].

Levonorgestrel (D-[*1*]-norgestrel) is a synthetic 13β-ethyl substituted 19-nor steroid (13β-ethyl-17α-ethynyl-17β-hydroxy-4-gonen-3-one) [12] used as a potent progestational agent [13, 14]. The aim of the present study was to evaluate, using this synthetic progestin, which does not interfere with progesterone measurement, the effect of progestins on LH-stimulated progesterone production by rat luteal cells.

EXPERIMENTAL

Animals

Pregnant rats bred in our laboratory (originally Wistar strain; day 0 = sperm positive), with free access to standard rat chow (Nutric, Córdoba, Argentina) and water, kept under controlled conditions of light (lights on from 06.00 to 20.00 h) and temperature (22–24°C), were used throughout.

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Materials

[1,2,6,7-^{3}H]progesterone (98 Ci/mmol) was purchased from Amersham Life Science (U.K.). Levonorgestrel (LNG) was a gift from Shering (Buenos Aires, Argentina) and medium 199 was from Gibco BRL (Grand Island, U.S.A.). Pregnenolone, dibutyryl-adenosine 3′:5′-cyclic monophosphate ($[Bu]_2$-cAMP), bovine serum albumin fraction V, EDTA and collagenase (type IV) were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Ovine LH (oLH-26) was a generous gift from NIDDK (Bethesda, MD). Other reagents and chemicals were of analytical grade. Luteal cells were incubated in 24-well plastic tissue culture dishes (Corning Laboratory Sciences Co., U.S.A.).

Preparation of collagenase-dispersed cells

On day 9 of pregnancy, the rats were killed by decapitation and the ovaries were collected immediately and placed in PBS–BSA 1% (pH 7.4). The corpora lutea were dissected under a stereoscopic microscope by means of small forceps and a needle; they were placed in a flask containing gassed PBS–BSA 1% with 1 mg/ml of collagenase, using 2 ml for the corpora lutea obtained from six rats. The dissected corpora lutea were maintained in a shaking, 37°C waterbath and stirred at 100 rpm for 1 h. After incubation, the tissue was centrifuged 5 min at 800 *g* and the supernatant was discarded. The cell pellet was suspended in 2 ml of PBS–BSA 1%-EDTA 1 mM for 3 min and again centrifuged for 5 min at 800 *g*. The new pellet was resuspended in 2 ml of medium 199, the cell suspension was filtered through Nytex mesh and counted in a Neubauer counting chamber. The viability of the cells was in the 90% range as determined by trypan blue staining. All experiments using dispersed luteal cells were performed at 37°C under an atmosphere of 95% air:5% CO_2 for 4 h and using viable cells (3×10^5) incubated in 1 ml culture media. At the end of the incubation period, cells were harvested and media were frozen at −20°C for subsequent RIA of progesterone.

Incubation of whole ovaries

Ovaries obtained from 9-day pregnant rats as explained above, were weighed and placed in flasks containing gassed medium 199; then the flasks were incubated in a 37°C waterbath under an atmosphere of 95% O_2:5% CO_2 for 2 h. After incubation, 1 ml aliquots of the medium were stored at −20°C until determination of progesterone.

Radioimmunoassay of progesterone and statistics

Progesterone was assayed in unextracted incubation medium using a radioimmunoassay developed in our laboratory with an antiserum raised against progesterone-11-bovine serum albumin conjugate in rabbits. The sensitivity and variability of this RIA has been previously reported [15]. The antiserum to progesterone cross-reacted 100% with progesterone; 5% with 20α-dihydroprogesterone, Δ^4-pregnan-17α-ol-3,20-dione and deoxycorticosterone; 0.5% with pregnenolone and testosterone; 0.05% with androstenedione, corticosterone and dehydroepiandrosterone; 0.01% with levonorgestrel; and <0.005% with oestradiol and cortisol.

Medium controls were run in triplicate for each treatment and these background levels were subtracted from each sample. Progesterone values measured in the absence of hormonal addition varied considerably between incubations. However, fold-effects of hormone additions were consistent. All results are given as the mean ± SEM of quadruplicate determinations and are representative of 3 different experiments. Statistical comparisons were made by one-way analysis of variance (ANOVA I) followed by Duncan's test for multiple comparisons. When variances were not homogeneous, $1/\sqrt{x}$ transformation of data was applied.

RESULTS

oLH Stimulation of progesterone synthesis in cultured luteal cells (Fig. 1)

To assess the steroidogenic capacity of the luteal cells obtained from 9-day pregnant rats, the cells were incubated for 4 h in culture media in the absence or presence of different concentrations of oLH. As shown in Fig. 1, maximal stimulation was obtained at a concentration of 100 ng oLH/ml.

Effect of LNG on progesterone production induced by oLH in cultured luteal cells and whole ovaries (Figs 2 and 3)

To evaluate the effects of treatment with LNG on LH-stimulated progesterone production, luteal

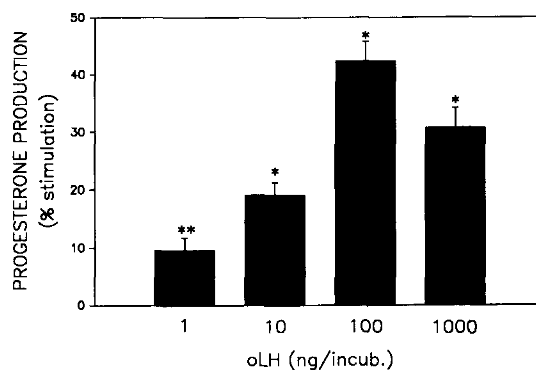

Fig. 1. Progesterone production by luteal cells obtained from 9-day pregnant rats in the presence of different concentrations of LH (oLH-26). Cells were incubated with oLH at 37°C under an atmosphere of 95% air:5% CO_2 for 4 h. Data are expressed as the percentage of progesterone stimulation compared to increase over basal values obtained in the absence of oLH. Values are the mean ± SEM of quadruplicate determinations of 3 different experiments. *$P < 0.01$ and **$P < 0.05$ vs basal.

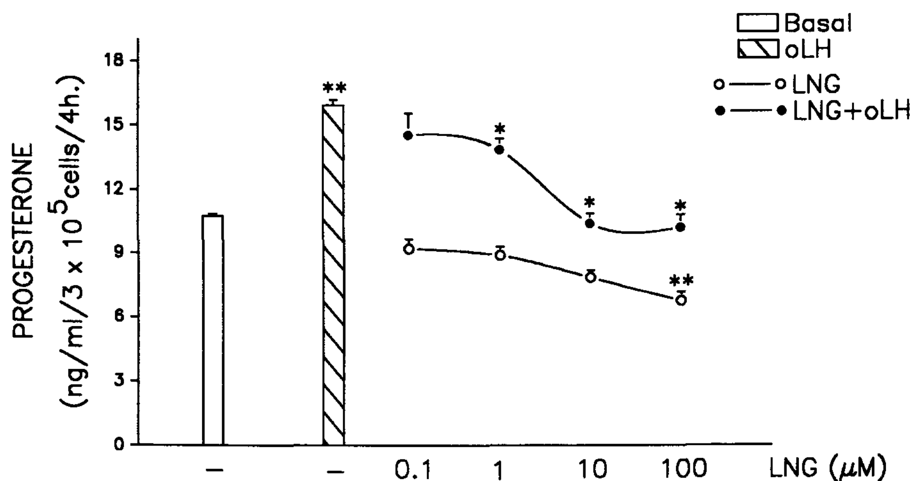

Fig. 2. Effect of increasing doses of LNG on basal (open circles) and LH-stimulated progesterone production (closed circles) in luteal cells of 9-day pregnant rats. Cells were incubated with oLH (100 ng/ml) at 37°C under an atmosphere of 95% air:5% CO_2 for 4 h and LNG was added to the culture medium 30 min before oLH. Results are expressed as ng/ml progesterone/3 × 10^5 cells/4 h ± SEM. Incubations were performed in quadruplicate determinations of 3 different experiments. *$P < 0.01$ vs oLH and **$P < 0.01$ vs basal.

cells were incubated in the presence or absence of LH with increasing doses of LNG (Fig. 2). Basal production of progesterone was unaffected by LNG at 0.1–10 μM. However, LNG at 100 μM inhibited basal progesterone production. Furthermore, pre-treatment with 1 μM of LNG significantly inhibited progesterone production induced by LH. When LNG was added to the incubation medium at concentrations greater than 1 μM (10 and 100 μM), it completely suppressed the LH-induced stimulatory effect of oLH on progesterone production.

As shown in Fig. 3, 100 ng/ml of oLH induced a more than 100% increase in progesterone production from whole ovaries obtained on day 9 of pregnancy, when compared with basal production of progesterone. When the whole ovaries were incubated in the presence of oLH with 10 μM of LNG, oLH-induced progesterone production was significantly reduced to basal production.

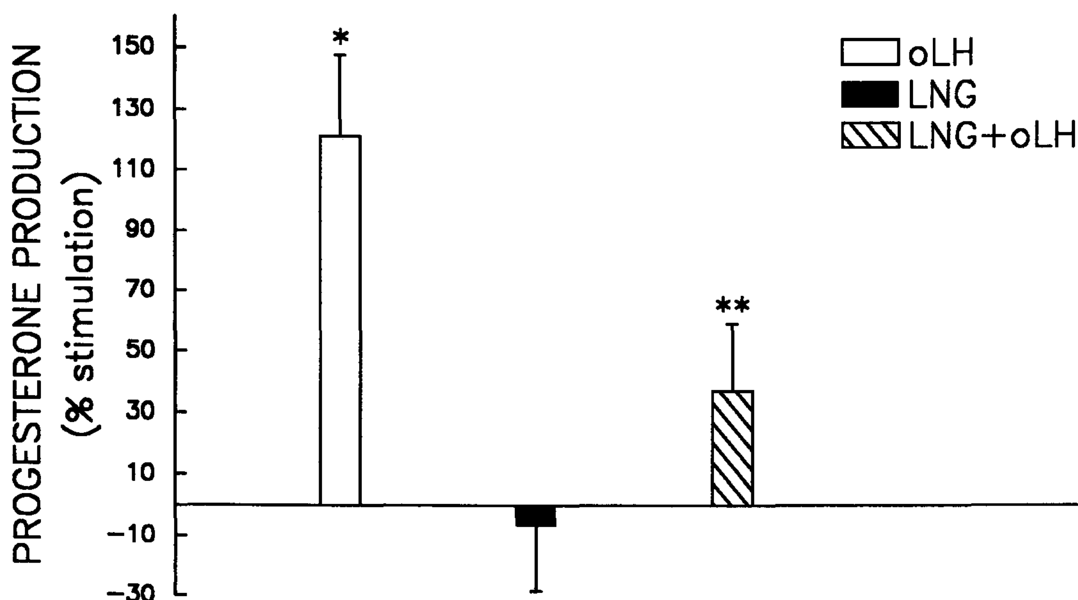

Fig. 3. Effect of LNG (10 μM) on basal and LH-stimulated progesterone production in whole ovaries of 9-day pregnant rats. Ovaries were incubated with oLH (100 ng/ml) at 37°C under an atmosphere of 95% O_2:5% CO_2 for 2 h and LNG was added to the culture medium 30 min before oLH. Values are expressed as the percentage of progesterone stimulation compared to increase over basal levels. Each bar is the mean ± SEM of quadruplicate determinations of 3 different experiments. *$P < 0.01$ vs basal and **$P < 0.01$ vs oLH.

Effect of LNG on pregnenolone-induced progesterone production (Table 1)

A significant increase in progesterone production was obtained from luteal cells incubated in the presence of 100 μM of pregnenolone. This evidence was significantly reduced when the luteal cells were cultured in the presence of pregnenolone with 10 μM of LNG. The basal production of progesterone was not modified when the cultured luteal cells were treated only with 10 μM of LNG.

Effect of $[Bu]_2cAMP$ on oLH-induced progesterone production (Fig. 4)

It is well recognized that the steroidogenic effect of LH on luteal cells is connected with the stimulation of the adenylate cyclase-cAMP pathway. The inhibition by LNG of LH-stimulated progesterone production prompted us to check the effects of LNG in the presence of exogenous cAMP. We used an analogue with the capacity to cross the cellular membrane, $[Bu]_2$cAMP, that by itself was capable of enhancing progesterone production. The addition of 10 μM of LNG to the incubation medium, totally inhibited the $[Bu]_2$cAMP-induced progesterone production.

DISCUSSION

In the rat, even though the large luteal cell population has the capacity to produce more progesterone than the small cells in the absence of LH, both cell types respond to LH with a similar dose-dependent increase in progesterone biosynthesis [10]. Our results are consistent with these studies since we obtained a maximal steroidogenic effect with a dose of 100 ng/ml of oLH, although in our experimental model we have a mixture of small and large luteal cells. The luteotrophic effect of oLH was obtained in whole ovaries as well as in dispersed luteal cells and it is compatible with the presence of LH receptors in the pregnant rat ovary [16], particularly in small and large luteal cells [10]. It is noteworthy that the steroidogenic effect obtained by treatment with oLH was greater in whole ovaries than in isolated luteal cells. The amplitude of this effect allows us to suggest that tissular integrity is necessary to obtain a maximal steroidogenic response to LH.

The present study shows that the synthetic progestin LNG inhibits LH-stimulated progesterone production

Table 1. Effect of levonorgestrel (LNG) on progesterone production stimulated by pregnenolone (P_5) in luteal cells obtained from 9-day pregnant rats

Group	Progesterone (ng/ml/3 × 10^5 cells/4 h)
Basal	2.48 ± 0.09
LNG (10 μM)	2.09 ± 0.07
P_5 (100 μM)	120.8 ± 12.6[a]
LNG + P_5	51.7 ± 4.6[a,b]

[a] and [b] = significant difference in comparison with the basal or P_5-group ($P < 0.01$), respectively. Each value is the mean ± SEM of quadruplicate determinations and are representatives of 3 different experiments.

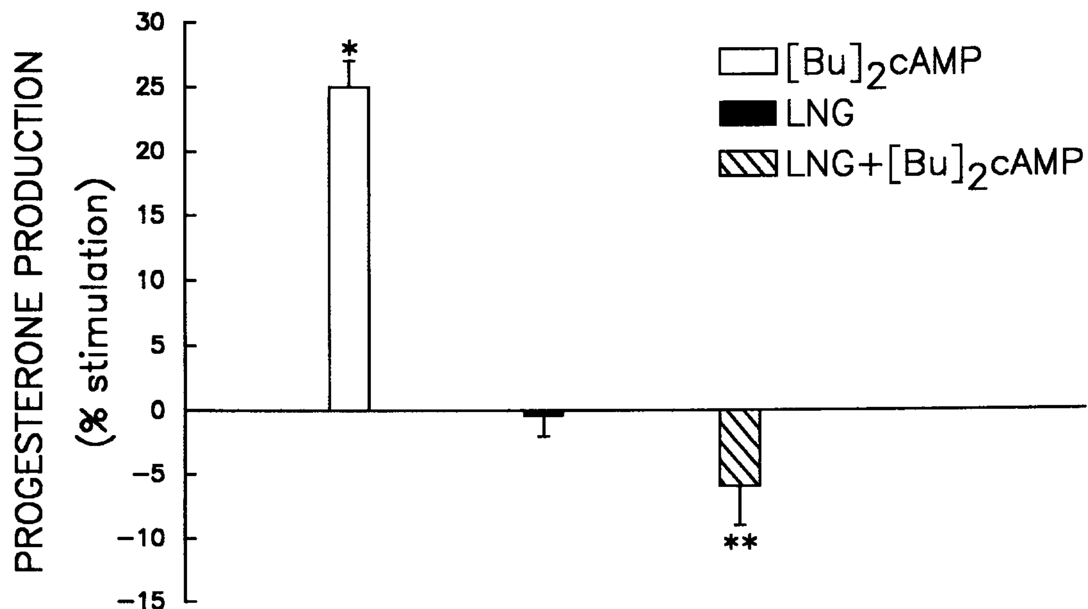

Fig. 4. Effect of LNG (10 μM) on basal and $[Bu]_2$cAMP-stimulated progesterone production in luteal cells of 9-day pregnant rats. Cells were incubated with $[Bu]_2$cAMP (100 μM) at 37°C under an atmosphere of 95% air:5% CO_2 for 4 h and LNG was added to the culture medium 30 min before $[Bu]_2$cAMP. Results are expressed as the percentage of progesterone stimulation compared to increase over basal values. Each bar is the mean ± SEM of quadruplicate determinations of 3 different experiments. *$P < 0.01$ vs basal and **$P < 0.01$ vs $[Bu]_2$cAMP.

in rat luteal cells and whole ovaries obtained from 9-day pregnant rats. Furthermore, the inhibitory action of LNG seems to be at a post-cAMP site, considering that the synthetic progestin also suppressed progesterone production induced by $[Bu]_2cAMP$. Moreover, LNG significantly inhibited progesterone production stimulated by pregnenolone indicating that most probably the synthetic steroid interferes with the last step of progesterone biosynthesis, the oxidation and isomerization of the precursor pregnenolone. This key enzymatic step is catalyzed by 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4 isomerase (3β-HSD), activity that is controlled at the level of 3β-HSD gene expression and/or 3β-HSD mRNA stability [17]. Recent studies suggest that expression of this enzyme is hormonally modulated [17–19]. Particularly, the activity of ovarian 3β-HSD depends on progesterone concentrations [20], therefore one possibility by which LNG could inhibit luteal cell progesterone production is that this agent interacts with intracellular progestin receptors to initiate genomic events important for progesterone biosynthesis. Another possibility is that LNG may directly modulate 3β-HSD activity, by a non-genomic mechanism.

In our study, the dose of LNG used to inhibit luteal cell steroidogenesis was high ($\approx 10^{-5}$ M), this is consistent with the effects of another synthetic progestin, R5020, that caused a 90% decrease in FSH-enhanced estrogen and progesterone secretion by cultured rat granulosa cells [21]. On the other hand, the corpus luteum produces large quantities of progesterone and it is likely that intraovarian levels of progesterone may be considerably high.

It has been reported that LNG acting *in vitro* on target organs displays potent progestational, and also androgenic, effects without estrogen-like activity [22]. The possibility that the action of progestin was mediated by nonspecific binding to androgen receptors must be considered; however, Hsueh *et al.*[23] demonstrated by using antiandrogens, that the modulatory action of R5020 on gonadotrophin-stimulated progesterone production by rat granulosa cells was not due to binding of the progestin to androgen receptor.

Although there is an extensive collection of findings that support the theory on the existence of a positive autocrine feedback of progesterone on its own biosynthesis in different mammalian species [2, 3, 20, 24], some results are contradictory. Thus, the spontaneous induction in isolated rat corpora lutea of 20α-HSD, an enzyme that transforms progesterone into a derivative devoid of progestational activity[25], is inhibited by the addition of the progesterone antagonist RU-486 to the culture medium [8]. This result indicates a possible indirect negative steroidogenic action of progesterone by means of an increase in conversion of progesterone to an inactive compound. Another controversial result is that the, synthetic progestin R5020 has the capacity to inhibit gonadotrophin-stimulated progesterone production in rat granulosa cells [21].

In conclusion, this study provides a novel point of view supporting a probably autocrine control mechanism in which progesterone, the primary steroidogenic product of the luteal cells exerts a negative ultrashort loop regulation on its own production. If we keep in mind that during pregnancy in the rat, progesterone biosynthesis depends on corpora lutea, the intraovarian actions of progestins may constitute an important local control of the mechanisms regulating luteal steroidogenesis.

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