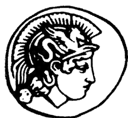

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Steroid regulation of progesterone synthesis in a stable porcine granulosa cell line: a role for progestins

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Abstract

The objective of this investigation was to determine the effect of steroid hormones on the synthesis of progesterone in a stable porcine granulosa cell line, JC-410. We also examined the effect of steroid hormones on expression of the genes encoding the steroidogenic enzymes, cytochrome P450-cholesterol side chain cleavage (P450scc) and 3β-hydroxy-5-ene steroid dehydrogenase (3β-HSD). We observed that 48 h exposure of the JC-410 cells to estradiol-17β (estradiol), androstenedione, 5α-dihydrotestosterone, levonorgestrel, and 5-cholesten-3β, 25-diol (25-hydroxycholesterol) resulted in stimulation of progesterone synthesis. 25-Hydroxycholesterol augmented progesterone synthesis stimulated by estradiol, 5α-dihydrotestosterone, levonorgestrel and 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP). This increase in progesterone synthesis was additive with estradiol, 5α-dihydrotestosterone and levonorgestrel, and synergistic with 8-Br-cAMP. Cholera toxin, progesterone, levonorgestrel and androstenedione increased P450scc mRNA levels, whereas estradiol had no effect. Cholera toxin, progesterone and levonorgestrel increased 3β-HSD mRNA levels, but estradiol and androstenedione had no effect. The results were interpreted to mean that estrogens, androgens and progestins regulate progesterone synthesis in the JC-410 cells. The effect of androgens appears to be mediated by stimulation of P450scc gene expression while progestins stimulate both P450scc and 3β-HSD gene expression. Our results support the concept that progesterone is an autocrine regulator of its own synthesis in granulosa cells. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Estrogens, androgens and progestins are hormonal steroids produced in the ovary under the control of the pituitary gonadotropins. Ovarian steroids have well defined endocrine actions in a variety of tissues and also act within the ovary in a paracrine and autocrine manner [1,2]. Estradiol, synthesized by aromatization of androgens by granulosa cells, represents the main estrogen produced by the ovary. Estradiol has recognized autocrine and paracrine effects in the ovary, including growth and differentiation of the follicles and regulation of steroidogenesis. In cultured granulosa cells, estradiol stimulates or inhibits progesterone synthesis production, depending on the duration of exposure to the steroid [3,4]. Androgens are synthesized in the theca cells of the ovarian follicles and also play an important role by promoting follicular growth and modulating steriodogenesis [5]. Progesterone is produced by the granulosa cells and represents the main substrate for the synthesis of all ovarian steroids. Progesterone may also play an important role in ovulation, since progesterone receptors are expressed in granulosa cells of preovulatory follicles [6,7]. Studies conducted using synthetic progestins have suggested that progesterone may stimulate its own synthesis in granulosa cells [8,9]. The results of these studies support the hypothesis of Rothchild [10,11], who suggested that progesterone plays an im-

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portant regulatory role by modulating its own synthesis in the ovary.

We previously reported the establishment and characterization of a steroidogenic stable porcine granulosa cell line, JC-410 [12]. These cells produce progesterone and are functionally responsive to the activators of protein kinase-A, cholera toxin, forskolin and cAMP [13]. In this study, we used JC-410 cells to investigate the effect of estrogens, androgens, and progestins on the synthesis of progesterone and on the expression of the steroidogenic enzymes.

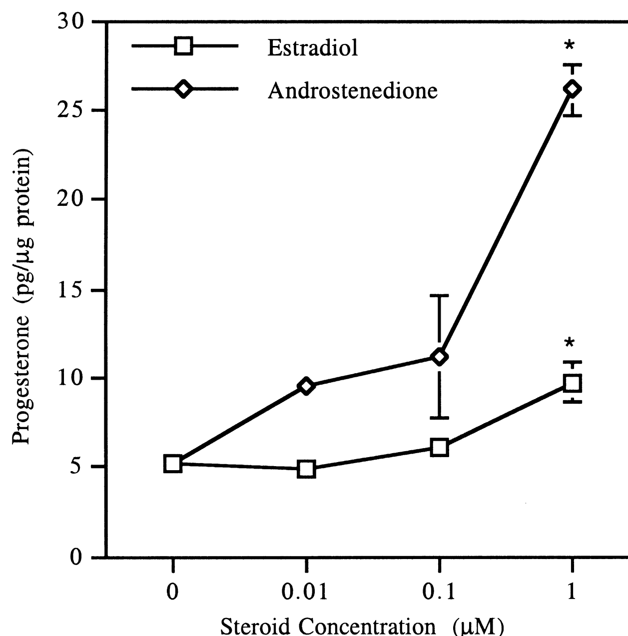

Fig. 1. Effect of estradiol and androstenedione on progesterone accumulation. Cells were cultured for 48 h in the presence of increasing concentrations of estradiol or androstenedione. Each point represents the means ±SEM of three independent replications. Stars indicate significant differences.

2. Materials and methods

2.1. Reagents

Reagents for tissue culture were purchased from Gibco, Burlington, ON. 25-hydroxycholesterol was purchased from Steraloids, Wilton, NH. Iodinated progesterone (11-α-hydroxyprogesterone 11-βD-glucuronide-^{125}I-iodotiramine) and Hybond-N nylon membrane were purchased from Amersham Pharmacia Biotech, Baie d'Urfé, PQ. ^{32}P was purchased from NEN, Boston, MA. The Bio-Rad DC Protein Assay kit was purchased from Bio-Rad Laboratories, Hercules, CA. 8-Br-cAMP, estradiol, pregnenolone (5-pregnen-3β-ol-20-one), androstenedione (4-androstene-3,17-dione) and 5α-dihydrotestosterone were purchased from Sigma, St. Louis, MO. Plastic culture plates were purchased from Falcon, Lincoln Park, NJ. Levonorgestrel was a generous gift of Wyeth-Ayerst, Montreal, PQ.

2.2. Cell culture

The immortalized porcine granulosa cell line, JC-410, was cultured as described [12]. Cells were grown in M199 supplemented with 5% newborn calf serum (NBCS), 5 μg/ml insulin, 100 IU/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml Fungizone® (culture media). Experiments were performed at 70% cell confluency in serum-free culture media.

2.3. Progesterone and protein assays

Progesterone content was determined by radioimmunoassay (RIA) in 100 μl of culture medium, as described [14]. In our radioimmunoassay, levonorgestrel cross-reacted with the progesterone antibody. Therefore, cross-reactivity was subtracted from the progesterone levels obtained. At the end of each experiment, cells were solubilized with 0.1% sodium dodecyl sulfate and protein content was determined with the Bio-Rad DC Protein Assay Kit.

2.4. Northern blot analyses

RNA was isolated by acid phenol/chloroform extraction [15]. Total RNA was denatured, then size-fractionated by electrophoresis on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane by diffusion blotting. Total RNA was cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). cDNAs for human type II 3β-HSD [16], porcine P450scc [17], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [18] were used as probes. cDNAs were labeled by primer extension [19] with [α-^{32}P] dCTP (>3000 Ci/mmol; New England Nuclear, Boston, MA) to a specific activity of $1.5–3.0 \times 10^9$ dpm/mg DNA. Membranes were hybridized and autoradiographed as previously described [20].

2.5. Statistical analysis

Data are presented as the mean +SEM of three independent experiments. Data were subject to two-way analysis of variance. When a significant F value was present, Fisher's Least Significant Difference test was used for individual comparison of means [21]. Significant differences were established as $P < 0.05$.

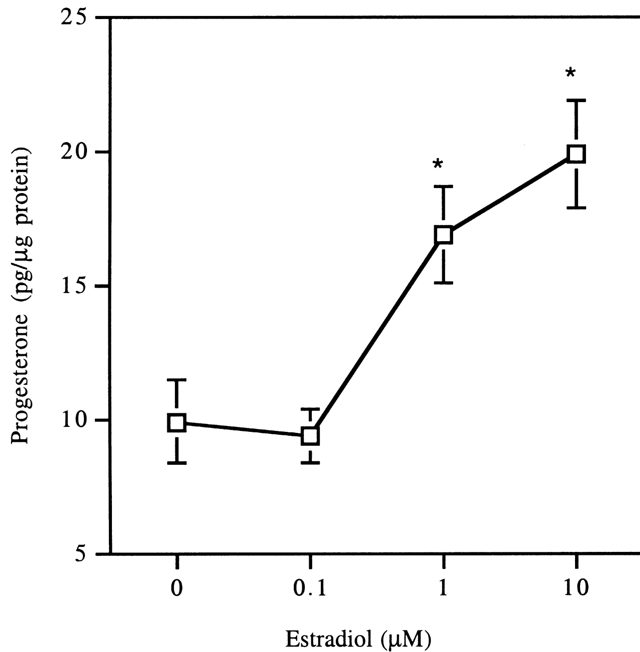

Fig. 2. Effect of estradiol on progesterone accumulation. Cells were cultured for 48 h in the presence of increasing concentrations of estradiol. Each point represents the means ±SEM of three independent replications. Stars indicate significant differences.

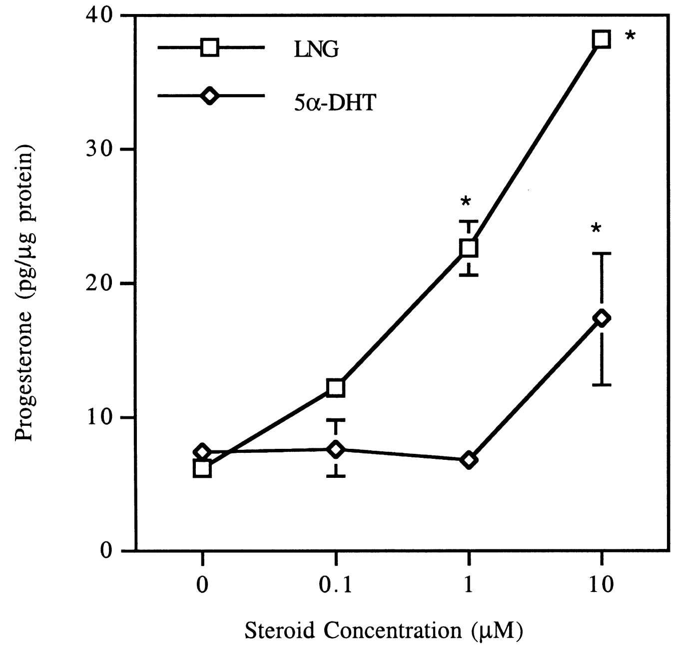

Fig. 4. Effect of levonorgestrel and 5α-dihydrotestosterone on progesterone accumulation. Cells were cultured for 48 h in the presence of increasing concentrations of levonorgestrel (LNG) or 5α-dihydrotestosterone (5α-DHT). Each point represents the mean ± SEM of three independent replications. Stars indicate significant differences.

3. Results

Estradiol, 0.1 and 1 µM, increased progesterone accumulation by 1.2- and 2-fold, respectively, after 48 h of culture (Fig. 1). Androstenedione, 0.01, 0.1 and 1 µM, increased progesterone accumulation by 1.9-,

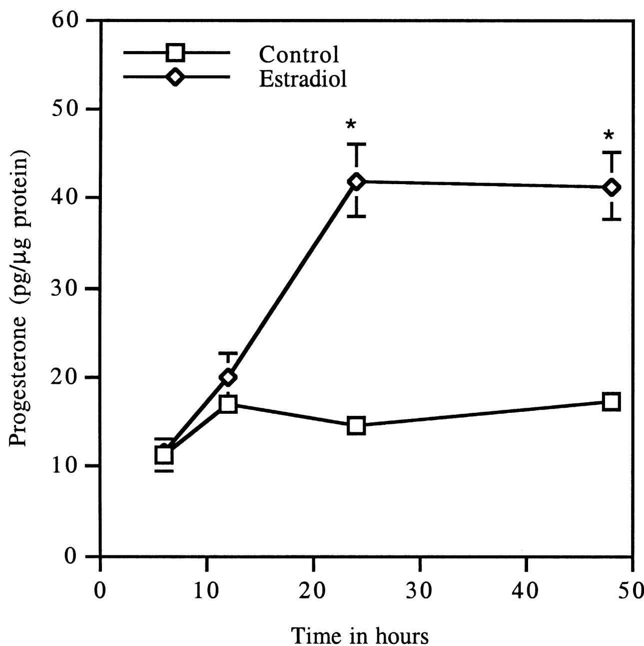

Fig. 3. Effect of time on estradiol-induced progesterone accumulation. Cells were cultured for up to 48 h in the presence or absence of 1 µM estradiol. Each point represents the mean ±SEM of three independent replications. Stars indicate significant differences.

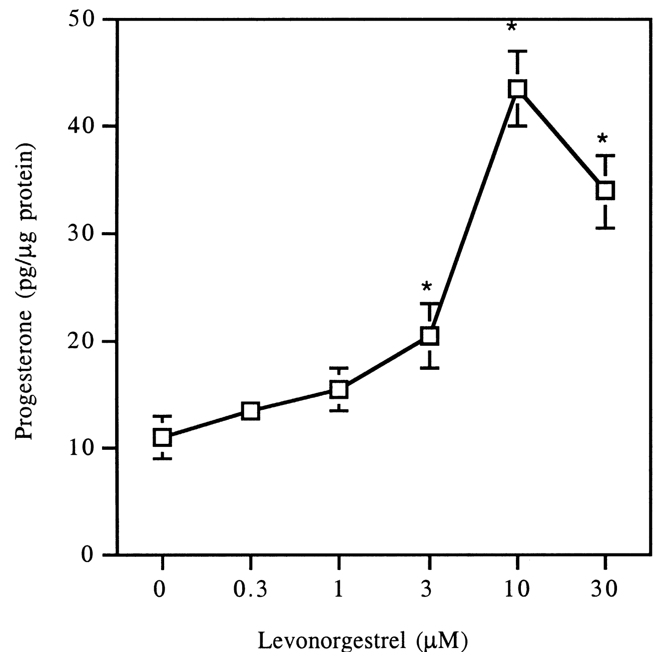

Fig. 5. Effect of levonorgestrel on progesterone accumulation. Cells were cultured for 48 h in the presence of increasing concentrations of levonorgestrel. Each point represents the mean ± SEM of three independent replications. Stars indicate significant differences.

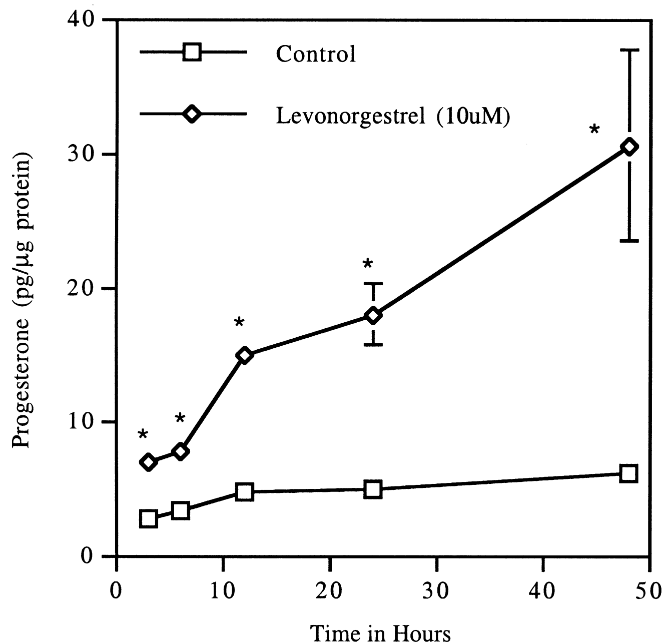

Fig. 6. Effect of time on levonorgestrel stimulation of progesterone accumulation. Cells were cultured for up to 48 h in the presence or absence of levonorgestrel. Each point represents the mean ± SEM of three independent replications. Stars indicate significant differences.

2.2- and 5.1-fold, respectively, after 48 h of culture (Fig. 1). Estradiol, 1 and 10 µM, increased progesterone accumulation by 1.7- and 2-fold, respectively, after

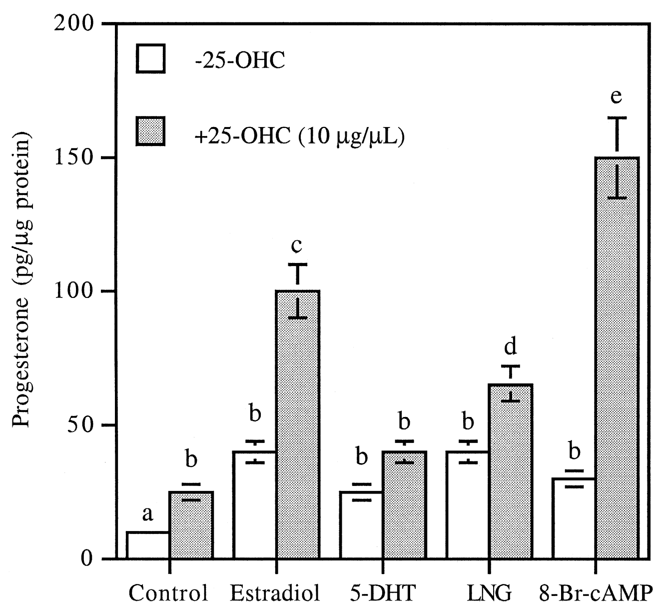

Fig. 7. Effect of 25-hydroxycholesterol on hormonal steroids- and cAMP-stimulated progesterone accumulation. Cells were cultured for 48 h with 10 µM estradiol, 5α-dihydrotestosterone (5-DHT), levonorgestrel (LNG), or 1 mM 8-Br-cAMP in the presence or absence of 25-hydroxycholesterol (25-OHC). Each point represents the mean ± SEM of three independent replications. Stars indicate significant differences.

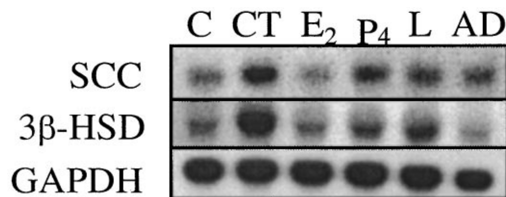

Fig. 8. Effect of cholera toxin, estradiol, progesterone, levonorgestrel and androstenedione on 3β-HSD and P450scc mRNA. Cells were cultured for 6 h in the presence or absence of 30 ng/ml cholera toxin (CT), or 10 µM estradiol (E2), progesterone (P4), levonorgestrel (L) or androstenedione (AD). Total RNA was analyzed by Northern blot, GAPDH was used as a control for RNA loading.

48 h of cultures (Fig. 2). Estradiol, 1 µM, increased progesterone accumulation by 1.5- and 3.5-fold at 24 h and 48 h, respectively (Fig. 3). The non-aromatizable androgen, 5α-dihydrotestosterone, 10 µM, increased progesterone accumulation by 2-fold, after 48 h of culture (Fig. 4). Levonorgestrel, 0.1, 1 and 10 µM, increased progesterone accumulation by 1.6-, 3.1- and 5-fold, respectively, after 48 h of culture (Fig. 4). Levonorgestrel, 3, 10 and 30 µM, increased progesterone accumulation by 1.9-, 4- and 3.1-fold, respectively, after 48 h of culture (Fig. 5). Levonorgestrel, 10 µM, increased progesterone accumulation by 2.6-, 2.3-, 3.2-, 3.7- and 5.0-fold, after 3, 6, 12, 24 and 48 h in culture, respectively (Fig. 6). 25-hydroxycholesterol increased progesterone accumulation by 1-fold, and increased estradiol-, 5α-dihydrotestosterone- and levonorgestrel-stimulated progesterone accumulation by 1.5-, 0.5- and 0.7-fold, respectively (Fig. 7). 8-Br-cAMP increased progesterone accumulation by 1.5-fold, and 25-hydroxycholesterol increased the 8-Br-cAMP-stimulated progesterone accumulation by 4-fold (Fig. 7). The effect of progesterone, levonorgestrel, estradiol, androstenedione, and the protein kinase-A activator, cholera toxin, on mRNA steady-state levels of 3β-HSD and P450scc, determined by Northern blot analysis, is shown in Fig. 8. Cholera toxin, progesterone and levonorgestrel increased 3β-HSD mRNA levels by 6-, 2- and 3-fold, respectively. Estradiol and androstenedione did not change 3β-HSD mRNA levels. Cholera toxin, progesterone, levonorgestrel and androstenedione increased P450scc mRNA levels by 3-, 2-, 2- and 2-fold, respectively. Estradiol did not change P450scc mRNA levels.

4. Discussion

Previous studies have demonstrated that ovarian hormonal steroids regulate ovarian steroid hormone biosynthesis [2]. In this study, we investigated the role of estrogens, androgens and progestins in a novel in vitro model of stable steroidogenic porcine granulosa

cells, JC-410. This is a cell line that originated from a primary culture of porcine granulosa cells. As the JC-410 cells do not respond to the gonadotropins [12], the steroid effects were observed without gonadotropin influences.

We found that estradiol stimulated progesterone synthesis in a concentration- and time-dependent manner in the JC-410 cells. Concentrations of estradiol were in the same range as those used in primary cultures of porcine granulosa cells [3,4,22,23]. The time of exposure was also in agreement with observations in primary cultures of porcine granulosa cells, in which exposure to estradiol for 48 h stimulated progesterone synthesis [4,24,25,26]. Other reports indicate that progesterone synthesis is inhibited when primary cultures of porcine granulosa cells were exposed to estradiol for less than 48 h [3]. We found no change in progesterone synthesis at 6 and 12 h exposure to estradiol; but at 24 and 48 h exposure to estradiol a consistent elevation in progesterone synthesis was observed. Discrepancies in the response to estradiol by porcine granulosa cells have been attributed to the presence of serum in the culture media [27]. The JC-410 cells were grown in the presence of 5% NBCS and the experiments were conducted in serum-free media. Thus, the stimulatory effect of estradiol on progesterone synthesis was observed under conditions in which the influence of serum was minimized.

The mechanism(s) by which estrogens stimulate progesterone synthesis in granulosa cells are unknown. In the rabbit corpus luteum, estradiol stimulated expression of the steroid acute regulatory protein gene (StAR), which is followed by an increase in progesterone synthesis [28]. StAR gene expression was not detected by Northern blot analysis in the JC-410 cells [13]. It is possible that StAR is expressed at low levels in the JC-410 cells, as described in primary cultures of porcine granulosa cells [29]. The increased progesterone observed in estradiol-treated JC-410 cells may also be the result of inhibited progesterone degradation. However, in primary cultures of porcine granulosa cells, estradiol increased, or did not change synthesis of 20α-hydroxypregn-4-en-3-one, a product of progesterone degradation [4,22,23]. Therefore, it is unlikely that estradiol inhibits progesterone degradation in the JC-410 cells, which are originated from granulosa cells.

Androstenedione and 5α-dihydrotestosterone, stimulated progesterone synthesis in JC-410 cells. These results agree with previous studies conducted in primary cultures of granulosa cells, in which stimulatory effects of androgens on progesterone synthesis were observed [30–33]. Other reports indicate that androgens inhibit or have no effect on progesterone synthesis in granulosa cells [34–36]. The JC-410 cells have an active aromatase system [13] and respond to both estradiol and the non-aromatizable androgen, 5α-dihydrotestosterone, with increased progesterone synthesis. Therefore, the stimulatory effect of androstenedione on progesterone synthesis is probably due to the combined effects of the androgen itself and the product of aromatization.

Previous reports indicate that gonadotropin-stimulated progesterone synthesis was augmented by the synthetic progestins R5020 and medroxyprogesterone acetate, in rat granulosa cells [8,9]. Higher concentrations of progesterone or medroxyprogesterone acetate inhibited progesterone production in the presence or absence of gonadotropins [9,37]. Our results suggest that progesterone plays a role in regulating its own synthesis independent of gonadotropin effects. Levonorgestrel is a synthetic progestin that binds to the progesterone receptor [38] and has low cross-reactivity with the antibody used in the RIA for testing progesterone [39]. This feature makes levonorgestrel a useful tool to study the effect of progestins on progesterone synthesis. Although levonorgestrel also binds to androgen receptors [38], it had a stimulatory effect at a much lower dose than 5α-dihydrotestosterone in the JC-410 cells. The results were interpreted to mean that levonorgestrel is acting as a progestin rather than an androgen. This interpretation supports previous findings in which the anti-androgen cyproterone acetate failed to block the stimulatory effect of R5020 on progesterone synthesis [40]. Levonorgestrel also stimulated progesterone synthesis in primary cultures of porcine granulosa cells (data not shown), but the effect was never greater than 2-fold, compared with the 5-fold stimulation seen in the JC-410 cells. The levels of progesterone in primary cultures of granulosa cells are much higher than the levels in cultures of JC-410 cells [12]. Therefore, we speculate that the effect of the progestins is masked by the high levels of progesterone present in the primary cultures of granulosa cells.

25-Hydroxycholesterol increased progesterone synthesis in JC-410 cell cultures. This increase was synergistic with 8-Br-cAMP and additive with estradiol, 5α-dihydrotestosterone and levonorgestrel. We have previously reported an increase in progesterone synthesis in the presence of 25-hydroxycholesterol and synergism with 8-Br-cAMP in the JC-410 cells [13]. These results agree with studies conducted in primary culture of porcine granulosa cells in which 25-hydroxycholesterol enhanced gonadotropin-stimulated progestin production [41]. However, they are contrary to the classical concept that gonadotropins or PKA activators do not alter 25-hydroxycholesterol-induced progesterone synthesis [42–44]. These discrepancies may be due to differences in the mechanisms by which steroidogenesis is regulated in granulosa and luteal cells. 25-Hydroxycholesterol may have a synergistic effect with activators of the PKA pathway only in granulosa cells,

where regulation of P450scc expression is cAMP dependent. In the corpus luteum, where expression of P450scc is cAMP independent [45], cAMP may not have any further effect on 25-hydroxycholesterol-increased progesterone synthesis. Regulation of the activity of the steroid hydroxylases by 25-hydroxycholesterol has been a subject of recent debate [46,47]. In our investigations, the additive effect of 25-hydroxycholesterol with estradiol agrees with previous reports conducted with primary cultures of porcine granulosa cells [22,23,41]. To our knowledge there is no information on the effects of progestins or androgens in combination with 25-hydroxycholesterol. We speculate that the steroid hormones stimulate progesterone synthesis by a mechanism that may not involve activation of the steroid hydroxylases.

We observed that progesterone, levonorgestrel and androstenedione, but not estradiol, increased P450scc mRNA levels in JC-410 cells. Estradiol has been reported to stimulate progesterone synthesis in porcine granulosa cell cultures without affecting expression of the P450scc gene [48,49]. We also found that levonorgestrel and progesterone, but not estradiol or androstenedione, increased 3β-HSD mRNA levels. Progesterone and levonorgestrel both stimulated expression of P450scc and 3β-HSD genes indicating that the action of levonorgestrel is mainly due to a progestin-like, rather than an androgen-like effect. Overall, the results were interpreted to mean that progesterone stimulates its own secretion by a mechanism involving increased expression of the P450scc and 3β-HSD genes. This interpretation is supported by previous reports indicating that 3β-HSD is the autocrine target of progesterone [50–52,39]. Taken together, this information supports the hypothesis that progesterone stimulates its own synthesis in the ovary [10,11].

Although receptors for androgens, estrogens and progestins were reported to be expressed in granulosa cells of several species, it is not possible from the available information to definitely conclude that the observed effects were mediated through their specific steroid receptors. However, there is information that supports the role of estrogens, androgens and progestins on granulosa cell physiology [2]. It is known that the concentration of estradiol and androgens in the follicular fluid may reach as high as 10 and 3 μM, respectively [53]. Therefore, it is likely that the concentrations of estradiol and androgens used in this study are physiologically relevant. Taking into consideration that progesterone is present in high concentrations in the ovary, up to 25 μg/g [54], it is feasible to speculate that the mechanism by which progesterone modulates its own synthesis may not necessarily be mediated through its classical receptor [11].

In summary, we found that estradiol, androstenedione, 5α-dihydrotestosterone and levonorgestrel stimulated progesterone synthesis. The mechanism by which androgens and progestins stimulate progesterone synthesis involves stimulation of P450scc gene expression. Whereas, progestins also stimulate expression of the 3β-HSD gene. This report supports the autocrine role of estrogens and progestins, and a paracrine role for androgens in the ovary. Overall, the result of this investigation can be interpreted to mean that the effects of hormonal steroids in the JC-410 cells are similar to those observed in primary cultures of granulosa cells. Therefore, it is likely that the mechanisms of action are comparable. Thus, the JC-410 cells may represent an ideal model to investigate the paracrine effects of steroid hormones in granulosa cells.

Acknowledgements

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