Pergamon

0960-0760(94)00126-X

J. Steroid Biochem. Molec. Biol. Vol. 51, No. 3/4, pp. 191–197, 1994

0960-0760/94 $7.00 + 0.00

Androstenedione Stimulates Progesterone Production in Corpora Lutea of Pregnant Rats: an Effect not Mediated by Oestrogen

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To determine if androstenedione, an aromatizable androgen, has a direct effect on luteal progesterone secretion, collagenase-dispersed luteal cells or whole corpora lutea from pregnant rats were incubated in the presence of the androgen. Luteal cells from 15-day pregnant rats responded to androstenedione in a dose-dependent manner, with an increase in progesterone output at doses of 1 and 10 μM, but with no effect at minor doses of the androgen. Luteal cells obtained from animals on day 4, 9, 15 or 19 of pregnancy and incubated with 10 μM of androstenedione, increased progesterone production by 243, 39, 84 and 146%, respectively. Androgens (androstenedione, testosterone or dihydrotestosterone) but no oestrogens (oestradiol or diethylstilboestrol) at a dose of 10 μM, stimulated progesterone production in incubated luteal cells obtained from 15-day pregnant rats. The time-course pattern of androstenedione-induced progesterone production was studied by superfusion experiments using corpora lutea from rats on day 15 of pregnancy. A significant progesterone output was observed when androstenedione, but not oestradiol, was perfused through the luteal tissue. Intrabursal ovarian administration of androstenedione (10 μM) to 19-day pregnant rats induced a significative increase in serum progesterone levels 8 and 24 h after treatment. These *in vivo* results confirm the stimulatory effect of androstendione on progesterone production obtained in incubated luteal cells from pregnant rats. This study reports a direct luteotrophic effect of androstenedione in rat corpus luteum, not mediated by previous conversion to oestrogens.

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INTRODUCTION

In the rat, the corpus luteum is the primary source of progesterone throughout pregnancy [1]. The rat placenta secretes progesterone [2], but is insufficient to sustain pregnancy in the absence of the ovaries [3]. The luteotrophic requirements of the rat corpus luteum change throughout gestation [4–6]. Prolactin or prolactin-like hormones from placental tissue, and oestradiol formed in the corpus luteum from aromatizable androgen, seem to regulate luteal cell steroidogenesis [7]. Transformation of aromatizable androgens to oestradiol has been reported as a property of luteal cells [8]. In pregnant rats, androstenedione production exceeds that of testosterone [9, 10] being consequently the principal substrate proposed for intraluteal oestrogen biosynthesis [11–13]. The source of androgenic substrate for the biosynthesis of oestradiol in luteal cells originates from the ovaries until mid-pregnancy [14] and thereafter from the placenta [8, 10, 15].

It has been shown that androgens have a synergistic effect on the stimulation of progesterone secretion by FSH in cultured rat granulosa cells [16–23]. Dihydrotestosterone, a steroid incapable of undergoing aromatization, is as effective as testosterone in enhancing FSH-stimulated progesterone production by rat granulosa cells [20], through a true androgenic effect rather than an effect mediated by conversion to oestrogens [16, 19, 24]. A recent paper demonstrated that androgens, but not oestrogens, progestins or glucocorticoids, augment vasoactive intestinal peptide- and

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Received 13 Dec. 1993; accepted 5 July 1994.

GH-releasing hormone-stimulated progestin production by cultured rat granulosa cells [25].

The aim of this study was to evaluate the capacity of androstenedione to exert a direct effect on ovarian progesterone production in pregnant rats, not mediated by the well-known intraluteal aromatization to oestradiol.

EXPERIMENTAL

Animals

Adult Holtzman rats housed at 22–24°C with a 14 h light/10 h dark photoperiod (lights on from 06.00–20.00 h) were used. Rat chow and water were available *ad-libitum*.

Vaginal smears were taken daily and only rats with 4-day cycles were used. Female rats were caged individually with fertile males on the night of the pro-oestrous day, and the presence of spermatozoa was checked in the vaginal smear the following morning. This day was designated day 0 of pregnancy. Our colony usually gave birth on day 22.

Materials

The following drugs were purchased from Sigma Chemical Co. (St Louis, MO): androstenedione, testosterone, oestradiol, diethylstilboestrol, dihydrotestosterone, progesterone, HEPES, medium 199, bovine serum albumin fraction V (BSA), EDTA, collagenase type IV (570 U/mg), methyl cellulose, 2,5-diphenyloxazole, 1,4-bis-2-(5-phenyloxazolil)-benzene, Sephadex-G50.

[1,2,6,7-^{3}H]progesterone was from New England Nuclear (Boston, MA). Luteal cells were incubated in 24-well plastic tissue culture dishes (Corning Laboratory Sciences Co., U.S.A.).

Preparation of collagenase-dispersed luteal cells

Luteal cells from pregnant rats were isolated as described previously [26]. In brief, ovaries from rats decapitated on days 4, 9, 15 or 19 of pregnancy, were collected and placed in phosphate-buffered saline (PBS, pH 7.4). The corpora lutea were dissected and cleaned of other ovarian tissues under a stereoscopic microscope. In a flask containing gassed PBS, collagenase (1 mg/ml) and BSA 1%, the corpora lutea were incubated for 1 h at 37°C in a stirred waterbath (100 rpm). At the end of incubation, the content of the flask was centrifuged for 5 min at 800 *g*. The supernatant was discarded and the cells were washed in PBS containing EDTA 1 mM for 3 min under agitation, and again centrifuged for 5 min. The cell pellet was resuspended in medium 199 containing HEPES (10 mM) and passed through nylon mesh. Before being used in the experiments, the cells were pre-incubated for 1 h at 37°C under an atmosphere of 95% air/5% CO_2 in a water-jacked incubator. The viability of the cells was in the 90% range as determined by trypan blue staining. Cell incubation was performed at a final concentration of 3×10^5 viable cells/ml in medium 199 for 4 h. Steroids were dissolved in absolute ethanol and added to the medium in a volume of 10 μl. At the end of the incubation period, cells were harvested and the media were frozen at −20°C until measurement of the progesterone content.

Superfusion system

We used the superfusion model described previously by Spinedi *et al.* [27], which was performed with a 2.5 ml plastic syringe. A rubber tap, which was passed through with a point end removed 23-gauge disposable needle, was placed at the open end of the syringe. The superfusion chamber was filled with three layers, done by adding 3 or 4 corpus luteum on a bed of 0.5 ml of pre-soaked Sephadex-G50. The medium containing reservoir was maintained at 37°C and constantly gassed with 95% O_2/5% CO_2. A fluid flow rate of 150–200 μl/min was passed through the superfusion chamber, the fluid came from the reservoir and entered via a peristaltic pump before being raised to the chamber. The fluid entered at the top of the chamber and left by the bottom, and was finally recovered by a multifraction collector. After 20 min stabilization period, fractions were collected at 5 min intervals and frozen until progesterone assay. When hormones were added to the media, they were dissolved in absolute ethanol.

Intrabursal administration of androstendione

Androstenedione was administered locally into the ovarian bursa according to the method of Kannisto *et al.* [28]. This intrabursal method is useful for examination of direct action of agents on the ovary [29]. This methodology has been previously validated by a push–pull method [30] showing that steroids reached ovarian cells when applied into the ovarian bursa. Under ether anaesthesia, the ovaries were exposed through lateral incisions. A pellet containing 86 ng of the androgen in a volume of 30 μl (10 mM), was implanted under the ovarian capsule. The hormone was mixed with methyl cellulose gel (4%, used as a vehicle) to minimize leakage from the ovarian bursa. Each animal received a bilateral intrabursal injection of the gel solution (gel control group) or gel solution plus androstenedione between 09.00–10.00 h on day 19 of pregnancy by using a Hamilton microliter syringe (705-N). The rats were decapitated at 8, 24, 30 or 48 h after treatment. Blood was allowed to clot at room temperature and the serum separated and stored frozen at −20°C until assayed for progesterone.

Progesterone assay

Progesterone was measured by a radioimmunoassay (RIA) using antiserum provided by Dr R. P. Deis (Laboratorio de Reproducción y Lactancia, Mendoza, Argentina). The sensibility, variability and

cross-reaction of this RIA, has been reported previously [26, 31].

Medium controls were run in triplicate for each treatment and these background levels were subtracted for each sample. The ethanol used for dissolving steroids added to the culture media (10 μl) does not affect progesterone production.

Progesterone values measured in the absence of hormonal addition varied considerably between incubations. However, fold-effects of hormone additions were consistent.

Statistical analysis

Results are presented as mean ± SEM of quadruplicate determinations and are representative of 3 different experiments. Statistical comparisons were performed using Student's t-test to assay significant differences between means of two groups and analysis of variance (ANOVA) followed by Duncan's multiple range test for multiple comparisons [32]. A level of $P < 0.05$ was accepted as statistically significant.

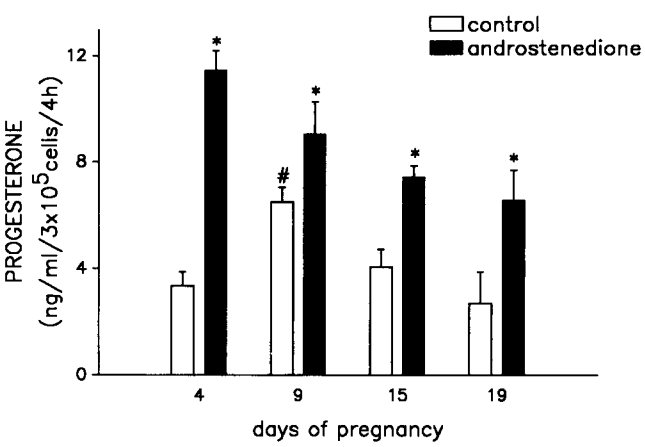

Fig. 2. Effect of androstenedione (10 μM) on progesterone production by luteal cells obtained from rats on different days of pregnancy. Cells were incubated in the presence or absence of androstenedione at 37°C under an atmosphere of 95% air/5% O_2 for 4 h. Values are the mean ± SEM of quadruplicate determinations of 3 different experiments. *$P < 0.05$ vs its respective basal group; #$P < 0.05$ vs basal from days 4, 15 or 19 of pregnancy (ANOVA followed by Duncan's multiple range test).

RESULTS

Effect of androstenedione on progesterone production by cultured luteal cells obtained from rats on different days of pregnancy

A dose–response assay was performed in collagenase-dispersed luteal cells in order to evaluate the effect of different concentrations of androstenedione on progesterone production. The luteal cells were obtained from ovaries of rats on day 15 of pregnancy, a time of gestation when serum progesterone levels present the highest values [33].

As Fig. 1 shows, incubation of the luteal cells in the absence or presence of androstenedione (0.01–10 μM) resulted in a significant increase in progesterone accumulation at doses of 1 and 10 μM of the androgen.

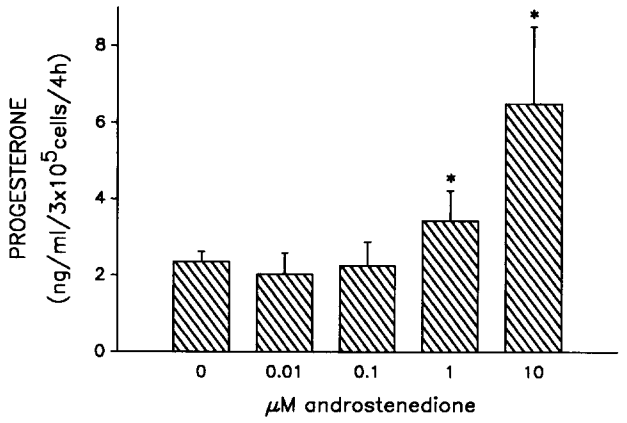

Fig. 1. Progesterone production by luteal cells from 15-day pregnant rats in the presence of different concentrations of androstenedione. Cells were incubated with androstenedione at 37°C under an atmosphere of 95% air/5% O_2 for 4 h. Values are the mean ± SEM of quadruplicate determinations of 3 different experiments. *$P < 0.05$ vs basal (ANOVA followed by Duncan's multiple range test).

To evaluate the luteotrophic effect of androstenedione during pregnancy, luteal cells from rats on days 4, 9, 15 or 19 of gestation were incubated for 4 h in the presence of the androgen at a dose of 10 μM (Fig. 2). Androstenedione stimulated progesterone release from luteal cells on all days of pregnancy, when compared with basal secretion. On day 9 of pregnancy, basal accumulation of progesterone in the incubation medium was higher than that observed in the other days. However, androstenedione was less effective in stimulating progesterone production on day 9 of pregnancy when compared with the response obtained on days 4, 15 or 19.

Effect of androgens and oestrogens on progesterone production from dispersed luteal cells

Since aromatase activity has been reported in rat luteal cells [34, 35] and taking into account that androstenedione is an aromatizable androgen, the possibility that androstenedione could be converted to oestradiol in our system, was evaluated. Dispersed luteal cells obtained from ovaries on day 15 of pregnancy were incubated in the presence of oestrogens (oestradiol, diethylstilboestrol), aromatizable androgens (testosterone, androstenedione) and a non-aromatizable androgen (dihydrotestosterone). As Fig. 3 shows, basal progesterone accumulation during 4 h of incubation was not modified by oestradiol or by diethylstilboestrol added to the incubation medium. However, the release of progesterone was increased 4 h after incubation with androgens, this response was more notable with the addition of testosterone

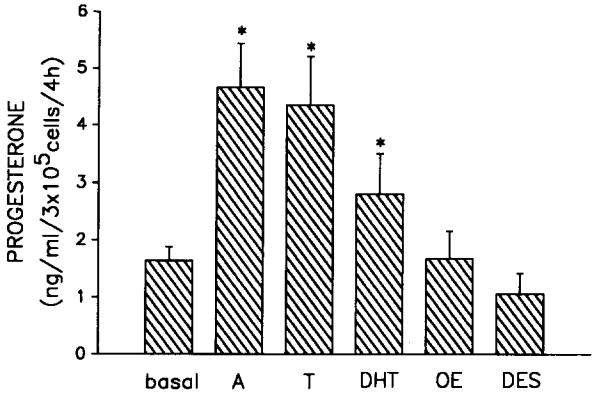

Fig. 3. Effect of androstenedione (A), testosterone (T), dihydrotestosterone (DHT), oestradiol (OE) and diethylstilboestrol (DES) on the accumulation of progesterone in the medium during a 4 h culture of luteal cells obtained from rats on day 15 of pregnancy. All steroids were used at a dose of 10 μM. Values are the mean ± SEM of quadruplicate determinations of 3 different experiments. *$P < 0.05$ vs basal progesterone production (ANOVA followed by Duncan's multiple range test).

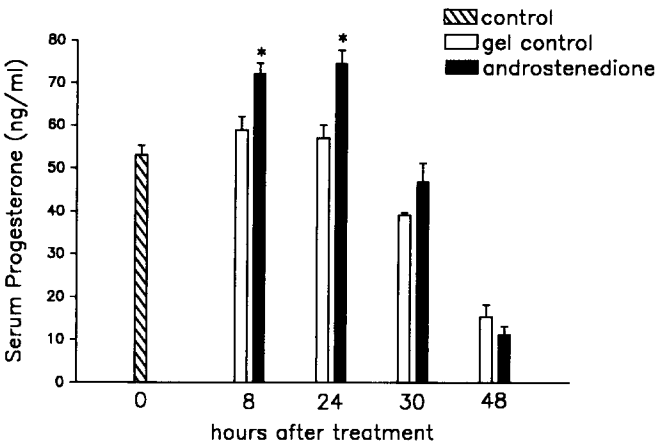

Fig. 5. Effect of intraovarian androstenedione administration (10 μM, solid bars) or gel control (open bars) at 09.00–10.00 h in 19-day pregnant rats, on serum progesterone levels measured 8, 24, 30 or 48 h after treatment. The hatched bar (control) represents progesterone values from intact animals sacrificed at 09.00 h on day 19 of pregnancy. Values are mean ± SEM from 6–9 animals per experimental group. *$P < 0.01$ compared with its respective control groups (Student's t-test).

or androstenedione than after incubation with dihydrotestosterone.

Effect of androstenedione on progesterone production from perfused corpora lutea

Superfusion of corpora lutea was performed in order to study the time-course of androstenedione effect on progesterone production (Fig. 4). In the perfusate, progesterone remained virtually unchanged throughout the time of superfusion with the medium, reaching values of 4–5 ng/5 min.

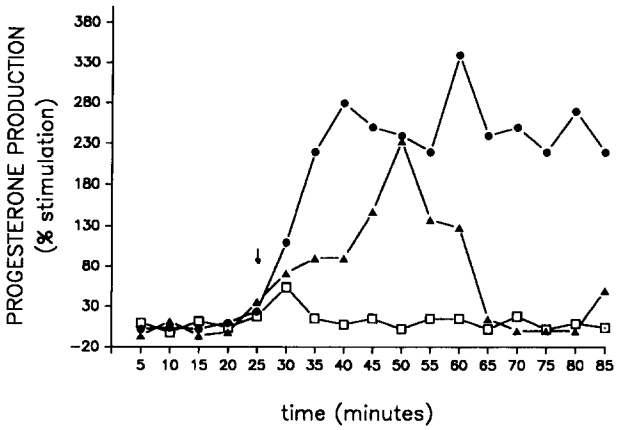

Fig. 4. Time-course for progesterone accumulation into culture media when corpora lutea from 15-day pregnant rats were perfused with (●) androstenedione (10 μM) continuously for 60 min; (▲) androstenedione (10 μM) or (□) oestradiol (10 μM) for 5 min after control medium was perfused. The arrow indicates the time of administration of the steroids to the superfusion system. Data are expressed as the percentage of progesterone stimulation compared to increase over basal values obtained in the absence of steroids. Each graph represents 4 different experiments.

When a continuous infusion of the medium containing androstenedione (10 μM) was performed, a significant increase, around 3-fold, in progesterone production was obtained throughout the time of superfusion.

When androstenedione (10 μM) was added to the superfusion medium for only 5 min, an increase, around 200%, in basal progesterone production was obtained and persisted for 35 min. However, when oestradiol (10 μM) was added to the medium for 5 min, progesterone production was not increased in the period studied.

Effect of intrabursal ovarian administration of androstenedione on serum progesterone concentration (Fig. 5)

To determine if androstenedione stimulates ovarian progesterone production in an *in vivo* model, 19-day pregnant rats were injected intrabursally with androstenedione (10 μM). Eight or 24 h after intraovarian androstenedione administration, serum progesterone concentration was significantly higher than the control group receiving gel solution without androgen. However, 30 and 48 h after treatment, serum progesterone concentration returned to normal.

DISCUSSION

Aromatizable androgens are well known as substrates for aromatase activity at the corpus luteum level in pregnant rats [34–37]. This enzyme converts both androstenedione and testosterone to oestradiol within the luteal cells [8–10]. Even though both luteal cell types are capable of synthesizing oestradiol, the large one appears to be the major source of oestradiol

[38, 39], which by an intracrine mechanism stimulates luteal progesterone biosynthesis [7, 38]. Taken together, these facts indicate that the androgens exert an important oestrogen-mediated luteotrophic action in pregnant rats. The present results confirm both *in vitro* and *in vivo*, a luteotrophic effect of androstenedione on different days of pregnancy in the rat. It is interesting to note that androstenedione stimulated progesterone production from luteal cells to a different degree according to the day of pregnancy studied. Thus, luteal cells obtained from animals on day 4, 9, 15 or 19 of pregnancy, increased progesterone production by 243, 39, 84 or 146%, respectively, when incubated in the presence of androstenedione. Moreover, basal not stimulated progesterone secretion fluctuated during pregnancy, reaching a maximum value on day 9. This pattern is correlated with ovarian progesterone content during pregnancy [40]. It appears clear from our results that on the days of pregnancy when basal progesterone production is low, androstenedione is more capable of stimulating progesterone secretion in luteal cells. Taking into account basal and androstenedione-stimulated progesterone production obtained during different days of pregnancy, it can be suggested that the availability of progesterone to be released from luteal cells, is independent of the luteal cells capacity to synthesize progesterone.

In addition to the *in vitro* experiments, intrabursal ovarian injection of androstenedione stimulated progesterone production by the ovary 8 and 24 h after treatment on day 19 of pregnancy. The decline in the levels of serum progesterone observed 24 and 30 h after intraovarian administration of androstenedione or gel vehicle, could be considered as the physiological prepartum decrease of progesterone. Although implants of androstenedione remains under the ovarian capsule, it is possible that the levels of the androgen released were not enough to prevent the luteolytic events occurring at the end of pregnancy [31]. This is consistent with results obtained in our laboratory showing that when 19-day pregnant rats were injected subcutaneously with a high dose of androstenedione (10 mg/rat), parturition was retarded for at least 24 h, and the physiological decrease in serum progesterone concentration previous to parturition was prevented (unpublished data).

The effect of androgens on progesterone accumulation observed by us, may indicate a direct effect of androstenedione at the corpora lutea and not be due to a previous conversion to oestrogens, since (i) the effect was not mimicked by either oestradiol or diethylstilboestrol, both in dispersed luteal cells and in perfused corpora lutea; and (ii) dihydrotestosterone, which is not a substrate of aromatase, showed the same stimulatory effect on progesterone production when induced by androstenedione or testosterone. A similar direct effect of testosterone, dihydrotestosterone and androstenedione on progesterone production was obtained in cultured granulosa cells stimulated either by FSH [16], vasoactive intestinal peptide or GH-releasing hormone [25].

The increase in progesterone production obtained by us in collagenase-dispersed luteal cells using doses of 1 or 10 μM of androstenedione, could be considered a pharmacological effect. However, in physiological conditions, the ovarian concentration of androstenedione produced by luteal, tecal or interstitial ovarian cells [41–43], may reach higher values to induce progesterone secretion, suggesting an autocrine or paracrine effect similar to that observed at the follicular level [44].

We must consider that the observed stimulation in progesterone production by androstenedione, testosterone or dihydrotestosterone, may be mediated by androgen receptors. The rat ovary is a target for androgen action as shown by the presence of receptors in granulosa cell cytosol [45, 46]. It has been reported that androgens are able to specifically regulate oestrogen biosynthesis by rat granulosa cells acting as substrates for the FSH-induced aromatase enzyme activity and by participating in the mechanism by which FSH increases the activity of the enzyme [16–20, 47]. The inhibition of endogenous androgens with a non-steroidal anti-androgen, SCH-16423, enhances the biological effects of FSH in oestrogen-treated hypophysectomized immature female rats [48], suggesting that the inhibitor interferes with androgen activation of receptor [49]. It has recently been shown that progesterone production by human placenta is enhanced by androgens acting selectively on cholesterol side chain cleavage enzymes ($P450_{scc}$) [50]. This modulatory effect of androstenedione, that affects the activity of $P450_{scc}$, has also been suggested for the androgen-stimulated progesterone production in porcine granulosa cells [51]. However, on immature rat granulosa cells, it has been proposed that the stimulatory action of androgens on progesterone production may occur via a receptor-mediated suppression of cAMP catabolism [52]. In our study, the short time–response to androstenedione-stimulated progesterone production from perfused corpora lutea, suggest a non-genomic rapid mechanism. In accordance with this, in early human placental explants, an initial rapid progesterone stimulation by 19-nortestosterone within 2 h of incubation was not effected by cycloheximide, an inhibitor of protein biosynthesis [50]. This result may indicate that androgens act at the cellular level by a mechanism that does not require synthesis of new proteins.

Our results suggest the existence of a rapid direct luteotrophic action of androstenedione in rat corpus luteum, not mediated by previous conversion to oestradiol. Further studies are necessary to define the mechanism responsible for the direct luteotrophic effect of androstenedione.

Acknowledgements—This work was supported by Grant No. 075900/85 from CONICET (Consejo Nacional de Investigaciones Cientificas y Técnicas, Argentina) and in part by Grant No. 8706 from Universidad Nacional de San Luis, Argentina. D.G.C., A.M.R. and L.I.A. are Professors at the Universidad Nacional de San Luis. D.G.C. and L.I.A. are also career scientists from the Centro Regional de Estudios Avanzados (San Luis, Argentina) and CONICET, respectively. C.M.T. is a fellow from CONICET. The authors are very grateful to Dr R. P. Deis for his critical revision of the manuscript.

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