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Dual Regulation of Luteal Progesterone Production by Androstenedione During Spontaneous and RU486-induced Luteolysis in Pregnant Rats

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The effect of androstenedione on luteal progesterone production was studied during luteolysis preceding parturition as well as that induced by the antiprogestin RU486 in late pregnant rats. Luteal cells from animals on days 19, 20 or 21 of pregnancy and incubated with 10 μM androstenedione increased progesterone production by 99, 136, and 277%, respectively. The animals receiving androstenedione (10 mg/rat s.c.) on day 19 of pregnancy showed an increase in serum progesterone levels, a decline in luteal 3β-hydroxysteroid dehydrogenase (3β-HSD) activity and an increase in corpus luteum weight without modifying 20α-hydroxysteroid dehydrogenase (20α-HSD) activity on day 21 of pregnancy. Androstenedione and testosterone but not dihydrotestosterone were able to prevent the decrease in serum progesterone concentration and corpus luteum weight observed 58 h after treatment with RU486 (2 mg/kg) on day 18 of pregnancy. However, the three androgens studied inhibited the luteal 3β-HSD activity but 20α-HSD activity was not affected, when compared with animals receiving RU486 alone. The co-administration of androstenedione with the aromatase inhibitor 4-hydroxyandrostenedione or with the specific antioestrogen ICI 164,384 did not modify the effects induced by androstenedione in RU486-treated rats, indicating that the action of androstenedione on progesterone production and secretion at the time of luteolysis seems to occur through an androgenic mechanism and is not mediated by previous conversion of the androgens to oestrogens. In all experiments the high luteal 20α-HSD activity, that characterizes a luteolytic process, was not modified by androgens. Androstenedione administered to adrenalectomized rats was also able to prevent the decrease in serum progesterone concentration observed in spontaneous or RU486-induced luteolysis. The administration of androstenedione to RU486-treated rats induced a decrease in luteal progesterone content concomitant with an increase in serum progesterone levels. These studies demonstrate that androgens during luteolysis, are able to stimulate luteal progesterone secretion, prevent the loss in corpora lutea weight and enhance the decrease in 3β-HSD activity, without affecting the increase in 20α-HSD activity.

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INTRODUCTION

Androgens play an important role in follicular development [1, 2] and steroidogenesis [2–9]. Androgenic steroids have a marked synergistic effect on the stimulation of progestin production by gonadotrophin in rat granulosa cells [3–5, 9] via a receptor-mediated mechanism [10–13] involving the suppression of cAMP catabolism [14]. This effect does not seem to be mediated by oestrogen formation from the androgens [15]. Conversely, there is less information concerning the effect of androgens on luteal progesterone production.

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Androstenedione and testosterone, produced by placental or ovarian tissues, stimulate progesterone production by a mechanism involving the previous intraluteal aromatization to oestradiol, that potentiates luteal steroidogenesis acting by an intracrine mechanism through its nuclear receptor [reviewed in ref. 16]. It was recently demonstrated that androgens such as androstenedione, testosterone or dihydrotestosterone may stimulate progesterone production from rat corpus luteum through a direct androgenic rapid effect, not mediated by previous conversion to oestradiol [17]. It was also demonstrated that the capacity of androstenedione to stimulate progesterone production from luteal cells is enhanced when basal progesterone production is low [17]. On the basis of these results, the present work was designed to evaluate the effect of androstenedione on luteal progesterone production and secretion: at the time of the physiological luteolytic process that precedes parturition and during the luteolysis induced by the antiprogesterone RU486 in late-pregnant rats [18, 19].

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.

Materials

RU486 (17β-hydroxy-11β-[4-dimethyl-aminophenyl]-17α-[1-propynyl]-estra-4,9-diene-3-one; mifepristone) was a gift from Roussell-Uclaf (Romainville, France). ICI 164,384 was provided by ICI Pharmaceuticals (Macclesfield, Cheshire, England). The following drugs were purchased from Sigma Chemical Co. (St Louis, U.S.A.): androstenedione, 4-hydroxyandrostenedione, dihydrotestosterone, HEPES, medium 199, bovine serum albumin fraction V (BSA), EDTA, collagenase type IV (570 U/mg). [1,2,6,7-^{3}H]Progesterone was purchased from Amersham Life Science (U.K.). Luteal cells were incubated in 24-well plastic tissue culture dishes (Corning Laboratory Sciences Co., U.S.A.).

Cell culture

Ovaries from rats decapitated on days 19, 20 or 21 of pregnancy were collected and placed in phosphate-buffered saline (PBS, pH 7.4). The corpora lutea were dissected and luteal cells were dispersed and incubated as described previously [17, 20]. Androstenedione was dissolved in absolute ethanol and added to the medium in a volume of 10 μl.

Surgical and hormonal treatments

In the experiments using animals at the time of the spontaneous luteolytic process, rats on days 17, 18 or 19 of pregnancy were injected subcutaneously dorsally at the back of the neck at 11.00 h with 10 mg androstenedione/rat dissolved in sunflower seed oil. The rats were killed at 11.00 h on days 19, 20 or 21, respectively. Rats injected with oil were used as controls.

An additional group of rats was adrenalectomized immediately before injecting androstenedione on day 19 of pregnancy and a group of sham-operated rats was included as control. These two groups of animals were sacrificed at 11.00 h on day 21 of pregnancy.

A group of rats on day 18 of pregnancy was treated with the progesterone antagonist RU486 (2 mg/kg) dissolved in sunflower seed oil and injected s.c. at 10.00 h. Groups of RU486-treated rats were injected s.c. with a dose of 10 mg of the androgens androstenedione, testosterone or dihydrotestosterone at 20.00 h on day 19 of pregnancy. This amount of androgens was administered considering that a great amount of the steroid in an oily solution would not be absorbed from the injection site. Two additional groups of animals receiving RU486 and androstenedione were bilaterally implanted intrabursally with 10 μg of the aromatase inhibitor 4-hydroxyandrostenedione or 8 μg of the specific antioestrogen ICI 164,384 at 20.00 h on day 19 of pregnancy. The drugs administered intrabursally were vehiculized in 30 μl of methyl cellulose gel (3% in saline) to minimize leakage from the ovarian bursa. Control RU486-treated rats were injected with vehicle. A group of RU486-treated rats was adrenalectomized immediately before injecting androstenedione (day 19 at 20.00 h) and a group of RU486-sham-operated rats was included as control. The rats were killed at 20.00 h on day 20 of pregnancy (58 h after treatment with RU486).

All rats were killed by decapitation; troncal blood was collected to determine serum progesterone concentrations and corpora lutea were dissected from adhering ovarian follicles and interstitial tissues, weighed, and stored at −75°C until processed for enzyme activities and progesterone content.

Enzyme activities

Luteal 3β-HSD and 20α-HSD activities were assayed spectrophotometrically as described previously [18, 19].

Progesterone assay

Progesterone was measured using a radioimmunoassay (RIA) developed in our laboratory with an antiserum raised against progesterone-11-bovine serum albumin conjugate in rabbit. The sensitivity, variability and cross-reaction of this RIA, have been previously reported [20, 21].

In the experiments performed in collagenase-dispersed luteal cells, 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.

The progesterone content of corpora lutea was measured with prior extraction according to the methodology described by Sanchez-Criado *et al.* [22] with a slight modification. The corpora lutea were thawed and homogenized in 1 ml 100% ethanol, centrifuged for 10 min at 2500 *g* and the pellet extracted twice with 500 μl acetone. The combined supernatants were evaporated to dryness and redissolved in 1 ml phosphate-buffered saline (0.01 mol/l, pH 7.0) containing 0.15% (w/v) gelatin. Thereafter, the progesterone concentration was measured by RIA.

Statistical analysis

Student's *t*-test was used to assay differences between means of two groups. One-way or two-way analysis of variance (ANOVA), followed, respectively, by the Duncan's multiple-range test or the *Tau* test, were used for multiple comparisons [23]. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Effect of androstenedione on progesterone production by cultured luteal cells obtained from rats on days 19, 20 or 21 of pregnancy

To evaluate the luteotrophic effect of androstenedione at the end of pregnancy, luteal cells from rats on days 19, 20 or 21 of gestation were incubated for 4 h in the presence of the androgen at a dose of 10 μM (Fig. 1).

A progressive decrease in basal accumulations of progesterone was observed in controls from days 19 to 21 of pregnancy. However, androstenedione stimulated progesterone release from luteal cells in all days studied when compared with basal secretion. A progressive increase in the percentage of stimulation of androstenedione on progesterone production was observed on days 19, 20 and 21 of pregnancy (99, 136 and 277%, respectively). The production rate of progesterone was not different between the three androstenedione-treated groups.

Serum progesterone levels, luteal 3β-HSD and 20α-HSD activities, and corpus luteum weight in rats on days 19, 20 or 21 of pregnancy measured 48 h after s.c. administration of androstenedione (Fig. 2)

Serum progesterone concentration was not modified by androstenedione on days 19 or 20 of pregnancy when compared with controls. However, on day 21 of pregnancy, the circulating concentrations of progesterone were significantly higher in the androstenedione-treated group than in the control group, and not different from the levels obtained in the previous days [Fig. 2(a)].

A progressive significant decrease in corpus luteum weight was observed in control animals between days 19 and 21 of pregnancy. On day 19 corpus luteum weight was similar in control and androstenedione-treated groups. However, androstenedione prevented the progressive decrease in corpus luteum weight described in control rats on days 20 and 21 of pregnancy [Fig. 2(b)].

There was no difference in 3β-HSD activity on day 19 of pregnancy between control and androstenedione-treated groups. However, on days 20 and 21 of pregnancy 3β-HSD activity was slightly lower in androstenedione-treated groups when compared with their respective controls. In control animals a decline in luteal 3β-HSD activity was observed on day 21 of gestation when compared with values on day 20 [Fig. 2(c)].

There was no detectable activity of 20α-HSD in control and androstenedione-treated animals on day 19 of pregnancy. Detectable and similar 20α-HSD

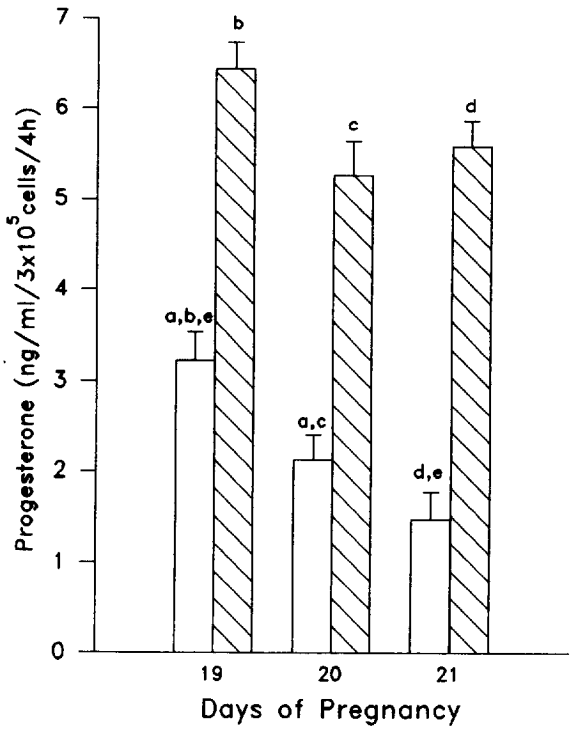

Fig. 1. Effect of androstenedione (▨, 10 μM) or vehicle (□) on progesterone production by luteal cells obtained from rats on days 19, 20 or 21 of pregnancy. Cells were incubated in the presence or absence of androstenedione at 37°C under an atmosphere of 95% air: 5% O_2 for 4 h. Values are the mean + SEM of quadruplicate determinations of 3 different experiments. Columns with the same letter differ significantly (a, $P < 0.01$; b, c, d, e, $P < 0.001$; two-way ANOVA followed by the *Tau* test).

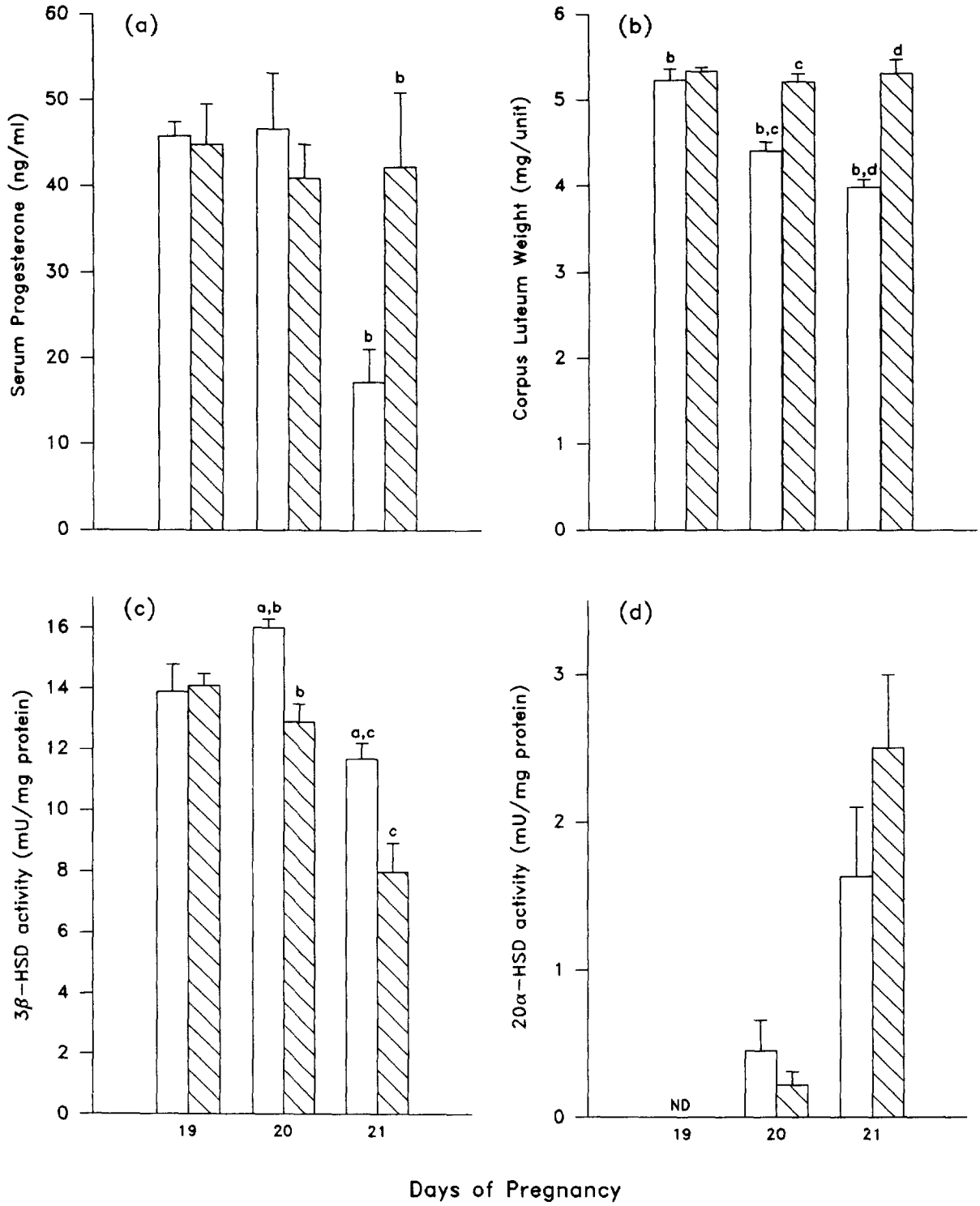

Fig. 2. Effect of androstenedione (▨) or vehicle (□) on serum progesterone concentration (a), corpus luteum weight (b), luteal 3β-HSD activity (c), and luteal 20α-HSD activity (d), 48 h after administration (10 mg/rat s.c. at 11.00 h on days 17, 18 or 19 of pregnancy). Values are mean ± SEM of groups of 6–9 animals. Columns with the same letter differ significantly (a, $P < 0.02$; b, $P < 0$ 01; c, d, $P < 0.001$; two-way ANOVA followed by the *Tau* test). ND indicates not detectable enzyme activity.

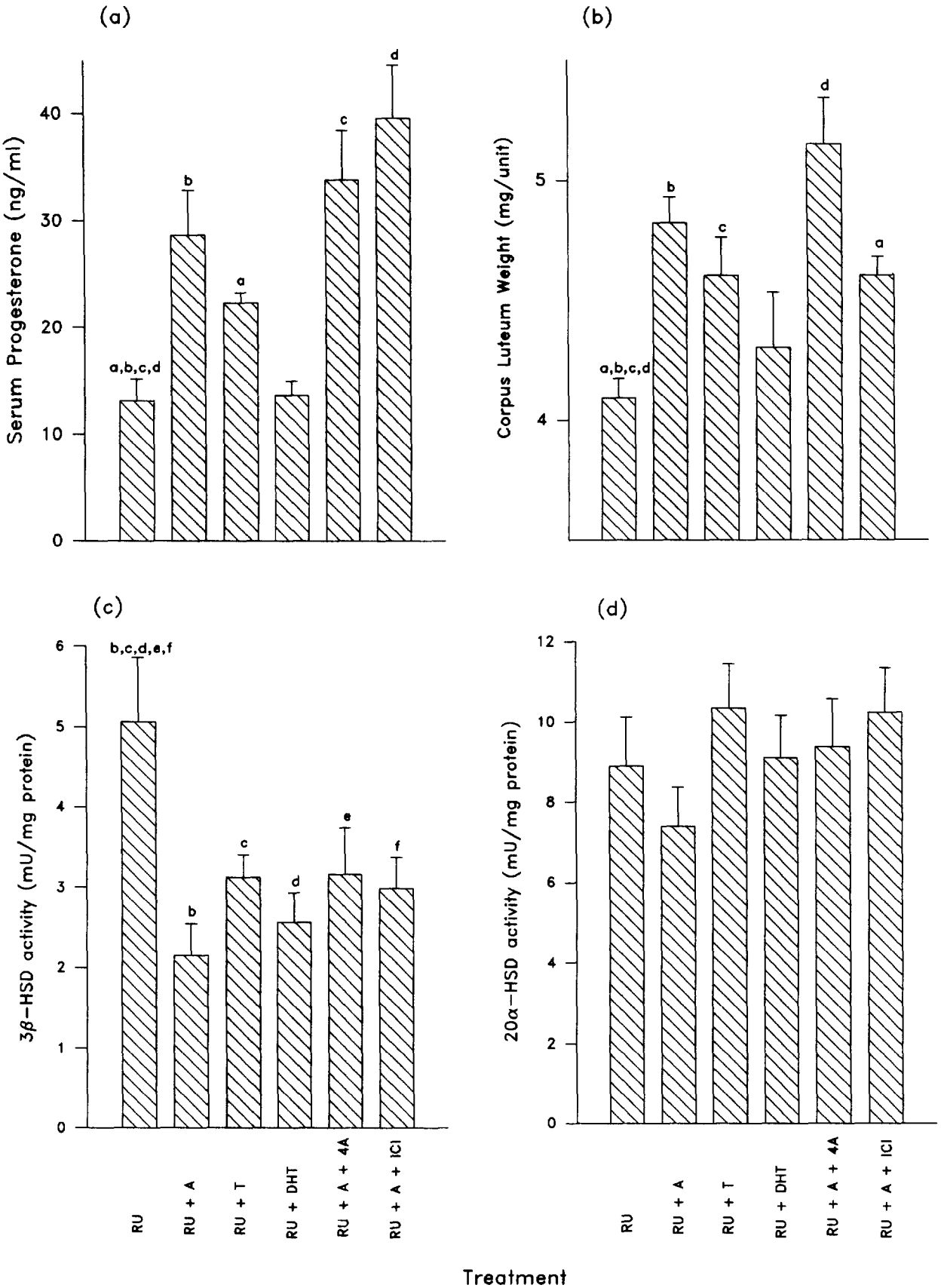

Fig. 3. Effect of androstenedione (A), testosterone (T), dihydrotestosterone (DHT), androstenedione plus 4-hydroxyandrostenedione (4A) and androstenedione plus ICI 164,384 (ICI), on serum progesterone concentration (a), corpus luteum weight (b), luteal 3β-HSD activity (c) and luteal 20α-HSD activity (d), 58 h after administration of RU486 (2 mg/kg s.c.) at 10.00 h on day 18 of pregnancy. The androgens (10 mg/rat s.c.), the aromatase inhibitor 4A (10 μg intrabursally) and the specific antioestrogen ICI (8 μg intrabursally) were injected on day 19 at 20.00 h. Values are mean ± SEM of groups of 6–9 rats. Columns with the same letter differ significantly (a, $P < 0.05$; b, c, d, e, f, $P < 0.01$; ANOVA followed by Duncan's multiple range test).

activities were observed on day 20 but a great increase of the enzyme activity in both control and androstenedione-treated rats occurred on day 21 [Fig. 2(d)].

Effect of in vivo administration of androgens (androstenedione, testosterone and dihydrotestosterone) and the co-administration of androstenedione with the aromatase inhibitor 4-hydroxyandrostenedione or with the antioestrogen ICI 164,384, on serum progesterone levels, corpus luteum weight and luteal 3β-HSD and 20α-HSD activities in RU486-treated rats (Fig. 3)

In these experiments we determined the effect of the *in vivo* administration of androstenedione on corpus luteum function when a luteolytic process was induced by the administration of the antiprogesterone RU486 on day 18 of pregnancy [18]. Taking into account that androstenedione is an aromatizable androgen, the possibility that androstenedione could be converted to oestradiol *in vivo*, was also evaluated. Pregnant rats treated with RU486 received the aromatizable androgens testosterone or androstenedione, the non-aromatizable dihydrotestosterone, the aromatase inhibitor 4-hydroxyandrostenedione plus androstenedione or the specific antioestrogen ICI 164,384 plus androstenedione.

Androstenedione, testosterone, 4-hydroxyandrostenedione + androstenedione, ICI 164,384 + androstenedione but not dihydrotestosterone, administered at 20.00 h on day 19 of pregnancy, prevented the decreases in serum progesterone levels and corpus luteum weight observed 58 h after treatment with the antiprogesterone RU486 (2 mg/kg, day 18, 10.00 h) [Fig. 3(a, b)]. 3β-HSD activity was significantly lower in the groups treated with RU486 plus androstenedione, testosterone, dihydrotestosterone, androstenedione with 4-hydroxyandrostenedione or androstenedione with ICI 164,384 when compared with the group treated with RU486 alone [Fig. 3(c)]. In contrast the 20α-HSD activity was not affected by the same treatments [Fig. 3(d)].

Source of androstenedione-stimulated progesterone production in rats at the time of luteolysis

It is well known that, in the rat, the corpus luteum is the primary source of progesterone throughout pregnancy [24], but it has been also suggested that the adrenals and the feto–placental units contribute to the progesterone pool during pregnancy in rats [25]. Therefore, to assess the target tissue of androstenedione for stimulating progesterone production in rats at the time of luteolysis, we studied the effect of the androgen in rats without placentae and/or adrenal glands. We used the two experimental models of luteolysis previously described.

Physiological luteolysis. The physiological decrease in serum progesterone concentrations observed on day 21 of pregnancy at 11.00 h (17.2 ± 3.9 ng/ml; $n = 7$) was not modified by adrenalectomy performed on day 19 at 11.00 h (15.4 ± 1.4 ng/ml; $n = 6$). However, treatment with androstenedione on day 19 of pregnancy at 11.00 h to adrenalectomized rats, prevented the physiological decrease in serum progesterone levels, and indeed caused an increase in the circulating values of the steroid to 35.9 ± 3.5 ng/ml ($n = 7$; $P < 0.01$).

Luteolysis induced by the antiprogesterone RU486. Rats treated with RU486 on day 18 of pregnancy showing premature delivery the day after [18], were adrenalectomized after abortion (day 19, 20.00 h). This experimental model was used to determine the effect of androstenedione on progesterone production in animals without placental and adrenal tissues. It was noted that adrenalectomy did not modify the decrease in serum progesterone levels observed 58 h after antiprogesterone treatment. Interestingly, androstenedione treatment prevented the decrease in circulating progesterone induced by RU486 administration in adrenalectomized rats (Fig. 4).

Effect of androstenedione administration on corpus luteum progesterone content and serum progesterone concentration in pregnant rats treated with RU486 (Table 1)

The administration of androstenedione on day 19 at 20.00 h, a few hours after the abortion induced by

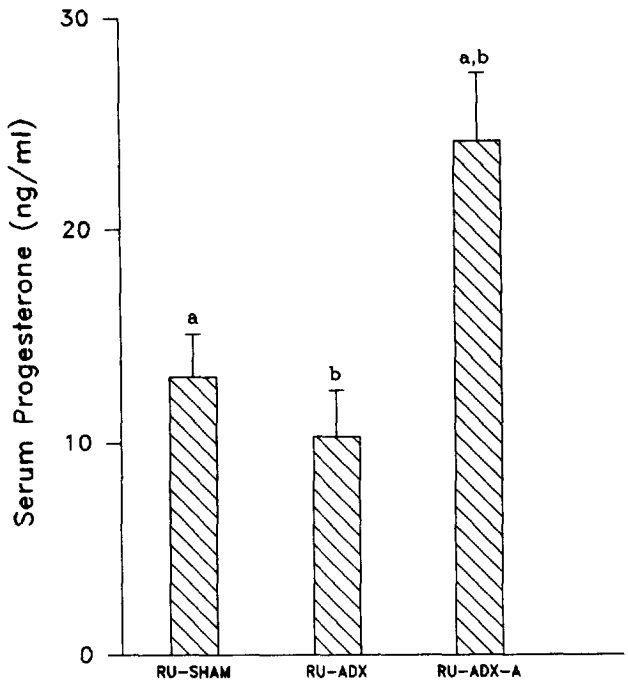

Fig. 4. Serum progesterone levels in RU486-sham operated rats (RU-SHAM; $n = 8$), RU486-adrenalectomized rats (RU-ADX; $n = 6$), and RU486-adrenalectomized rats treated with androstenedione (10 mg/rat s.c. at 20.00 h on day 19 of pregnancy; RU-ADX-A; $n = 5$). RU486 (2 mg/kg s.c.) was administered at 10.00 h on day 18 of pregnancy, and ADX were performed immediately before injecting androstenedione. Results are means ± SEM. Columns with the same letter differ significantly (a, b, $P < 0.01$; ANOVA followed by Duncan's multiple range test).

Table 1. Effect of androstenedione (A, 10 mg/rat s.c. at 20.00 h on day 19 of pregnancy) on corpus luteum progesterone content and serum progesterone concentration measured 58 h after treatment with RU486 (2 mg/kg s.c. at 10.00 h on day 18 of pregnancy)

Treatment	Corpus luteum progesterone content (ng/mg tissue)	Serum progesterone concentration (ng/ml)
RU486	4.53 ± 0.14 (4)	11.13 ± 0.85 (6)
RU486 + A	3.30 ± 0.20 (5)*	34.10 ± 5.78 (5)*

*$P < 0.01$ compared with RU486 group according to Student's t-test. Results are mean ± SEM, number of rats in parentheses. Corpus luteum progesterone content is the mean of 7 corpora lutea per rat.

RU486 treatment, provoked a significant decrease in luteal progesterone content with a simultaneous increase in serum progesterone concentration when compared with values obtained in rats treated only with the antiprogesterone.

DISCUSSION

It has recently been demonstrated that androstenedione, testosterone and dihydrotestosterone stimulate progesterone production, by a direct luteotrophic action, in incubated rat luteal cells obtained in different days of pregnancy [17]. It appears from these results that, on the days of pregnancy when basal progesterone production is low, androstenedione is more effective in stimulating progesterone secretion in luteal cells [17]. Our present results are consistent with these studies since the maximal steroidogenic response to androstenedione was obtained in corpora lutea from day 21 of pregnancy, when the physiological luteolytic process takes place. This pattern of androgen-stimulated luteal progesterone production was also observed in animals in which the luteolytic process was induced by treatment with the antiprogesterone RU486. Androstenedione administered to rats treated with RU486 was capable of preventing the decrease in serum progesterone induced by the antiprogesterone. We may consider that the ovarian response to androgens could be conditioned by the intraovarian progesterone content since it is evident that, when the intraovarian progesterone concentration is low during luteolysis at the end of pregnancy, luteal cells are more responsive to androgens.

A study performed in primates showed that the developing and functional corpora lutea contain receptors for both androgen and progestin, but, at the onset of luteolysis, progestin receptors diminish whereas those of androgens are maintained [26]. Furthermore, the presence of androgen receptors in rat corpora lutea has been demonstrated [27]. We may suggest from our results that androgens, acting at the receptor level, may regulate the luteolytic process.

In the present study there are some conflicting results on the effect of androgens on progesterone biosynthesis and serum progesterone concentration. The increase in serum progesterone levels in response to androstenedione was not correlated with an increase in the progesterone-synthesizing capability of the luteal cells. On the contrary, androgens inhibited luteal 3β-HSD activity. It has been shown that this enzyme catalyzes the last step in progesterone biosynthesis, is hormonally modulated [28–30] and its mRNA declines in corpora lutea together with a decrease in serum progesterone concentration during the initial stage of luteolysis [31]. Our results may indicate a dissociation between progesterone biosynthesis and progesterone secretion, suggesting that the availability of progesterone to be released from corpora lutea may be independent of the corpora lutea capacity to synthesize the steroid. The inhibition of luteal 3β-HSD activity concomitant with the decrease in corpus luteum progesterone content and the increase in serum progesterone concentration observed in the groups of rats treated with RU486 plus androstenedione, support this hypothesis. The dissociation between progesterone biosynthesis and secretion may also be explained by the existence of progesterone-binding sites, recently demonstrated in the cytoplasm of luteal cells in some mammalian species [32–34]. These binding sites could be involved in the sequestration of newly-synthesized progesterone to be released by the luteal cells when progesterone synthesis is impaired. On the other hand, recent evidences obtained in primates and bovines suggest an autonomous pulsatile luteal progesterone secretion, indicating the existence of a local control by the presence of an intraluteal oscillator or pulse generator for progesterone secretion [35, 36].

The decrease in luteal 3β-HSD activities after androgen treatments suggests that androgens may inhibit progesterone synthesis in the corpus luteum. Our results are in accordance with Polan *et al.* [37] who observed that testosterone and dihydrotestosterone antagonized gonadotrophin-stimulated progesterone production and LH-receptor expression in human luteinized granulosa cells. Moreover, it was noted that the non-aromatizable androgen dihydrotestosterone rises in non-luteal tissue of rat ovaries between days 18 and 22 of pregnancy [38] and may be involved in the lysis of the corpora lutea before parturition. Veldhuis *et al.* [39] found an inhibition of progesterone production when cells were incubated with dihydrotestosterone or oestradiol and suggested that it may be due to a blockade of the conversion of endogenous pregnenolone to progesterone by the enzyme 3β-HSD.

We showed that the decrease in corpus luteum weight observed in control rats on days 20–21 of pregnancy was prevented by androstenedione. However, it is interesting to mention that this androgen did not modify the high luteal activity of 20α-HSD, an enzyme that transforms progesterone into a metabolite which is known to be devoid of progestational action

and is also considered a good marker of luteolysis [21, 40]. Preliminary results show that some morphological changes that characterize luteolysis are prevented by androstenedione treatment (data not shown). These results could indicate that androgens may prevent the morphological changes that characterize structural luteolysis [41] without modifying the biochemical changes occurring during the functional luteolytic process.

In animals receiving the aromatase inhibitor 4-hydroxyandrostenedione to prevent conversion of androgens to oestrogens or the specific antioestrogen acting at the receptor level ICI 164,384, androstenedione also stimulated progesterone production. This response indicates that the effect of androgens on progesterone secretion is not due to its conversion to oestrogens, at least when the luteolytic process is induced by RU486.

The results obtained by using the non-aromatizable androgen dihydrotestosterone are not clear. Dihydrotestosterone administration showed an effect similar to that of androstenedione or testosterone only in terms of luteal 3β-HSD activity, but it did not prevent the decrease in serum progesterone levels nor in corpora lutea weight as did the treatment with the other androgens. The partial effects of dihydrotestosterone affecting only luteal 3β-HSD activity may be explained by its rapid metabolism [42] and/or the low effectiveness on luteal progesterone production when compared with the effect of androstenedione and testosterone [17].

Under our experimental conditions, it is clear that the high serum progesterone levels obtained in response to treatment with androgens, are provided by the corpora lutea. The lack of participation of the adrenal glands and the feto–placental units as a source of progesterone after androstenedione treatment, was clearly demonstrated in the adrenalectomized rats and in the rats having abortion after RU486 administration. These results also confirm that the corpora lutea are the principal and essential source of progesterone maintaining pregnancy in rats as indicated by Csapo [24].

In conclusion, our results demonstrate that androgens are able to stimulate luteal progesterone secretion, prevent the loss in corpora lutea weight and enhance the decrease in 3β-HSD activity without affecting the increase in 20α-HSD activity during luteolysis. These androgenic effects suggest that serum progesterone levels may reflect only a component of androgen-supported steroidogenesis.

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