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Luteolytic Action of RU486: Modulation of Luteal 3β-Hydroxysteroid Dehydrogenase and 20α-Hydroxysteroid Dehydrogenase Activities in Late Pregnant Rats*

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The effect of the synthetic antiprogestin RU486 on luteal function in late pregnant rats was studied by evaluating the activities of the enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 20α-hydroxysteroid dehydrogenase (20α-HSD). RU486 (2 mg/kg) administered to rats on day 18 of pregnancy at 10.00 h induced preterm delivery 26.4 ± 0.35 h ($n = 8$) after treatment. Luteal 3β-HSD activity increased 24 and 34 h after RU486 injection, but a significant and progressive decrease started at 48 h with the maximal reduction 72 h after RU486 treatment, when compared with controls. Serum progesterone concentration decreased at the time of 3β-HSD activity reduction. Interestingly, 20α-HSD activity started to increase 58 h after RU486 injection. The administration of the cyclooxygenase inhibitor, diclofenac (1.3 mg/kg), on days 17–19 of pregnancy to RU486-treated rats, delayed abortion and the duration of delivery, and prevented the decrease in 3β-HSD and the increase in 20α-HSD activities observed 58 h after antiprogesterone treatment. RU486 administered intrabursally (1 μg per ovary) on day 20 (14.00–15.00 h) increased 3β-HSD and decreased 20α-HSD luteal activities at 18.00 h on day 21 of pregnancy, without modifying serum progesterone concentration, when compared with normal pregnant rats. In conclusion, the luteolytic process after preterm delivery induced by RU486 administration in late pregnant rats is characterized by a decrease in luteal 3β-HSD activity and circulating progesterone, which may trigger the increase in luteal 20α-HSD activity. Prostaglandins seems to be involved in the increase of 20α-HSD activity and therefore, in the demise of corpora lutea.

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INTRODUCTION

It is well documented that the synthetic steroid RU486, that binds to progesterone receptors acting as a progesterone antagonist [1], induces preterm delivery in the rat [2–4] being the action of RU486 on labour induction mediated by prostaglandins [4]. Less information is available about luteal function following treatment with RU486. It has been proposed that the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), which is responsible for the conversion of pregnenolone to progesterone, is inhibited by RU486 in human granulosa cells [5], rat preovulatory follicles [6, 7] or rat corpora lutea [8]. Moreover, this enzyme is hormonally regulated [9–11] and particularly dependent on progesterone concentrations [7].

The enzyme 20α-hydroxysteroid dehydrogenase (20α-HSD), that converts progesterone into 20α-dihydroprogesterone, a derivative devoid of progestational activity, has been proposed as a good marker of luteolysis [12–15]. The corpora lutea of pregnancy that actively secretes progesterone, expresses no such activity [14, 16]. Luteal cells acquire extensive 20α-HSD activity in a period of less than 24 h at the end of pregnancy by a rapid massive expression of the 20α-HSD enzyme [17], concomitant with an increase in 20α-dihydroprogesterone secretion and a corresponding fall in serum progesterone concentration [12, 14]. The induction of 20α-HSD activity is not only indicative of a luteolytic process previous to parturition, but also in other steps of pregnancy. Thus, corpora lutea of early pregnant rats that received an anti-implantational

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dose of RU486, show an increase in the activity of the enzyme [8].

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is considered one of the principal factors that induces luteolysis in mammals [18]. In pregnant rats, $PGF_{2\alpha}$ induced abortion [19] which is the consequence of the luteolytic effect of $PGF_{2\alpha}$ [20–22]. A rapid induction of 20α-HSD activity after $PGF_{2\alpha}$ treatment suggests an effect of $PGF_{2\alpha}$ on the synthesis of this enzyme [23].

The following experiments were undertaken to ascertain the effect of RU486 on luteal function in rats at the end of pregnancy, through an evaluation of 3β-HSD and 20α-HSD enzyme activities as well as serum progesterone concentrations. The possible participation of prostaglandins mediating the action of RU486 was also examined by using a cyclooxygenase inhibitor.

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. In our laboratory, rats usually give birth on day 22.

Experimental procedures

The progesterone antagonist RU486 (17β-hydroxy-11β-[4-dimethyl-aminophenyl]-17α-[1-propynyl]-estra-4,9-diene-3-one; mifepristone, Roussel-Uclaf, Romainville, France) was dissolved in sunflower seed oil and injected s.c. at 10.00 h on day 18 of pregnancy at a dose of 2 mg/kg. The rats were killed 24, 34, 48, 58 or 72 h after treatment. Rats receiving oil were used as controls.

A group was designed to determine the influence of oestrogen on luteal function in late pregnant rats pretreated with RU486. Animals receiving RU486 (2 mg/kg, day 18, 10.00 h) were treated per os with tamoxifen citrate vehiculized in NaCl 0.14 M, 0.5% tween 80 (Gador, Buenos Aires, Argentina) at a dose of 500 μg/kg, at 10.00 h on day 19 of pregnancy. Animals were killed 34 h after RU486 injection.

A group of animals treated with RU486 on day 18 of pregnancy (2 mg/kg) were injected with diclofenac (Ciba-Geigy, Buenos Aires, Argentina; 1.3 mg/kg s.c.) vehiculized with 0.5 ml of saline solution on days 17 (20.00 h), 18 (08.00 and 20.00 h), 19 (08.00 and 20.00 h) and 20 (08.00 h) of pregnancy. The control group was injected with RU486 on day 18 of pregnancy at 10.00 h and saline solution at the same time of diclofenac administration. The animals were killed 58 h after RU486 administration.

A group of RU486 treated animals was injected with diclofenac (1.3 mg/kg) on days 17 (20.00 h), 18 (08.00 and 20.00 h) and 19 (08.00 h) of pregnancy. A control group was injected with RU486 on day 18 of pregnancy at 10.00 h and saline solution at the same time of diclofenac administration. The interval between RU486 treatment and abortion and the duration of delivery were evaluated. The animals were killed 12 h after abortion to determine the presence or absence of pups inside the uterus. In a group of pregnant rats, RU486 was administered locally into the ovarian bursa according to the methodology described previously [24]. The RU486 was mixed with methyl cellulose gel (4%) as vehicle to minimize leakage from the ovarian bursa. Under ether anaesthesia, the ovaries were exposed through lateral incisions. Each animal received a bilateral intrabursal injection of 30 μl of gel solution (control group) or gel solution with RU486 (1 μg per ovary), on day 20 of pregnancy (14.00–15.00 h) by using a Hamilton microliter syringe (705-N). The rats were killed at 18.00 h on day 21 of pregnancy.

All the rats were killed by decapitation; troncal blood was collected to determine serum progesterone concentrations and corpora lutea were enucleated from the excised ovaries to measure 3β-HSD and 20α-HSD activities.

Determination of abortion

The rats were checked several times a day between 08.00 h and 22.00 h for signs of abortion. As soon as the first foetus was delivered the duration of parturition was carefully determined (time elapsed between the delivery of the first and last foetus).

Enzyme activities

The activities of the enzymes were measured according to Kawano *et al.* [8] with a slight modification. Corpora lutea from each animal were homogenized in 0.7 ml of Tris–HCl, 0.1 M-EDTA 1 mM (pH 8) at 0°C with a glass homogenizer. The homogenates were centrifuged at 105,000 *g* for 60 min. The supernatant fluids were used for the assay of 20α-HSD activity. The precipitates were rehomogenized with 0.7 ml of 0.25 M sucrosa and centrifuged at 800 *g* for 5 min. The supernatants were used as the enzyme solution for the assay of 3β-HSD activity. Both enzyme activities were assayed spectrophotometrically, dependent on the increase in NADH or NADPH in 1 min at 37°C and values were expressed as mU/mg protein. The method of Lowry *et al.* [25] was used for the protein determination with bovine serum albumin as the standard.

Radioimmunoassay of progesterone

Serum progesterone was measured using a radioimmunoassay developed in our laboratory [23] with an antiserum raised against progesterone-11-bovine serum albumin conjugate in rabbits. The sensitivity of the assay was less than 16 nmol/l of serum and the inter- and intra-assay coefficients of variation were less than 10%. Added RU486 had no effect on the progesterone radioimmunoassay.

Statistics

Statistical evaluations of the results were done with unpaired Student's t-test to assay significant differences between means of two groups. One-way analysis of variance (ANOVA) followed by the Duncan's test was used for multiple comparisons. A P value < 0.05 was considered statistically significant.

RESULTS

Luteal 3β-HSD and 20α-HSD activities and serum progesterone concentrations in RU486 treated and control pregnant rats

Treatment with the antiprogesterone changed the activity of 3β-HSD significantly increasing the values with respect to controls 24 and 34 h after treatment and thereafter 3β-HSD activity declined progressively 48, 58 and 72 h after RU486 administration [Fig. 1(a)].

There was no detectable activity of 20α-HSD in controls and RU486 treated animals 24 and 34 h after treatment. A similar detectable 20α-HSD activity was observed 48 h after oil or RU486 administration. A detectable but small increase was observed in the control groups 58 and 72 h after oil administration. On the contrary, a progressive and significant increase in the enzyme activity was obtained in the RU486 groups [Fig. 1(b)].

Serum progesterone concentration was not modified by RU486 24 or 34 h after treatment with respect to controls. Thereafter, a significant decrease in serum progesterone concentration was observed 48,58 and 72 h after treatment with the antiprogestin [Fig. 1(c)].

Effect of tamoxifen on the changes in luteal 3β-HSD and 20α-HSD activities, and in serum progesterone values in pregnant rats treated with RU486

The increase in 3β-HSD activity obtained 34 h after RU486 treatment (18.1 ± 1.6 mU/mg protein, $n = 6$) was significantly reduced (10.5 ± 1.2 mU/mg protein, $n = 8$) ($P < 0.01$) to values of the control group (10.5 ± 1.3 mU/mg protein $n = 5$). The non-detectable 20α-HSD activity was not modified by tamoxifen treatment. Serum progesterone concentration in RU486 treated rats was not affected by treatment with the antioestrogen.

Luteal 3β-HSD and 20α-HSD activities and serum progesterone concentrations in rats treated with RU486 and diclofenac

The administration of the cyclooxygenase inhibitor, diclofenac to pregnant rats treated with RU486, partially prevented the decrease of 3β-HSD activity observed 58 h after RU486 administration [Fig. 2(a)]. On the other hand, the increase in the activity of 20α-HSD obtained after RU486 treatment was prevented by the administration of diclofenac associated with RU486 [Fig. 2(b)]. Simultaneously, the decrease in serum progesterone concentration observed 58 h after treatment with RU486 was partially prevented by diclofenac administration [Fig. 2(c)].

Parturition in pregnant rats after treatment with RU486 and diclofenac

RU486 administration on day 18 of pregnancy to 8 rats induced preterm delivery. Abortion ocurred 26.4 ± 0.35 h after RU486 administration. Treatment with diclofenac significantly delayed parturition and abortion occurred 29.3 ± 0.94 h ($n = 7$) after RU486 treatment ($P < 0.01$). In the rats treated with the antiprogesterone, parturition was completed in 77 ± 5.3 min, while in the group receiving RU486 and

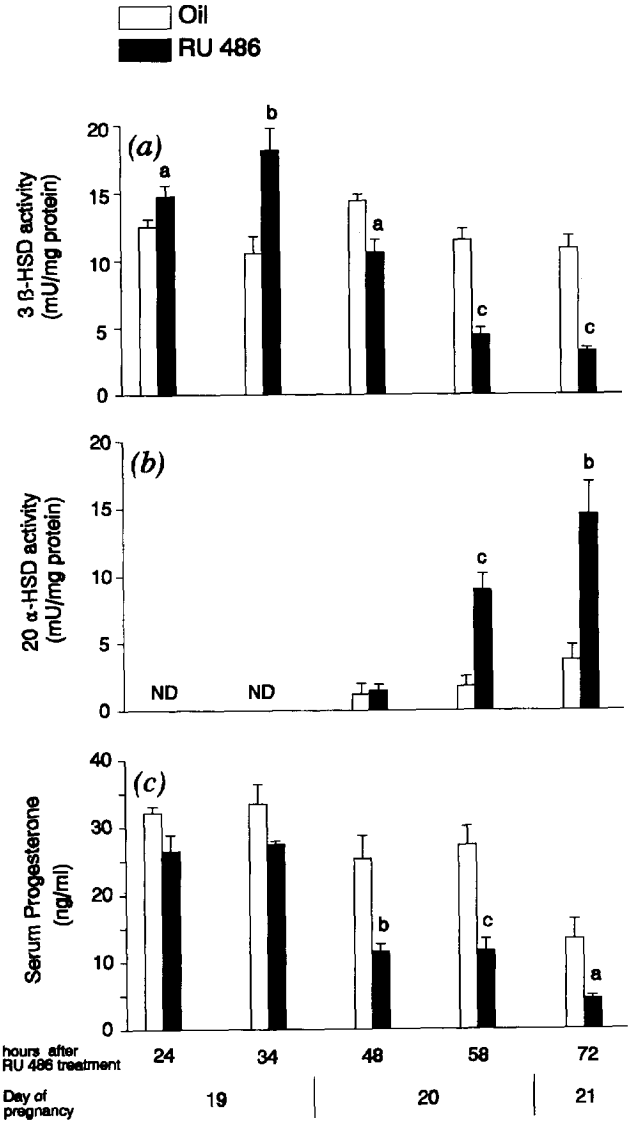

Fig. 1. Time-course variation in luteal 3β-HSD activity (a), luteal 20α-HSD activity (b) and serum progesterone concentration (c) in rats receiving oil or RU486 (2 mg/kg) at 10.00 h on day 18 of pregnancy. Results are mean ± SEM of groups of 6–9 animals. (a) $P < 0.05$, (b) $P < 0.01$ and (c) $P < 0.001$ compared with the respective control values (unpaired Student's t-test).

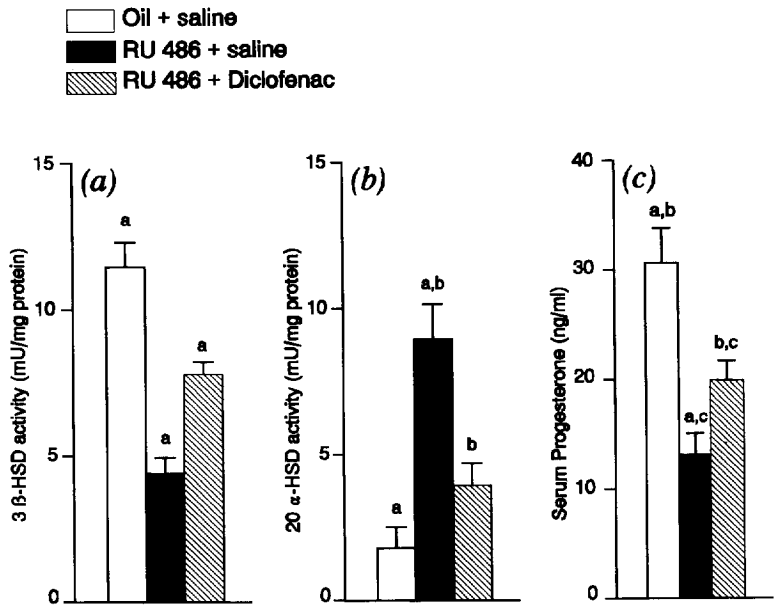

Fig. 2. Effect of diclofenac on luteal 3β-HSD activity (a), luteal 20α-HSD activity (b) and serum progesterone concentration (c), 58 h after administration of RU486 (2 mg/kg) at 10.00 h on day 18 of pregnancy. Diclofenac (1.3 mg/kg) was injected on days 17 (20.00 h), 18 (08.00 and 20.00 h), 19 (08.00 and 20.00 h) and 20 (08.00 h). Values are mean ± SEM of groups of 6–9 animals. Columns with the same letter differ significantly (a, b: $P < 0.01$; and c, $P < 0.05$; ANOVA followed by Duncan's multiple range test).

diclofenac, all rats had incomplete parturition showing inside the uterus 1–5 dead foetuses when killed 12 h after delivery of the first foetus.

Luteal 3β-HSD and 20α-HSD activities and serum progesterone concentrations on day 21 of pregnancy after intrabursal ovarian administration of RU486

To assess if the stimulatory effect of RU486 on 3β-HSD activity observed 24 and 34 h after treatment [see Fig. 1(a)] was due to a direct ovarian action of the antiprogesterone, the drug was administered locally into the ovarian bursa on the afternoon of day 20 of pregnancy. The low 3β-HSD activity observed at 18.00 h on day 21 of pregnancy in control rats, was significantly increased by the intrabursal administration of RU486 [Fig. 3(a)]. On the contrary the physiological increase in 20α-HSD activity was inhibited by the antiprogestin [Fig. 3(b)]. Serum progesterone concentration was similar in both groups [Fig. 3(c)].

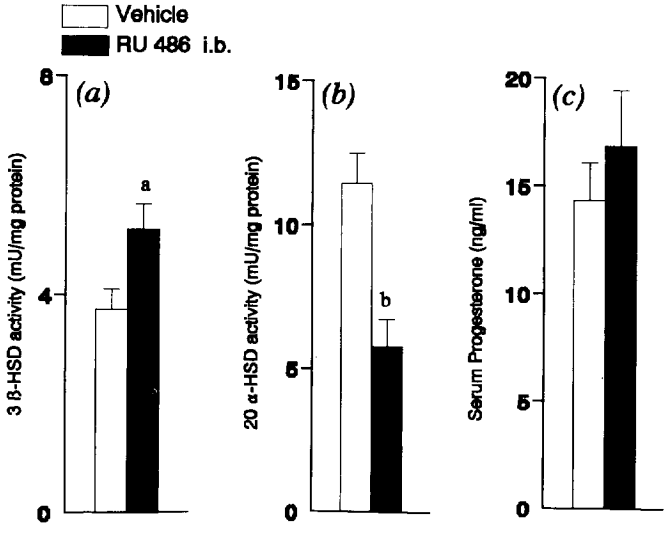

Fig. 3. Effect of RU486 or vehicle administered intrabursally (i.b., 1 μg per ovary, at 14.00–15.00 h on day 20 of pregnancy) on luteal 3β-HSD activity (a), luteal 20α-HSD activity (b) and serum progesterone concentration (c) at 18.00 h on day 21 of pregnancy. Values are mean ± SEM of groups of 6–9 animals. (a) $P < 0.05$ and (b) $P < 0.01$ compared with vehicle (unpaired Student's t-test).

DISCUSSION

The observations reported here on the efficiency of RU486 in inducing preterm delivery acting directly on uterine cells, are in agreement with previous reports [2–4]. Confirming the observation of Cabrol *et al.* [4] suggesting the participation of prostaglandins in the induction of labour by RU486, we show that the cyclooxygenase inhibitor, diclofenac, delays not only the interval between the administration of RU486 and parturition but also the duration of delivery. These results are compatible with clinical studies performed in humans and in other primates where the association of RU486 and prostaglandin derivatives improved the efficacy of RU486 for the medical termination of early pregnancy [26].

In pregnancy, the demise of corpora lutea precedes parturition, the withdrawal of serum progesterone being an essential step in the initiation of both lactogenesis and parturition [23, 27–30]. However, when uterine progesterone receptors were blocked by RU486 on day 18 of pregnancy, delivery occurred approx. 24 h after treatment, but the enzymatic changes which characterize luteolysis became evident 58 h after RU486 with the concomitant decrease in serum progesterone concentration. On the contrary in the control pregnant rats, luteolysis and changes in luteal enzyme activities occurred immediately before parturition [19].

It is amply recognized that the increase in luteal 20α-HSD activity indicates that the process of luteolysis is occurring [23, 31]. The decrease in 3β-HSD activity and the subsequent decline in serum progesterone concentration observed by us 48 h after RU486 injection, without increase in 20α-HSD, may suggest that the reduction in 3β-HSD activity previous to activation of 20α-HSD could be an early marker of luteolysis. Although 3β-HSD appears to be constitutively expressed in the rat corpus luteum [32–34], recent studies indicated that this enzyme can be modulated by hypophysial hormones at the level of gene expression [33, 35]. A recent work performed in cattle [36] demonstrated that the initial stage of luteolysis induced by $PGF_{2\alpha}$ is correlated with the decline in plasma progesterone and mRNA for 3β-HSD, without modifications in the mRNA for cytochrome *P*-450 side-chain cleavage, the enzyme complex involved in the conversion of cholesterol to pregnenolone. These results suggest that the decrease in mRNA of 3β-HSD may be implicated in the decline in plasma progesterone during luteolysis. It has also been shown that the inhibition of 3β-HSD gene expression and activity observed in rat corpora lutea, occurs early in the luteolytic process induced by prolactin, in hypophysectomized rats even before gross morphologic changes could be detected [35].

One striking result was the increase in luteal 3β-HSD activity observed 24 and 34 h after RU486 treatment. The changes in enzymatic activities obtained after treatment with the antiprogestin administered intrabursally may indicate a direct stimulatory action of RU486 at the ovarian level. Most probably the increase in 3β-HSD activity may have prevented or delayed the increase in 20α-HSD activity when compared with the physiological luteolysis process occurring in control pregnant rats in the afternoon of day 21 of pregnancy. Curiously, despite the changes in 3β-HSD activities after intrabursal RU486 treatment on day 20, no differences from controls were observed in serum progesterone concentration. This apparent discrepancy between steroidogenic enzyme activities and serum progesterone concentration, may be interpreted as an independent regulation of progesterone biosynthesis and secretion. It was recently reported that in corpora lutea from pigs and sheep, progesterone is compartmentalized in a unique subcellular fraction [37, 38] which also contains specific binding sites for progesterone [38, 39]. These luteal progesterone binding sites may be involved in the sequestration of newly-synthesized steroid for secretion by the luteal cells.

On the other hand, treatment with the oestrogen antagonist, tamoxifen on day 19 of pregnancy, prevented the stimulatory effect of RU486 on 3β-HSD activity 34 h after RU486 administration. A possible oestrogenic action of tamoxifen has been suggested at uterine level, but no evidence exists of an oestrogen effect of tamoxifen at ovarian level. Therefore, these results may suggest that the circulating oestrogen at this time of pregnancy [40, 41] may exert its well characterized luteotrophic effect [42] when progesterone is not acting on its ovarian receptors [43, 44] after RU486 administration. This result may also suggest that at the end of pregnancy the luteotrophic effect of oestrogen is in some way regulated by the ovarian progesterone concentration. Recently, we observed that RU486 is a useful tool to demonstrate an inhibitory direct effect of progesterone on ovarian steroidogenesis at the end of pregnancy [45]. This inhibitory effect of progesterone seems to be mediated by oestrogen, indicating the interaction between oestrogen and progesterone in the regulation of ovarian steroidogenesis.

The results obtained by using a cyclooxygenase inhibitor, indicate that prostaglandins may mediate RU486-induced luteolysis in late pregnant rats. Prostaglandin biosynthesis during luteolysis has been demonstrated in rat uterus [46] and corpora lutea [47–49]. However, the origin of the prostaglandins involved in the luteolysis induced by RU486 remains to be elucidated.

The cyclooxygenase inhibitor partially prevented the decrease in 3β-HSD activity, but 20α-HSD activity was not different from control values. Therefore, the withdrawal of luteotrophic factors from placental tissue [42] may be considered as being responsible for the decrease in luteal 3β-HSD activity and circulating progesterone. The progressive decline in 3β-HSD activity and consequently progesterone production, may

trigger the synthesis of prostaglandins and thereafter the increase in luteal 20α-HSD activity to complete luteolysis.

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