Activity of peroxidase and Δ^5 -3 β -hydroxysteroid dehydrogenase in the ovary of LH-treated cyclic female rats

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Summary. Rats were treated with LH at 08:00 h on the first day of dioestrus or on Days 1 and 2 of dioestrus. Peroxidase activity increased within 3 h in females injected with LH on Day 1 and was associated with a depletion of ascorbate that lasted until the afternoon of Day 1. Values of both substances then returned to basal values by the morning of Day 2. A second LH injection on Day 2 produced effects similar to those seen after LH on Day 1. Females treated with LH did not display greater Δ^5 -3 β -HSD activity than did controls on Day 1 after one LH injection, but did on Day 2 after two LH injections. Nonetheless, the changes were only modest by comparison with those of peroxidase. The peroxidase-ascorbate system appears to be involved in the mechanism responsible for the increased secretion of ovarian progesterone resulting from LH stimulation.

Introduction

Numerous investigations have shown that luteinizing hormone (LH) is luteotrophic in the rat, since it can prolong the life of 'non-functional' corpora lutea (CL) of the oestrous cycle and convert them into 'functional' CL, even in hypophysectomized animals (Huang & Pearlman, 1962; Armstrong, O'Brien & Greep, 1964; Channing & Villet, 1966; Armstrong, 1968; Moudgal *et al.*, 1974). It has also been shown that LH injected on the morning of dioestrus (Day 1) in 4-day cyclic female rats increases ovarian progesterone secretion (Roser & Buffler, 1972; Buffler & Roser, 1974) as well as peripheral blood progesterone concentrations (Barracrough, Collu, Massa & Martini, 1971; Roser, 1974) on the morning of Day 2. Armstrong & Greep (1962) have shown that LH can increase progesterone synthesis by luteinized rat ovarian tissue *in vitro*, whereas prolactin is without effect; the well-known depletion of ascorbate and cholesterol in the luteinized rat ovary in response to LH may be related to this steroidogenic action (Parlow, 1961). However, LH-dependent depletion of ascorbic acid may be due to the induction of peroxidase in the CL of pseudopregnant hCG-primed immature rats, and the conversion of pregnenolone to progesterone may be achieved by peroxidase by a free radical mechanism (Agrawal & Laloraya, 1977). Peroxidase can be demonstrated histochemically in the CL, but not in the growing follicles, of rat ovaries (Agrawal & Laloraya, 1978a) while Δ^5 -3 β -hydroxysteroid dehydrogenase (Δ^5 -3 β -HSD) was found in the granulosa cells of developing follicles, the hypertrophied interstitial cells of thecal origin and also in luteal cells (Agrawal & Laloraya, 1978b). Similarly, in rabbits, peroxidase was present only in the CL, while Δ^5 -3 β -HSD was present in the theca interna of mature follicles, CL and interstitial cells, but not in the granulosa cells (Agrawal & Laloraya, 1980a). The action of LH in inducing peroxidase activity and associated depletion of ascorbate in the CL of rats can be blocked by treatment with antiserum to LH

(Agrawal & Laloraya, 1980b). It therefore appeared likely that the increased luteotrophic action of LH on the ovary during the oestrous cycle might be regulated by the same mechanism. The present study was therefore undertaken (a) to examine the changes in activity of Δ^5 -3 β -HSD [EC 1.1.1.51] and peroxidase [EC 1.11.1.7], in the ovary of the rat during the oestrous cycle after LH administration on the mornings of Days 1 and 2; (b) to measure the associated depletion of ascorbate; (c) to test whether the enzyme changes are correlated with variations in the plasma progesterone concentrations; and (d) to determine whether the changes induced by a single injection of LH would be repeated following a second injection. Such information would be further evidence that the observed changes had physiological, rather than just pharmacological, significance.

Materials and Methods

After completing at least 3 consecutive 4-day oestrous cycles, 150 Sprague–Dawley female rats (CD (SD)BR, COBS, Charles River Breeding Laboratories, Wilmington, Massachusetts) weighing 250–300 g were selected when the vaginal smear was first indicative of dioestrus, i.e. dioestrous Day 1 was always considered as the first day of the cycle.

Experiment 1. The changes in blood progesterone concentration and in ovarian Δ^5 -3 β -HSD and peroxidase activity and ascorbate levels after LH injection on Day 1 were investigated. A group of 45 animals served as controls, each receiving 0.25 ml 0.9% (w/v) NaCl solution injected subcutaneously (s.c.) on the morning of Day 1 at 08:00 h. Venous blood was collected from the left ovarian vein during a period of 15 min under pentobarbitone anaesthesia, following an intravenous injection of heparin (5000 i.u./kg) into the same vein. About 1 ml plasma was obtained from each animal. The individual plasma samples were stored at -20°C until analysis. Blood was collected from groups of 5 rats at the times indicated in Table 1. The ovaries were then removed, dissected free from adjacent tissue, weighed and processed; the left ovary from each animal was analysed for peroxidase and Δ^5 -3 β -HSD, and the right ovary for ascorbate.

Another group of 40 animals received a single dose of LH (NIH-LH-S4) s.c. (70 μg in 0.25 ml 0.9% NaCl) on the morning of Day 1 at 08:00 h. Subgroups of the injected females were killed at the same hours as controls. Blood and ovaries were collected as described above.

Experiment 2. LH was injected on Days 1 and 2 (70 μg LH (NIH-LH-S4) in 0.25 ml 0.9% NaCl s.c. at 08:00 h). The 35 control rats received vehicle only. Groups of 5 rats were killed at the same times as in Exp. 1, except that none were killed at 08:00 on Day 2. Blood and tissue were collected and processed as in Exp. 1.

Progesterone assay. Plasma progesterone was estimated after hexane extraction by radioimmunoassay using [1,2,6,7- ^3H]progesterone (New England Nuclear, Boston, Massachusetts) and an antibody (P-11-192, Endocrine Sciences, Tarzana, California) for progesterone as described by Pauerstein *et al.* (1978). This antibody has cross-reactions of 100% for progesterone; 20.6% for 5 β -pregnan-3,20-dione; 15.3% for 5 α -pregnan-3,20-dione; 6.3% for 4-pregnen-17 α -ol-3,20-dione; 5.3% for 4-pregnen-21-ol-3,20-dione; 5.0% for 4-pregnen-20 β -ol-3-one; and <2.3% for all other steroids tested.

Aliquants (50 μl) of each control and experimental plasma sample were extracted with hexane:ethyl acetate (90:10 v/v, 3 ml) and agitated for 5 min; 0.5 ml of the extraction fluid was added in duplicate to glass mini-vials (Beckman Instrument Co., Fullerton, California) and dried. Anti-progesterone antiserum (100 μl at a dilution of 1:800 of stock in 0.01 M-phosphate-buffered saline containing 0.15 M-NaCl, 0.05 M-EDTA, 0.1% NaN_3 and 0.5% bovine serum albumin [PBS-BSA]) and [^3H]progesterone (20 pg/100 μl in PBS containing 0.1% bovine gamma globulin) were then added to each mini-vial. After overnight incubation at 25°C , separation of bound and free hormones was performed with 200 μl of 90% saturated ammonium sulphate. The bound hormone was pelleted by centrifugation at 4500 *g* for 15 min. The pellet was dissolved in the mini-vial in 0.1 ml 0.1 M-HCl, and 2.5 ml of scintillation cocktail (4 g PPO/l toluene containing 2% methanol) were

added. The vials were counted in a liquid scintillation spectrometer (460C Tri-Carb; Packard Instrument Co., Downers Grove, Illinois). An external recovery was used. Recovery label (0.1 ml of a solution containing 20 pg [1,2,6,7- ^{3} H]progesterone and 0.1% crystalline bovine gamma globulin) was added to the recovery vials and mixed. Scintillation fluid was added and the vials counted in the same manner in the control and unknown samples. The assay sensitivity was 3 pg/vial, and the inter- and intra-assay coefficients of variation were 14.3% and 3.7% respectively. The progesterone values were expressed as ng steroid/ml plasma after correction for recovery losses.

Enzyme assay. The ovaries were weighed and homogenized with a Polytron PT10 homogenizer (Brinkmann Instrument Co., Westbury, New York) in 2.5 ml sucrose (0.25 M) at 4°C. The homogenate was centrifuged at 105 000 *g* for 30 min in a Beckman L5-65 ultracentrifuge. The supernatants were discarded. The pellets were resuspended in 2.5 ml sucrose (0.25 M) and used for assay of Δ^5 -3 β -HSD (Kuhn & Briley, 1970) and peroxidase (Maehly & Chance, 1954). The particulate fraction was used because the highest specific activities of peroxidase activity are found in the pellets comprising the nuclear, mitochondrial and microsomal fractions (Agrawal & Harper, 1983). In the present experiments no initial low-speed centrifugation was performed, so all these elements were present in the pellet used. According to Beyer & Samuels (1956), Δ^5 -3 β -HSD activity is also greatest in the microsomal fraction with a lesser amount in the mitochondria.

Ascorbate was determined by the colorimetric method of Mindlin & Butler (1937).

Statistical analysis. All data were subjected to an analysis of variance followed by a Student-Newman-Kuels test (SPSS Statistical Algorithms). A *P* value of <0.05 was considered significant.

Results

Progesterone concentrations

In Exp. 1 there was no significant difference between progesterone levels at any time during Day 1 or Day 2 (Text-fig. 1a), but concentrations at or after 11:00 h on Day 2 were significantly (*P* < 0.05) less than those measured at equivalent times on Day 1. The value for pro-oestrous animals was significantly lower than those for all other groups except those examined on the afternoon of Day 2. LH injection on Day 1 caused significantly elevated progesterone levels until 14:00 h on Day 2. At and after this time, progesterone values did not vary between control and treated groups.

In Exp. 2 progesterone levels were lower in control animals on Day 2 than on Day 1, and LH injection on Days 1 and 2 led to significantly elevated levels at all times on Day 2 and at 11:00 h on Day 1 (Text-fig. 1b).

Peroxidase activity

Peroxidase activity was not detectable in control animals, but was significantly elevated by LH injection. In Exp. 1 this elevation persisted until 14:00 h on Day 2 and in Exp. 2 until at least 17:00 h on Day 2 (Table 1).

Ascorbate levels

Significant reduction after LH occurred only at 11:00 h on Day 1 (Exp. 1, Table 1) and at 14:00 h on Day 2 (Exp. 2, Table 1). Despite this lack of significance between control and treated animals at most times, after a second injection of LH on the morning of Day 2, the ascorbate levels were reduced to the same values as seen on Day 1 after the first LH injection (see Table 1). Ascorbate levels did not differ in control animals between 11:00 h on Day 1 and 11:00 h on the day of pro-oestrus.

Text-fig. 1. Changes in peripheral blood progesterone concentrations in 4-day cyclic female rats injected with LH at 08:00 h on (a) the first day of dioestrus (Exp. 1), and (b) on Days 1 and 2 of dioestrus (Exp. 2). Each column represents the mean $\pm$ s.e.m. from a group of 5 animals. *Significantly different from control value ($P < 0.05$).

Δ^5 - 3β -HSD activity

In control animals this activity did not vary on Day 1 or Day 2, although it was higher at 11:00 h on the day of pro-oestrus than at 11:00 or 14:00 h on Day 1. In Exp. 1, LH injection did not significantly increase Δ^5 - 3β -HSD activity over the control value at any time (Table 1). In Exp. 2 (Table 1), however, significant elevations were seen at 14:00 h on Day 1 and at 14:00 and 17:00 h on Day 2. In both experiments, mean Δ^5 - 3β -HSD activity after LH injection tended to be greater at all times studied.

Discussion

The observation that the induction of peroxidase activity in the ovary of the rat after LH injection on the morning of Day 1 was accompanied by the depletion of ascorbic acid and a modest increase in the activity of Δ^5 - 3β -HSD agrees with previous findings that normal luteal activity in the rat is also characterized by the presence of peroxidase and Δ^5 - 3β -HSD activities (Agrawal & Laloraya,

Table 1. Changes in peroxidase and Δ^5 - β -hydroxysteroid dehydrogenase activity and ascorbate levels in 4-day cyclic female rats injected with LH at 08:00 h on the morning of dioestrus 1 (Exp. 1) or on the mornings of Days 1 and 2 of dioestrus (Exp. 2)

Exp.	Time killed (h)	Peroxidase ($\mu\text{mol/min/g wet wt}$)		Ascorbate (mg/g wet wt)		Total Δ^5 - β -HSD ($\mu\text{mol/min/g wet wt}$)	
		Vehicle	LH	Vehicle	LH	Vehicle	LH
1	<i>Dioestrus 1</i>						
	11:00	ND ^a	1100.8 $\pm$ 11.1 ^a	13.7 $\pm$ 1.34 ^{hi}	5.7 $\pm$ 0.89 ^j	0.22 $\pm$ 0.013 ^m	0.29 $\pm$ 0.023 ^{klm}
	14:00	ND ^a	800.6 $\pm$ 13.4 ^b	14.2 $\pm$ 1.42 ^h	7.1 $\pm$ 2.23 ^{hij}	0.22 $\pm$ 0.025 ^m	0.32 $\pm$ 0.035 ^{klm}
	17:00	ND ^a	420.8 $\pm$ 8.9 ^c	12.0 $\pm$ 1.89 ^{hij}	6.5 $\pm$ 1.78 ^{ij}	0.23 $\pm$ 0.031 ^{lm}	0.31 $\pm$ 0.026 ^{klm}
	<i>Dioestrus 2</i>						
	08:00	ND ^a	250.6 $\pm$ 11.1 ^d	12.3 $\pm$ 1.78 ^{hij}	10.2 $\pm$ 1.34 ^{hij}	0.23 $\pm$ 0.022 ^{lm}	0.29 $\pm$ 0.017 ^{klm}
	11:00	ND ^a	50.9 $\pm$ 7.6 ^f	12.5 $\pm$ 2.23 ^{hij}	11.0 $\pm$ 1.38 ^{hij}	0.27 $\pm$ 0.015 ^{klm}	0.30 $\pm$ 0.022 ^{klm}
	14:00	ND ^a	80.2 $\pm$ 6.2 ^e	13.2 $\pm$ 0.89 ^{hij}	10.8 $\pm$ 1.25 ^{hij}	0.27 $\pm$ 0.009 ^{klm}	0.33 $\pm$ 0.017 ^{klm}
	17:00	ND ^a	ND ^a	12.1 $\pm$ 1.69 ^{hij}	11.7 $\pm$ 1.11 ^{hij}	0.26 $\pm$ 0.013 ^{klm}	0.34 $\pm$ 0.026 ^{kl}
	<i>Pro-oestrus</i>						
	11:00	ND	ND ^a	11.1 $\pm$ 0.84 ^{hij}	10.1 $\pm$ 1.60 ^{hij}	0.34 $\pm$ 0.026 ^{kl}	0.35 $\pm$ 0.022 ^k
2	<i>Dioestrus 1</i>						
	11:00	ND ^a	1150.5 $\pm$ 8.90 ^b	12.7 $\pm$ 2.23 ^{hi}	5.1 $\pm$ 0.89 ^{hi}	0.21 $\pm$ 0.026 ^m	0.27 $\pm$ 0.009 ^{klm}
	14:00	ND ^a	800.9 $\pm$ 8.04 ^d	13.1 $\pm$ 2.68 ^h	4.8 $\pm$ 1.34 ⁱ	0.22 $\pm$ 0.013 ^m	0.38 $\pm$ 0.022 ^{kl}
	17:00	ND ^a	890.5 $\pm$ 6.70 ^c	12.1 $\pm$ 1.34 ^{hi}	5.1 $\pm$ 1.78 ^{hi}	0.22 $\pm$ 0.017 ^m	0.33 $\pm$ 0.026 ^{klm}
	<i>Dioestrus 2</i>						
	11:00	ND ^a	1230.9 $\pm$ 9.39 ^a	12.2 $\pm$ 1.36 ^{hi}	6.5 $\pm$ 1.25 ^{hi}	0.24 $\pm$ 0.026 ^{lm}	0.35 $\pm$ 0.022 ^{kl}
	14:00	ND ^a	650.8 $\pm$ 7.6 ^f	11.7 $\pm$ 1.78 ^{hi}	6.3 $\pm$ 1.38 ^{hi}	0.25 $\pm$ 0.022 ^{lm}	0.38 $\pm$ 0.013 ^k
	17:00	ND ^a	700.4 $\pm$ 6.74 ^e	11.3 $\pm$ 2.01 ^{hi}	5.6 $\pm$ 1.45 ^{hi}	0.26 $\pm$ 0.031 ^{lm}	0.32 $\pm$ 0.259 ^k

Values are mean $\pm$ s.e.m. for 5 animals.

* ND = not detectable ($<20 \mu\text{mol/min/g wet weight}$).

Within each experiment, means with a superscript containing the same letter do not differ significantly ($P > 0.05$).

1978a, b). The injection of LH on the morning of Day 2 further stimulated these two enzymes and the increases coincided with a further elevation of plasma progesterone levels. However, the increase in peroxidase activity was very much greater than that of Δ^5 - β -HSD.

LH increases progesterone synthesis by luteinized rat ovaries *in vitro* (Armstrong & Greep, 1962) and the cellular structures involved in the synthesis of progesterone appear to be stimulated (Savard, 1973). Boehm, Hassani, Kerdellue & Aron (1980) convincingly demonstrated that the changes in plasma progesterone concentration caused by LH (64 μg NIH LH S3 given s.c. between 08:00 and 09:00 h on Day 1) are the consequence of the luteotrophic action of LH on the ovary, and that the LH-induced ovarian progesterone increase on Day 2 results from the activity of the CL formed at the end of the preceding cycle. Our values for ovarian venous blood levels of progesterone were approximately twice those reported by Boehm *et al.* (1980) for the vena cava, but the pattern of secretion over Day 1 and Day 2 in control and LH-injected animals was very similar. The fall in the concentration of blood progesterone after injection of LH on Day 1 occurs by the early afternoon of Day 2, and this was accompanied by a precipitous drop in the activity of peroxidase to low and then undetectable levels. After the second injection of LH on Day 2, progesterone levels and peroxidase activity both remained elevated to the same extent as occurred on Day 1 after the first injection of LH. The final fall in the concentration of blood progesterone occurred by early afternoon of Day 2, and in our study this decrease correlated well with a pronounced decrease in the activity of peroxidase to low and then non-detectable levels. It has been suggested that Δ^5 - β -HSD and 20α -hydroxysteroid dehydrogenase are involved in the regulation of progesterone synthesis during late pregnancy, parturition and lactation in rats (Kuhn & Briley, 1970), although the activity of the 20α -HSD varied very greatly before parturition (90% increase), while the activity of Δ^5 - β -HSD hardly

changed during late pregnancy and then fell abruptly (50% decrease) at parturition. The drop in plasma progesterone was attributed to diversion of pregnenolone to pregnenediol and consequent competition between this compound and pregnenolone for the limited Δ^5 - β -HSD, the pregnenediol being preferentially converted to 20α -hydroxypregn-4-en-3-one. Kuhn & Briley (1970) suggested that ovarian Δ^5 - β -HSD resembles the corresponding enzyme of the adrenal cortex described by Beyer & Samuels (1956) which is important in the conversion of pregnenolone to corticosteroids. Kuhn & Briley (1970) did not measure ovarian peroxidase activity, and another explanation for their results could involve changes in this enzyme rather than those in Δ^5 - β -HSD, which did not seem well correlated with changes in progesterone levels. In the ovary, therefore, peroxidase may be more important than Δ^5 - β -HSD for the conversion of pregnenolone to progesterone.

A high activity of peroxidase has been shown in the CL at oestrus (Agrawal & Laloraya, 1977). The presence of Δ^5 - β -HSD and peroxidase in the same cell types, i.e. luteal cells, probably indicates that, in the rat ovary, both enzymes may be induced by the action of LH and that either or both of these enzymes could regulate the biosynthetic machinery controlling luteal steroidogenesis. Since ovarian and horseradish peroxidase can convert pregnenolone to progesterone very efficiently and this conversion is stimulated 200% by the presence of ascorbic acid (Agrawal & Harper, 1983), and since the absolute level of and changes in Δ^5 - β -HSD activity in the present experiments were small compared to those of peroxidase, we propose that peroxidase may be the key enzyme involved in induced luteal steroidogenesis. Peroxidase acting in peroxisomes may also have other functions, for example disposing of H_2O_2 arising from cholesterol side chain cleavage (de Duve, 1979). The relationship between such activity and the catalysis of pregnenolone to progesterone remains to be elucidated. The present experiments also suggest that Δ^5 - β -HSD may still be involved in basal production of progesterone in the cyclic rat.

This work was supported in part by a WHO fellowship (P.A.) and NIH grant HD10202 (Radioimmunoassay Core). We thank Dr T. M. Siler-Khodr for instruction on the progesterone RIA.

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Received 16 August 1982