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THE APPEARANCE OF 20α-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN PREOVULATORY FOLLICLES OF IMMATURE RATS TREATED WITH PREGNANT MARE SERUM GONADOTROPIN

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SUMMARY

20α-Hydroxysteroid dehydrogenase (20α-SDH) activity, believed to be present exclusively in regressing corpora lutea, was demonstrated in preovulatory follicles of immature rats exposed 52 h previously to pregnant mare serum gonadotropin (PMSG). Production of progesterone from 20α-dihydroprogesterone was proportional to enzyme concentration and time of incubation, and obeyed Michaelis–Menten kinetics.

Induction of follicular 20α-SDH activity is not dependent on the LH surge, since the same activity was found in follicles before and after exposure to the first LH surge.

INTRODUCTION

The enzyme 20α-hydroxysteroid dehydrogenase (EC 1.1.1.149; 20α-SDH) that converts progesterone to dihydroprogesterone has been extensively studied in the rat ovary (see review by Wiest and Kidwell[1]). In the prepubertal rat ovary approaching the first ovulation, enzyme activity remains restricted to the interstitial tissue and theca interna, although weak activity can be detected in large atretic follicles [2]. After the first ovulation, ovarian enzyme activity is restricted to the corpora lutea [3]. Marked variations of 20α-SDH activity are observed during the estrous cycle, with the lowest activity found during diestrus and maximum activity at proestrus and estrus [4]. Histochemically, the enzyme activity has been localized in involuting corpora lutea of the preceding cycle. Granulosa cells and developing graafian follicles were consistently found to be devoid of enzyme activity [5, 6]. The enzyme was absent in newly formed corpora lutea till the end of the first cycle, and then it became moderately active during the following proestrus. Enzyme activity increased continuously throughout the involution of the corpus luteum [7, 8]. In the first generation of corpora lutea from prepubertal rats treated with pregnant mare serum gonadotropin (PMSG), 20α-SDH activity appeared only 10 days after corpus luteum formation [9]. From these and similar studies it became accepted that 20α-SDH activity resides almost exclusively in regressing corpora lutea [1, 6, 10].

In a previous paper we have shown that preovulatory follicles of immature rats posses the ability to produce large quantities of 5α-pregnane-3α,20α-diol, at a time when no luteal tissue is present in the ovary [11]. This indicated the presence of 20α-SDH activity in non-luteal tissue. In the findings described here, the presence of 20α-SDH activity in preovulatory follicles of immature rats treated with PMSG is demonstrated.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade. Most organic solvents were redistilled before use. The sodium salt of NADP was purchased from Sigma Chemical Company, St Louis, MO. Crystalline steroids were obtained from Ikapharm, Ramat Gan, Israel. [1,2-^{3}H]-20α-hydroxy-4-pregnen-en-3-one (20α-dihydroprogesterone; S.A. 45 Ci/mmol) and [1,2-^{3}H]-progesterone (S.A. 50 Ci/mmol) were obtained from NEN, Boston MA and rechromatographed before use.

Animals and homogenization procedure. Female albino rats of the Hebrew University (Wistar derived) strain, weaned at 21 days, were housed in temperature-controlled quarters illuminated for 14 h per day (5:00–19:00 h) and allowed free access to a pelleted food mixture and tap water. For the following experiments immature and adult rats were used. 24-Day-old rats were divided into two groups: one was injected (at 08:00–09:00 h) s.c. with 25 i.u. PMSG in 0.5 ml 0.9% saline and the other group received 0.5 ml saline. On day 26 at 12:00 or 24:00 h, the rats were killed and the ovaries used for the experiments described. In adult rats, vaginal smears were examined daily at 08:00–10:00 h. On the day of proestrus, the rats were killed by cervical dislocation, the ovaries dissected out, weighed on a torsion balance and homogenized in ice-chilled homogenization fluid: 1 ml fluid to 100 mg ovarian tissue for the immature ani-

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mals and 20 ml fluid to 100 mg tissue for the mature rats. Homogenization was accomplished with 10 strokes in an all-glass tight-fitting hand homogenizer. Ovarian weight of the PMSG-treated rats was 39.2 ± 4.0 mg (S.E.M.; $n = 9$), of the immature controls 21.5 ± 0.9 mg ($n = 6$) and of the proestrus rats 72.5 ± 2.2 ($n = 6$). The homogenizing fluid consisted of 0.01 M nicotinamide, 0.001 M cysteine and 0.001 M ETDA in 0.1 M Tris buffer of pH 7.4.

Assay of enzyme activity. The homogenate was centrifuged at 20,000 *g* and the supernatant used for the enzyme assay. 20α-SDH activity was determined as the rate of conversion of 20α-dihydroprogesterone to progesterone in the presence of NADP. The assay was performed in 5 ml test tubes, of 10 mm internal diameter. To each tube 50 nmoles of 20α-dihydroprogesterone (6.4×10^4 d.p.m.) dissolved in ethanol were deposited, and the ethanol evaporated to dryness under a stream of nitrogen. To the tubes (kept in ice) were added 0.3 ml of homogenizing fluid containing 0.5 μmol of NADP and 0.2 ml of the 20,000 *g* supernatant. The incubations were carried out at 37°C in a Dubnoff shaker, in air, and were stopped by the addition of 4 vol. of dichloromethane. Five μg of progesterone were added to each sample and the mixture vigorously shaken on a Vortex mixer and centrifuged at 2000 rev/min for 5 min. The organic phase was removed and the aqueous phase extracted again with 4 vol. of dichloromethane. The combined organic phase was evaporated to dryness under a stream of dry air. Radiometabolites were separated by thin layer chromatography (t.l.c.) in the system chloroform:ethyl acetate 13:1 (v/v). Progesterone and 20α-dihydroprogesterone zones were located on the t.l.c. plates under u.v. light, scraped off the plates and eluted with methanol. Aliquots of the eluates were taken for counting in a Packard liquid scintillation spectrometer in the toluene PPO-POPOP system.

The protein content in each tissue preparation was determined by the method of Lowry *et al.*[12] using crystalline albumin as standard.

Enzyme kinetic constants were calculated by fitting the data to the Michaelis–Menten equation, using the statistical method of Wilkinson[13].

RESULTS

Spectrophotometric determination of 20α-SDH activity by the method of Wiest *et al.*[14] showed low but definite enzyme activity in ovaries of immature rats treated 52 h previously with PMSG, while no activity could be detected by this method in untreated immature rats. Histological examination of the PMSG-treated ovaries revealed well-developed follicles but no corpora lutea. Thus, the difference in enzyme activity has to be attributed to the development of follicular tissue. The low activity in these ovaries necessitated the use of the radiological method of enzyme determination described here, instead of the more convenient spectrophotometric method.

Table 1. Identification of progesterone produced from 20α-dihydroprogesterone by 20α-SDH of preovulatory follicles of immature rats

Crystallization no.	S.A. (d.p.m./mg) Crystals	Mother liquor
1	2630	3260
2	2480	2500
3	2380	2420

The lipid extract of the reaction mixture was chromatographed on t.l.c. in the system chloroform:ethyl acetate 13:1 (v/v) and the radioactivity associated with progesterone (72,000 d.p.m.) mixed with 30 mg of authentic progesterone and recrystallized 3 times from aqueous methanol.

Identification and quantitation of the reaction products. After incubation and separation of the products (see Methods section), the radioactivity was localized exclusively in the zones of progesterone and 20α-dihydroprogesterone on the t.l.c. plates (more than 85%). Radiochemical homogeneity of progesterone on the plates was ascertained by pooling aliquots from different runs and recrystallizing with authentic progesterone to constant S.A. As evident from Table 1, the specific activities of all samples (crystals and mother liquor) remained constant throughout 3 recrystallizations with no appreciable fall in S.A. after the first crystallization.

Optimal conditions for enzyme determination. The effect of enzyme concentration on the rate of conversion of 20α-dihydroprogesterone to progesterone in the presence of NADP was determined. By varying the amount of the 20,000 *g* supernatant added to the standard assay system it was found that up to a certain level (6.5 nmol progesterone produced/10 min/mg protein) initial rates were linear with the amount of enzyme added. Formation of progesterone from 20α-dihydroprogesterone by the 20,000 *g* supernatant as a function of time is shown in Fig. 1. The reaction was linear from 0–40 min (correlation coefficient, $r = 0.961$). Enzyme preparations prepared from ovaries of untreated 26-day-old rats gave a value of 0.28 ± 0.03 nmol progesterone produced per mg protein (mean ± S.E.M.; $n = 6$). This basal value did not increase with increasing time of incubation.

Comparison of 20α-SDH activity derived from immature PMSG-treated and proestrous rat ovaries. Substrate–velocity kinetics of the enzyme preparation from immature PMSG-treated rats were compared to those of a similar preparation from proestrous rats (Fig. 2). The K_m values obtained, after fitting of the data to the Michaelis–Menten equation, were statistically identical for the two preparations (16.2 ± 4.5 μM for the PMSG-treated and 17.9 ± 4.2 μM for the proestrous rats; mean ± S.E.M.). The concentration of the enzyme, however, was about 40 times lower in the PMSG-treated than the proestrous ovaries,

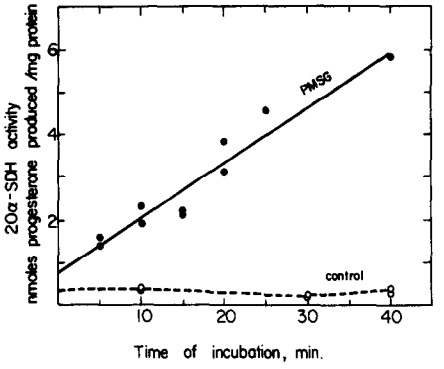

Fig. 1. Time course of 20α-SDH activity in PMSG-treated immature rat ovarian 20,000 *g* supernatant fraction. Aliquots of the fraction containing 1.80 mg protein were incubated with 100 μM of 20α-dihydroprogesterone (6.4 × 10^4 d.p.m.) and 1 mM of NADP in a final vol. of 0.5 ml homogenizing fluid, in a Dubnoff metabolic shaker at 37°C. Progesterone produced was measured as described under the Method section. Each point represents the mean duplicate determinations.

as expressed by the different values of maximal velocities (9.3 ± 0.7 nmol/20 min/mg protein, and 411 ± 48 nmol/20 min/mg protein, respectively).

The effect of varying the temperature of incubation on 20α-SDH activity in both preparations is shown in Fig. 3. Under these experimental conditions, both enzyme preparations exhibited maximal activity at 37°C.

Determination of 20α-SDH activity before and after the first LH surge. In order to find out whether the first LH surge has an effect on enzyme activity, ovaries that were removed at noon on day 26, i.e. two h before the LH surge [15], were compared with those removed at midnight of the same day, namely

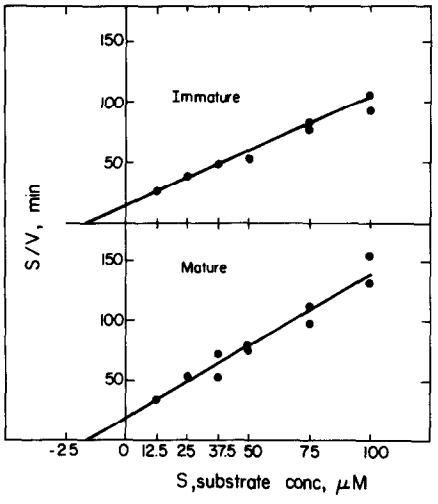

Fig. 2. Kinetic data obtained with enzyme preparations of mature rat ovaries at proestrus and ovaries of PMSG-treated immature rats. 20α-dihydroprogesterone concentrations varied over the range of 12.5–100 μM. The lines representing the Michaelis–Menten equations were fitted statistically to the experimental values [13].

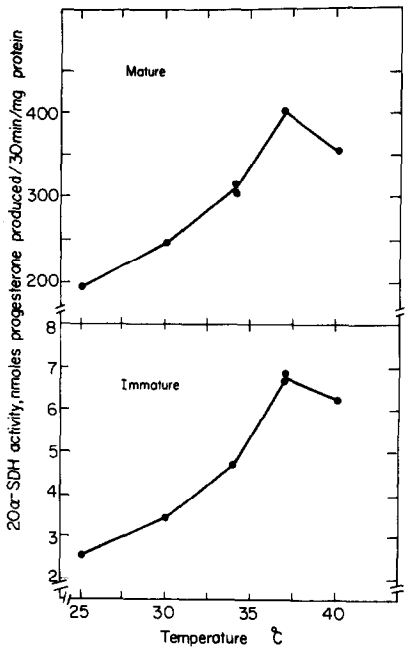

Fig. 3. Effect of temperature of incubation on 20α-SDH activity in mature proestrus and PMSG-treated immature rat ovarian 20,000 *g* supernatant fractions. The enzyme preparations were incubated with 100 μM of [^{3}H]-20α-dihydroprogesterone (6.4 × 10^4 d.p.m.) in a Dubnoff metabolic shaker at various temperatures (20°, 25°, 34°, 37°, and 40°). Data points shown represent the mean of duplicate determinations.

7 h after the peak of the LH surge. No difference in activity was found.

DISCUSSION

The results of the present study demonstrate that an active 20α-SDH is present in preovulatory follicles of immature rats treated with PMSG. This finding contradicts the accepted concept that the enzyme resides almost exclusively in regressing corpora lutea [1,7, 10] and that no enzyme activity can be detected in follicular cells [6, 7, 16]. Histological examination of the ovaries at 52 h after the gonadotropin administration revealed only well developed follicles, but no corpora lutea. Even if some fresh corpora lutea (which could be no more than 10 h duration) has escaped our histological examination, such structures do not contain enzyme activity as shown recently by Lamprecht *et al.*[9].

The discrepancy between the finding of 20α-SDH activity in the present study in follicular structures, and the accepted concept that the enzyme activity resides almost exclusively in the regressing corpus luteum, can be explained by the relatively weak activity that was associated with the ovaries of PMSG-treated rats; this low activity is likely to escape detection by the methods of enzyme assay routinely employed for measuring 20α-SDH activity. Nevertheless, this low activity plays an essential role in pro-

gesterone metabolism: the 1000 *g* supernatant of immature ovaries exposed 52 h previously to 25 i.u. PMSG converted up to 37% of added [^{3}H]-progesterone to 5α-pregnane-3α,20α-diol [11].

The appearance of 20α-SDH activity in the ovaries of immature rats was associated with proliferation of the follicles after stimulation with PMSG, since the enzyme reached its maximal activity before the first LH surge. This finding rules out the function of the LH surge in regulating 20α-SDH activity in this system, in contrast to the role of LH in the regulation of luteal 20α-SDH activity [1]. This does not exclude the possibility that the LH-like activity of PMSG is responsible for the induction of enzyme activity in these ovaries. A more likely possibility is that the induction of 20α-SDH in the proliferating follicles is associated with the action of FSH-like activity of the PMSG preparation.

We do not have definitive proof that the 20α-SDH enzyme found in ovaries of PMSG treated rats is identical with the enzyme that is associated with the regressing corpus luteum. However, both preparations showed the same Michaelis–Menten constant, and had the same temperature maximum under our experimental conditions.

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