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Temporal Changes in Ovarian Steroid-17α-hydroxylase in Immature Rats Treated with Pregnant Mare's Serum Gonadotropin[1] (40375)

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Ovaries in immature rats increase their production of androgens and estrogens in response to pregnant mare's serum gonadotropin (PMS) but only after a lag period of several hours; in contrast, progesterone production increases within a few minutes (1–3). This delay suggests that the enzymes necessary for production of androgen and estrogen, i.e. 17α-hydroxylase, 17–20 lyase, aromatase, and 17β-steroid dehydrogenase, must be induced by gonadotropic action. Indeed, Suzuki *et al.* (4) have demonstrated that the activity of each of these enzymes was increased in ovaries when measured 48 hr after exposure of immature rats to PMS.

Steroid 17α-hydroxylase (EC 1.14.99.9) would appear to be an especially important enzyme in steroidogenesis because it could control androgen and estrogen production via either the 3-oxo-4-unsaturated pathway from progesterone or the 5-unsaturated pathway from pregnenolone. The latter pathway has been shown to predominate in the rabbit ovarian follicle (5) but for the rat the 4-unsaturated pathway may be preferred (4). The present study was undertaken to define the quantitative and temporal changes in hydroxylase of intact and hypophysectomized immature rats associated with exposure of the ovary to PMS. The results indicate that changes in this enzyme are related to secretory patterns of ovarian androgens and estrogens.

Materials and methods. Holtzman strain female rats were maintained in temperature (23 ± 1°) and light (14 hr light: 10 hr dark) controlled quarters and given free access to water and Purina laboratory chow. In some experiments animals were injected with 2mg diethylstilbestrol (DES) dissolved in 0.1 ml of sesame seed oil on the 25th and 26th days of age; controls received only oil. These animals were hypophysectomized via the parapharyngeal approach using ether anesthesia when they were 27 days old. After hypophysectomy a solution of 5% glucose was used for drinking water.

Pregnant mare's serum gonadotropin (PMS) (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.15 M NaCl and injected (20 IU in 0.2 ml, iv) on the 28th day of age. At various times after this injection animals, in groups of 10–15, were killed by decapitation; animals with incomplete hypophysectomy were discarded. Ovaries were removed, cleaned of adhering fat and oviduct, pooled, weighed and then homogenized (100mg wet wt/ml) in cold 0.15 *M* KCl. The homogenate was centrifuged for 20 min at 10,000*g* and then at 105,000*g* for 1 hr. The microsomal pellet from the latter centrifugation was resuspended in 0.15 *M* $NaKPO_4$ buffer (pH 7.4) and used for assay of 17-hydroxylase activity. The protein concentration of the microsomal suspension was determined using the Bio-Rad kit (Bio-Rad Laboratories, Richmond, CA). When in this dilution (≅1mg protein/ml) the hydroxylase is very labile and storage for 12 hr at −20° removes more than half of its activity. In contrast, microsomal suspensions with protein concentrations of 10 mg/ml or greater retain activity for several weeks when stored at −20°.

Hydroxylase activity was determined by the method of Kremers (6). This tritium exchange assay depends upon the reaction: $17\alpha^3$H-pregnenolone + NADPH + H^+ + O_2 ⟶ 17α-hydroxypregnenolone + ^{3}HOH + $NADP^+$. Specifically labelled pregnenolone (15 mCi/mmole) was kindly prepared and characterized by Dr. P. Kremers (University of Liege, Belgium). Unlabelled pregnenolone (Sigma) was used to reduce the specific activity of the label to 2.4 μCi/ μmole.

Generally 0.2 ml of the ovarian microsomal

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suspension (1 ml = 100 mg wet weight of ovary) was incubated in a 20 ml glass scintillation vial. The medium (final volume = 1 ml) contained 100 or 200 nmole of $17\alpha^3H$-pregnenolone (0.25 μCi), 0.5 mg tween 80 to solubilize the steroid, 5 μmole glucose-6-PO_4, 1 IU glucose-6-PO_4 dehydrogenase, 1 μmole NADP, 4 μmole Mg Cl_2 and 0.7 ml $NaKPO_4$ buffer (pH 7.4); all chemicals were obtained from Sigma. Vials were incubated in a Dubnoff shaking water bath at 37°. Preliminary experiments had confirmed Kremers's (6) results in that the enzyme activity is a function of the incubation time, up to 60 min, the amount of ovarian homogenate incubated, up to 0.5 ml, and that 100 nmole of substrate saturated the enzyme. In the present experiments the incubation time was 40 min.

The incubation was stopped by adding 1 ml of ice-cold distilled water followed quickly by a 4 mg pellet of dextran-coated charcoal (IEM Screening System Inc., North Hollywood, CA). We found this method as efficient as the addition of 20% trichloroacetic acid (6) and it has the advantage of removing the substrate radioactivity. The charcoal was separated by centrifugation at 2000*g* for 30 min. The supernatant was transferred to a 25 × 200 mm glass tube and the water distilled under reduced pressure at a temperature of 40°. A 1 ml aliquot of the distillate was placed in a scintillation vial along with 10 ml of instagel (Packard Instrument Co., Downers Grove, IL). The mixture was counted in a Packard Scintillation counter with an efficiency of 64% for tritium. The enzyme activity was expressed as nmole of pregnenolone converted per mg protein per hour. In all experiments the homogenates were incubated in triplicate and in some cases at two dose levels; each series was repeated. The details for special treatments are given with their results. Statistical analyses of enzyme determinations were done using Student's *t* test: $p < .05$ was considered significant.

Results. Ovaries of intact immature rats have considerable 17-hydroxylase activity (Fig. 1). Two groups of animals injected with saline and killed 48 or 96 hr later had enzyme activities which were not significantly different from that of starting controls. Eight hours after injection of 20 IU PMS the activity was reduced by about 90%. The enzyme level remained low through 24 hr and then began an increase so that by 36 hr the activity was the same as that found in starting controls. The enzyme activity continued to increase to a peak level at 48 hr, but it then decreased drastically during the next 12 hr. Activity continued to decrease to almost undetectable levels by 72 hr. Histological examination of the ovaries at 48 and 60 hr revealed many large antral follicles and a highly stimulated theca and interstitium but little luteinization of granulosa. To insure ovulation and luteinization animals were given (sc) 10 IU of human chorionic gonadotropin (hCG) (Antuitrin-"S", Parke-Davis & Co., Detroit, Mich) 48 hr after the injection of PMS. The ovaries were assayed 12, 24, 48 and 72 hr after the hCG (Fig. 1). This treatment did not alter the pattern of decline in hydroxylase activity to any extent.

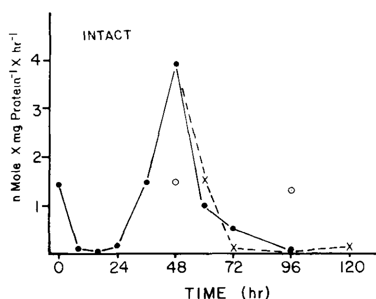

FIG. 1. 17α-hydroxylase activity, determined by a tritium exchange assay, in the ovaries of immature (28 day) rats. PMS(20 IU) was injected (iv) at time 0. Groups of 10–15 rats were killed at various times, the ovaries pooled, homogenized and centrifuged. The 105,000*g* pellet from the homogenate was incubated for 40 min with $17\alpha^3H$-pregnenolone and the tritiated water produced distilled under reduced pressure; the enzyme activity is expressed as nmole of substrate converted per hour per mg protein. Each point represents the mean activity in at least 6 samples; SEM does not exceed the area covered by the symbols. Solid circles (●) indicate values for animals given PMS; single points (◎) indicate enzyme activity for ovaries from control animals given 0.2 ml normal saline. The dotted line indicates changes in enzyme activity in ovaries from animals injected with PMS at time 0 and 10 IU human chorionic gonadotropin (sc) at 48 hr to insure ovulation in all animals.

Ovarian hydroxylase levels in hypophysectomized animals are shown in Fig. 2. The starting controls (24 hr posthypophysectomy) had an activity which was 31% less than that found in uninjected intact females (1.42 ±

0.03 vs 0.98 ± 0.03 nmole × mg protein^{-1} × hr^{-1}). In an additional 24 hr the activity in hypophysectomized animals declined to 0.76 ± 0.08 which indicated a decay rate or half-life of more than 48 hr when endogenous gonadotropins were removed.

Ovaries in rats given DES were 36% heavier at the time of PMS administration than those given oil; the increase was due to larger numbers of granulosa cells in the DES-treated animals. However, hydroxylase activity in the enlarged ovaries was only 4% of the level found in oil-treated animals. The enzyme activity increased in the ovaries of both oil and DES-treated animals between 12 and 24 hr after PMS. In the oil-treated animals enzyme activity had returned to the preinjection control level by 24 hr, remained at this level until 36 hr and then increased sevenfold in the next 12 hr (Fig. 2). Hydroxylase also increased in the DES-treated animals but to a somewhat lesser extent. However, even in the latter, enzyme activity exceeded that found at 48 hr in intact immature females. The enzyme activity of ovaries in hypophysectomized oil-treated animals decreased 27% between 60 and 72 hr (NS $P > .05$) after PMS but it was still 63% higher than the peak level found in intact animals at 48 hr.

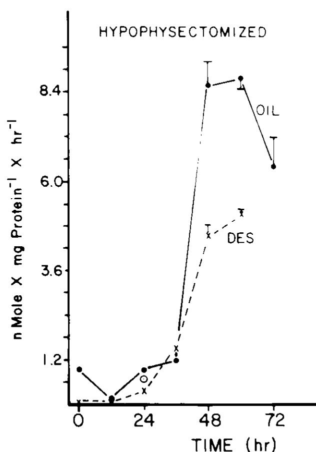

FIG. 2. Hydroxylase activity in the ovaries of hypophysectomized animals; methods indicated in legend for Fig. 1. Animals were injected with 0.1 ml of sesame seed oil (●——●) or 2mg diethylstilbestrol (DES) (×- - -×) on the 25th and 26th days of age; hypophysectomy was performed on day 27 and PMS was injected 24 hr later. The vertical line at each point indicates the SEM for at least 6 samples. The single point (⊙) indicates the enzyme level in a group pretreated with oil and injected with normal saline.

Discussion. The reason for the initial decrease in hydroxylase activity in the ovaries of intact or oil-treated hypophysectomized animals after the injection of PMS is unknown. A similar phenomenon occurs in testicular hydroxylase after administration of hCG (7). The drop in enzyme activity is too rapid (90% in 8 hr) to be accounted for by inhibition of further production and suggests that some other process of inactivation is involved. Testicular hydroxylase has a half-life of 2.5 days (7); the ovarian enzyme seems to disappear at about the same rate when gonadotropin is removed.

Attention has recently been focused again upon the kinds of cells which contain 17-hydroxylase (8). Presumably this enzyme is largely, if not entirely, restricted to thecal and interstitial tissues of the mammalian ovary (8, 9). The present results are consistent with this view; that is, ovaries which were increased in size and weight by increasing the number of granulosa cells with DES did not have a proportionate increase in hydroxylase activity (Fig. 2). We must be careful in interpreting these results however because the ovaries were exposed to high levels of a potent estrogen and this may have had an effect upon hydroxylase or upon the cellular response to gonadotropin. Some suggestion of such an effect, although not necessarily upon granulosa, is gained from the finding that the enzyme level at time 'O' in animals given DES was very low; the enzyme would not be expected to disappear this quickly in animals lacking a pituitary.

If the granulosa cell does not have hydroxylase then luteinization would not be expected to increase the enzyme level in the ovary. Actually luteinization was associated with a drastic decrease in hydroxylase (Fig. 1); as with the initial decrease this one also appeared to be due to an inactivation process considering the rate of decrease. This decrease in hydroxylase may be causally related to a decrease in ovarian androgen and estrogen secretion seen between 48 and 60 hr after PMS administration to immature rats (10, 11). A similar decrease is found with the LH surge on proestrus in the rat and has prompted several speculations into possible

mechanisms. While intra-ovarian autoregulation by steroids or their metabolites has received the most attention (see ref in 11) Katz and Armstrong (12) suggested that LH caused a decrease in aromatase and this was responsible for the drop in estrogen production. The surge in LH associated with PMS treatment may also be responsible for the decrease in hydroxylase found in the intact animals of present study. Further support for this is obtained from the finding that injection of 10 IU hCG into hypophysectomized rats 48 hr after giving PMS resulted in a 90+% decrease in hydroxylase activity within 12 hr (unpublished data). However, this may not be the only factor involved since prolactin caused a 70% reduction in the enzyme activity stimulated by PMS and a 94% reduction in that stimulated by hCG in hypophysectomized animals (unpublished data). Considerably more study is needed for clarification of the control of 17-hydroxylase.

The increase in hydroxylase activity found in hypophysectomized animals coincides with an increase in serum estradiol and testosterone (1, 2). However, the amounts of these steroids in DES or oil-treated animals does not correlate with the enzyme levels found; DES-treated animals had significantly more estrogen and testosterone than did oil-treated controls (3). Perhaps the enzyme levels found in DES-treated animals are inaccurate due to the large volume of granulosa present which does not contribute to enzyme activity, or equally likely, the amount of enzyme present may not indicate the amount of function possible.

Summary. Steroid 17α-hydroxylase was measured, using a tritium exchange assay, in the microsomal fraction of ovaries from immature intact or hypophysectomized rats exposed to 20 IU pregnant mare's serum gonadotropin. The hypophysectomized animals were pretreated with diethylstilbestrol (DES) to increase the ratio of granulosa:theca + interstitium in the ovary; controls received oil vehicle. In intact animals hydroxylase levels decreased within 8 hr after injecting PMS but by 48 hr the concentration was more than 3 times that found in starting controls; after 48 hr the enzyme level decreased drastically and remained low through 120 hr. In oil-treated hypophysectomized rats hydroxylase activity decreased within 12 hr after PMS but in DES-treated animals the enzyme was already extremely low. In both, the enzyme level reached much higher levels than in intact animals and it did not decrease significantly through 72 hr after PMS. The results indicate that 17-hydroxylase activity is induced by PMS treatment but that the enzyme is actively destroyed beginning at 48 hr in intact animals; this could account for the decrease in estrogen and androgen production associated with the ovulatory surge in gonadotropins which occurs on the second day after PMS injection.

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