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The Estrogen 2-Hydroxylase Activity of the Gonadotropin-Stimulated Hypophysectomized Immature Rat Ovary (42796)

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Abstract. Using high-performance liquid chromatography and a combination of electrochemical and radiometric flow detection for 2-[^{14}C]hydroxyestradiol, changes in estrogen 2-hydroxylase activity in the microsomal fraction of rat ovarian homogenates were followed. Injection of human chorionic gonadotropin (hCG) at 12-hr intervals to hypophysectomized immature rats stimulated hypertrophy of the theca-interstitial tissue and produced a profound increase in enzyme activity. With the last injection of hCG at 96 hr the peak serum concentration of hCG was reached 12 hr later and then decreased exponentially with a half-time of 13 hr. However, enzyme activity remained elevated for at least 60 hr before beginning to fall. Pregnant mare's serum gonadotropin (PMSG) also produced an increase in activity, which was apparently limited to the thecal-interstitial tissue because freshly removed granulosa cells from the mature follicles had undetectable activity levels. Administration of anti-PMSG antiserum after enzyme activity had been increased resulted in a prompt fall in activity, as did injection of hCG to mimic an ovulatory surge of LH. The results indicate that the thecal-interstitial tissue of the rat ovary has estrogen 2-hydroxylase activity that is dependent upon gonadotropic stimulation for expression.

Ovarian follicular fluid contains relatively large amounts of hydroxylated metabolites of estradiol (E_2) and estrone (E_1) (1, 2). Included in these compounds are two catechol estrogens, 2-hydroxyestradiol and 2-hydroxyestrone, products of a specific cytochrome *P*-450-dependent monooxygenase (3). Although studied in some detail in only the porcine ovary, mature follicles are presumed to contain the cytochrome *P*-450 and to be the site of catechol estrogen production (4). Furthermore, the activity was found in the follicular wall, with the granulosa layer having the most activity (4). In contrast to results obtained with porcine ovaries, attempts to detect estrogen 2-hydroxylase activity in the rat ovary have had little success (5, 6). However, in the latter studies no attempt was made to correlate enzyme activity with ovarian morphology or hormonal stimulation.

Regulation of estrogen 2-hydroxylase in the ovary has not been examined, but because increased activity can be correlated with increased size of follicles (4) it was assumed that gonadotropins and/or estrogens are involved. Also the role of the enzyme in the economy of the ovary has not been established but catechol estrogens have been implicated as possible autocrine factors for control of steroidogenesis (4). In the present study the hypophysectomized immature rat was used as a model to examine the control of the estrogen 2-hydroxylase activity by gonadotropins. The results indicate that the thecal-interstitial tissue is the major site of enzyme activity and that its specific activity can be dramatically increased by exogenous gonadotropic stimulation.

Materials and Methods. Estradiol-17β (E2), Hepes, Tris, ascorbic acid, NADPH, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). Authentic 2-hydroxyestradiol was obtained from Steraloids (Wilton, NH). Methanol and sodium acetate (HPLC grade) were obtained from Fisher Scientific (St. Louis, MO), and glacial acetic acid was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). The [4-^{14}C]E2 (56.4 mCi/mmole) and [6,7-^{3}H]E2 (45 Ci/mmole) were obtained from Dupont-NEN (Wilmington, DE). The latter label was purified by HPLC before use.

Immature (27–28 day) rats of the Holtzman strain were hypophysectomized by the parapharyngeal approach, using ether anesthesia. The animals were given a 5% solution

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of glucose for drinking water after this operation. Twenty units of pregnant mare's serum gonadotropin (PMSG; Calbiochem, San Diego, CA) was administered as a single intraperitoneal (ip) dose the day prior to hypophysectomy in animals used for harvesting granulosa cells. In another series, 25 IU PMSG was injected the day before hypophysectomy. Chronic treatment with human chorionic gonadotropin (hCG; Serono Laboratories, Randolph, MA) was begun the day following hypophysectomy; the first dose (10 IU) was injected ip, but subsequent doses (10 IU) were injected subcutaneously at 12-hr intervals. The concentration of hCG in the serum resulting from this treatment was determined using the Abbott βhCG 15/15 diagnostic kit (Abbott Laboratories, North Chicago, IL). The mean sensitivity of the assay is 0.76 mIU/ml, evaluated by determining the least detectable dose, defined as the 95% confidence limit (2 SD) at 0 mIU/ml. The interassay coefficient of variation was 6.4% and the intraassay coefficient was 4.1%.

The animals (groups of 4–6) were decapitated, after brief ether anesthesia at various times after hCG, and the ovaries quickly removed, cleaned of fat and oviducts, pooled, weighed, and stored (−75°C) in a solution of 5×10^{-4} *M* NADPH and 0.5 *M* EDTA. In the first PMSG-treatment series the animals were killed at 72 hr and the large preovulatory follicles in 20 ovaries were punctured with a 30-gauge needle. The granulosa cells were expressed by gentle pressure applied by a watchmakers forceps into Hanks' balanced salt medium without calcium or magnesium (GIBCO, Grand Island, NY). The cells were centrifuged at 1000*g* for 5 min, washed with fresh medium, lysed by sonication (Kontes cell disrupter, Vineland, NJ), and processed in the same manner as the ovaries. In the second PMSG-treatment series animals were injected (ip) with 0.1 ml of rabbit anti-PMSG antiserum, or 0.1 ml of normal rabbit serum, 48 hr after administration of PMSG; groups of five rats were killed 6, 12, or 24 hr later. Other animals were injected (ip) with 10 IU hCG, or 0.1 ml saline, 72 hr after the administration of PMSG and killed at various intervals thereafter. Whole ovaries (8–12) were homogenized (glass/Teflon) in a solution of 0.15 *M* KCl containing 0.1 *M* EDTA and centrifuged at 10,000*g* for 15 min. The supernatant fluid was centrifuged at 105,000*g* for 60 min. The pellet was resuspended in the homogenizing buffer and again centrifuged at 105,000*g* for 60 min. The final pellet was resuspended in TEDG buffer (10 m*M* Tris–HCl, 1.5 m*M* EDTA, 1.5 m*M* dithiothreitol, and 10% glycerol) and aliquot quantities were stored at −70°C until assayed for enzyme activity. The protein content was measured by the Bradford (7) method using bovine serum albumin as a standard.

Estrogen 2-hydroxylase was measured by a product isolation method (8). An aliquot quantity of the microsomal fraction of the ovaries, usually equivalent to 50 μg protein, was incubated for 30 min with 100 μl of a reaction mixture containing 10 μ*M* estradiol (E2), 0.075 μCi [4-^{14}C]E2, 10 m*M* ascorbic acid, 1 μ*M* cold 2-hydroxyestradiol, and 1.5 m*M* NADPH in Hepes/Tris (0.05 *M*/0.05 *M*) buffer at pH 8.0. Some assays were terminated by adding 100 μl of 1 *N* HCl containing 2000 cpm of [^{3}H]2OH-E2 for detection of procedural losses. Recovery was consistently between 80 and 85% and therefore no correction was made for losses. Further, to determine the amount of catechol estradiol that might be oxidized under the assay conditions, the same amount of 2-OH-E2 that was used as a carrier was injected directly into the HPLC column; there was no detectable oxidation and thus no correction was needed. The reaction products and unreacted substrate were extracted with ethyl acetate saturated with ascorbic acid, evaporated to dryness, and redissolved in methanol for HPLC analysis. Blank values were obtained by using samples not containing NADPH.

Separation of 2-OH-E2 was performed using an LC 300 liquid chromatograph (Bioanalytical System, West Lafayette, IN) equipped with a flow detector (Flo-One Model IC, Radiomatic, Tampa, FL) as well as an LC-4B amperometric detector, LC-17 glassy carbon flow cell, Ag/AgCl reference RE-1 electrode, PM-30A dual piston pump, and Rheodyne 7125 sample injector with 20-μl injection loop. A 5-μm econosphere ODS column, 25 cm × 4.6 mm (Altech Assoc. Inc., Deerfield, IL) was used, with a

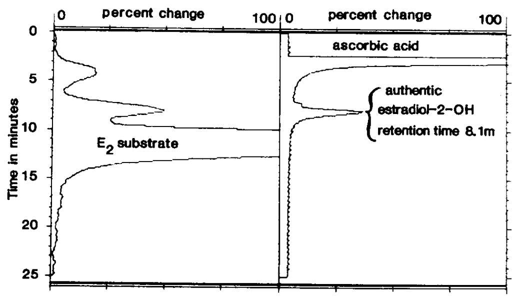

FIG. 1. Pattern of electrochemical (EC channel—right tracing) and radiometric (left tracing) changes with the chromatographic separation of products obtained by reacting rat ovarian microsomes with [^{14}C]estradiol. The first peak in the EC channel corresponds to that of ascorbic acid used to prevent oxidation of the catechol estrogen. The first peak in the radiometric channel represents two compounds, one of which has the retention time of authentic estriol (4.3 min); only the second peak (8.1 min), that of authentic 2-hydroxyestradiol, was used for quantitation. In this system authentic 4-OH-estradiol has a retention time of 6.9 min.

mobile phase of methanol/0.1 *M* sodium acetate/glacial acetic acid (50/42.5/7.5, v/v, pH 4.0) with a flow rate of 1 ml/min and backpressure of 2800 psi. Presence of the analog input option with the radioactive flow detector allows combination with the analog signal of the electrochemical (EC) detector. Thus the ^{14}C and EC channels can be used simultaneously (Fig. 1). The integrated peak area of the product found in the ^{14}C channel corresponding to the authentic cold 2-OH-E2 in the EC channel is used for calculation. Results were expressed as picomoles per milligram protein per 30 min.

Results. Preliminary trials indicated that the mitochondrial fraction of the ovarian homogenates contained little estrogen 2-hydroxylase activity. Furthermore, under the conditions of the present study neither mitochondria nor microsomes produced detectable amounts of 4-OH-E2. However, the incubation of the microsomal fraction resulted in three main peaks as measured by the radiometric detector (Fig. 1). The first peak had a retention time very close to that of estriol (4.3 min), while the second, with a retention time of 8.1 min, was fourfold larger than the first and corresponded to that of authentic 2-hydroxyestradiol (labeled and unlabeled). The third and largest peak eluted at 11.1 min and corresponded with that of labeled and unlabeled estradiol. The first peak was not completely resolved by the present system and the material was not quantitated. Thus all subsequent studies used the microsomal fraction of the ovaries for measurement of only 2-OH-E2 production. The optimal pH for the enzyme was between 8 and 8.5 (Fig. 2); all assays in the present study were run at pH 8.0. Figure 3 indicates that the enzyme is substrate-dependent and using the best-fit curve from the plot of the reciprocal of substrate concentration vs reciprocal of activity yields an appar-

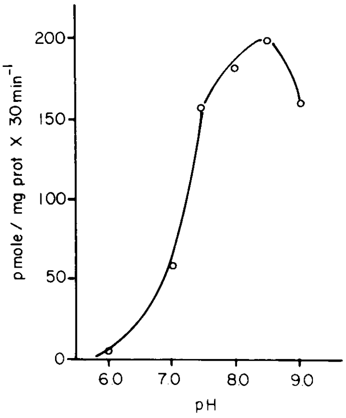

FIG. 2. The effect of pH on estrogen 2-hydroxylase activity obtained with 50 μg of rat ovarian microsomes. All other assays were run at pH 8.0.

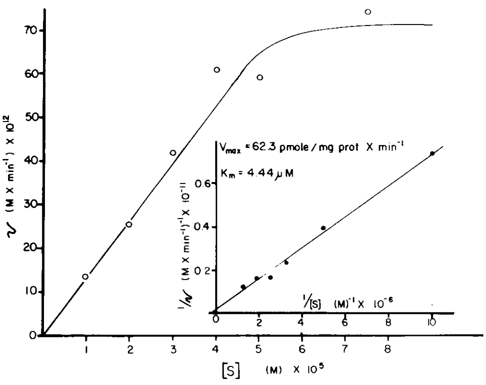

FIG. 3. Changes in estrogen 2-hydroxylase activity of rat ovarian microsomes with increasing substrate concentration. The insert shows the best-fit line for reciprocal velocity vs reciprocal substrate concentration.

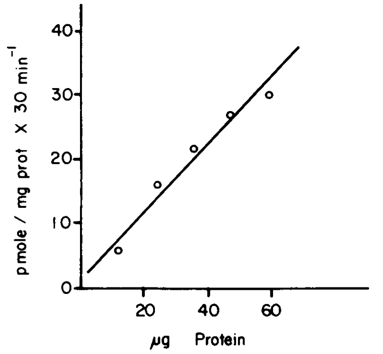

FIG. 4. Estrogen 2-hydroxylase activity increased with increasing amounts of microsomal protein; 50 μg was used for all other assays.

ent K_m of 4.44 μM and a V_{max} of 62.3 pmole/mg protein × min^{-1}. In all subsequent assays 10 μM substrate was used and with this the reaction was linear with the amount of microsomal protein used to at least 60 μg (Fig. 4); all assays were run using 50 μg of protein/assay tube.

Changes in estrogen 2-hydroxylase activity with time after injection of hCG are shown in Fig. 5. The activity decreased during the first 12 hr and remained at about this level at 24 hr. However, within another 24 hr the activities had increased dramatically and by 72 hr, i.e., after six injections of hCG, appeared to approach the maximum; a similar amount of activity was found in ovaries obtained at 96 hr, or 12 hr after the eighth injection of hCG. The maximal serum concentration of hCG was reached by about the same time; i.e., hCG at 24 hr, after two doses of the hormone, was 144.3 ± 4.1 mIU/ml, 176.1 ± 20.7 mIU at 48 hr, 234.7 ± 12.4 mIU at 72 hr, and 283.5 ± 28 mIU/ml at 96 hr, 12 hr after the eighth dose.

If nine doses of hCG were given, i.e., last dose at 96 hr and ovaries removed at varying times thereafter, activity remained elevated for another 60 hr even though the hCG level had fallen by nearly 90%, to 32.3 ± 6.1 mIU/ml by this time (Fig. 6). After 60 hr, however, enzyme activity declined quite rapidly. hCG disappeared from the serum exponentially, i.e., half lost every 13 hr, while enzyme activity decreased more slowly.

PMSG also increased enzyme activity in the ovaries (Fig. 5). There was no detectable activity in 50 μg of microsomes of granulosa cells obtained from the large preovulatory follicles. The ovarian remnant, obtained after removal of the granulosa cells, which contained a few unruptured follicles plus thecal and interstitial tissue, had slightly more specific activity than did ovaries containing the full complement of granulosa cells. These results were obtained on only two groups of ovaries and no statistical analyses are possible.

Figure 7 shows the changes in estrogen 2-hydroxylase activity in animals injected with 25 IU PMSG one day before hypophysectomy. Activity more than tripled between 24 and 48 hr after the dose of PMSG. Injection of anti-PMSG antiserum at the latter time

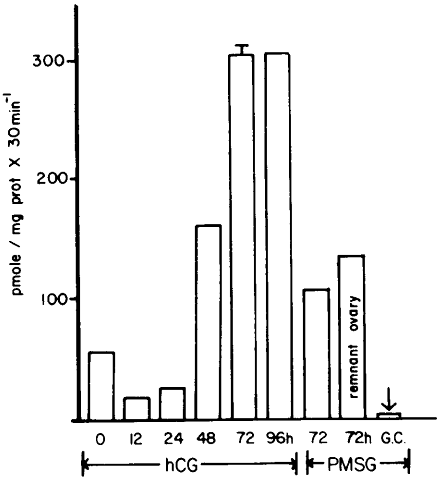

FIG. 5. Estrogen 2-hydroxylase activity in the microsomal fraction of ovaries (8–12 ovaries/homogenate) at varying times after injection of gonadotropins. hCG (10 IU) was injected (ip) at time 0 and sc every 12 hr thereafter to hypophysectomized immature rats. PMSG (20 IU) was injected 24 hr before hypophysectomy and the ovaries were removed 48 hr after the latter operation. Mature follicles from 20 ovaries were punctured to obtain granulosa cells (GC), which had undetectable activity in 50 μg of microsomal protein. The remnant ovary, which contained a few unruptured follicles as well as thecal-interstitial tissue, had slightly more activity than did the whole ovaries in which granulosa cells were not harvested. Each group shown in this figure was represented in the same assay. The activity from several pools was assayed in duplicate; variation did not exceed 6%. Interassay variation (SEM shown as vertical line) was checked only for the homogenate of ovaries removed at 72 hr.

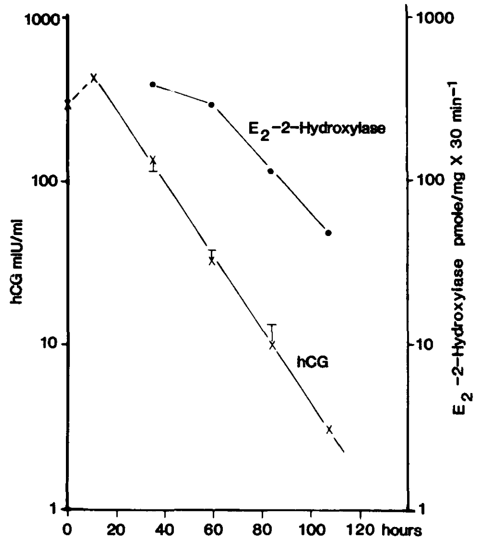

FIG. 6. Changes in serum concentration of hCG and ovarian microsomal estrogen 2-hydroxylase activity of hypophysectomized immature rats. Animals were given hCG (10 IU) every 12 hr for nine doses; the last dose was given at time 0, i.e., which was 96 hr after the first dose. After an initial increase, hCG decreased with a half-time of 13 hr. Enzyme activity remained elevated for about 60 hr and then began to decrease exponentially: Note the log scales for hormone (left) and enzyme (right) values. Vertical lines indicate SEM for the hormone level in groups of at least four animals. Enzyme activity was determined in the homogenate prepared from at least eight ovaries and all were measured in the same assay.

resulted in a distinct fall in activity 6 hr later; activity did not change between the 6th and 12th hr but it declined further between the 12th and 24th hr. Other animals of this series were injected with 10 IU hCG, or saline, 72 hr after the administration of PMSG and were killed 2, 4, 6, or 12 hr later (Fig. 7). The hCG produced an increase in activity within the first 24 hr but this was followed by a dramatic decrease in activity that persisted through the 12th hr.

Discussion. The present study has demonstrated that (i) the rat ovary contains estrogen 2-hydroxylase activity, (ii) this activity is increased by exposure to gonadotropin, (iii) the continual presence of gonadotropin is necessary to maintain activity, and (iv) the activity is probably largely localized in the thecal-interstitial tissue. The ovary of the hypophysectomized rat treated only with hCG does not have maturing follicles but only highly hypertrophied theca and interstitium. The 32-day-old animals that had received nine doses of hCG, at 12-hr intervals, did not have open vaginae nor stimulated uteri, indicating a lack of estrogen; attempts to detect estradiol in the serum by radioimmunoassay were unsuccessful (data not shown). Therefore, the increase in enzyme activity seen at 48 hr and later is not correlated with follicular development nor with ovarian estrogen level, as was the case for the porcine ovary (4). Stopping treatment resulted in an exponential fall in serum hCG level, but enzyme activity did not change for at least 2 days (Fig. 6). On the other hand, when gonadotropin stimulation was removed by an antibody to PMSG (Fig. 7) enzyme activity decreased by 42% in the first 6 hr; by 24 hr only 23% of the activity remained. The serum level of estradiol decreased by 93% within 6 hr and was undetectable in four of six rats at 12 hr after anti-PMSG treatment (data not shown).

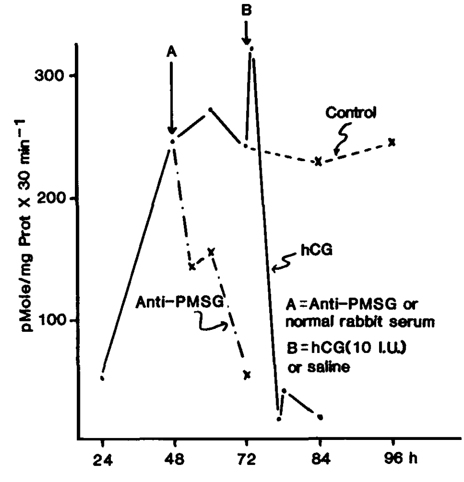

FIG. 7. Loss of ovarian estrogen 2-hydroxylase activity with antigonadotropin or an ovulating dose of hCG. Immature rats were injected with 25 IU PMSG; they were hypophysectomized 24 hr later and the ovaries from a group removed for assay. At 48 hr post-PMSG (A) 0.1 ml of anti-PMSG antiserum, or normal rabbit serum, was injected (ip) and groups were killed at 6, 12, or 24 hr (controls only at 12 and 24 hr). Six other groups were injected (ip) 72 hr post-PMSG (B) with either 10 IU hCG or 0.1 ml saline, and groups were killed 2, 4, 6, or 12 hr later. Each point represents the value obtained for microsomes of ovaries from 4 to 6 rats.

The microsomes of granulosa cells, expressed from mature follicles in ovaries exposed for 72 hr to PMSG, did not contain detectable enzyme activity. Furthermore, removal of most of the granulosa cells from the ovary appeared to increase specific activity suggesting, but certainly not proving, that this ovarian component does not contribute appreciably to the total estrogen 2-hydroxylase activity of the rat ovary. Aspirated granulosa cells from porcine ovaries, as well as dissected interstitial cells, had only a small amount of estrogen 2-hydroxylase activity, but the "follicle wall" containing both granulosa and theca was rich in activity (4). In the latter study separation of the wall into granulosa and thecal layers indicated that both had activity, with the former having the most when calculated on a per follicle basis. Porcine corpora lutea did not contain activity (4). Corpora lutea were not assayed in the present study, but estrogen 2-hydroxylase activity was quickly lost following exposure of the PMSG-stimulated ovary to hCG (Fig. 7). Interestingly, activity increased immediately after injection of hCG in the PMSG-primed animals, but the significance of this is unknown.

The rat ovary, in contrast to the porcine gland (4), did not produce detectable amounts of 4-OH-estradiol. This only indicates that with the amount of microsomes used to measure 2-hydroxylase activity, 4-hydroxylase activity was not detected. Subsequent trials with much larger amounts of microsomes (100+ μg) have yielded measurable amounts of 4-OH-estradiol; quantitation must await further studies. However, another metabolite, which on the basis of retention time in the HPLC could be estriol, was produced (Fig. 1). Further evidence to indicate that this material was estriol was obtained following its separation by column chromatography on Sephadex LH-20 and measurement by a specific radioimmunoassay for estriol (data not shown). Recently a microsomal cytochrome *P*-450 that has 2- and 16α-hydroxylase activity was isolated from male and female rat liver (9). The hCG-stimulated ovary of the hypophysectomized rat has a protein that, when separated by SDS-PAGE and used in immunoblots, binds to the purified antibody to that cytochrome *P*-450 (unpublished data). The hepatic enzyme would appear to play a role in the metabolism of estradiol or estrone, i.e., for production of less potent estrogens. A role for the ovarian enzyme has not been established. A possible paracrine function for catechol estrogen in the porcine follicle has been suggested (4) on the basis of the ability of these metabolites to increase progestin production by porcine granulosa cells. A similar role has been proposed for 2-OH-estradiol in the rat (10, 11). However, several other possibilities require investigation. The catechol estrogens, particularly the 2-hydroxyestrogens, have been shown to be potent biological antioxidants (12) and they may play some role in protecting steroidogenic enzymes. More attractive is the view that the catechol estrogens stimulate the synthesis of arachidonic acid metabolites (13). 2-OH-E2 stimulates prostaglandin synthetase activity and in addition is an excellent cosubstrate for hydroperoxidase (13). Cooxidation with prostaglandin destroys the catechol estrogen, thus removing the latter from further physiologic function (13). Gonadotropic stimulation of the ovary would thus lead to increased synthesis of estrogen 2,16-hydroxylase which could act upon estrogen synthesized by the granulosa to produce catechol estrogens. The latter would stimulate prostaglandin synthesis, and perhaps direct which PGs would be produced (13), and be consumed in the process.

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