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Cellular Origin of Prolonged Induction of Ornithine Decarboxylase in the Rat Ovary[1] (42675)

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Abstract. The temporal changes of ornithine decarboxylase (ODC) activity were investigated in the immature rat ovary following a single subcutaneous injection of pregnant mare serum gonadotropin (PMSG). A dose–response relationship was established. Maximal ODC activity was obtained at a dose of 25 IU of PMSG. This increase in ODC activity was accompanied by an increase of ovarian weight before reaching a maximum. A 250-fold increase of ODC activity was observed 1 day following a single dose of PMSG (50 IU). The enzyme specific activity only returned to the control level 4–5 days after hormone treatment. Immunoreactive ODC in immature, PMSG-primed rat ovaries and in heavily luteinized rat ovaries was localized utilizing the immunoperoxidase method and an antibody to ODC. Immunoreactive enzyme was confined to the cytoplasm of the granulosa cells but was not present in luteal cells. Thecal cells showed only weak immunostaining. This study provides clear evidence that the granulosa cell is the unique source of ODC activity in response to PMSG treatment. Furthermore, these data support the concept that polyamines play a role in granulosa cell proliferation and hence follicular development. © 1988 Society for Experimental Biology and Medicine.

Changes in cellular polyamine content are associated with cell proliferation and differentiation (1). Ornithine decarboxylase (ODC) is a key enzyme in polyamine biosynthesis (2). The activity of ODC is highly regulated and can be stimulated by hormones and growth factors (1). Studies *in vivo* have demonstrated that either pregnant mare serum gonadotropin (PMSG) or luteinizing hormone (LH) stimulates rat ovarian ODC activity (3, 4). Osterman *et al.* (5) have reported that FSH and LH can stimulate ODC activity of porcine granulosa cells *in vitro.* Those studies suggested that the granulosa cell is one potential source of polyamines in the ovary under direct hormone control. However, interactions between different cell types in the developing follicle are complex, and it was not known whether the granulosa cell was the sole source of polyamines in the follicular development phase of the ovary. Since ODC is the first step and therefore a key enzyme in the polyamine biosynthetic pathway in eukaryotes, measurement of this activity serves as a useful indicator of the induction of the pathway. The present study was therefore undertaken to examine temporal changes and the dose–response of ODC in response to exogenous gonadotropin and to assess the cellular locale of the activity *in vivo* by immunohistochemical localization of ODC before and after gonadotropin treatments.

Materials and Methods. *Materials.* Partially purified PMSG and ovine luteinizing hormone (oLH) were obtained from The National Hormone and Pituitary Program, University of Maryland (Baltimore, MD) and the NIDDK, NIH (Bethesda, MD). L-[^{14}C]Ornithine was purchased from Amersham Corp. (Chicago, IL). Vectastain avidin–biotin Complex (ABC) kit for immunoperoxidase staining was purchased from Vector Laboratories (Burlingham, CA). Anti-ODC antiserum raised in rabbits against mouse kidney ODC (6) was a gift from Dr. Chaim Kahana, Department of Virology, the Weizmann Institute of Science, Israel.

Hormone treatments and tissue collections. To study the dose–response relationship of ODC activity to PMSG stimulation, groups of animals were treated with various

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doses of a single injection (sc) of PMSG. Groups of 3–4 immature female Sprague–Dawley rats were injected subcutaneously at 25 days of age with 50 IU of PMSG. In order to determine the effect of luteinization on ODC activity, similarly treated animals were injected with 125 μg of oLH (NIH-LH-S13; 1 U/mg) 56 hr after PMSG. The control groups received saline injections. Animals were sacrificed at various time intervals. Ovaries were quickly removed, trimmed, and frozen in liquid nitrogen. The frozen ovaries were stored in liquid nitrogen until processed.

Ornithine decarboxylase assay. Ornithine decarboxylase was extracted from ovaries after thawing, by homogenizing in 0.5 ml of buffer utilizing a ground glass homogenizer. The buffer used for homogenization was also the ODC assay buffer. The ODC assay buffer contained 50 mM Na_2HPO_4–KH_2PO_4 (pH 7.2) containing 0.1 mM EDTA, 1.0 mM dithiothreitol, 5.0 mM NaF, 1.0 mM phenylmethylsulfonyl fluoride, and 30 mM pyridoxal phosphate. The homogenate was then sonicated in an ice water bath at 50 W for two 10-sec bursts. The sonicated suspension was then centrifuged at 16,000g for 5 min. The clear supernatant fluid was utilized for the assay of ODC activity, performed as previously described (7).

Procedures for immunohistochemistry. Both frozen and paraffin sections were used for ODC immunoperoxidase staining. Fresh ovaries obtained without freezing were fixed 3–5 hr in 10% neutral buffered formalin and embedded in paraffin. Tissue sections from paraffin blocks were deparaffinized and rehydrated. Frozen and paraffin-embedded tissue specimens were sectioned at 5 μm. The frozen sections were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 15 min at room temperature (RT) (8). After fixation the sections were extensively washed in 0.01 M phosphate-buffered (pH 7.4) normal saline (PBS). All the immunostaining procedures were performed at RT.

Paraffin and frozen sections were immersed for 10 min in normal goat serum at 1:40 dilution in PBS to minimize nonspecific binding of the subsequent reagents. After blotting to remove excess normal goat serum, the sections were incubated with anti-ODC serum (1:200 dilution) overnight. The antiserum was diluted in 0.1% saporin in PBS. After washing 3 times in PBS for 10 min, the sections were incubated for 30 min with biotinylated goat anti-rabbit antibody diluted in PBS. After washing in PBS for 10 min, a Vectastain ABC reagent (avidin and biotinylated horseradish peroxidase) was then applied to the sections and they were incubated for 60 min. After incubation the sections were washed in PBS, incubated for 2–7 min in 0.1 M acetate buffer containing 3-amino-9-ethylcarbazole (AEC) and H_2O_2, and washed in water.

Some of the sections were counterstained with hematoxylin. The controls for ascertaining the specificity of the immunoperoxidase staining included the following: (a) the primary antibody was replaced by normal rabbit serum; (b) the primary and secondary antibodies were deleted and avidin–biotinylated horseradish peroxidase complex was added; and (c) the avidin–biotinylated horseradish peroxidase complex was replaced by normal rabbit serum.

Results. The dose–effect relationship of PMSG treatment upon ODC activity is illustrated in Fig. 1. A 40-fold increase in the enzyme activity over the control was noted after a single dose of 10 IU PMSG/rat. Maximal enzyme activity was obtained with 25 IU PMSG/rat. There was no further incre-

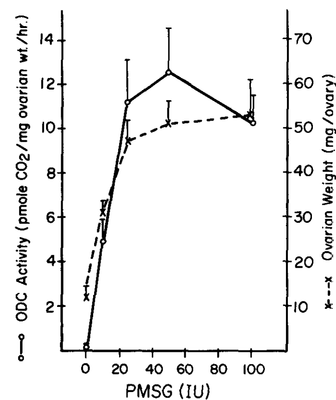

FIG. 1. Changes in ornithine decarboxylase activity (O) and ovarian weights (×) of the ovaries of immature rats treated with various doses of PMSG for 56 hr. Each bar represents the mean ± SE from 4 to 10 rats.

ment with higher doses of PMSG. Ovarian weight also showed a parallel increase following 10 IU PMSG/rat and reached a maximum at 25 IU PMSG/rat.

The effect of PMSG (with or without oLH) injections on ODC activity is illustrated in Fig. 2. One day after a single dose (50 IU) of PMSG administration the enzyme specific activity increased from a control value of 0.13 ± 0.03 (SE) to 32.1 ± 1.1 (SE) pmole/hr/mg wet wt, representing a 250-fold increase in activity. The enzyme activity then began to decrease but only returned to the control level approximately 4 days after the hormone injection. The control groups injected with saline showed no significant change in ODC activity when examined at Days 0, 1, and 2 (Fig. 2).

In order to determine the effect of luteinization on the level and distribution of ODC activity in the ovary, PMSG-primed immature rats were injected with 125 μg oLH 56 hr after PMSG treatment. The oLH produced no significant changes in ODC activity (Fig. 2) when compared with the group of rats which received PMSG without oLH.

Fifty-six hours after a single injection of PMSG (50 IU/rat), the ovaries showed a large increase in the number of secondary follicles and a proliferation of granulosa cells. When immunoperoxidase staining was performed using frozen sections, a distinct brown peroxidase staining appeared in the granulosa cells of PMSG-primed ovaries. There was only a weak staining in the thecal cells (Fig. 3a). At higher magnification, the staining product was found to be confined to the cytoplasm of the granulosa cells (Fig. 3b). All the controls (first antibody deleted) showed negative staining (Fig. 3c). Positive staining was also obtained only in the granulosa cells of nonprimed immature ovaries. However, there are only a small number of granulosa cells in the small follicles of nonprimed immature ovaries. Formalin-fixed and paraffin-embedded sections failed to show staining. The ovaries were heavily luteinized following treatment with PMSG–oLH for 1 week and contained predominantly luteal cells. There was no peroxidase staining in luteal cells. However, positive staining was observed in the granulosa cells of the few small follicles that were present in the luteinized ovaries.

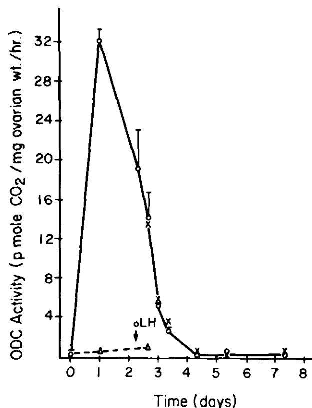

FIG. 2. Changes in ornithine decarboxylase activity in the ovaries of immature rats injected subcutaneously with 50 IU PMSG at Time 0, with (×) or without (O) 125 μg of oLH 56 hr later. Control groups (Δ) received saline injections. Each bar represents the mean ± SE from three to four animals.

Discussion. The present study demonstrates that ODC is localized in the cytoplasm of granulosa cells of immature and PMSG-primed ovaries and that the proliferation of granulosa cells is linked to the increase in ODC activity after PMSG treatment. Since PMSG stimulates the growth of ovarian follicles in part by inducing proliferation of the granulosa cells, and since only weak peroxidase staining of thecal cells in PMSG-primed ovaries was observed, we conclude that the elevated ODC activity after PMSG administration is associated, in part, with the increase in number of granulosa cells. Since the specific activity of the enzyme increased, synthesis of large amounts of the enzyme must occur. Therefore, not all of the increase in ODC activity is due solely to an increase in cell number. Luteal cells of the heavily luteinized ovaries showed no immunoperoxidase staining. In the heavily luteinized ovaries positive staining of granulosa cells was observed only in a few small follicles present. The observation correlates well with the low level of ODC activity in the luteinized ovary 7 days after PMSG (Fig. 1). Osterman *et al.* (5) have shown that either FSH or LH induced ODC activity of granulosa cells isolated from small porcine follicles. It is intriguing that in the present study

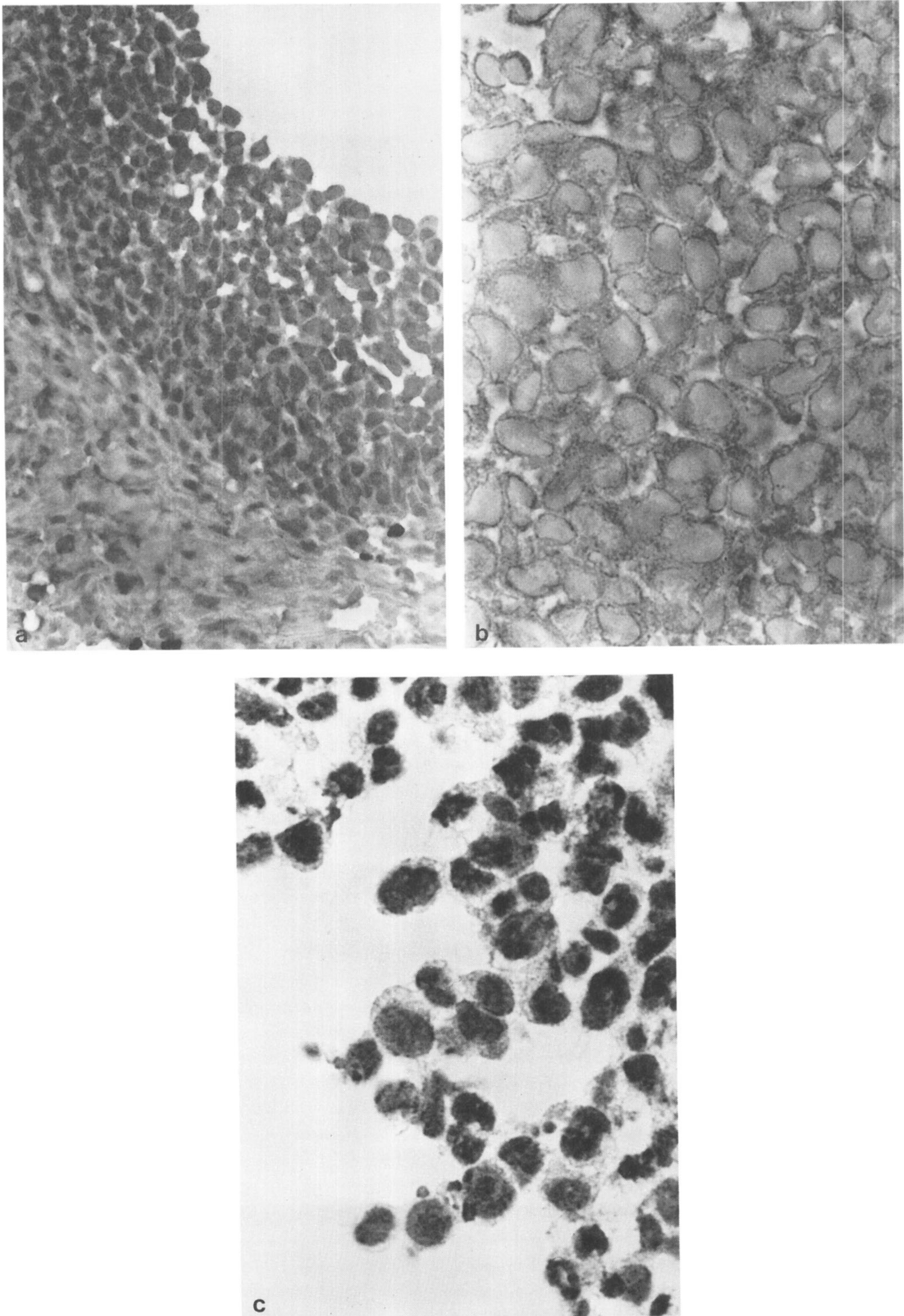

FIG. 3. Frozen sections of rat ovaries stained for ornithine decarboxylase (ODC) utilizing anti-ODC serum and avidin–biotin complex (ABC) technique. The animals were injected with a single subcutaneous dose (50 IU) of PMSG and were killed 56 hr later. The low-power view (a) shows intensely stained granulosa cells and in the left lower part of the micrograph one can see weakly stained thecal cells. Cells were counterstained with hematoxylin (×100). The high-power view (b) shows that the staining is located in the cytoplasm of granulosa cells. No counterstain (×600). The control (c) was stained using normal goat serum instead of anti-ODC serum. The control was counterstained with hematoxylin. The nuclei were stained with hematoxylin but no staining was present in the cytoplasm (×600).

oLH did not cause any further increase in ODC activity during luteinization (Fig. 2) despite the fact that LH receptors increase during luteinization (9) and, therefore, cAMP would have been increased in these cells following LH stimulation. Icekson *et al.* (10) have observed that the Graafian follicle, in contrast to the corpus luteum, was the source of increased ODC activity in response to LH.

Studies *in vivo* using PMSG have demonstrated stimulation of ODC activity in the rat ovary. Johnson and Sashida have demonstrated that intravenous injection of PMSG (20 IU) to immature rats caused a maximal (70-fold) increase in ovarian ODC activity at 3 hr and the enzyme activity declined to a low level by 9 hr (3). In this report, with 50 IU of PMSG, ovarian ODC activity also reached a maximum at 24 hr and sustained a long period of high activity (Fig. 2). The difference in the timing of the responses between our and other investigators' results could be attributed to different routes of injection and doses utilized.

It has been demonstrated that polyamines are required for optimal cell growth and differentiation (1). In the present study, PMSG (10–25 IU) caused a dose-dependent stimulation of ODC activity which was closely correlated with increase in ovarian weight (Fig. 1). Kobayashi *et al.* (4) observed a rise in ovarian ODC activity on the afternoon of proestrus, which may be related to the ovulatory process. Gonadotropin treatment has been shown to increase the secretion of estrogen of the immature rat ovary (11). The concomitant induction of ODC suggests that polyamine biosynthesis is a finely modulated process closely associated with steroidogenic activity of growing granulosa cells that are under hormonal regulation.

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