Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 770-777

ROLE OF ORNITHINE DECARBOXYLASE IN GRANULOSA-CELL REPLICATION AND STEROIDOGENESIS IN VITRO

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Received October 2, 1979

SUMMARY: We investigated the role of ornithine decarboxylase in ovarian steroidogenesis and granulosa-cell replication under basal and hormone-stimulated conditions in vitro. Enzyme activity was markedly (>95 or >99%) reduced by DL-difluoromethyl-ornithine or 1,3-diaminopropane, which significantly impaired granulosa-cell replication in log-phase cultures. However, inhibition of ornithine decarboxylase activity augmented basal and hormone-stimulated steroid production per cell, an effect abolished by cyanoketone, a specific inhibitor of steroid synthesis. Both the anti-proliferative and the steroidogenic effects of enzyme inhibition were substantially reversed by putrescine, the end-product of the reaction. Thus, ornithine decarboxylase, or polyamines, may be required for granulosa-cell replication, while deprivation of these compounds facilitates the expression of more differentiated cell function, such as steroid synthesis.

INTRODUCTION

Tissue growth is closely associated with an increase in intracellular polyamine concentrations, and ornithine decarboxylase activity (ODC,(l-ornithine carboxy)lyase, E.C.4.1.1.17), the rate-limiting enzyme in polyamine synthesis (1). In the ovarian follicle, striking proliferation of granulosa cells occurs during follicular maturation prior to the achievement of maximum steroidogenic capacity (2,3). The role of polyamines or ODC in such follicle growth has not yet been investigated. However, a relationship of ODC to steroid production has also been suggested by certain recent observations. There is a striking correlation between hormonal agents such as gonadotropins, prostaglandins, and catecholamines that stimulate ODC activity and also enhance steroidogenesis (4-7). For example, luteinizing hormone is

ABBREVIATIONS - ODC: ornithine decarboxylase; DFO: DL-alpha-difluoromethyl-ornithine; LH: luteinizing hormone; FSH: follicle-stimulating hormone; DAP: 1,3-diaminopropane

0006-291X/79/230770-08$01.00/0

a potent inducer of ovarian ODC activity in the endogenous proestrus surge (8), and *in vitro* (5), and also markedly enhances follicular steroid secretion (3,4,8). Other *in vivo* observations in the rat suggest that blockade of ODC activity impairs progesterone production (9), and inhibits induced ovulation (10).

To examine the relationship of ODC activity to ovarian steroidogenesis and granulosa-cell replication without potentially indirect effects *in vivo*, we have utilized granulosa cells maintained in acute and long-term monolayer cultures. We have investigated the effects of direct and indirect inhibitors of ODC activity on basal and hormone-stimulated progesterone secretion, and on granulosa-cell replication *in vitro*.

MATERIALS AND METHODS

Ovine LH (NIH-LH-S20), FSH (NIH-FSH-S11) and prolactin (NIH-oPRL-S11) were obtained from the Hormone Distribution Office, NIAMDD, NIH, Bethesda, MD. L-1-[^{14}C]-ornithine monohydrochloride (specific activity: 45 mCi/mM) was purchased from New England Nuclear Corp., Boston, MA; putrescine, diaminopropane, and L-ornithine hydrochloride from Sigma Chemical Corp., St. Louis, MO; and tissue culture medium 199 from Grand Island Biological Corp., New York.

Granulosa cells were isolated nonenzymatically from porcine ovaries as previously described (5), and cultured in 10 cm tissue-culture dishes, or in quadruplicate Falcon multiwells at a density of 5 x 10^5 viable cells/cm^2 in 95% humidified air and 5% CO_2. Tissue culture medium 199 was supplemented with 10% fetal calf serum (Gibco), bicarbonate buffer, and antibiotics (11).

Granulosa cells were mechanically scraped from cultures in ice-cold hypotonic buffer (25 mM Tris HCl, pH 7.4, 0.1 mM EDTA, and 2.5 mM dithiothreitol), and lysed by 2 cycles of freeze-thawing. After centrifugation at 20,000 x g for 30 min, the supernatant was assayed for ornithine decarboxylase activity as previously described (5), and protein determined by the method of Lowry (12). ODC activity is expressed as pmol of CO_2 released/mg protein per 30 min of incubation, with samples assayed in duplicate. The intra-assay coefficient of variation was ≤10%.

Medium was removed from cultures and frozen for the subsequent determination of progesterone by radioimmunoassay after hexane extraction, as previously characterized (13). To express progesterone secretion on a per-cell basis in individual wells, quadruplicate cultures were trypsinized (0.25% trypsin x 20 min at 23°C) and mechanically dispersed with a rubber policemen for electronic particle (Coulter) counting. DFO was dissolved in phosphate buffered saline prior to addition to culture medium. Control cultures received an equal volume of pH-adjusted solvent.

Data are expressed as mean ± SEM. Statistical analysis employed unpaired, two-tailed Student's *t* testing or one-way analysis of variance by the Neumann-Keul's technique when multiple comparisons among means were required.

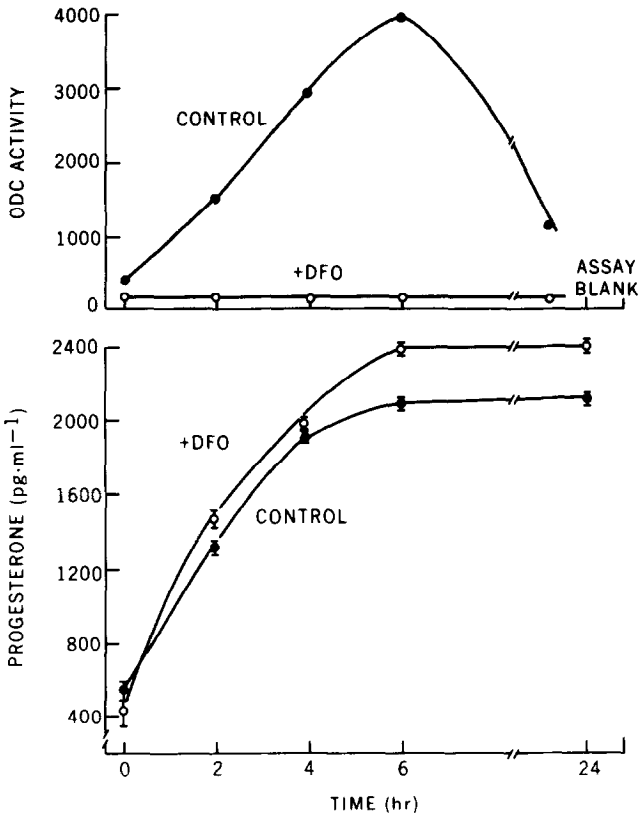

Figure 1. Cultured porcine granulosa cells were treated at time 0 with DFO, 5 mM (o——o) or control solvent (●——●) in the absence of hormonal stimulation.
Upper panel. ODC activity ($pmCO_2$/mg protein·30 min) was assayed in duplicate cultures at 1/2, 2, 4, 6, and 24 hr after medium change.
Lower panel. Progesterone accumulation (pg/ml) was assayed pari passu at each time point.

RESULTS

Effects of Acute Inhibition of ODC: When established monolayer cultures of immature porcine granulosa cells were treated with DFO (5 mM), profound (>99%) suppression of ODC activity occurred, and was maintained for 1/2-24 hr (Fig. 1, upper panel). However, acute steroid production was unimpaired initially (0-4 hr) and tended to increase thereafter ($p < 0.05$, Fig. 1, lower panel). Subsequently, the indirect inhibition of ODC, DAP (1 mM) (14,16), was compared with DFO in gonadotropin-stimulated cells. Both compounds reduced ODC activity after a medium change by >95%, but did not impair progesterone production over 1/2-24 hr (data not shown). Thus, in acute studies, both

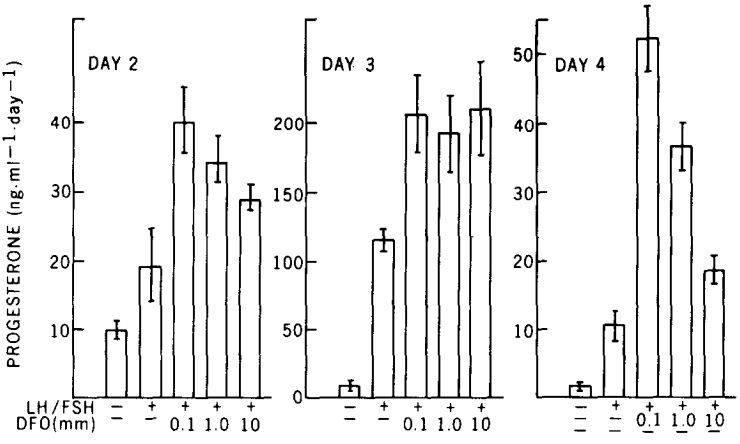

Figure 2. Granulosa cells in monolayer culture were treated for 24 hr (day 2), 48 hr (day 3), or 72 hr (day 4 in culture) with 0.1, 1.0, or 10 mM DFO in the presence or absence of LH and FSH (200 ng/ml). Data are mean and range of duplicate cultures assayed for progesterone production ($ng \cdot mg^{-1} \cdot day^{-1}$).

direct and indirect inhibitors effectively block ODC activity in cultured granulosa cells, but do not reduce either spontaneous or hormone-stimulated progesterone secretion.

Effects of Prolonged DFO Exposure on Gonadotropin-Stimulated Granulosa Cells: After 1 day in monolayer culture, immature granulosa cells were treated with DFO (0.1, 1.0, 10 mM), added every 24 hr when spent medium was removed for progesterone determinations. With prolonged inhibition by DFO (Fig. 2), significant enhancement of gonadotropin-stimulated progesterone accumulation occurred ($p < 0.01$ by one-way analysis of variance). This stimulatory effect was particularly notable after 2 or 3 days of exposure to DFO, i.e., on day 3 and 4 of culture. After 3 days exposure, the 10 mM concentration of DFO was toxic.

Effect of Prolonged DFO Exposure on Untreated or Prolactin-Stimulated Granulosa Cells: After establishing mature granulosa cell (isolated from >6 mm porcine follicles) in monolayer culture for 4 days, DFO (5 mM) was administered to quadruplicate cultures in log-phase growth, in the presence or absence of putrescine hydrochloride (10 μM). DFO treatment for 48 hr produced striking diminution in cell multiplication (Fig. 3, upper panel),

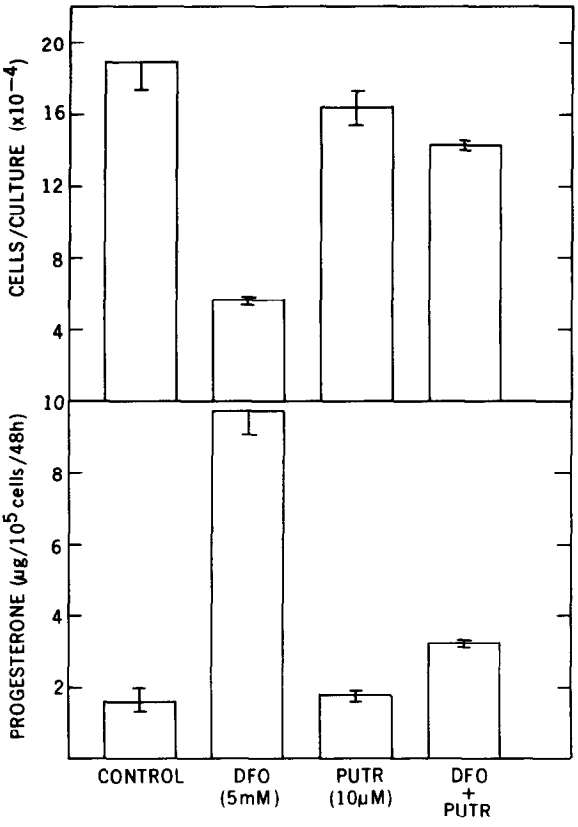

Figure 3. Granulosa cells in log-phase growth were treated for 48 hr with DFO (5 mM), putrescine HCl (10μM, Putr), or both.
Upper. DFO markedly impaired cell proliferation ($p < 0.01$), except in the presence of putrescine.
Lower. Progesterone secretion per 10^5 cells was significantly enhanced by DFO ($p < 0.01$), but putrescine suppressed this facilitatory effect.
Data are mean ± SEM (n=4 cultures).

but increased total progesterone accumulation per culture by approximately 2-fold ($p < 0.02$). When the mass of progesterone was more appropriately expressed per cell, DFO treatment increased progesterone production approximately 6-fold (Fig. 3, lower panel, $p < 0.01$). The co-administration of putrescine to DFO-treated cultures significantly reversed both the antiproliferative and the steroidogenic responses to DFO ($p < 0.05$). Putrescine alone in these concentrations exhibited no significant effects on progesterone secretion or cell growth.

Similar observations were made in prolactin-treated granulosa cells, in which DFO produced an 8-fold increase in progesterone accumulation (from

998 ± 88 to 8250 ± 143 ng/10^5 cells·48 hr, $p < 0.01$). This stimulatory effect was reversed approximately 85% by the co-administration of putrescine HCl.

We considered the possibility that increased steroid accumulation in culture medium reflected release of presynthesized progesterone by DFO-treated cells. However, a specific inhibitor of steroid synthesis, cyanoketone (10^{-6} M) (17), completely abolished progesterone secretion in such cultures (80 ± 7 pg/ml, at 3,8, and 22 hr after treatment with cyanoketone and DFO, vs. 150, 680 and 940 pg/ml progesterone concentrations at corresponding time points after DFO treatment alone). Thus, the DFO effect requires active steroid synthesis.

DISCUSSION

We have examined the putative role of ODC in mediating basal and hormone-stimulated progesterone secretion in the mammalian ovary. To circumvent indirect effects introduced *in vivo* by inhibitors of ODC, we have utilized steroidogenically active granulosa cells maintained *in vitro*. Under these conditions, ODC activity is strikingly suppressed (>99%) by DFO, a catalytic, irreversible inhibitor. The indirect inhibitor, DAP (14,16), also effectively (>95%) reduced ODC activity.

Acute progesterone secretion measured *pari passu* with ODC activity over 1/2-24 hr in culture is not impaired by the action of these ODC inhibitors, in the presence or absence of hormonal stimulation of steroidogenesis. However, more prolonged (24-72 hr) exposure of replicating granulosa cells to DFO clearly enhances progesterone production. The stimulatory effect is significant even when progesterone accumulation is expressed as the total mass of steroid accumulated. However, the facilitation of steroidogenesis is even more striking when the anti-proliferative effects of DFO are considered. This increase in progesterone production cannot be attributed to cell lysis and the release of stored progesterone, since cyanoketone, an inhibitor of ovarian steroid synthesis (17), blocks the action of DFO. The specificity of the DFO effect in stimulating progesterone secretion is supported further by a

significant reversal of DFO action by putrescine co-administration. These concentrations of exogenous putrescine will increase intracellular polyamine levels in monolayer cell cultures (14,18), and do not appear to impair intracellular DFO accumulation (since ODC activity remains inhibited in the presence of combined DFO and putrescine).

A reduction in intracellular polyamine levels has not been documented following DFO or DAP administration in the current studies in granulosa cells, but has been demonstrated under similar in vitro conditions in Chinese hamster ovary cells (19), rat hepatoma cells and mouse leukemia cells (18). With this reservation, our observations make an obligatory role for ODC and polyamines in ovarian steroidogenesis unlikely. However, these data support a permissive role of ODC and polyamines in the replication of granulosa cells, and further suggest that suppression of ODC activity, and cell growth, facilitates the expression of more differentiated cell function, such as steroid synthesis.

ACKNOWLEDGEMENTS

DL-alpha-difluoromethyl-ornithine was provided generously by Centre de Recherche, Merrell International, Strasbourg (France). Cyanoketone was a gift from the Upjohn Co. (Thomas J. Lobl, Ph.D.), Kalamazoo, MI. We thank the Shamokin Packing Corp. for porcine ovaries, Marlene Thompson for the preparation of this manuscript, and Patricia Klase for radioimmunoassay of steroids. This work was supported by an NIH Grant (#HD10122) and a National Research Service Award (#1F332HD05755) (to J.D. Veldhuis) from NICHD.

REFERENCES

1. Jänne, J., Pösö, H., and Raina, A. (1978) Biochim. Biophys. Acta 473, 241-293.

2. Edwards, R.G., Fowler, R.E., Gore-Langton, R.E., Gosden, R.G., Jones, E.C., Readhead, C., and Steptoe, P.C. (1977) J. Reprod. Fertil. 51, 237-263.

3. McNatty, K.P., and Sawers, R.S. (1975) J. Endocrinol. 66, 391-400.

4. Guha, S.K. and Jänne, J. (1976) Acta Endocrinol. 81, 793-800.

5. Osterman, J. and Hammond, J.M. (1977) Endocrinology 101, 1335-1338.

6. Osterman, J., Demers, L.M., and Hammond, J.M. (1978) Endocrinology 103, 1718-1724.

7. Veldhuis, J.D., Harrison, T., and Hammond, J.M. (1979) Biochim. Biophys. Acta (in press).

8. Kobayashi, Y., Kupelian, J., and Maudsley, D.V. (1971) Science 172, 379-380.

9. Guha, S.K., and Jänne, J. (1977) Biochem. Biophys. Res. Commun. 75, 136-142.

10. Tsafriri, A., and Graff, G. (1978) Program and Abstracts of the 60th Annual Meetings of The Endocrine Society, Miami, p. 258 (Abst. #366).

11. Channing, C.P. (1970) Rec. Prog. Horm. Res. 26, 589-595.

12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

13. Thorneycroft, I.H., and Stone, S.C. (1972) Contraception 5, 129-136.

14. Maudsley, D.V. (1979) Biochem. Pharmac. 28, 153-161.

15. Clark, J.L., and Fuller, J.L. (1976) Biochem. Biophys. Res. Commun. 73, 785-791.

16. Heller, J.S., Fong, W.F., and Canellakis, E.S. (1976) Proc. Natl. Acad. Sci. (U.S.A.) 73, 1858-1862.

17. Graves, P.E., Uzgiris, V.I., Querner, M., Kashinagi, K., McIntosh, E.N., and Salhanick, H.A. (1978) Endocrinology 102, 1077-1087.

18. Mamont, P.S., Duchesne, M.C., Grove, J., and Bey, P. (1978) Biochem. Biophys. Res. Commun. 81, 58-66.

19. Sunkara, P.S., Potu, N.R., and Nishioka, K. (1977) Biophys. Res. Commun. 74, 1125-1130.