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SPECIFIC NUCLEAR UPTAKE OF INTRACELLULARLY-PRODUCED ESTROGEN BY RAT GRANULOSA CELLS

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Summary—Granulosa cells of the ovarian follicle are unique in that they both synthesize steroid hormones and respond to exogenously-administered steroids. Isolated granulosa cells from ovaries of gonadotropin-primed rats were incubated in the presence of [^{3}H]testosterone, which the cells convert to [^{3}H]estradiol. Nuclear extracts of these cells were analyzed by high-performance liquid chromatography in a system of 40% acetonitrile. When cells were incubated in the presence of [^{3}H]testosterone alone, a significant portion of the radioactivity present in nuclei co-eluted with authentic estradiol. The nuclear binding was considered to be specific, since 50–75% of total binding was suppressed when the incubation medium contained excess unlabeled estrogen. Moreover, when an antibody to estradiol was included in the medium, specific nuclear uptake of [^{3}H]estradiol was not abolished, but rather was increased. Granulosa cells may, therefore, directly utilize endogenously-produced estradiol, a mechanism which may play a role in the regulation of ovarian cells.

INTRODUCTION

The traditional concept of the endocrine system required that hormones be synthesized in specific tissues, secreted and transported via the bloodstream to other, target, tissues, where they exerted their effects. More recently, "hormone action" has come to include not only this classic model, but also the action of substances which act on neighboring cells without entering the bloodstream ("paracrine" activity). A further broadening of the boundaries of hormone action includes "autocrine" activity, which describes the release by a cell of a hormone for which the cell itself has receptors and can, therefore, respond.

The granulosa cells of the ovary participate in all levels of hormone action as defined above: they respond to, among others, the trophic hormones LH and FSH, which arrive through the bloodstream in the endocrine system [1]. Thecal cells secrete androgens for which the neighboring granulosa cells have receptors [2], a paracrine effect. And estrogens act within granulosa cells themselves in a sort of "ultra-short loop stimulation" [3], or autocrine, pathway. An autocrine pathway could include a situation in which hormone is secreted and subsequently re-enters before exerting an effect on the cells from which it originally came. The results presented here suggest that newly-synthesized estradiol may in fact bind to nuclear receptors before leaving the cell. Such a mechanism would allow a cell or cells to become autonomous and to continue to grow and develop even in the absence of external stimulation.

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EXPERIMENTAL

Materials

Radiolabeled steroids were purchased from Dupont-NEN Research Products (Boston, Mass). McCoys 5a Medium (modified) was obtained from Gibco Laboratories (Grand Island, N.Y.) and antibodies to total estrogens and to testosterone were from Radioassay Systems Laboratories (Carson, Calif.). The antiserum against total estrogens was characterized by the supplier as having a cross reactivity of 100% towards estradiol-17β and estrone, of 60% towards estrone sulfate, 6.9% towards estradiol-17α, and 4.5% towards estriol. There was less than 0.01% cross reactivity observed towards all other steroids tested, including androgens. Sch164231 was the generous gift of Schering Corporation. Solvents for high-performance liquid chromatography (HPLC) were of HPLC grade. All other chemicals, including gonadotropin from pregnant mares (PMS) were supplied by Sigma Chemical Co. (St Louis, Mo.).

Radioactive steroids

[7-^{3}H(*N*)]testosterone (24.5 Ci/mmol) and [6,7-^{3}H(*N*)]estradiol-17β (48.2 Ci/mmol) were obtained from Dupont-NEN (Boston, Mass). [^{3}H]testosterone was further purified by thin-layer chromatography in benzene:ethyl acetate, 2:1.

Animals

Female Sprague–Dawley rats 21–23 days old were obtained from Charles River Laboratories (Wilmington, Mass). Each received one s.c. injection of PMS (8 IU) 48 h prior to sacrifice.

Isolation of granulosa cells

The method used was that of Campbell[4]. This method has the advantage of preserving intercellular gap junctions, and therefore results in higher cell viability than is seen for other methods. Cell viability was assessed by tryphan blue exclusion and was typically 40–50%. In some cases, cells were prepared by the method of Zeleznik *et al.*[5]. For these cells, viability was 15–20%.

Incubation of cells

Granulosa cells, prepared as described above, were resuspended in McCoys 5a Medium ($4–6 \times 10^6$ live cells/ml). The medium for all incubations contained 1 μM Sch16423, a non-aromatizable ligand for the androgen receptor [6]. Other additions were ^{3}H-steroids, non-radioactive steroids and/or anti-serum at the appropriate concentrations. The total incubation volume was 2 ml. A mixture of O_2–CO_2, 95%–5% was bubbled through the medium, and the suspensions were incubated with constant shaking at 37°C for 60 min.

Isolation of steroids from nuclei

At the end of the incubation period, tubes containing the cells were placed on ice. All subsequent procedures up to the extraction of steroids from nuclei were carried out at 0–4°C. The cells were collected by centrifugation at 1000 ***g*** for 5 min, and washed twice with SMT (0.25 M sucrose, 3 mM $MgCl_2$, 10 mM Tris, pH 7.4). The cells were lysed by sonication at 15 W (three 10-s pulses). Nuclei were pelleted by centrifugation at 800 ***g*** for 20 min, washed once with 1% Triton X-100 in SMT and twice with SMT. Steroids were then extracted from the nuclear pellet with ethanol (containing 1 mg/ml estradiol as carrier) for 24 h at room temperature. The ethanol was evaporated, and the extracts resuspended in acetonitrile, ethanol or methanol, as appropriate, for HPLC analysis.

The medium and post-nuclear supernatant (cytoplasm) were extracted with ethyl acetate for 1 h at room temperature, and the exacts analysed in the same manner as for the nuclear extracts.

HPLC

Steroids were separated using a Perkin-Elmer Series 10 liquid chromatography system with a Perkin-Elmer analytical C18 column. The solvent system was either 40% acetonitrile or 67% methanol. Each injected sample contained 1000–2000 cpm in 20 μl. The flow rate was 1 ml/min, with a maximum pressure of 30 MPa. 1-ml fractions were collected and aliquots analyzed for radioactivity.

Other methods

Radioimmunoassay was carried out according to the protocol recommended by the supplier of anti-sera, with the modifications that all steroids were added prior to the addition of the antibody, and that samples were incubated for 30 min at 37°C, followed by 16 h at 0°C. DNA was determined by the method of Burton[7]. Radioactivity was measured in a Packard 2000CA Tri-Carb liquid scintillation counter, using Econofluor (NEN). The efficiency for tritium was 50%.

RESULTS

Production of [^{3}H]estradiol from [^{3}H]testosterone

As expected, when granulosa cells were incubated in the presence of [^{3}H]testosterone, [^{3}H]estradiol was produced. In incubations containing 10 nM [^{3}H]-testosterone, the total concentration of radioactive estradiol in medium, cytoplasm and nuclei was approximately 2 nM (data not shown). Radioimmunoassay of the media used to wash cells immediately after isolation also demonstrated that approximately 20% of the available testosterone (120 nM) was converted to estrogens (22 nM).

Appearance of [^{3}H]estradiol in nuclear fractions

When nuclear extracts from cells incubated with [^{3}H]testosterone were analyzed by HPLC in 40% acetonitrile, a peak whose position corresponded to an estradiol standard was observed. A respresentative chromatogram is shown in Fig. 1 (solid line).

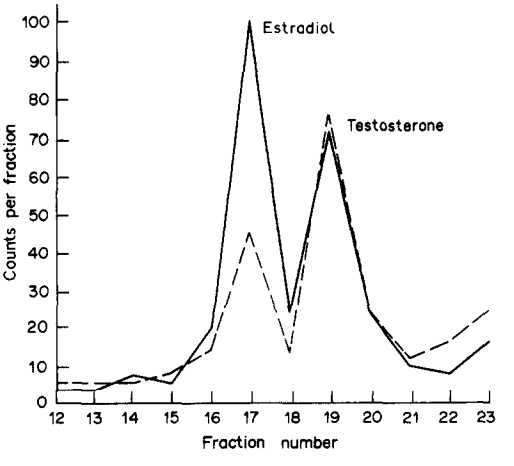

Fig. 1. Nuclear uptake of [^{3}H]estradiol. The ethanol extracts from nuclei of granulosa cells incubated in the presence of 10 nM [^{3}H]testosterone alone or in the presence of 10 nM [^{3}H]testosterone + 1 μm diethylstilbesterol (DES) were dried and resuspended in 100 μl ethanol. 20 μl of each extract was analyzed by HPLC in 40% acetonitrile. Such a separation was carried out for each incubation described in the text. Solid line, [^{3}H]testosterone alone; dotted line, [^{3}H]testosterone + DES.

When this radioactive peak was collected, and re-injected, it continued to co-elute with authentic estradiol in a system of either 40% acetonitrile or 67% methanol.

Suppression of nuclear uptake of [^{3}H]estradiol by excess unlabeled estrogen

Addition of 100-fold excess unlabeled estradiol or diethylstilbesterol resulted in lowered levels of radioactive estradiol appearing in the nucleus (Fig. 1, dotted line). The residual, or non-specific, binding in the presence of unlabeled estrogen was 45–55%. This is comparable to the level of non-specific binding observed when cells were incubated directly with 0.5 nM [^{3}H]estradiol (Fig. 2). When cells were incubated with 5 nm [^{3}H]estradiol, non-specific binding was sufficiently high to obscure any specific nuclear binding (data not shown).

It is notable that, although [^{3}H]testosterone appeared in all nuclear samples analyzed, the amount of [^{3}H]testosterone found in nuclei was not affected by excess unlabeled estrogen (Fig. 1), indicating that the labeled testosterone was not bound to estrogen receptors or to other proteins which bind estradiol specifically.

Kinetics of nuclear uptake

When nuclear samples were analyzed at several time points after addition of labeled testosterone, a peak corresponding to labeled estradiol appeared at 30 min. The number of counts in the estradiol peak increased at 60 min, and continued to be present at 120 min. (Data not shown) In separate experiments, specific binding of radioactive steroid in the nucleus

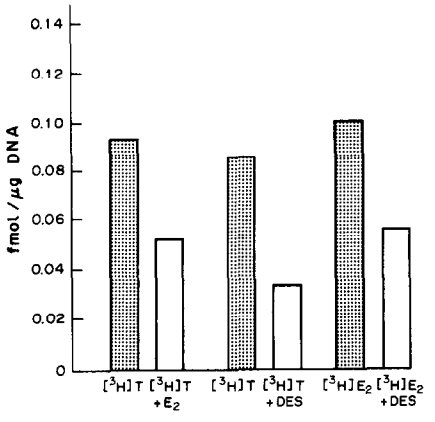

Fig. 2. Non-specific binding of [^{3}H]estradiol to granulosa cell nuclei. Granulosa cells were incubated in the presence of 10 nM [^{3}H]testosterone alone, 10nM [^{3}H]testosterone + 1 μM unlabeled estradiol or DES, 0.5 nM [^{3}H]estradiol alone or 0.5 nM [^{3}H]estradiol + 50 nM unlabeled estradiol. Nuclei were isolated and extracts analyzed for the presence of [^{3}H]estradiol, as described in the text. Bars represent the amount of radioactive estradiol found in the nuclei per μg DNA. Nuclear suppression with unlabeled estradiol or DES was observed in 4 sets of experiments.

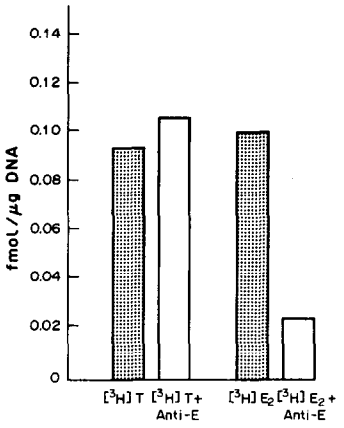

Fig. 3. Nuclear uptake of [^{3}H]estradiol with antibody to estrogens in the medium. Cells were incubated in the presence of 10 nM [^{3}H]testosterone or 5 nM [^{3}H]estradiol in the presence or absence of antiserum to total estrogens (Anti-E). Nuclear extracts were prepared and analyzed for [^{3}H]estradiol on HPLC, as described in the text. Bars represent the amount of radioactive estradiol found in the nuclear extract per microgram DNA.

occurred at 1 h, and by 3 h was reduced almost to background levels.

Effect of antibody to estrogens on nuclear uptake of [^{3}H]estradiol

If the [^{3}H]estradiol synthesized from [^{3}H]testosterone were secreted before re-entering the cell, and subsequently the nucleus, the presence of an antibody to estradiol in the medium should abolish any nuclear uptake of [^{3}H]estradiol. In fact, just the opposite was observed: when the incubation medium contained [^{3}H]testosterone and sufficient antiserum to bind approximately 100 nM estradiol (20–50 times the concentration estimated to be present), a 1:100 final dilution, there was no decrease in the level of nuclear [^{3}H]estradiol observed, but rather a slight increase was seen (Fig. 3). In contrast, cells incubated in the presence of [^{3}H]estradiol and antibody displayed only 25% of the binding observed in the absence of antibody (Fig. 3). In separate experiments, it was observed that the total amount of estradiol present in the nucleus when cells were incubated in the presence of [^{3}H]testosterone and antibody was 3 times that observed when the cells were incubated with [^{3}H]estradiol and antibody. When the steroids extracted from cytoplasm and medium were analyzed by HPLC, no difference was observed for the samples from cells incubated in the presence or absence of antibody (data not shown). The cells, therefore, appeared to be capable of normally taking up testosterone, converting it to estrogens and sequestering it in nuclei even when the antibody was present in the medium.

Recovery of estradiol and testosterone during isolation and analysis

When [^{3}H]estradiol was added to nuclear preparations, 70% of the radioactivity could be recovered

after ethanol extraction. The recovery after drying, resuspension in ethanol and analysis by HPLC was between 45 and 57%, whereas for standard [^{3}H]testosterone, 69–84% of the radioactivity was accounted for after HPLC analysis.

DISCUSSION

The experiments presented in this paper were designed to trace directly the path of newly-synthesized estrogens in granulosa cells in short-term suspension. The results demonstrate: (1) specific nuclear uptake of a radioactive compound presumed to be newly-synthesized estradiol, which is suppressed by the addition of excess estrogen and (2) continued nuclear uptake of newly-synthesized estrogen even when re-entry of secreted estradiol was prevented by the presence of an antibody to estrogens in the medium. While the total amount of radioactive estradiol observed in the preparations is very small, it is specific, as demonstrated by its suppression in the presence of excess unlabeled estrogen. There are several possible reasons for the low levels of [^{3}H]estradiol detected: the [^{3}H]testosterone added to the incubation medium was diluted with endogenous testosterone, present even after isolation of cells. This endogenous steroid is present in approximately 10-fold greater concentration than the tracer added. In addition, the overall recovery of [^{3}H]estradiol during analysis may be as low as 35%. It is, of course, possible that the nuclear uptake we have described is a minor pathway for newly-synthesized estrogens. The results do indicate, however, that at least some of this steroid is accessible to nuclear receptors.

It is well-known that granulosa cells contain estrogen receptors [8, 9] and that exogenously-administered estrogens have numerous effects on granulosa cells, *in vivo* and *in vitro* (for a review, see [10]). It is, therefore, not surprising that these receptors may bind the steroid synthesized in granulosa cells. Based on the ability of estrogens to act synergistically with trophic hormones to increase estrogen biosynthesis in granulosa cells, Zhuang *et al.*[3] and Kessel *et al.*[11] have proposed that estradiol has local regulatory effects on the follicle.

The autocrine model does not require that the hormone exert its effects on the same cell in which it is synthesized, merely on the same cell type. The results of Kessel *et al.*[11], in fact, suggests that estradiol *is* secreted prior to utilization. Our results, particularly those from incubations including anti-serum to estrogens, imply that the steroid is *not* secreted before it enters the nucleus and is specifically bound. The disparity between our results and those of Kessel *et al.*[11] may be due to the fact that those investigators used induction of LH receptors as a measure of estradiol action, whereas we looked directly at estradiol binding in nuclei. Nuclear binding is a first step in estrogen action, and a later step, leading to LH receptor induction, may indeed require secretion of the estradiol. The difference may also be a result of the difference in cell preparation: Kessel *et al.* used cultured granulosa cells, while our studies were done on cells in short-term suspensions.

The slight increase in nuclear uptake of estrogens when the medium included antibody to estrogens is intriguing. The decreased availability of estradiol to the cell may result in a sequestering of whatever estradiol is present, as has been observed for some cells in culture [12]. Alternatively, although product inhibition has not been reported for the aromatase enzyme which catalyzes the conversion of testosterone to estradiol, it is conceivable that such inhibition does normally exist, and that in the face of lowered levels of estradiol, the enzyme becomes more active.

It is now known that estrogen receptors are located in the nucleus even in the absence of steroid [13, 14]. We have previously postulated [15] that steroidogenesis occurs along an organized array of enzymes, with little free cytoplasmic diffusion of intermediates or products. If this is true, and if newly-synthesized estrogen is found specifically-bound in the nucleus, there must be a mechanism for transfer of the hormone from the membrane-bound enzymes to the nucleus. By extension, such a mechanism could play a role in the development of a dominant follicle: a follicle would become dominant by virtue of sequestering its own, newly-formed estrogen.

Autocrine control, that is, control of a function for a tissue by a hormone produced within that tissue, was first proposed by Sporn and Todaro[16] as a mechanism regulating malignant transformation of cells. These authors suggested that one way in which normal cells are released from exogenous controls is by endogenous production of growth factors for which they have their own receptors and to which they are capable of responding. The analogy between a transformed cell and the dominant follicle is clear; one population of cells is rapidly dividing at the expense of surrounding cells. These studies confirm that intracellular utilization of synthesized hormones is one possible pathway of hormone action.

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