Synthesis of Retinoic Acid by Rat Ovarian Cells That Express Cellular Retinoic Acid-Binding Protein-II1

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ABSTRACT

The induction of pseudopregnancy by the injection of eCG in rats results in the appearance of cellular retinoic acid-binding protein type II (CRABP[II]) in the granulosa cells of the ovary and the lining epithelium of the uterus within 48 h. This expression pattern is also seen in the normal mature female rat, in which CRABP[II] is expressed in the uterine epithelium during estrus (but not diestrus) and in the granulosa and luteal cells of the ovary. We have previously demonstrated that the uterine epithelial cells from the pseudopregnant rat have gained the ability to synthesize retinoic acid from retinol, in correlation with the induced expression of CRABP[II]. If this is true for other sites of CRABP[II] expression, then local production of retinoic acid is intimately connected with various stages of reproduction in the female. Here we report that granulosa cells from the ovary of the eCG-treated immature rat and luteal cells from the ovary of the eCG/hCG-treated immature rat (both of which express CRABP[II]) synthesized markedly higher amounts of retinoic acid when cultured, compared to granulosa cells cultured from the ovary of the prepubertal rat treated with control vehicle. Culturing the granulosa cells from either control or eCG-treated animals had no effect on the expression of CRABP[II] cells. These data are consistent with our hypothesis that CRABP[II] expression is associated with retinoic acid synthesis and strengthen the case that local generation of retinoic acid plays an important role in reproduction.

INTRODUCTION

Vitamin A is essential for the maintenance of normal reproductive function in both male and female rats [1]. Early studies showed the presence of vitamin A in the ovary and its fluctuation in serum during the menstrual cycle, indicating that vitamin A may play a role in ovarian function [2, 3]. It is known that retinoic acid, the primary active metabolite of vitamin A, mediates biological effects on target cells through nuclear receptors (retinoic acid receptors and retinoid-X receptors [RARs/RXRs]) that are members of steroid/thyroid hormone nuclear receptor superfamily. These receptors interact with retinoic acid response elements in the promoter region of various target genes [4]. The ovary has been shown to express RARs, consistent with its a target for retinoic acid action [5].

Although previous studies have demonstrated that retinoic acid regulates the expression of specific genes, there is still little known about which cells produce retinoic acid and under what circumstances. Our recent findings [6] suggest that certain sites of production may be indicated by the expression of a specific retinoic-acid binding protein. There are two small cytosolic proteins that specifically bind retinoic acid: cellular retinoic acid binding protein (CRABP) and cellular retinoic acid binding protein-II (CRABP[II]), which belong to a large, well-described family that includes a number of fatty acid binding proteins [7, 8]. The function for this family of proteins is to aid in the solubilization and transportation of their hydrophobic ligand and, in some cases—particularly for the retinoid binding proteins—to direct metabolism of the ligand by restricting access to certain enzymes [7–10]. In addition, it has been shown that CRABP functions in regulating access of retinoic acid to the nuclear receptors by mechanisms of sequestration and/or acceleration of catabolism to inactive forms [11–14].

In contrast, we propose that the function of CRABP[II] does not appear to be in aiding catabolism of retinoic acid but rather in its production and/or secretion by certain cells for action as a paracrine signal. We have recently reported that CRABP[II] expression correlates with retinoic acid synthesis in epithelial cells cultured from the rat uterus after eCG injection or implantation of estrogen pellets [6]. Subsequent injection of hCG stops expression of CRABP[II] in the uterine epithelium, and when those cells are cultured, they are no longer able to synthesize retinoic acid. We have also observed that CRABP[II] is expressed in the uterine epithelium during estrus, but not diestrus, in the mature female [15]. In the normal pregnant female, CRABP[II] expression continues in the uterine epithelium during early pregnancy but is asymmetric, being predominant on the mesometrial side. Upon decidualization of stromal cells at the site of embryo implantation, CRABP[II] expression is also induced [16]. If our hypothesis is correct, then local generation of retinoic acid plays a signalling role that is spatially and temporally regulated throughout the female reproductive system.

To test this hypothesis, we sought to extend this correlation between CRABP[II] expression and the ability of cells to synthesize retinoic acid. CRABP[II] is expressed at high levels in the granulosa cells and luteal cells of the pseudopregnant rat ovary [17]. In this report, we lend support to this hypothesis by demonstrating that these CRABP[II]-expressing cells can also produce retinoic acid.

MATERIALS AND METHODS

Materials and Animals

Female Sprague-Dawley rats (17 days old on delivery; Hrlan, Indianapolis, IN) were housed in a temperature- and light-controlled room (21 ± 1°C, lights-on 0700–1900 h). Rats were fed rat chow (Ralston-Purina Co., St. Louis, MO), provided with water ad libitum, and allowed to acclimate for 1 wk before use in experiments. These studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the oversight of a specific retinoic-acid binding protein. There are two small cytosolic proteins that specifically bind retinoic acid: cellular retinoic acid binding protein (CRABP) and cellular retinoic acid binding protein-II (CRABP[II]), which belong to a large, well-described family that includes a number of fatty acid binding proteins [7, 8]. The function for this family of proteins is to aid in the solubilization and transportation of their hydrophobic ligand and, in some cases—particularly for the retinoid binding proteins—to direct metabolism of the ligand by restricting access to certain enzymes [7–10]. In addition, it has been shown that CRABP functions in regulating access of retinoic acid to the nuclear receptors by mechanisms of sequestration and/or acceleration of catabolism to inactive forms [11–14].
Hormone Treatments

Treatments of prepubertal rats to induce pseudopregnancy have been described previously [6, 17]. The controls and some of the eCG-treated rats were killed, and their ovaries were removed for isolation of granulosa cells. Two days after eCG injection, the remaining eCG-primed rats received an ovariolytic dose of hCG (10 IU), and ovaries were collected after 1 day, 3 days, and 14 days for isolation of luteal cells. Ovaries were collected from 3–5 rats for each time point.

Cell Preparations

Granulosa cells were isolated, by the method of Campbell et al. [18] with modifications, from the ovaries of saline-injected control rats and rats that had received injections of eCG 24 h and 48 h previously. All procedures were done under sterile conditions. Briefly, after fat was removed from ovaries in Dulbecco’s Modified Eagle’s medium (DMEM/F12) with 1.8 mM EGTA and gentamycin sulfate (50 μg/ml), the cleaned ovaries were transferred to DMEM/F12 with 6.8 mM EGTA and gentamicin sulfate (50 μg/ml) and incubated for 15 min at room temperature. Ovaries were then placed in DMEM/F12 with 15% sucrose, 1.8 mM EGTA, and gentamicin sulfate (50 μg/ml) for 5 min at room temperature. The ovaries were subsequently punctured with a 27-gauge needle, granulosa cells were expelled by mild pressure with a flat spatula blade, and cells were centrifuged at 350 x g for 5 min. The cells were cultured in DMEM/F12 with 0.05% ITS (insulin-transferin-sodium selenite), 18 ng/ml corticosterone, 0.1% BSA, and gentamicin sulfate (5 μg/ml). Cell viability was determined by trypan blue dye exclusion. Viability of 70–80% was routinely obtained by this method. One to two million viable cells were plated per well, in 6-well Corning plates (Corning Glass Works, Corning, NY).

Luteal cells were separated according to a modification of the method described by Osteen et al. [19]. Briefly, after eCG/hCG injection, ovaries from Day 1, Day 3, and Day 14 rats were minced into 1- to 2-mm pieces, and cells were isolated after enzymatic dissociation in a 37°C shaker water bath for 45 min. The digest consisted of calcium- and magnesium-free Hanks’ balanced salt solution (Gibco/BRL, Gaithersburg, MD) containing 0.4% type IV collagenase (Worthington, Freehold, NJ), 0.1% hyaluronidase (Sigma), 0.1% pronase (Sigma) and 2% chicken serum (Sigma). Once a large number of single cells had been released, the digest was spun at 220 relative centrifugal force (rcf) for 5 min, and the pellet was resuspended into 5 ml of Hanks’ balanced salt solution and passed through an 88-μm filter unit. The filtrate was centrifuged for 5 min as above. The pellet was resuspended in 5 ml DMEM/F12 and passed through a 20-μm filter. Contaminating red blood cells were removed by using a 66% Percoll gradient. Cells were resuspended in DMEM/F12, counted, and plated onto 24-well culture dishes (Falcon; Becton Dickinson, Rutherford, NJ) at a concentration of 2.5 x 10⁵ cells/ml per well. Some of the cells were cultured in a slide chamber for immunohistochemistry studies. Cell viability was assessed by trypan blue dye exclusion. For the first 24 h in culture, the cells were maintained in DMEM/F12 with 1% ITS, 0.1% ExcyteR (Miles Inc., Kankakee, IL), and 5% calf serum.

For the remainder of the culture period, the cells were in serum-free medium. All cultures were maintained at 37°C in a humidified chamber with 95% air:5% CO₂.

Preparation of Immune Reagents and Western Analysis

Antibodies to CRABP(II) were generated, in collaboration with Robert Seitz at Research Genetics (Huntsville, AL), as described previously [20]. The specificity of these antibodies for the binding protein has been reported elsewhere [15, 20]. The antibody against rat relaxin was a gift from Professor O. David Sherwood at the University of Illinois, Urbana.

After removal of the medium for extraction of retinoic acid, cells from two to three wells were lysed by addition of the SDS loading buffer and the lysate combined. Western blots were conducted as previously described [6, 17]. Primary affinity-purified antibody (OD₂₈₀ = 0.36, 1:2500 dilution) was incubated with the transferred proteins overnight at 4°C in blocking solution. Secondary antibody and subsequent visualization steps were performed according to protocols in the Amersham ECL (enhanced chemiluminescence) kit (Amersham, Arlington Heights, IL).

HPLC Analysis of Retinoic Acid

The procedures used for retinoic acid analysis have been described in detail [6]. The culture medium was made to 8 μM BSA/2 μM retinol. After incubation for 12 h, the medium from three wells was transferred to a 50-ml conical tube (Sarstedt, Inc., Newton, NC) for extraction, 2–3 tubes for each group. The cells were washed with PBS before lysis and Western analysis, as described above.

Under the methods employed here, no detectable retinoic acid was observed if the medium containing 8 μM BSA/2 μM retinol was incubated in the absence of cells. Recovery of retinoic acid was determined by the addition of known amounts of all-trans-retinoic acid to mock sample groups for analysis. Recovery was about 70%, and numbers were corrected by this factor.

RESULTS

Expression of CRABP(II) by Cultured Granulosa and Luteal Cells

Because the culture of these cell types is not routine, we first established that appropriate expression of CRABP(II) was retained during culture. No changes were noted, and expression was identical to that established by immunohistochemistry for the intact ovary; i.e., granulosa cells cultured from the control ovary did not express CRABP(II), whereas expression of this protein was retained in both granulosa and luteal cells from the hormone-treated animals (Fig. 1). The purity of the cultured luteal cell fraction was
FIG. 2. Analysis of retinoic acid production by cultured ovarian cells. HPLC chromatogram of material extracted from the culture medium revealed very little all-trans-retinoic acid produced by granulosa cells from controls (prepubertal) (b), but obvious production by granulosa cells cultured from the ovary after eCG (c) and by luteal cells from the ovary at 3 days post-hCG (d). All-trans-retinoic acid standard is shown in a.

FIG. 3. Quantitation of retinoic acid synthesis by cultured cells. Cells were cultured for two days before addition of 2 μM retinol/8 μM BSA. Values shown are the average for the amounts recovered from three separate samples for each time point (± SD) for one series of animals. Granulosa cells: 1, control; 2, 24 h after eCG; 3, 48 h after eCG. Luteal cells: 4, 1 day after hCG; 5, 5 days after hCG; 6, 14 days after hCG.

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determined by immunohistochemical detection of relaxin; nearly 90% of the cells exhibited staining (data not shown). The isolation protocol for granulosa cells typically yields 98–99% granulosa cells because of the nonenzymatic methodology used [18].

Synthesis of Retinoic Acid by Cultured Cells

Retinoic acid production was initiated by the addition of retinol to the culture medium. After an additional 12 h of culture, the medium was extracted and analyzed for the presence of retinoic acid by HPLC (Fig. 2). Little retinoic acid (less than 1 pmol/10⁶ cells) was produced by the cultured granulosa cells isolated from prepubertal controls (Figs. 2b and 3). However, cultured granulosa cells (Fig. 2c) from animals 24 and 48 h after eCG, as well as luteal cells (Fig. 2d) cultured from animals treated with eCG/hCG at 1 day, 3 days, and 14 days after the hCG injection produced levels of retinoic acid ranging from 15 to 25 pmol/10⁶ cells (Fig. 3). Two additional sets of animals were prepared to confirm that the hormone treatment induced the ability to synthesize retinoic acid. Synthesis by granulosa cells from the controls in both sets remained below 1 pmol/10⁶ cells. At 24 h after eCG injection, values for the granulosa cells were 44.5 ± 9.5 and 15.0 ± 3.7 pmol/10⁶ cells (from a total of 20 rats). One additional time point at 48 h after eCG injection gave a value of 44.5 ± 7.5 pmol/10⁶ cells (from a total of 10 rats). Luteal cells were not examined in these additional sets.

DISCUSSION

The results presented here are consistent with the hypothesis that CRABP(II) is associated with retinoic acid production in certain cells of the female reproductive tract. It is important to note that the synthesis of retinoic acid reported here is a gain of function for the cell. Immature granulosa cells, before the appearance of CRABP(II), were not able to synthesize retinoic acid. This mirrors our results for cultured uterine epithelial cells expressing CRABP(II) [6] and our recent observation that both human and rat uterine stroma cells induced to decidualize in vitro gained the ability to synthesize retinoic acid from retinol, coincident with the appearance of CRABP(II) ([21], unpublished data). As this correlation of CRABP(II) expression with retinoic acid synthesis now appears to be generally true, then the presence of CRABP(II) in certain cells of the adult animal is an important clue to sites of retinoic acid production. This production may be, in part, for paracrine function since the retinoic acid was found in the medium. Thus, this would establish a role for retinoid signaling in the uterus during early pregnancy and later at the site of implantation of the embryo.

It is clear that retinoic acid production does not always require the presence of CRABP(II). There is no correlation of CRABP(II) expression at certain sites in the embryo...
where production of retinoic acid is believed to occur [22]. A possible explanation for this is that there may be two different systems for retinoic acid production: one for an autocrine purpose, as described for the embryo [22] and the other for paracrine action, as observed for cells of the uterus and ovary. The presence of CRABP(II) may be to facilitate synthesis/secretion of the larger amounts of retinoic acid that would be necessary for a paracrine system.

It is interesting that CRABP(II) is expressed in both granulosa cells and luteal cells in the ovary of the hormone-primed as well as the normal cycling animal. A number of other genes demonstrate differential expression for these two cell types, including aromatase, 17α-hydroxylase, 3β-hydroxysteroid dehydrogenase, P450 side-chain cleavage, LH receptor, and the R11β subunit of protein kinase A [23]. The expression pattern for CRABP(II) suggests some potentially unique regulatory mechanisms.

The target cells for retinoic acid produced by the granulosa and luteal cells may be the thecal cells. There is good evidence for granulosa cell-thecal cell interaction in androgen synthesis of the preovulatory follicle [24] and in the production and action of growth factors [25]. In the case of androgen synthesis, the coordinate production of androgen precursors in theca cells for conversion to estrogen by granulosa cells may involve local bidirectional communication [24]. In the case of growth factors, transforming growth factor β (TGF-β) is expressed by the thecal cells and has dramatic effects on granulosa cells in the developing follicles [26, 27]. Synthesis of TGF-β has been shown to be dependent on the retinoic acid signal in some systems (e.g., [28]). Retinoic acid could thus serve as another important molecular signal from mature granulosa cells or luteal cells to the thecal cells.

The presence of RARs in ovary cells, including most granulosa cells, germinal epithelial cells, and some luteal cells, indicates that these cells would also be targets for retinoic acid [5]. Studies have shown that retinol-deprived animals have significantly lower activity levels of the enzymes involved in progesterone synthesis, resulting in lower levels of progesterone [29, 30]. In vitro, both retinol and retinoic acid can increase progesterone production in lutetinizing granulosa cells [31]. These data suggest that one way in which retinoic acid may regulate the ovary and the female reproductive tract may be by modulating steroidogenesis within the ovary. However, an additional possibility is that the corpus luteum serves as an endocrine organ for the production of retinoic acid, which can then act at a site other than the ovary itself, just as is the case for progestosterone.

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