RAT SERTOLI CELL AROMATASE CYTOCHROME P450: REGULATION BY CELL CULTURE CONDITIONS AND RELATIONSHIP TO THE STATE OF CELL DIFFERENTIATION

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SUMMARY

Primary cultures of immature rat Sertoli cells in plastic dishes are highly responsive to follicle stimulating hormone (FSH) and its second messenger, cAMP, in metabolizing testosterone to estradiol, thus indicating the presence of an active, hormone-regulated aromatase cytochrome P450 (P450arom). However, in vivo studies indicated that P450arom is FSH-responsive only in very young animals, where the cells have not yet differentiated, but they lose this ability later on in development. Sertoli cells grown on Matrigel (a reconstituted basement membrane), laminin (a basement membrane component), or in bicameral chambers coated with Matrigel, assume structural and functional characteristics more similar to that of in vivo differentiated Sertoli cells. When the cells were cultured on laminin or Matrigel, the FSH- and cAMP-induced estradiol production was greatly reduced by 30% and 60%, respectively. When Sertoli cells were cultured in bicameral chambers coated with Matrigel, no induction of testosterone aromatization by FSH or cAMP was observed. However, FSH-induced cAMP formation was greater when the cells were cultured on basement membrane or in the chambers than on plastic dishes. These results suggest that culture conditions favoring the assumption by Sertoli cells of a phenotype closer that of the differentiated cells in vivo (tall columnar and highly polarized) suppress the induction of P450arom by FSH and cAMP. We then examined the mechanism(s) by which cell phenotype affects p450arom activity.

Key words: Sertoli cells; extracellular matrix; laminin; aromatase activity; differentiation.

INTRODUCTION

Estrogen secretion is a well-recognized function of the mammalian testis. The enzyme responsible for estrogen synthesis, named aromatase or estrogen synthetase, belongs to the cytochrome P450 family of enzymes, and catalyzes the conversion of C19 androgens (androstenedione and testosterone) to the corresponding estrogens (estrone and estradiol). In vivo observations suggested that in immature rats both Sertoli and Leydig cells synthesized estradiol (28). Treatment of immature rats with follicle stimulating hormone (FSH) and leutinizing hormone (LH) suggested that in the very young rat (before 15 days of age) aromatization is a function of both Sertoli and Leydig cells and that after this age, Sertoli cells no longer respond to FSH in aromatizing androgens (28-30). In vitro studies demonstrated that aromatization of androgens to estrogens increased in Leydig cells but decreased in the Sertoli cells during testicular development; in the adult only Leydig cells maintain this capacity (7,27,30,32,36,39,40). FSH stimulation of immature Sertoli cells obtained from rats up to 30 days of age stimulated androgen metabolism to estrogen, showing that these cells have an active and hormone-dependent P450arom activity (7,27,32,36). Further in vitro studies demonstrated that the cellular distribution of the gonadotropin stimulated aromatase activity changed during testicular maturation (27,32). In the immature animals, both Leydig
and Sertoli cells synthesize estrogens but only Leydig cells have this ability after puberty. All these data taken together indicate that there is a discrepancy between the in vitro and the in vivo data, namely, that the in vivo Sertoli cell FSH-induced aromatization of androgens occurs in rats up to 15 days of age whereas in vitro, cultured Sertoli cells prepared from up to 30-day-old rats maintain their responsiveness to FSH. Presumably, Sertoli cells from the 30-day-old animals are more differentiated than the 15-day-old Sertoli cells.

Recent studies from this and other laboratories demonstrated that morphologic differentiation could be induced in vitro when Sertoli cells were cultured on a laminin substrate or on Matrigel, a reconstituted basement membrane, or even better in bicameral chambers coated with Matrigel. Sertoli cells from 10-day-old rats cultured on plastic exhibit a flattened morphology and generally lack the polarity observed in vivo. On a laminin substrate the Sertoli cells become somewhat more differentiated and assume a cuboidal to low columnar shape. When cultured on a Matrigel substrate the Sertoli cells adopt a phenotype very similar to the in vivo 10-day-old Sertoli cells. They appear tall columnar and are highly polarized with basally located nuclei, abundant supranuclear cytoplasm, and the typical Sertoli-Sertoli tight junctions. Sertoli cells cultured on Matrigel-coated bicameral chambers also appeared almost identical to the in vivo "differentiated" Sertoli cells.

Taking into account the findings presented above, we examined whether the FSH-stimulated aromatization of androgens to estrogens found in cultured, immature Sertoli cells, but absent in vivo from rats of the same age, may be an artifact introduced by the cell culture conditions that favor Sertoli cell dedifferentiation. By culturing 10-day-old immature rat Sertoli cells that are highly responsive to FSH, on laminin, Matrigel, or on Matrigel-coated bicameral chambers, we demonstrate that Sertoli cells lose their ability to metabolize androgens to estrogens as they acquire a more differentiated cell morphology.

**MATERIALS AND METHODS**

**Materials.** 
[4,6,7,8H]Estradiol (E2) (specific activity 96.1 Ci/mmol) and [1,2,5,6H]Nandrost-4-ene-3,17-dione (specific activity 55.3 Ci/mmol) were purchased from Amersham International (Arlington Heights, IL). Matrigel was obtained from Collaborative Research Inc. (Lexington, MA). Bicameral chambers (Millicell-HA, 12 mm) were purchased from Millipore Corp. (Bedford, MA). Laminin, steroids, N'2'-O-dibutyryl cAMP, methyl-3-isobutyl-1-xanthine (MIX), epidermal growth factor (EGF), salmon testes DNA, bovine serum albumin, human transferrin, vitamin A, vitamin E, glutamine, sodium selenate, sodium pyruvate, and sodium lactate were purchased from Sigma (St. Louis, MO). FSH (NIADDK-oFSH-17) was a gift from NIADDK, National Institutes of Health, Bethesda, MD. All cell culture supplies were purchased from GIBCO (Grand Island, NY). Trypsin- Versene solution was obtained from Collaborative Research Inc. (Lexington, MA). Bicameral chambers (Millicell-HA, 12 mm) were purchased from Millipore Corp. (Bedford, MA). Laminin, steroids, N'2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate ([cyclicAMP]), methyl-3-isobutyl-1-xanthine (MIX), epidermal growth factor (EGF), salmon testes DNA, bovine serum albumin, human transferrin, vitamin A, vitamin E, glutamine, sodium selenate, sodium pyruvate, and sodium lactate were purchased from Sigma (St. Louis, MO). FSH (NIADDK-oFSH-17) was a gift from NIADDK, National Institutes of Health, Bethesda, MD. All cell culture supplies were purchased from GIBCO (Grand Island, NY). Trypsin- Versene solution was obtained from Bioproducts, Inc. (Walkersville, MD). Centricon-10 concentrators (M, cut off 100 000) were obtained from Amicon (Beverly, MA). Electrophoresis reagents and materials as well as Bio-Gel HTP hydroxylapatite were supplied from Bio-Rad (Richmond, CA). Nitrocellulose (0.45 μm) was obtained from Hoefer Scientific (San Francisco, CA). Rainbow protein molecular weight markers in the range of 14 500 to 200 000 Da were obtained from Amersham Co. All other chemicals were of analytical quality and were obtained from various other commercial sources.

**Sertoli cell preparation.** Sertoli cells were isolated from 10-day-old male Sprague-Dawley rats as described previously (8,12). Cells were plated in 35-mm culture dishes in serum-free defined media (SDFM, Dulbecco's minimal essential medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml insulin, 10 ng/ml EGF, 5 μg/ml human transferrin, 50 ng/ml vitamin A, 200 ng/ml vitamin E, 10-9 M hydrocortisone, 10-8 M testosterone, 10-9 M FSH, 2 μm glutamine, 5 ng/ml sodium selenite, 1 μM sodium pyruvate, and 22 μM sodium lactate). Sertoli cells were plated at a density of 1 X 10^6 cells on plastic, and 0.5 X 10^6 cells on the laminin (50 μg/well) coated or Matrigel (thin-layer <50 μm) coated 24-well dishes in a final volume of 1 ml SFDM. Sertoli cells (3 X 10^6 cells) were also plated in bicameral chambers (Millicell-HA, 12 mm) as described by Byers et al. (4) and modified by Onoda et al. (24). In some experiments we varied the number of Sertoli cells plated. Sertoli cells were cultured at 32°C in a mixture of 5% CO2:95% air for 2 days, the medium was replaced with fresh media, and the culture was continued for 1 or 2 more days. Then the medium was changed and fresh medium was added containing 0.5 X 10^-6 M testosterone, 0.5 X 10^-7 M MIX, and increasing concentrations (0.1 to 500 ng) of FSH or [cyclicAMP (0.01-1 μM). When Sertoli cells cultured in the Matrigel coated chambers were used, concentrations of testosterone and MIX were the same as above. However, saturating amounts of FSH (500 ng) or [cyclicAMP (0.5 μM) were used. After another 24 h culture the medium was collected for FSH measurement, and the Sertoli cells were harvested from the wells with a trypsin-verseolin solution for DNA content determination.

**DNA measurement.** The procedure for determination of DNA was a modification of Burton's method (3). Briefly, harvested Sertoli cells were centrifuged and the cell pellet was resuspended in 150 μl distilled water and left for 10 min. Standards, made of salmon testes DNA (type III), in the range of 1 to 100 μg were used. Then 100 μg of bovine serum albumin and 150 μl of 0.6 N perchloric acid were added to each sample. After centrifugation the pellets were washed twice with 0.3 N perchloric acid, resuspended in 150 μl 0.3 N perchloric acid, and incubated in a 90°C water bath for 20 min. At the end of the incubation, the samples were centrifuged and 100 μl of the supernatant was mixed with 200 μl of diphenylcarbazide reagent (180 mg diphenylamine, 6 ml of glacial acetic acid, 90 μl of sulfuric acid, 30 μl of acetaldehyde (1:50 dilution) in glass tubes. After 12 h, absorbance at 600 nm was determined. Triplicate determinations were performed for each sample.

**Estradiol measurement.** Sertoli cell estradiol synthesis was determined using a specific radioimmunoassay. Antiserum to 17β-estradiol (E26-47) was obtained from Endocrine Sciences (Tarzana, CA) and the assays were performed as described by the manufacturer. The sensitivity of the assays was 10 pg. Analysis of the radioimmunoassay data was performed using the IBM-PC RIA Data Reduction program (version 4.1) obtained from Jaffe and Assoc. (Silver Spring, MD).

**Sertoli cell P-450arom enzyme solubilization and immunoblot analyses.** Immature rat Sertoli cells were cultured on plastic, laminin, or Matrigel-coated dishes and Matrigel-coated bicameral chambers. After a 3-day culture period, the medium was changed and 24 h later cells were harvested from the dishes and the chambers. Microsomes were then prepared using an established procedure (22). P450arom enzyme was then solubilized for 1 h at 4°C in an ice bath, in 50 mM potassium phosphate (pH 7.5) containing 1 mM dithiothreitol, 0.1 mM EDTA, and 1% [3-(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). A soluble extract was obtained by centrifugation for 60 min at 12 000 g at 4°C. The samples were then concentrated and the buffer was exchanged to 100 mM KPB containing 1 mM diethitolreitol, 0.1 mM EDTA, and at the end completed to 10% glycerol. The samples were then loaded onto hydroxyapatite columns, equilibrated with the same buffer, and P450arom was eluted with the same buffer containing 300 mM KPB. Eluted fractions were concentrated/dialyzed against 50 mM KPB containing 1 mM dithitolreitol and 0.1 mM EDTA using centrifugation on centrifron-10. Proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide (18). The proteins were electrophoretically transferred to nitrocellulose paper (38) and blotted with the rabbit IgG using a specific radioimmunoassay. Antiserum to 17β-estradiol (E26-47) was used to detect the rabbit IgG using an enzyme-linked immunosorbent assay (ELISA).

**Microsomal P450arom activity measurement.** Microsomes from Sertoli cells cultured on different substrates or in bicameral chambers were prepared as described above. Incubations with various amounts of microsomal proteins (50 to 400 μg) were performed in 50 mM KPB (pH 7.4) in the presence of NADPH-dependent reductase and 1 μCi[1,2,5,6H]Nandrost-4-ene-3,17-dione at a final concentration of 2 μM was used. Reactions were preincubated for 5 min at 30°C, and the reaction was started with 1 μM NADPH and continued for 30 min at 30°C. The incubation was