GYNECOLOGY

Expression of the estrogen receptors and steroidogenic enzymes involved in estradiol formation in the monkey vagina

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OBJECTIVE: Estrogens are well recognized to have beneficial effects on vulvovaginal atrophy because of menopause. The distribution of estrogen receptors and enzymes responsible for estradiol (E_2) formation within the vagina may provide insight into how dehydroepiandrosterone, a precursor of both estrogens and androgens, improves vulvovaginal atrophy.

STUDY DESIGN: The purpose of the study was to determine where the steroidogenic enzymes responsible for E_2 formation as well as estrogen receptors are localized in vaginal specimens collected from cynomolgus monkeys (*Macaca fascicularis*), the closest model to the human. HSD3B1, HSD17B1, HSD17B5, HSD17B12, aromatase (CYP19A1), estrogen receptor (ER)-α, and ER-β were measured or localized by quantitative real-time polymerase chain reaction, immunohistochemistry, and immunofluorescence. Estrogens were quantified by liquid chromatography/tandem mass spectrometry.

RESULTS: All steroidogenic enzymes and estrogen receptors are localized mainly in the superficial layer of the stratified squamous epithelium, blood vessel walls, and muscle fibers of the vagina. Immunolabeling of HSD17B5 and HSD17B12 shows that these enzymes are uniformly distributed from the basal membrane to the superficial keratinized cells, whereas HSD3B1 and aromatase are particularly localized in the outer (external) portion of the epithelial layer. ER-α and ER-β are also distributed within the vaginal epithelium, with expression especially elevated at the basal membrane level.

CONCLUSION: The enzymes responsible for E_2 formation as well as ERs are expressed mainly in the superficial layer of the stratified epithelium as well as the muscle layer of the vagina. The present data provide morphologic and biochemical support for the role of local dehydroepiandrosterone transformation into estrogens in regulating epithelial cell maturation, pH, fluid secretion, smooth muscle activity, and blood flow regulation in the primate vagina.

Key words: dehydroepiandrosterone, stratified epithelium, vagina, vaginal blood vessel walls, vaginal muscularis, vulvovaginal atrophy

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The gonads synthesize active steroid hormones from cholesterol and send them into the circulation, from which they act upon all tissues containing the appropriate receptors. The adrenals, on the other hand, secrete inactive precursors, especially dehydroepiandrosterone (DHEA), which are converted intracellularly into active sex steroids in the target tissues, possessing the required steroidogenic enzymes by the mechanisms of intracrinology (Figure 1).[1,2] The local biosynthesis of active sex steroids in most peripheral tissues is supported by the local presence of enzymes required for the conversion of the inactive adrenal precursor DHEA into estradiol (E_2) and androgens,[1-6] which exert their activity locally by means of the estrogen receptors (ERs) present in estrogen-sensitive cells or androgen receptors expressed in androgen-sensitive cells.[7]

Much information is available on the effects of estrogens on female urogenital tissue (cell maturation, pH, lubrication, and vaginal blood flow), in which E_2 influences the superficial vulvar and vaginal epithelium/mucosa.[8-11] Vulvovaginal atrophy (VVA), a typical example of a consequence of hormone deficiency in postmenopausal women, is strongly correlated with low serum DHEA coupled with the arrest of E_2 secretion by the ovaries at menopause.[7] In fact, 50% of postmenopausal women 50-60 years of age and 72% of women 70 years of age and older experience urogenital dysfunction.[12,13] Common symptoms include dyspareunia, vaginal dryness, irritation, burning, itching, inflammation, and bleeding.

Because estrogen secretion by the ovaries ceases at menopause, investigators developed intravaginal estrogen formulations to minimize the systemic exposure to estrogens compared with the oral formulations.[8-11] However, studies have demonstrated that all intravaginal

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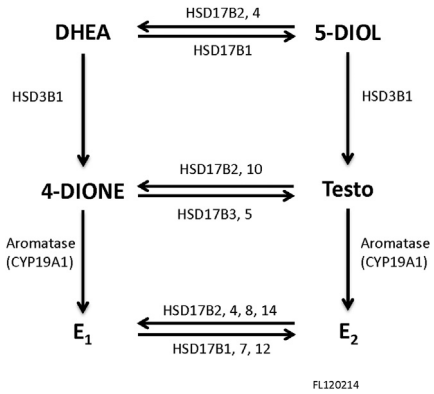
FIGURE 1
Local pathways leading to estrogen formation from DHEA



Schematic representation of the local pathways leading to estrogen formation from DHEA in peripheral tissues by the mechanisms of intracrinology.

DHEA, dehydroepiandrosterone; *5-diol*, androst-5-ene-3α,17β-diol; *4-dione*, androstenedione; *5α-dione*, androstanedione; E_1, estrone; E_2, 17β-estradiol; *testo*, testosterone.

Bertin. ERs and steroidogenic enzymes involved in estradiol formation. Am J Obstet Gynecol 2014.

estrogen formulations lead to significantly increased serum estrogen levels.[14-17] Nevertheless, recent data clearly show that serum DHEA becomes the exclusive source of sex steroids after menopause.[5,7,18] Moreover, recent clinical data show high efficacy and safety of DHEA (prasterone) when used to treat VVA in postmenopause.[19-21]

Preclinical data have clearly shown the beneficial effects of steroids made locally from DHEA in the vagina, not only in the superficial epithelial layer but also most notably on collagen fibers of the lamina propria and on components of the muscularis.[22-24] These data clearly indicate the importance of DHEA and its local transformation into androgens, which may then be aromatized into estrogens, to support normal vaginal physiology that estrogen replacement therapy alone cannot achieve.[22-24]

For the reasons mentioned in previous text, the evaluation of the treatment of VVA has to date focused exclusively on the effect of estrogens on the superficial epithelial layer of the human vagina, namely the maturation index, pH, lubrication, dryness, irritation/itching, and dyspareunia. Little is known, however, about changes in the other layers of the vagina.

Because DHEA is the exclusive precursor of sex steroids after menopause, we set out to determine the localizaton of the route of estrogen formation in the vagina by measuring HSD3B1, HSD17B1, HSD17B5, HSD17B12, aromatase, and ER expression by quantitative real-time polymerase chain reaction (RT-PCR), immunohistochemistry, and immunohistofluorescence as well as estrogens by liquid chromatography/tandem mass spectrometry (LC/MS/MS) in nonhuman primate vaginal tissue, the model closest to the human.

Materials and Methods

All animals in this study were maintained and handled in accordance with the guidelines of the Canadian Council on Animal Care and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Tissues from cynomolgus monkeys aged 6-7 years were collected for quantitative RT-PCR. For microscopy assays, animals aged 5-7 years were killed under isoflurane anesthesia by intracardiac perfusion with a saline solution or 4% paraformaldehyde (PFA) neutral buffer.

The vaginas collected from PFA-perfused animals were immersed in 4% PFA neutral buffer for 12 hours, routinely processed, and embedded in paraffin for immunofluorescence and immunohistochemistry analysis. The vaginas collected from animals perfused with the saline solution alone were immediately snap frozen in liquid nitrogen for quantitative RT-PCR and steroid assays.

Antibodies

The antibody against the sequence of 13 amino acids located at positions 175−187 of aromatase, a sequence homologous for human and *M fascicularis* aromatase, was synthesized and purified with high-performance liquid chromatography by Dr Serge St-Pierre (INRS-Santé, Pointe-Claire, QC, Canada).[25] The peptide sequences 217−312 and 297−320 were selected for preparation of antibodies to HSD17B5 and HSD17B12, respectively.[26-28] For this purpose, New Zealand rabbits received a subcutaneous injection of 85 mg peptide solubilized in 1 mL phosphate-buffered saline (PBS) containing 50% Freund's complete adjuvant.

The animals were boosted twice with 40 mg peptide in 50% Freund's incomplete adjuvant at 1 month intervals. Two weeks after the last injection, the animals were killed by decapitation and the blood was collected. Antiserum was obtained by decantation and separation by centrifugation and was stored at −80°C. Expression of ER-β, HSD17B1, and HSD3B1 (monoclonal) were determined with antibodies purchased from Abcam Inc (Toronto, ON, Canada). ER-α was detected with the use of antibodies obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

Immunohistochemistry

Paraffin sections (5 mm thick) were deparaffinized, hydrated, and treated with 3% H_2O_2 in methanol for 20 minutes. The sections were blocked before incubation overnight at 4°C with antibodies (HSD17B1 at a dilution of 1:50; HSD17B5, HSD17B12, and HSD3B1 at a dilution of 1:500; ER-α at 1:250; and ER-β at 1:500 in blocking solution). We studied negative controls by incubating tissues with rabbit isotypic control (Abcam Inc) and a commercial detection system kit (Covance, Inc, Montreal, QC, Canada) using the streptavidin-biotin peroxidase amplification method. Finally, we visualized the antigen-antibody complex with a solution of PBS 1× containing 5 mg/mL of 3,3-diaminobenzidine and 0.012% H_2O_2. Sections were lightly counterstained with Gill's modified hematoxylin (EMD Serono, Billerica, MA). Images were generated with the use of a ×10 or ×20 objective on the Leica DMRB fluorescence microscope (Concord, Ontario, Canada).

Immunohistofluorescence

Deparaffinized sections were blocked (0.5% BSA, 0.4% Triton X-100, and 10% normal goat serum) and incubated overnight at 4°C with antibodies (HSD17B1 at a dilution of 1:50, HSD17B5 and HSD17B12 at 1:500, ER-α at 1:250, ER-β at 1:200, and HSD3B1 at 1:100 in blocking solution) and