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Mimicking postmenopausal steroid metabolism in breast cancer cell culture: Differences in response to DHEA or other steroids as hormone sources

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ABSTRACT

Following menopause virtually 100% of estrogens are synthesized in peripheral target tissues from precursor steroids of adrenal origin. These steroids are the unique source of sex steroids in these women. This positions some steroid metabolizing enzymes as primary targets for novel therapies for estrogen receptor-positive (ER+) breast cancer. However, previous research on the steroid-converting enzymes has been performed using their direct substrate as a hormone source, depending on the facility where studied and the robust signal obtained. These experiments may not always provide an accurate reflection of physiological and post-menopausal conditions. We suggest providing dehydroepiandrosterone (DHEA) as an intracrinological hormone source, and comparing the role of steroid-converting enzymes using DHEA and their direct substrates when an extensive mechanistic understanding is required. Here, we present a comparative study of these enzymes with the provision of DHEA and the direct substrates, estrone (E1) or dihydrotestosterone (DHT), or additional steroids as hormone sources, in breast cancer cells. Enzyme knockdown by respective specific siRNAs and observations on the resulting differences in biological function were carried out. Cell biology studies showed no difference in biological function for 17β-HSD1 and 17β-HSD7 when cultured with different steroid hormones: cell proliferation and estradiol levels decreased, whereas DHT accumulated; cyclinD1, PCNA, and pS2 were down-regulated after knocking down these two enzymes, although the quantitative results varied. However, culture medium supplementation was found to have a marked impact on the study of 3α-HSD3. We demonstrated that provision of different steroids as a substrate or hormone sources may promote modified biological effects: provision of DHEA is the preferred choice to mimic postmenopausal steroid metabolism in cell culture.

1. Introduction

There is evidence that sex hormones play a significant role in the etiology of breast cancer [1–2]. The concentration of estradiol is significantly higher in malignant compared with nonmalignant human breast tissue from pre- and postmenopausal women [3]. Estradiol (E2) can enhance risk by stimulating proliferation of breast epithelial cells through a nuclear receptor-mediated signaling pathway involving the estrogen receptor (ER) [4].

Estrogens are derived from both ovaries and adipose tissue in premenopausal women: E2 is the dominant circulating estrogen and is principally secreted by the ovaries [5]. The ovaries atrophy in postmenopausal women and cease function to such an extent that nearly all estrogen is synthesized in peripheral target tissues from precursor steroids originating from the adrenal glands (75% before menopause) [6–7]. Dehydroepiandrosterone (DHEA) is an important precursor for steroid metabolism in estrogen related diseases with postmenopausal women [8]. Human steroidogenic enzymes in peripheral intracrine tissue play important roles in the main biosynthetic and inactivating pathway of androgens and estrogens (Fig. 1. Simplified pathway showing human steroidogenic and steroid-inactivating enzymes involved in the steroid metabolism in peripheral intracrine tissues). Therefore, these enzymes are positioned as major targets for novel therapies for steroid-sensitive diseases, particularly breast and prostate cancers [6–7].

The reductive 17β-hydroxysteroid dehydrogenase (17β-HSD) family comprises key enzymes involved in the formation of estradiol [9]. The reductive 17β-HSD1 and 17β-HSD7 enzymes

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have critical roles in the regulation of estradiol (E2) synthesis from estrone (E1) and a role in DHT inactivation in mammalian cells [10–12]. The importance of 17β-HSD1 stems from its efficient synthesis of the most potent estrogen, E2, in addition to other estrogens such as 5-androstene-3β, 17β-diol and 5α-androstane-3β, 17β-diol, and inactivation of the most active androgen, DHT. All these contribute to the stimulation and development of breast cancers and demonstrate a dual function in the promotion of breast cancer cell proliferation [13–14]. 17β-Hydroxysteroid dehydrogenase 7 also converts E1 to E2 and possesses a 3-keto-reductase activity, which inactivates DHT [9,15]. Human 3-alpha hydroxysteroid dehydrogenase type 3 (3α-HSD3, AKR1C2) also inactivates DHT [16–17]. The expression of 3α-HSD3 was inversely correlated to apoptosis-inducing factor (AIF) in non-small cell lung cancer (NSCLC) cells [18]. Apoptosis-inducing factor is also expressed in MCF7 cells [19] and may be under negative regulation by 3α-HSD3 in this cell type. The function of these three enzymes is of great importance for the development and aggression of breast cancer. Thus, 17β-HSD1 and 17β-HSD7 were identified as interesting therapeutic targets for estrogen receptor-positive (ER +)-breast cancer and for the development of potent selective inhibitors. The function of 3α-HSD3 is currently under research.

In studies addressing 17β-HSD1 and 17β-HSD7 function and inhibition, E1 is primarily used as the substrate. However, E2 synthesis can involve multiple pathways in addition to synthesis from E1, and the origins of estrogen differ before and after menopause. In order to validate the effects of 17β-HSD1 and 17β-HSD7 inhibitors, DHEA and E1S are supplied as representative substrates. The majority of recent studies used direct substrates as the hormone source (E1 for 17β-HSD1 and 17β-HSD7, DHT for 3α-HSD3); therefore, our study aims to compare the effects of providing DHEA and the direct substrates E1 or DHT as hormone sources in order to reveal the mechanism of steroid synthesis in postmenopausal breast cancer.

2. Materials and methods

2.1. Cell culture

MCF-7 cells were from the American Type Culture Collection (ATCC) and were maintained in phenol red-free DMEM low glucose medium supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Charcoal-treated medium was used to plate cells to eliminate exogenous hormones and to permit the application of alternative hormone sources.

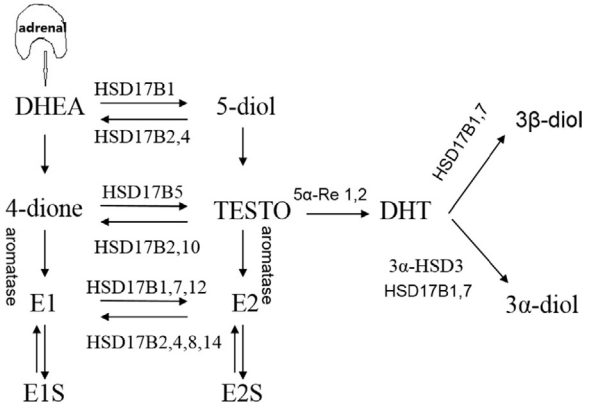

Fig. 1. Simplified pathway showing human steroidogenic and steroid-inactivating enzymes involved in the steroid metabolism pathway in peripheral intracrine tissues.
DHEA: dehydroepiandrosterone; 4-dione: androstenedione; 5-diol: androst-5-ene-3 α, 17 β-diol; E1: estrone; E1-S: estrone sulfate; E2: 17 β-estradiol; E2-S: estradiol sulfate; HSD: hydroxysteroid dehydrogenase; testo: testosterone; DHT: dihydrotestosterone; 5α-Re: 5α-reductase. (Modified from Labrie and Labrie, CLIMACTERIC 2013:16:205-213).

2.2. siRNA synthesis and transfection

The sense and antisense sequences of three 17β-HSD1 siRNAs, two 17β-HSD7 siRNAs, and one 3α-HSD3 siRNA sequence were selected and synthesized as previously described in Table 1. Transfection of MCF7 cells with siRNA was carried out in 6-well plates using Lipofectamine 2000 (Invitrogen), 2.5×10^5 cells per well and 100 nM mixed 17β-HSD1-specific siRNAs (siRNA1 + siRNA2 + siRNA3), 100 nM mixed 17β-HSD7-specific siRNAs (siRNA1 + siRNA2), or 100 nM 3α-HSD3 siRNA. Control cells were transfected with control siRNA (Table 1).

2.3. Semi-quantitative RT-PCR

Total RNA was isolated from cells using the RNeasy Plus mini kit (Qiagen): 1 μg of total RNA was subjected to a one-step semiquantitative reverse transcription (RT)-PCR using the Titanium One-Step RT-PCR kit (Clontech). The primer sets included four primers for human 18S (used as an internal control) and the following: 17β-HSD1 primers, 17β-HSD7 primers or 3α-HSD3 primers as listed in Table 2. The RT-PCR program was carried out in an Eppendorf Mastercycler Gradient (Eppendorf, Mississauga, Ontario, Canada). The RT was performed at 50 °C for 60 min followed by initial denaturation at 94 °C for 5 min. The PCR program was as follows: 30 s at 94 °C for denaturation, 30 s at 65 °C for annealing, and 1 min at 68 °C for elongation, followed by a 2-min final elongation. The number of cycles was 35. The PCR products were separated on a 1% agarose gel with RedSafe Nucleic Acid Staining Solution (20,000×). Bands were viewed and photographed under UV light.

2.4. Western blot

Total proteins were extracted from cells with RIPA buffer (Invitrogen) supplemented with a 1% protease inhibitor cocktail (EMD Chemicals, Gibbstown, NJ). Proteins were quantified by the Bradford method and 40 μg total proteins from each sample were separated on a 12% SDS-polyacrylamide gel and then electroblotted onto nitrocellulose membrane overnight. The membranes were blocked with 5% skimmed milk in TBS-Tween (TBST) 20 for 1 h at room temperature. Thereafter, the membranes were hybridized to a polyclonal antibody directed against rabbit cyclinD1 (Abcam 1:10,000 dilution), pS2 (Santa Cruz 1:500 dilution), PCNA (Santa Cruz 1:500 dilution), Bcl-2 (Abcam 1:1000 dilution), and AIF (Abcam 1:1000 dilution) for 2 h at room temperature. The membranes were subsequently incubated with a goat anti-rabbit IgG peroxidase conjugated secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:2000. A 1:5000 dilution of monoclonal anti-β-actin antibody produced in mouse (Sigma) was used as the loading control. Membranes were washed with TBST and proteins were visualized using Western Lightning™ Plus ECL (PerkinElmer), followed by exposure of the membranes to X-ray films for 1 s, 5 s, 1 min and 10 min. The radiographic films were scanned and the Image program (Molecular Dynamics, Sunnyvale, CA) was used to quantify the band density.

2.5. Cell proliferation

Cell proliferation was determined by CyQuant cell proliferation kit. MCF-7 cells (3000) were plated into 96-well plates containing 100 μl hormone-free culture medium and were transfected with