Maturitas 71 (2012) 376–384

Contents lists available at SciVerse ScienceDirect

Maturitas

journal homepage: www.elsevier.com/locate/maturitas

Impact of testosterone on the expression of organic anion transporting polypeptides (OATP-1A2, OATP-2B1, OATP-3A1) in malignant and non-malignant human breast cells *in vitro*

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ARTICLE INFO

Article history:
Received 15 August 2011
Received in revised form
27 December 2011
Accepted 29 December 2011

Keywords:
OATP
Mammary gland
Breast cancer
Testosterone
Anastrozole
Androgen

ABSTRACT

Objectives: Postmenopausal hormone therapy (HT) increases local estrogen formation in breast tissue. The enzymatic substrates depend on transmembrane anion transporting polypeptides (OATPs) to reach intracellular enzymes. The aim of this study was to investigate the effect of testosterone (T) on the expression of OATP-1A2, OATP-2B1, and OATP-3A1 in malignant (MCF-7, BT-474) and non-malignant (HBL-100) breast cells *in vitro*.
Study design: Cells were incubated in RPMI 1640 medium containing 5% steroid-depleted fetal calf serum for 3d, and subsequently incubated in the absence or presence of T, anastrozole (A), and T+A (10^{-6} M) for 24 h at 37 °C.
Main outcome measures: OATP expression was determined by immunocytochemical staining. Expression intensity was graded as low, moderate, or strong. Hormone receptor (AR, PR, ESR1, ESR2) expression was investigated by qPCR and Western blotting. Rank variance analysis was performed for statistical analysis ($p \leq 0.05$).
Results: OATP-1A2, OATP-2B1, and OATP-3A1 expression was present in all untreated breast cell lines examined, with OATP-1A2 and OATP-3A1 being the predominant ones. There was a trend for a higher baseline expression in untreated HBL-100 and BT-474 in comparison to MCF-7 cells, which was significant for OATP-2B1. T treatment led to decreased OATP-1A2, -2B1, and -3A1 expression in BT-474 and HBL-100 cells, respectively. In contrast, in MCF-7 cells, OATP-2B1 expression was significantly increased. T-induced upregulation of AR and PR protein expression in BT-474 and MCF-7 cells was reduced by A treatment.
Conclusions: T may constitute a signal for differential regulation of mammary OATP expression. In non-malignant breast cells T seems to have a beneficial effect by reducing the availability of substrates for the intracellular formation of potent estrogens.

1. Introduction

Since decades, women with loss of libido have been treated with androgens. However, only in 2006 the first testosterone (T) product for women was approved by the EMA. Since then, there has been a tremendous debate addressing the safety of T therapy. With conventional postmenopausal hormone therapy (HT), especially combined estrogen plus progestin therapy (EPT), being associated with an increased breast cancer (BC) risk [1], the issue of T breast safety remains within the focus [2]. Clinical data are controversial. Serum androgen levels have been linked to BC risk in postmenopausal women in some but not all studies [3–5]. In women with polycystic ovary syndrome frequently associated with hyperandrogenemia [6] as well as in women-to-men transsexuals treated with supra-physiological androgen dosages [7] BC risk was not increased. Mammographic density, a risk factor for BC, did not correlate with androgen serum levels [8]. Likewise, addition of T to EPT did not further increase mammographic density in postmenopausal women [9]. Quite the contrary, T was shown to prevent breast tissue from EPT induced proliferation in women and non-human primates [10,11].

Abbreviations: A, anastrozole; AR, androgen receptor; BC, breast cancer; CK, cytokeratin; DHEAS, dehydroepiandrosterone sulfate; EGFR, epidermal growth factor receptor; EPT, estrogen plus progestin therapy; ER, estrogen receptor; ESR1, estrogen receptor alpha; ESR2, estrogen receptor beta; HT, hormone therapy; HER2, human epidermal growth factor receptor-2; 17ßHSD-1, 17ß-hydroxysteroid dehydrogenase type 1; 3ß-HSD, 3ß-hydroxysteroid dehydrogenease; OATP, organic anion transporter peptide; PR, progesterone receptor; STS, steroid sulfatase; T, testosterone.

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doi:10.1016/j.maturitas.2011.12.020

One way of how EPT affects the mammary gland is by modulating the local endocrine milieu [12,13]. Breast tissue and BC cells possess the enzymes necessary for biosynthesis of estrogens from precursor molecules circulating in the serum. Three main enzymes are important in this process: aromatase converting androgens to estrogens, estrone sulfate (E1S)-sulfatase (STS) hydrolyzing E1S to estrone (E1), and 17ß-hydroxysteroid dehydrogenase type 1 (17ßHSD-1) reducing E1 to estradiol (E2) [14]. However, the majority of estrogens are stored as conjugates such as E1S. Thus, the hydrophilic conjugates need a transport system for crossing the double lipid layer cell membrane to reach the intracellular compartment where the enzymes are localized. Organic anion transporting polypeptides (OATP) are membrane transport glycoproteins that mediate the sodium-independent, bi-directional transport of amphipathic organic compounds, e.g. bile salts, drugs, and steroid conjugates. In 2004, a new OATP (protein)/SLCO (gene) nomenclature was introduced since the traditional SLC21 gene classification did not permit an unequivocal and species-independent identification of genes and gene products [15,16]. We have previously demonstrated that the impact of T on the local endocrine milieu in breast cells varied depending on treatment duration *in vitro* while STS and 17ßHSD-1 mRNA and STS protein expression were not altered [17]. This finding led us to the hypothesis that T may alter the transmembrane transport system of substrates, e.g. OATP expression, rather than modulating the transcription and translation of the enzymes involved in E2 biosynthesis. Therefore, the aim of our current study was to investigate the effect of T on the expression of OATP-1A2, OATP-2B1, and OATP-3A1 in malignant (MCF-7, BT-474) and non-malignant (HBL-100) breast cells *in vitro*.

2. Materials and methods

2.1. Chemicals, reagents, and steroids

T was obtained from Sigma–Aldrich (Deisenhofen, Germany). The nonsteroidal aromatase inhibitor (AI) anastrozole (A) was obtained from AstraZeneca (London, UK). Unless stated otherwise, all chemicals were from Sigma–Aldrich.

2.2. Cell culture and hormone treatments

The cell lines MCF-7, HBL-100, and BT-474 were donated by Dr. C. Poremba (Düsseldorf, Germany). Cells were routinely cultured in RPMI 1640 medium (Gibco, Karlsruhe, Germany) containing 10% fetal calf serum (FCS; PAA, Cölbe, Germany), 2 mM L-glutamine, and 1% penicillin–streptomycin, in an atmosphere containing 5% CO_2, and 95% air at 37 °C, resulting in a physiological pH. For culture of BT-474 cells, 0.1% insulin was added to the media, and the percentage of FCS was increased to 20%. Prior to, and during hormonal treatments, phenol-red free medium containing 5% dextran-coated charcoal (DCC) steroid-depleted FCS was used. Hormonal treatments and immunostainings were performed after seeding the cell lines into 8-well chamber slides (Nunc, Wiesbaden, Germany) at a density of 5000 cells/well (MCF-7, HBL-100) or 20,000 cells/well (BT-474), followed by culturing for 3 days without hormonal treatment, and an additional 24 h in the presence of hormones. Before adding hormones, the culture medium was replaced for 72 h by a medium containing 5% steroid-depleted FCS. T and/or A were added from a stock solution, and diluted in culture medium resulting in a final concentration of less than 0.1% ethanol. T and A were used at a concentration of 10^{-6} M, according to the results of a previous study. [17] All experiments were performed independently at least three times.

2.3. Immunocytochemistry

Cells cultured in chamber slides were fixed with −20 °C cold methanol for 10 min, washed, and subjected to a peroxidase block (3% Tris-buffered H_2O_2, DAKO, Glostrup, Denmark) treatment. Non-specific antibody binding was blocked with 10% BSA (Aurion) in PBS for 30 min at RT. Cells were subsequently incubated with the following monospecific primary goat antisera directed against human OATPs (Santa Cruz, Santa Cruz, USA) diluted 1:200 in PBS (1% BSA) or with control goat antiserum (DAKO) over night at 4 °C: OATP-1A2 (C-15: sc-18428, 200 μg/ml); OATP-2B1 (S-17: sc-66561, 200 μg/ml), and OATP-3A1 (L-16: sc-66566, 200 μg/ml). Following 3 × 5 min washing steps with PBS, the cells were incubated with the biotinylated secondary reagent (Universal LSAB™ + Kit/HRP, Rabbit/Mouse/Goat, DAKO) for 20 min, washed, and incubated with streptavidin peroxidase (DAKO) for 20 min. After three washing steps, the substrate DAB+ (DAKO) was developed for 5 min. Samples were counterstained with Mayer's Hemalum (Merck, Darmstadt, Germany), and coverslips were mounted with Faramount (DAKO).

2.4. Evaluation of immunohistochemical staining results

Stained cells were photographed using a Zeiss Axiovert 100 microscope equipped with an Axiophot Mrc camera (Zeiss, Göttingen, Germany) at 100× magnification, and the staining intensity was evaluated for 300 cells/treatment. Staining intensity was graded as strong (2), moderate (1), weak, or absent (0). Staining results were evaluated by two observers (AR and TS). For statistical analysis, strong and moderate stainings were grouped in one category (positive staining) and weak or absent staining into a second category (negative staining). Final staining intensities are given as the percentage of positively stained cells. At least 6 individual staining results were analyzed per treatment. Examples for immunohistochemical grading and for negative control stainings can be found in Supplemental files 1–4.

2.5. Quantitative TaqMan® real-time PCR (qPCR) analysis of hormone receptor and aromatase expression

qPCR was performed essentially as previously described [17]. Total cellular RNA of the cell lines was prepared using the RNeasy reagent system (Qiagen Inc., Hilden, Germany) and reverse-transcribed using the RT-for-PCR Kit (BD Clontech, Heidelberg, Germany). cDNA corresponding to 0.5 ng total RNA was used as a template in the PCR reaction consisting of ABI Master-Mix (Applied Biosystems, Darmstadt, Germany), and pre-designed TaqMan gene expression systems (Applied Biosystems) according to the manufacturer's instructions. For detection of aromatase, primer hs00240671m1, for the androgen receptor (AR), primer hs00171172m1, for estrogen receptor alpha (ESR1), primer hs00174860m1, for estrogen receptor beta (ESR2) primer hs00230957m1, and for progesterone receptor (PR) were employed. Target gene expression was normalized to the expression of mammalian 18S rRNA (Hs99999901 s1, all primers by Applied Biosystems). Detailed sequence information is available under http://www.allgenes.com. qPCR was performed using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems) by using the default thermal cycling conditions (10 min at 95 °C, and then 40 cycles of 15 s at 95 °C plus 1 min at 60 °C). Relative quantitation was performed using the comparative cycle threshold method. At least three biological replicates were used for each timepoint investigated.