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Effect of testosterone on E1S-sulfatase activity in non-malignant and cancerous breast cells *in vitro*

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ABSTRACT

Introduction: Testosterone (T) is a therapeutic option for women with hypoactive sexual desire disorder. T may have an impact on the mammary gland by altering local estrogen synthesis. The aim of the present study was to measure the effect of T on estrone-sulfate (E1S)-sulfatase (STS) expression, and activity using hormone-dependent BC cells with high and low aggressive potential (BT-474, MCF-7), and HBL-100 as a breast cell line of non-malignant origin.
Methods: Cells were incubated in RPMI 1640 medium containing 5% steroid-depleted fetal calf serum for 3 d, and subsequently incubated in absence or presence of T alone, and combined with anastrozole (A) at 10^{-8} M, and 10^{-6} M at 37 °C for either 24 h or directly in cell extracts ("direct"). STS protein expression was measured by dot-blot (immunoblotting), and STS, HSD17B1 and HSD17B2 mRNA levels by quantitative RT-PCR. STS activity was evaluated by incubating homogenized breast cells with [^{3}H]-E1S and separating the products E1, and E2 by thin layer chromatography.
Results: Basal STS mRNA expression did not reveal group differences. However, STS mRNA was decreased by T + A in MCF-7 cells. 17HSDB1 expression was decreased by T + A in BT-474 cells, and 17HSDB2 expression was decreased by A and T + A treatment in MCF-7 cells. Basal and T treated STS protein expression was significantly higher in malignant compared to non-malignant breast cells. However, T did not induce significant intra-cell line differences. Similarly, basal and T treated STS activity was significantly higher in highly malignant compared to non-malignant breast cells. Regardless of cell lines, T slightly decreased STS activity after "direct" incubation, but led to an increase of local estrogen formation after 24 h which was attenuated, and partly reversed by A, respectively.
Conclusions: The more aggressive the breast cell line, the higher the local estrogen formation. The transition from normal to malignant seems to be accompanied by an altered autoregulation. The given local endocrine milieu seems to be essential for response to T.

1. Introduction

Low sexual desire is the most prevalent female sexual problem [1], and it is estimated that 40% of postmenopausal women experience decreased libido [2]. About 50% of those women reporting low sexual desire experience distress due to this loss (hypoactive sexual desire disorder (HSDD)) [3] which leads to a substantial decrease of health-related quality of life [4]. For women with HSDD due to oophorectomy transdermal testosterone (T) replacement therapy is available which has been shown to improve various domains of sexuality [5–8]. However, several clinical trials have indicated an increased risk of breast cancer (BC) associated with combined estrogen plus progestin hormone therapy (HT) [9–11]. Since transdermal T for women with HSDD is new on the market, data on its impact on BC risk is limited. Previous data from observational studies in women using androgens in different dosages, application modi, and combined with estrogens have yielded partly contradicting results [12–16]. A recent study in postmenopausal women using transdermal T alone reported three cases of BC compared to none in the placebo group [5]. However, if this is a finding by chance or due to T therapy remains unclear. The addition of T during combined HT in postmenopausal women has not been shown to influence mammographic density for a period of 6 months [17]. Furthermore T, has been shown to inhibit estrogen/progestogen-induced breast cell proliferation [18]. But do these findings already allow to conclude that T is safe for the breast? T is the substrate

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of the enzyme aromatase and thereby catalyzed to estradiol (E2). It is well accepted that estrogens play a major role in the genesis of BC [19]. The most biologically active estrogen in breast tissue is E2. However, the majority of women developing BC is postmenopausal, when plasma levels of E2 have decreased by 90% due to ovarian exhaustion. Moreover, the predominant circulating estrogen in postmenopausal women is estrone-sulfate (E1S). Thus, the uptake of E2 from the circulation does not appear to contribute significantly to the total content of estrogens in breast tumors. It has been demonstrated that breast tumors and mammary cancer cells possess the enzymatic systems necessary for the intratumoral biosynthesis of estrogens from precursor molecules circulating in the plasma. Three main enzymatic pathways are considered to be implicated in this process: aromatase, which converts androgens to estrogens [20,21], E1S-sulfatase (STS) which hydrolyzes E1S to estrone (E1) [22–24], and 17ß-hydroxysteroid dehydrogenase type 1 (17ßHSD-1) which reduces E1 to E2 [25,26]. STS activity in breast tumors has been shown to be 10–500-fold higher than aromatase activity [27–30]. In BC, STS mRNA has been shown to be an independent prognostic indicator in predicting relapse-free survival [31] and to correlate positively with tumor size and lymph node metastasis [32]. Thus, STS seems to play a crucial role in local biosynthesis of estrogens in breast (cancer) tissue.

The aim of this study was to evaluate the effect of T on local estrogen formation in malignant and non-malignant human breast cells *in vitro*. For this purpose, we studied STS activity, and its expression in a breast cell line of non-malignant origin, and in breast cancer cells with high, and low aggressive potential. The human breast epithelial cell line HBL-100, originally derived from milk secretion of a nursing mother without detectable breast lesion, has been extensively characterized as immortal but not tumorigenic in nude mice due to the presence of a replication deficient Simian virus 40 (SV40) large T antigen [33,34]. Although the SV40 large T antigen is known to contribute to virus induced tumorigenesis by interacting with, and altering the function of key cellular regulatory proteins, a true neoplastic conversion of HBL-100 cells requires the activation of additional oncogenes, such as Ki-ras [35]. The human breast cancer cell line MCF-7, derived by pleural effusion from a BC patient [36], is frequently used as a model cell line to demonstrate the estrogen dependency of BC growth. It represents a luminal epithelial phenotype [37], is weakly invasive *in vitro*, and depends on estrogen supplementation, and on the injection of high cell numbers for tumorigenesis in nude mice [38,39]. BT-474 cells were established from a solid, invasive ductal breast carcinoma, and represent a more advanced stage of tumor progression compared to MCF-7 cells [37]. In nude mice, BT-474 cells are tumorigenic, tend to metastasize to the regional lymph nodes, are capable of forming micrometastatic lesions in the lung, and do not depend on estrogen supplementation [40]. In this study, we focused on STS mRNA, and protein expression, as well as on its enzyme activity in order to distinguish between posttranscriptional, and posttranslational effects of T. We also measured 17ßHSD-1, and -2 mRNA expression to better understand the impact of treatment on the reductive and oxidative 17ßHSD.

2. Materials and methods

2.1. Chemicals, reagents, and steroids

The radioactively labeled steroid [^{3}H]-E1S (specific activity, 57.30 Ci/mmol) was purchased from Perkin Elmer Life, and Analytical Sciences (Boston, MA, USA). Unlabeled T, E2 and E1 were obtained from Sigma, anastrozole was obtained from AstraZeneca (London, UK). Unless stated otherwise, all chemicals were from Sigma–Aldrich (Deisenhofen, Germany).

2.2. Culture procedures of human breast cell lines

The cell lines MCF-7 [36], HBL-100 [33], and BT-474 [41] were donated by Dr. C. Poremba (Düsseldorf, Germany). Cells were 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. Prior to, and during hormonal treatments, phenol-red free medium containing 5% dextran-coated charcoal (DCC) steroid-depleted FCS was used.

2.3. Hormone treatments

Before adding hormones, the culture medium was replaced for 72 h by a medium containing 5% steroid-depleted FCS. T and/or the nonsteroidal aromatase inhibitor (AI) anastrozole (A) were added from a stock solution, and diluted in culture medium resulting in a final concentration of less than 0.1% ethanol. T was used at a concentration of 10^{-8} M, and 10^{-6} M. Cells were incubated in the presence of T for either 24 h, or enzyme activity was determined directly in cell extracts ("direct"). In order to avoid aromatization of T, cells were incubated accordingly with A at 10^{-8} M, and 10^{-6} M alone, and combined with T, respectively.

2.4. Immunocytochemistry

For immunocytochemistry, cells were grown to 80% confluence in 8-well-chamber slides (Corning Costar). Cells were fixed with −20 °C cold methanol for 10 min, washed, and blocked with PBS/2% goat serum for 1 h at room temperature. For conventional immunocytochemistry, cells were incubated with a monospecific rabbit-anti-human STS antiserum [42], diluted 1:1000 in phosphate buffered saline (PBS) containing 1% bovine serum albumine (BSA), or with a control rabbit serum over night at 4 °C. Endogenous peroxidase was quenched with methanol/0.6% H_2O_2, followed by 3 washes with PBS. STS was detected using the DAKO cytomation rabbit-EnVision plus-horseradish peroxidase (HRP) system, and the AEC+ substrate (DAKO, Glostrup, Denmark), followed by counterstaining with Mayer's Hemalum (Merck, Darmstadt, Germany). Sections were observed with a Zeiss Axiovert 100 microscope equipped with an Axiophot Mrc camera.

2.5. Thin layer chromatography for STS enzyme activity

Breast cells were homogenized on ice, in 2 ml 0.06 M Tris–HCl-buffer pH 7.0 (assay buffer throughout the entire procedure) for approximately 1 min at maximum speed using a Polytron homogenizer. STS activity was assessed as previously described [43–45]. In brief, all incubations were carried out in duplicates. The incubation mixtures consisted of 200 μl homogenate, 19.39 pmol [^{3}H]-E1S (specific activity 57.30 Ci/mmol, Perkin Elmer Life, and Analytical Sciences, Boston, MA, USA) in 100 μl of buffer, and 100 μg of NADH, and NADPH in 100 μl of buffer. Simultaneous background incubations using buffer instead of tissue homogenate were performed in order to correct for non-enzymatic transformation of the substrate. Besides measuring STS activity in breast cells having been incubated for 24 h, STS activity was studied by incubating cell extracts "directly" with the respective hormone treatment. After 15 min of incubation at 37 °C in a water bath, 200 μg of non-radioactive E1, and E2 were added in 100 μl of ethanol together with 0.5 ml of 0.5 M sodium phosphate buffer, pH 7.0. The unconjugated steroids were extracted with 2.0 ml toluene. After inverting the tubes 100 times they were centrifuged at 1500 × *g* for 10 min. The lower aqueous phase was frozen in liquid nitrogen. 100 μl of the upper toluene phase was removed, and added to 100 μl of buffer, and 3.0 ml of scintillation fluid to determine total hydrolysis. The remaining

toluene phase was evaporated to dryness in nitrogen atmosphere, and dissolved in 100 μl Folch-Solution (2:1 chloroform:methanol). Thin layer chromatography (TLC) on Silica gel G/UV 254-Alugram (Fa. Roth; Macherey-Nagel Düren GmbH) was performed using 13% ethanol in toluene as solvent. After visualization in 254 nm ultraviolet (UV) light, the zones in the plate corresponding to the estrogens as well as the non-fluorescent zones were cut apart, and separately eluted with 3 ml ethanol, and 6 ml scintillation fluid. Aliquots were taken from each fraction from the TLC plate for counting of the [^{3}H] activity. A Wallac 1409 (ß-counter) liquid scintillation spectrometer was used for the radioactivity measurements, and Riafluor Perkin Elmer Life, and Analytical Sciences (Boston, MA, USA) was used as scintillation fluid. The enzyme activity was expressed as the amount of unconjugated estrogen (E1 + E2) formed per minute, and per mg of protein. The total protein was determined by the method of Bradford [46].

2.6. Dot-blot for STS protein expression

Cells were lysed in RIPA buffer (20 mM Tris–HCl pH 7.4, 137 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM EDTA) containing protease inhibitors (Sigma) on a rocking platform on ice for 30 min, and removed from the culture dish with a cell scraper. Extracts were centrifuged for 10 min at 10,000 × *g*, 4 °C, and the protein content of the supernatants was determined by BCA assay (Pierce). 30 μg of protein/well/600 μl Tris-buffered saline (TBS) were subjected to a dot-blot as follows: a nitrocellulose membrane was activated with ddH_2O, and preincubated in Tris-buffered saline (TBS) pH 7.4 for 10 min. The membrane was placed on Whatman 3 mm paper soaked in TBS, and assembled in a Bio-dot microfiltration apparatus (Bio-Rad). The apparatus was washed with 200 μl TBS/well prior to loading 200 μl of sample diluted in TBS in triplicates, followed by washing with 200 μl TBS. Subsequently, the membrane was blocked with 5% nonfat dry milk in TBS containing 1% (v/v) Tween 20 for 1 h at room temperature, washed 3 × 5 min with TBS, and incubated with anti-STS antiserum [42] diluted 1:1500 in TBS containing 5% BSA, and 1% (v/v) Tween at 4 °C for 16 h. The membrane was washed 3 × 5 min with TBS (1% Tween), and incubated with a peroxidase-conjugated goat-anti-rabbit IgG antibody (Calbiochem) diluted 1:2000 in TBS containing 5% BSA, and 1% (v/v) Tween at room temperature for 1 h. The blot was washed 3 × 5 min with TBS (1% Tween), followed by an enhanced chemoluminescence reaction (Super Signal, Pierce, Rockford, MA), and exposition to ECL-hyperfilm (Amersham, Braunschweig, Germany). For normalization, blot membranes were stripped with 0.2 M glycine, pH 2.5 containing 150 mM NaCl for 2 × 10 min, neutralized with TBS, and subjected to the procedure described above using mouse-anti-tubulin primary antibodies (Sigma, 1:4000), and peroxidase-conjugated goat-anti-mouse IgG antibodies (Calbiochem, diluted 1:10,000). Digital images of the ECL-hyperfilms were generated using a BioDoc Analyze system (Biometra, Göttingen, Germany), and subjected to a quantitative analysis using the Image J software (NIH, Bethesda, USA).

2.7. Quantitative TaqMan® real-time PCR for STS mRNA expression

Total cellular RNA of the cell lines was prepared using the RNeasy reagent system (Qiagen Inc., Hilden, Germany). RNA was 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 MasterMix (Applied Biosystems, Darmstadt, Germany), and pre-designed TaqMan gene expression systems (Applied Biosystems) according to the manufacturer's instructions. For detection of STS, primer Hs00165853_m1, for 17HSDB1, primer HS00166219_g1, and for detection of 17HSDB2 primer Hs00157993_m1 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. Quantitative real-time PCR 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. Three biological replicates were used for each timepoint investigated.

2.8. Statistical methods

Statistical analysis was performed at the "Services in Statistics", Würzburg, Germany, using exact, and non-parametrical statistical methods (SPSS module, Exact Tests", SPSS 2003) since essential assumptions of underlying data distributions ("normality"), and type of variances ("homoscedasticity assumption") were not known to be fulfilled. Thus, for pairwise group comparisons the exact Mann–Whitney-*U*-test [47] was applied. All *p*-values will be given two-tailed, and *p*-values $p < 0.05$ will be considered as a descriptive difference. Experiments were repeated independently three (immunocytochemistry, dot-blot, rtPCR) and five times (TLC), respectively.

3. Results

3.1. Immunolocalization of STS in HBL-100, MCF-7, and BT-474 cells

First, we intended to confirm STS expression in HBL-100, MCF-7, and BT-474 cells. Therefore we performed immunocytochemistry using a monospecific antiserum directed against human STS [42]. In contrast to HBL-100, and MCF-7 cells, BT-474 breast cancer cells showed a much stronger expression of STS protein (Fig. 1). After that, we tested for immunolocalization of STS in HBL-100, MCF-7, and BT-474 cells subjected to T, or combined T + A treatment. Treatment with T at 10^{-8} M (not shown), or 10^{-6} M, and combined T + A treatment (both 10^{-6} M) for 24 h did not induce changes in STS expression, respectively (Fig. 1).

3.2. Local E1, and E2 formation as indicative for STS activity

In a next step we focused on the effect of T on local STS enzyme activity differentiating between "direct" incubation of cell extracts, and 24 h incubation of cultured cells in the presence of T. In order to avoid aromatization of T to E2 we also incubated cells with T combined with A (T + A). Thus we made sure the observed effects by T were not indirectly induced by E2. With the differences for E1, and E2 formation after treatment with T, and T + A for 24 h at different concentrations (10^{-6} M, and 10^{-8} M) being negligible the respective data were combined.

E1 was the most abundant labeled estrogen found in breast cells after incubation with labeled E1S. Across the whole data set the median concentration of locally formed E1 was 90 times higher than that of E2. Basal total estrogen formation (E1 + E2) in untreated breast cells decreased significantly with increasing time of incubation ($p = 0.05$). Except for HBL-100 cells, a reduction of total estrogen formation after 24 h of incubation was also found in treated breast cells (Fig. 2).

After "direct" incubation with T E1, E2, and total estrogen formation (E1 + E2) were slightly, but not significantly, decreased in all three breast cell lines. The ratios of E1 + E2 after "direct" treatment with T to baseline were 0.83 for HBL-100, 0.67 for MCF-7, and 0.82 for BT-474 cells, respectively. The ratios of E1 + E2 after "direct"

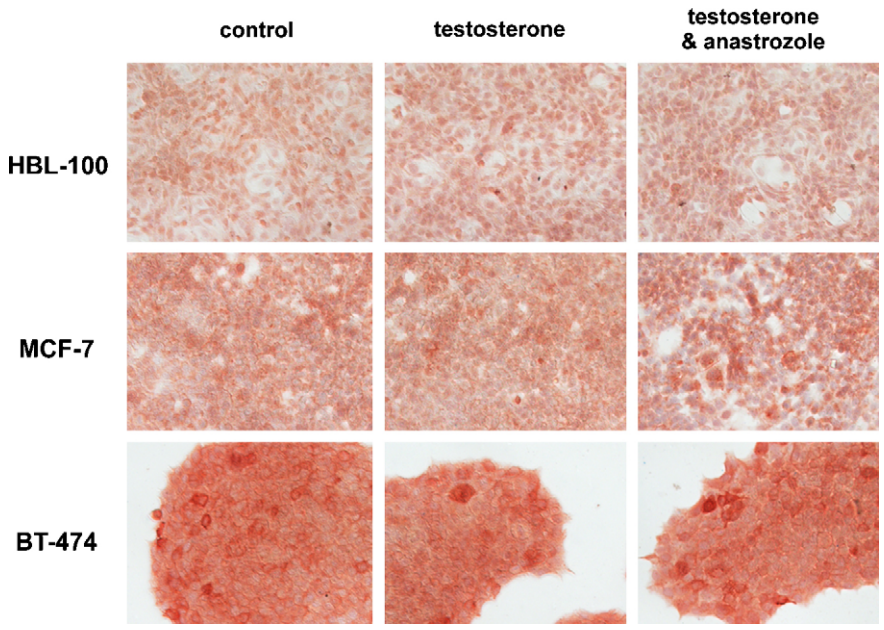

Fig. 1. Immunolocalization of STS in HBL-100, MCF-7, and BT-474 cells subjected to T or T + A treatment. Cells were fixed with methanol and immunostained with a monospecific antiserum against STS [42] as described in Section 2. STS expression in HBL-100, and MCF-7 cells was low compared to BT-474 cells. Treatment with T at 10^{-8} M (not shown) or 10^{-6} M, or combined treatment with T + A (both 10^{-6} M) for 24 h did not induce changes in STS expression. 32× magnification shown for all panels.

treatment with A to baseline were 1.18 for HBL-100 ($p = 0.121$), 0.81 for MCF-7 ($p = 0.02$), and 0.90 for BT-474 cells ($p = 0.606$), respectively. The combination of T + A only revealed minor changes in comparison to T alone, but turned the T induced reduction of E1 (not shown), and total estrogen formation into a significant one at least in MCF-7 cells ($p = 0.02$). Within HBL-100, and MCF-7 cells the ratios of E2 + E2 were higher in cells treated with A only in comparison to T + A treatment ($p < 0.05$).

Relative to controls treatment with T for 24 h induced a significant increase of E1, and total estrogen formation regardless of cell line ($p = 0.02$). The ratios of E1 + E2 after 24 h treatment with T to baseline were 5.53 for HBL-100, 2.00 for MCF-7, and 1.59 for BT-474 cells, respectively. As for treatment with A alone a similar direction of change for total estrogen formation was observed. The ratios of E1 + E2 after 24 h treatment with A to baseline were 1.78 for HBL-100 ($p = 0.302$), 3.57 for MCF-7 ($p = 0.02$), and 0.61 for BT-474 cells ($p = 0.02$), respectively. The combination of T + A attenuated the effects by T observed in HBL-100 cells, and MCF-7 cells, and even counteracted the increase of total estrogen formation in BT-474 cells. The ratios of E1 + E2 after 24 h treatment with T + A to baseline were 2.21 for HBL-100 ($p = 0.02$), 1.83 for MCF-7 ($p = 302$), and 0.48 for BT-474 cells ($p = 0.02$), respectively. Within cell lines the increase of total estrogen formation in HBL-100, and BT-474 cells by T was significantly counteracted by T + A ($p = 0.004$) (Fig. 3).

However, the individual capability of the different cell lines for estrogen formation was never remarkably abolished regardless of treatment. Thus, local total estrogen formation in the more aggressive BT-474 cell line was always higher than in MCF-7 (basal "direct", and 24 h $p = 0.05$; "direct", and 24 h T, A, A + T $p = 0.04$, respectively), and HBL-100 cells (basal "direct", and 24 h $p = 0.05$; "direct" T $p = 0.004$, and 24 h T $p = 0.016$; "direct" A $p = 0.004$, and 24 h A $p = 0.054$; "direct" A + T $p = 0.004$, and 24 h A + T $p = 0.873$). When comparing MCF-7, and HBL-100 cells total estrogen formation was significantly different at baseline ($p = 0.05$), after "direct" incubation with A ($p = 0.016$), and after 24 h incubation with T, A, and A + T ($p = 0.04$).

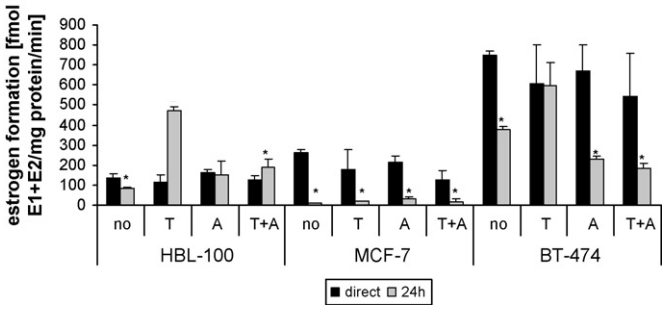

Fig. 2. Hormone-dependent local estrogen formation in breast cells. STS activity was determined in non-malignant (HBL-100), and malignant (MCF-7, BT-474) breast cells after "direct" and 24 h incubation with T, A, and T + A, respectively. STS activity was evaluated as indicated in the text. Values (in fmol of estrogen/mg of protein/min) are expressed as means and SD. $^*p \leq 0.05$ when comparing direct and 24 h incubation. Abbreviations: T = Testosterone, A = Anastrozole, T + A = Testosterone + Anastrozole. $n = 3$ (controls), and $n = 6$ (treated cells).

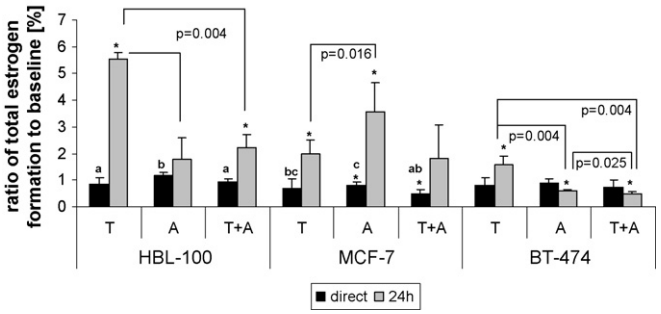

Fig. 3. Relative STS activity in non-malignant and malignant breast cells. Relative STS activity in non-malignant (HBL-100), and malignant (MCF-7, BT-474) breast cells was determined after "direct", and 24 h incubation with T, A, and T + A, respectively. STS activity was evaluated as indicated in the text. Values are expressed as means and SD of the ratio of total estrogen formation to baseline (%). $^*p \leq 0.05$ when comparing treated cells with the respective controls. Different letters indicate significant differences within cell lines at $p \leq 0.05$. Significant differences between treatment groups at 24 h are presented separately as indicated. Abbreviations: T = Testosterone, A = Anastrozole, T + A = Testosterone + Anastrozole. $n = 3$ (controls), and $n = 6$ (treated cells).

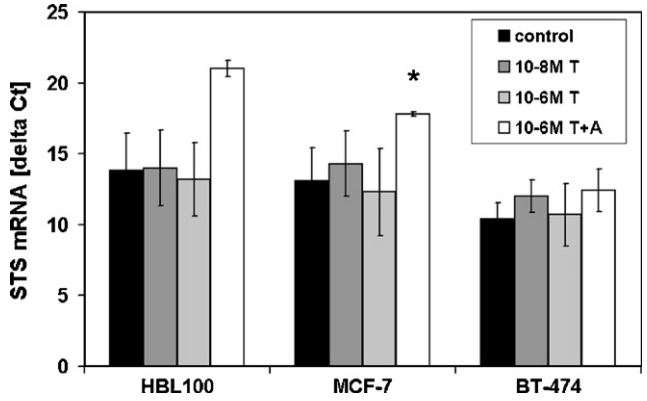

Fig. 4. STS mRNA expression in HBL-100, MCF-7, and BT-474 cells subjected to T, or T + A treatment. HBL-100, MCF-7, and BT-474 cells were treated with a solvent control, T at 10^{-8} M, T at 10^{-6} M, or T + A at 10^{-6} M each for 24 h, followed by RNA isolation and quantitative real-time PCR analysis. STS mRNA expression was evaluated as indicated in the text. Expression data are given as delta *Ct* values normalized to 18S rRNA expression. Note that a low delta *Ct* value corresponds to high expression levels. Error bars = SEM, $n = 3$–5.

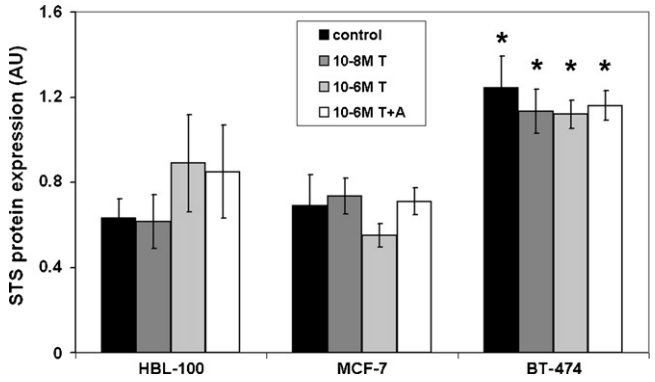

Fig. 6. STS protein expression in HBL-100, MCF-7, and BT-474 cells subjected to T, or T + A treatment. HBL-100, MCF-7, and BT-474 cells were treated with a solvent control, T at 10^{-8} M, T at 10^{-6} M, or T + A at 10^{-6} M each for 24 h, followed by preparation of cell lysates as described in Section 2. 30 μg protein/well were employed in a dot-blot assay using a monospecific antiserum to STS. Scanned images of the blots were analyzed by densitometry and normalized to the expression of tubulin. *Significantly different from HBL-100 and MCF-7 at $p \leq 0.05$, error bars = SEM, $n = 3$.

3.3. Quantitative RT-PCR analysis of STS, hydroxysteroid (17-beta) dehydrogenase (17HSDB)1, and 17HSDB2 mRNA expression following treatment with T

We investigated if the observed differences in STS activity could be attributed to differential changes in STS mRNA expression induced by T treatment in HBL-100, MCF-7, and BT-474 cells. Quantitative real-time PCR revealed no significant difference in basal STS mRNA expression between malignant, and non-malignant breast cell lines (Fig. 4). Furthermore, stimulation with either T at 10^{-8} M or 10^{-6} M for 24 h did not induce significant changes in STS mRNA expression compared to unstimulated controls (Fig. 4). Combined treatment with T + A at 10^{-6} M for 24 h led to increased STS ΔCt values in MCF-7 cells ($p < 0.05$), and HBL-100 cells ($p = 0.08$), corresponding to reduced mRNA expression, while no major expression changes were noted in BT-474 cells. The mRNA expression of both 17HSDB1 and 17HSDB2 was at the limit of detection and could not be reliably determined in HBL-100 cells (not shown). In BT-474 cells, combined T + A treatment lead to a significant reduction of 17HSDB1 mRNA expression (increased ΔCt values, Fig. 5A), whereas 17HSDB2 expression was significantly decreased by T, and T + A treatment in MCF-7 cells (Fig. 5B).

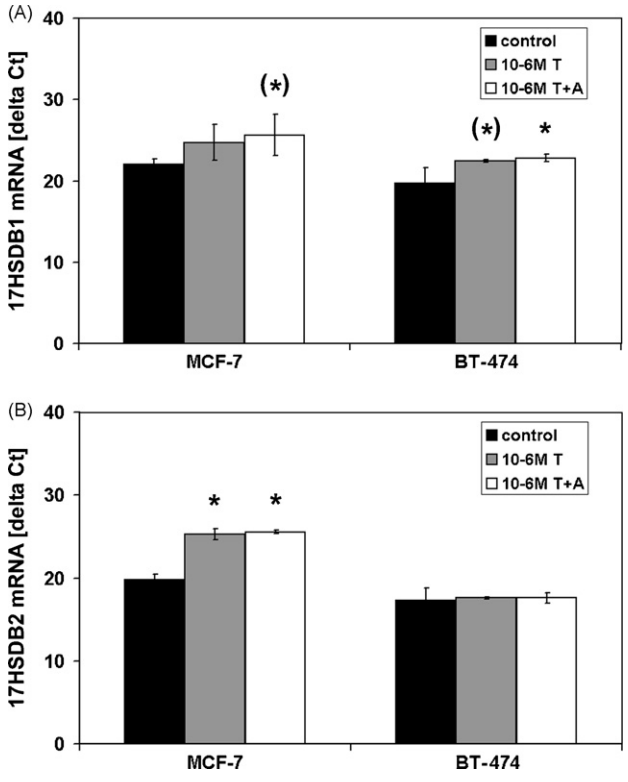

Fig. 5. mRNA expression of 17HSDB1 and 17HSDB2 in MCF-7 and BT-474 cells subjected to T or T + A treatment. HBL-100, MCF-7, and BT-474 cells were treated with a solvent control, T at 10^{-6} M, or T + A at 10^{-6} M each for 24 h, followed by RNA isolation and quantitative real-time PCR analysis. mRNA expression was evaluated as indicated in the text. 17ßHSD isoform expression was at the limit of detection in HBL-100 cells, and could not be reliably evaluated. Expression data are given as delta *Ct* values normalized to 18S rRNA expression. Note that a low delta Ct value corresponds to high expression levels. Error bars = SD, $n = 3$–4. *$p < 0.05$, *$p = 0.06$.

3.4. Analysis of STS protein expression following treatment with T

Since the observed differences in STS activity could have been due to changes in STS protein expression potentially caused by posttranscriptional regulation, we aimed at complementing our immunohistochemical data (Fig. 1) with a semiquantitative analysis of STS protein expression. For this purpose, we performed a dot-blot analysis on cell lysates of T-, and vehicle-treated HBL-100, MCF-7, and BT-474 cells. In accordance with our immunostaining results, BT-474 cells showed a significant, about 2-fold increased expression of STS protein compared to HBL-100, and MCF-7 cells (Fig. 6). However, T treatment did not result in significant STS protein expression changes in any of the tested cell lines.

4. Discussion

In this study, we investigated the effect of T on local estrogen formation in malignant, and non-malignant human breast cells *in vitro*. Prior to that, we confirmed STS expression in HBL-100, MCF-7, and BT-474 cells by immunocytochemistry. Basal STS activity in the more aggressive BT-474 cell line was significantly higher than in HBL-100, and MCF-7 cells, with MCF-7 cells still displaying a higher basal STS activity in comparison to HBL-100 cells *in vitro*. Furthermore regardless of treatment STS activity was always highest in the most aggressive BC cell line. To our knowledge we are the first to study the effect of T on local estrogen formation *in vitro*. We added an AI in order to avoid aromatization, and thus the results being biased by indirect E2 formation. “Direct” incubation, which

eliminates cellular regulation of metabolism, led to a reduction of estrogen biosynthesis regardless of cell line. However, when incubating breast cells with T for 24 h the effect on STS activity differed from what was found after "direct" incubation. Regardless of cell line total estrogen formation was significantly increased by T alone. Most likely this effect was due to aromatization of T to E2 at least to some extent since the addition of A attenuated, and partly reversed the observed effect of T alone, respectively. Surprisingly 24 h treatment with T and A + T resulted in higher local estrogen formation in HBL-100 cells in comparison to MCF-7 cells.

By "direct" incubation, we believe to obtain more valid information about regulation of T at the level of the enzyme's catalytic activity. In contrast, longer incubation in intact cells also includes complex effects on STS expression, and on downstream processing of metabolites, making it difficult to differentiate between the respective effects. Finally, the stimulating effect of T (and to some extent A + T, respectively) on STS activity in the breast cells observed in the 24 h assay could have been influenced by secondary factors: Organic anion transporting polypeptides (OATPs) have been shown to mediate the transmembrane transport of E1S in human breast tissue, and in a variety of human breast cancer cell lines [48–50]. In living cells (24 h assay), OATPs could increase the availability of E1S, whereas this mechanism is not available in the "direct" assay, potentially contributing to the different outcomes of our assays. Our findings support previous hypotheses suggesting a modification of estrogen metabolism accompanying the tumoral process of the breast [51–53] which might also be reflected in different effects of steroids on normal, and malignant human breast cells, and tissue, respectively.

Compared to the changes in the enzyme activity, we observed no effects of T treatment on STS mRNA, and protein expression regardless of cell line, whereas T itself (as demonstrated by aromatase inhibition) seemed to display a protective effect by reducing STS mRNA expression in HBL-100 ($p = 0.08$), and MCF-7 cells. However, this effect might be negligible since STS protein expression was higher in the more aggressive BT-474 cells in comparison to non-malignant, and less aggressive breast cells although no differences were found on STS mRNA level implying a posttranscriptional modulation of STS. These results are largely comparable to a previous study on the effect of HT on STS expression in HBL-100, and MCF-7 cells, where either no or only moderate changes in STS expression were observed upon treatment with E2, Tibolone (OrgOD14), and its metabolite Org4094 [43]. These data further suggest that the effects of steroids on human breast cells are largely due to posttranslational alterations of STS activity. Apart from our recent study [43] this view is supported by the finding that the progestogen medroxyprogesterone acetate [54], estrone-3-O-sulfamate (EMATE) [55], and interleukin-6 [56] are all capable of modulating STS activity, but do not change its mRNA expression level (cf. [44] for a more extensive discussion).

Expression of STS mRNA in BC has a prognostic impact: STS mRNA expression is an independent prognostic factor in predicting relapse-free survival, with high expression levels being associated with poor prognosis [31,32,57]. Moreover, mRNA expression levels of STS are up-regulated in soft tissue metastases compared to those in the primary tumors [58]. While no significant differences in STS mRNA expression were observed in the breast cell lines studied, it is striking that STS protein expression was significantly higher in the more aggressive breast cancer cell line BT-474 compared to the less invasive MCF-7 cell line, and non-malignant HBL-100 cells. This finding may underline the importance of generating estrogen from sulfated precursors in order to promote tumor progression.

First, our finding of an stimulatory effect of "longterm" treatment with T on local estrogen formation in non-malignant, and malignant breast cells *in vitro* possibly due to posttranslational modifications of STS activity is somewhat contradictory to previous studies showing no adverse effect of T on proliferation in human breast cancer cell lines *in vitro* [59,60], normal human breast tissue *in vivo* [18], and on mammographic breast density [17]. However, most studies were performed in the presence of estrogens by for example adding conventional HT to T treatment in postmenopausal women [6–9,17,61]. Therefore our study design is better comparable to the Aphrodite trial in which T alone was offered to postmenopausal women with HSDD for 52 weeks. In this study invasive BC was diagnosed in two, and ductal carcinoma in situ of the breast in one women in the T groups after 4, 7, and 12 months of treatment, respectively, while none was affected in the placebo arm. However the authors report that in retrospect one woman reported a bloody nipple discharge before randomization [5]. Furthermore it is commonly accepted that the time period for a cancerous breast cell to progress to an identifiable tumor takes several years. Thus, secondly, our finding that the origin of breast cells has a greater impact on local estrogen formation than hormonal treatment supports this hypothesis. The more aggressive BT-474 cell line displayed higher basal and treated STS enzyme activity, and protein expression than MCF-7, and HBL-100 cells. Therefore, the transition from normal to malignant, and even more aggressive malignant breast cells seems to be accompanied by an altered autoregulation. However, the answer to the question as to whether a basal estrogenic milieu ("priming") has an impact on how additive T modulates breast cells, and tissue, respectively, remains speculative [62]. It might be that in an estrogen-deprived milieu (postmenopause) with an increased estrogen receptor expression in normal breast tissue in comparison to premenopausal breast tissue [63]. T is rapidly aromatized to E2 (and not artificially inhibited like in our study). In contrast, breast tissue having been treated with conventional HT prior to T treatment has already reacted with specific responses like an increased local estrogen formation [45,64], and the additional T is not aromatized to E2 due to product inhibition. This hypothesis is supported by Sikora et al. reporting an up-regulation of steroidogenic enzymes under profound estrogen-deprived conditions *in vitro*. Furthermore the downstream metabolite of 5a-dihydrotestosterone was found to be estrogenic in breast cancer cells, and inducing growth and ER-signaling via activation of estrogen receptor alpha [65].

However, our study has its limitations: other human breast cell lines may respond differently to hormonal treatment. The duration of hormonal treatment may lead to varying results. Similarly, other steroids may alter STS activity by affecting the transcriptional, and/or translational level as it was demonstrated for different chemokines [54,56]. The different effect of T on STS mRNA, and protein expression, and local estrogen formation might also be due to T modifying other enzymes being involved in local estrogen formation. This view is supported by our finding of a reduced mRNA expression of 17HSDB1 in BT-474 cells treated with T + A, and of 17HSDB2 in MCF-7 cells treated with T, or T + A, respectively. However, while a differential expression of 17BHSD2 between normal and malignant breast tissue was recently described, no correlations regarding clinicopathological parameters were found [66]. Moreover, the contribution of E2 to the combined E1 + E2 values was very small in our experimental system, suggesting only a minor influence of 17ßHSD1/2 in our study. Furthermore, complexity increases when investigating tissue samples. In our previous study in postmenopausal monkeys, STS activity in the mammary gland varied depending on treatment, and breast fat content [64]. Since STS is predominantly found in epithelial breast tissue this might explain the increased STS activity in epithelial-rich breast samples. Thus, further studies are needed to characterize the molecular level at which STS expression is regulated, since the unusual organization of the promotor region of the STS gene does not allow to easily predict which factors may be involved in this process [67,68].

5. Conclusions

In conclusion, T decreases STS (and possibly 17ßHSD-1) catalytic activity for local estrogen formation *in vitro*. However, downstream processing of metabolites appears to counteract the immediate inhibitory effect since local estrogen information increased after 24 h treatment. Despite the modulating effect of T on local estrogen formation STS protein, and mRNA expression remained unchanged suggesting a posttranslational effect of T on STS. Our findings seem to contradict previous studies showing no adverse effect of T on breast cells, and tissue. However, most studies also used estrogen combined with or without progestin in parallel. The given local endocrine milieu seems to be essential for response to T. Furthermore STS enzyme activity, and protein expression seem to be more dependent from the cells' character than from hormonal treatment. Thus, the transition from normal to malignant, and even more aggressive malignant breast cells seems to be accompanied by an altered autoregulation.

Competing interests

The author(s) declare that they have no competing interests.

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