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17 β HSD are enzymes catalysing the conversion between oestrogens

We found that progestins affect the expression of specific 17 β HSD-enzymes

We propose a mechanism for increased breast cancer risk after HRT use with progestins

Progesterone and Levonorgestrel Regulate Expression of 17 β HSD-enzymes in
Progesterone Receptor Positive Breast Cancer Cell Line T47D

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Abstract

The use of combined hormone replacement therapy (HRT) with oestrogens and progestins in postmenopausal women has been associated with an increased risk for developing breast cancer. The reasons are not fully understood, but influence of HRT on endogenous conversion of female sex hormones may be involved. The expression of 17 beta hydroxysteroid dehydrogenases (17 β HSD), which are enzymes catalysing the conversion between more or less potent oestrogens, may partly be regulated by progestins. The breast cancer cell lines T47D, MCF-7 and ZR75-1 were treated with progesterone, medroxyprogesterone acetate (MPA) or levonorgestrel for 48 and 72 hours at 10^{-7} M and 10^{-9} M to investigate influence on 17 β HSD1, 17 β HSD2 and 17 β HSD5 mRNA expression measured by real time PCR. The expression of 17 β HSD1 increased in progesterone and levonorgestrel treated T47D cells (48h 10^{-7} M $P=0.002$; $P<0.001$) and 17 β HSD5 increased after progesterone treatment (48 h 10^{-7} M $P=0.003$), whereas the expression of 17 β HSD2 decreased after the (48 h 10^{-7} M $P=0.003$; $P<0.001$). Similar, but less prominent effects were seen in MCF7 and ZR75-1. The progestin effects on 17 β HSD-expression were lost when T47D-cells were co-treated with progestins and the progesterone receptor (PgR) inhibitor mifprestone. We show that both reductive (17 β HSD1 and 17 β HSD5) and oxidative (17 β HSD2) members of the 17 β HSD-family are under control of progesterone and progestins in breast cancer cell lines. This is most clear in T47D cells which have high PgR expression. 17 β HSD enzymes are important players in the regulation of sex steroids locally in breast tumours and tumoural expression of various 17 β HSD-enzymes have prognostic and treatment predictive relevance. We propose a mechanism for increased breast cancer risk after HRT in which hormone replacement affects the expression of 17 β HSD-enzymes, favouring the expression of reductive enzymes, which in turn could increase levels of bioactive and mitogenic estrogens in local tissue, e.g. breast tissue.

Keywords: Breast cancer, Progestin, progesterone, 17 β HSD1, 17 β HSD2

Introduction

Hormone replacement therapy (HRT), which typically contains oestrogens or oestrogens in combination with progestin, is given to women to ease discomfort associated with reduced circulating female sex hormones at menopause. The use of HRT was greatly reduced after a report from the Women's Health Initiative which showed an increased risk of breast cancer for women receiving progestins in combination with oestradiol compared to oestradiol treatment alone [1]. This association has since been confirmed in several other studies. The breast cancer risk associated with HRT is higher for oestrogen receptor (ER)-positive cancers than for ER-negative cancers [2-5].

One in ten women will be affected by breast cancer during their life time. Several risk factors have been proposed, among them exposure to female sex hormones. The importance of oestrogens is illustrated by the fact that approximately 80% of all breast cancers express high levels of ER. Although progesterone is vital for normal growth and development of the breast, the role of this female sex hormone in the development of breast cancer, either as a single actor or as a regulator of oestrogen signalling, is under debate [6,7]. The progesterone receptor (PgR) is an oestrogen responsive gene, and the presence of PgR is often used as a marker for functional ER signalling. Progestins are designed to mimic progesterone but with a longer half-life and better availability, however, their mechanism of action is not fully understood. It is clear that different progestins exert different effects that may be due to metabolism, dose variation, pharmacokinetics and effects mediated by steroidogenic enzymes [8]. Even though the main targets of progestins are steroid receptors, they may also bind mineralocorticoid receptors (MR), androgen receptors (AR) or glucocorticoid receptors (GR) [9].

17 β hydroxysteroid dehydrogenases (17 β HSD), is a family of enzymes involved regulating the availability of more or less biologically potent sex hormones. 17 β HSD1 efficiently catalyses the reduction of oestrone to oestradiol, whereas 17 β HSD2 catalyses the oxidation

of oestradiol to oestrone, testosterone to androstendione and dihydrotestosterone to 5 α -androstendione. 17 β HSD5 converts androstendione to testosterone and oestrone to oestradiol. We have previously shown that 17 β HSD1, 17 β HSD2, 17 β HSD5 and 17 β HSD14 are important as prognostic and treatment predictive factors in breast cancer [10-13]. It has been shown that progestins influence the oestradiol and testosterone levels, and these effects have been presumed to be an effect caused by altered regulation of 17 β HSD enzymes; however the contribution of separate family members of the 17 β HSD-family has not been addressed [14-19].

Our aim was to investigate potential roles/contributions of individual 17 β HSD-enzymes for the altered sex hormone conversion seen after treatment with progesterone/progestins in vitro as well as in vivo. We investigated the effect of progesterone or progestins (levonorgestrel and medroxyprogesterone acetate (MPA)) on the expression levels of 17 β HSD1, 17 β HSD2 and 17 β HSD5 in breast cancer cell lines T47D, MCF7 and ZR75-1 measured with semi-quantitative realtime-PCR. Furthermore, in order to study whether detected changes were mediated by PgR, cells were treated with progesterone and progestins in combination with the PgR inhibitor mifepristone.

Material and methods

Cell culture and treatment

T47D, MCF7 and ZR75-1 breast cancer epithelial cells (American Type Culture Collection, Manassas, VA) were cultured in phenol-red free Opti-Mem I (Invitrogen, Carlsbad, CA) supplemented with 4% foetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and incubated at 37°C, 5% CO₂. In all experiments charcoal/dextran treated serum (CTS; HyClone, Utah, USA) was used to control the levels of hormones. The experiments with T47D were performed between passage 100 and 104, MCF7 between passage 151 and 157 and ZR75-1 between passage 91 and 93. The cells were seeded in twelve-well plates in 1mL/well in culture medium 30 000/well for T47D cells, 60 000/well MCF7 and 30 000/well for ZR75-1. Twenty-four hours after seeding, the cells were treated with either progesterone 10⁻⁷ M or P 10⁻⁹ M, MPA 10⁻⁷ M or 10⁻⁹ M and levonorgestrel 10⁻⁷ M or 10⁻⁹ M (Sigma-Aldrich, St Louis, MO) for 48 and 72 hours, a vehicle control was treated in the same way. The cells received new hormone medium every 24 hour. All experiments were performed in triplicate and repeated three times.

Inhibition with Mifprestone

To identify changes mediated via PgR, T47 D cells were treated with mifprestone 10⁻⁷ M (Sigma-Aldrich); mifprestone 10⁻⁷ M and progesterone 10⁻⁷ or 10⁻⁹ M, mifpresone 10⁻⁷ M and levonorgestrel 10⁻⁷ or 10⁻⁹ M; and mifprestone 10⁻⁷ M and MPA 10⁻⁷ and 10⁻⁹ M for 48 hours, a vehicle control was included. The cells received new hormone medium every 24 hour. The experiment was performed in triplicate and repeated three times.

Western blot

To compare protein expression of PgR and ER, cultured MCF7, T47D and ZR75-1 cells were lysed. The same amount of protein/lane was loaded onto 4-15% gradient precast gels (Criterion, Bio-Rad, Hercules, CA). The proteins were transferred to PVDF membranes that were further incubated overnight with ER antibody (Mus ERα/NR3A1 (Clone H4624), 1 mg/ml, R&D systems, Minneapolis, MN, USA) or PgR antibody (Rabbit anti Pgr 1:1000, Cell

signalling, Beverly, MA, USA). To control for equal loading the membrane was incubated with β -actin antibody (1:2000, Cell signalling). Binding of the antibodies to the membranes was detected using a commercial ECL-Plus kit (GE Health care UK limited, Little Chalfont, UK). Results were visualised using LAS1000 CCD-camera detection system (FujiFilm, Tokyo, Japan).

Semi-quantitative Real time-PCR

RNA was extracted 48 and 72 hours after treatment using SV Total RNA Isolation System (Promega, Madison, WI). The RNA was stored at -70°C . Synthesis of cDNA from total RNA was performed using the 1st strand cDNA Synthesis kit with random hexameres (Roche Diagnostics Corporation, Indianapolis, IN, USA). mRNA expression of 17 β HSD1 and 17 β HSD2 was analysed according to Gunnarsson *et al.* [11] and 17 β HSD5 according to Jansson *et al.* [13]. β -actin was used as endogenous reference gene and the assay was purchased from Applied Biosystems (Warrington, UK). Standard curves for all analysed genes were run on each plate, using serially diluted cDNA to normalise the runs. The obtained data from β -actin was used to normalise the sample variation in the amount of input cDNA. All samples were run as triplicates and in all experiments samples without template were used as control. The relative quantification of 17 β HSD1, 17 β HSD2, and 17 β HSD5 were performed according to the manufacturer's description (Protocol P/N 4303859, Pearkin Elmer).

Statistical evaluation

Students t-test was used for comparison between treated samples and the control. The results are expressed as $\pm$ SE of the mean values, obtained from three replicates repeated three times. All P-values are two sided, and $p < 0.05$ was considered to be statistically significant. All the procedures were comprised in the statistical package STATISTICA 8.0 (StatSoft Scandinavia AB, Sweden).

Results

17 β HSD1, 17 β HSD2 and 17 β HSD5 expression in T47D, MCF7 and ZR75-1

The relative expression of 17 β HSD1, 17 β HSD2 and 17 β HSD5 were analysed in untreated breast cancer cell lines, T47D, MCF7 and ZR75-1. T47D cells expressed high levels of 17HSD β 1; intermediate levels of 17 β HSD2, and intermediate e 17 β HSD5 levels; MCF7 expressed low levels of 17 β HSD1, low levels of 17 β HSD2 and intermediate levels of 17 β HSD5; and ZR75-1 expressed intermediate levels of 17HSD β 1, low levels of 17HSD β 2 and intermediate levels of 17 β HSD5 (Table 1).

PgR and ER expression in T47D, MCF7 and ZR75-1

The highest protein expression level of PgR was detected in T47D, intermediate/low expression in ZR75-1 and MCF-7, in line with previous measurements. Further, ER expression was high in T47D and MCF-7, and intermediate in ZR75-1.

17 β HSD1, 17 β HSD2 and 17 β HSD5 expression after progesterone, levonorgestrel and MPA treatment in T47D cells

Progesterone induced expression of 17 β HSD1 in T47D cells (48 h 10^{-7} M $P=0.002$, 10^{-9} M $P=0.03$; 72 h 10^{-7} M $P=0.005$ and 10^{-9} M $P>0.05$). The same was found after treatment with levonorgestrel (48 h 10^{-7} M $P=0.0007$, 10^{-9} M $P=0.0004$; 72 h 10^{-7} M $P=0.0006$ and 10^{-9} M $P=0.01$) (Fig 1a). No changes were detected after MPA treatment. Further, the expression of 17 β HSD2 was reduced when treated with progesterone (48 h 10^{-7} M $P=0.003$, 10^{-9} M $P=0.0004$; 72 h 10^{-7} M $P=0.01$ and 10^{-9} M $P>0.05$), levonorgestrel (48 h 10^{-7} M $P=0.0001$, 10^{-9} M $P=0.005$; 72 h 10^{-7} M $P>0.05$ and 10^{-9} M $P=0.04$) and MPA (48 h 10^{-7} M $P=0.009$, 10^{-9} M $P=0.003$; 72 h 10^{-7} M $P=0.02$ and 10^{-9} M $P=0.01$) (Fig. 1b). 17 β HSD5 expression increased after 48 hours of progesterone (10^{-7} M $P=0.003$, 10^{-9} M $P>0.05$) and MPA treatment (48 h 10^{-7} M $P>0.05$, 10^{-9} M $P=0.001$). After 72 hours, expression levels of 17 β HSD5 decreased in cells treated with progesterone (10^{-7} M $P=0.01$ 10^{-9} M $P>0.05$), levonorgestrel (10^{-7} M $P=0.02$, 10^{-9} M $P=0.03$) and MPA treatment (48 h 10^{-7} M $P=0.01$, 10^{-9} M $P=0.03$) (Fig.1c).

17 β HSD1, 17 β HSD2, and 17 β HSD5 expression after progesterone, levonorgestrel and MPA treatment in MCF7 cells

In MCF7 cells 17 β HSD1 expression levels increased after progesterone (48 h 10⁻⁷ M P=0,001, 10⁻⁹ M P=0.0004; 72 h 10⁻⁷ M P>0.05 and 10⁻⁹ M P>0.05) (Fig. 2a) and MPA treatment (48 h 10⁻⁷ M P=0.01, 10⁻⁹ M P=0.004; 72 h 10⁻⁷ M P>0.05 and 10⁻⁹ M P>0.05), but not after treatment with levonorgestrel (P>0.05). Expression levels of 17 β HSD2 were not affected. 17 β HSD5 expression increased after progesterone (48 h 10⁻⁷ M P>0.05, 10⁻⁹ M P>0.05; 72 h 10⁻⁷ M P=0.02 and 10⁻⁹ M P>0.05) and MPA treatment (48 h 10⁻⁷ M P>0.05, 10⁻⁹ M P>0.05; 72 h 10⁻⁷ M P=0.02 and 10⁻⁹ M P=0.004) (Fig. 2b), but not after levonorgestrel exposure.

17 β HSD1, 17 β HSD2 and 17 β HSD5 expression after progesterone, levonorgestrel and MPA treatment in ZR751 cells

No changes in expression levels of neither 17 β HSD1 nor 17 β HSD2 at any time point was detected in ZR75-1 cells when treated with progesterone, levonorgestrel or MPA (P>0.05). However, 17 β HSD5 expression increased after progesterone (48 h 10⁻⁷ M P>0.05, 10⁻⁹ M P>0.05; 72 h 10⁻⁷ M P>0.05 and 10⁻⁹ M P=0.0001), levonorgestrel (48 h 10⁻⁷ M P=0.003, 10⁻⁹ M P=0.01; 72 h 10⁻⁷ M P=0.004 and 10⁻⁹ M P>0.05) and MPA treatment (48 h 10⁻⁷ M P>0.05, 10⁻⁹ M P>0.05; 72 h 10⁻⁷ M P=0.001 and 10⁻⁹ M P=0.04) (Fig. 3).

Mifepristone inhibits the induction of 17 β HSD1 and 17 β HSD5 in T47D

To investigate if the changed expression levels detected were mediated by the progesterone receptor, T47D cells were treated with mifprestone, vehicle, progesterone and mifprestone, levonorgestrel and mifprestone or MPA and mifprestone for 48 h. No changes in expression levels of 17 β HSD1, 17 β HSD2 or 17 β HSD5 were detected when the different treatments were compared (P>0.05).

Discussion

Women receiving HRT run an increased risk of developing breast cancer when progestins are combined with oestradiol [1-5]. 17 β HSD-enzymes have been found to play important roles in the development of breast cancer, possibly through their impact on the fine tuning of sex steroid levels. Progestins have been shown to induce conversion of oestrone to oestradiol and vice versa in breast cancer cell lines, and this alteration in sex steroid conversion has been assumed to be a regulatory effect of 17 β HSD-enzymes caused by progestins [14-19].

To study the effect of progesterone and progestins on the expression of 17 β HSD-enzymes, we used progesterone and the most commonly used progestin in Europe and the US which are levonorgestrel and MPA respectively. The physiological concentration detected in serum ranges from 0.1-10 nM in women using HRT [8,20,21]. We treated the cells with two different concentrations of progesterone/progestins reflecting the serum levels detected after HRT use. We could show that progesterone and progestins in deed does affect the levels of individual 17 β HSD-enzymes in breast cancer cell lines, but that the degree to which individual 17 β HSD-enzymes were affected and by which progesterone/progestin, varied to some extent. The expression level of PgR and ER varied in the cell lines, with the highest expression of PgR in T47D, intermediate in MCF7 and low in ZR75-1, this is in agreement with previous findings. [22]. The effect was most apparent in T47D which has a high PgR expression. In this cell line, treatment with levonorgestrel and progesterone led to the up-regulation of reductive 17 β HSD-enzymes 17 β HSD1 and 17 β HSD5 whereas the oxidative 17 β HSD2 was down-regulated. MPA did not affect 17 β HSD1-expression in this cell line, although 17 β HSD5 was upregulated. The absence of effects when co-treating T47D-cells with progestins and the PgR-inhibitor mifepristone indicates that the regulation of 17 β HSD-enzymes in this cell line is mediated via activation of PgR. This is in agreement with previous results showing that mifepristone blocks progestin induced steroid conversion in T47D cells [18]. Alterations were also seen in MCF7 and ZR75-1 with lower PgR expression. Adams *et*

a/ [14] showed progestin induced changes in both reductive (oestrone to oestradiol) and oxidative (oestradiol to oestrone) activity in MCF-7. An increase in oestrone to oestradiol conversion could, at least partly, be explained by increases in 17 β HSD1 and 17 β HSD5 gene expression seen after progesterone and MPA treatment of MCF7, although, no changes were seen in 17 β HSD2-expression which could explain the altered oestradiol to oestrone conversion. Couture *et al* [16] reported MPA induced changes in both oxidative and reductive activity in ZR75-1 cells, and furthermore, that the MPA-induced changes were inhibited by an anti-androgen, suggesting involvement of male sex hormones. In our hands, levonogestrel, but not MPA, increased the expression of 17 β HSD5, which converts androstenedione into more potent testosterone, in ZR75-1, whereas MPA failed to induce this change. Although PgR seems to be important for the effects mediated by progestins, at least in a cell line harbouring high expression levels of the receptor such as T47D, there is a possibility that other steroid receptors could be involved when PgR is low or absent, and this may in part explain why the different cell lines respond differently to progesterone and different progestins. In addition to PgR, progestins have been reported to bind to GR, AR and MR, and also ER, and the extent to which these receptors are activated by different progestins may be dose dependent [23-27]. The failure of MPA to induce 17 β HSD1-expression in T47D could be an indication that this progestin operates on other steroid receptors, e.g. the AR. There are studies indicating that MPA in fact inhibits the proliferation in MCF-7, ZR75-1 and T47D cells through GR and AR [28-30].

We have investigated enzymes which have so far been attributed most relevance in breast cancer; however other 17 β HSD-enzymes may also be of importance. A reason for inconsistencies between our data and previously published data on the effects of progestin stimulation on 17 β HSD-activity could be generated by the relative redundancy within the 17 β HSD-family, where it is unknown to what extent individual enzymes are actually involved in affecting the levels of bioactive sex steroids. Data concerning 17 β HSD14, which was

assessed but not presented, revealed no changes in any cell line analysed which indicates this enzyme to be of minor importance in the current setting.

Our data suggest that systemic treatment with progesterone and progestins could alter the expression levels of individual 17 β HSD-enzymes, in general favouring the expression of reductive enzymes over oxidative enzymes in local tissue, e.g. breast tissue. Unopposed, this change in enzyme expression would lead to an increase in bioactive female sex steroids, e.g. oestradiol, which through its mitogenic interaction with the ER could enhance proliferation of breast epithelial cells. Schonnen *et al* [31] showed that levonorgestrel stimulated proliferation in MCF-7 cells through ER. It had been suggested that metabolites to progestins may bind directly to ER. In our experiments levonorgestrel increased expression of 17 β HSD1, decreased 17 β HSD2 and increased 17 β HSD5 expression, showing that progestins can indirectly influence ER activity through the regulation of steroid converting enzymes which in turn would lead to altered activity of ER.

In summary, we show that both reductive (17 β HSD1 and 17 β HSD5) and oxidative (17 β HSD2) members of the 17 β HSD-family are under control of progesterone and progestins in breast cancer cell lines. This is most clear in T47D cells which have high PgR expression. 17 β HSD enzymes are important players in the regulation of sex steroids locally in breast tumours and tumoural expression levels of various 17 β HSD-enzymes have been found to be of prognostic and treatment predictive relevance. We propose a mechanism for increased breast cancer risk after HRT in which hormone replacement affects the expression of 17 β HSD-enzymes, favouring the expression of reductive enzymes, which in turn could increase levels of bioactive and mitogenic estrogens in local tissue, e.g. breast tissue. Given this background, inhibition of PgR may be beneficial for breast cancer patients.

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Figure legends

Figure 1. Progestin treatment of T47D cells

T47 D cells treated with progesterone (P), levonorgestrel (L) or medroxyprogesterone acetate (MPA) for 48 or 72 hours in relation to untreated control, concerning A) 17 β HSD1; B) 17 β HSD2 and C) 17 β HSD5. The results represent mean of a representative experiment $\pm$ SE, * < 0.05, ** < 0.01, *** < 0.001

Figure 2. Progestin treatment of MCF7 cells

MCF7 cells treated with progesterone (P), levonorgestrel (L) or medroxyprogesterone acetate (MPA) for 48 or 72 hours in relation to untreated control, concerning A) 17 β HSD1 and B) 17 β HSD5. The results represent mean of a representative experiment $\pm$ SE, * < 0.05, ** < 0.01, *** < 0.001

Figure 3. Progestin treatment of ZR75-1 cells

ZR75-1 cells treated with progesterone (P), levonorgestrel (L) or medroxyprogesterone acetate (MPA) for 48 or 72 hours in relation to untreated control, concerning A) 17 β HSD5. The results represent mean of a representative experiment $\pm$ SE, * < 0.05, ** < 0.01, *** < 0.001

Figure 1
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Figure 2
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Figure 3
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Table

Table 1. Endogenous expression levels of 17βHSD1, 17βHSD2 and 17βHSD5 in untreated breast cancer cells, presented as mean of a representative experiment ±SE.

	17βHSD1	17βHSD2	17βHSD5
T47D	7.42 ± 0.35	0.52 ± 0.06	0.10 ± 0.01
MCF7	0.05 ± 0.02	0.003 ± 0.002	0.095 ± 0.01
ZR75-1	0.81 ± 0.19	0.11 ± 0.07	0.11 ± 0.006