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Estradiol Decreases Xanthine Dehydrogenase Enzyme Activity and Protein Expression in *Non-Tumorigenic* and Malignant Human Mammary Epithelial Cells

Gennaro Taibi,[1*] Giuseppe Carruba,[2] Vitale Miceli,[2] Letizia Cocciadiferro,[2] and Concetta M.A. Nicotra[1]

[1]*Department of Experimental Oncology and Clinical Applications, University of Palermo, Palermo, Italy*
[2]*Experimental Oncology, Department of Oncology, ARNAS-Civico, Palermo, Italy*

ABSTRACT

The retinoic acid deficiency in breast tumour epithelial cells has been ascribed to an insufficient expression of either the enzyme(s) involved in its biosynthesis or the cellular retinol binding protein (CRBP) or both. In an attempt to define the mechanisms underpinning retinoic acid deficiency in these cell model systems, we have investigated the potential regulatory effect of oestrogen (17β-estradiol) on one key player in retinoic acid biosynthesis, the xanthine dehydrogenase (XDH). This enzyme is consistently expressed and very active in *non-malignant* human mammary epithelial cells (HMEC), as opposed to tumour MDA-MB231 and MCF7 cells. In these latter two cell lines, as opposed to HMEC cells, we observe a residual ability of XDH to produce retinoic acid from retinaldehyde and the inability to use retinol, as a consequence of a deficit in CRBP. In addition, estradiol treatment of MDA-MB231 and MCF7 cells decreases protein expression and activity of the enzyme, with no modification of the mRNA transcript levels, eventually leading to deteriorate further retinoic acid production. J. Cell. Biochem. 108: 688–692, 2009.

KEY WORDS: RETINOIC ACID; XANTHINE DEHYDROGENASE; XANTHINE OXIDASE; ESTRADIOL

Retinoids, alone or in combination with anti-estrogens, inhibit growth of human breast cancer cells [Sporn et al., 1976; Lotan, 1980; Marth et al., 1985; Fontana, 1988; Moon and Metha, 1990; Koga and Sutherland, 1991]. Since their effects are mainly mediated by retinoic acid (RA), it is important to establish the pattern of enzymes involved in RA biosynthesis and their regulatory mechanisms. The production of such an essential differentiation factor is accomplished by several dehydrogenase and oxidoreductase enzymes working in the cytoplasm, mitochondria, endoplasmic reticulum and peroxisomes [Ottonello et al., 1993; Napoli, 1996; Niederreither et al., 1999; Baker, 2001; Zhao et al., 2004; Lidén et al., 2005]. As a substrate, these enzymes can use retinol, retinol bound to cellular retinol binding protein (CRBP) and retinaldehyde [Ottonello et al., 1993; Boerman and Napoli, 1996; Niederreither et al., 1999; Zhao et al., 2004]. Among the enzymes involved in the cytoplasmic synthesis of RA, we have investigated the xanthine dehydrogenase (XDH), since it very actively oxidizes retinol-CRBP or retinaldehyde to retinoic acid in mammary epithelial cells [Taibi et al., 2008]. The significant role of this enzyme in cell differentiation prompted us to assess its implication in human breast cancer by using tumor mammary epithelial cells, wherein RA deficit has been extensively reported and ascribed to the lack of expression of CRBP, of aldehyde dehydrogenase, or both [Kuppumbatti et al., 2000; Mira-Y-Lopez et al., 2000; Rexer et al., 2001; Hayden and Satre, 2002]. Aiming to define the pattern and bio-availability of proteins required for RA production, we inspected the XDH, its cellular protein content, and the potential effect of estradiol on transcription and expression of the enzyme by comparing *non-malignant* HMEC cells and tumorigenic, hormone-dependent MCF7 and hormone-refractory MDA-MB231, breast epithelial cells, that contain the enzyme in the oxidase form (XO) [Taibi et al., 2009].

MATERIALS AND METHODS

CELL CULTURES

Normal human mammary epithelial cells (HMEC), obtained from Clonetics (Lonza, Walkersville, MD), were grown in MEBM serum-free medium supplemented with EGF, insulin, hydrocortisone, gentamycin and BPE (Clonetics single quot reagent), MCF7 cells and MDA-MB231 cells were grown in RPMI-1640 medium containing

*Correspondence to: Prof. Gennaro Taibi, Department of Experimental Oncology and Clinical Applications, University of Palermo, Palermo, Italy. E-mail: gtaibi@unipa.it
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5 μg/ml phenol red and supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 U/ml penicillin, 1 μg/ml streptomycin. Cells were harvested using trypsin–EDTA (Gibco-BRL, 1:5, v/v) and subcultured in 75 cm^2 cell culture flasks (BD Biosciences, CA) at 37°C in a humidified atmosphere of 5% CO_2 in air.

HORMONE TREATMENT OF CULTURED CELLS

Cells at 50% confluence were exposed to 10 pM–1 nM estradiol in RPMI medium supplemented with 5% charcoal treated-FCS. Control cells received only the vehicle (ethanol). After 48 h treatment, subconfluent cell monolayers were rinsed twice in PBS-A and harvested using a cell scraper. Cell pellets were either immediately processed as described below or stored at −80°C until analysis.

PREPARATION OF CELL EXTRACT

Pellets of HMEC, MDA-MB231 and MCF7 cells (about 20×10^6 cells), were suspended in 2 ml of 0.25 M sucrose–50 mM Trizma, pH 7.4, containing 2 mM EDTA, 5 mM glutathione, and protease inhibitors, and homogenized by cavitation at 600 psi nitrogen pressure for 10 min in a precooled cell disruption bomb (Parr Instrument Company). Cell homogenates were then centrifuged at 110,000*g* in a Beckman 70 Ti-rotor (L8-M Beckman ultracentrifuge) for 60 min and the resulting supernatants (cytosols) collected and saved at −70°C. Protein content of each sample was measured by Sigma FluoroProfile Protein quantification Kit.

ENZYME ASSAY

Cytosol preparations were assayed for XDH activity using 250 nM all-*trans*-retinaldehyde as described elsewhere [Taibi et al., 2008]. To evaluate the formation of RA, extraction of retinoids and HPLC chromatography analysis were performed on one third aliquots of samples, as previously reported [Taibi and Nicotra, 2007]. Enzyme activity was expressed as nanomoles of RA formed min^{-1} mg^{-1} protein.

WESTERN BLOT ANALYSIS

For the analysis of XDH/XO protein expression, equal amount of cytosol samples of treated (1 nM β-estradiol) and untreated cells (100 μl) were boiled in 1× sample treatment buffer (125 mM Tris–Cl pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol) and run electrophoretically on a 8% SDS–polyacrylamide gel. Cytosol proteins after electro migration were directly blotted to nitrocellulose and blocked for 1 h with non fat dry milk in TBS containing 0.05% Tween 20 and subsequently incubated for 3 h with Abs specific to XDH/XO (Ab, mouse IgM; United States Biological, DBA Italia). After washing 3 times in PBS-T, the blot was incubated for 1 h with horseradish peroxidase-conjugated anti mouse Ab (Amersham) and visualized using an enhanced chemiluminescence detection system (SuperSignal West Dura Extended duration Substrate; Pierce Chemical Co., Rockford, IL). Blots were stripped and reblotted with anti-actin antibody to determine equal loading of samples. Stripping was accomplished using the Re-Blot Western Blot Recycling kit (Chemicon International, Temecula, CA). The blots were submerged into stripping solution and incubated at room temperature with continuous rocking for 10–15 min. The blots were then washed twice in blocking solution and reprobed with anti-actin antibody. Bands analysis and quantification were performed using the software Image J 1.42k (National Institutes of Health, USA).

RNA EXTRACTION AND SEMI-QUANTITATIVE RT-PCR

Total RNA was isolated using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. The extracted amounts of RNA were determined by measuring the absorbance at 260 nm, and RNA integrity was assessed by *non-denaturing* agarose gel electrophoresis. RNA was treated with RNase-free DNase (Promega, Madison, WI) to remove potential contamination of genomic DNA. Total RNA (1 μg) was reverse transcribed into cDNA in 20 μl of reverse transcription reaction mixture (Invitrogen) containing 0.1 μg random hexamer primer, 5× buffer, 0.1 M DTT, 10 mM of each dNTP, 40 U RNase inhibitor and 200 U of SuperScript™ II Reverse Transcriptase. PCRs (cycles of 94°C for 45 s, annealing at 58–60°C for 45 s and extension at 72°C for 45 s) were performed for a number of cycles corresponding to high end of the range in which a linear increase in products could be detected. The primers sequences for PCR were: XDH, forward ACC CCT TCC ACT ACT ACT TCA GCT AT and reverse TTA GAC TGG AGC CAA CAT CCA TG; β-actin, forward CTG GCA CCA CAC CTT CTA C and reverse GGG CAC AGT GTG GGT GAC, used as control. The PCR products were electrophoresed on a 2% agarose gel and the bands were analyzed and quantified using the software Image J 1.42k (National Institutes of Health, USA).

STATISTICAL ANALYSIS

Statistical significance of the data was evaluated using Student's *t*-test [Snedecor and Cochran, 1967] and probability values below 0.05 ($P < 0.05$) were considered significant. Results are expressed as mean ± SD from the indicated set of experiments.

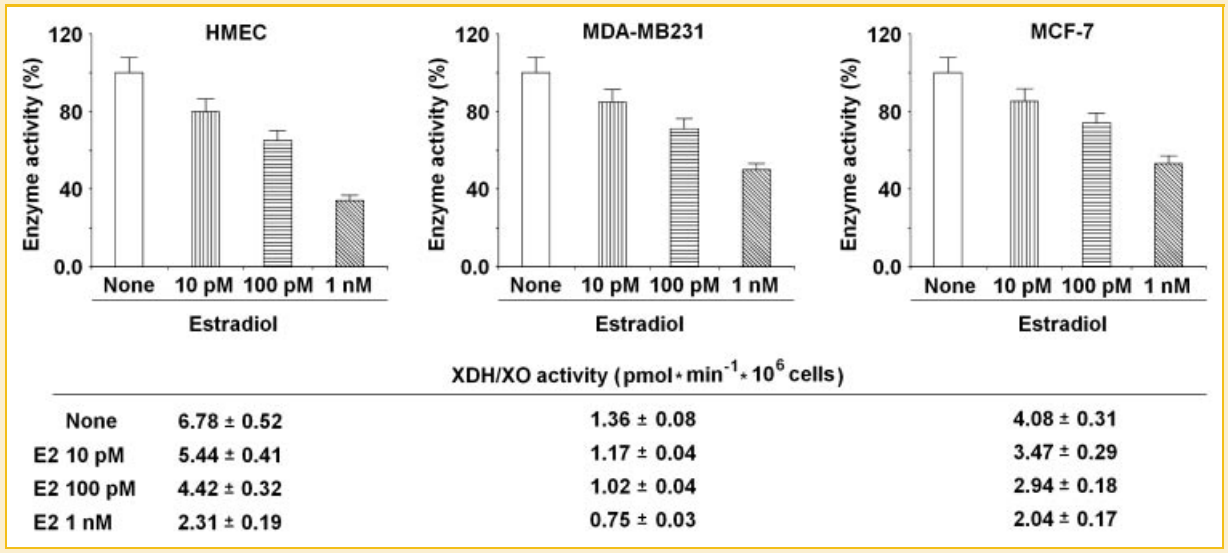
RESULTS

ESTRADIOL DECREASES PRODUCTION OF RA BY XDH/XO IN HUMAN BREAST EPITHELIAL CELLS

The RA biosynthesis was assayed in non-malignant HMEC, and tumorigenic MDA-MB231 and MCF7 human breast epithelial cells using retinaldehyde instead of retinol as a XDH substrate.

As previously reported [Taibi et al., 2009], both MDA-MB231 and MCF7 cells showed a reduced conversion of retinaldehyde to retinoic acid, as compared to HMEC cells.

To test whether oestrogen influences this process, XDH/XO activity levels were measured by assaying cytosol fractions of the three cell lines after treatment with 10 pM–1 nM estradiol for 48 h. A significant, dose-dependent reduction of XDH/XO enzyme activity was observed in HMEC cells, with a decrease of 28% and 70% being found using 10 pM and 1 nM estradiol, respectively (see Fig. 1). The low XDH/XO activity, commonly detected in MDA-MB231 and MCF7 cells in standard conditions, was further reduced by exposure to estradiol, though this decrease was smaller than that observed in non-malignant HMEC cells.



XDH/XO activity (pmol · min^{-1} · 10^6 cells)

None	6.78 ± 0.52	1.36 ± 0.08	4.08 ± 0.31
E2 10 pM	5.44 ± 0.41	1.17 ± 0.04	3.47 ± 0.29
E2 100 pM	4.42 ± 0.32	1.02 ± 0.04	2.94 ± 0.18
E2 1 nM	2.31 ± 0.19	0.75 ± 0.03	2.04 ± 0.17

Fig. 1. Estradiol (E2) causes about 20–50% inhibition of retinoic acid biosynthesis catalyzed by XDH/XO in human non-malignant and cancer breast epithelial cells. The source of XDH/XO was cytosol fraction of HMEC, MDA-MB231, and MCF7 cells, treated with 10 pM–1 nM β-estradiol for 48 h. Enzyme assays were performed with 250 nM retinaldehyde, as described in Materials and Methods Section. Data represent means ± SD of three independent experiments conducted in triplicate.

THE ESTRADIOL-INDUCED DECREASE OF XDH PROTEIN EXPRESSION CONCURS TO WORSEN THE RETINOIC ACID DEFICIENCY IN MAMMARY TUMOUR EPITHELIAL CELLS

In control untreated cell cultures, the XDH expression was consistently lower in MDA-MB231 cells (14%) and MCF7 cells (53%), as compared to HMEC cells (Fig. 2). Treatment of cells with 1 nM estradiol for 48 h resulted in a significant decrease of XDH protein level, that was 31% in both HMEC and MDA-MB231 cells and 15% in MCF7 cells, when compared with untreated cell cultures.

ESTRADIOL DOES NOT ALTER THE XDH mRNA LEVELS IN NON-MALIGNANT AND TUMORIGENIC HUMAN BREAST EPITHELIAL CELLS

The XDH mRNA levels were measured using a semi-quantitative PCR method, as described in Materials and Methods Section. As shown in Figure 3, levels of XDH transcript were markedly lower in MCF7 cells and, to a lesser extent, in MDA-MB231 cells than in HMEC cells. Treatment of HMEC, MDA-MB231, and MCF7 cells with 1 nM estradiol in oestrogen-free experimental culture medium, did not result in any measurable effect on XDH mRNA levels in any cell line (Fig. 3).

DISCUSSION

Several studies exploring the deficiency of retinoic acid in tumor breast epithelial cells have repeatedly emphasized a down-regulation of the dehydrogenases involved in the oxidation of retinol and retinaldehyde, and the lack of the retinol binding protein (CRBP) as potential underlying cause [Kuppumbatti et al., 2000; Mira-Y-Lopez et al., 2000; Rexer et al., 2001; Hayden and Satre, 2002; Taibi et al., 2009; Lu et al., 2005]. We have previously observed that the XDH enzyme, in cooperation with CRBP, is

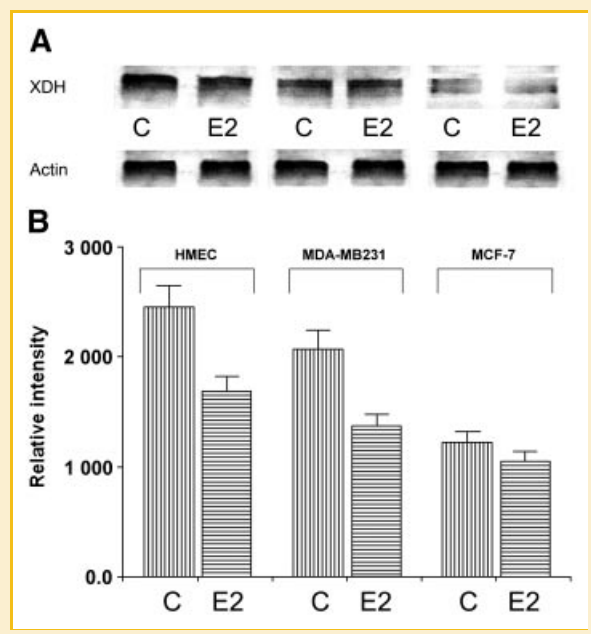


Fig. 2. Estradiol decreases XDH protein expression in both human *non-tumoral* and malignant breast epithelial cells. XDH protein levels were measured in MDA-MB231 and MCF7 breast cancer cell lines and in HMEC immortalized human mammary epithelial cells. A: Western blot analysis of XDH protein before (C) and after treatment of cultured cells with 1 nM estradiol (E2) for 48 h. The same membrane was stripped and re-blotted with anti-actin antibody. The actin expression was determined as a control for equal loading of protein samples. B: Densitometric analysis of the protein patterns shown in (A).

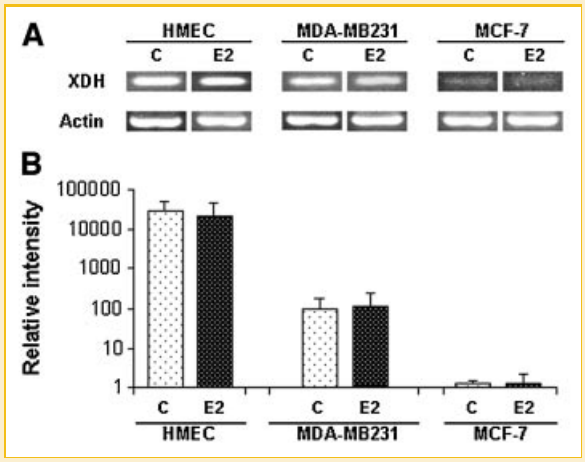


Fig. 3. XDH mRNA levels in human breast cancer cells are not modified by estradiol. The amounts of XDH mRNA were normalized by comparison with β-actin expression and measured in MDA-MB231 and MCF7 human breast cancer cell lines and HMEC human non-tumoral breast epithelial cells. A: Expression of XDH mRNA transcript before and after treatment of cultured cells with 1 nM estradiol for 24 h. B: Graphical representation of all densitometric data, with results presented as fold stimulation of mRNA expression over baseline (control). Data are the means ± SD of four independent experiments. C, control; E2, 17-β estradiol. Bars, SD.

required for retinoic acid production in *non-malignant* breast epithelial cells (HMEC) [Taibi et al., 2008] and that the cytosolic enzyme of MDA-MB231 and MCF7 breast tumour cells has almost no activity when assayed with retinol in the 0.25–2 μM concentration range, although it can oxidize retinaldehyde to retinoic acid [Taibi et al., 2009]. This combined evidence has led us to reconsider retinoic acid biosynthesis in these cell model systems in the light of the influence of a likely effector, such as the estradiol, on the XDH expression.

Estradiol, in a sub-physiological range of concentrations (10 pM–1 nM), induced a significant inhibition of the XDH/XO-driven conversion of retinaldehyde to retinoic acid in HMEC, MDA-MB231, and MCF7 human breast epithelial cells. This effect was associated with a corresponding decrease of the XDH protein expression in HMEC and MDA-MB231 cells, and, to a lesser extent, in MCF7 cells. Low levels of both protein expression and enzyme activity of XDH/XO (XOR, Xanthine oxidoreductase) have been reported in 50% of human breast cancer and were undetectable in another 7%. This decrease has been associated with an adverse prognosis of breast cancer patients [Linder et al., 2005].

As far as the XDH mRNA expression is concerned, we found that the XDH transcript levels are significantly higher in *non-malignant* HMEC cells, as opposed to tumorigenic MDA-MB231 and MCF7 cells. No effect of estradiol on XDH mRNA transcript levels could be detected both in *non-tumoral* and malignant breast epithelial cells, suggesting that this hormone has a post-transcriptional effect on XDH expression and activity. Similarly, in rat pulmonary micro vascular endothelial cells, the inhibitory effect exerted by estradiol stereo isomers on XDH/XO activity has been ascribed to a post-transcriptional, receptor-independent mechanism [Budhiraja et al., 2003].

We hypothesize here that the reduced concentration of the enzyme protein induced by oestrogen could be a consequence of the conversion of XDH to its oxidase form, leading to alterations in the kinetic properties of the enzyme and to its subsequent translation on the outer-face of the cellular membrane. It has been reported that the basis of the conversion of XDH to XO is the oxidation of cysteine thiols to form disulfide bonds [Stirpe and Della Corte, 1969; Waud and Rajagopalan, 1976; McManaman and Bain, 2002], associated with conformational change at the flavin-binding site [Massey et al., 1989; Saito et al., 1989], resulting in the loss of NAD^+ binding affinity [Hille and Nishino, 1995] and in alterations of the redox and kinetic properties of the enzyme [Saito and Nishino, 1989; Hunt and Massey, 1992]. These structural modifications of XDH become irreversible when proteolytic cleavage of the protein occurs [Kooij et al., 1994], making it unable to oxidize *t*-ROL while maintaining the capacity of oxidizing *t*-RAL.

In addition, the concurrent induction of eNOS, through nongenomic effects of E2 bound to ERα, could explain the inactivation of XDH/XO. In fact, it is well known that XOR can reduce $NO^{\bullet}$ to the nitroxyl anion (NO^- or HNO) and that this in turn inactivates XOR by reacting with the cysteine residues of the enzyme [Saleem and Ohshima, 2004].

In this framework, the potential effect of oestrogen on CRBP expression should also be considered, as the CRBP expression represents an essential prerequisite in XDH enzyme assays performed at *quasi*-physiological retinol concentrations [Taibi et al., 2008]. Mira-Y-Lopez et al. [2000] have reported that decreased CRBPI levels are apparently associated with the inability to oxidize retinol to RA in breast cancer cells, although the restored expression of CRBP did not result in the restoration of RA synthesis in these systems [Kuppumbatti et al., 2001]. This last evidence can be explained by the presence, in breast cancer cells [Taibi et al., 2009], of the oxidase instead the dehydrogenase form of the enzyme that is unable to oxidize directly *t*-ROL to *t*-RA also in the presence of CRBP [Taibi and Nicotra, 2007]. The functional relationship between RA and CRBPI should also be taken into consideration, as the CRBPI gene transcription in human mammary epithelial cells is triggered by RARβ2 through a RA → RARα signaling [Bistulfi et al., 2006].

The relationship between retinoids and oestrogen has also been considered very important for mammary development and cell differentiation. An antagonistic effect of retinoid X receptor β (RXRβ) on oestrogen receptor function and the inhibition of ERE promoter activity have been previously observed in MCF7 cells [Segars et al., 1993]. However, a mutual, inverse relationship between oestrogen and retinoids has not been considered to date.

The present evidence that estradiol exerts an inhibitory effect on both protein expression and enzymatic activity of XDH, eventually leading to a significant decrease of RA production, without affecting XDH gene transcription, appears to be intriguing. One could speculate that estradiol affects the intracellular kinetics of XDH and its translocation outside the mammary epithelial cell. If this is the case, the effects of estradiol might be mediated by a plasma membrane oestrogen receptor, presumably ERα [Levin, 2002]. In this context, the estradiol-induced secretion of the 160 and 52 kDa proteins in MCF7 cells deserves attention [Fontana et al., 1990].

Further studies should explore and define the conditions that could restore a sufficient extent of bio available xanthine dehydrogenase and the supporting CRBP in human mammary epithelial cancer cells.

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