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Involvement of caspase-9 but not caspase-8 in the anti-apoptotic effects of estradiol and 4-OH-Estradiol in MCF-7 human breast cancer cells

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Objectives. Evidence is accumulating that certain estradiol metabolites may play a more important role in enhancing breast cancer risk than their parent substance - 17 β-estradiol (E2). Of special interest are the metabolites 2-hydroxyestradiol (2-OH-E2), which can show anticarcinogenic effect, while that of 4-hydroxyestradiol (4-OH-E2) may be rather procarcinogenic. We suggest that local activation of cytochrome P450 enzymes - CYP1A1 and/or CYP1B1 - by E2 could generate active metabolites that affect the apoptosis and thereby promote mammary carcinogenesis. Over the last several years, there has been accumulating evidence that, apart from the receptor-mediated (extrinsic) pathway, also the mitochondrial (intrinsic) pathway plays a role in E2-induced apoptosis. In the present study, we have compared the effect of these metabolites and their parent substance E2 on caspase-8 and caspase-9 activity as well as on the end step of apoptosis DNA fragmentation.

Methods. MCF-7 human breast cancer cells (ATCC) were routinely cultured in DMEM supplemented with 10 % heat-inactivated FBS. Forty-eight hours before experiments, the medium was removed and replaced by DMEM without phenol red supplemented with 5 % heat-inactivated fetal bovine serum. For determination of caspase-8 and caspase-9 activities, MCF-7 cells were seeded in 48-well culture plates at a density of 15 x 10^4 cells/well and incubated with 1 nM E2 and its metabolites for 24 h. DNA fragmentation, caspase-8 and caspase-9 activities were determined in cell lysates by ELISAs. The CYP1A1 and CYP1B1 protein expression was evaluated by Western blotting.

Results. E2 had no effect on CYP1A1 protein levels. However an increase in CYP1B1 protein expression was observed within 48 hrs of exposure. None of the compounds tested changed caspase-8 activity as compared to the controls. Statistically significant decrease in caspase-9 activity and DNA fragmentation was observed in the presence of E2 and 4-OH-E2, but no significant effect was found for the metabolite 2-OH-E2.

Conclusions. It was found that local activation of cytochrome P450 enzyme CYP1B1 by E2 may change the local metabolic activation pathway into 4-OH-E2 as well as the activation of caspase-9 (a part of the intrinsic mitochondrial apoptotic pathway) in the antiapoptotic effect of E2 and 4-OH-E2.

Keywords: estradiol, 2-OH-estradiol, 4-OH-estradiol, MCF-7 cell line, CYPs activity, apoptosis, mammary cancer

Estrogens are believed to be involved in breast cancer development possibly via several different mechanisms and reactive metabolites. An early suggestion that some of estrogen's effects in target tissues may be due to the metabolism came from studies by Fishman and Norton (1975). Recently, the concept of "intracrinology" has become important for estrogen activity in local tissues. Thus, inactive steroids in plasma are locally converted

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to bioactive estrogens in the reproductive tract as an intracrine activity (Sasano et al. 2000), and the shift in the metabolic pathways of A- or D-ring hydroxylation in favor of the D-ring has been regarded, by some authors, as a biological marker of cancer risk (Feigelson and Henderson 1996).

The first objective of our research was to demonstrate the local activation of the cytochrome P450 enzymes CYP1A1 and CYP1B1 by estradiol (E2) which may generate active metabolites further affecting the apoptosis and thereby promote mammary carcinogenesis. The cytochrome P450-dependent monooxygenases (CYP) are responsible for the biosynthesis and metabolism of endogenous compounds such as steroid hormones. In fact, CYP1A1 primarily acts as an E2 2-hydroxylase, whereas CYP1B1 is primarily an E2 4-hydroxylase, with a lesser activity at C-25 (Liehr et al. 1995; Hayes et al. 1996).

Apoptosis is recognized as a major barrier that must be circumvented by tumor cells to allow them to survive and proliferate under stressful conditions (Hanahan et al. 2000). Moreover, resistance to apoptosis plays an important role in tumorigenesis (Johnstone et al. 2002; de Bruin et al. 2008). Actually, two most common mechanisms of apoptosis inside the cell are operating either via an extrinsic pathway involving the activation of plasma membrane death receptors (Fas receptor/caspase-8 pathway) or via an intrinsic pathway which depends on mitochondrial release of cytochrome c (cytochrome c/caspase-9 pathway). Actually, there are conflicting reports concerning the effect of E2 on the Fas receptor/caspase-8 pathway. Thus, Mor et al. (2000) demonstrated a time dependent up-regulation of FasL by estradiol, while Seeger et al. (2006) showed that E2 and 4-OH-E2 did not change the concentration of FasL.

To gain further insight into the role of hydroxylated E2 metabolites in this process, we analyzed the effect of E2 hydroxylated metabolites on the activity of caspase-8 and caspase-9 as well as the end step of apoptosis DNA fragmentation.

Materials and Methods

Reagents. 17-β-estradiol (E2), 2-hydroxy-17-β-estradiol (2-OH-E2) and 4-hydroxy-17-β-estradiol (4-OH-E2) were obtained from Steraloids, Inc.(Newport, RI) and dissolved in absolute ethanol. The final concentration of ethanol in the medium in each case was 0.1 %. Ethanol at this concentration did not show any effect on cell viability (data not shown). DMEM medium without phenol red, Fetal Bovine Serum (FBS, heat inactivated), penicillin, streptomycin, Trypan blue and charcoal-dextran were obtained from Sigma Chemical Co. (MO, USA).

Cell culture. MCF-7 human breast cancer cells (ATCC) were routinely cultured in DMEM supplemented with 10 % heat-inactivated FBS (Sigma Chemical Co., MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown in 75 cm^2 tissue culture dishes (Nunc, Denmark) in a 37 °C incubator with a humidified mixture of 5 % CO_2:95 % air. Forty-eight hours before experiments, the medium was removed and replaced by DMEM without phenol red supplemented with 5 % heat-inactivated FBS. For determination of caspase-8 and caspase-9 activities, MCF-7 cells were seeded in 48-well culture plates at a density of 15 x 10^4 cells per well and incubated with 1 nM E2 and its metabolites for 24 h. The media was removed and the cells were lysed in the kit buffer. The protein concentration of lysates was determined by Bradford assay (Bio Rad Protein, USA). Equal amounts of cytosolic extract (50 μg protein) from each sample were used for analysis.

CYP1A1 and CYP1B1 protein levels. For the determination of CYP1A1 and CYP1B1 protein levels, cells were seeded in 24-well culture plates at a density of 50 x 10^4 cells per well and incubated with 1 nM E2 for 6, 24 and 48 h. The cells were then transferred to ice-cold lysis buffer and stored at –20 °C for Western blot analysis. The protein concentration of the lysates was determined by the Bradford assay (Bio Rad Protein, USA). Equal amounts of protein (20 μg) from each treatment group were separated by 10 % SDS-PAGE and transferred to PVDF membranes using a Bio-Rad Mini-Protean 3 apparatus (Bio-Rad Laboratories, Inc., USA). The blots were blocked overnight with 5 % dry milk and 0.1 % Tween 20 in 0.02 M TBS buffer. Blots were incubated overnight (4 °C) with antibodies specific for CYP1A1 (sc-9828) and CYP1B1 (sc-31667) (Santa Cruz Biotechnology Inc., CA, USA) and β-actin (A5316) (Sigma Chemical Co., MO, USA). After incubation with the primary antibody, the membranes were washed three times and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies: P0447 (DakoCytomation, Denmark) for β-actin and sc-2020 (Santa Cruz Biotechnology Inc., CA, USA) for CYP1A1 and CYP1B1. Immunopositive bands were visualized using the Amplified Opti-4CN Kit (Bio-Rad Laboratories, Inc., USA) and were quantified using a densitometer (EasyDens, Cortex Nowa, Poland).

Caspase-8 activity. The Caspase-8/FOLICE Colorimetric Protease Assay Kit (BioSource International, Inc. USA) is based on the hydrolysis of the peptide substrate Ile-Glu-Thr-Asp-p-nitroanilide (IETD-pNA) by caspase-8, which results in the release of a p-Nitroaniline (p-NA) moiety. p-NA was measured colorimetrically at 405 nm.

Caspase-9 activity. The Caspase-9/FOLICE Colorimetric Protease Assay Kit (BioSource International, Inc. USA) is based on hydrolysis of Leu-Glu-His-Asp-p-nitroanilide (LEHD-pNA) by caspase-9, which results in the release of a p-nitroaniline (p-NA) moiety. p-NA was measured colorimetrically at 405 nm, similar to the caspase-8 assay. The assays were performed as per manufacturer's instructions using appropriate controls.

DNA fragmentation was determined using the Cellular DNA Fragmentation ELISA kit (Roche Molecular Biochemicals) which is based on the quantitative detection of bromodeoxyuridine (BrdU)-labeled DNA fragments. After exposure to BrdU for 18 h, cells were reseeded onto a 96-well microplate (10^5 cells/well) and treated for 24 h with the test compound. The cells were then incubated for an additional 3 h with Staurosporine (0.1μM). The supernatant was then removed and the remaining cells were lysed in the kit buffer. The cytoplasmic fraction was transferred separately into an anti-DNA pre-coated microtiter plate and analyzed using the ELISA procedure, according to the kit instructions. DNA fragmentation was measured spectrophotometrically at 450 nm. The results presented are the average value of three independent experiments.

Statistical evaluation. Each treatment was repeated three times (n=3) in quadruplicates. The average of the quadruplet values was used for statistical calculations. Statistical analysis was performed using Statistica 6.0. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey honestly significant difference (HSD) multiple range test. Groups that were significantly different from controls are indicated in the figures with *($p<0.05$), **($p<0.01$) or ***($p<0.001$).

Results

Efect of estradiol on CYP1A1 and CYP1B1 protein expression. CYP1A1 and CYP1B1 protein levels were measured by immunoblot analysis in control cells and cells exposed to 1 nM estradiol for 3, 6, 24, or 48 hours. β-actin was used as control for equal loading of the lanes. The endogenous level of CYP1A1 in untreated cells was low. Estradiol had no effect on CYP1A1 protein expression (Fig.1), while CYP 1B1 protein expression was high in cells treated with E2 after 48 h of culture (Fig. 2).

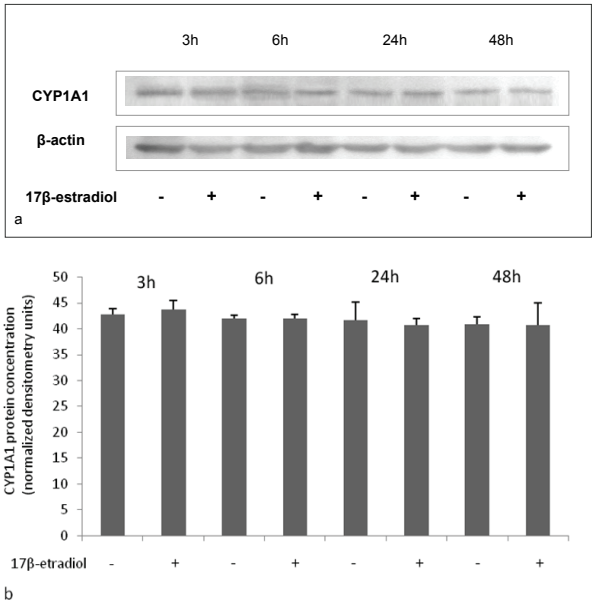

Fig. 1. Effect of 17β-estradiol (1nM) on protein expression of the CYP 1A1: a. representative Western blot analysis of CYP 1A1 protein levels; b. densitometry analysis of Western blot, in MCF-7 cells without treatment and in 17β-estradiol-treated cells after 3, 6, 24 and 48 h of incubation.

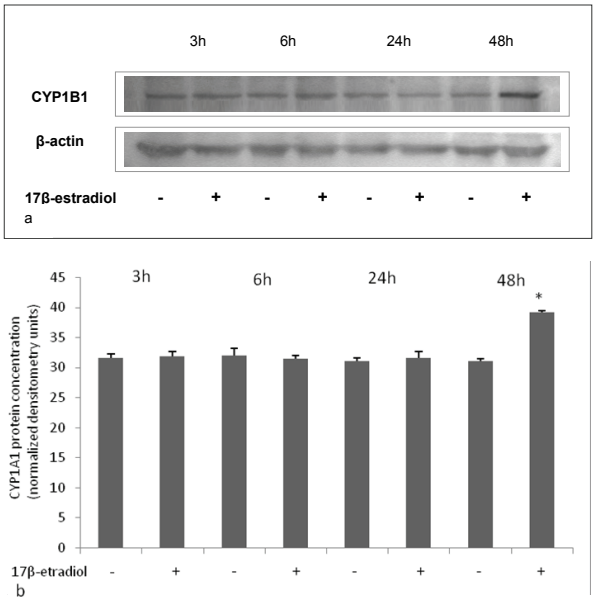

Fig. 2. Effect of 17β-estradiol (1nM) on expression of the CYP 1B1 protein: a. representative Western blot analysis of CYP 1B1 protein levels; b. densitometry analysis of Western blot, in MCF-7 cells without treatment and in 17β-estradiol-treated cells after 3, 6, 24 and 48 h of incubation. All means marked with *($p<0.05$), are significantly different from the control.

Effect of estradiol, 2-OH-E2 and 4-OH-E2 on MCF-7 cells apoptosis. A. DNA fragmentation. Estradiol and 4-OH-E2 decreased DNA fragmentation within the range of 38 and 24.5 % respectively ($p<0.01$ and $p<0.001$, resp.; Fig. 1), while no effect was found for 2-OH-E2 (Fig. 3).

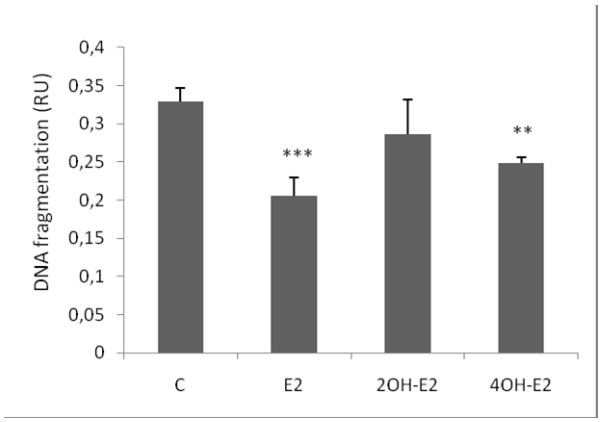

Fig. 3. Effect of 17β-estradiol, 2-OH-E2 and 4-OH-E2 on apoptosis in MCF-7 cells growing in the medium supplemented with 5 % FBS. Apoptosis was measured by DNA fragmentation EIA Staurosporine (St; 0.1μM) was added during the last 3 h to induced apoptosis. All means marked by **($p<0.01$), *($p<0.001$), are significantly different from the control.**

B. Caspase-8 activity. Fig. 4 shows that none of the compounds tested changed the activity of caspase-8 as compared to controls (Fig. 4);(Table 1).

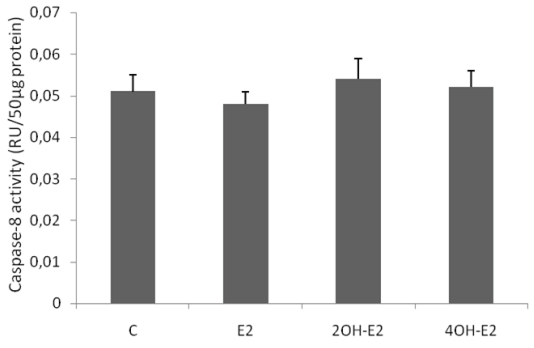

Fig. 4. Effect of 17β-estradiol, 2-OH-E2 and 4-OH-E2 on apoptosis in MCF-7 cells growing in the medium supplemented with 5 % FBS. Apoptosis was measured by caspase-8 activity Staurosporine (St; 0,1μM) was added during the last 3 h to induced apoptosis.

C. Caspase-9 activity. The intracellular concentration of caspase-9 has been found significantly decreased by estradiol as well as 4-OH-E2, which was in the range of 28.8 % and 24.3 %, respectively ($p< 0.001$; Fig. 5). No effect was found for 2-OH-E2 (Table 1).

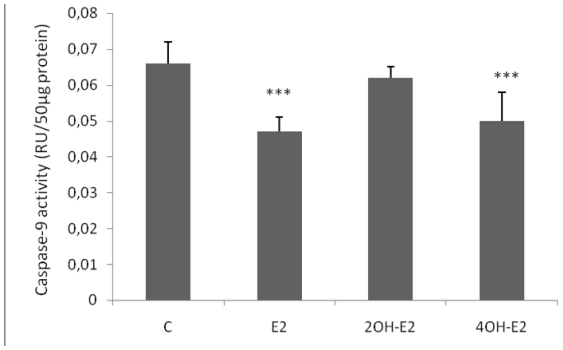

Fig. 5. Effect of 17β-estradiol, 2-OH-E2 and 4-OH-E2 on apoptosis in MCF-7 cells growing in the medium supplemented with 5 % FBS. Apoptosis was measured by caspase-9 activity Staurosporine (St; 0,1μM) was added during the last 3h to induced apoptosis. All means marked by *($p<0.001$), are significantly different from the control.**

Table 1

Percent changes of apoptosis markers in MCF-7 cells after addition of estradiol (E2), 2-hydroxyestradiol (2-OH-E2) and 4-hydroxyestradiol (4-OH-E2) as compared to control values considered as 100%

	E2	2-0H-E2	4-OH-E2
DNA fragmentation	62.0 ***	96.95	75.5**
Caspase-8 activity	94.1	105.8	101.9
Caspase-9 activity	71.2**	93.9	75.7**

- $p<0.01$; * - $p<0.001$

Discussion

Concerning the physiological concentration ranges of various estradiol metabolites, analytical studies in early 1970s provided the evidence that a majority of such metabolites circulate in the serum in a comparable range as 17β-estradiol, i.e. between 0.1 and 1 nM (Merriam et al. 1983). Moreover, it was also found that local activation of cytochrome P450 enzymes CYP1A1 and CYP1B1 by E2 may generate active metabolites that affect the apoptosis and thereby promote mammary carcinogenesis. Actually, the major metabolite of 17β-estradiol, 2-hydroxyestradiol (2-OH-E2), was found mainly produced by CYP1A1 in extrahepatic tissues (Aoyama 1990).

The results we presented here show that 17β-estradiol had no effect on CYP1A1 protein levels. On the other hand, an increase in CYP1B1 protein expression was observed after 48 h of cell culture. Thus, CYP1B1 has been suggested to play key roles in initiating breast cancer in humans, since this enzyme is active in catalyzing 17β-estradiol to a 4-hydroxylated metabolite (4-OH-E2) (Spink 1998). As high as three-fold induction of CYP1B1 mRNA after 17β-estradiol treatment has been noted in ER-positive MCF-7 cells (Tsuchiya et al. 2004), thus highlighting the effect of 17β-estradiol on gene levels. Moreover, our data also suggest the effect on protein levels.

17β-estradiol has been also reported to prevent apoptosis in breast cancer cells (Perillo et al. 2000) and this anti-apoptotic effect has been linked to a rapid post-translational (non-genomic) effect of estrogen as elicited at the plasma membrane level (Farach-Carson 1998; Razandi et al. 2000).

Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway. The extrinsic pathway is initiated through the stimulation of transmembrane death receptors (such as the Fas receptors), located in the cell membrane. In contrast, the intrinsic pathway is initiated through the release of signaling factors by mitochondria within the cell.

We showed here that both 17β-estradiol and its hydroxylated metabolites had no effect on caspase-8 activity, whereas a decrease in caspase-9 activity and DNA fragmentation was observed under the influence of 17β-estradiol and 4-OH-E2 suggesting apoptotic action via the mitochondrial or intrinsic pathway. Our suggestions are in accordance with Johnstone et al. (2002) who noted that, due to the sensitivity of intrinsic pathway, tumors arise more often through the intrinsic than the extrinsic pathway. Moreover, we also confirmed the suggestion by Seeger et al. (2006) who showed that 17β-estradiol and 4-OH-E2 decreased the concentration of cytochrome C (marker of intrinsic pathway) but did not change the concentration of FasL. Moreover, as noted in our data, no effect was found for 2-OH-E2. Indeed, the data of Seeger et al. (2006) and our results presented here are contrasting to what has been found by Mor et al. (2000) who demonstrated a time-dependent up-regulation of FasL by 17β-estradiol. They showed maximal FasL mRNA levels after 3 h of estrogen stimulation, followed by a 40 % decrease from 6 to 12 h, with a definite but smaller increase at 24 h. Seegal et al. (2006), suggested that one reason for this discrepancy was due to the incubation time of 96 h in his experiments which, according to Mor et al. (2000) may be too long to demonstrate an effect of estradiol on Fas. In our experiment, although the incubation time has been as long as 24 h, we did not observe any activation of caspase-8. It is likely that the lack of effect on caspase-8 activity, as observed in our experiment after 24 h incubation with 17β-estradiol, has been due to the consequence of 40 % reduction of FasL mRNA levels at 6 and 12 h incubation time as observed by Mor et al (2000).

In summary, it was found that 17β-estradiol increases CY1B1, but not CYP1A1 protein levels in MCF-7 cells, an alteration that may change local metabolic activation pathway into 4OH-E2. In addition, the data presented here showed the lack of caspase-8 activation (part of the extrinsic receptor pathway) by 17β-estradiol and both its metabolites as well as the involvement of caspase-9 (part of the intrinsic mitochondrial apoptotic pathway) in the antiapoptotic effect of 17β-estradiol and 4-OH-E2 thus confirming the suggestion of Seegal et al. (2006).

Acknowledgement

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