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The Ah receptor inhibits estrogen-induced estrogen receptor β in breast cancer cells

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Abstract

We have studied the effect of the aryl hydrocarbon receptor ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on estrogen receptor (ER) β gene expression in the human breast cancer cell line, T47D. TCDD inhibited 17β-estradiol (E_2)-induced up-regulation of both ER β wild type and ER β cx mRNA. Cycloheximide pre-treatment had no inhibitory effect, and the estimated half-life of ER β mRNA of about 33 min was not changed by any hormone administration. Chromatin immunoprecipitation experiments showed recruitment of ER α to the ER β promoter. Gel mobility shift experiments revealed an E_2-induced protein binding to a half site estrogen response element in the ER β promoter, and TCDD reduced that binding. These results show that ER α regulates the expression of its own heterodimerization partner, ER β, in T47D cells. TCDD, an anti-estrogenic compound, inhibits ER α-mediated induction of ER β mRNA. These findings add to our understanding of cross talk between dioxin and estrogen signaling in human cells.

Keywords: Estrogen receptor; Gene expression; Aryl hydrocarbon receptor; Human; Breast cancer

The estrogen receptor (ER) ligand 17β-estradiol (E_2) plays an important role in a variety of physiological processes, including growth and development of the mammary gland, but can also act as a promoter of breast cancer [1]. Expression of the estrogen receptor together with expression of the progesterone receptor is used as a prognostic marker in breast cancer and is important in the decision of adjuvant hormone therapy [2]. Since the discovery of the second estrogen receptor, ER β, in 1996 [3] many studies have been carried out monitoring ER β expression in breast cancer patients. It is still not clear, however, whether the expression of ER β indicates good or bad prognosis in breast cancer [4]. In cancers of prostate and colon, for example, ER β protein expression disappears during cancer progression, while its mRNA is still present [5,6]. At present, 10 isoforms, constituting splicing/deletion variants of the ER β gene, have been identified [7], making the study of ER β mRNA quite complex. Some of the isoforms seem to be more highly expressed in cancer than in non-cancer tissue [8]. At least some of them can be translated into an active protein. One of the isoforms, ER β cx, is particularly interesting, since it seems to be able to inhibit ER α transactivation [9]. The structural difference between ER β wild type (wt) and ER β cx is caused by an exchange of exon 8, which is part of the ligand-binding domain. ER β seems to be up-regulated in breast tumors resistant to tamoxifen [10], the most common anti-estrogen used in adjuvant hormone therapy. Since many cancers develop resistance to anti-estrogen therapy, and because of crucial side effects of tamoxifen with regard to the uterus, numerous studies are ongoing to find out the underlying mechanisms and to develop new drugs. Within the last few years, several selective ER α/ER β agonists/antagonists have been developed, which specifically activate/inhibit only one of the ER subtypes. This creates an opportunity to

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influence the transactivation of one of the ER subtypes independently of the other one. In some tissues, ER β can counteract ER α transactivation activity, whereas in other tissues, ER β may, at least partially, compensate for the function of ER α in ER α deleted mice [11]. Studies in mice, disrupted with respect to one or both ER's, have revealed target genes specific for ER α and ER β, respectively [11].

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and other ligands of the aryl hydrocarbon receptor (AhR) exhibit anti-estrogenic and anti-tumorigenic activities in the mammary gland and uterus [12,13]. The AhR is, like ER, a ligand-inducible transcription factor, but an endogenous ligand has yet to be identified. Some metabolites of sterols (7-ketocholesterol) [14] and heme (bilirubin, biliverdin) [15] can bind to and activate the AhR, but the most potent ligands are environmental contaminants, such as TCDD and related polychlorinated dibenzo-*p*-dioxins, dibenzofurans, polycyclic aromatic hydrocarbons, and naturally occurring phytochemicals [16]. It has been shown in several studies that TCDD inhibits E_2-induced responses in the rodent mammary gland and uterus and in breast and endometrial cancer cell lines through complex inhibitory AhR-ER α cross talk [17]. TCDD does not compete with E_2 for binding to ER [18]. Instead, AhR-ligands mediate their antagonistic effects on ER signaling by several separate pathways: (1) TCDD increases the metabolism of E_2 by inducing the cytochrome P450 monooxygenases CYP 1A1 and CYP 1B1 [19,20]. (2) TCDD decreases the level of ER α protein by inducing a proteasome-dependent pathway [21]. (3) TCDD suppresses the transcription of many E_2-induced genes by blocking or disrupting binding of ER α to DNA at ERE, Sp1, and AP-1 sites, adjacent to or overlapping with AhR-binding sites (XRE) [22–24]. (4) AhR and ERs compete for shared cofactors [25]. (5) TCDD opposes E_2-mediated effects on the cell cycle regulator Hairy and Enhancer of Split homolog-1 (HES-1) [26].

The influence on ER α signaling pathway by AhR agonists has been extensively investigated, whereas little is known about cross talk between AhR and ER β. In this study, we have investigated the regulation of ER β wt and ER β cx by estrogens and the anti-estrogen TCDD in the human breast cancer cell line T47D. Different treatment regimes, quantitative real-time PCR, chromatin immunoprecipitation, and electrophoretic mobility shift assay have been used to identify the mechanism by which ER α mediates the E_2-induced up-regulation of ER β wt mRNA and ER β cx mRNA. TCDD opposes this effect and inhibits the estrogen-mediated induction of ER β. The changes in ER β expression occur at the transcriptional level, and a specific element in the 5′-regulatory region of the ER β gene seems to be responsible.

Materials and methods

Cell culture. The human breast cancer cells T47D (ATCC) were cultured in a 1:1 mixture of Ham's nutrient mixture F12 and DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) (Life Technologies) and 1% PEST (100 U penicillin/ml and 100 μg streptomycin/ml) (Gibco-BRL). The cells were seeded on plates 3 days before hormone treatment in a 1:1 mixture of phenol-red free DMEM (Gibco-BRL) and F12 containing 5% dextran-coated charcoal-treated FBS and 1% PEST (DCC-medium).

hRNA isolation, DNA digestion, and cDNA synthesis. Total RNA was extracted using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The extracted total RNA was digested with DNaseI (Roche). Then 3 μg of digested RNA was reverse-transcribed using random hexamer priming and the SuperScriptII reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Quantitative real-time PCR (QRT-PCR). Fifty nanograms of cDNA was amplified in a real-time polymerase chain reaction (PCR) using TaqMan Universal Master Mix (PE Applied Biosystems), 100 nM primer for ER β, and 100 nM fluorogenic ER β probe. The real-time PCRs were performed in an ABI PRISM Model 7700 sequence detector (PE Applied Biosystems). The optimum concentration of primers and probe was determined in preliminary experiments. The sequences of ER β primers and probe are as follows: forward primer: TCC ATG CGC CTG GCT AAC, reverse primer ER β wt: CAG ATG TTC CAT GCC CTT GTT A, reverse primer ER β cx: CCA TCG TTG CTT CAG GCA A, and probe TCC TGA TGC TCC TGT CCC ACG TCA. Real-time PCR was done in duplicate. The 18S rRNA (PDAR, PE Applied Biosystems) was used as reference gene. The specificity of the amplified products was confirmed by sequencing. Standard curves were generated using serially diluted solutions of cDNA derived from untreated T47D cells. The target gene transcripts in each sample were normalized on the basis of its 18S rRNA transcript content.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays (EMSAs) were performed utilizing the 26 bp half ERE/XRE composite site (−97/−71 with regard to start of exon 0N) 5′ of human ER β gene: CCA CTT AGA GGT CAC GCG CGG CGT CG. Ten picomoles of synthetic double-stranded oligonucleotide was end labeled using [γ-^{32}P]ATP and a 5′-end labeling kit (Amersham–Pharmacia Biotech). In the binding reaction, nuclear extracts (20 μg) from various treatment groups were incubated with 0.50 μg poly(dA–dT) (Amersham–Pharmacia Biotech) for 15 min at room temperature. Following addition of [γ-^{32}P]-labeled probe, the mixture was incubated for an additional 15 min at room temperature. Reaction mixtures were loaded onto a 5% polyacrylamide gel and electrophoresed at 225 V in 1× Tris–borate and EDTA. Following gel drying, retarded protein–DNA complexes were visualized by autoradiography.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assay (ChIP) assays were performed as described [27] with minor modifications. Briefly, T47D cells were cross-linked with 1% formaldehyde for 10 min at room temperature and then quenched with 125 mM glycine. The cell pellets were washed with phosphate-buffered saline, re-pelleted by centrifugation, resuspended in 50 mM Tris (pH 8.0), 1% Triton X-100, 0.1% deoxycholate, 1 mM EDTA, 0.5 mM EGTA, and 140 mM NaCl, and incubated for 10 min at 4 °C. Samples were sonicated (5 × 20 s), centrifuged and the supernatant containing the soluble chromatin was flash-frozen and stored at −80 °C until use. Immunoprecipitations and subsequent washes were as described [28]. The washed resin was resuspended in 120 μl of elution buffer (TE, 1% SDS) and reverse-cross-linked overnight at 66 °C. DNA fragments were isolated and purified (QIAquick Spin Kit, Qiagen), and the PCR-amplified (human ER β promoter region −500/+353 with regard to start of exon 0N) fragments were separated on 2% agarose gels.

Results and discussion

Inhibition of estrogen-induced ER β mRNA expression by TCDD

In order to determine the levels of ER β mRNA, we carried out QRT-PCR. By designing primers situated in exon 7 and 8, we were able to differentiate between ER β wt and the isoform ER β cx, since the structural difference between ER β wt and ER β cx is caused by an exchange in exon 8. The exon 8 is part of the ligand-binding domain of ER β. ER β cx does not bind ligand, but may inhibit ER α transactivation [9]. In our T47D cells, the constitutive level of ER β cx mRNA is about threefold higher than that of ER β wt mRNA. It has previously been shown that the isoform ER β cx is more highly expressed in breast cancer than normal tissue [8].

Treatment of T47D cells up to 24 h with 10 nM TCDD alone had no effect on ER β wt or ER β cx mRNA expression (Fig. 1). As a control for TCDD treatment, we determined the expression of the AhR target gene cytochrome P450 1A1 (CYP 1A1). CYP 1A1 mRNA was up-regulated about 800-fold by TCDD (data not shown). Although TCDD alone did not change ER β mRNA at any time point measured, co-treatment with E_2 and TCDD partially inhibited the E_2-mediated induction of both ER β wt and ER β cx mRNA during a treatment period of 18 h. After 24 h co-treatment, TCDD had completely abolished the induction of ER β mRNA by E_2 (Fig. 1). Treatment of the cells with E_2 alone induced ER β mRNA expression time-dependently up to 30-fold, which is in accordance with a previous study [29].

Neither actinomycin D nor cycloheximide influences TCDD suppression of E_2-induced ER β mRNA

To clarify if the changes in ER β mRNA levels were due to changes in mRNA stability or due to changes in transcription rate of the ER β gene, we used the RNA synthesis inhibitor actinomycin D. T47D cells were treated with actinomycin D in the presence or absence of E_2 and/or TCDD. In the absence of hormones, the half-life of ER β wt mRNA was approximately 33 min. Treatment with E_2 and/or TCDD did not significantly change the turnover rate of ER β wt mRNA (Table 1), indicating that the regulation of the ER β gene expression by these hormones occurs at the transcriptional level.

The estimated half-life of ER β mRNA of 33 min is very short and is in contrast to the one observed in

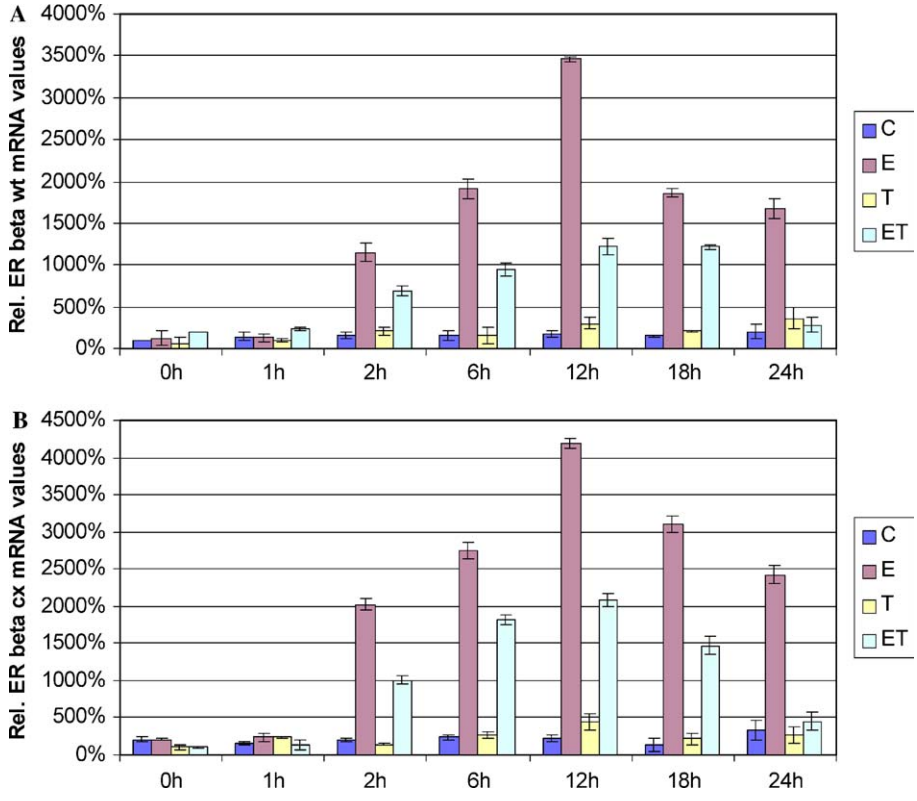

Fig. 1. TCDD alone did not change ER β mRNA at any time point measured, whereas co-treatment of E_2 and TCDD partially inhibited the E_2-mediated induction of ER β wt (A) and ER β cx (B) mRNA. The level of quantitative ER β mRNA in T47D cells was analyzed by real-time PCR, and the 18S rRNA was used as internal standard. Cells were treated for the given time points (hours) with vehicle [C], 1 nM E_2 [E], 10 nM TCDD [T], or co-treated TCDD + E_2 [ET].

Table 1
The half-life of ER beta wt mRNA is not affected by ligands

actD + vehicle	33 (±8) min
actD + E_2	37 (±7) min
actD + TCDD	31 (±4) min
actD + E_2+TCDD	29 (±3) min

The stability of ER β wt mRNA in T47D cells was investigated using real-time PCR, and the 18S rRNA was used as internal standard. T47D cells were treated with 10 μg/ml actinomycin D (actD) in the presence or absence of E_2 and/or TCDD for several time points. The estimated half-life is the time point with 50% decline of ER β wt mRNA.

primary cultured rat granulosa cells [30]. In those cells, the half-life of ER β mRNA was determined to be about 17 h. The difference may be due to species and/or tissue specificity, because the assay using actinomycin D worked well in T47D cells as seen from GAPDH mRNA, which was stable for more than 24 h (as a control experiment, data not shown). To our knowledge, there is no other report about ER β mRNA half-life, to which we could compare our results. Interestingly, in T47D cells grown in 10% normal FBS supplemented medium (instead of charcoal-treated serum), ER β mRNA half-life was stabilized at about 4 h (data not shown). Probably, this was caused by some factor(s) in the serum.

In order to determine whether de novo synthesis of proteins is required for the regulation of ER β mRNA, we treated the cells with or without the protein synthesis inhibitor cycloheximide in the presence or absence of hormones. Cycloheximide (100 μg/ml culture medium) had no effect on the basal levels of ER β wt mRNA expression, and did not prevent the inhibitory effect of TCDD on E_2-induced ER β mRNA (Fig. 2). Moreover, in the presence of E_2, cycloheximide caused a super-induction of ER β wt mRNA, which was still partially

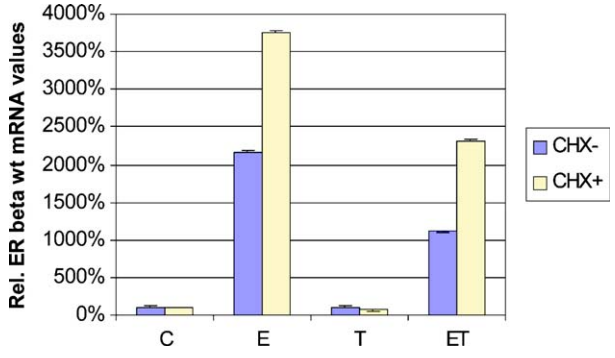

Fig. 2. The protein synthesis inhibitor cycloheximide did not prevent the inhibitory effect of TCDD on E_2-induced ER β mRNA. Moreover, in the presence of E_2, cycloheximide caused a super-induction of ER β wt mRNA. The level of quantitative ER β mRNA in T47D cells was analyzed by real-time PCR, and the 18S rRNA was used as internal standard. Cells were treated for 12 h with vehicle [C], 1 nM E_2 [E], 10 nM TCDD [T], or co-treated TCDD + E_2 [ET] in the presence or absence of cycloheximide (100 μg/ml culture medium).

inhibited by co-treatment with TCDD (Fig. 2). These results indicate that the regulation of ER β wt mRNA by E_2 and TCDD does not require de novo protein synthesis.

Moreover, the cycloheximide-induced super-induction of ER β mRNA by E_2 might indicate that a repressor of estrogen activation is involved. Proteins, which can repress ER transactivation, have been described in the literature [31]. And at least for one other, newly identified, ER α target gene, the same observation of super-induction by cycloheximide has been reported [32].

Involvement of ER α in the regulation of ER β mRNA

The endogenous ligand E_2 binds to both receptors with similar affinity, and leads to their transactivation, although the transactivation potential of ER β is usually lower than that of ER α [33]. It is known that ER α can influence the transcription of its own gene [34], but it is not clear, if ER β does the same. T47D cells have been described to express small amounts of ER β protein [35]. At the mRNA level, our T47D cells express about 12-fold higher amounts of ER α than ER β wt mRNA (data not shown), suggesting a role for ER α in regulation of ER β by estrogens and anti-estrogens. Genistein did not alter ER β mRNA expression (data not shown). On the other hand, the selective ER α agonist propylpyrazole triol (PTT) [36] induced the expression of ER β gene in a similar manner as E_2 (Fig. 3). The induction of ER β mRNA by this selective ER α ligand was again partially blocked by TCDD (Fig. 3). These results indicate that ER α plays a central role in the regulation of ER β gene.

Several studies have documented that exposure to estrogens in vivo and in vitro can influence ER β expression [37–39]. However, there are differences in ER β regulation with regard to tissue- and species-specific effects. In human cancer cell lines, derived from prostate and breast, ER β mRNA is also up-regulated by E_2 [29]. The molecular mechanisms behind the E_2-mediated induction of ER β mRNA have not been identified so far.

The results from the actinomycin D treatment implicate that the regulation of the ER β gene by E_2 and TCDD occurs at the transcriptional level, and therefore suggest the presence of responsive elements in the 5′-regulatory region of the ER β gene. It has recently been shown that the ER β gene is under control of different promoters [40], leading to transcription of different untranslated exons of the ER β gene. Therefore, we first determined which promoter of the ER β gene is used in the T47D cells by employing QRT-PCR with primers and probes annealing the different untranslated exons. We found that our cells use the exon 0N (data not shown), suggesting an active 0N-promoter. This promoter contains at least two half-sites of estrogen responsive elements (ERE). The distal ERE is a so-called

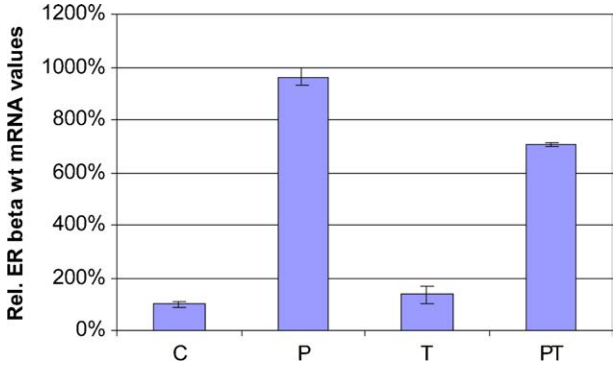

Fig. 3. The selective ER α agonist propylpyrazole triol (PTT, 4 g) induced the expression of ER β wt mRNA. The induction of ER β mRNA by this selective ER α ligand was partially blocked by TCDD. The level of quantitative ER β mRNA in T47D cells was analyzed by real-time PCR, and the 18 S rRNA was used as internal standard. Cells were treated for 12 h with vehicle [C], 1 nM PTT, 4 g [P], 10 nM TCDD [T], or co-treated TCDD + PTT, 4 g [PT].

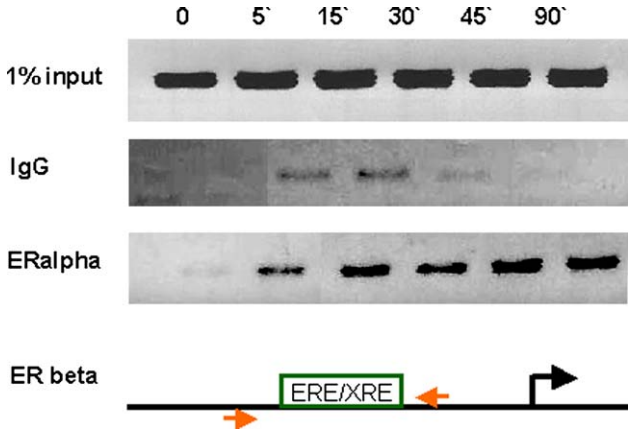

Fig. 4. ChIP assay experiments revealed that ER α is recruited to the ER β promoter in the presence of E_2. Chromatin immunoprecipitation assays were performed using extracts from T47D cells [input] treated with 1 nM E_2 for given time points (min). After sonication, samples were precipitated with anti-ER α rabbit polyclonal antibody H-184. DNA fragments were isolated and purified, and the PCR-amplified (human ER β promoter region −500/+353 with regard to start of exon 0N) fragments were separated on 2% agarose gels [ERα]. IgG was used as negative control [IgG].

Alu-ERE. ChIP assay experiments did not show any ER α binding to that part of the ER β promoter. The proximal half-site ERE is located at −89/−84 (with regard to the start of exon 0N), and, interestingly, that half-site ERE is overlapping with an imperfect AhR binding site (XRE). Imperfect XREs have been found in other genes exerting inhibitory effects [41]. ChIP assay experiments revealed that ER α is in fact recruited to that part of the ER β promoter in the presence of E_2 (Fig. 4). Gel mobility shift experiments utilizing the half ERE/XRE composite site showed an E_2-induced nuclear protein complex bound to that element (Fig. 5), which is diminished by co-treatment with TCDD.

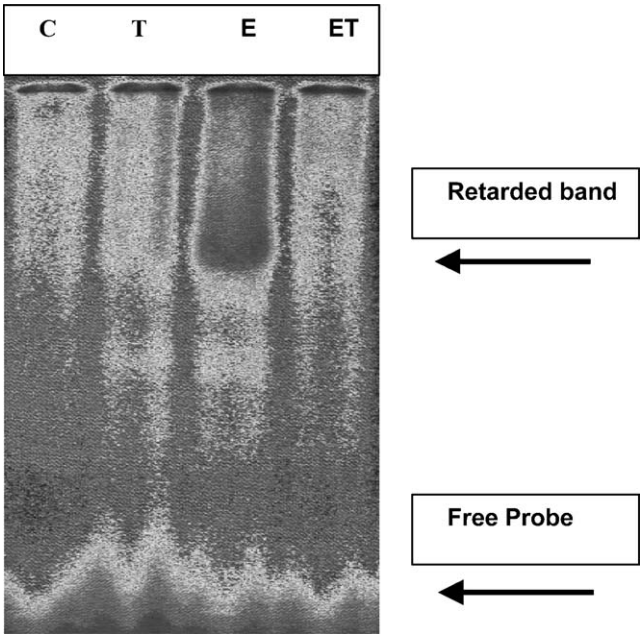

Fig. 5. An E_2-inducible protein binds the ERE half site element present in the ER β flanking region. E_2-inducible binding to that element is diminished by co-treatment with TCDD. Electrophoretic mobility shift assays were performed utilizing a 26 bp [γ-^{32}P]ATP labeled oligonucleotide containing the half ERE/XRE composite site from the 5′-regulatory region of human ER β. In the binding reaction, nuclear extracts (20 μg) from various treatment groups (vehicle [C], 10 nM TCDD [T], 1 nM E_2 [E], and co-treatment TCDD + E_2 [ET]) were loaded onto a 5% polyacrylamide gel.

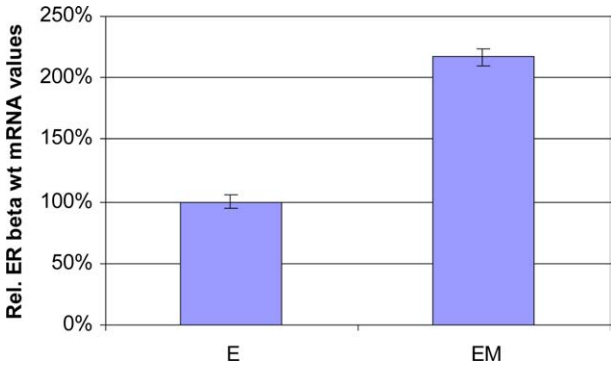

Fig. 6. Treatment of T47D cells with the proteasome inhibitor MG132 led to increased amounts of ER β wt mRNA. The level of quantitative ER β mRNA in T47D cells was analyzed by real-time PCR, and the 18S rRNA was used as internal standard. Cells were treated for 12 h with 1 nM E_2 [E] or co-treated MG132 + E_2 [EM].

TCDD has been shown to interfere with ER α signaling via different mechanisms; one of them is enhancement of ER α degradation by activation of the proteasome machinery [42]. Activation of the proteasome-dependent pathway leads to rapid decrease of ER α protein level [42]. This could be one mechanism whereby TCDD decreases the E_2-dependent binding of

ER α to the ER β promoter, since treatment of T47D cells with the proteasome inhibitor MG132 led to increased amounts of ER β mRNA (Fig. 6). On the other hand, it has been shown that TCDD suppresses the transcription of many E_2-induced genes by blocking or disrupting binding of ER α to DNA at ERE, Sp1, and AP-1 sites, adjacent to or overlapping with inhibitory XRE's [22–24]. A similar mechanism could operate in the present case of the human ER β gene.

This study shows that there exists, in fact, a cross talk between ER β and the AhR in T47D cells. To our knowledge, this is the first report about the interference between both pathways in breast cancer cells. In cultured rat granulosa cells, it has been described that ER β mRNA increased after TCDD treatment [43]. This might indicate the existence of a tissue- and/or species-specificity of TCDD/ER β cross talk.

We have shown here that ER α plays a central role in regulation of the ER β gene by estrogens and the anti-estrogenic compound TCDD. The expression of ER α is one of the prognostic markers used in breast cancer. In the normal mammary gland, ER β is the predominant ER subtype with only a few cells expressing ER α [44]. During breast carcinogenesis, the ratio between ER α and ER β increases [45], at least one mechanism being down-regulation of ER β by gene hypermethylation [46]. We have recently shown that overexpression of ER β in T47D cells leads to inhibition of cell proliferation [47]. In the present study, our data implicate that the ER β gene is a target gene for both ER α and dioxin, indicating that ER β may be an important component in the cross talk between dioxin and estrogen signaling in human cells.

Acknowledgments

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