MECHANISMS OF INHIBITORY ARYL HYDROCARBON RECEPTOR (AhR)-ESTROGEN RECEPTOR A CROSSTALK

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Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces multiple tissue-specific biochemical, toxic, genotoxic and anticarcinogenic responses in cells and laboratory animals, and our research has focused on TCDD-induced inhibitory aryl hydrocarbon receptor (AhR)-estrogen receptor a (ERa) crosstalk [1,2]. TCDD inhibits 17â-estradiol (E2)-induced responses/gene expression in the rodent uterus, breast cancer cells, and rodent mammary tumors. The indirect antiestrogenic effects of TCDD in these models is consistent with the lower incidence of endometrial and mammary tumors in women exposed to high levels of TCDD in Seveso, Italy [3]. Studies in this laboratory have investigated the mechanisms of inhibitory AhR-ERa crosstalk and development of selective AhR modulators for treating breast and endometrial cancers [1,2,4]. Mechanistic studies suggest that TCDD inhibits expression of some E2-resonsive genes through direct interaction of the AhR with inhibitory dioxin responsive elements (iDREs) in their gene promoters. In contrast, inhibition of other E2-responsive genes is iDRE-independent and is due, in part, to limiting levels of ERa in breast cancer cells cotreated with TCDD plus E2. This paper will describe one mechanism important for inhibitory AhR-ERa crosstalk, namely TCDD-induced degradation of ERa through activation of proteasomes [5].

Materials and Methods

Human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). E2, chlroquine, calpain inhibitor II, EST, MB132 and PSI were the highest quality available from commercial sources. Antibodies for ERa (sc-544), AhR (sc-8088), and Sp1 proteins were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Human ERa, AhR and AhR nuclear translocator (Arnt) full length cDNA probes were cloned into the pcDNA3.1 expression vector (Invitrogen) and used to translate *in vitro* standards for Western blots using a reticulocyte lysate method. Cells were grown on monolayer cultures in Sigma MEM alpha modification or DMEM F12 HAM media with phenol red supplemented with 2.2 g/L sodium bicarbonate, 0.2 g/L bovine serum albumin (bovine, fraction V), 0.01 g/L apo-transferrin (human), 5% fetal bovine serum (Intergen, Purchase, NY), and antibiotic-antimycotic solution, pH 7.4. Cells for experiments were seeded into 35 mm 6-well tissue culture plates in phenol-free media (DMEM F-12 HAM, Sigma) containing 2.5 % charcoal-stripped fetal bovine serum. The following day, the cells were treated and harvested at designated time points as follows. Cells were washed once in ice cold phosphate-buffered saline and collected by scraping in 0.25 ml ice cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 % (v/v) glycerol, 1 % Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mg/ml aprotinin, 50 mM phenylmethylsulphonylflouride, 50 mM sodium orthovanadate. The lysates were incubated on ice for 1 h with intermittent vortexing followed by centrifugation (15,000 *g*, 5 min, 4°C). Equal amounts of protein from each treatment group were separated by SDS-PAGE and electrophoresed to PVDF membrane using a BioRad Trans-Blot Electrophoretic Transfer Cell (BioRad, Hercules, CA); transfer

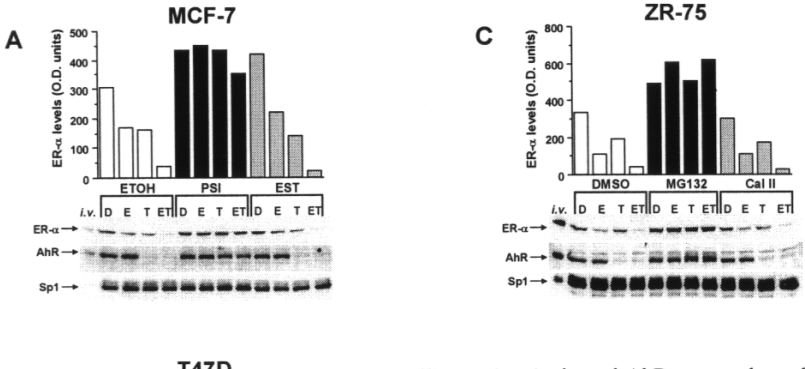

Figure 1. Activated AhR causes degradation of ERα in multiple breast cancer cell lines through the proteasome pathway. MCF-7 [A], T47D [B] and ZR-75 [C] cells were treated for 3 h with DMSO (D), 10 nM E, 10 nM TCDD (T), or their combination (ET) following a 30 min pretreatment with vehicle control (ETOH or DMSO), proteasome (PS1, PS11 or MG132), or protease (EST, Chlor, Cal II) inhibitors at 10 μM concentrations for each. Whole cell extracts from the different treatment groups were analyzed by Western blot analysis for ERα (G-20), AhR (N-19) and Sp1 (PEP2) proteins as described in the Materials and Methods. Relative ERα protein levels for each cell line are illustrated in bar graphs.

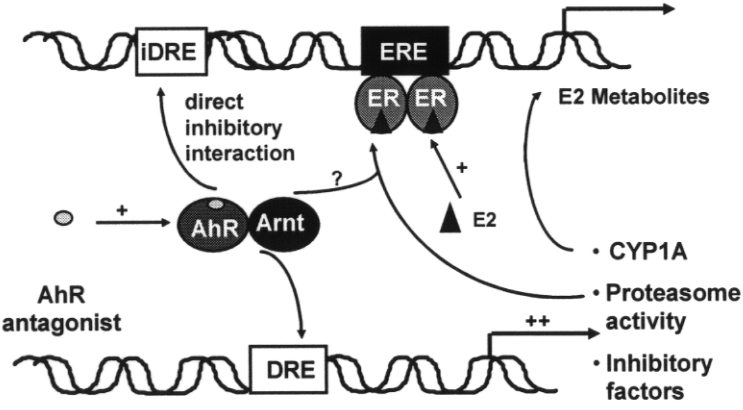

Figure 2. Proposed mechanisms for inhibitory AhR-ER crosstalk in the rodent uterus/mammary and human breast cancer cells.

buffer: 48 mM Tris, 39 mM glycine, 0.025 % sodium dodecyl sulfate (SDS). Membranes were blocked for 30 min in Blotto [5% milk + TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl), 0.05% Tween-20], probed with the polyclonal antibodies for ERa (1:1000 dilution in Blotto), AhR (1:200), or Arnt (1:200) (see "Chemicals" above) for 5 h, washed 2 ´ 5 min in TBS + 0.05 % Tween 20, and probed with

secondary peroxidase-conjugated antibody (1:5000 in Blotto) for 2 h. The membranes were then washed 3 ´ 5 min in TBS + 0.05% Tween-20, 1 ´ 5 min in TBS, and visualized using the ECL detection system (New England Nuclear, Boston, MA). Quantitation of Western blots was performed as described [5].

Results and Discussion

The results illustrated in Figure 1 show that both TCDD, E2 and TCDD+E2 downregulate ERa and the combined treatment results in exceeingly low levels of ERa. TCDD also induces downregulation of the AhR, whereas E2 treatment decreases ERa but not the AhR. In addition, we also observed the following: (a) TCDD-mediated inhibition of E2-induced transactivation correlated with the decreased ERa levels in cotreated cells; (b) TCDD also enhanced ubiquitination of ERa; and (c) results of mammalian two hybrid assays indicate that TCDD (but not E2) induces ERa-AhR interactions. These data suggest that the mechanisms of inhibitory AhR-ERa crosstalk on some genes is due to enhanced proteasome-dependent degradation of ERa which becomes limiting in cells cotreated with TCDD+E2 (Fig. 2).

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