NON-AH RECEPTOR MECHANISMS UNDERLYING IMMUNOTOXICITY AND NEUROTOXICITY

GROWTH REGULATION BY AhR LIGANDS IN THE HUMAN MAMMARY EPITHELIAL CELL LINE MCF-10A

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Introduction

Mammary tumorigenesis is a complex process in which the dysregulation of signaling pathways has been implicated.[1] It has been postulated that environmental pollutants may play a part in the etiology of this disease. The exposure of women to ubiquitous environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other halogenated aromatic hydrocarbons (HAHs), and polycyclic aromatic hydrocarbons (PAHs) have been well-documented.[2] Previous studies in our lab have demonstrated that TCDD mimics growth factor stimulation and cell growth in the human mammary epithelial cell line, MCF-10A.[3] TCDD was found to increase total tyrosine phosphorylation and phosphatidylinositol 3-kinase (PI3K) activity. In addition, withdrawal of epidermal growth factor (EGF) resulted in the induction of apoptosis, while treatment with TCDD prevented apoptosis and increased Akt phosphorylation.[4] These results argue that TCDD could act as a mammary tumor promoter by over-stimulating the EGF signal transduction pathway thus inhibiting apoptosis. The inhibition of apoptosis is widely accepted as one possible mechanism of tumor promotion/progression.[5] Growth factors are able to inhibit apoptosis through the regulation of Akt and ras/raf/mitogen-activated protein kinase (MAPK) pathways.[6] The present studies were initiated to begin to delineate the mechanism(s) through which TCDD inhibits EGF withdrawal-induced apoptosis in the human mammary epithelial cell line, MCF-10A. These studies were designed to test the hypothesis that inhibition of apoptosis in MCF-10A cells occurs through an EGF receptor (EGFR)-dependent pathway.

Methods and Materials

Chemicals and reagents

All chemicals were purchased from Sigma (St. Louis, MO), unless otherwise indicated. TCDD was obtained from Cambridge Isotopes Laboratories (Andover, MD) at >99% purity, and was maintained as a stock solution (300 μM) in anhydrous tissue culture grade dimethyl sulfoxide (DMSO). AG1478, AG825, PD98059, and Ly294002 were purchased from Calbiochem (La Jolla, CA) and stored at -20°C in DMSO. The final concentration of DMSO in experiments was 0.1%.

MCF-10A cell culture

MCF-10A cells, a non-transforming, estrogen receptor-negative human mammary epithelial cell line were grown on Vitrogen-coated (Collagen Corp., Palo Alto, CA) 100x20 mm dishes (Corning Glass, Corning, NY) in a 10% CO_2 incubator as previously described.[3,4]

Detection of Apoptosis

Apoptosis was determined by flow cytometry using a kit that employs Annexin V conjugated to *FITC (PharMingen, San Diego, CA) and has been used previously.*[4] To distinguish between apoptosis and necrosis, cells that stained for propidium iodide (PI) or PI and Annexin V were determined to be necrotic and not counted as apoptotic.

Determination of Erk Activation by Western Blot

MCF-10A cells were plated on Vitrogen-coated 100x20mm dishes at 3.5 x 10^5 cells per dish in SFIHE plus 2% FBS. The cells were re-fed the following day and thereafter every 3 days with SFIHE media without FBS. Sub-confluent cells 5 days after plating were starved for 18 hours in media without insulin or EGF (SFH) and were treated as indicated. Cells were washed twice with ice-cold phosphate buffered saline (PBS) and 200 µL of lysis buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol) was added. Cells were then scraped into Eppendorf tubes and soluble membranes and DNA were sheared by pulling the lysates through an 18-gauge needle. 10 µL of the lysate was set aside for protein concentration determination using Pierce's micro-BCA protein assay reagent (Rockford, IL) and to the remaining sample dithiothreitol (DTT) and bromophenol blue was added to a final concentration of 50 mM and 0.1%, respectively. 50 µg of protein was boiled and separated on 12% Tris-glycine gels using standard conditions. Proteins were transferred overnight to PolyScreen® PVDF membrane (NEN Life Sciences, Boston, MA). Phosphorylated Erk1,2 was detected using New England BioLabs' (Beverly, MA) PhosphoPlus® Erk1,2 (Thr202 / Tyr204) antibody kit as per supplied instructions and visualized with NEN's Renaissance® Western Blot Chemiluminescence Reagent directed towards a HRP-conjugated secondary antibody (Promega).

Transient Transfections and Reporter Assays

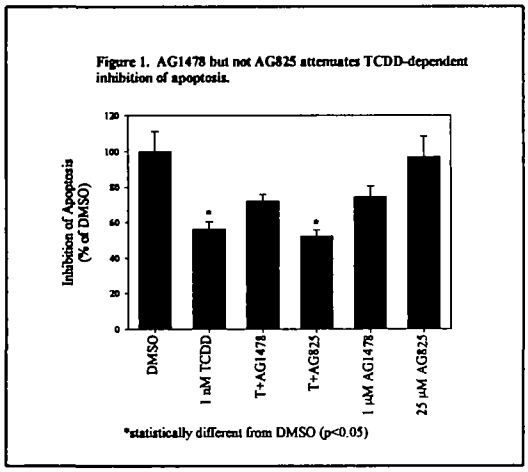
Sub-confluent cultures if MCF-10A cells were transiently co-transfected with pGudLuc1.1 or 6.1 (pGL1.1 and 6.1 respectively) and pTK-RL (Promega Corp.) to control for transfection efficiency using SuperFect tranfection reagent (Qiagen, Germany) as per supplied instruction. Cells were allowed to recover for 12 hrs in SFIHE media followed by treatment with 1 nM TCDD (or 0.1% DMSO) for 3 hrs. Cells were harvested and luciferase activities determined using Promega's Dual-Luciferase Reporter Assay kit.

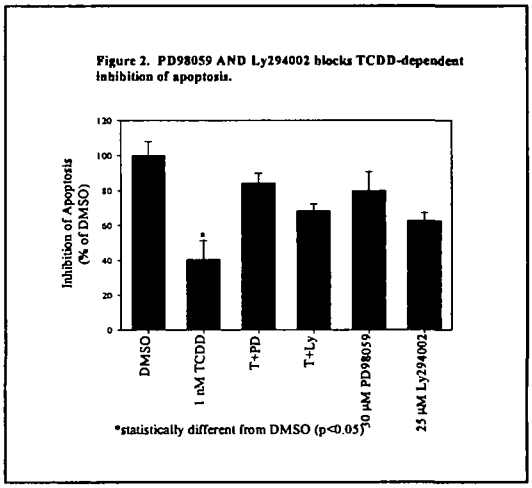
Statistical Analysis

Data were analyzed for statistical difference ($P < 0.05$) between control and treated groups using SigmaStat statistical software (Jandel Scientific, San Rafael, CA). ANOVA followed by Dunnett's *t*-tests were performed on sample means.

Results and Discussion

Growth factors withdrawal has been used as a model to induce apoptosis in many cell types in an





effort to study the signaling pathways responsible for controlling apoptosis. A recent publication from our lab demonstrated that TCDD was able to inhibit apoptosis induced by EGF withdrawal.[4] In an effort to analyze the signaling pathways responsible for this effect, MCF-10A cells were cultured in the absence of growth factors and co-treated with TCDD and either AG1478, a specific inhibitor of the EGFR or AG825, an erbB2 inhibitor. As shown in Figure 1, treatment with TCDD for 3 days inhibited apoptosis by 50%. When cells were co-treated with AG1478 there was no difference from DMSO. However, co-treatment with AG825 did not have an affect on TCDD-dependent inhibition of apoptosis. This data suggests that the ability of TCDD to inhibit apoptosis is dependent on EGFR activity but not erbB2. Since inhibition of apoptosis by growth factors is dependent on both the PI3K/Akt and the MAPK pathway[6] we next wanted to determine which of these pathways was responsible to TCDD's effects. Surprisingly, TCDD's ability to inhibit apoptosis was dependent on both PI3K and MEK1 activity. Co-treatment with PD98059, a MEK1 inhibitor, or Ly294002, a PI3K inhibitor almost completely reversed TCDD-dependent inhibition of apoptosis (Figure 2). TCDD is able to activate Akt under conditions that lead to inhibition of apoptosis,[4] and since EGFR signaling is able to control both Akt and Erk,[6] we next wanted to determine if TCDD is able to activate Erk. Treatment with 10 nM TCDD for 6 hours resulted in an increase in Erk phosphorylation (Figure 3). In addition, this effect is specific for EGFR since AG1478 but not AG825 was able to almost completely block the response. The ability of TCDD to stimulate Erk activity was similar to that of EGF and TGFα known EGFR ligands. This is further evidence for an EGFR pathway.

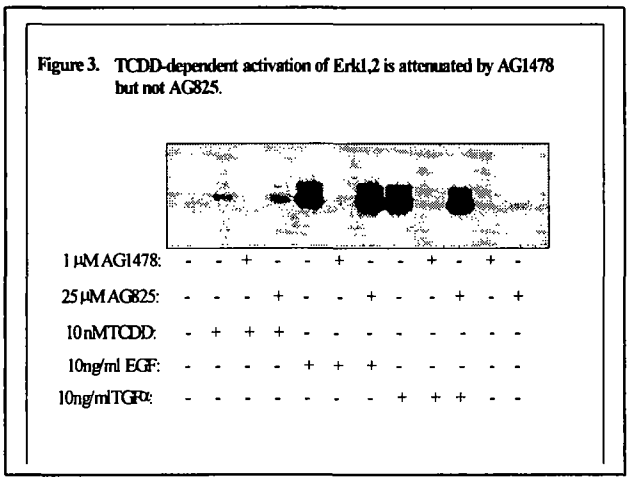


Figure 3. TCDD-dependent activation of Erk1,2 is attenuated by AG1478 but not AG825.

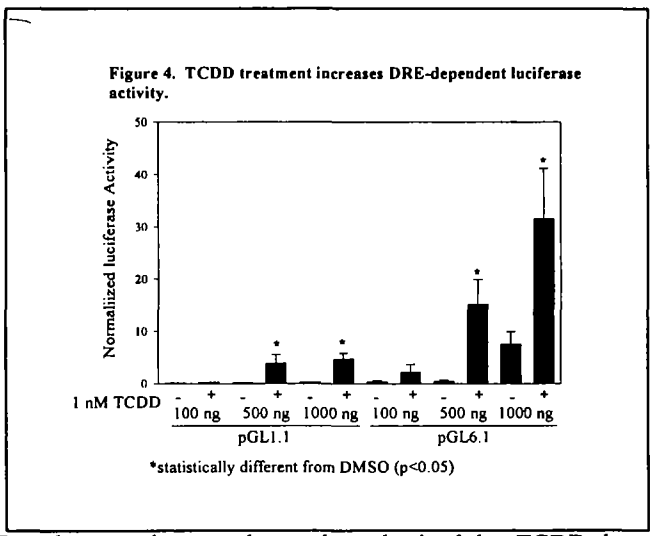


Figure 4. TCDD treatment increases DRE-dependent luciferase activity.

Based on previous results, we hypothesized that TCDD-dependent inhibition of apoptosis is mediated by the aryl hydrocarbon receptor (AhR). The MCF-10A cell line expresses the mRNA for AhR and its heterodimerization partner, the AhR nuclear translocator (ARNT).[7] In addition, TCDD is able to inhibit apoptosis at 1 nM (Figures 1 and 2), which is in the range of the K_d for the receptor.[8] In order to examine whether an active AhR pathway exists in this cell line, MCF-10A

cells were transfected with increasing amounts of one of two reporter vectors that under the control of dioxin response elements luciferase activity (Figure 4). This, along with the observation that benzo(a)pyrene (BaP) treatment increased cytochrome P450 1A1 and 1B1 mRNA,[7] genes known to be transcriptionally activated by AhR[8], does indeed suggest that MCF-10A cells possess an intact, functional AhR pathway. In summary, TCDD appears to inhibit apoptosis by activating the EGFR. Furthermore, TCDD treatment also results in the activation of numerous kinases that are downstream of the EGFR and known to regulate apoptosis through various mechanisms.

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