APPLICATION OF A HUMAN OVARIAN CELL BIOASSAY FOR THE DETECTION AND ANALYSIS OF ESTROGEN-TOXICANT INTERACTIONS

Jane M. Rogers and Michael S. Denison

Department of Environmental Toxicology, University of California, Davis, CA 95616 USA

Introduction

Environmental contaminants which can disrupt the hormonal homeostasis of an organism have gained widespread attention in recent years, and their detrimental effects on the reproductive success of many wildlife populations have been well documented[1,2]. Although many of these compounds can act as estrogen agonists, the dioxin-like halogenated aromatic hydrocarbons (HAHs) are a class of chemicals which have been shown to interact in an inhibitory manner with the estrogen system, and yet the mechanism for these interactions has still not been resolved. There appear to be several mechanisms by which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent HAH and aryl hydrocarbon receptor (AhR) ligand, may exert its anti-estrogenic action. It has been reported that inhibition of induction of some estrogen dependent genes such as cathepsin-D and pS2 is due to binding of the transformed TCDD:AhR complex to an imperfect dioxin responsive element which overlaps the estrogen responsive element (ERE) and prevents DNA binding of the estrogen receptor (ER)[3,4]. However, this mechanism does not appear to be responsible for the TCDD induced repression of other estrogen dependent genes[5]. Additional mechanisms, which involve changes in ER phosphorylation, down regulation of ER gene expression and increased metabolism and clearance of estradiol, have also been proposed, however, the role of these events remains to be firmly established[6,7]. The majority of the detailed mechanistic work on the anti-estrogenic effect of TCDD has focused on examining its effect on the promoter and upstream regulatory elements of a specific estrogen responsive gene[3,4]. However, the presence of overlapping and/or adjacent DNA binding sites for transcription factors and hormone receptors can complicate assessment as to whether TCDD can directly affect the ER dependent signaling mechanism. To minimize these potential interference's we have developed a recombinant, human ovarian, cell line (BG1Luc4E$_2$), which has been stably transfected with a luciferase reporter plasmid that is under the estrogen inducible control of four EREs[8]. In addition to its use as a detection tool for the identification of estrogenic and anti-estrogenic compounds in environmental samples, the development of this recombinant cell line will also provide us with a controlled system with which to study the mechanism by which TCDD and related HAHs disrupt the estrogen signaling pathway.

Materials and Methods

Luciferase Expression Assay for BG1Luc4E$_2$ Cells: Cells were grown in either regular media (DMEM with 10% fetal bovine serum (FBS)) or for 7 days in estrogen stripped media (ESM) (phenol red free MEM with 5% dextran-coated charcoal stripped FBS). Unless otherwise stated the cells were exposed to the indicated chemicals for 24 hours, followed by lysis and measurement of luciferase activity as described in detail[8].

Western Blot Analysis: Whole cell extracts, cytosolic protein or nuclear protein (5μg), was separated by SDS PAGE, followed by electroblotting to nitrocellulose membranes. Blots were probed with ERα antibody (Santa Cruz Biotechnology, Inc.) as previously described[8].

ER Ligand Binding Analysis: Whole cell extracts (0.5 mg) were incubated with 2 nM ^{3}H estradiol for 2 hr at 4°C. Extracts were then incubated with a charcoal pellet for 15 min. at 4°C and then centrifuged at 3500 rpm for 15 min. The supernatant was then added to scintillation

cocktail and counted in a liquid scintillation counter. Non-specific binding was determined by addition of 100 fold excess of the synthetic estrogen diethylstilbesterol.

Results and Discussion

The development of this recombinant human ovarian cell line provides us with a novel bioassay for detecting estrogenic and anti-estrogenic compounds. We have optimized growth and assay conditions so as to essentially eliminate background luciferase activity attributed to endogenous estrogenic chemicals[8]. BG1Luc4E$_2$ cells have previously been shown to respond to estrogen in a time-, dose-, and chemical-specific manner, with a detection limit of as low as 0.1 – 1 pM estradiol[8].

BG1Luc4E$_2$ cells are currently being utilized to determine which step(s) in the estrogen signaling pathway is disrupted by TCDD. Growth of these cells in regular media, containing phenol red and 10% FBS, results in high constitutive luciferase activity (due to estrogens within the media) which is not further increased by estradiol but is decreased in a dose-dependant manner by TCDD. To show a more direct effect of TCDD on estrogen signaling the cells were grown in ESM for several days to reduce background luciferase activity. Co-treatment of these cells with 1 nM estradiol and 10 nM TCDD results in a 30% - 60% decrease in estrogen induced luciferase. Pre-treatment of the cells with TCDD for 24 h or 48 h fails to further reduce the estrogen response, compared to simultaneous treatment (Figure 1A), and an excess of estradiol (1000 fold) does not eliminate the effect of TCDD (Figure 1B). Taken together these data suggest that the anti-estrogenic effect of TCDD in this system is not due to enhanced estrogen metabolism by TCDD induced enzymes.

We next examined the effect of TCDD on the amount of ER in this cell line. Radioactive ligand-binding analysis of whole cell extracts (WCE) from treated cells grown in regular media shows that TCDD reduces the amount of ER able to bind ^{3}H estradiol by about 30% - 60%, a similar amount of inhibition as seen with the luciferase assay (Figure 2A). In addition, both WCE and nuclear and cytosolic protein from cells grown in regular media and treated for 24 h with 10 nM TCDD show a decrease in the amount of ERα compared with DMSO treated cells, as seen by Western blot (Figure 2B). However, both ligand-binding and Western blot analyses show a substantial decrease in the amount of ER in cells grown in ESM for several days compared with regular media. A time course of days grown in ESM shows a correlation between the decrease in ERα and an increase in estrogen inducibility of luciferase which implies the presence of spare receptors that are not required for the response of these cells to estrogen, or for inhibition of that response by TCDD. Although we detect only low levels of ERα from cells grown in ESM these levels do not appear to be decreased further by TCDD treatment. These data indicate that although TCDD is able to reduce the amount of ER in cells grown under conditions which result in maximal levels of ER, only a fractional amount of that ER is apparently required for an estrogen response in this system. Therefore, the observed decrease in ERα does not appear to be responsible for the inhibition of estrogen induced luciferase activity seen in BG1Luc4E$_2$ cells.

Acknowledgments

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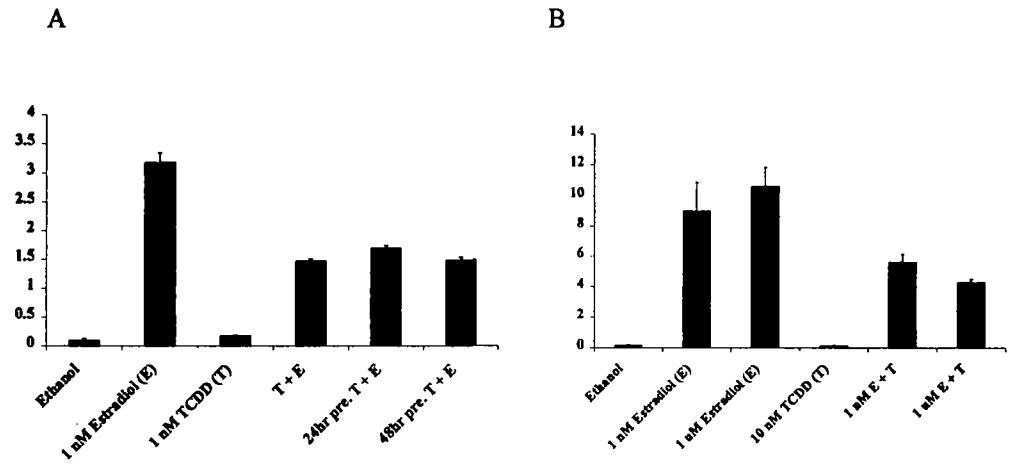

Figure 1. A. Effect of TCDD co-treatment and 24 hr and 48 hr pre-treatment on the estrogen induced luciferase activity in BG1Luc4E$_2$ cells. **B.** Effect of 1000 fold excess of estradiol (1 μM) on the anti-estrogenic effect of TCDD in BG1Luc4E$_2$ cells. Cells were grown for one week in estrogen stripped media and exposed for 24 hrs (unless otherwise indicated) to the indicated compounds. Values represent mean relative light units/mg protein ± SD of at least triplicate determinations.

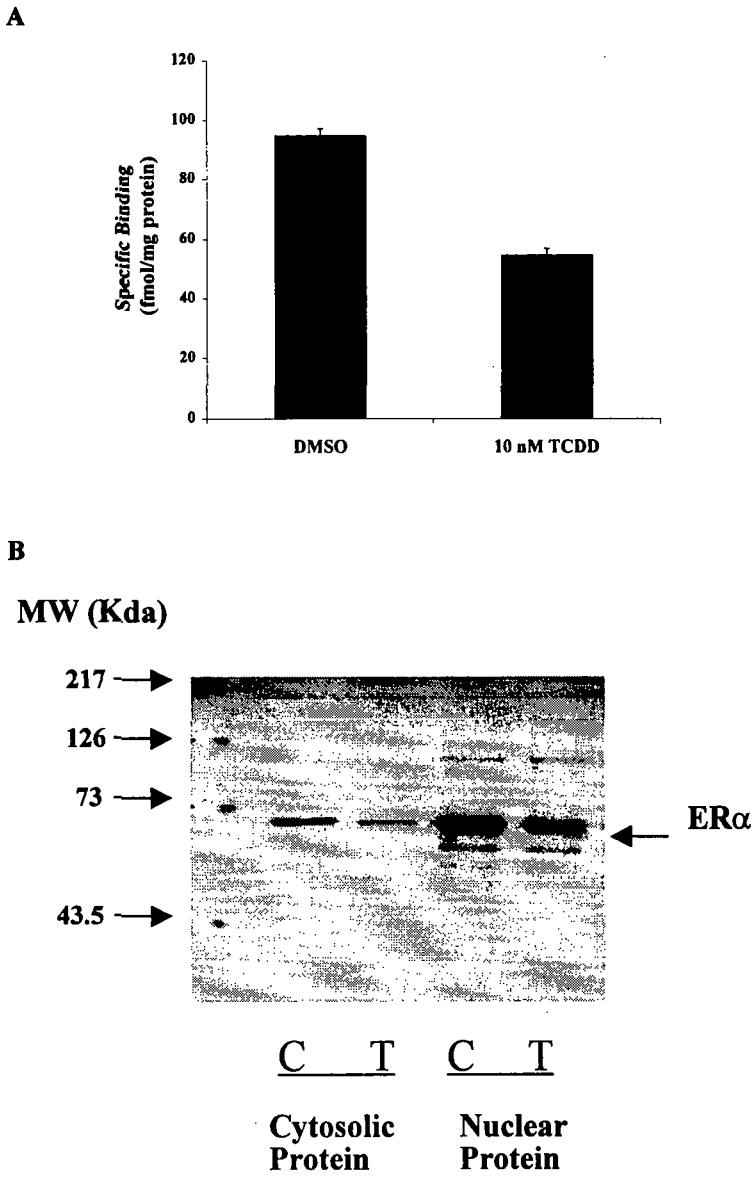

Figure 2. **A.** Specific binding of ^{3}H estradiol in whole cell extracts of BG1Luc4E$_2$ cells treated for 24 hrs with DMSO or 10 nM TCDD. **B.** Western blot showing the decrease in ERα in both cytosolic and nuclear fractions of BG1Luc4E$_2$ cells treated for 24 hrs with DMSO (C) or 10 nM TCDD (T).