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Estrogen Inhibits Bcl-2 Expression and Stimulates Apoptosis Mediated by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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ABSTRACT. The effects of estrogen on apoptosis induced by 2,3,7,8-tetrachlorodibenzo-*p*-doxin (TCDD) were examined in cultured MCF-7 cells. TCDD stimulated apoptosis and inhibited the expression of bcl-2 gene in MCF-7 cells grown in the media supplemented with 10% fetal bovine serum. However, TCDD failed to induce apoptosis if cells were grown in the media deprived of all estrogen-like compounds. Removal of estrogen-like compounds from the growth media also led to the activation of bcl-2 gene expression in cells treated with TCDD. Combined treatment of estrogen with TCDD abrogated the binding of Aryl hydrocarbon Receptor (AhR)-TCDD complex to Dioxin response element (DRE) of bcl-2 gene leading to the inhibition of bcl-2 gene expression as well as stimulation of apoptosis. The present study suggests that the binding of estrogen receptor (ER)-estrogen complex to the estrogen responsive element (E) interferes with the binding of AhR-TCDD complex to the DRE and inhibits the bcl-2 expression.

Keywords: Estrogen, Tetracholorodibenzo-*p*-dioxin, TCDD, Bcl-2.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most toxic halogenated hydrocarbon compounds that elicit immune, reproductive and developmental toxicity and tumor promotion. TCDD suppresses T cell functions and reduces T cell numbers in multiple models of immune stimulation. TCDD-induced suppression of CD4 (+) T cells involves the increased cell death mediated by Fas/FasL interaction (Dearstyne and Kerkvliet, 2002). Reproductive toxicity of TCDD has been also reported and the reduced number of spermatids/testis was reported in TCDD treated animals (Chahoud *et al.*, 1992). Tumor promotion by TCDD has been believed to be mediated by Aryl hydrocarbon receptor (AhR) (Schwarz *et al.*, 2000). TCDD binds to an intracellular cytosolic protein called AhR, a ligand-dependent transcription factor, which is a member of the basic helix-loop-helix-PAS family (Burbach *et al.*, 1992; Ema *et al.*, 1992). Binding of TCDD to AhR complex results in the release of heat shock protein 90 (Hsp90), enabling the receptors to heterodimerize with the AhR nuclear translocator protein. The AhR-TCDD complex has been shown to interact with dioxin responsive elements (DRE). The DRE sequence (5'-T/GNG-CGTGA/CG/CA-3') includes the essential sequences (5'-GCGTG-3') necessary for the binding with the AhR-TCDD complex and the flanking sequences required for the transcriptional activation of the adjacent gene (Whitlock, 1993). AhR activation by TCDD can induce apoptosis in the central nervous system during developmental stage (Dong *et al.*, 2001). TCDD is also known to induce apoptosis mediated by c-Jun N-terminal kinases in human T cells (Hossain *et al.*, 1998). However, controversial roles of TCDD on apoptosis have been presented, for example, the positive role of TCDD on apoptosis has been reported in human T cells (Hossain *et al.*, 1998), while the negative role of TCDD on apoptosis has been presented in rat hepatocytes treated with diethylnitrosamine (Stinchcombe *et al.*, 1995).

Estrogen has been known to inhibit apoptosis in MCF-7 cells by activating the expression of bcl-2. Estrogen binding to Estrogen Receptor (ER) induces structural changes in ER conformation, and allows its

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interaction with estrogen-responsive element (ERE) located within the coding region of bcl-2 (Perillo *et al.*, 2000). Studies have demonstrated that the expressions of estrogen-responsive genes including c-fos, cathepsin D, Hsp 27 and pS2 are inhibited after the treatment with TCDD in animal cells. (Safe *et al.*, 1998; Gillesby *et al.*, 1997).

In this study we found that the estrogen treatment triggers stimulation of TCDD-induced apoptosis as well as the inhibition of bcl-2 expression in human breast cancer MCF-7 cells. Based on these observations we propose that there is a competition between the binding of ER-estrogen complex to the E element and the binding of AhR-TCDD complex to DRE within bcl-2 gene.

MATERIALS AND METHODS

Materials

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from AccuStandard Inc., and 17β-Estradiol (E2) was from Sigma.

Vectors, Transfection and Luciferase Assays

The enhancer region of bcl-2 was cloned in pGL3 promoter vector (Promega) using the sequence, 5'-GGGCACACGCCCCATCCAGCCGCATCCCGCGACCCGGTCGCCAGGACCTCGCCGCTGCAGACCCCGGCTGCCCCCGGCGCCGCCGCGGGGCCTGCGCTCAGCCCGGTGCCACCTGTGGTCCACCTGGCCCTCCGCCAAGCCGGCGACGACTTCTCCCGCCGCTACCGCGGCGACTTCGCCGAGATGTCC-3'. The binding site of AhR-TCDD complex was 5'-CACGCCC-3'. The binding sites of ER-estrogen complex were 5'-GGTCGCCAGGACC-3' and 5'-GGTCCACCTGGCC-3'. D5-E3 was constructed using the oligomer of 5'-GGGCACACGCCCCATCCAGCCGCATCCCGCGACCCGGTCGCCAGGACCTCGCA-3' in pGL3 promoter vector. The mutant vector, mutD5T-E3 (+157~+208) lacking the transcriptional activity of bcl-2 was constructed in pGL3 promoter vector using the oligomer of 5'-GGG**A**ACACGCCTCATCCAGCCGCATCCCGCGACCCGGTCGCCAGGACCTCGCA-3'. C at 160 was replaced by A while C at 168 was replaced by T in mutD5T-E3. The mutant vector mutD5B-E3 (+157~+208), which lacks the ability to bind AhR-TCDD complex was consturcted in pGL3 promoter vector using the oligomer of 5'-GGGCACTCGCCCCATCCAGCCGCATCCCGCGACCCGGTCGCCAGGACCTCGCA-3'. A at 163 was replaced by T in mutD5B-E3. MCF-7 cells were transfected using LipofectAMINE according to the manufacturer's procedure (Life Technologies, Inc.). After 24 hr of transfection, luciferase activities were then assessed by Luciferase assay system (Promega).

Cell Culture

Human breast cancer MCF-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), sodium bicarbonate, HEPES, 0.1% gentamicin at 37°C under 5% CO_2. To eliminate the effects of estrogen-like compounds on TCDD mediated apoptosis, MCF-7 cells were grown in phenol red-free Dulbecco's Minimal Essential Medium (Gibco/BRL) containing 5% charcoal-dextran FBS for 7 days as described (Perillo *et al.*, 2000).

Apoptosis Assay

After incubation with TCDD for 24 hr, cells (1.0×10^8) were collected and suspended in 1 ml phosphate-buffered saline (PBS). To each cell samples was added 2.5 ml Ethanol and the cell preparation was incubated on ice for 2 hr, followed by washing three times with phosphate buffered citric acid containing 96% 0.2 M Na_2HPO_4 and 4% 0.1 M citric acid (pH 7.8). Cells (1.0×10^8) were collected and suspended in 1 ml PBS containing 1 mg RNase A and incubated for 10 min at 37°C. Cells were stained with propidium iodide (250 μg/ml, Sigma) for 10 min at room temperature. Cell death was assessed by FACS as described (Telford *et al.*, 1991).

Bcl-2 Expression

MCF-7 cells were treated with various concentrations of TCDD for 24 hr. Cells were dissolved in a lysis buffer containing 50 mM Tris, 120 mM NaCl, 0.5% NP-40, 100 mM NaF, 200 mM sodium orthovanadate and 1 mM serine protease inhibitor (pH 8.0), incubated on ice for 20 min and centrifuged at 13,000 ×g. The resulting supernatant was collected, mixed with 4 volume of sample buffer containing 250 mM Tris, 40% glycerol, 8% SDS, 4% S-mercaptoethanol and 0.002% bromophenol blue (pH 6.8). Expression of bcl-2 was analized by 10% SDS-PAGE gel electrophoresis. Primary antibody was mouse IgG specific to Bcl-2 (Santa Cruz) and secondary antibody was anti-mouse IgG HRP (Santa Cruz). Expression of Bcl-2 proteins was assessed by ECL (Amersham).

RESULTS AND DISCUSSION

Stimulation of TCDD-Induced Apoptosis by Estrogen

MCF-7 cells were maintained in the media supplemented with 10% FBS. After TCDD treatment for 24 hr, TCDD-induced cell death was examined (Fig. 1A).

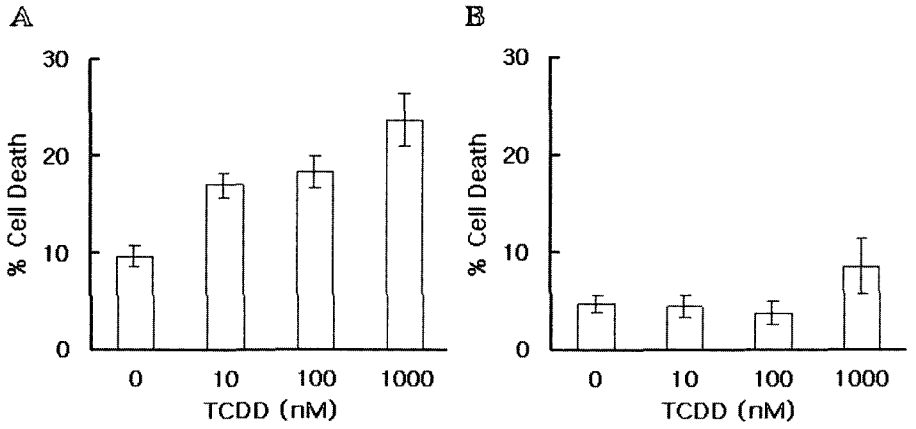

Fig. 1. Effects of estrogen on TCDD-induced apoptosis. (A) MCF-7 cells grown in DMEM containing 10% FBS were treated with TCDD for 24 hr and apoptosis was measured by FACS analysis after staining cells with propidium iodide. (B) MCF-7 cells were grown in phenol red-free DMEM in which all estrogen-like compounds were depleted. The experiment was repeated by three times and the error bar shows the standard deviation.

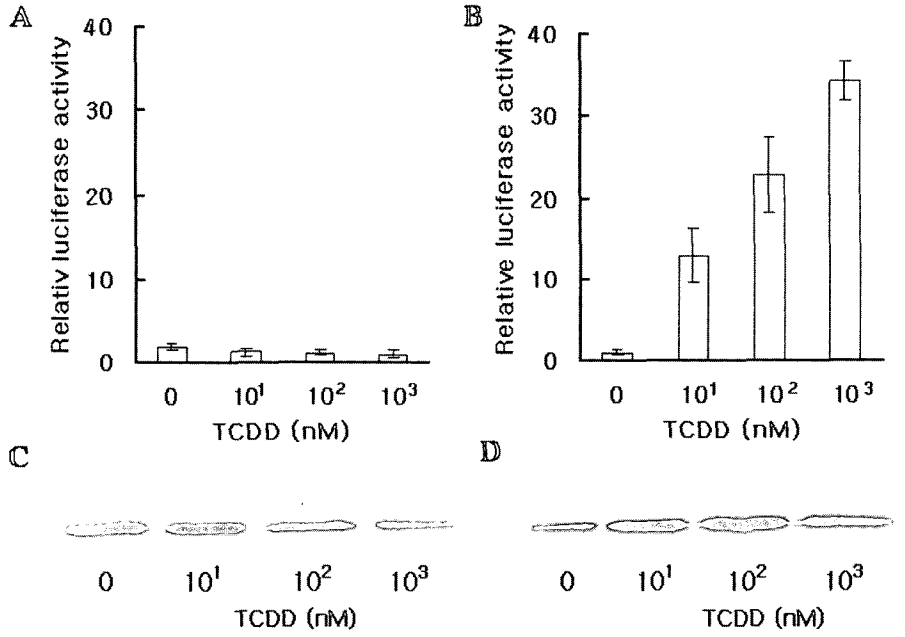

Fig. 2. Effects of estrogen on Bcl-2 expression. MCF-7 cells were maintained in DMEM containing 10% FBS (A, C) or phenol-red free DMEM in which all estrogen-like compounds were depleted (B, D). (A) Cells grown in the media containing 10% FBS were treated with specified concentrations of TCDD for 4 hr and the expressions of bcl-2 gene were examined by measuring the activity of reporter luciferase in cells transfected with the construct harboring bcl-2 enhancer elements and the reporter gene. (B) Cells were grown in the media depleted of all estrogen-like compounds. (C) Expression of Bcl-2 proteins in cells received serum components was analyzed after TCDD treatment by immunoblot analysis. (D) Expression of Bcl-2 proteins in TCDD treated cells maintained in the media depleted of all estrogen-like compounds was analyzed by immunoblot analysis.

TCDD, however, fails to induce apoptosis if cells were maintained in the media depleted of all estrogen-like compounds (Fig. 1B). Since Bcl-2 protein is anti-apoptotic protein, the relationship between the expression level of bcl-2 enhancer and cellular levels of Bcl-2 protein were assessed. The enhancer region of bcl-2 gene was subcloned in a reporter vector containing luciferase gene, and the transcriptional activity was monitored (Figs. 2A and 2B). The inhibition of Bcl-2 expression was observed in cells grown in the media containing 10 % FBS (Fig. 2C). Interestingly the expression of Bcl-2 was elevated after TCDD treatment if cells were cultured in the media in which all estrogen-like compounds were depleted (Fig. 2D).

Inhibition of the Enhancer Activity of bcl-2 Gene by co-Treatment of TCDD and Estrogen

As many as seven possible DREs that might bind with AhR-TCDD complex were estimated to reside in bcl-2 gene. Three of them were between promoter 1 and promoter 2 at positions -909, -865, and -148. Two of DREs were in the coding region at positions +7 and +165. The rest of DREs seemed to be located in 3' UTR at positions +1227 and +1381. D5, one of the three complete DRE sequence was estimated to be located in 30 bp upstream of E3. Among DREs, D5 was anticipated to be most sensitive to the binding of ER-estrogen complex to E3, estrogen receptor element since D5 is located in only 30 bp upstream of E3. Therefore, mutant enhancer vectors, mutD5B-E3 and mutD5T-E3 (Fig. 3B) were constructed and were examined for its ability to activate the transcription of bcl-2 in cells co-treated with TCDD and estrogen.

Cell were maintained in the media containing 10 nM estrogen (17β-Estradiol) and were transfected with D5-E3 construct harboring E3. Cells were then treated with 10 nM TCDD or TCDD plus 10 nM estrogen for 4 hr and luciferase activity was assessed as a function of bcl-2 enhancer activity. Data in Fig. 4 shows that TCDD treatment elevated the transcriptional activation of bcl-2 in transfected cells. Co-treatment of TCDD with estrogen abolished the activation of bcl-2 expression by TCDD. Data suggest that binding of AhR-TCDD complex to the D5 sequence might hinder the ER-estrogen complex binding to E3 element. The two elements of D5 and E3 are so close that AhR-TCDD complex and ER-estrogen complex may not bind to their corresponding *cis* elements simultaneously. Bcl-2 expression was not inhibited after the co-treatment of TCDD with estro-

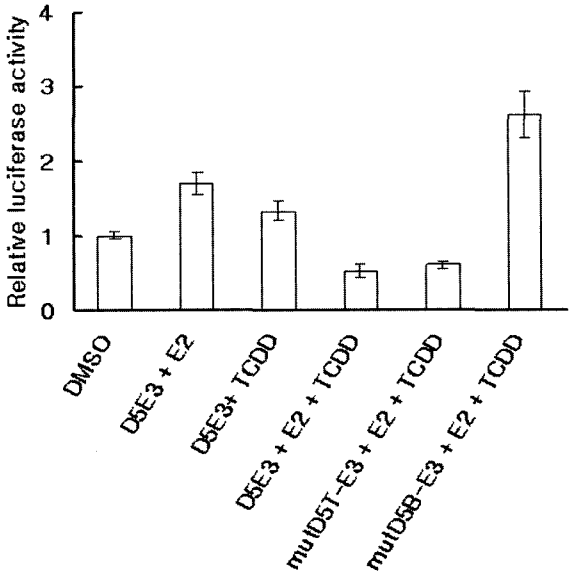

Fig. 4. Estrogen inhibition of TCDD-induced apoptosis in MCF-7 cells. Cells were transfected with wild-type D5-E3 or mutated enhancer elements. Cells were then maintained in the media depleted of all estrogen-like compounds for 7 days. Cells were treated with 10 nM TCDD either in presence (D5-E3+E2+TCDD) or in the absence of 17-β estradiol (E2) for 4 hr (D5-E3+TCDD). Cells were transfected with the mutant vector, mutD5B-E3 which lacks either its binding ability with the AhR-TCDD complex or transcriptional activity after TCDD treatment (mutD5-E3+E2+TCDD).

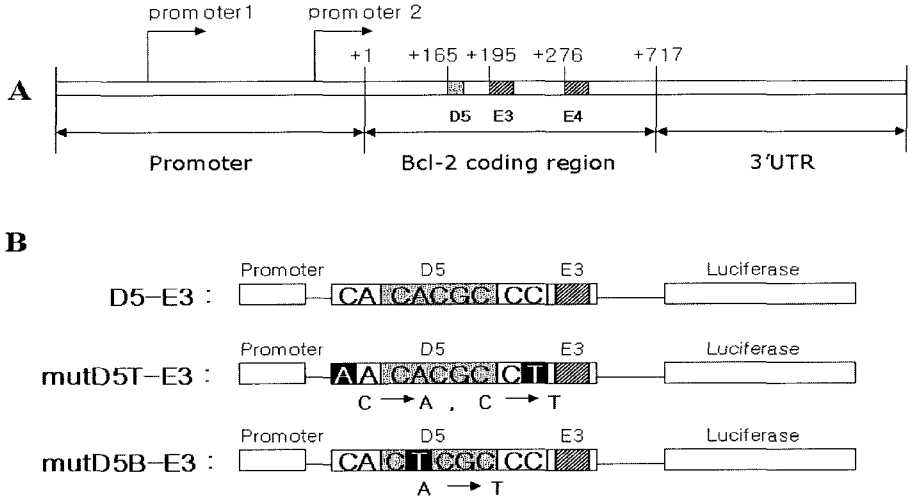

Fig. 3. Construction of mutant enhancers of bcl-2. (A) Schematic structure of human bcl-2 gene. E3 is an enhancer element that binds with estrogen. D5; DRE sequence is located 30 bp in front of E3. (B) D5-E3; wild-type D5 and E3. MutD5T-E3; mutD5-E3 was made by PCR using mutant oligomers to obliterate its transcriptional activity. MutD5B-E3; mutD5B-E3 lacks its binding activity to D5. The genes were inserted into between restriction enzyme site Sma I and Bgl II.

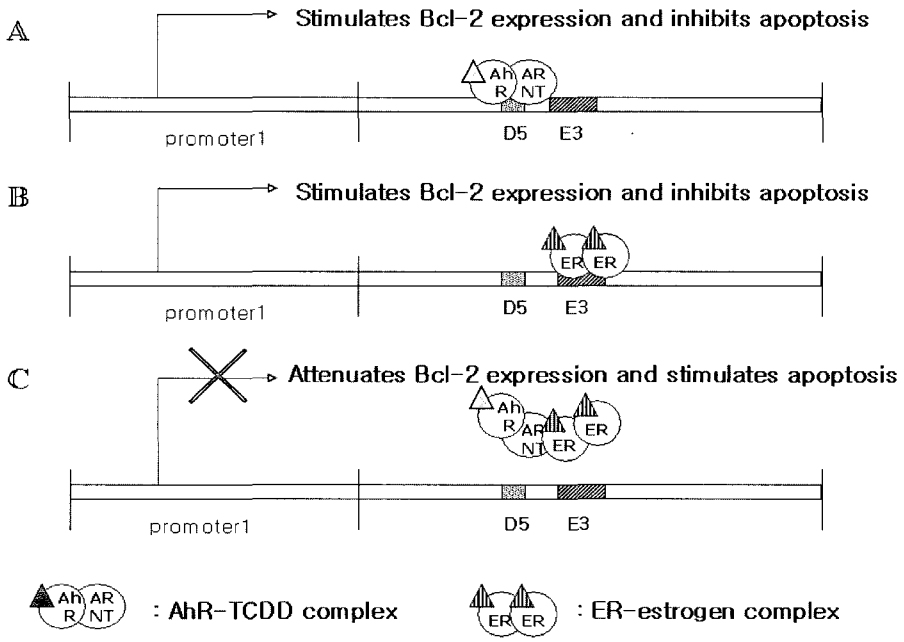

Fig. 5. Schematic representation of estrogen inhibition of Bcl-2 expression during TCDD-induced apoptosis. (A) AhR-TCDD complex binds to the D5 to activate Bcl-2 expression. (B) ER-estrogen complex binds to the E3 to activate Bcl-2 expression. (C) Competition between the estrogen complex to the D5 and the AhR-TCDD complex to E3 was shown.

gen in cells transfected with a mutant D5, mutD5B-E3, which lacks the ability to bind with AhR-TCDD complex, suggesting that the binding of AhR-TCDD complex to D5 element is essential for the regulation of bcl-2 expression in cells treated with TCDD or co-treated with TCDD and estrogen. This result is supported by the work of Shen and Whitlock (1992) who showed the indispensable role of the receptor-enhancer interaction at DRE on the transcriptional activation. In this study, we propose that the binding of ER-estrogen complex to E3 element compete with the binding of AhR-TCDD complex to DRE sequence in bcl-2 gene (Fig. 5). This explanation may advocate the suggestion that the binding of AhR-TCDD complex to enhancer element bend DNA at the site of interaction (Elferink and Whitlock, 1990).

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