TRANSCRIPTIONAL ACTIVATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-4 (IGFBP-4) BY 17β-ESTRADIOL AND INHIBITION BY TCDD

Chunhua Qin and Stephen Safe

Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University, TX 77840 USA

Introduction

IGFBP-4 is one of a family of proteins that bind IGFs and regulate their action in multiple tissues. IGFBP-4 is expressed in breast cancer cells and 17β-estradiol (E2) induces IGFBP-4 mRNA and protein levels in estrogen receptor (ER) positive breast cancer cells and antiestrogens inhibit these responses. A recent study in our laboratory has investigated the mechanism of ER action; analysis of the 5'-promoter region of IGFBP-4 has identified GC-rich sites at -559 to -553 and -72 to -64 that bind Sp1 protein and are important for E2-responsiveness[1]. ER/Sp1 action at GC-rich sites does not require direct interaction of ER with 5'-promoter genomic DNA, and IGFBP-4 is one of a growing number of E2-responsive genes regulated by this transcription factor complex in breast cancer cells. This study reports that like a number of other E2-regulated genes, TCDD inhibits E2-induced IGFBP-4 gene and protein expression and also inhibits reporter gene (luciferase) expression in MCF-7 cells transfected with construct (pIGFBP4-1) containing the -1245 to +45 region of the IGFBP-4 gene promoter.

Materials and Methods

Chemicals and Cell Culture. All chemicals and biochemicals were purchased from commercial sources. Cells were obtained from American Type Culture Collection (ATCC). MCF-7 cells were maintained in MEM media with phenol red and supplemented with 2.2 g/L sodium bicarbonate, 10% FBS and 10 ml/L antibiotic solution (Sigma). Cells were grown in 100-cm^2 culture plates in an air:carbon dioxide (95:5) atmosphere at 37°C. Cells were maintained in phenol red free DME/F12 media with 5% stripped FBS for at least 48 hr prior to treatment with DMSO, E2, E2 + TCDD, and TCDD alone.

Northern Blot Analysis. Total RNA was isolated using STAT-60™ Kit; 20 μg of total RNA was separated on a 1.2% agarose/1 M formaldehyde gel, transferred onto nylon

membrane, and hybridized at 65°C with ^{32}P-labeled IGFBP-4 cDNA probe. The cDNA probe was made by RT-PCR using IGFBP-4 primers (forward 5'-TGCAGAAGCACTTCGCCAAA-3', reverse 5'-ACAGGACTCAGACTCAGACT-3'). Blots were visualized by autoradiography and quantitated using a densitometer. The membrane was then stripped and re-probed with β-tubulin as control.

Western Blot Analysis. MCF-7 cells were seeded in DME/F12 media with 5% charcoal-dextran stripped serum, and treated hormone/ chemicals. Media were collected after 16~24hr and concentrated using Microcon-10 microconcentrators. At the same time, cells in each well were counted to normalize the final results. Protein samples were heated at 100°C for 2 min, separated by 12% SDS-PAGE, and transferred to Hybond ECL NC membrane (Amersham Life Sci.). The NC membrane was probed with 1:500~1000 primary IGFBP-4 antibody (Santa Cruz Biotech) and 1:1000~2000 secondary antibody. Blots were visualized by autoradiography and quantitated using a densitometer.

Transient Transfection and Luciferase Activity Assay. The IGFBP-4 gene promoter (from -1245 to +45) was cloned into the *Kpn*I and the *Xho*I polylinker site immediately upstream from firefly luciferase expression gene in pGL2 vector. Five μg of this plasmid DNA, 2.5 μg hER, and 1.0 μg β-galactosidase (β-Gal) expression plasmids were co-transfected into MCF-7 cells using calcium-phosphate co-precipitation method. After transfection for 16-20 hr, cells were washed with PBS and treated with DMSO, E2, E2 + TCDD, and TCDD alone. After treatment for 40 hr, Cells were harvested and assayed for luciferase (Promega) and β-Gal (Tropix) activities. LumiCount (Packard Instrument Co.) was used to quantitate luciferase and β-Gal activities, and β-Gal activity was used to normalize luciferase activity.

Results and Discussion

The results summarized in Figure 1 show that 10 nM E2 induced IGFBP-4 mRNA levels (>2 fold) in MCF-7 cells whereas 10 nM TCDD did not affect expression of this gene. In contrast, after co-treatment with TCDD plus E2, the hormone-induced response was completely inhibited by TCDD. In a parallel study, the effects of the same treatments on immunoreactive IGFBP-4 protein were also determined and gave similar results. E2 induced a >4-fold increase in immunoreactive IGFBP-4 protein and this response was inhibited by TCDD (Figure 2).

A recent study has demonstrated that GC-rich sites within the IGFBP-4 gene promoter are required for ER action[1] and in MCF-7 or T47D breast cancer cell lines transiently transfected with pIGFBP4-1 (containing a -1245/+45 promoter insert), E2 induced reporter gene activity (2- to 3-fold). TCDD alone was inactive, however, in cells

cotreated with TCDD plus E2 there was a significant decrease in hormone-induced luciferase activity (Figure 3). These results suggested that promoter elements within -1245 to +45 region of this promoter are required for Ah receptor-mediated inhibition. Previous studies with the Cathepsin D and pS2 gene promoters[2,3] have identified pentanucleotide (GCGTG) inhibitory dioxin response elements (iDREs) that are required for the antiestrogenic effects of TCDD and an iDRE has also been identified in the IGFBP-4 gene promoter at -1170. Ongoing studies are investigating the functional role of this iDRE and possibly other genomic sequences that are required for Ah receptor-mediated inhibition of E2-induced IGFBP-4 gene expression in breast cancer cell lines.

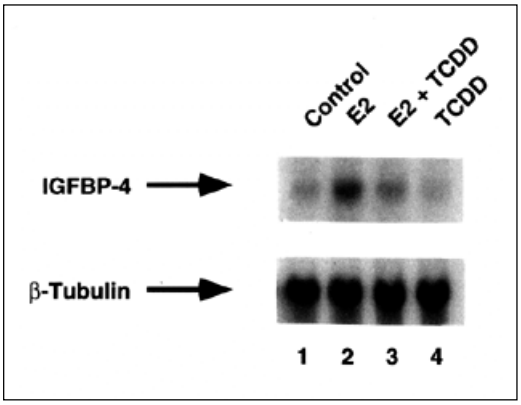
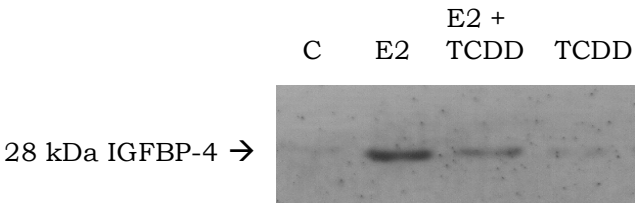


Figure 1. Induction of IGFBP-4 mRNA levels by E2 (24 hr), and inhibition after cotreatment with TCDD.



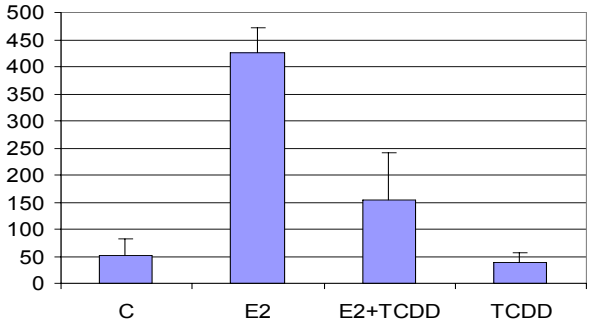


Figure 2. IGFBP-4 protein levels in MCF-7 cell conditional media after treatment with E2 for 36 hr.

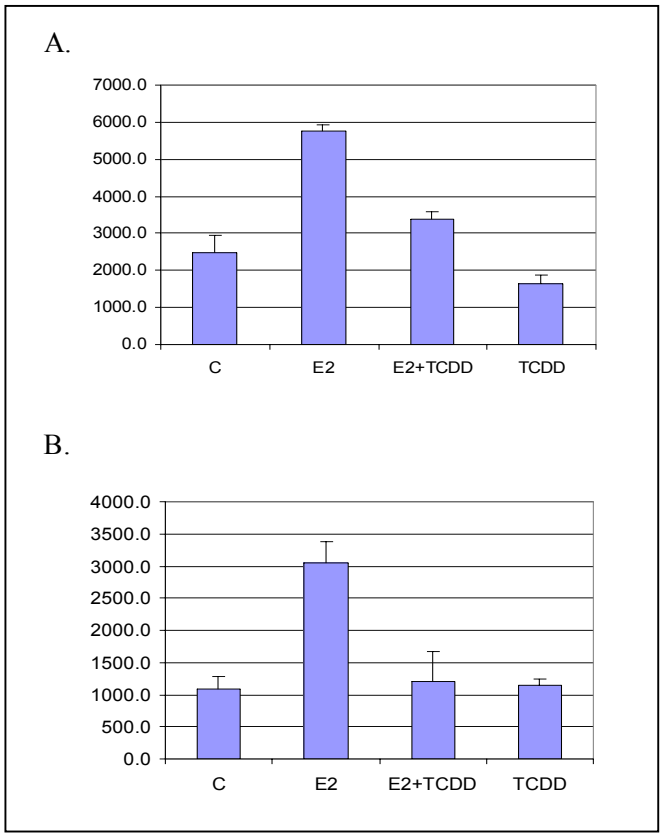


Figure 3. Inhibition of E2-induced luciferase activity in MCF-7 (A) and T47D (B) cells cotransfected with pIGFBP4-1 and hER.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health (ESO4176 and ESO9106), the Texas Agricultural Experiment Station and the Sid Kyle chair endowment.

REFERENCES

1. Qin, C., Singh, P., and Safe, S. (1999) *Endocrinology* **140**, 2501-2508
2. Krishnan, V., Porter, W., Santostefano, M., Wang, X., and Safe, S. (1995) *Mol. Cell. Biol.* **15**, 6710-6719.
3. Gillesby, B., Santostefano, M., Porter, W., Wu, Z. F., Safe, S., and Zacharewski, T. (1997) *Biochemistry* **36**, 6080-6089.