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Transcriptional Suppression of Estrogen Receptor Gene Expression by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

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TCDD, the most potent congener of the polychlorinated dioxins, has been shown to be an antiestrogen. The mechanisms of TCDD-induced antiestrogenicity are still under investigation. In this study, we investigated the effects of TCDD on the expression of the estrogen receptor (ER) gene. We studied the levels of un-spliced ER transcript (hnRNA) as well as the ER mRNA in ovary, uterus and liver of TCDD-treated mice with different genetic backgrounds. To quantitate the ER hnRNA levels, the intron and exon boundary of ER hnRNA was amplified by competitive RT-PCR. The ER mRNA from these mice was quantitated by competitive RT-PCR amplifying exons separated by an intron. ER hnRNA and ER mRNA levels were quantitated 4 days after a single i.p. dose of TCDD (5 μg/kg) in female C57BL/6J (B6) mice, which carry the responsive allele to TCDD. TCDD treatment significantly ($p < 0.05$) suppressed the levels of ER hnRNA in the ovary (27.4%) and uterus (21.9%). The decreases in ER hnRNA were coordinated with significant ($p < 0.01$) decreases in ER mRNA in ovary (57.7%) and uterus (37.6%). There was a significant decrease (20.3%, $p < 0.05$) in liver ER mRNA, however, the changes of ER hnRNA in liver were not significant. The coordinated decreases in ER hnRNA and mRNA in TCDD-treated mice suggest a suppression of transcription of the ER gene. We performed the same study on DBA/2J (D2) mice, which possess the "non-responsive" allele of the aryl hydrocarbon receptor (AhR). These mice demonstrated no significant decrease in either the ER mRNA or hnRNA after TCDD treatment. Overall, these results suggest that TCDD suppresses the gene expression of the ER receptor by decreasing its transcription, and the AhR plays an important role in mediating this response. © 1998 Elsevier Science Ltd. All rights reserved.

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INTRODUCTION

TCDD is an ubiquitous environmental contaminant of significant public concern. It is a prototype of polyhalogenated aromatic hydrocarbons and the most potent congener of these compounds. Many of these compounds are persistent contaminants in the environment and in the mammalian body, and have been shown to cause various toxic responses in humans and animals. TCDD causes toxic responses mainly by altering normal expression of many critical genes. The commonly recognized model proposes that TCDD exerts its toxic effects by first binding specifically to a cytosolic aryl hydrocarbon receptor (AhR) [1,2], which is a basic helix–loop–helix (bHLH) transcription factor [3,4]. Upon binding to TCDD, the AhR translocates into the nucleus and dimerizes with another bHLH protein, the aryl hydrocarbon receptor nuclear translocator (ARNT) [5,6]. The heterodimer then binds to a consensus sequence (T/ANCGTG) known as the xenobiotic response element (XRE) in the promoter regions of genes (notably P450 1A1), thereby altering gene expression [7]. The toxic effects caused by TCDD include chloracne, wasting syndrome, birth defects, immune suppression and endocrine disruptions, such as the antiestrogenic effects [8–13].

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The antiestrogenicity of TCDD has been studied by several investigators and the following lines of evidence have been considered to be important: (1) TCDD-activated AhR interferes with the binding of ER to its cognate enhancer sequences, i.e. estrogen response element (ERE) [14]; (2) TCDD treatment reduces the nuclear translocation of the ligand-activated ER [12, 15]; (3) TCDD treatment decreases ER protein and ligand binding [16, 17], which may result from a reduced ER mRNA level [18]; (4) TCDD-activated AhR interferes with the transcription of the ER gene by binding to its promoter resulting in steric hindrance of transcription [19]; (5) TCDD induces cytochrome P450s which accelerate the metabolism of estrogens [13, 20].

The expression of the ER gene is autoregulated [21]. It has been shown that the promoter region of the human ER gene contains XREs and also potential *Sp*1 sites [19], which are known to interact with AhR [22]. Nonetheless, experimental evidence is lacking regarding the effects of TCDD on the transcription of ER gene. In an earlier study, we showed that TCDD decreases ER mRNA levels in several tissues [18]. A reduced pool of the steady-state level of mRNA could result from reduced gene transcription, or alternatively, it could be due to accelerated mRNA turnover or both. In this study, we examined the mechanism of this reduction.

Study of transcriptional regulation of ER gene requires measurement of the steady-state level of mRNA as well as the kinetics of output of hnRNA from pre-assembled cellular transcriptional machinery. The techniques that are often used include Northern blot and nuclear run-on assays. These techniques are neither quantitative nor sensitive enough for studying transcription of ER gene which is expressed at low levels in many tissues. In this study, we extended the competitive RT-PCR procedure to evaluate the changes of the primary, un-spliced ER transcript, the ER hnRNA. Similar PCR-based methods have been used by other investigators [23–27] to study transcriptional regulation in place of the conventional run-on assay which is laborious, less accurate and requires handling large amounts of radioisotopes.

In this study, we measured the relative changes of ER mRNA and hnRNA in TCDD-treated C57BL/6J (B6) mice and DBA/2J (D2) mice. The B6 mice express the AhR from the Ah^b allele that has a high binding affinity and capacity for TCDD, while D2 mice express AhR from Ah^d allele with much lower binding affinity and capacity for TCDD [28]. These allelic differences provide the genetic backdrop for testing the involvement of the AhR in the TCDD-induced antiestrogenicity.

MATERIALS AND METHODS

Chemicals and reagents

Restriction enzymes, T4 ligase, 100 bp DNA ladder, SuperScript II RNase H^- reverse transcriptase, Taq DNA polymerase, Random primer and custom-made oligonucleotide primers were purchased from Gibco (Gaithersburg, MD). GENECLEAN II kit was from Bio101 (La Jolla, CA), spin columns were obtained from Qiagen (Chatsworth, CA), TA cloning vector (pCRII) was from Invitrogen (San Diego, CA). The sequence analysis was performed in the core facility at UMDNJ-Robert Wood Johnson Medical School. TCDD was kindly provided by Dr Safe at Texas A&M.

Animals

Female C57BL/6J and DBA/2J mice (6–8 weeks old) were purchased from Charles River Laboratories (Wilmingon, MA) and were housed under standard conditions. The mice were acclimated to the animal facility for 7–10 days prior to the experimentation. The animals were given a single dose of TCDD (5 μg/kg, i.p.) or an equivalent volume of vehicle (corn oil) and sacrificed 4 days post-dosing. All animal studies were approved by the Institutional Animal Care and Use Committee of UMDNJ-Robert Wood Johnson Medical School.

Inverse PCR to amplify the DNA sequences across intron–exon boundary

In order to quantitate the hnRNA by RT-PCR, it was first necessary to obtain an intron sequence of the ER gene. The mouse ER intron sequences were not available to us, so we elected to use an inverse PCR scheme to obtain a segment of the intron for its sequence. The general scheme for inverse PCR is illustrated in Fig. 1. Mouse genomic DNA was digested with several restriction enzymes including *Bam*H I (one microgram of each digestion in total 20 μl) under the standard conditions. One microliter of each digested product was ligated with T4 ligase (total 10 μl contained 1 × ligation buffer, 1 units T4 ligase) at 14°C overnight. The ligated products were amplified with primers P1 (AGCTCAGCTCCTTCTCATTCTTTCC) and P2 (TGGATGTGGTCCTTC–TCTTCCAG) and the PCR conditions were 94°C for 45 s, 56°C for 60 s, and 72°C for 60 s, which was repeated for 30 cycles. One microliter of the PCR product was further amplified with P1 and nested primer P3 (GTGCTGGACAGAAACGTGTA) under the following conditions: 94°C for 45 s, 56°C for 60 s and 72°C for 60 s, which was repeated for 30 cycles. The PCR products were then cloned into the TA cloning vector for sequence analysis. The intron sequences obtained were used together with the down-stream

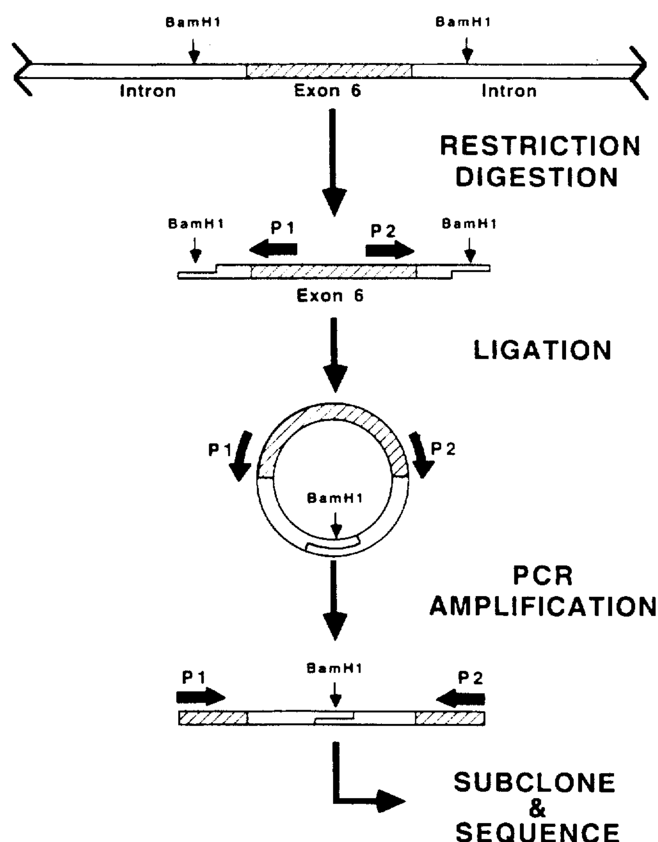

Fig. 1. Schematic illustration of the inverse PCR for cloning intron sequences bordering a known exon. Mouse genomic DNA is digested by restriction enzyme (*Bam*H I) and circularized by ligation with T4 ligase. The ligated DNA is amplified in PCR reaction with the exon primers P1 and P2 facing outward as shown. The PCR products are then cloned into a plasmid cloning vector for sequence analysis.

exon sequences to design a primer pair for RT-PCR amplification of the ER hnRNA.

Generation of DNA competitors

Generation and use of the competitors for ER mRNA, β-actin mRNA and ER hnRNA were based on a procedure described by Überla *et al.* [29]. The generation of ER and β-actin mRNA has been described in our earlier study [18]. Briefly, ERint-1 (GTGTC-AAACATGTCCACTG) and ERint-2 (GGGAAAG-AATGAGAAGGAG), which bracket DNA sequences crossing an intron–exon boundary of the mouse ER genomic sequences were used to randomly amplify the mouse total genomic DNA under the following conditions: $10 \times$ T*aq* buffer (5 μl), 200 μM dNTP, 0.1 μg mouse genomic DNA, 2.5 units T*aq* polymerase, 0.2 μM each of primers in total 50 μl. The PCR amplification cycles were 94°C for 40 s, 37°C for 60 s, and 72°C for 45 s, the amplification was repeated for 30 cycles. The PCR products were separated on a 1.2% agarose gel and the products, in the range of 200 to 500 bp, were isolated using a GENECLEAN II kit following the manufacturer's instructions. The isolated products were inserted into a TA cloning vector and the recombinant plasmids were purified using a Qiagen spin column. The primer binding sites in the competitor fragments were checked by sequence analysis to eliminate the competitors that contained incorrect primers, and the concentrations of the DNA constructs were measured by UV-spectroscopy. Finally, the constructs were linearized by restriction enzyme digestion and were serial-diluted for use as the DNA competitor.

Competitive RT-PCR

Mice were sacrificed by cervical dislocation and total RNA was isolated from selected tissues [30]. RT-PCR quantitation of ER mRNA has been described elsewhere [18]. To quantitate hnRNA, each sample of isolated total RNA was first digested with *Eco*R I (5 μg total RNA, 20 units of the restriction enzyme, 20 μl total volume at 37°C for 15 min). The *Eco*R I digests the potential DNA template to prevent it from interfering with the PCR quantitation. The RNA samples were then reverse-transcribed into first strand cDNA under the following conditions: 500 ng random primer, 4 μl of total RNA, 4 μl of $5 \times$ first strand buffer, 4 μl of 0.1 mM DTT, 4 μl of 10 mM dNTP mixture, 200 Units of SuperScript II RNAse H^- reverse transcriptase, and the reaction was incubated at 42°C for 60 min. Equal aliquots of the reverse-transcribed product were co-amplified with serial-diluted competitor in the following PCR reaction containing $1 \times$ T*aq* buffer, 200 μM dNTP, 4 μl of reverse-transcribed product and 4 μl of the competitor. The PCR conditions were: 92°C for 45 s, 56°C for 45 s and 72°C for 45 s, which was repeated for 30 cycles. The PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining. The results were recorded on Polaroid Positive/Negative film and the intensity values were obtained by scanning with a densitometer (BioImage, Ann Arbor MI). The results were analyzed using linear regression.

RESULTS

Animals and TCDD treatment

Four days post-dosing, there were no overt signs of toxicity and no significant changes in body weight associated with TCDD treatment. However, there was a significant increase in the liver-to-body weight ratios in B6 mice (28%, $p < 0.01$, $n = 5$). TCDD-treated D2 mice exhibited a slight, yet statistically insignificant, increase (8.6%, $p > 0.1$, $n = 5$) in liver to body weight ratio (Fig. 4(D)).

Inverse PCR to amplify the DNA sequences across the intron–exon boundary

To develop a quantitative PCR method to measure ER hnRNA, we first had to clone the intronic

sequences adjacent to an exon. Our method of obtaining these sequences was modified from published protocols [31,32]. Mouse genomic DNA was digested with *Bam*H I and ligated to form circular DNA (Fig. 1). Although several PCR products were obtained after the nested PCR amplification, the product from initial *Bam*H I digested genomic DNA yielded a distinct PCR product (data not shown) and was subsequently cloned into the TA cloning vector. The cloned DNA fragments were then sequenced and found to contain the intron sequences bordering an exon starting at 1573 of the coding sequences of mouse ER gene (Fig. 2) [33]. The PCR primers (ERint-1 and ERint-2) were selected based on the newly obtained intron sequences. Care was taken to include a restriction site (*Eco*R I) during the primer design (Fig. 2). The *Eco*R I site was used to eliminate the contaminating genomic DNA by restriction digestion prior to the PCR quantitation.

Generation of DNA competitors

Generation of DNA competitors for quantitation of the ER mRNA were described previously [18]. The same procedure was used for creating the competitor for quantitating ER hnRNA. The essence of this method is that under conditions of low annealing temperature (37°C), primers anneal nonspecifically at different positions along template DNA. The PCR products generated in this manner are a mixture of DNA fragments of different lengths. However, all DNA fragments have the primer sequences at both ends allowing primers to bind. By cloning these DNA fragments into plasmid vectors, competitor DNAs are obtained. Using this method, a DNA fragment of 400 bp was isolated, serial-diluted and used as the competitor for quantitation of ER hnRNA (Fig. 3).

Fig. 2. Intron sequences and RT-PCR primers for quantitating ER hnRNA. The intron sequences (in small letter) were obtained by sequence analysis of the DNA fragments from the inverse PCR. Based on sequences of the intron and adjacent exon, PCR primers, ERint-1 and ERint-2, were selected to amplify the segment crossing the intron–exon boundary. The ERint-1 and 2 were also used for generating the competitors for quantitating the ER hnRNA. The *Eco*R I site was used for restriction digestion of the RNA samples prior to the reverse-transcription to eliminate the contaminating genomic DNA.

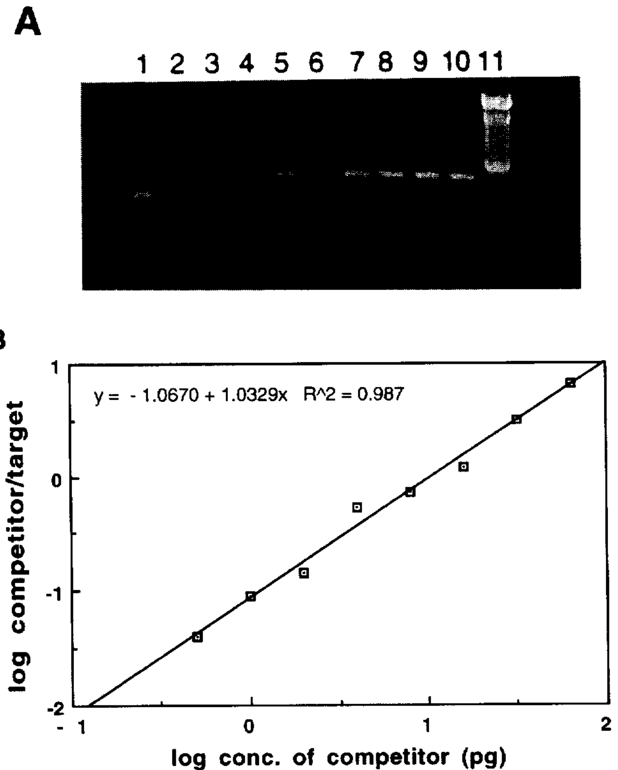

Fig. 3. Quantitation of ER hnRNA. (A) Reverse-transcribed ER total RNA samples were co-amplified with serial-diluted competitor. Lanes 1–10 correspond to PCR tubes containing 0.01, 0.5, 1, 2, 4, 8, 16, 32, 64 and 100 pg of the competitor, respectively, and lane 11 corresponds to 100 bp DNA marker. The PCR conditions are described in Section 2. The PCR products were separated by 1.5% agarose gel electrophoresis, and density of the bands was quantitated by scanning with a densitometer. The upper bands represent the PCR products of the competitor and the lower bands represent the PCR products of the ER hnRNA. (B) the ratios of competitor vs target were plotted against the dilution of competitor DNA. The standard curve was obtained by linear regression (r>0.98). This graph is representative of several experiments that always demonstrate nearly identical linear relation. On the standard curve, the point where the log of ratio equaled 0 (competitor/target = 1) is considered the concentration of the reverse-transcribed ER hnRNA. Samples were measured in triplicates, and the quantity of reverse-transcribed ER mRNA was calculated according the standard curve.

Quantitation of the decrease of the ER hn RNA and mRNA

To quantitate ER hnRNA, total RNA samples were first digested with *Eco*R I to eliminate the contaminating DNA templates prior to reverse-transcription. For ER mRNA quantitation, the primer-binding sites are on exons separated by an intron, therefore restriction digestion is not neccessary. After the reverse-transcription, equal quantities of the reverse-transcribed products were co-amplified with serial-diluted competitors for ER hnRNA and mRNA, respectively. The competitor fragments compete with the target (cDNA of ER hnRNA) for primers and the other substrates. A log–log plot of the ratios of the competitor and target (ER hnRNA) vs the concentration of

the competitors was linear (Fig. 3(B)). The point where the ratio of the competitor to target is equal to 1 represents the quantity of the reverse-transcribed ER hnRNA. To determine the percentage decreases of the ER hnRNA, the levels of ER hnRNA were normalized with the mRNA level of β-actin, which is a commonly used constant gene and did not change after TCDD treatment. Significant suppression ($p < 0.05$) of ER hnRNA in TCDD-treated female B6 mice was found in the ovary (27.4%), uterus (21.9%) (Fig. 5). The decrease in liver, which was not statistically significant, was 13.8%.

The standard curve for quantitating ER mRNA was described previously [18]. The changes in ER mRNA were normalized against β-actin. Reduction of ER mRNA in B6 mice was found in ovary (57.7%, $p < 0.01$), and uterus (37.6%, $p < 0.01$) and liver (20.3%, $p < 0.05$) (Fig. 4). There were slight changes (reduction) in both ER hnRNA and mRNA in tissues from the D2 mice, ranging from 1–3% and 5–8%, respectively. However, the decreases were not statistically significant (Figs 4 and 5).

DISCUSSION

In an earlier study, we reported that TCDD treatment suppressed levels of ER mRNA in multiple tissues of female CD-1 mice with the suppression in ovary and uterus being most pronounced [18]. The decreases in ER mRNA after TCDD treatment raised the possibility that expression of the ER gene is regulated by the AhR at the level of transcription. The current study was designed to investigate this possibility. By using competitive RT-PCR, we demonstrated TCDD treatment caused significant decreases in ER hnRNA levels with decreases in the ovary and uterus being 27.4%, and 21.9%, respectively (Fig. 5). There were coordinated decreases of ER mRNA in ovary (57.7%) and uterus (37.6%) of the B6 mice (Fig. 4). There was also a significant decrease (20.3%, $p < 0.05$) in liver ER mRNA, however, the decrease of the hnRNA in liver was not statistically significant (13.8%, $p > 0.05$). A reduced hnRNA, which is the primary transcript of the gene, strongly argues for transcriptional regulation.

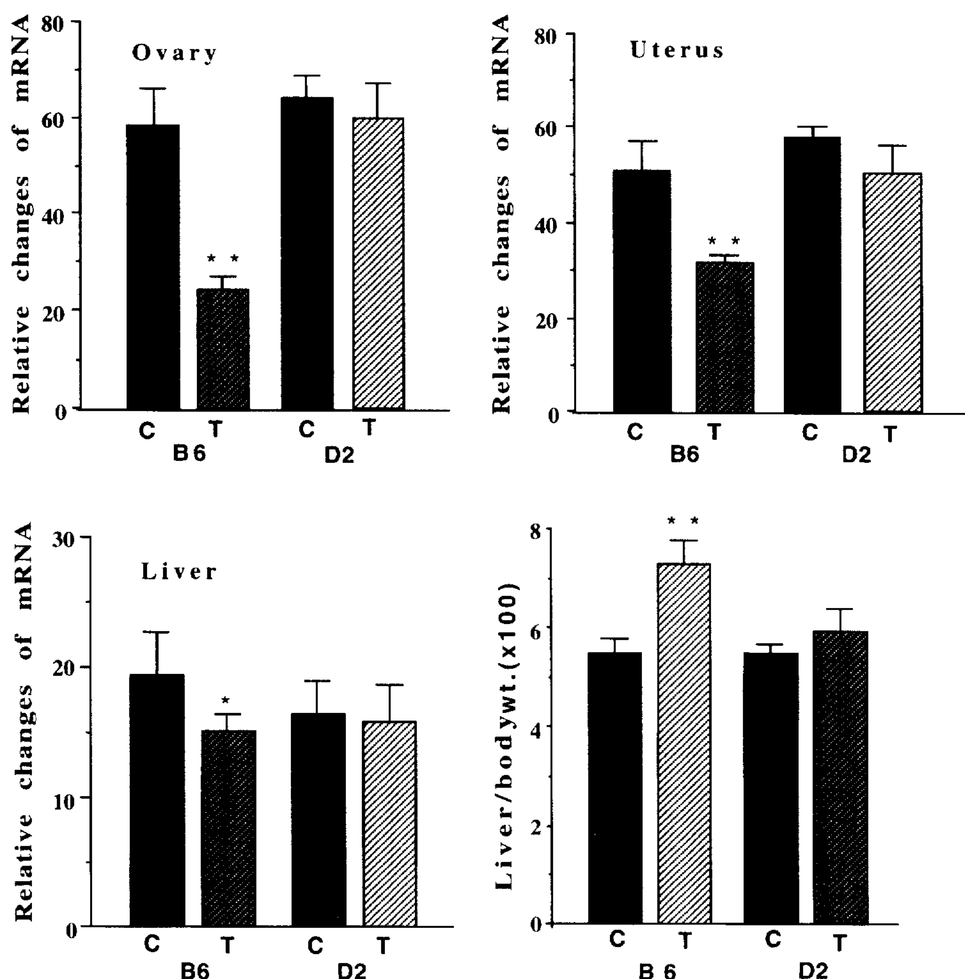

Fig. 4. Decreases of ER mRNA in tissues from TCDD-treated female B6 and D2 mice. (A), (B) and (C) TCDD-induced decreases of ER mRNA of the uterus, ovary, and liver of B6 mice. The ER mRNA changes were determined after normalization with constant genes (β-actin). (D) ratio of liver-body weight of control and TCDD treated mice (×100). *($p < 0.05$, $n = 5$); **($p < 0.01$, $n = 5$). C = control, T = TCDD treated.

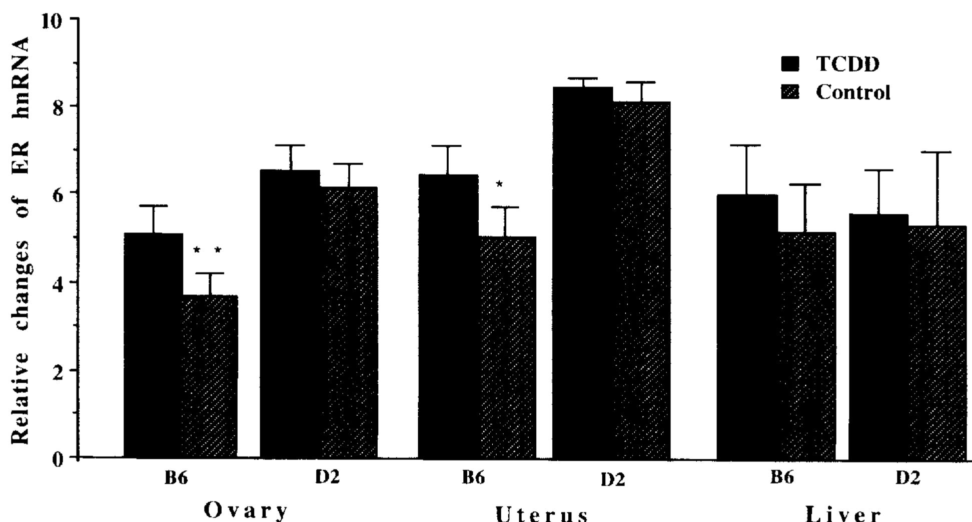

Fig. 5. Decreases of ER hnRNA in tissues from TCDD-treated female B6 and D2 mice. ER hnRNA in total RNA samples was quantitated by competitive RT-PCR using primer pair (ERint-1 and ERint-2) crossing intron–exon boundary of the hnRNA. The changes of ER hnRNA were determined based on the standard curve (Fig. 3(B)) by linear regression analysis. The relative changes of ER hnRNA of TCDD-treated vs control animals were determined after normalization against β-actin. *($p < 0.05$, $n = 5$); **($p < 0.01$, $n = 5$).

The mechanisms of the TCDD-induced suppression of the ER gene transcription are presently unknown. There is no evidence that TCDD or related compounds binds to ER, therefore, it is unlikely that TCDD competes for the ER ligand binding site with the estrogen. White *et al.* [19] uncovered several dioxin response elements in the promoter region of human ER gene, suggesting that a putative explanation for TCDD-induced toxicity is that the AhR complex may hinder the binding of transcription factor for ER gene transcription. It is also possible that the activated AhR competes with ER for some limiting transcription factor(s) as suggested by Kharat *et al.* [14].

Several steps of the ER signal transduction pathway have been shown to be affected by TCDD treatment. These include reduced translocation of ligand-activated ER to the nucleus, reduced protein levels of ER, and diminished binding of ER to EREs in estrogen-responsive genes. In addition, TCDD induces cytochrome p450 (notably CYP1A1, 1A2 and 1B1) which may result in accelerated metabolism of estrogens. Results of our earlier and present studies demonstrated that TCDD causes a down-regulation of transcription of the ER gene. While it is not known which of these effects are primarily responsible for the antiestrogenic action of TCDD, it is possible that the suppression of ER gene transcription contributes to the antiestrogenic outcomes. For example, down regulation of transcription would likely result in decreased ER protein levels which has been shown [16, 17]. It is conceivable that the decrease of ER protein, combined with a reduction of nuclear translocation of the ER, may cause significant reduction of the nuclear ER and, consequently, suppress ER-dependent gene expression.

By using competitive RT-PCR, this study clearly demonstrated that TCDD treatment decreased both ER hnRNA and mRNA levels, thereby suppressing transcription. The reductions of ER hnRNA in TCDD-treated B6 mice was less than those of ER mRNA. It is possible, for example, that by four days after TCDD treatment, the ER gene is beginning to recover toward its normal expression levels and the hnRNA-to-mRNA ratios have simply not reached equilibrium. Alternatively, it is possible that the down-regulation of ER gene transcription by TCDD also decreases the rate of splicing, which would in turn increase the hnRNA/mRNA ratio, or that TCDD increases mRNA turnover in addition to decreasing transcription. Further studies need to be conducted to address these specific aspects of TCDD control of ER transcription.

The most common method used for assessing transcriptional regulation is the nuclear run-on assay which measures the amount of hnRNA being produced from pre-assembled transcriptional machinery to reflect the actual rate of gene transcription. In this study, however, we found it necessary to employ our quantitative RT-PCR assay instead of nuclear run-on assays because of the low levels of ER transcription present in the tissues and because we were trying to detect a decrease in the already low level of transcripts. For a gene with low levels of transcription, nuclear run-on assay would require large amounts of nuclei which makes it impractical to perform. Changes in the levels of hnRNA determined by PCR-based methods have been used by many investigators to gauge the transcriptional regulation [23–27]. The sensitivity and quantitative nature of the PCR-based method makes it an attractive alternative to the nuclear run-on assay.

In order to design an intron primer for PCR amplification, one needs to know at least partial intron sequences. However, very often this information is not available for the gene of interest in the current data bases. The inverse PCR procedure described in this study makes it possible to isolate sequences bordering the intron and exon boundary relatively easily. The sequence information can then be obtained by sequencing the subcloned PCR products. This method circumvents the need to screen a genomic library for the relevant genomic clones when what is needed is only a small portion of the intron sequence.

Our data support the hypothesis that the AhR mediates the TCDD-induced down-regulation of the ER gene expression. Different alleles have been found for the AhR gene [34]. These allelic differences result in great variations in susceptibility to TCDD toxicity among different species and also among different strains of the same animal species [35]. The pivotal role of the AhR in mediating the toxicity of TCDD was recently confirmed by use of AhR "knock-out" mice (Ah −/−) [36]. It was found that they were relatively unaffected by a dose of 2000 μg TCDD/kg, which is 10 fold higher than that found to induce severe toxic and pathologic effects in their wild type littermates (Ah +/+) [37] and 400 fold greater than the TCDD dose used in our study. Mice responsive to TCDD, such as the B6, were found to express Ah^b alleles, and the non-responsive strains, such as the D2 mice, were found to express Ah^d alleles [34, 38]. The AhR expressed by the Ah^b alleles was found to have about 10 fold higher binding affinity than that expressed by the Ah^d alleles [28]. In the present study, the rather large difference between B6 and D2 mice in the levels of ER mRNA and hnRNA are consistent with the hypothesis that the AhR regulates the antiestrogenic effects of TCDD.

In conclusion, the results of this study demonstrated that mRNA of the ER receptor is suppressed by TCDD at the level of transcription via an AhR-mediated process. The significant decrease of ER in ovary suggests that ER-dependent ovarian function is particularly sensitive to the antiestrogenic effects of TCDD at a low dose.

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