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EVIDENCE FOR THE MECHANISM OF ACTION OF THE 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN-MEDIATED DECREASE OF NUCLEAR ESTROGEN RECEPTOR LEVELS IN WILD-TYPE AND MUTANT MOUSE HEPA 1c1c7 CELLS

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Abstract—Treatment of wild-type Hepa 1c1c7 cells with 1 nM [^{3}H]-17β-estradiol resulted in the rapid accumulation of the nuclear estrogen receptor complex whose levels were maximized within 1 hr. Cotreatment of the cells with 10 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and [^{3}H]-17β-estradiol did not affect the nuclear estrogen receptor levels 1 hr after addition of the radioligand; however, pretreatment of the cells for 1, 6, 24 or 42 hr with 10 nM TCDD prior to the addition of the radiolabeled hormone caused a greater than 50% decrease in nuclear estrogen receptor levels (determined by velocity sedimentation analysis) 1 hr after the addition of [^{3}H]-17β-estradiol. In parallel experiments in which 10 nM TCDD was added 6 hr prior to the radiolabeled hormone, TCDD caused a 63 and 74% decrease in immunodetectable cytosolic and nuclear estrogen receptor protein levels, respectively, in the wild-type Hepa 1c1c7 cells. The nuclear estrogen receptor was also detected in two Hepa 1c1c7 mutant (class 1 and class 2) cell lines which have been characterized previously as TCDD non-responsive due to either decreased *a*ryl *h*ydrocarbon (*Ah*) receptor levels or a defect in the accumulation of transcriptionally active nuclear *Ah* receptor complexes, respectively. Treatment of these mutant cell lines with TCDD and [^{3}H]-17β-estradiol (as described above) caused only a minimum (class 1) or non-detectable (class 2) decrease in nuclear estrogen receptor binding activity or immunodetectable protein levels. These results, coupled with the structure-dependent differences in the activities of TCDD (a strong *Ah* receptor agonist) and 2,8-dichlorodibenzo-*p*-dioxin (a weak *Ah* receptor agonist) in this assay system, support a role for the *Ah* receptor in the TCDD-mediated decrease of the nuclear estrogen receptor in mouse Hepa 1c1c7 cells. In addition, actinomycin D and cycloheximide both inhibited the TCDD-mediated decrease of nuclear estrogen receptor levels in the Hepa 1c1c7 wild-type cells, and these results suggest that TCDD may induce specific gene products which are involved in this process.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD†) has been used extensively as a prototype for investigating the cellular and molecular mechanisms of action of the toxic halogenated aromatic hydrocarbons. TCDD elicits a broad spectrum of toxic and biochemical responses in laboratory animals and mammalian cells in culture, and these include dermal toxicity, reproductive and endocrine effects, hepatotoxicity and porphyria, immunosuppressive effects, carcinogenesis and the modulation of phase I and phase II drug-metabolizing enzymes [1–5]. Kociba *et al.* [6] reported that chronic treatment of female Sprague–Dawley rats with TCDD causes a dose-dependent decrease in the development of spontaneous mammary and uterine tumors in aged animals. Subsequent studies have confirmed that TCDD exhibits a broad spectrum of antiestrogenic properties in the rodent and in human breast cancer cells in culture [7–15]. The rat uterus and MCF-7 cells contain the *a*ryl *h*ydrocarbon (*Ah*) receptor and the cells are *Ah* responsive with respect to the induction of cytochrome P450IA1 gene expression [16–20]. In the immature female rat, TCDD inhibits the 17β-estradiol-induced increase in uterine wet weight, uterine peroxidase activity and levels of the estrogen and progesterone receptor [7–9, 14]. Moreover, in MCF-7 cells, TCDD inhibits several 17β-estradiol-induced responses including cell growth, the secretion of tissue plasminogen activator activity and the 52-kDa protein (procathepsin D) [11, 12, 15]. TCDD also causes a dose- and time-dependent decrease in the levels of nuclear estrogen receptor binding and protein [14].

Whitlock, Hankinson and coworkers have characterized several mutant Hepa 1c1c7 cell lines which have been used extensively for studying the mechanism of TCDD-induced cytochrome P450IA1 gene transcription [21–25]. The wild-type Hepa 1c1c7 cells are TCDD responsive, whereas class 1 and class 2 variant cells are TCDD non-responsive due to decreased *Ah* receptor levels or a defect in the accumulation of transcriptionally active nuclear receptor complexes, respectively. Preliminary studies in this laboratory have identified the estrogen receptor in the wild-type and mutant Hepa 1c1c7 cells, and the present study investigated the mechanism of the TCDD-induced decrease of the

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† Abbreviations: *Ah*, *a*ryl *h*ydrocarbon; DCDD, dichlorodibenzo-*p*-dioxin; DES, diethylstilbestrol; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and TCDF, 2,3,7,8-tetrachlorodibenzofuran.

Table 1. Levels and sedimentation coefficients for the nuclear *Ah* and estrogen receptors in mouse wild-type, class 1 and class 2 mutant Hepa 1c1c7 cells*

Cells	Nuclear *Ah* receptor		Nuclear estrogen receptor	
	Sediment coeff. (*S*)	Levels (fmol/mg protein)	Sediment coeff. (*S*)	Levels (fmol/mg protein)
Hepa 1c1c7	5.4 ± 0.6	720 ± 12	5.4 ± 0.7	44 ± 7.0
Class 1 variants	5.9 ± 0.7	32 ± 2.2	4.8 ± 0.6	32 ± 1.5
Class 2 variants	5.4 ± 0.6	31 ± 1.9	5.4 ± 0.7	28 ± 1.8

* Data are means ± SD from at least three separate determinations; the nuclear *Ah* receptor levels were determined in cells treated with 1 nM [^{3}H]-TCDD and the nuclear estrogen receptor levels were determined in cells treated with 1 nM [^{3}H]-17β-estradiol.

occupied nuclear and cytosolic estrogen receptor in these cell lines.

METHODS

Chemicals and biochemicals. Unlabeled TCDD, 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 2,8-dichlorodibenzo-*p*-dioxin (DCDD) were synthesized in this laboratory to >99% purity. [^{3}H]-17β-Estradiol (150 Ci/mmol) was purchased from New England Nuclear, Boston, MA; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and Tris–HCl were purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest quality from commercial sources.

Growth of Hepa 1c1c7 cells. The wild-type Hepa 1c1c7 cell line was obtained from the ATCC, Rockville, MD, and the class 1 and class 2 mutant cell lines were provided by Dr. J. Whitlock, Jr., Stanford University. The cells were grown as a continuous cell line in α-Minimum Essential Medium without ribonucleosides, deoxyribonucleosides and sodium bicarbonate, but with L-glutamine. The medium was supplemented with 10% fetal bovine serum, gentamycin sulfate (50 μg/mL) and Fungizone (22.5 μg/mL). Stock culture cells were grown in 150 cm^2 culture flasks in a humidified air:carbon dioxide (95:5) atmosphere at 37°. After reaching confluency, the cultures were trypsinized and seeded in a 150 cm^2 culture at 10^6 cells/mL in 50 mL of medium. The test solutions in dimethyl sulfoxide (DMSO) were added to the cell culture system so that the final concentration of DMSO in the culture medium was 0.5%. [^{3}H]-Estradiol (1 nM) was added to the culture medium 1 hr before harvest unless otherwise stated.

Isolation of nuclear and cytosolic receptor fractions. Cells in 150 cm^2 flasks were treated with [^{3}H]-TCDD (1×10^{-9} M) or [^{3}H]-17β-estradiol (1×10^{-9} M) 60 min before harvest. For immunoquantitation studies unlabeled TCDD and 17β-estradiol were used. After incubation of the cells with the test compounds for various time periods, the medium was removed and the cultures were washed twice thoroughly with phosphate-buffered saline (PBS), pH 7.4. To harvest cells, plates were exposed briefly to trypsin with the trypsin quickly removed. The cells were then collected in phosphate-buffered saline and centrifuged at 800 *g* for 10 min. The resulting pellet was resuspended in HEGD or TEGD buffer [25 mM Hepes for *Ah* receptor or 25 mM Tris–HCl for estrogen receptor assays, 1.5 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, pH 7.6]. The suspension was then washed twice under the same centrifuge conditions. Following the last wash, the pellet was resuspended in HED or TED buffer (HEGD without glycerol) and homogenized with a Wheaton homogenizer and pestle attached to a drill. The highest yield of nuclei was obtained with five passes of the pestle/drill apparatus. The homogenate was placed in a 15-mL conical tube and centrifuged at 800 *g* for 10 min. The resulting pellet was resuspended in HEGDM or TEGDM [HEGD or TEGD + 20 mM sodium molybdate (VI)] and washed two additional times in the same buffer. After the final wash, the nuclear pellet was resuspended in HEGDM or TEGM + 0.5 M KCl to extract the nuclear receptor–ligand complex. This "nuclear extract" was allowed to incubate on ice for 60 min. At the end of this incubation period, the nuclear extract was centrifuged at 105,000 *g* for 60 min. The resulting supernatant was then used for immunoquantitation purposes. However, for sucrose density gradient analysis [26], a 1-mL aliquot of the supernatant containing the nuclear receptor–ligand complex was treated briefly with a dextran/charcoal solution (0.2 mg charcoal and 0.02 mg dextran) for 15 min. The dextran/charcoal was removed by centrifugation and 300 μL of the supernatant was layered carefully onto a 5–25% sucrose density gradient prepared in HEG or TEG + 0.4 M KCl. The gradient was centrifuged in a Beckman VTI-65.2 rotor at 435,000 *g* at 2° for 150 min. The gradient was then fractionated into 30 fractions with 4 drops/fraction. Scintillation fluid was added to the samples (5 mL/fraction), and the samples were measured for radioactivity. Protein concentrations were determined by the method of Lowry *et al.* [27].

For both sucrose density gradient analysis and immunoquantitation, three measurements were obtained for each extract (cytosolic and nuclear), and the results are reported as means ± SD for each sample. The specific binding for the estrogen receptor was the total binding determined after incubation of 1 nM [^{3}H]-17β-estradiol minus the non-specific binding obtained after incubation of 1 nM [^{3}H]-17β-estradiol with 200-fold excess of unlabeled

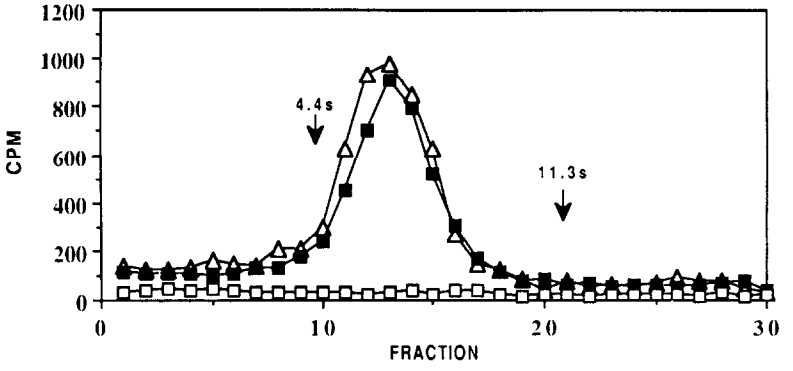

Fig. 1. A representative sucrose density gradient profile of the nuclear estrogen receptor complex from wild-type Hepa 1c1c7 cells. Cells were incubated for 1 hr with 1 nM [^{3}H]-17β-estradiol in the presence (■) or absence (△) of a 200-fold excess of 2,3,7,8-tetrachlorodibenzofuran (TCDF) or diethylstilbestrol (DES) (□), and the nuclear extract was isolated as described in Methods. The sedimentation coefficient for this complex was 5.4 ± 0.7 (mean ± SD from three determinations); the results obtained for the mutant cell lines were comparable (Table 1).

diethylstilbestrol. Specific binding of [^{3}H]-TCDD was similarly determined using an unlabeled 200-fold excess of TCDF. For the inhibitor studies, actinomycin D (10^{-6} M) or cycloheximide (2×10^{-5} M) was added to the culture medium in the presence or absence of TCDD 6 hr prior to the addition of [^{3}H]-17β-estradiol.

Immunoquantitation. The level of receptor protein was assayed using an EIA kit from Abbott Laboratories (North Chicago, IL) containing monoclonal antibodies D547 and H222. To obtain total ER, the cells were homogenized by a drill/pestle apparatus in high salt buffer (0.4 M KCl in HEGDM) following treatment and a 1-hr incubation with 17β-estradiol. The homogenate was incubated on ice for 30 min and centrifuged at 100,000 g for 1 hr at 4°. Aliquots of the total extracts were then analyzed according to the instructions of the manufacturer [28].

Statistics. The statistical differences in the estrogen receptor levels between treatment groups were determined by analysis of variance.

RESULTS

The data reported in Table 1 summarize the results obtained after incubation of the mouse hepatoma cells for 1 hr with either [^{3}H]-TCDD (1 nM) or [^{3}H]-17β-estradiol (1 nM). The total levels of nuclear *Ah* receptor detected in wild-type Hepa 1c1c7 cells (720 fmol/mg protein) were over twenty times higher than those observed in either the class 1 or class 2 mutant cells; however, the sedimentation coefficients for the nuclear *Ah* receptor complexes from the three cell lines were not significantly different. The levels of occupied nuclear estrogen receptor complexes varied from 28 to 44 fmol/mg protein in the wild-type and mutant cell lines, and the sedimentation coefficients for these complexes were not significantly different. A profile of the mouse wild-type Hepa 1c1c7 nuclear estogen receptor complex eluted from a sucrose gradient after velocity sedimentation is illustrated in Fig. 1. Incubation of

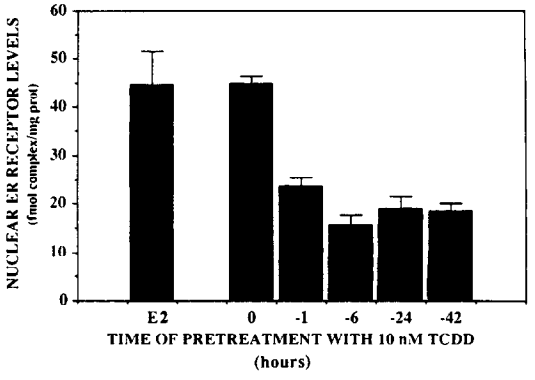

Fig. 2. Effects of different pretreatment times on the TCDD-mediated decrease in nuclear estrogen receptor levels in wild-type Hepa 1c1c7 cells. At time 0 the cells were treated with 1 nM [^{3}H]-17β-estradiol and in separate experiments 10 nM TCDD was also added 0, 1, 6, 24 and 42 hr prior to the radiolabeled hormone. The nuclear estrogen receptor complex was determined 1 hr after addition of the radioligand by velocity sedimentation as described in Methods. There were significant ($P < 0.01$) decreases in nuclear estrogen receptor levels at the −1, −6, −24 and −42 hr time points. The data are means ± SD from at least three determinations.

the cells with 1 nM [^{3}H]-17β-estradiol plus a 200-fold excess of diethylstilbestrol completely eliminated the specifically-bound radiolabeled nuclear estrogen receptor peak, whereas the specifically-bound peak was not affected after cotreatment of the cells with 1 nM [^{3}H]-17β-estradiol plus a 200-fold excess of TCDF.

The results illustrated in Fig. 2 summarize the time-dependent effects of TCDD (10 nM) on the nuclear estrogen receptor levels in wild-type Hepa 1c1c7 cells 1 hr after treatment with 1 nM [^{3}H]-17β-estradiol. Cotreatment of the cells with [^{3}H]-17β-estradiol plus TCDD did not affect the levels of the nuclear estrogen receptor complex, whereas when

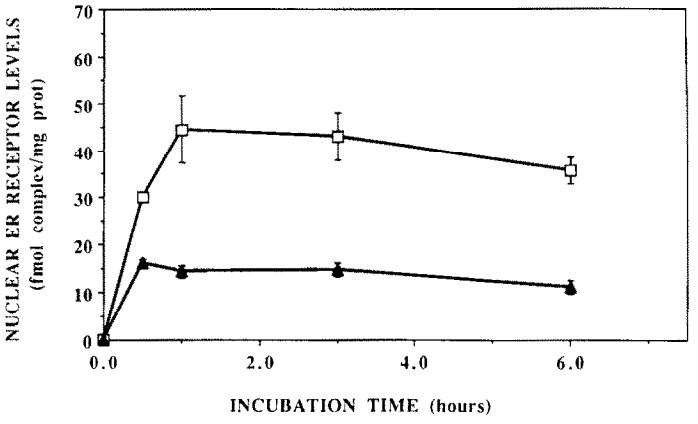

Fig. 3. Time–course effects of TCDD on nuclear estrogen receptor levels in wild-type Hepa 1c1c7 cells. The cells were incubated with 1 nM [^{3}H]-17β-estradiol for 0.5, 1.0, 3.0 or 6.0 hr in the presence (▲) or absence (□) of 10 nM TCDD which was added to the medium 6 hr prior to the addition of the radiolabeled hormone. The nuclear estrogen receptor levels were determined as described in Methods, and the results are means ± SD for at least three separate determinations. At all time points the nuclear estrogen receptor levels in the TCDD-treated cells were significantly lower ($P < 0.01$) than the corresponding levels treated with only the hormone.

the cells were treated with TCDD 1, 6, 24 or 42 hr prior to the addition of the radioligand there was a significant ($P < 0.01$) decrease in occupied nuclear estrogen receptor levels. The results in Fig. 3 summarize the time-dependent levels of the nuclear estrogen receptor after treatment of the cell with [^{3}H]-17β-estradiol or [^{3}H]-17β-estradiol plus 10 nM TCDD (note: the TCDD was added to the cell culture medium 6 hr before the radioligand). The results show that in the cells treated with TCDD plus 17β-estradiol the nuclear estrogen receptor levels were significantly lower ($P < 0.01$) at all time points than those in the cells treated only with the hormone.

The results in Fig. 4 summarize the concentration-dependent effects of TCDD and 2,8-DCDD on occupied nuclear estrogen receptor levels in wild-type Hepa 1c1c7 cells 1 hr after the addition of [^{3}H]-17β-estradiol to the culture medium. TCDD and 2,8-DCDD were added 6 hr prior to the addition of the radioligand. TCDD significantly ($P < 0.01$) decreased occupied nuclear estrogen receptor complex levels at all concentrations from 10^{-7} to 10^{-11} M, whereas 2,8-DCDD was significantly active at concentrations of 10^{-6} and 10^{-7} M but inactive at 10^{-8} M. The potency differences in these two congeners roughly parallels their differences as *Ah* receptor agonists and as competitive binding ligands for the *Ah* receptor [2].

The effects of 10 nM TCDD on the levels of the nuclear estrogen receptor complex were determined in wild-type, and class 1 and class 2 mutant Hepa 1c1c7 cells (Fig. 5). The levels of the nuclear estrogen receptor complexes were measured following a 1-hr incubation with 1 nM [^{3}H]-17β-estradiol and a 6-hr pretreatment with 10 nM TCDD. The TCDD-mediated decrease in levels of the occupied nuclear estrogen receptor complex was noted in the wild-type cells; however, no significant effects were

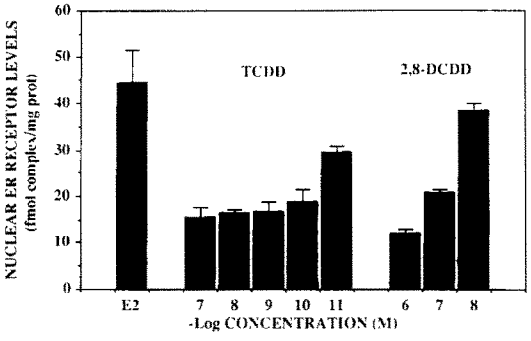

Fig. 4. Concentration–response decrease of nuclear estrogen receptor levels in wild-type Hepa 1c1c7 cells treated with TCDD or 2,8-DCDD. The experimental protocols were identical to those described in Fig. 2; however, nuclear estrogen receptor levels were determined only at the 1-hr time point. The results are means ± SD for at least three separate determinations; TCDD significantly ($P < 0.01$) decreased nuclear estrogen receptor levels at all concentrations from 10^{-7}–10^{-11} M whereas 2,8-DCDD caused these effects only at concentrations of 10^{-6} and 10^{-7} M.

observed in the class 2 variant cells and only a small decrease was detected in the class 1 cell line. These results paralleled the *Ah* responsiveness of these cells to the induction of CYP1A1 gene expression by TCDD [16–20].

The results in Table 2 summarize the effects of 10 nM TCDD on levels of the cytosolic and nuclear estrogen receptor in the wild-type and mutant cell lines using an immunoassay procedure with estrogen receptor antibodies purchased from Abbott Laboratories. The nuclear and cytosolic estrogen receptor levels were determined in the three cell

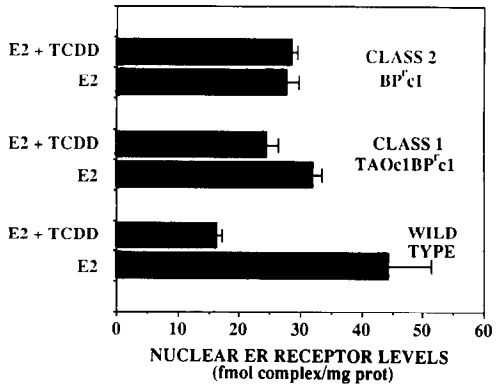

Fig. 5. Effects of TCDD on nuclear estrogen receptor levels in wild-type, class 1 and class 2 mutant Hepa 1c1c7 cells. The experimental conditions were identical to those described in the legend of Fig. 4 except that the concentration of TCDD was 10 nM in all experiments. The results are means ± SD for at least three separate determinations. TCDD significantly ($P < 0.01$) decreased nuclear estrogen receptor levels in the wild-type and class 1 mutant cells.

lines following the 6-hr pretreatment with 10 nM TCDD and a 1-hr incubation with 1 nM 17β-estradiol. The nuclear estrogen receptor levels were 40.3, 35.6 and 55.5 fmol/mg protein in the wild-type, class 1 and class 2 variant cells, respectively (Table 2). These results were similar but not identical to the levels which were determined by velocity sedimentation analysis (Fig. 5). Pretreatment of the cells with 10 nM TCDD significantly decreased the cytosolic, nuclear and total estrogen receptor levels in the wild-type cells (i.e. < 40% of the levels observed in untreated cells), whereas no significant effects were observed in the class 2 variant cells. A less than 25% reduction in estrogen receptor levels was observed in the TCDD-treated class 1 variant cells. Thus, the results in Fig. 5 and Table 2 were complementary and confirm that TCDD decreased both estrogen receptor binding activity and immunoreactive estrogen receptor protein levels in *Ah*-responsive wild-type Hepa 1c1c7 cells.

Wild-type Hepa 1c1c7 cells were also treated with 10 nM TCDD in the presence or absence of cycloheximide or actinomycin D (Fig. 6). Actinomycin D alone caused an increase in the accumulation of occupied nuclear estrogen receptor levels in wild-type cells 1 hr after incubation with [^{3}H]-17β-estradiol. These results were in contrast to the TCDD-mediated decrease in nuclear estrogen receptor levels. In cells treated with both actinomycin D and TCDD, it was apparent that the former compound inhibited the TCDD-induced response. Similarly, cycloheximide blocked the decrease of the nuclear estrogen receptor by TCDD in the wild-type Hepa 1c1c7 cells.

DISCUSSION

There is extensive evidence which supports the role of the *Ah* receptor in mediating the biochemical and toxic responses elicited by TCDD and related compounds [1–5]. However, the molecular mechanisms of action of TCDD have been elucidated for only one response, namely the induction of CYP1A1 gene expression [5, 29–32]. The nuclear TCDD–*Ah* receptor complex acts as a transcriptional enhancer and interacts with specific dioxin responsive elements (DREs) which have been identified upstream from the CYP1A1 and glutathione *S*-transferase Ya subunit [33] genes. It has been suggested that the other primary effects caused by TCDD in target cells may also be due to the altered transcription of specific genes which have hitherto not been identified [1–5].

The wild-type and mutant Hepa 1c1c7 cells have been used extensively to investigate the molecular mechanisms associated with the induction of CYP1A1 gene expression by TCDD. The results in Table 1 confirm that after treatment with [^{3}H]-TCDD there is a rapid accumulation of nuclear TCDD–*Ah* receptor complex in the *Ah*-responsive wild-type Hepa 1c1c7 cells. In contrast, relatively low levels

Table 2. Effects of TCDD pretreatment on immunodetectable cytosolic and nuclear estrogen receptor levels*

		Control (DMSO)		TCDD (10 nM)	
Cell line	Class	Estrogen receptor (fmol/mg protein)	%	Estrogen receptor (fmol/mg protein)	%
Cytosolic					
Hepa 1c1c7	Wild type	12.4 ± 0.9	100	4.6 ± 0.3†	37
TAOc1BP^rc1	Class 1	5.8 ± 0.8	100	5.0 ± 0.5	86
BP^rc1	Class 2	14.4 ± 1.6	100	17.1 ± 0.4	119
Nuclear					
Hepa 1c1c7	Wild type	40.3 ± 4.0	100	10.5 ± 0.7†	26
TAOc1BP^rc1	Class 1	35.6 ± 3.4	100	26.8 ± 1.6†	75
BP^rc1	Class 2	55.5 ± 7.4	100	53.0 ± 1.9	95

* Data are means ± SD from at least three separate determinations.
† Significantly lower ($P < 0.01$) than levels observed in the control cells.

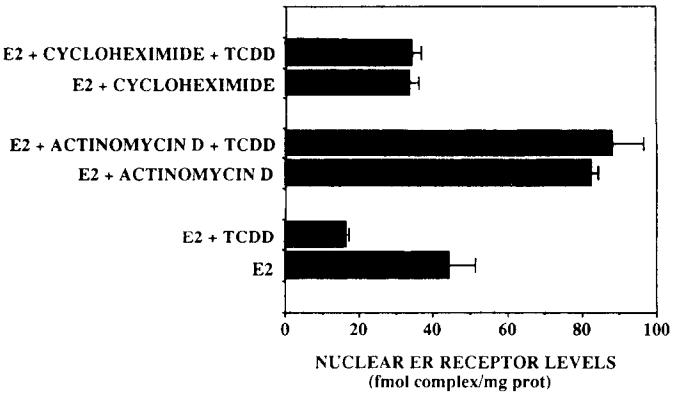

Fig. 6. Effects of cycloheximide (100 μM) and actinomycin D (10 μM) on the TCDD-mediated decrease of the nuclear estrogen receptor complex in wild-type Hepa 1c1c7 cells. The experimental protocols were identical to those described in the legend of Fig. 5; the cycloheximide and actinomycin D were added to the cells along with TCDD 6 hr prior to the addition of the [^{3}H]-17β-estradiol. The results are means ± SD for at least three separate determinations.

of the nuclear *Ah* receptor complex are detected in *Ah* non-responsive class 1 and class 2 mutant cell lines. These observations are consistent with results from previous studies which suggested that the failure of these cells to respond to TCDD was due to the relatively low levels of the *Ah* receptor (class 1 mutant cells) or a defect in the accumulation of transcriptionally-active nuclear *Ah* receptor complex (class 2 mutant cells) [23–25]. In contrast, treatment of the wild-type and mutant cell lines with 1 nM [^{3}H]-17β-estradiol resulted in the rapid formation of occupied nuclear estrogen receptor complex (e.g. Fig. 3), and the levels and sedimentation coefficients for these complexes were similar in all three cell lines (Table 1, Fig. 1). In addition, TCDD or its structural analogs did not compete with 17β-estradiol for estrogen receptor binding sites (Fig. 1).

Previous studies have reported that progestins decrease occupied nuclear estrogen receptor levels in uterine cells and it has been suggested that this may contribute to the antiestrogenic activity of this class of hormones [34–37]. TCDD also exhibits a broad spectrum of antiestrogenic effects in the rodent uterus and in MCF-7 human breast cancer cells and, like progesterone, causes a decrease in nuclear estrogen receptor levels [14]. The mechanism of action of TCDD as an antiestrogen has not been delineated; however, at least two possible pathways have been proposed. Gierthy and coworkers have shown that the induction of the cytochrome P450IA1-dependent monooxygenase, aryl hydrocarbon hydroxylase (AHH), is paralleled by increased 17β-estradiol metabolism and their results suggested that the antiestrogenic effects of TCDD may be due to the induced metabolism of 17β-estradiol [38, 39]. It has also been proposed that the antiestrogenic effects of TCDD occur via a direct receptor-mediated mechanism which would involve the altered transcription of specific target genes [7–9, 13, 14]. This study was designed to further investigate the mechanism of action of TCDD as an antiestrogen using the wild-type and mutant Hepa 1c1c7 cells and reports the effects of TCDD on the occupied nuclear estrogen receptor complex.

In the wild-type Hepa 1c1c7 cells, TCDD caused a concentration- and time-dependent decrease in occupied nuclear estrogen receptor levels. Cotreatment of the wild-type cells with TCDD plus [^{3}H]-17β-estradiol did not result in significantly decreased occupied nuclear estrogen receptor levels (1 hr after treatment with the radioligand); however, cells treated with 10 nM TCDD 1, 6, 24 or 42 hr prior to the addition of the radioligand exhibited significantly decreased levels of the occupied nuclear estrogen receptor (Fig. 2). Moreover, levels of the occupied nuclear estrogen receptor complex were decreased significantly for up to 6 hr in wild-type cells exposed to 10 nM TCDD 6 hr prior to the addition of [^{3}H]-17β-estradiol (Fig. 3). The results indicate that the TCDD-mediated decrease in occupied nuclear estrogen receptor levels can be observed within 1.5 hr whereas previous studies show that only minimum induction of AHH activity occurs within this time-frame [25]. This suggests that in the wild-type Hepa 1c1c7 cells, the effects of TCDD on occupied nuclear estrogen receptor levels are not due to increased metabolism of 17β-estradiol. It is possible that TCDD may cause a rapid induction of proteases which can degrade the estrogen receptor. A similar hypothesis has been proposed for the progestin-mediated decrease of the nuclear estrogen receptor [33–36]; however, the proteins associated with this effect have not been characterized. TCDD also causes a decrease of uterine and liver estrogen receptor and estrogen receptor mRNA levels in CD-1 mice and this is not accompanied by changes in serum levels of 17β-estradiol [40]. These results also suggest that TCDD-induced *in vivo* metabolism of 17β-estradiol is not associated with the antiestrogenic activity of TCDD.

A comparison of the concentration-dependent decrease in levels of the occupied nuclear estrogen receptor complex by TCDD and 2,8-DCDD (Fig. 4) shows that the former compound was at least

1000 times more active than the 2,8-DCDD congener. Thus, the relative potency of these two congeners as antiestrogens is comparable to their *Ah* receptor binding avidities and *Ah* receptor agonist activities [1, 2]. Additional support for the role of the *Ah* receptor was obtained by comparing the effects of TCDD on the occupied nuclear estrogen receptor complex in wild-type and mutant Hepa 1c1c7 cells (Fig. 5). In contrast to the TCDD-mediated decrease of the occupied nuclear estrogen receptor complex in the wild-type cells, no significant effects were observed in class 2 mutant cells and only a small decrease in hormone receptor levels was observed in the class 1 cells. The results observed in this study paralleled the differential induction of CYP1A1 gene expression by TCDD in the wild-type and mutant Hepa 1c1c7 cells. Thus, both the structure–activity relationships (Fig. 4) and the genetic studies (Fig. 5) suggest that the antiestrogenic effects of TCDD are mediated through the *Ah* receptor.

TCDD treatment causes a decrease in nuclear estrogen receptor levels in the wild-type Hepa 1c1c7 cells, MCF-7 human breast cancer cells and in the female rat uterus. In addition, TCDD also decreases progesterone receptor [7–9], epidermal growth factor receptor [41] and glucocorticoid receptor binding activity [42–44] in diverse target organs or cells. Although TCDD and related aromatic hydrocarbons decrease hepatic glucocorticoid receptor binding activity, the levels of immunodetectable glucocorticoid receptor protein were essentially unchanged [41–43]. In MCF-7 cells, TCDD decreases both nuclear estrogen receptor binding activity and immunodetectable protein levels [14]. The results summarized in Table 2 show that TCDD caused a 74 and 63% decrease in immunodetectable nuclear and cytosolic estrogen receptor, respectively, in wild-type Hepa 1c1c7 cells, and these results are similar to those observed using velocity sedimentation analysis of the nuclear estrogen receptor complex (Fig. 2). TCDD either did not decrease or slightly decreased immunodetectable estrogen receptor in the *Ah* non-responsive class 2 and class 1 mutant cells, respectively and these data also paralleled the results obtained using velocity sedimentation analysis (Fig. 5). Thus, the TCDD-mediated decrease in estrogen receptor binding activity was paralleled by a comparable decrease in immunodetectable protein.

The effects of the transcriptional and translation inhibitors actinomycin D and cycloheximide on the TCDD-mediated decrease in occupied nuclear estrogen receptor levels in wild-type cells are summarized in Fig. 6. Treatment of the cells with actinomycin D or cycloheximide alone resulted in a significant increase or decrease in occupied nuclear estrogen receptor levels, respectively. Both actinomycin D and cycloheximide cause a large increase in nuclear estrogen receptor levels in MCF-7 breast cancer cells [14, 15] and TCDD decreases the effects of both compounds [14]. The mechanism of action of cycloheximide and actinomycin D on estrogen receptor levels in these cells has not been delineated. In contrast to the results observed in MCF-7 cells, both actinomycin D and cycloheximide blocked the TCDD-mediated decrease in nuclear estrogen receptor levels in Hepa cells and this suggests that TCDD may cause this effect through altered gene transcription. The identity of the gene(s) involved in TCDD-mediated antiestrogenicity is unknown. Moreover, the physiological significance of the 60–70% decrease in nuclear estrogen receptor levels on estrogen-induced gene transcription is also unknown. However, the results from this study suggest that future research on the molecular biology of the genes associated with this response may provide new insights not only on the mechanism of action of TCDD but also on the processes associated with antiestrogenic action.

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