Effects of 2,3,7,8-Tetrachlorodibenzop-dioxin and Related Compounds on the Occupied Nuclear Estrogen Receptor in MCF-7 Human Breast Cancer Cells

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ABSTRACT

Treatment of MCF-7 human breast cancer cells with 17β-[3H]estradiol resulted in a rapid accumulation of occupied nuclear estrogen receptor complex in which levels were maximized within 1 h and decreased after 3 h. Pretreatment of the cells with 2,3,7,8-tetrachlorodibenzop-dioxin (TCDD) 6 or 12 h prior to the addition of the radiolabeled hormone resulted in a 38 and 63% reduction in the levels of the occupied nuclear estrogen receptor, respectively, whereas addition of TCDD 1 h prior to the radioligand did not cause any significant change in the levels of the occupied nuclear receptor using velocity sedimentation analysis. Moreover, it was also shown with estrogen receptor antibodies that the TCDD-mediated dose-dependent decrease in occupied nuclear receptor levels was paralleled by a comparable decrease in immunoreactive protein at concentrations of 10^{-8} to 10^{-11} M TCDD. The reduction in levels of the occupied nuclear estrogen receptor was not due to increased estradiol metabolism since a significant reduction was observed at TCDD concentrations (10^{-11} to 10^{-12} M) which do not induce cytochrome P-450-dependent monooxygenase enzyme activities in MCF-7 cells. Treatment of the MCF-7 cells with actinomycin D or cycloheximide resulted in a greater than 2-fold increase in levels of the occupied nuclear estrogen receptor, and cotreatment of the cells with both TCDD and these inhibitors significantly decreased levels of the nuclear estrogen receptor complex. The structure-activity relationships for TCDD and several congeners were similar for both the reduction of occupied nuclear estrogen receptor levels and several aryl hydrocarbon (Ah) receptor agonist activities, and these results support a role of the Ah receptor in these processes.

INTRODUCTION

TCDD and related toxic halogenated aromatic hydrocarbons elicit a diverse spectrum of biochemical and toxic responses in laboratory animals and mammalian cells in culture (reviewed in Refs. 1 to 3). The effects and relative potency of TCDD are dependent on several factors including the age, sex, and species of animal used and the genetics of both animal and cellular models. The cellular and molecular mechanisms of TCDD-induced toxicity have not been delineated; however, a mechanistic model has been developed from extensive research on the induction of CYP1A1 gene expression by TCDD (4-10). These studies show that the induction response is dependent on the initial interaction of TCDD with an intracellular protein, designated the Ah receptor. The induction of CYP1A1 gene expression also requires the interaction of activated nuclear TCDD-Ah receptor complexes with specific genomic “dioxin” or xenobiotic regulatory elements which have been identified in the 5'-upstream region of the CYP1A1 gene in human and rodent cells.

The proposed receptor-mediated mechanism of action of TCDD and related compounds is similar to the mechanisms proposed for steroid hormone-induced gene transcription (11-13). Steroid hormones and polyhalogenated aromatics are polycyclic organic compounds which are highly lipophilic; however, competitive binding studies have shown that TCDD does not bind to the steroid hormone receptors nor do the steroid hormones bind to the Ah receptor (14-17). Although there is no direct interaction between TCDD and the estrogen receptor, several studies have reported that TCDD and related compounds are antiestrogens (14-16, 18-22). Kociba et al. (22) reported that long-term feeding of TCDD caused a dose-response reduction of spontaneous mammary and uterine tumors in female Sprague-Dawley rats. Moreover, in rodents TCDD also inhibited the estrogen-induced increase in uterine weight and uterine peroxidase activity (21) and caused a decrease in estrogen-induced estrogen and progesterone receptor levels (14-16). TCDD also inhibited the growth and postconfluent foci production in MCF-7 human breast cancer cells (20) and suppressed the estrogen-induced secretion of tissue plasminogen activator activity in the cell line (19).

This study will report the effects of TCDD and structural analogues on the nuclear estrogen receptor in MCF-7 cells and utilize this response as a model for investigating the mechanism of antiestrogenic action of the toxic halogenated aromatic hydrocarbons.

MATERIALS AND METHODS

Chemicals and Reagents. [3H]TCDD (32 Ci/mmol) was prepared by the chlorination of 1,6-[3H]dibenzop-dioxin, and the final product was purified by high-pressure liquid chromatography to greater than 95% purity. Unlabeled TCDD, TCDF, PeCDF, 1,2,3,7,9-PeCDF, and 1,3,6,8-TCDF were prepared in this laboratory (23, 24), and their purities were greater than 98% as determined by gas chromatographic analysis. 17β-[3H]Estradiol (150 Ci/mmol) was purchased from New England Nuclear, Boston, MA; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO); and all other chemicals were of the highest quality available from commercial sources.

Cell Culture Growth. Cells were grown at 37°C in 95% air and 5% CO2. The MCF-7 cell line was grown in minimal essential medium from Gibco. This medium was supplemented with insulin (6 μg/liter), 10% fetal calf serum, sodium bicarbonate (2.2 g/liter), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (2.383 g/liter), glucose (1 g/liter), sodium pyruvate (1 × 10^{-4} mol), and essential (50×) amino acids from Gibco. The medium was also supplemented with gentamycin (2.5 mg/liter), penicillin/streptomycin (10000 units/liter of penicillin and 10 mg/liter of streptomycin), and amphotericin B (1.25 mg/liter). Cells for use in nuclear translocation assays were plated onto 150-cm² flasks and allowed to grow for 60 h before treatment with the 17β-[3H]Estradiol. The halogenated aromatic hydrocarbons in dimethyl sulfoxide were added as described in “Results.” The dimethyl sulfoxide concentrations in the control and treated cells were 1% in all the experiments.

Isolation of the Occupied Nuclear Estrogen Receptor Complex. Cells in 150-cm² flasks were treated with 17β-[3H]Estradiol (1 × 10^{-4} mol) or

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3 The abbreviations used are: TCDD, tetrachlorodibenzo-p-dioxin; TCDF, tetrachlorodibenzofuran; Ah receptor, aryl hydrocarbon receptor; AHH, aryl hydrocarbon hydroxylase; CYP1A1, cytochrome P-450; EC50, 50% effective concentration.

5'-upstream region of the CYP1A1 gene in human and rodent cells.

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17β-[3H]estradiol (1 x 10^-9 mol) plus a 200-fold excess of diethylstilbestrol (for determination of nonspecific binding) 60 min before harvest with each treatment group consisting of 5 flask. To harvest cells, plates were briefly exposed to trypsin with the trypsin quickly removed. The cells were then collected in phosphate-buffered saline, pooled in 50-ml conical tubes, and centrifuged at 800 x g for 10 min. The resulting pellet was resuspended in TEGDM buffer. The suspension was then washed twice by centrifugation (800 x g for 10 min). The pellet, following the last wash, was resuspended in TEGDM buffer and homogenized with a Wheaton homogenizer and pedestal attached to a drill. The highest yield of nuclei was obtained with 5 passes of the pedestal/di

RESULTS

Treatment of MCF-7 cells with 17β-[3H]estradiol (10^-9 mol) resulted in the rapid formation of occupied nuclear estrogen receptor complexes (see Fig. 1) within 0.5 h of treatment (114 ± 3.06 fmol of nuclear complex/mg of protein). The maximal level of the occupied nuclear estrogen receptor complex (162 ± 3.14 fmol of nuclear complex/mg of protein) was observed 1 h after treatment with 17β-[3H]estradiol, and these levels subsequently decreased by >50% within 3 h. A summary of the time-dependent accumulation of occupied nuclear estrogen receptor complex is illustrated in Fig. 2. Fig. 3 summarizes the time-dependent effects of TCDD (10^-9 mol) on levels of the nuclear estrogen receptor complex 1 h after treatment of the cells with 17β-[3H]estradiol. The results showed that levels of the occupied nuclear estrogen receptor complex were unchanged in cells pretreated with TCDD 6 h prior to the addition of the labeled hormone. In contrast, pretreatment of the cells with TCDD 6 and 12 h prior to the addition of the 17β-[3H]estradiol (10^-9 mol) was added at time 0, and the levels of occupied nuclear estrogen receptor were determined at various time points by velocity sedimentation analysis as described in "Materials and Methods." This experiment was also carried out in cells pretreated with 10^-9 M TCDD 6 h prior to the addition of the 17β-[3H]estradiol. Each experiment the cells from at least five 150-cm² flasks were harvested and pooled. The results shown are the mean ± SD for nuclear estrogen receptor levels determined in triplicate on the pooled nuclear extracts by velocity sedimentation analysis.
The levels of the occupied nuclear estrogen complex after treatment with different concentrations of TCDD were also determined using the commercially available estrogen receptor antibody kit. Treatment of the cells with TCDD 12 h prior to incubation of the cells for 1 h with 17β-[3H]estradiol resulted in the following concentration-dependent decrease in immunoreactive nuclear estrogen receptor protein: untreated cells, 86 ± 2.2 fmol/mg of protein; TCDD (10⁻⁶, 10⁻⁷, 10⁻⁸, and 10⁻¹¹ mol), 36 ± 2.2, 48 ± 0.37, 58 ± 1.6, and 66 ± 4.6 fmol/mg of protein, respectively; the EC₅₀ value for this response was 2.6 × 10⁻⁹ mol, and this was similar to the EC₅₀ value for the TCDD-mediated decrease in levels of the occupied nuclear estrogen receptor which was determined by velocity sedimentation analysis (Fig. 4).

Fig. 5 summarizes the effects of actinomycin D (10⁻⁶ mol) and cycloheximide (2 × 10⁻⁵ mol) in the presence or absence of TCDD on levels of the occupied nuclear estrogen receptor 1 h after the addition of 17β-[3H]estradiol (10⁻⁶ mol) to the MCF-7 cells in culture. Treatment of the cells with cycloheximide or actinomycin D resulted in a 204 or 395% increase in the levels of the occupied nuclear estrogen receptor complex, respectively, compared with control cells. In the cotreatment studies, it was apparent that TCDD significantly inhibited the cycloheximide- and actinomycin D-mediated increases in the accumulation of occupied nuclear estrogen receptor complexes.

**DISCUSSION**

Human breast cancer cell lines, such as the estrogen receptor-rich MCF-7 cells, have been extensively utilized to investigate the cellular and molecular mechanism of estrogen-induced gene transcription (28-33). These cells also serve as useful models for investigating the effects and mechanisms associated with antiestrogens, such as tamoxifen, related triphenylethylene analogues, and progestins (34-37). Recent studies have reported that TCDD exhibits antiestrogenic activity in rodents and in human breast cancer cells in culture (14-16, 18-22). Moreover, the Ah receptor has recently been characterized in human breast cancer cell lines (38, 39), and compounds such as TCDD and 2,3,7,8-dioxin on levels of the nuclear estrogen receptor complex 1 h after the cells were treated with [3H]estradiol are summarized in Table 1. The cells were pretreated with these congeners 12 h prior to addition of the radiolabeled hormone. TCDD significantly reduced occupied nuclear receptor complex levels at all concentrations between 10⁻¹³ and 10⁻⁸ mol, whereas no significant decreases were observed using 2,8-dichlorodibenzo-p-dioxin (10⁻⁹ to 10⁻¹¹ mol). The dose-response effects of several polychlorinated dibenzo-p-dioxins and dibenzofurans on nuclear estrogen receptor levels in the untreated cells for each experiment, the effects of TCDD (10⁻⁸ mol) on occupied nuclear estrogen receptor levels were relatively constant (i.e., 41.8 ± 6.66% mean ± SD reduction for all the experiments). The results shown in the figure are means for the nuclear estrogen receptor levels determined in triplicate on the pooled nuclear extracts for each concentration of the congeners used in these experiments. SDs were less than ± 8% for all the means, and the EC₅₀ values for the antiestrogenic effects (see "Results") were determined by logit plots of the data.
related polycyclic aromatic and halogenated aromatic hydrocarbons induce CYP1A1 gene expression and related cytochrome P-450-dependent monooxygenase enzyme activities (AHH and EROD) in these cells (20, 38-44).

Although the nonsteroidal triphenylethylene-derived antiestrogens are used in the treatment of hormone-responsive mammary tumors, their mechanisms of action are not fully understood (34-37). Structure-activity studies indicate that the relative potencies of these drugs can be correlated, in part, by their respective binding affinities to the estrogen receptor (45). Progestins also exhibit antiestrogenic activities (35, 46-50), and these responses appear to be mediated through the progesterone receptor. Like the nonsteroidal triphenylethylenes analogues, the molecular mechanisms of action of progestin-induced antiestrogenic activity have not been delineated.

TCDD and related halogenated aryl hydrocarbons represent a new class of antiestrogens which inhibit a broad spectrum of estrogen-induced responses. The mechanism of TCDD-mediated antiestrogenicity has not been determined; however, at least two hypotheses have been proposed. Gierthy and coworkers (20, 40) have suggested that TCDD-induced hydroxylation of estradiol may play a role in the antiestrogenic responses observed in the MCF-7 human breast cancer cell line. In contrast, Safe and coworkers (14-16, 21) have reported that the observed structure-dependent effects of TCDD and related compounds as antiestrogens in the female rat uterus support a more direct role of the Ah receptor in mediating the antiestrogenic responses.

Several studies have reported that progestins decrease occupied estrogen receptor levels in uterine cells (52-54), and this is accompanied by progestin-induced increases in nuclear estrogen receptor turnover and decreases in estrogen-induced receptor replenishment (53). Although the mechanism of these effects has not been determined, it has been suggested that the effects of progestin on nuclear estrogen receptor levels and turnover may contribute to the antiestrogenic actions of progestins. Romkes and coworkers (14, 16) have previously reported that treatment of female rats with either progesterone or TCDD caused a decrease in uterine cytosolic and nuclear estrogen receptor levels. The results in Figs. 1 and 2 illustrate the rapid formation of occupied nuclear estrogen receptor complexes in MCF-7 complexes treated with 10^{-9} M 17β-[3H]-estradiol and their subsequent decrease after 3 h. These observations were comparable to results previously reported in this cell line (28, 55, 56). Treatment of the cells with 10^{-9} M TCDD resulted in a significant decrease in occupied nuclear estrogen levels (Figs. 2 and 3); however, the effects of TCDD were observed only when the chemical was administered 6 and 12 h prior to addition of 17β-[3H]estradiol; addition of the chemical 1 h prior to the addition of the hormone did not result in any significant decrease in levels of the occupied nuclear estrogen receptor. In contrast, Gierthy and Lincoln (20) did not observe any effects of TCDD (10^{-9} mol) on estrogen receptor levels after preincubating MCF-7 cells with TCDD prior to the addition of 17β-[3H]estradiol. However, in these latter experiments the medium was removed prior to addition of the radiolabeled hormone, and the total (not nuclear) cellular estrogen receptor levels were determined. In contrast, the results reported in this study clearly show that TCDD decreases nuclear estrogen receptor levels in a dose- and time-dependent manner. Previous studies have reported that TCDD and related aromatic hydrocarbons decrease hepatic glucocorticoid receptor binding activity but not the levels of immunodetectable glucocorticoid receptor protein (57-59). Therefore, the effects of TCDD on nuclear estrogen receptor levels were determined using a commercially available estrogen receptor antibody kit (27). The results clearly showed that TCDD (10^{-9} to 10^{-11} mol) caused a concentration-dependent decrease in both immunodetectable estrogen receptor protein and levels of the occupied nuclear estrogen receptor which were determined by velocity sedimentation analysis.

As noted above, at least 2 mechanisms have been proposed for TCDD-induced antiestrogenicity, namely (a) induction of monooxygenases which result in increased metabolism of estradiol (20, 40) and (b) a more direct Ah receptor-mediated response which may be related to the induction of factors which inhibit estrogen-induced gene transcription (14, 16). Both mechanisms would be Ah receptor mediated; however, the second process would be analogous to mechanisms proposed for progestin-induced antiestrogenicity (51-54). At a concentration of 10^{-8} mol TCDD readily induces AHH and EROD activities and CYP1A1 mRNA levels (20, 38-40). Moreover, Giertych et al. (40) have demonstrated a parallel between TCDD-induced AHH activity and 17β-estradiol-2- and 16α-hydroxylase activities in MCF-7 cells. The effects of TCDD as an inducer of AHH and related monooxygenase enzyme activities in MCF-7 cells are concentration dependent, no induction was observed at a concentration < 10^{-11} mol, and only minimal induction responses were observed at 10^{-11} M TCDD (39). It is apparent from the results in Table 1 that TCDD caused a concentration-dependent (10^{-8} to 10^{-11} M) decrease in levels of the occupied nuclear estrogen receptor and significantly decreased these levels at concentrations as low as 10^{-11} mol. Therefore these results indicate that the reduction in levels of the occupied nuclear estrogen receptor by TCDD does not occur via a mechanism which involves induction of cytochrome P-450-dependent enzyme activities.

The biochemical and toxic effects of TCDD and related halogenated aromatics are highly structure dependent, and these structure-activity relationships provide support for the proposed Ah receptor-mediated mechanism of action for these compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (M)</th>
<th>Nuclear estrogen receptor levels (fmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD</td>
<td>10^{-9}</td>
<td>124 ± 3.91*</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>273 ± 2.42</td>
</tr>
<tr>
<td>2,8-Dichlorodibenzo-p-dioxin</td>
<td>10^{-7}</td>
<td>307 ± 14.3</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>184 ± 3.18</td>
</tr>
<tr>
<td>TCDD</td>
<td>10^{-9}</td>
<td>306 ± 6.30</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>300 ± 6.30</td>
</tr>
</tbody>
</table>

*The levels of occupied nuclear estrogen receptor in the MCF-7 cells treated with TCDD (10^{-9} to 10^{-11} mol) were all significantly lower than those observed in the untreated cells (P < 0.01).
Ah receptor in modulating nuclear estrogen receptor levels. The TCDD (18-22). The structure-activity relationships observed compounds significantly increase nuclear estrogen receptor complex in MCF-7 cells due to the direct effects of both previous study which reported that actinomycin D prevented nu estrogen receptor. Both actinomycin D and cycloheximide in these data and results summarized in Fig. 5 may be due to the higher concentration of cycloheximide (2 x 10^-5 mol) used in this study. In the interactive studies, it was apparent that TCDD significantly inhibited the actinomycin D- and cycloheximide, respectively. These results were not readily interpretable due to the effects of these inhibitors on levels of the occupied nuclear estrogen receptor. Both actinomycin D and cycloheximide treatment of MCF-7 cells caused a significant increase in levels of the occupied nuclear estrogen receptor (Fig. 5); the results observed for the former compound are consistent with a previous study which reported that actinomycin D prevented nuclear processing of the estrogen receptor in MCF-7 cells (28). Comparable effects were not previously observed for cycloheximide at a concentration of 10^-6 mol (60), and the differences in this data and results summarized in Fig. 5 may be due to the higher concentration of cycloheximide (2 x 10^-5 mol) used in this study. In the interactive studies, it was apparent that TCDD significantly inhibited the actinomycin D- and cycloheximide-mediated increase in nuclear estrogen receptor levels. This observation was comparable with the interaction of TCDD and 17β-estradiol summarized in Figs. 2 and 3. Thus, it was not possible to utilize the transcriptional and translation inhibitors to further probe the mechanism of the TCDD-induced decrease in the levels of the occupied nuclear estrogen receptor complex in MCF-7 cells due to the direct effects of both actinomycin D and cycloheximide in this cell line. Currently, other cellular models in which actinomycin D and cycloheximide do not increase levels of the occupied nuclear estrogen receptor are being investigated. In summary, this study suggests that TCDD and related compounds significantly increase nuclear estrogen receptor processing in MCF-7 cells in culture, and this effect may influence, in part, the antiestrogenic responses reported for TCDD (18-22). The structure-activity relationships observed in this study (Fig. 4, Table 1) are consistent with a role for the Ah receptor in modulating nuclear estrogen receptor levels. The molecular mechanism of this process is currently being investigated.

REFERENCES

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