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Partial Antagonism of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-Mediated Induction of Aryl Hydrocarbon Hydroxylase by 6-Methyl-1,3,8-trichlorodibenzofuran: Mechanistic Studies

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SUMMARY

6-Methyl-1,3,8-trichlorodibenzofuran (MCDF) binds with moderate affinity to the aryl hydrocarbon (Ah) receptor protein (4.9×10^{-8} M) but is a weak Ah receptor agonist. Cotreatment of male Long Evans rats with MCDF (50 μmol/kg) and a dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) that causes a near-maximal induction of hepatic microsomal aryl hydrocarbon hydroxylase and ethoxyresorufin *O*-deethylase activities resulted in a significant inhibition of these activities for up to 96 hr. Comparable results were obtained with MCDF (10^{-7} M) and TCDD (10^{-8} M) in rat hepatoma H-4-II E cells in culture over 36 hr. TCDD treatment of rats resulted in an initial decrease of hepatic cytosolic Ah receptor within 6 hr and this was followed by a subsequent 138% increase in cytosolic receptor levels 72 hr after treatment. Although MCDF (50 μmol/kg) did not significantly alter rat hepatic cytosolic Ah receptor levels in animals cotreated with TCDD plus MCDF, the latter compound significantly inhibited TCDD-mediated replenishment of the cytosolic Ah receptor. In contrast, treatment of rat hepatoma H-4-II E cells with TCDD (10^{-8} M) resulted in the rapid (within 1 hr) depletion of cytosolic Ah receptor, which remained undetectable for up to 36 hr; cotreatment of the cells with MCDF (10^{-7} M) and TCDD (10^{-8} M) resulted in cytosolic Ah receptor levels that were similar to those observed after treatment with TCDD alone. The effects of MCDF on the uptake and persistence of nuclear [^{3}H]TCDD-Ah receptor complex levels were also determined in rat liver and rat hepatoma H-4-II E cells in culture. MCDF did not significantly decrease levels of occupied nuclear Ah receptor complexes in the rat or rat hepatoma cells. Moreover, using the sucrose density gradient assay procedure, the sedimentation coefficients of the cytosolic and nuclear TCDD-Ah receptor complexes in the presence or absence of MCDF were comparable. The results of these and other related studies with 6-substituted-1,3,8-trichlorodibenzofurans suggest that MCDF may act as a partial TCDD antagonist by competing with TCDD for nuclear binding sites.

TCDD elicits diverse tissue-, species-, strain-, and age-specific biologic and toxic responses in animals and mammalian cells in culture (reviewed in Refs. 1–4). One response, namely the induction of cytochrome P-4501A1 gene expression, has been extensively investigated at the cellular and molecular level and the results of these studies have formed the basis for the proposed mechanism of action of TCDD and related compounds. This mechanism involves the initial binding of TCDD to a soluble intracellular protein, designated the Ah receptor, followed by the accumulation of the occupied receptor in the nuclei of target cells. The subsequent interaction of the occupied Ah receptor complexes with specific nuclear binding sites or "dioxin regulatory elements," which are located upstream from the 5′ end of the cytochrome P-4501A1 gene, is required for increased gene transcription (4–6).

Several compounds, including 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin, 1,3,6,8-tetrachlorodibenzofuran, 2,3,6,8-tetrachlorodibenzofuran, MCDF, Aroclor 1254, and α-naphthoflavone antagonize the induction of AHH and EROD enzyme activities and cytochrome P-4501A1 in both *in vivo* and *in vitro* systems (7–11). All of these chemicals are typically weak Ah receptor agonists and bind with moderate affinity to the Ah receptor protein. Previous studies in our laboratory (11) have shown that MCDF partially antagonizes the TCDD-mediated induction of AHH and EROD enzyme activities in rat hepatoma H-4-II E cells and rat hepatic microsomes and the induction of cytochromes P-4501A1 and P-4501A2 in the latter system. This study investigates the mechanism of the MCDF-mediated antagonism of the induction of AHH and EROD activities by TCDD, by measuring the effects of the partial antagonist on cytosolic Ah receptor levels and their replenishment and on occupied nuclear [^{3}H]TCDD receptor complexes.

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AHH, aryl hydrocarbon hydroxylase; EROD, 7-ethoxyresorufin *O*-deethylase; MCDF, 6-methyl-1,3,8-trichlorodibenzofuran; TCDF, 2,3,7,8-tetrachlorodibenzofuran; Ah receptor, aryl hydrocarbon receptor; HEPES, *N*-2-hydroxylethylpiperazine-*N*′-2-ethanesulfonic acid.

Materials and Methods

Chemicals and biochemicals. [^{3}H]TCDD (37 Ci/mmol), MCDF, ethoxyresorufin, and TCDF have previously been prepared in this laboratory, as described (11). Benzo[*a*]pyrene, NADP, NADPH, rhodamine B, bovine serum albumin, and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO). Hydroxylapatite was purchased from Bio-Rad Laboratories (La Jolla, CA). ^{14}C-labeled bovine serum albumin and catalase (prepared in this laboratory) were used as external standards for determination of sedimentation coefficients (*S* values). All other chemicals and biochemicals used in these studies were the highest quality available commercial products.

***In vivo* animal treatment and isolation of rat hepatic cytosol.** Immature male Long Evans rats were obtained from Harlan (Houston, TX). The animals were housed in polycarbonate cages, fed Ralston Purina rat chow *ad libitum*, and maintained on a daily 12-hr diurnal light/dark cycle. The animals were sacrificed either by ether anesthesia or cervical dislocation and were killed at approximately the same time each day to minimize diurnal differences in hormone levels. The livers were perfused *in situ* via the portal vein with HEDGM buffer (25 mM HEPES, pH 7.4, 15 mM EDTA, 1 mM dithiothreitol, 20 mM molybdate) and 10%, v/v, glycerol. The blanched livers were placed in HEDGM buffer and kept on ice. All remaining procedures were carried out at 0–4°. The livers were finely minced with scissors and rinsed with 2 volumes of buffer. The livers were homogenized to a uniform homogenate using a Teflon-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 105,000 × *g* in a Beckman L8 70 ultracentrifuge for 60 min. The supernatant cytosolic fraction was removed and care was taken to exclude the upper lipid layer. The cytosol was stored in liquid nitrogen and used within 2 weeks after preparation. The concentration of protein in the cytosol was determined on the day of sacrifice. On the day of the assay, the cytosol was diluted with HEDGM buffer (containing freshly added dithiothreitol) and an aliquot of the diluted protein was used to redetermine protein concentration. This protocol was used to isolate cytosol from rats treated with TCDD, MCDF, and TCDD plus MCDF. The levels of Ah receptor in the cytosolic fraction were determined by measuring the specific binding of [^{3}H]TCDD using the hydroxylapatite assay, as outlined below.

Isolation of rat hepatic nuclear receptor-ligand complexes. The rats were injected intraperitoneally with the [^{3}H]TCDD (16 nmol/kg) and sacrificed at several time points up to 72 hr after initial treatment. The perfused liver was removed, weighed, and placed in a beaker containing 5 ml of HEDGM buffer. The liver was then homogenized in a 35-ml Wheaton homogenizing tube with a Teflon pestle; 5 passes with the pestle resulted in the highest yield of nuclei as determined by light microscopy. This homogenate was centrifuged at 1000 × *g* for 15 min. The pellet was termed the nuclear fraction and washed three additional times with HEDGM buffer, each time with a 10-min spin at 1000 × *g*. After the third wash, the nuclear pellet was resuspended in HEGDM containing 0.5 M KCl. This homogenate was subjected to six or seven passes with the Teflon pestle/Wheaton apparatus. The homogenate was then allowed to incubate for 1 hr at 4° and was centrifuged at 105,000 × *g* for 1 hr. The resulting pellet was used to quantitate total DNA. The supernatant was collected and 1 ml was treated with 0.01 charcoal/0.001% dextran solution for 15 min. The charcoal was removed by centrifugation and 300 μl of this nuclear extract was placed onto a 5–25% sucrose gradient made in 0.4 M KCl. The gradient was centrifuged at 435,000 × *g* for 2.5 hr and fractionated with an LKB fractionator. Four drops were collected per fraction. Specific binding of the nuclear fraction was determined by a comparable experiment using [^{3}H]TCDD and a 200-fold molar excess of unlabeled TCDD or TCDF. The amount of radioactivity in the specifically bound peak is utilized to calculate the levels of nuclear receptor complex per mg of nuclear protein or nuclear DNA. Protein and DNA concentrations were determined by the methods of Lowry *et al.* (12) and Labarca and Paigen (13), respectively. This approach can be used to determine the effects of MCDF (50 μmol/kg) on the levels of nuclear [^{3}H]TCDD-Ah receptor complexes and the rate of decomposition of these complexes in the presence or absence of MCDF.

Rat hepatoma H-4-II E cells: growth and isolation of nuclear and cytosolic receptors. Mammalian cells (H-4-II E) were grown as a continuous cell line in minimum essential medium without ribonucleosides, deoxyribonucleosides, and sodium bicarbonate, but with L-glutamine. The medium was supplemented with 10% fetal calf serum, 10% serum, 50 μg/ml gentamicin sulfate, and 22.5 μg/ml Fungizone. Stock cultures were grown in 150-cm^2 culture flasks in an humidified air/carbon dioxide (95:5) atmosphere at 37°. After reaching confluency, the cultures were trypsinized and seeded, in 150-cm^2 culture flasks, at 10^7 cells/plate in 50 ml of medium. MCDF (10^{-7} M) and/or [^{3}H]TCDD (10^{-8} mM) in dimethylsulfoxide were added to the cell culture flasks (eight flasks/point) so that the final concentration of dimethylsulfoxide in the culture medium was 0.5%. Nuclear extract baselines were obtained by co-administering a 200-fold excess unlabeled TCDF. After incubation, the medium was removed and the cell surface was rinsed twice with phosphate-buffered saline (pH 7.4, 0.90% NaCl). The cells were harvested 48 hr after plating by trypsinization and were pooled in two 50-ml disposable polypropylene tubes. This and all subsequent procedures were performed at 4°.

Isolation of cytosolic and nuclear fractions from rat hepatoma cells. Harvested cells were washed two times in 30 ml of HEGD buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, and 10%, v/v, glycerol, pH 7.6) by resuspending the pellet with a disposable pipette and pelleting the suspension by centrifugation for 10 min at 100 × *g*. The washed cell pellet was resuspended in 1.5 ml of HED buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol) and was transferred to a 15-ml Wheaton homogenizing tube. Cells were homogenized with five full speedstrokes of a Teflon pestle/drill apparatus. The homogenate was transferred to a 15-ml disposable polypropylene tube with an additional 1.5 ml of HEGDM and was centrifuged at 1000 × *g* for 10 min. The resulting pellet was used for the preparation of the nuclear extract. The supernatant fraction was centrifuged at 110,000 × *g* for 1 hr at 2° to yield the cytosolic fraction. Cytosolic and nuclear fractions were prepared and analyzed by the sucrose density gradient procedure on the same day. The 1000 × *g* pellet resulting from centrifugation of cell homogenates was washed two times, as described above, with 10 ml of HEGDM. The washed pellet was transferred in 3 ml of HEGDM buffer that contained 0.5 M KCl, pH 8.5, and was resuspended using a disposable pipette. The suspension was allowed to stand at 4° for 1 hr and then centrifuged at 105,000 × *g* for 1 hr. The resulting supernatant and pellet were saved for further investigation, as described above. Microscopic examination of nuclei prepared in this fashion were found to be intact and appeared to be greater than 90% free of extranuclear contamination. This assay was used for the time-course study of the effects of TCDD, MCDF, and TCDD plus MCDF on cytosolic Ah receptor levels.

Induction of AHH and EROD activities. Microsomes were isolated from rat liver homogenates by differential centrifugation, as described, and the determinations of AHH and EROD were carried out on the hepatic microsomes and cell preparations using the methods of Nebert and Gelboin (14) and Pohl and Fouts (15), respectively. Measurement of the induction of AHH and EROD in the rat hepatoma H-4-II E cells utilized the same enzyme assay as previously described (11).

Statistical analysis. The statistical differences between treatment groups were determined by the Student *t* test and the levels of probability are noted ($p < 0.05$ or $p < 0.01$). The data are expressed as means ± standard deviations.

Results

Studies with rat subcellular fractions. Fig. 1 summarizes the time course of induction of hepatic microsomal AHH and EROD activities by TCDD (16 nmol/kg) and TCDD plus MCDF (50 μmol/kg). MCDF (50 μmol/kg) alone was inactive

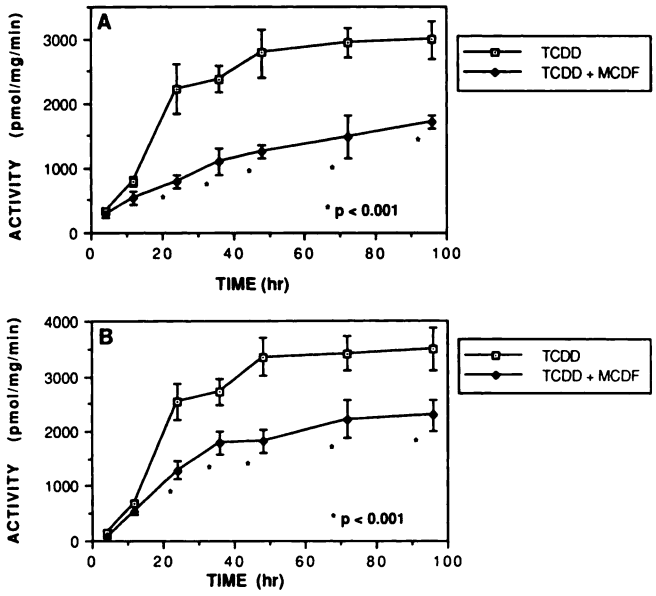

Fig. 1. Time course of induction of rat hepatic microsomal AHH (A) and EROD (B) by TCDD (16 nmol/kg) and TCDD (16 nmol/kg) plus MCDF (50 μmol/kg). The assay procedures are summarized in Materials and Methods and the results are expressed as means ± standard deviations.

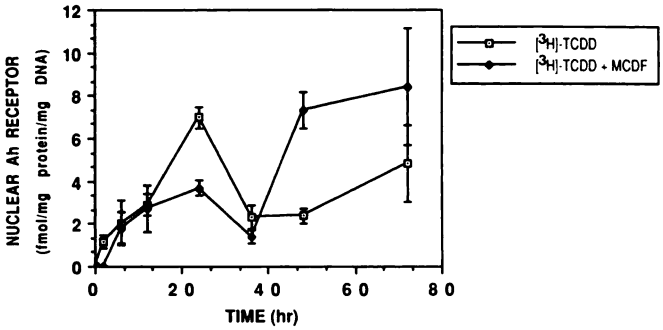

Fig. 2. Time course of accumulation of nuclear [^{3}H]TCDD Ah receptor complexes in rats treated with [^{3}H]TCDD (16 nmol/kg) and [^{3}H]TCDD (16 nmol/kg) plus MCDF (50 μmol/kg). The levels of nuclear receptor complexes were determined by the sucrose density gradient assay procedure as described. The data are expressed as means ± standard deviations. Significant differences in nuclear receptor levels were observed only at the 24- and 48-hr time points.

as an inducer. Maximal induction was observed between 24 and 36 hr after treatment with TCDD and the induction response persisted for 96 hr. MCDF at a dose of 50 μmol/kg was inactive as an inducer of the monooxygenase activities and cotreatment of the rats with MCDF plus TCDD resulted in significantly lower induction, compared with animals treated with TCDD alone; the antagonism was observed 24 to 96 hr after cotreatment. Fig. 2 summarizes the time course of accumulation of occupied [^{3}H]TCDD-Ah receptor complexes in rats treated with [^{3}H]TCDD alone and [^{3}H]TCDD plus MCDF. The levels of nuclear complexes were comparable in animals treated with either [^{3}H]TCDD or [^{3}H]TCDD plus MCDF after 72 hr; however, there were significant differences between the groups at the 24 and 48 hr time points.

The effects of TCDD treatment on hepatic cytosolic receptor levels (Fig. 3) were comparable to those reported by Sloop and Lucier (16). After treatment with TCDD, there was an initial decrease in the cytosolic receptor levels and this was followed by a time-dependent replenishment of cytosolic Ah receptors;

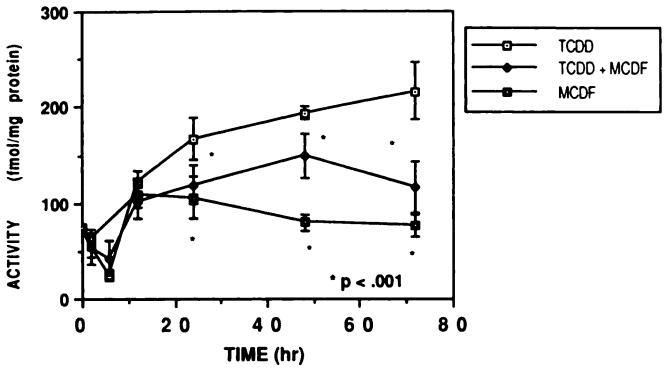

Fig. 3. Time course of effects of TCDD (16 nmol/kg), MCDF (50 μmol/kg), and TCDD (16 nmol/kg) plus MCDF (50 μmol/kg) on rat hepatic cytosolic Ah receptor levels using the hydroxylepatite assay procedure as described. The data are expressed as means ± standard deviations.

after 48–72 hr the cytosolic Ah receptor levels were 38% greater than those observed in the corn oil (control)-treated animals. MCDF treatment alone did not cause any major variation in cytosolic Ah receptor levels, compared with the corn oil-treated control rats; however, the results illustrated that, in the animals cotreated with TCDD plus MCDF, the latter compound partially inhibited the TCDD-mediated replenishment of the cytosolic receptor levels. The sedimentation coefficients for the rat hepatic cytosolic and nuclear receptor complexes are summarized in Fig. 4 and the results were comparable to those observed for the rat hepatoma H-E-II E Ah receptors. The sucrose density gradient sedimentation coefficients were identical in the presence or absence of MCDF.

***In vitro* studies.** Fig. 5 summarizes the time course of induction of AHH and EROD activities by TCDD (10^{-8} M), MCDF (10^{-7} M), and TCDD (10^{-8} M) plus MCDF (10^{-7} M). MCDF did not significantly induce AHH or EROD activities over the 36-hr time course, whereas TCDD caused maximal induction after 18 hr and these induced levels were maintained for the duration of the experiment. Cotreatment of the cells with TCDD plus MCDF resulted in a significant decrease in the enzyme induction responses, compared with the effects of treatment with TCDD alone. Significant antagonism of the monooxygenases was observed after 18 hr and was maintained for up to 36 hr. Table 1 summarizes the effects of staggered treatment of the cells with MCDF (10^{-7} M). At time 0, all cells were treated with TCDD (10^{-8} M) and MCDF was added at various times before and after the inducer. Significant antagonism of the induced monooxygenase enzyme activities by TCDD was observed when MCDF was added to the media at any of the time points from 2 hr before to 12 hr after the addition of TCDD.

Fig. 6 summarizes the time course of accumulation of the TCDD receptor complex in the nucleus in the presence or absence of MCDF. In the [^{3}H]TCDD-treated cells, an initial peak of occupied receptor complex was observed after 2 hr (approximately 250 fmol of nuclear complex/mg of DNA), which decreased substantially to 80–100 fmol of nuclear complex/mg of DNA after 4 hr and was then maintained for the remaining 36-hr treatment period. The effects of MCDF on nuclear TCDD receptor levels are also summarized in Fig. 6 and it was apparent that MCDF did not cause a significant decrease in occupied nuclear receptor levels throughout the total 36-hr incubation period. Fig. 7 illustrates that treatment

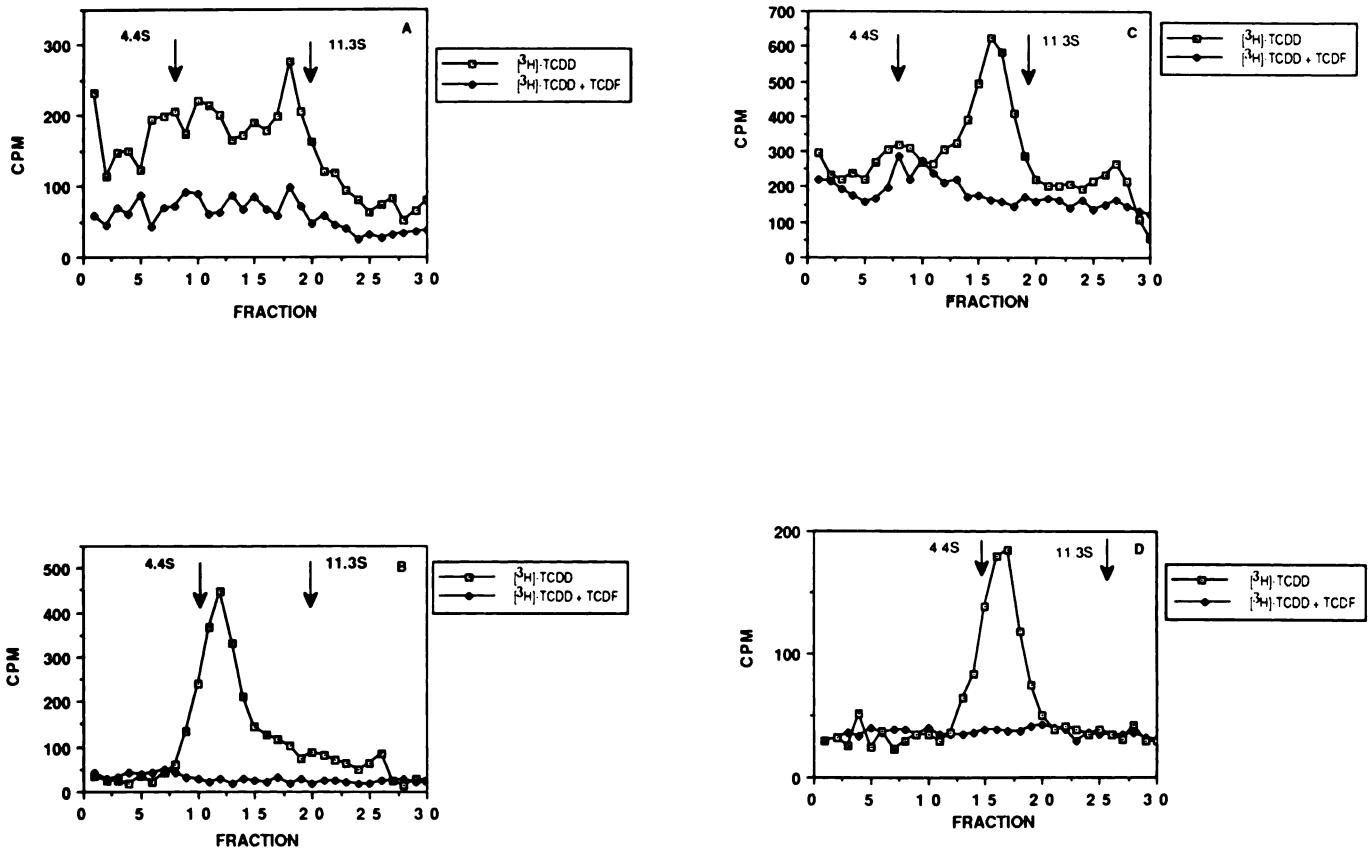

Fig. 4. Sucrose density gradient analysis of cytosolic Ah receptors from rat liver (*top right*) and rat hepatoma H-4-II E cells (*top left*) and nuclear Ah receptor complexes extracted from rat liver (*bottom right*) and rat hepatoma H-4-II E cells (*bottom left*). A 200-fold excess of TCDF was used to determine specific binding and ^{14}C-labeled bovine serum albumin and catalase were used as external standards to determine sedimentation constant (*S*).

with either TCDD or TCDD plus MCDF rapidly depleted cytosolic receptor levels within the first hour after treatment and these levels remained depressed for the duration of the study. Fig. 4 illustrates the sucrose density gradient profiles of the cytosolic and nuclear TCDD receptor complexes from rat hepatoma H-4-II E cells; their sedimentation coefficients were 9.21 ± 1.01 and 5.46 ± 1.04 S, respectively.

Discussion

MCDF binds with moderate affinity to the Ah receptor and is a weak Ah receptor agonist in rats and mice (11, 17). Cotreatment of male rats with a subeffective dose of MCDF (e.g., 50 μmol/kg) plus TCDD (16 nmol/kg) resulted in partial antagonism of the induction of AHH and EROD activities and cytochromes P-4501A1 and P-4501A2, compared with the effects observed after treatment of the rats with TCDD alone (11). MCDF also partially antagonized TCDD-mediated immunotoxicity (inhibition of the splenic plaque-forming cell response to sheep red blood cells) teratogenitcity (cleft palate), and AHH induction in C57BL/6J mice (17); however, partial antagonism of the latter response was less dramatic in mice than in rats. Double-reciprocal plot analysis of the saturation binding isotherms obtained with [^{3}H]TCDD in both rat and mouse hepatic cytosol in the presence of different concentrations of MCDF gave linear plots that intersected on the *y*-axis, suggesting that MCDF acts as a competitive inhibitor (11, 17). Comparable results have been reported for Aroclor 1254, α-naphthoflavone, and 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin (8–10).

The *in vivo* time course of induction of rat hepatic microsomal AHH and EROD activities by TCDD is summarized in Fig. 1 and demonstrates that both enzymes are rapidly induced over a period of 24–36 hr and are maintained at a maximally induced level for up to 96 hr. This rapid and persistent increase in enzyme activities by TCDD has previously been noted for TCDD and related halogenated aryl hydrocarbons (18–20). Polynuclear aromatic hydrocarbons such as 3-methylcholanthrene also cause a rapid induction response (18, 19); however, the induced enzyme activities do not persist, presumably due to rapid metabolism of the parent hydrocarbons. In contrast, MCDF (50 μmol/kg) does not significantly induce AHH or EROD activities over the 96-hr observation period and this was consistent with previous studies with this compound (11, 17). Cotreatment of the rats with MCDF plus TCDD resulted in significant partial antagonism of the induction responses within 24 hr and the antagonism was maintained for up to 96 hr. These results suggest that, at least over the limited duration of the *in vivo* study (i.e. 96 hr), hepatic levels of MCDF are sufficient to partially antagonize the induction of the monooxygenase activities. This is not surprising, because the 1,3,6,8-substituted dibenzofuran antagonists are substituted on alternate carbon atoms and, therefore, do not contain adjacent unsubstituted positions that would facilitate oxidative ring metabolism and excretion (21, 22). Thus, this class of TCDD

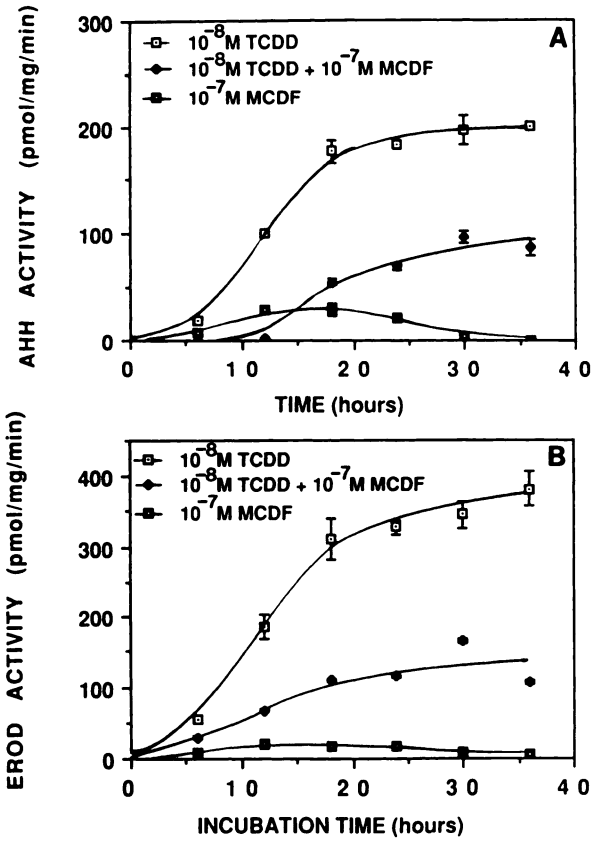

Fig. 5. Time course of induction of AHH (A) and EROD (B) by TCDD (10^{-8} M), MCDF (10^{-7} M), and TCDD (10^{-8} M) plus MCDF (10^{-7} M) in rat hepatoma H-4-II E cells. The results are expressed as means ± standard deviations.

TABLE 1

Induction of AHH and EROD by TCDD and TCDD plus MCDF: effects of timing on the partial antagonist activity of MCDF

All enzymes were assayed after 18-hr incubation with 10^{-8} M TCDD (added at time 0).

Time of MCDF (10^{-7} M) Addition	AHH	EROD
hr	*pmol/mg/min*	
−12	138 ± 11.7	245 ± 12.9
−6	141 ± 2.60	246 ± 17.0
−4	127 ± 9.75	223 ± 14.7
−2	101 ± 9.37*	169 ± 11.7*
−1	87.2 ± 3.70	154 ± 3.75*
−0.5	75.8 ± 4.15*	131 ± 15.8*
−0.25	67.0 ± 3.71*	111 ± 6.01*
0	66.7 ± 5.92*	109 ± 2.66*
0.25	68.7 ± 6.68*	125 ± 6.02*
0.5	62.3 ± 4.52*	114 ± 2.72*
1	68.9 ± 3.32*	118 ± 6.63*
2	73.4 ± 4.33*	131 ± 6.31*
4	76.2 ± 2.00*	114 ± 11.0*
6	75.7 ± 2.31*	121 ± 7.48*
12	75.0 ± 3.85*	105 ± 3.52*
TCDD (10^{-8} M)	153 ± 8.11	264 ± 17.2
Control (dimethylsulfoxide)	0 ± 0	4.63 ± 0.39

* Significantly different ($p < 0.01$) from cells treated with TCDD (10^{-8} M) alone.

antagonists is ideally suited for more long term *in vivo* studies and this was confirmed by the results shown in Fig. 1. Not surprisingly (11), the results from the *in vitro* studies in rat hepatoma H-4-II E cells were comparable to those observed in the rat; MCDF (10^{-7} M) exhibited minimal Ah receptor agonist

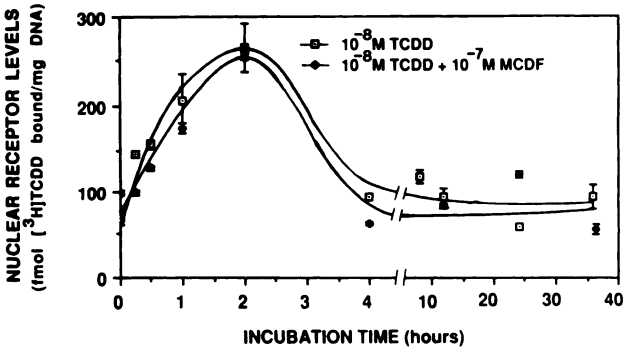

Fig. 6. Time course of accumulation of nuclear [^{3}H]TCDD-Ah receptor complexes in rat hepatoma H-4-II E cells treated with [^{3}H]TCDD (10^{-8} M) and [^{3}H]TCDD (10^{-8} M) plus MCDF (10^{-7} M), using the sucrose density gradient assay procedures. The data are expressed as means ± standard deviations.

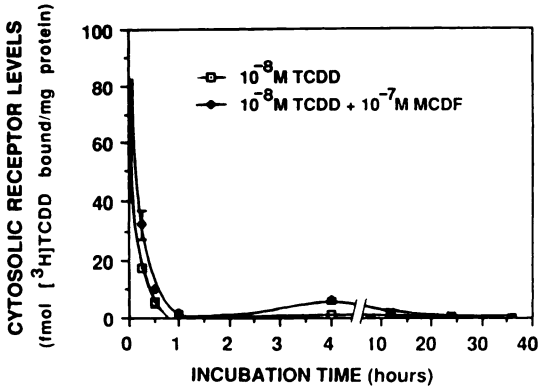

Fig. 7. Time course of effects of TCDD (10^{-8} M) and TCDD (10^{-8} M) plus MCDF (10^{-7} M) on cytosolic receptor levels in rat hepatoma H-4-II E cells. Data are expressed as means ± standard deviations.

activity as an inducer of AHH and EROD enzyme activities and, in the cotreatment experiments (Fig. 5), MCDF partially antagonized the induction of both monooxygenases. Partial antagonism was observed 12 hr after the initial treatment with TCDD and MCDF and persisted for 36 hr.

Sloop and Lucier (16) first noted that treatment of rats with TCDD resulted in a persistent elevation of hepatic Ah receptor levels. In other studies, it has also been reported that TCDD treatment also decreases cellular levels of other receptors [(e.g., progesterone (23, 24), estrogen (23, 24), and epidermal growth factor [25, 26)]. Fig. 3 illustrates the effects of TCDD (16 nmol/kg) on rat hepatic cytosolic Ah receptor levels for 72 hr after treatment. After an initial rapid decrease in receptor levels over the first 6 hr, there was a rapid replenishment of the cytosolic Ah receptor and, compared with the untreated animals, the receptor levels remained elevated for up to 72 hr. Sucrose density gradient analysis of hepatic cytosolic Ah receptors from the control or TCDD-treated rats gave peaks that sedimented at 9–10 S (see Fig. 4) and this was consistent with *S* values that have been previously reported (27–29). It is noteworthy that *in vivo* treatment with other Ah receptor agonists such as β-naphthoflavone (29), and 3,3′,4,4′,5-pentachlorobiphenyl (30) did not elevate hepatic Ah receptor levels in C57BL/6 mice and Wistar rats, respectively, whereas cytosolic Ah receptor levels were significantly elevated after treatment of rats with 2,2′,4,4,′,5,5,′-hexachlorobiphenyl (30). Thus, two halogenated aryl hydrocarbons, namely TCDD and 2,2′,4,4′,5,5′-

hexachlorobiphenyl, both elevated hepatic Ah receptor levels in rats; however, only TCDD was an Ah receptor agonist.

The results summarized in Fig. 3 also show that MCDF treatment does not significantly alter hepatic cytosolic Ah receptor levels in rats; however, in the cotreatment studies (TCDD plus MCDF), it was evident that MCDF significantly inhibited TCDD-mediated replenishment of the cytosolic receptor. It is possible that TCDD-induced elevation of hepatic Ah receptor levels may represent another Ah receptor-mediated process that is also partially antagonized by MCDF. Previous studies on the interactions of estradiol and partial estrogen antagonists have reported similar results and it was suggested that inhibition of cytosolic receptor replenishment may play a role in the activity of an antagonist (31). The biological importance of cytosolic Ah receptor replenishment and the inhibition of this process by MCDF is unknown and further studies are required to determine the significance of these observations.

The effects of TCDD and TCDD plus MCDF on cytosolic Ah receptor levels in rat hepatoma H-4-II E cells (Fig. 3) contrasted dramatically with the observed *in vivo* data (i.e., Fig. 3). Within 1 hr after treatment with either TCDD or TCDD plus MCDF, cytosolic Ah receptor levels rapidly decreased to nondetectable levels and remained depressed for up to 36 hr. Thus, the mechanisms associated with cytosolic Ah receptor replenishment observed in rats are not functional in the cells and constitute a major difference between the two systems.

The observation that TCDD causes a rapid decrease in apparent cytosolic Ah receptor in the rat hepatoma H-4-II E cells was utilized in a study that investigated the importance of measurable cytosolic receptor levels and timing in the action of MCDF as an antagonist (Table 1). Treatment of the cells for 18 hr with TCDD (10^{-8} M) resulted in the induction of AHH and EROD activities. As noted previously (Fig. 5), simultaneous cotreatment of the cells with TCDD plus MCDF (10^{-7} M) resulted in significant partial antagonism of the enzyme induction responses. Moreover, significant partial antagonism by MCDF was observed when the compound was added to the cells 2 and 1 hr before treatment with TCDD or 0.25, 0.5, 1, 2, 4, 6, and 12 hr after treatment with TCDD. These results show that, in the absence of measurable cytosolic Ah receptor (i.e., after 1 hr), the activity of MCDF as a partial antagonist was retained. However, based on the measured levels of cytosolic Ah receptor in this study, there are approximately 1600 Ah receptor binding sites/cell. In contrast, if the observed nuclear Ah receptor complex levels are used for the calculation, there are approximately 4200 Ah receptor binding sites/cell. These results suggest that there may be cellular reservoirs of Ah receptor that are available for interaction with MCDF after the apparent TCDD-mediated depletion of cytosolic Ah receptors. Thus, these results do not preclude the possibility that MCDF may act as a partial antagonist via binding to the Ah receptor; however, the reasons for the low partial antagonist activities observed when MCDF is added before TCDD have not been determined. The role of the Ah receptor in this process is supported by results obtained for a series of 6-substituted-1,3,8-trichlorodibenzofurans (32), in which only those congeners that exhibited moderate affinity for the Ah receptor protein and contained linear alkyl substituents (i.e., methyl, ethyl, propyl, isopropyl, and *t*-butyl) exhibited partial antagonist activity. In contrast, the 6-cyclohexyl analog was a poor Ah receptor binding ligand and exhibited no activity as an antagonist of TCDD-mediated AHH/EROD induction in rats or rat hepatoma H-4-II E cells.

Fig. 2 summarizes the levels of hepatic nuclear [^{3}H]TCDD Ah receptor complexes in rats treated with [^{3}H]TCDD (16 nmol/kg) and [^{3}H]TCDD (16 nmol/kg) plus MCDF (50 μmol/kg). With the exception of one time point, namely 24 hr, there were no significant differences in the levels of occupied nuclear receptor complexes in the presence or absence of MCDF. Moreover, if the receptor levels after 24 hr are calculated in terms of fmol of receptor complex/mg of nuclear protein (data not shown), there were no significant differences in occupied nuclear [^{3}H]TCDD Ah receptor complexes in the presence or absence of MCDF. The *in vitro* results summarized in Fig. 6 also showed that the levels of hepatic nuclear [^{3}H]TCDD Ah receptor complexes were not significantly different in the presence or absence of MCDF. Previous studies of genetically inbred mice by Tukey and co-workers (20) have reported a correlation between the levels of nuclear [^{3}H]TCDD receptor complex and the percentage of maximally induced cytochrome P-4501A1 mRNA, which also correlated with the per cent induction of cytochrome P-4501A1 and dependent enyzme activities. The results obtained in this study and in previous work (11) clearly show that MCDF can reduce the TCDD-mediated induction of AHH and EROD activities in rat liver and rat hepatoma H-4-II cells; however, the occupied nuclear levels are not significantly altered after cotreatment with the partial antagonist. Moreover, the sucrose density gradient sedimentation coefficients for nuclear [^{3}H]TCDD-Ah receptor complexes (5–6 S from rat liver or rat hepatoma H-4-II E cells) were comparable in the presence or absence of MCDF (Fig. 4). These data can be interpreted in several ways; however, based on the results presented herein and on related studies (7, 8, 11, 17, 30), it is hypothesized that the antagonist activity of MCDF is associated with initial formation of an Ah receptor complex, followed by competition for nuclear binding sites, which may include the dioxin regulatory elements located in the 5′-upstream region for the cytochrome P-4501A1 gene (4–6). Current research is focused on the preparation of radiolabeled analogs of MCDF that can be utilized to further probe the cellular interactions of the partial antagonist with the Ah receptor (or other proteins) and the disposition of the compound/complex in target cells.

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