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INHIBITION OF ORNITHINE DECARBOXYLASE ACTIVITY BY 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN*

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Abstract—The effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the activity of rat liver ornithine decarboxylase (ODC) were investigated. Sixteen hours after partial hepatectomy, rats that had been pretreated with TCDD for 1 week exhibited a 3- to 4-fold increase in ODC activity, while vehicle controls exhibited an 8- to 10-fold increase. This inhibition of ODC induction by TCDD was time dependent since TCDD administration at the time of partial hepatectomy did not produce inhibitory effects on the subsequent ODC induction. ODC induction after either aminophylline or dexamethasone administration, agents which act via cAMP-mediated and direct nuclear events, respectively, also was inhibited by pretreatment with TCDD. It was concluded that TCDD pretreatment decreased the ability of the liver to respond to hormonal stimulation as reflected in the attenuation of ODC induction. RNA polymerase I activity, which positively correlates with ODC activity in growth and development, decreased concomitantly with decreased induction of ODC. In unstimulated liver, RNA polymerase I activity, as well as protein, DNA, and RNA levels, remained unchanged 1 week after TCDD. However, TCDD administration resulted in decreased liver concentrations of putrescine and spermidine, but not spermine. This suggests that TCDD pretreatment results in a time-dependent decrease in hormone responsivity.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a highly toxic contaminant formed during the commercial production of 2,4,5-trichlorophenol [1], has been detected as a widespread environmental contaminant [2]. Although the toxicity of TCDD has been well characterized [1, 3, 4], the mechanism(s) by which TCDD exerts its biological toxicity remains unknown. TCDD has been shown to induce a large number of enzymes [5], to produce liver hypertrophy [1], and to result in a characteristic wasting syndrome in laboratory animals [1, 3].

This report discusses the effects of TCDD on ornithine decarboxylase (EC 4.1.1.17, ODC) and RNA polymerase I (EC 2.7.7.6) activities. ODC, the rate-limiting enzyme in polyamine biosynthesis [6–8], exhibits increased activity in response to various hormones, drugs, and hypertrophic stimuli [9, 10]. Polyamines, the organic cations of the cell, accumulate as a function of increased tissue mass and increased proliferation [9–12]. ODC may be the labile initiation factor of RNA polymerase I and has been shown to increase in activity prior to induction of the monooxygenase system [9, 13–21].

In this study, we have evaluated the ability of pretreatment with TCDD to inhibit both ODC induction and the concomitant RNA polymerase I activity following partial hepatectomy and after dexamethasone or aminophylline administration, all systems in which ODC activity is markedly stimulated. Pretreatment with TCDD markedly altered the ability of the liver to respond to hormonal challenge as assessed by the extent of ODC induction and RNA polymerase I activity.

METHODS

Chemicals. L-[1-^{14}C]Ornithine hydrochloride (54 mCi/mmole) was purchased from the Amersham/Searle Corp. (Arlington Heights, IL). Dithiothreitol (DTT), pyridoxal phosphate, phenylmethylsulfonylfluoride, EDTA, bovine serum albumin, dexamethasone acetate, aminophylline, NADH, NADPH, benzo[*a*]pyrene, and all buffers and metals employed in this study were obtained from the Sigma Chemical Co. (St. Louis, MO). TCDD was a gift from Dr. H. B. Matthews, NIEHS, Research Triangle Park, NC. 3-Hydroxybenzo[*a*]pyrene was provided by Dr. D. Nebert, National Institutes of Health.

Protein assay. Protein was determined by the method of Lowry *et al.* [22], with bovine serum albumin used as the reference standard.

Animals. Male Sprague–Dawley rats (80–100 g) were obtained from the University of Arizona breeding colony and were administered TCDD i.p. (dissolved in acetone–corn oil, 1:17) at doses and times described in the figure legends. Partial (70%) hepatectomies were performed under light ether anesthesia on both TCDD-treated and vehicle control animals [23]. Dexamethasone was administered i.p. (0.5 mg/kg) in normal saline (2 ml/kg), and aminophylline was administered i.p. (0.2 mmole/kg) in

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normal saline (4 ml/kg). Animals were killed by cervical dislocation. Tissues were removed and homogenized in 4 parts of the buffer required for the individual assays. Homogenates were centrifuged for 5 min at 50,000 *g*. The supernatant fraction was used for all cytosolic enzyme assays.

Ornithine decarboxylase assay. ODC activity was measured as the liberation of $^{14}CO_2$ from L-[1-^{14}C]ornithine [8]. Incubations were conducted at 37° in a medium containing 50 mM sodium–potassium phosphate buffer (pH 7.2), 0.1 mM EDTA, 2 mM dithiothreitol, 0.013 mM pyridoxal phosphate, 0.04 mM phenylmethylsulfonylfluoride, 5 mM NaF, 1.0 μCi L-[^{14}C]ornithine (sp. act. = 5 mCi/mmole), and 0.5 mM L-ornithine, in a final volume of 0.2 ml.

The reaction was initiated by the addition of 0.05 ml of tissue supernatant fluid to the reaction mixture. After 30 min, the reaction was terminated by the addition of 0.5 ml of 1 M citric acid. The samples were allowed to stand for another 20 min, and the CO_2 evolved was trapped by 0.02 ml of 2 N NaOH on a 3 MM filter paper (Whatman, Clifton, NJ) suspended above the reaction mixture in a plastic well (Kontes, Vineland, NJ). The filters were counted in toluene–Omnifluor scintillation mixture (New England Nuclear Corp., Boston, MA). Activity is expressed as pmoles of CO_2 evolved per min per mg of cytosolic protein at 37°. The enzyme activity was linear with respect to incubation time and enzyme concentration.

Aryl hydrocarbon hydroxylase assay. Aryl hydrocarbon hydroxylase (AHH) activity was determined by a modification of the procedure of Nebert and Gelboin [24]. In a total reaction volume of 1.0 ml, 0.2 ml of tissue supernatant fluid was incubated in the presence of 0.03 M Trizma buffer (pH 7.4), 5 mM $MgCl_2$, 0.3 mg NADPH and 0.3 mg NADH. The reaction was initiated by adding 0.08 mmole benzo[*a*]pyrene (in 0.04 ml methanol). The mixture was shaken at 37° for 10 min. The reaction was stopped by the addition of 4.25 ml of cold hexane–acetone solution (3.25 ml hexane : 1 ml acetone). Samples were then vortexed and incubated with shaking at 37° for 10 min. A 1.0-ml sample of the organic phase was extracted with 3.0 ml of 1 N NaOH. The concentration of hydroxylated benzopyrene extracted in the alkaline phase was determined spectrophotofluorometrically with activation at 396 nm and fluorescence at 522 nm. Activity is expressed as pmoles of 3-hydroxybenzo[*a*]pyrene formed per min per g of tissue.

RNA polymerase I assay. Nuclei were isolated by the procedures of Blobel and Potter [25] and Busch *et al.* [26]. The nuclear aliquots were adjusted to approximately equal amounts of protein (0.6 mg/0.15 ml) before use in the RNA polymerase assay. RNA polymerase I activity was determined [15] by measuring the incorporation of [^{3}H]UTP (37.4 mCi/mmole, New England Nuclear) into RNA in the presence of α-amanitin. The RNA polymerase assay mixture contained in a volume of 0.375 ml: 7.5 μg pyruvate kinase; 18.8 μmoles Tris–HCl (pH 7.9); 0.675 μg α-amanitin; 1.5 μmole phosphoenol pyruvate; 0.6 μmole mercaptoethanol; 3.0 μmoles KCl; 0.6 μmole $MnCl_2$; 2.25 μmoles NaF; 21.75 μmoles $(NH_4)_2SO_4$; 8×10^{-5} μmoles [^{3}H]UTP; 0.0375 μmole unlabeled UTP; and 0.225 μmole each of GTP, ATP and CTP. Activity is expressed as trichloroacetic acid-precipitable cpm.

RNA and DNA determinations. Hepatic RNA and DNA contents were determined by the methods of Schneider [27]. An aliquot (1.0 ml) of a 20% liver homogenate in 0.05 M Tris–HCl (pH 7.9) was extracted sequentially with 10% trichloroacetic acid and 95% ethanol to remove acid-soluble compounds and lipids. Remaining precipitates were dissolved in hot trichloroacetic acid, and supernatant aliquots were taken separately for the determination of total RNA and DNA.

Polyamine determinations. Polyamine analysis was performed with a Durrum D-500 amino acid analyzer (Dionex Corp., Sunnyvale, CA) as described previously [28].

Statistics. The two-sided Student's *t*-test was used to determine the level of significance. Values of $P < 0.05$ were considered significant.

RESULTS

No increase in ODC activity in rat liver was observed at 2, 4, 6, 12, 16, 24, 48 or 72 hr after i.p. administration of TCDD at doses ranging from 5 to 135 μg/kg. However, these same doses of TCDD produced large increases in renal and hepatic AHH activity, which may serve as a positive control for TCDD biological activity in the rodent [29]. Table 1 summarizes the effects of a single dose of TCDD (135 μg/kg) administered i.p. 16 hr before sacrifice on the activities of hepatic and renal ODC and AHH. The kidney exhibited higher ODC activity than did the liver. Interestingly, ODC activity in the kidney appeared to be inhibited at 16 hr following TCDD administration, although a large S.E.M. precludes a definitive statement. Based on this observation, it

Table 1. Ornithine decarboxylase and aryl hydrocarbon hydroxylase activities 16 hr after TCDD (135 μg/kg body weight, i.p.)*

	Liver		Kidney	
	ODC†	AHH‡	ODC†	AHH‡
Control	1.0 ± 0.2	2,360 ± 740	10.1 ± 2.0	38 ± 15
TCDD	0.9 ± 0.3	27,119 ± 3,974§	6.2 ± 2.2	4,665 ± 1,492§

* Each value is the mean of five animals ± S.E.M.
† Expressed as pmoles · min^{-1} · (mg protein)$^{-1}$.
‡ Expressed as pmoles · min^{-1} · (g tissue)$^{-1}$.
§ Significantly different from control ($P < 0.05$).

Table 2. Effects of TCDD on ornithine decarboxylase and aryl hydrocarbon hydroxylase activities 16 hr after partial hepatectomy*

TCDD treatment	ODC†	AHH‡
At hepatectomy		
Control	1.0 ± 0.2	2,360 ± 740
Hepatectomy alone	12.5 ± 2.2	1,200 ± 140
TCDD plus hepatectomy§	10.7 ± 1.6	12,000 ± 220‖
7 Days pretreatment		
Control	1.2 ± 0.1	2,400 ± 350
Hepatectomy alone	12.5 ± 2.1	3,500 ± 165
TCDD plus hepatectomy¶	4.3 ± 1.0‖	29,000 ± 1,600‖
14 Days pretreatment		
Control	0.9 ± 0.1	3,480 ± 490
Hepatectomy alone	8.5 ± 0.9	6,700 ± 470
TCDD plus hepatectomy¶	3.4 ± 1.0‖	34,000 ± 6,000‖

* A single dose of TCDD was administered to the rats in the TCDD groups at the times indicated prior to partial hepatectomy. Each value is the mean ± S.E.M. for five rats.

† Expressed as pmoles·min^{-1}·(mg protein)$^{-1}$.

‡ Expressed as pmoles·min^{-1}·(g liver)$^{-1}$.

§ Thirty μg/kg body weight, i.p.

‖ Significantly different from hepatectomy alone ($P < 0.05$).

¶ Five μg/kg body weight, i.p.

was considered that TCDD may actually decrease ODC activity in the liver, but that such a decrease would be difficult to substantiate because of the low basal ODC activity.

Therefore, the effects of TCDD pretreatment on the effects of hormones or of partial hepatectomy, regimens known to enhance hepatic ODC activity [7, 9, 30, 31], were investigated. Following partial hepatectomy, hepatic ODC activity increases in a biphasic manner with peak activities occurring at 4 and 16 hr post-surgery [8, 9]. In the present experiments, similar elevations of ODC activity were found at both 4 and 16 hr, and we monitored TCDD effects at 16 hr post-hepatectomy. Rats pretreated with TCDD for 7 or 14 days and then hepatectomized exhibited 3- to 4-fold increases in ODC activity (Table 2) compared to 8- to 10-fold elevations by 16 hr post-hepatectomy in those not pretreated with TCDD. Thus, TCDD partially inhibited the ODC activity induced by partial hepatectomy. When TCDD was administered at the time of partial hepatectomy, no difference in the 16-hr ODC activity appeared between TCDD-treated and control animals (Table 2). This suggested that TCDD required a certain amount of time following administration to produce inhibitory effects on hepatic ODC induction. Aminophylline that was administered 24 hr after administration of TCDD induced ODC activity to the same extent as aminophylline alone (data not presented). In all cases, TCDD-treated animals exhibited AHH activity 5- to 10-fold higher than controls (Table 2).

Since ODC is known to be induced either by direct nuclear action, as with certain steroid hormones, or via the activation of cAMP-dependent protein kinase(s) in response to increased intracellular levels of cAMP, the effects of TCDD on ODC activity induced by either pathway were investigated [9, 18, 32–38]. The inducing agents were dexamethasone, which is believed to act directly at a nuclear site [33], and aminophylline, a phosphodiesterase inhibitor which acts by increasing intracellular levels of cAMP. Both agents produced substantial increases in ODC activity by 4 hr after i.p. injection (Table 3). TCDD pretreatment totally abolished the effects of aminophylline to induce ODC (Table 3) and substan-

Table 3. Effects of TCDD pretreatment on hepatic ornithine decarboxylase activity induced by aminophylline or dexamethasone*

	ODC activity [pmoles·min^{-1}·(mg protein)$^{-1}$]	
	Control	TCDD
Saline	0.9 ± 0.05	
Aminophylline†	13.1 ± 1.2	1.4 ± 0.3‡
Dexamethasone§	11.0 ± 1.5	4.8 ± 1.2‡

* TCDD (5 μg/kg) in acetone–corn oil (1:17) was administered to the rats in the TCDD groups 1 week prior to challenging them with saline, aminophylline, or dexamethasone. Each value is the mean ± S.E.M. for five rats.

† Dose of 0.2 mmole/kg body weight, i.p.

‡ Significantly different from control ($P < 0.05$).

§ Dose of 0.5 mg/kg body weight, i.p.

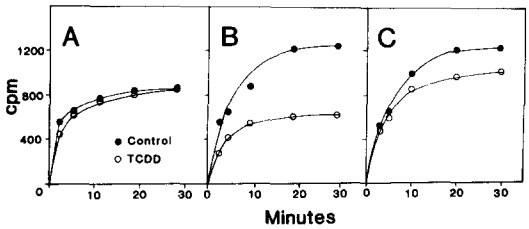

Fig. 1. RNA polymerase I activities 1 week after TCDD (5 μg/kg). Values represent the time-dependent incorporation of [^{3}H]uridine into rRNA in rat liver (A) without stimulation, (B) 5 hr after partial hepatectomy, and (C) 16 hr after partial hepatectomy.

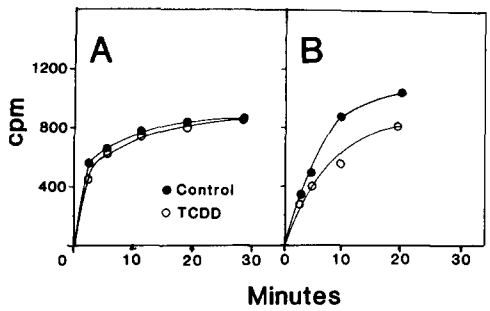

Fig. 2. RNA polymerase I activities 1 week after TCDD (5 μg/kg). Values represent the time-dependent incorporation of [^{3}H]uridine into rRNA in rat liver (A) without stimulation, and (B) 4 hr after dexamethasone.

tially reduced the induction of ODC in response to dexamethasone.

Because increased ODC activity has been correlated with a subsequent increase in RNA polymerase I activity [13–15, 19], we examined the effects of TCDD on RNA polymerase I activity at times when ODC activity was inhibited. RNA polymerase I activities in unstimulated rat liver or following partial hepatectomy (Fig. 1) or dexamethasone (Fig. 2) were evaluated 1 week after TCDD. When hepatic ODC activity was found to be inhibited by TCDD, 20–50% decreases in RNA polymerase I activity also were detected. RNA polymerase I activities in unstimulated rat liver (Figs. 1A and 2A) were essentially the same for TCDD-treated and control animals.

TCDD-treated animals exhibited lower hepatic levels of putrescine and spermidine, relative to controls, 1 week after treatment (Table 4). No significant differences in liver protein, DNA, RNA or spermine appeared between TCDD-treated and control animals at 1 week.

Table 4. Liver content of protein, DNA, RNA and polyamines 1 week after TCDD (5 μg/kg body weight, i.p.)*

	Control	TCDD
Liver weight†	4.6 ± 0.2	5.8 ± 0.1‡
Total protein§	223.2 ± 3.2	210.0 ± 6.5
Total DNA§	2.6 ± 0.2	2.7 ± 0.1
Total RNA§	9.2 ± 0.6	8.7 ± 0.6
Total polyamines‖		
Putrescine	11.0 ± 2.5	5.7 ± 1.0‡
Spermidine	942.5 ± 130.6	644.5 ± 76.8‡
Spermine	612.5 ± 73.4	687.0 ± 101.0

* Each value is the mean ± S.E.M. of five animals.
† Expressed as g/100 g body weight.
‡ Significantly different from controls ($P < 0.05$).
§ Expressed as mg/g wet weight of liver.
‖ Expressed as nmoles/g wet weight of liver.

DISCUSSION

The ability of TCDD to inhibit hormonally-induced ODC activity depended upon a pretreatment period. TCDD administration at the same time as dexamethasone, aminophylline, or partial hepatectomy did not result in the inhibition of ODC induction in liver detected in animals that had been pretreated for 1 week with a single dose of TCDD. The requirement for a pretreatment period suggests that the inhibition of ODC induction by TCDD was not the result of direct inhibition, but resulted from an alteration in the responsiveness of the liver to hormonal challenge.

The fact that TCDD pretreatment inhibited the induction of ODC activity by either direct nuclear (dexamethasone) or cAMP-mediated (aminophylline) events suggests that this phenomenon was related to marked alteration in hormone responsiveness of the animal rather than to a selective effect on a particular hormonal ODC-induction pathway. It could not be determined from this study whether the TCDD affected a point common to steroid hormone and cAMP-mediated induction of ODC, or if it possibly increased a posttranslational modification of the ODC molecule.

Since TCDD treatment resulted in large increases in AHH activity, it may be that the subsequent inhibitory effects on ODC induction and RNA polymerase I activity were due to increased metabolism (inactivation) of the aminophylline, dexamethasone or endogenous hormones involved in ODC induction following partial hepatectomy. This seems unlikely, however, since administration of aminophylline 24 hr after TCDD administration, a time when AHH activity was well induced, resulted in none of the inhibitory effects noted 1 week after TCDD (unpublished data).

Similar results have been reported by Bulger and Kupfer [39] using the rat uterus. They reported that treatment of ovariectomized female rats with 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane (*o,p'*DTT) was followed by the induction of ODC activity in 6–7 hr. However, pretreatment with *o,p'*DTT, for 48 hr resulted in an inhibition of rat uterine ODC induction by subsequent administration of estradiol, tamoxifen, or *o,p'*DTT. They also provided evidence that increased activity of the monooxygenase system was not responsible for the reported inhibition of ODC induction. Pretreatment of rats with tamoxifen, which does not induce the monooxygenase system, resulted in an inhibition of ODC induction by subsequent treatment with *o,p'*DTT or estradiol [39]. Furthermore, *p,p'*DDE, a structural analog of *o,p'*DTT, which induces the hepatic monooxygenase system, does not possess the

inhibitory effects of *o,p'*DTT on ODC induction [40]. Therefore, it appears that a factor(s) other than increased metabolism of the inducing agent is responsible for the inhibition of ODC induction produced by *o,p'*DTT and TCDD pretreatment in rat uterus and liver respectively.

Recently, it has been shown that conjugation of ODC to its product putrescine *in vitro* by the calcium-dependent enzyme, transglutaminase, results in a loss of ODC activity [21]. This ODC–putrescine conjugate has been suggested to be the rapidly turning over protein responsible for RNA polymerase I stimulation [20] that results in increased production of ribosomal RNA. This mechanism does not explain our results, however, because decreased, rather than increased, RNA polymerase I activity (Figs. 1 and 2) accompanied decreased ODC induction.

Pretreatment with TCDD did not significantly alter the basal levels of liver protein, DNA or RNA at 1 week post-treatment. These data support our findings that the basal level of RNA polymerase I activity was essentially unaltered in unstimulated rat liver 1 week after treatment with 5 μg/kg TCDD. However, there were decreased concentrations of putrescine and spermidine, but not of spermine, in unstimulated liver 1 week after TCDD. The decreased putrescine and spermidine reflect decreased polyamine biosynthesis or increased turnover following TCDD treatment. This suggests a time-dependent alteration, after TCDD treatment, in the ability to respond to hormonal stimulation.

Failure to observe increased ODC activity within 72 hr following TCDD administration does not rule out an early induction of ODC activity by TCDD. As pointed out by Nebert *et al.* [41], in order to rule out induction of ODC by TCDD, time points should be evaluated approximately every half-hour.

TCDD pretreatment results in a time-dependent inhibition of ODC induction and of RNA polymerase I activity following hormonally regulated growth stimulation. Since ODC and RNA polymerase I activities correlate positively with cell growth and metabolism, these results may reflect a TCDD-induced alteration in the ability of the cell to respond normally to growth and metabolic stimuli.

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