INDUCTION OF DT-DIAPHORASE BY 2,3,7,8-TETRA-CHLORODIBENZO-p-DIOXIN (TCDD)

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Received November 5,1975

SUMMARY

The compounds 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are both inducers of the enzyme system aryl hydrocarbon hydroxylase. It has recently been reported that 3-MC is also an inducer of DT-diaphorase activity in rat liver. In this report the ability of TCDD to induce hepatic DT-diaphorase activity was examined. The results indicate that TCDD is approximately 17,000 times more potent as an inducer of DT-diaphorase activity than 3-MC.

The compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)* is the most toxic member of the chlorinated dibenzo-p-dioxins, which are found as contaminants in some preparations of polychlorinated phenols (1). TCDD has an LD_{50} in the adult male guinea pig of 0.6 μg/kg (1). As well as exhibiting extreme toxicity, TCDD, like 3-methylcholanthrene (3-MC), is also capable of inducing the activity of the enzyme system aryl hydrocarbon hydroxylase (2). A significant degree of induction of the activity of this enzyme system in rats is maintained for up to 35 days after a single 10 μg/kg dose of TCDD (2).

Recently it has been reported (3) that rat liver DT-diaphorase (E.C.1.6.99.2) undergoes a several fold increase in activity after the *in vivo* administration of 20 mg/kg 3-MC. In view of the similarities of 3-MC and TCDD in AHH induction, it was of interest to examine the possibility of induction of DT-dipahorase activity by TCDD.

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* Abbreviations used are TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; 3MC, 3-methylcholanthrene; AHH, aryl hydrocarbon hydroxylase; and 2,6-dichlorophenolindophenol, DCIP.

METHODS AND MATERIALS

Male Sprague-Dawley rats were injected i.p. with varying doses of TCDD (98% pure, Dow Chemical Company) or 20 mg/kg 3-MC (Sigma) dissolved in olive oil. Controls received olive oil only. After sacrifice, the livers were removed and subcellular fractions were prepared according to Hogeboom (4) using 0.1M Tris-HCl buffer, pH 8.1, rather than sucrose.

Diaphorase activity was assayed by following the reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm. The assay system contained 0.06 mM DCIP (K&K Laboratories, Inc.), 50 mM Tris-HCl buffer, pH 7.5, 0.15 mM NADH or NADPH (Boehringer Mannheim) and sufficient of the enzyme solution to give a linear reaction for at least one minute at this reduced pyridine nucleotide concentration. The total volume of the incubations was 2.0 ml. In the assays using microsomes as the source of the enzyme activity and NADPH as the reductant, the cytochrome-c reductase activity in the microsomes was selectively inhibited by the addition of NADP (5). As verified by electron microscopy, the mitochondria prepared as described above were osmotically disrupted. Sonication of these mitochondrial preparations in 0.1M Tris-HCl buffer, pH 8.1, did not increase the DT-diaphorase activity. Protein was measured by the biuret method (6). The levels of significance between treatment groups were calculated using the Students' t-test.

RESULTS

A single injection of 3-MC (20 mg/kg) resulted in a steady rise in the NADPH diaphorase activity of both cytosol and microsomes reaching a maximum in approximately 72 hours in both cases (Figures 1 and 2). In the cytosol significantly ($P < 0.05$) higher levels than controls were maintained for at least fifteen days post-injection. A significant level of induction of the microsomal activity was also maintained fifteen days post-injection. By comparison a single i.p. dose of 90 μg/kg of TCDD resulted in a rapid rise in NADPH diaphorase activity in both cytosol and microsomes to a level approximately six fold above controls after 24 hours. In the microsomal fraction this activity continued to increase slowly, eventually achieving a thirteen fold induction after 15 days. The cytosol activity increased to a maximal value seventeen times greater than controls at seven days. This level of induction was maintained through the last time point in this experiment (15 days).

The short term induction of NADPH diaphorase activity in cytosol, microsomes, and mitochondria after a single 90 μg/kg dose of TCDD was also examined (Figure 3). After six hours the NADPH diaphorase activity in all

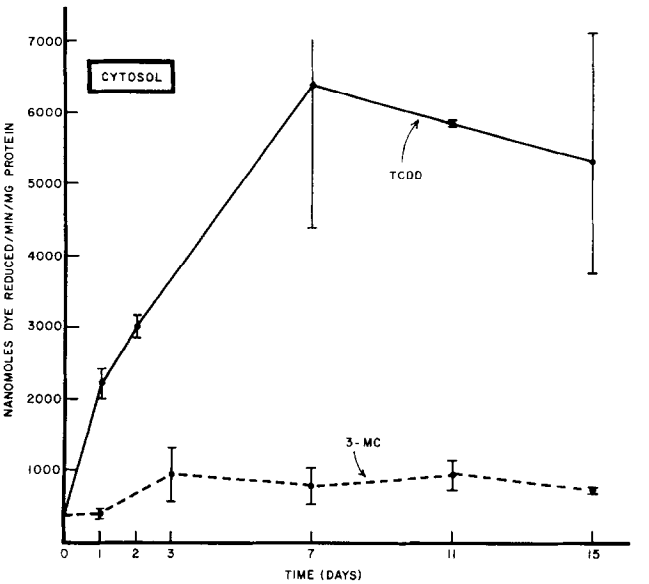

Figure 1. A comparison of the induction of cytosol NADPH diaphorase activity by TCDD or 3-MC.

Each value represents the mean of individual values from three rats; brackets indicating standard deviations. Rats, 250-300 gm, were injected i.p. with either 90 μg/kg TCDD or 20 mg/kg 3-MC both dissolved in olive oil.

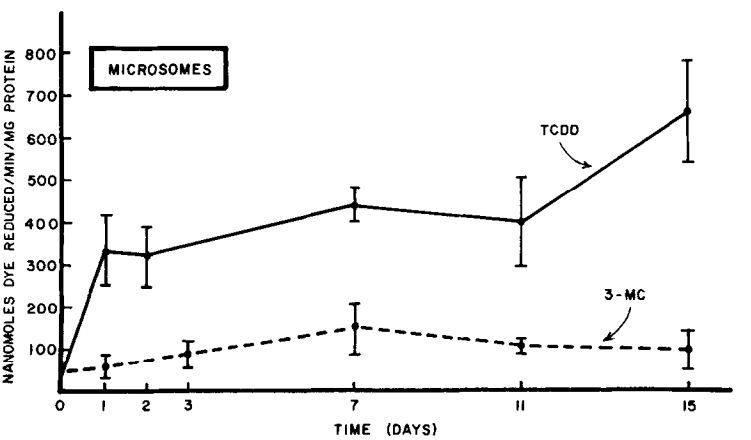

Figure 2. A comparison of the induction of microsomal NADPH diaphorase activity by TCDD or 3-MC.

Each value represents the mean of individual values from three rats; brackets indicating standard deviations. Rats, 250-300 gm, were injected i.p. with either 90 μg/kg TCDD or 20 mg/kg 3-MC both dissolved in olive oil. NADP (5×10^{-3}M) was used in these incubations to inhibit NADPH cytochrome c reductase.

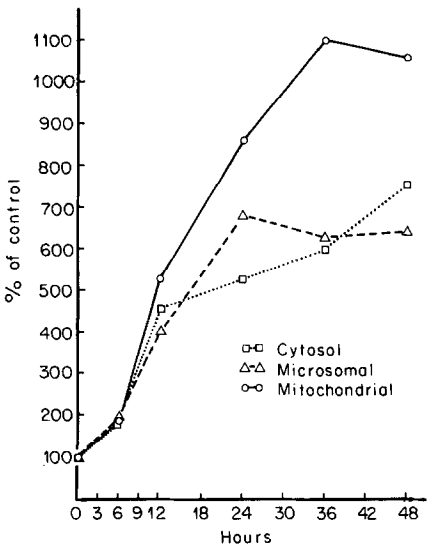

Figure 3. Short term induction of cytosol, microsomal and mitochondrial NADPH diaphorase activity by TCDD.

Each value represents the average of individual determinations from three animals. Rats 100-125 gm were injected i.p. with 90 μg/kg TCDD dissolved in olive oil. Control values were: (nanomoles of DCIP reduced/min./mg protein) cytosol 398, microsomes 49, mitochondria 34. NADP (5×10^{-3}M) was used in the incubations with microsomes to inhibit NADPH cytochrome c reductase.

fractions had approximately doubled and increased about four fold at twelve hours post-injection. Cytosol activity continued to rise, although at a somewhat reduced rate, to a value after 48 hours which was 700% that of control. Microsomal activity increased to 600% that of the controls at 48 hours. The most dramatic increase was in mitochondrial activity which rose to a maximum value eleven times that of controls at thirty-six hours and remained at this level forty-eight hours post-injection. Pretreatment of animals with actinomycin D (1 mg/kg) one hour prior to a dose of 45 μg/kg TCDD prevented the increase in diaphorase activity seen at 6 hours. It thus appears the increase in activity is the result of increased synthesis of enzyme.

Figures 4 and 5 present the data obtained from a combined dose response and time course study of the TCDD induced increase in NADPH diaphorase activity in the cytosol and microsomes respectively. Four doses (5.63, 11.25,

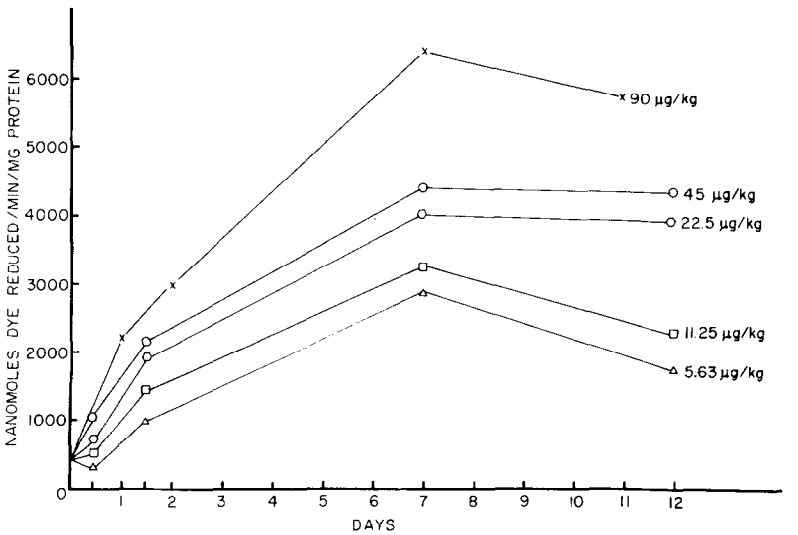

Figure 4. An examination of the kinetics of induction of NADPH-diaphorase activity in rat liver cytosol using various doses of TCDD.

Each value represents the average of the enzyme activities in 3 rats at each dose and at each time point. An approximation of the standard deviations of each value can be obtained by examining the data in Table 1. To have included the standard deviations in this figure would have rendered it less interpretable.

22.5 and 45 µg/kg) of TCDD were administered i.p. and the induction of NADPH diaphorase activity was determined at 12 hours, 36 hours, 7 days and 12 days post-injection. The degree of induction caused by a dose of 90 µg/kg (from Figures 1 and 2) is also included for comparison. As can be seen, with all doses the increase in enzyme activity appears to reach a maximum in approximately 7 days in both the cytosol (Figure 4) and microsomes (Figure 5). However, it is quite possible that the enzyme activity in animals receiving any one dose may have reached a maximum a few days earlier or later than the 7 day time point used in this experiment. The activity in the cytosol of animals receiving 45 and 22.5 µg/kg TCDD appeared not to have decreased from the 7th to the 12th day post-injection. On the other hand, the activity in the cytosol appears to have decreased from the 7th to the 12th day post-

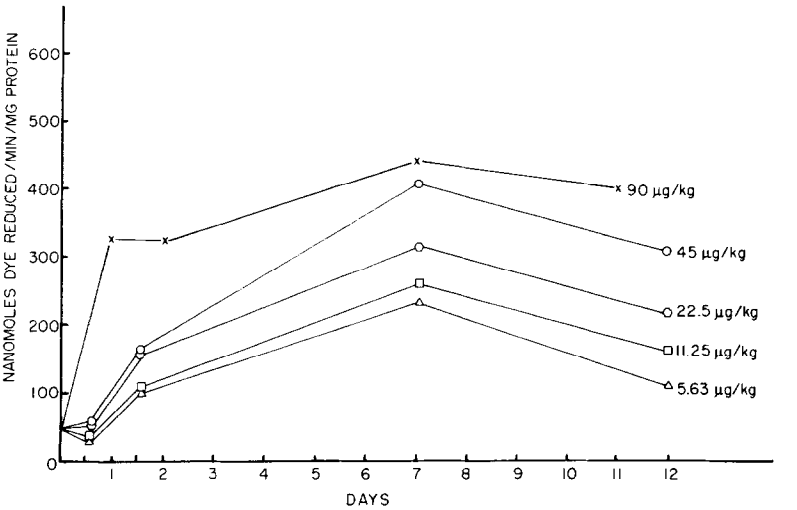

Figure 5. An examination of the kinetics of induction of NADPH-diaphorase activity in rat liver microsomes using various doses of TCDD.

Each value represents the average of the enzyme activities in 3 rats at each dose and each time point. An approximation of the standard deviations of each value can be obtained by examining Table 1. To have included the standard deviations in this figure would have rendered it less interpretable. NADP (5×10^{-3}M) was used in these incubations to inhibit NADPH cytochrome c reductase.

injection in the animals which received 11.25 and 5.63 µg/kg TCDD. At all doses, except 90 µg/kg (see Figure 5), the activity in the microsomes appears to have decreased from the 7th to the 12th day (11th day in the case of 90 µg/kg). However, the decrease seems to have been more pronounced in the animal receiving the lower doses of TCDD.

Table 1 presents a comparison of the activity of NADPH and NADH diaphorase activity in the cytosol at 7 days post-injection of the various levels of TCDD. In general, the lower doses of TCDD appeared to increase NADPH diaphorase activity to a greater degree than NADH diaphorase activity. However, these differences are not statistically significant. The large standard deviations in Table 1 illustrate that there was considerable animal variation in the degree of induction of the two diaphorase activities.

TABLE 1

DT-Diaphorase Activity in the Cytosol Seven Days After Administration of Various Dosages of TCDD[a]

Dose TCDD µg/kg	Reduced Pyridine Nucleotide	
	NADPH	NADH
45.0	4415 ± 271	4579 ± 414
22.5	4022 ± 598	3309 ± 645
11.25	3276 ± 256	2972 ± 354
5.63	2891 ± 1011	2505 ± 193
0[b]	366 ± 98	289 ± 133

[a] Nanomoles DCIP reduced/min/mg protein. Each value is the mean ± the standard deviations of individual values from three animals.

[b] Control values are the mean ± S.D. of three individual values from rats injected with olive oil and sacrificed 24 hours later.

DISCUSSION

The data presented here indicates that TCDD is an extremely potent inducer of the activity of hepatic DT-diaphorase enzymes in various subcellular fractions of the liver. Using a 90 µg/kg dose of TCDD the onset of induction is rapid, being detectable after 6 hours. At this dose, maximal induction of microsomal NADPH-diaphorase is achieved in 24-48 hours, whereas approximately 7 days are required for maximal induction of the activity in the cytosol. This maximal level of induction in both cytosol and microsomes appears to be maintained for at least 15 days post-injection, the last time point examined in these studies.

At lower doses of TCDD the onset of induction is less rapid. However, maximal induction was obtained at approximately 7 days with all doses ex-

amined. There appears to be a roughly linear relationship between NADH diaphorase and NADPH diaphorase activity at 7 days and the dose of TCDD administered (linear regression correlation coefficient of 0.895 and 0.725 respectively).

It has been reported that TCDD is an extremely potent inducer of AHH in rats (2). On a molar basis TCDD is 30,000 times more potent as an inducer of AHH activity than 3MC (2). TCDD is also a potent inducer of chick embryo δ-amino levulinic acid (δ-ALA) synthetase (7,8). The amount of TCDD required to produce a 35 fold induction of this enzyme is some 3 orders of magnitude less than any other known inducer of this enzyme (7,8). TCDD does not appear to induce δ-ALA synthetase in rats (8).

The results of the experiments reported here indicate that TCDD is also a potent inducer of hepatic DT-diaphorase activity. On a molar basis TCDD appears to be approximately 17,000 times more potent an inducer of DT-diaphorase than is 3-MC (3).

ACKNOWLEDGEMENTS

This work was supported by USPHS Grant ES 00267. The Training support of Patrick Beatty by ES 00112 and ES 07028 is also gratefully acknowledged.

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