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CHANGES IN HAMSTER HEPATIC CYTOCHROME P-450, ETHOXYCOUMARIN *O*-DEETHYLASE, AND REDUCED NAD(P):MENADIONE OXIDOREDUCTASE FOLLOWING TREATMENT WITH 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN

PARTIAL DISSOCIATION OF TEMPORAL AND DOSE–RESPONSE RELATIONSHIPS FROM ELICITED TOXICITY

THOMAS A. GASIEWICZ,* GEORGE RUCCI, ELLEN C. HENRY and RAYMOND B. BAGGS

Division of Toxicology, Department of Radiation Biology and Biophysics, and Division of Laboratory Animal Medicine, The University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642, U.S.A.

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Abstract—The temporal and dose-related characteristics of hepatic enzymes induced in the hamster by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were examined. Male Syrian golden hamsters received a single intraperitoneal injection of TCDD at a dose of 0–500 μg/kg. At various times up to 35 days, a number of variables were determined and compared: whole body, liver, and thymus weights; hepatic concentrations of cytochrome P-450 (P-450); and activities of 7-ethoxycoumarin *O*-deethylase (ECOD) and reduced NAD(P):menadione oxidoreductase (NMOR). Increased liver weights and decreased thymus weights were observed to be dose related. At day 7 following treatment, the approximate ED_{50} values for these responses were 15 and 100 μg/kg respectively. The ED_{50} values for the increase in hepatic P-450 concentrations and activities of ECOD and NMOR ranged from 0.5 to 2.0 μg/kg. At 10 and 500 μg/kg, NMOR activity remained maximally induced for up to 35 days. This was also the case for P-450 and ECOD activity at a dose of 10 μg/kg. At 500 μg/kg, both P-450 and ECOD demonstrated an induction up to day 4 followed by a decrease to near control levels by day 14. This decrease appeared to correlate with changes in hepatic morphology. These results demonstrate a dissociation of the induction of these hepatic enzymes from TCDD-induced lethality, in this species.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent member of a large family of halogenated aromatic and polycyclic aromatic hydrocarbons including other halogenated dibenzo-*p*-dioxins and dibenzofurans, polyhalogenated biphenyls, 3-methylcholanthrene, benzo[*a*]pyrene, and β-naphthoflavone. All of these compounds have been shown to be potent inducers of a number of enzymes in a variety of tissues and species [1]. Some of these enzymes include two or more forms of cytochrome P-450 (P-450) and associated monooxygenase activities such as 7-ethoxycoumarin *O*-deethylase (ECOD), as well as soluble enzymes including reduced NAD(P):menadione oxidoreductase (NMOR) (DT-diaphorase, EC 1.6.99.2) [1]. In certain strains of mice, the induction of these enzymes is determined by a set of regulatory, structural, and possibly temporal genes known as the *Ah* (*a*ryl *h*ydrocarbon) complex [1], and mediated by the stereospecific binding to the *Ah* receptor, a soluble intracellular protein conceived of as the regulatory gene product of the *Ah* complex [2–4].

Although many studies have focused on the role of the *Ah* receptor in the regulation of the cytochrome P-450 associated monooxygenases, it is clear that these are but a few of the many species- and tissue-specific responses controlled by the *Ah* complex [5]. Structure–activity studies, as well as those utilizing inbred strains of mice, implicate a role of the *Ah* receptor in the toxicity of TCDD and its congeners [5]. However, there exists a large species variability in susceptibility to the lethal effects of TCDD as well as in the signs of toxicity observed [5]. For example, the hamster is approximately 3000 times less sensitive to the lethal effects of TCDD than the guinea pig [6, 7]. The reason for these large differences in species susceptibility is presently unknown. Recent studies have indicated that a number of mammalian species possess *Ah* receptor molecules with similar biochemical properties, which translocate to the nucleus following TCDD exposure *in vivo*, and which have similar distributions within the animal [8]. These data suggest that qualitative or quantitative differences in the nature of the response to the TCDD–receptor complex may be contributive factors to species differences in sensitivity. However, the hamster liver also has been shown to possess higher basal activities of drug-metabolizing enzymes as compared to most other mammals [9–11]. Furthermore, the rate of elimination of TCDD and/or its metabolites from the hamster is considerably faster than that reported for more susceptible species such as the rat or guinea pig [12–14]. Therefore, it

* Author to whom all correspondence should be addressed at the Division of Toxicology, Department of Radiation Biology and Biophysics, The University of Rochester, Rochester, NY 14642.

is possible that the combination of increased elimination and localized metabolism of TCDD by the hamster may contribute to a more transient occupation of the receptor. Thus, in contrast to prolonged responses observed in other species [15, 16], the TCDD-induced modulation of gene expression may be less sustained in the hamster. Indeed, the fact that 3-methylcholanthrene and β-naphthoflavone are metabolized and eliminated to a much greater degree than TCDD has been proposed as the major reason for the dose- and time-related differences in the induction of the cytochrome P-450 associated enzymes as well as in qualitative differences in the toxic responses observed following a single exposure to these compounds [5].

It was the goal of this study to examine and compare the time- and dose-dependency of enzyme induction and toxic effects elicited by TCDD in the hamster and to compare these results to those which have been observed in other species. For this study we have chosen hepatic P-450 concentration and the activities of ECOD and NMOR to be representative of the pleiotropic biochemical response elicited by TCDD.

MATERIALS AND METHODS

Materials. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (>98% pure) was purchased from KOR Isotopes (Cambridge, MA); 7-ethoxy- and 7-hydroxycoumarin were from the Aldrich Chemical Co. (Milwaukee, WI); and cytochrome *c* (horse heart, type III), dicoumarol, and menadione were from the Sigma Chemical Co. (St. Louis, MO). All other reagents and chemicals used were of the highest grade and purity available. A stock solution containing 200 μg TCDD/ml of olive oil was prepared as described previously [14]. All subsequent dosing solutions were prepared from this stock such that 0.25 ml of olive oil was administered per 100 g body weight.

Animals. Male Syrian golden hamsters weighing 100–115 g were purchased from the Charles River Breeding Laboratories (Wilmington, MA). These animals were maintained under laboratory conditions with a 12-hr light cycle for at least 5 days prior to use. During this period they were fed commercial chow (Rodent Laboratory Chow No. 5001, Ralston Purina Co., St. Louis, MO) and water *ad lib.*

For the time course study, animals were randomly assigned to groups of three per cage and administered a single intraperitoneal dose of 0, 10, or 500 μg TCDD/kg body weight. Body weights were recorded on alternate days and on the day of sacrifice. TCDD-treated animals were killed on days 2, 4, 7, 14, 21, 28, and 35. Control animals were killed on days 1, 3, 13, 27, and 34. Animals were killed under carbon dioxide anesthesia by exsanguination via heart puncture between the hours of 8:00 and 10:00 a.m. Liver and thymus were excised and weighed, and a portion of the liver was prepared for biochemical and histopathological analysis. For the dose–response study, animals were randomly assigned to groups containing four per cage and administered 0, 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, 100 and 500 μg TCDD/kg. All animals were killed at day 7 following treatment.

Preparation of liver and enzyme activity assays. Livers were separately homogenized in 4 vol. of buffer containing 50 mM potassium phosphate, pH 7.6, and 1 mM EDTA. 7-Ethoxycoumarin *O*-deethylase activity was determined by the method of Greenlee and Poland [17] using the 10,000 *g* supernatant fraction. The activity of reduced NAD(P):menadione oxidoreductase in the 105,000 *g* supernatant fraction was determined using the method described by Kumaki *et al.* [16]. One enzyme unit (EU) is defined as the amount of enzyme necessary to catalyze the reduction of 1.0 nmole of cytochrome *c* per min at 30°. Cytochrome P-450 concentration in the microsomal pellet (105,000 *g*) was determined by the method of Omura and Sato [18]. Protein concentrations were determined by the method of Markwell *et al.* [19] using bovine serum albumin as a standard. Sections of thymus and liver were fixed in phosphate-buffered 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Hepatic sections were blindly scored on the basis of fat content (oil red-O staining), glycogen content (PAS staining), and necrosis.

RESULTS

Time course study. Figure 1A shows the time-related changes in body weight of hamsters administered 0, 10, or 500 μg TCDD/kg. A dose-dependent decrease in weight gain was observed. However,

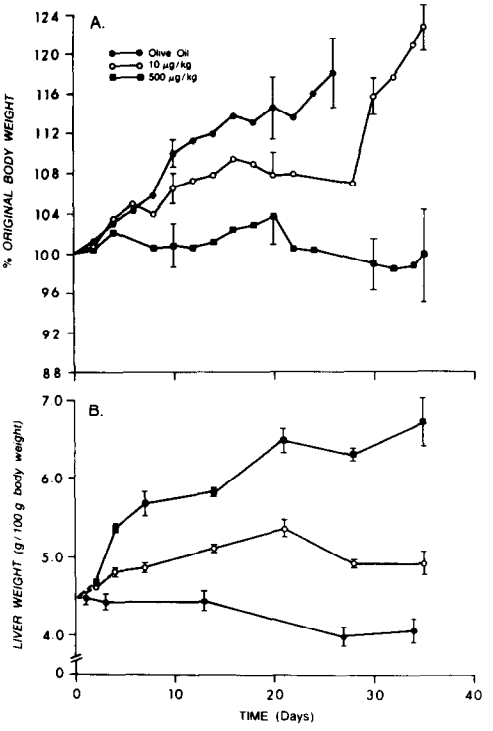

Fig. 1. Time-related changes in body (A) and liver (B) weights in hamsters administered single intraperitoneal doses of 0 (●), 10 (○), or 500 (■) μg TCDD/kg. Each point represents the mean ± S.E.M. of three to twenty-one animals. Data are presented as percent of original body weight. Initial body weights ranged from 100 to 115 g.

even at the highest dose, hamsters maintained their original body weight throughout the 35-day period, and no lethality was observed. An additional group of vehicle-treated animals was pair-fed to a group treated with 100 μg/kg TCDD. These results indicated that the decreased weight gain could be accounted for primarily by decreased food intake by the TCDD-treated group (E. C. Henry and T. A. Gasiewicz, unpublished results). A similar observation in TCDD-treated rats has been documented by other investigators [20, 21].

Relative liver weights (g wet weight per 100 g body weight) were also increased in a time- and dose-dependent manner by TCDD treatment (Fig. 1B). Marked increases in the relative liver weight were observed at all doses within 4 days following TCDD treatment. At day 35 the mean relative liver weight of the 500 μg/kg group was approximately 60% greater than that of the control group. Similar results were observed when absolute liver weights were compared (data not shown). In addition, the liver weights of the control group pair-fed to the 100 μg/kg treated group were similar to the vehicle-treated animals fed *ad lib.* (E. C. Henry and T. A. Gasiewicz, unpublished results). Thus, the increased relative liver weights were not due to the decreased body weight gain of the TCDD-treated groups.

No significant morphological alterations in the liver were observed by light microscopy in the 10 μg/kg treatment group at day 35 as compared to the vehicle-treated group. However, livers from the 500 μg/kg treated group demonstrated bile duct hyperplasia as well as the presence of numerous inflammatory cells including neutrophils, lymphocytes, and large mononuclear cells (not shown). Discrete clusters of hypertrophic hepatocytes appeared near the hyperplastic, disorganized bile ducts. An increase in the number of multinucleated cells was also observed.

The administration of 500 μg TCDD/kg to hamsters produced a decrease in thymus weight that appeared to be nearly maximal by day 7 (Table 1). This weight fell from a control value of approximately 47 to approximately 7 mg/100 g body weight by day 34. Histologic examination of the thymus tissue revealed the atrophy to be due mainly to loss of the cortical cells without obvious cell necrosis. This observation is in agreement with reports by other investigators [22].

Hepatic concentrations of P-450 were increased substantially within 2 days following TCDD treatment (Fig. 2A). At a dose of 10 μg TCDD/kg, P-450 concentrations reached a maximal level by day 7 and remained at this level for up to 35 days. Hamsters treated with 500 μg TCDD/kg demonstrated an increased concentration of P-450 up to day 4, followed by a return to near control levels by day 14. This decreased concentration (relative to the maximal level reached at day 4) persisted up to day 35 following treatment. The temporal pattern observed for the P-450-associated ECOD activity (Fig. 2B) was similar. At a dose of 10 μg TCDD/kg, the activity of ECOD was rapidly induced to approximately 4-fold its control value and remained at the maximal level up to day 35. At a dose of 500 μg TCDD/kg, the maximal level of ECOD activity was achieved by day 7; after day 14 this activity declined to, and remained at, a level below that observed for the 10 μg/kg group. In contrast to the pattern observed for P-450 and the associated ECOD activity, the activity of cytosolic NMOR reached its respective maximal level of induction by day 14 and remained at, or slightly below, this level for 35 days following treatment (Fig. 2C).

Table 1. Time-dependent alteration in thymus weights in hamsters administered 500 μg TCDD/kg

Day	Thymus weight (mg/100 g body wt)	Percent day 0 value
0	47.3 ± 3.5	100
7	12.5 ± 2.5	26
21	6.4 ± 2.7	14
34	7.1 ± 1.2	15

Each value is the mean ± S.E.M. of four animals. Thymus weights from control (untreated) animals at day 34 were approximately 92% of the day 0 value.

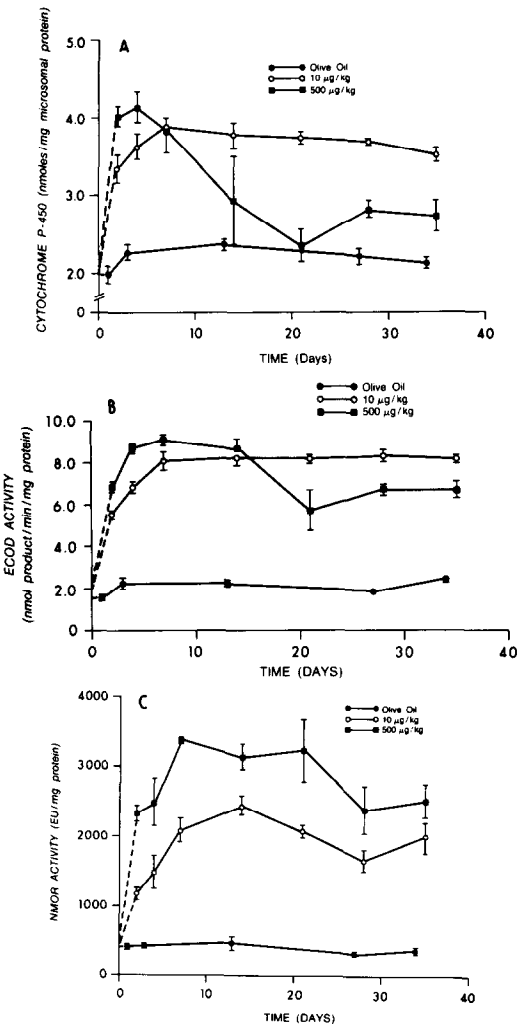

Fig. 2. Time-related changes in hepatic concentration of P-450 (A) and activities of ECOD (B) and NMOR (C) in hamsters administered single intraperitoneal doses of 0 (●), 10 (○), or 500 (■), μg TCDD/kg. Values are the mean ± S.E.M. of three animals.

Dose–response study. As noted in the initial time–course study, TCDD administration produced a dose-related increase in liver weight which appeared to be at or near maximal at a dose of 100 μg/kg (Fig. 3A). The estimated ED_{50} for this increase was approximately 15 μg/kg (Table 2). No necrosis or apparent alteration in hepatic morphology was observed at day 7 for any dose of TCDD up to and including 500 μg/kg. However, a dose-related decrease in hepatocellular lipid and increase in glycogen content was observed following histochemical staining of hepatic sections (data not shown). A slight increase in thymus weight was observed at TCDD doses of 0.1 and 0.5 μg/kg (Fig. 3B). At higher doses there was a dose-dependent atrophy of the thymus. The estimated ED_{50} for this response was approximately 100 μg TCDD/kg.

Hepatic concentrations of P-450 in the hamster were increased in a dose-dependent fashion up to a level which was approximately 90% greater than the control value (Fig. 4A). However, this elevation occurred only up to approximately 10 μg TCDD/kg, above which a slight decrease in P-450 concentrations was observed. In contrast, the hepatic activities of both ECOD and NMOR continued to be increased at doses greater than 10 μg TCDD/kg, reaching a maximal level at or near 100 μg/kg (Fig. 4, B and C). The estimated ED_{50} values for the increase in these activities were approximately 1 and 2 μg TCDD/kg respectively (Table 2).

DISCUSSION

Previous studies from this laboratory have shown that *Ah* receptor molecules from hepatic tissue of rats, mice, and hamsters are similar with regard to the relative affinity for TCDD [apparent equilibrium dissociation constants (K_D) range from 0.1 to 0.4 nM], hepatic concentrations (59 to 74 fmoles/mg cytosolic protein), and ability for nuclear association following TCDD administration *in vivo* [8]. Results from these and other investigations [2] predict that the K_D of TCDD binding approximates the ED_{50} for

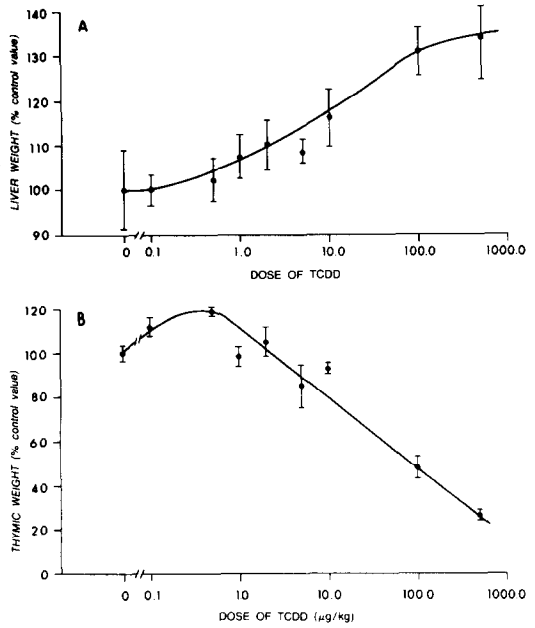

Fig. 3. Dose-dependent changes in relative liver (A) and thymus (B) weights in hamsters 7 days after a single intraperitoneal dose of TCDD. Results are expressed as percent of control (0 μg/kg) values, mean ± S.E.M. of four animals. Control values for liver and thymus weights were 3.86 ± 0.35 and 0.047 ± 0.003 g/100 g body weight respectively.

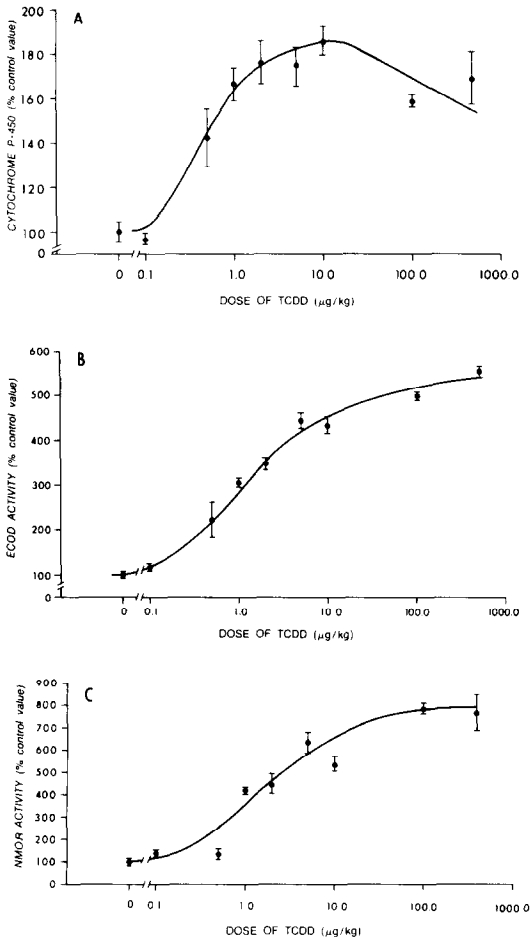

Fig. 4. Dose-dependent alterations in hepatic concentration of P-450 (A) and activities of ECOD (B) and NMOR (C) in hamsters 7 days after a single intraperitoneal dose of TCDD. Results are expressed as percent of control (0 μg/kg) values, mean ± S.E.M. of four animals. Control values for concentration of P-450 and activities of ECOD and NMOR were 1.66 ± 0.15 nmoles/mg microsomal protein, 1.84 ± 0.22 nmoles product/min/mg protein, and 302 ± 70 EU/mg protein respectively.

Table 2. Summary of ED_{50} data for the hamster following TCDD treatment

Response	Approximate ED_{50} (μg TCDD/kg)
Lethality	> 5000 [6]
Increased liver weight	15
Decreased thymus weight	100
Increased hepatic P-450	0.5
Increased hepatic ECOD	1.0
Increased hepatic NMOR	2.0

the modulation of certain biochemical responses. Indeed, the ED_{50} values observed for the induction of P-450 and the activities of ECOD and NMOR (Table 2; 1.6, 3.2, and 6.4 nM, respectively; calculated assuming uniform distribution of TCDD *in vivo*) are similar to the determined K_D values in the hamster (0.3 nM [8]), and to the relative ED_{50} values for the induction of aryl hydrocarbon hydroxylase (AHH) and ECOD activity in the C57BL/6J mouse (1.8 and 1.7 nM respectively [17]) and AHH activity in the rat (0.9 nM [15]). Furthermore, the duration of sustained induction of these enzymes in the hamster was similar to that previously observed in both rats [15] and mice [16].

The above results suggest that a faster rate of metabolism and elimination of TCDD and/or its metabolites by the hamster, relative to other species, is not a major factor in determining the dose- or time-related characteristics of TCDD-receptor-mediated modulation of gene expression. Furthermore, since the relative ED_{50} values for enzyme induction are approximately three orders of magnitude less than the reported LD_{50} value (Table 2), it appears that the induction of these hepatic enzymes cannot be directly associated with TCDD-induced lethality in the hamster. However, it is important to consider some qualifications to these tentative conclusions. First, biochemical responses were examined only in the liver in this study. Although the biochemical characteristics of the *Ah* receptor in different hamster tissues may be similar [8], many of the biochemical, biological, and toxicological responses to TCDD are tissue-specific [5]. Thus, the regulatory mechanisms controlling the action of the receptor, in addition to the battery of genes expressed, are likely tissue-specific as well. Furthermore, other factors such as an alteration in the number of receptors may influence the kinetics of TCDD as well as the consequences of the TCDD–receptor interaction within a given tissue. For example, the relatively low or undetectable concentration of receptors in the hamster thymus [8] may, in part, be responsible for the observation that the ED_{50} for TCDD-induced thymus atrophy (Table 2) is approximately 10-fold greater than that observed for rats [23] or C57BL/6J mice [24]. In addition, although the observation that TCDD treatment results in an altered set-point in body weight regulation (characterized by a wasting-type syndrome at higher doses of TCDD) may suggest a specific site of action for this response [20, 21], the actual tissue alteration and/or interaction of multiple alterations to produce such a response has yet to be determined. Finally, the events leading to a toxic effect, such as the generalized wasting syndrome, may only be subsequent to a prolonged, maximal biochemical alteration. In the present study, alterations in body weight (Fig. 1), liver morphology, and decreased P-450 concentrations (Fig. 4) occurred at doses of TCDD (>10 μg/kg) which produced sustained maximal or near maximal enzyme induction. Thus, it may be imprudent to strictly and quantitatively compare the dose- and time-related characteristics of defined biochemical processes with biological responses which may result from interactions of a multitude of these pathways.

Despite the above considerations, significant toxicity and lethality are known to occur in a variety of other mammalian species at doses of TCDD which are well below the reported LD_{50} dose for the hamster [5]. The present results imply that additional factors other than the ability of TCDD to interact with the receptor, or the ability of the TCDD–receptor complex in the hamster to invoke a prolonged modulation of gene expression, are responsible, in part, for these differences. Again, the tissue-specific control over the battery of genes expressed (or repressed) may be one determining factor. Alternatively, there may be species-specific biochemical differences which may modulate the response of the animal to the genes expressed following TCDD treatment. For example, it has been postulated recently that the greater regulatory role of brown adipose tissue in the hamster (a hibernating animal) for the control of energy metabolism via nonshivering thermogenesis contributes to the relative insensitivity of this species to the toxic effects of TCDD [25, 26].

Although the induced hepatic enzymes may not play a direct role in determining lethality in the TCDD-treated hamster, they may be associated with biochemical, morphologic, and functional changes found in this tissue. Previous studies have observed transient increases in the serum concentration of bilirubin as well as the activity of alkaline phosphatase in the hamster following the administration of TCDD [6]. In the present study, limited examination of liver sections by light microscopy revealed significant morphologic changes such as bile duct hyperplasia as well as hypertrophic and multinucleated hepatocytes. These morphologic changes appeared only in the day-35, 500 μg/kg treatment group and were concomitant with a lowering of the hepatic concentration of P-450 as well as one of its associated activities, ECOD. Notably, these changes were not observed in any dose group at day 7 following treatment. An association of a reduction in P-450 concentrations and mixed-function oxidase activities with liver injury produced by a variety of hepatotoxins has been well documented [27–29]. This may be due to a variety of mechanisms including enzyme inhibition, decreased enzyme synthesis, increased enzyme degradation, or enzyme-catalyzed peroxidation of associated microsomal membranes. The finding that there was no apparent reduction in the activity of the cytosolic enzyme NMOR suggests that general protein synthetic pathways were not compromised significantly at the higher doses of TCDD. However, several indirect lines of evidence do suggest that lipid peroxidation may occur in TCDD-treated animals. Lipofuscin pigments, which are considered to be by-products of peroxidative pathways, have been found in hearts of TCDD-treated animals [30]. In addition, a deficiency of iron has been shown to reduce TCDD-induced hepatic toxicity in mice [31, 32]. Iron has been determined to be involved in the production of lipid peroxidation and peroxidation-catalyzed destruction of P-450 in *in vitro* systems [33]. Furthermore, lipid peroxidation, as determined by a measurement of malondialdehyde and conjugated dienes, has been produced in hepatic tissue from rats following TCDD treatment [34, 35]. This latter study [35] did not, however, observe lipid peroxidation to occur in

hamster liver 6 days following treatment with a total dose of 600 μg TCDD/kg. In the present study, a significant decrease of hepatic P-450 concentrations (as compared to the maximal level reached at day 4) was not observed until day 14 following a single dose of 500 μg TCDD/kg (Fig. 2A). Thus, the previous study [35] may have examined hamster tissues prior to the actual onset of lipid peroxidative mechanisms. Although the exact role of induced enzymes in the hepatic damage elicited by TCDD has yet to be delineated, an increased oxidation or activation of endogenous substrates mediated by these enzymes may also contribute to these processes. Furthermore, all of these factors may be highly dependent upon the species- and tissue-specific pattern of P-450 isozymes induced, as well as their relative substrate specificities [11, 36]. For example, AHH activity, which is highly induced in rat and mouse liver following TCDD treatment [15, 17], is not induced in the hamster liver [35].

In summary, this study demonstrated similar dose- and time-related characteristics of hepatic enzyme induction by TCDD in hamsters as compared to other species which possess the *Ah* receptor. Although there is a lack of a quantitative association of these characteristics with the lethality in this species, further investigations are needed to determine the consequences of prolonged induction of these enzymatic activities as well as to identify other biochemical pathways which may be altered subsequent to TCDD-modulated gene expression. This is especially true for the relationship between induced hepatic enzymes and damage to this tissue. In this case, a closer, more direct association may be obtained.

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