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BIPHASIC RESPONSE FOR HEPATIC MICROSOMAL ENZYME INDUCTION BY 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN IN C57BL/6J AND DBA/2J MICE*

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Abstract—The induction of the murine hepatic microsomal cytochrome P-450 monooxygenase system by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was studied over a wide range of doses, including those associated with acute toxicity. Studies were conducted in two inbred strains of mice which vary at the Ah receptor and at a number of other genetic loci. C57BL/6J mice possess a high-affinity Ah receptor and are responsive to enzyme inductive effects of TCDD, whereas DBA/2J mice do not possess a high-affinity receptor and are less responsive to TCDD. In a dose–response study, 7-ethoxyresorufin *O*-deethylase (EROD) activity appeared to be maximally induced in C57BL/6J and DBA/2J mice at 7 days following exposure to 3 and 30 μg of TCDD/kg respectively. Very similar results were reported previously for the induction of aryl hydrocarbon hydroxylase activity in these strains of mice. However, at higher doses of TCDD (at least 45 μg/kg for C57BL/6J and 300 μg/kg for DBA/2J), EROD activity was further increased (2-fold) from the apparent maximal (plateau) level, resulting in an unusual biphasic log dose–response relationship. EROD activity remained at these elevated rates in both strains for doses approaching and exceeding the respective LD_{50} values for each strain. To further characterize this biphasic induction phenomenon, cytochrome P-450 content, benzo[*a*]pyrene metabolism, and EROD and NADPH–cytochrome P-450 reductase activities were measured 1, 3 and 7 days after TCDD administration to C57BL/6J (3 and 150 μg/kg) and DBA/2J (30 and 600 μg/kg) mice. Maximal responses occurred in both strains at 3 days for all doses. In both strains, TCDD produced a dose-dependent increase in cytochrome P-450 content, EROD, and benzo[*a*]pyrene metabolism. Furthermore, a 2-fold induction of reductase activity was observed in each strain following exposure to the respective high doses. Induction of cytochrome P_1-450 and P_3-450 was also measured by Western immunoblot, using antisera raised against the homologous rat isozymes. In both strains, TCDD produced a dose-related increase in two protein-staining bands recognized by anti-P-$450_{BNF\text{-}B}$ (P_1-450) and anti-P-$450_{BNF/ISF\text{-}G}$ (P_3-450) respectively. The extended induction of hepatic microsomal monooxygenase activities at the respective high doses of TCDD appears to be due, in part, to increases in NADPH–cytochrome P-450 reductase activity and cytochromes P_1-450 and P_3-450 content. Significant alterations in the expression of the cytochrome P-450 monooxygenase system following exposure to high doses of TCDD may be associated, in part, with the delayed acute toxicity reported at this level of exposure.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD‖) is recognized as a highly toxic and persistent environmental pollutant. Although the ultimate mechanism of TCDD-mediated toxicity remains unidentified, many biochemical responses have been associated with TCDD intoxication. One of the most well-understood actions of TCDD is its ability to induce the hepatic microsomal cytochrome P-450 monooxygenase system. Results from studies employing inbred mouse strains and murine cell cultures indicate that TCDD selectively induces cytochrome P_1-450 and P_3-450 and associated catalytic activities [1–3]. The induction of these forms of cytochrome P-450 by TCDD and other aromatic hydrocarbons such as 3-methylcholanthrene and β-naphthoflavone is regulated by the Ah locus and its major regulatory product the Ah receptor [4–6]. The association between the presence of the Ah receptor and TCDD-inducible AHH activity (a cytochrome P_1-450 mediated activity) has been documented for a variety of animal tissues, including those from rats, certain inbred strains of mice, guinea pigs, chick embryos, hamsters, and humans [1–3].

Although induction of AHH activity may not be directly responsible for TCDD lethality, some of the toxic responses produced by TCDD in the mouse have been found to segregate with the Ah receptor

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‖ Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; EROD, 7-ethoxyresorufin *O*-deethylase; AHH, aryl hydrocarbon hydroxylase; PBS, 10 mM sodium phosphate-buffered 0.9% (w/v) saline at pH 7.4; $UDPGT_1$, type 1 UDP-glucuronosyltransferase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and BNF or βNF, β-naphthoflavone.

and the induction of AHH activity, which are regulated by the Ah locus. C57BL/6J mice possess a high-affinity Ah receptor and are responsive to the enzyme inductive effects of 3-methylcholanthrene and TCDD, whereas DBA/2J mice do not possess a high-affinity receptor and are responsive to TCDD but not 3-methylcholanthrene [7, 8]. C57BL/6J mice are also more sensitive than DBA/2J mice to TCDD-mediated thymic involution, a hallmark of TCDD intoxication. TCDD-mediated induction of AHH activity and the Ah receptor are detected in the thymus of C57BL/6J but not DBA/2J mice [9]. TCDD-mediated hepatic porphyria has also been found to segregate with AHH responsiveness in C57BL/6J and DBA/2J mice [10, 11].

In an earlier study on the influence of the Ah phenotype on the hepatic metabolism of TCDD undertaken in our laboratory, the patterns of induction of hepatic cytochrome P-450-catalyzed AHH (benzo[*a*]pyrene metabolism) and EROD activities by TCDD were found to be different [12]. In agreement with other reports, doses of 3 and 30 μg of TCDD/kg were found to maximally induce benzo[*a*]pyrene metabolism (AHH activity) in hepatocytes from C57BL/6J and DBA/2J mice respectively [8]. Additionally, we found that hepatic EROD activity was not maximally induced by these doses in either the C57BL/6J or DBA/2J strain. For both strains, the respective LD_{50} doses of TCDD (150 μg/kg, C57BL/6J; 600 μg/kg, DBA/2J) resulted in at least a 50% greater induction of hepatocyte EROD activity than that produced by the lower doses. The current study was undertaken to characterize the induction of the hepatic microsomal cytochrome P-450 monooxygenase system over a wide range of doses of TCDD, including those associated with acute toxicity.

METHODS

Chemicals. TCDD was a gift from the Dow Chemical Co. (Midland, MI). [G-^{3}H]Benzo[*a*]pyrene (88 Ci/mmol) was purchased from the Amersham Corp. (Arlington Heights, IL). Aminopyrine, unlabeled benzo[*a*]pyrene, horse heart cytochrome *c*, tetrasodium NADPH (type I), monosodium NADP, glucose-6-phosphate dehydrogenase, monosodium glucose-6-phosphate, spectrophotometric grade *p*-nitrophenol, anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate, sodium uridine 5′-diphosphoglucuronic acid, glycine, ammonium persulfate, and *N,N,N′,N′*-tetramethylethylenediamine were obtained commercially from the Sigma Chemical Co. (St Louis, MO). Electrophoresis grade *N,N′*-methylene-bis-acrylamide, and sodium dodecyl sulfate were purchased from National Diagnostics (Highland Park, NJ). Nitrocellulose membrane (BA85; 0.45 μm pore size) was purchased from Schleicher & Schuell, Inc. (Keene, NH). Alkaline phosphatase substrate kit II was purchased from Vector Laboratories, Inc. (Burlingame, CA). Purified rat cytochrome $P\text{-}450_{BNF\text{-}B}$ and $P\text{-}450_{BNF/ISF\text{-}G}$ and rabbit antisera to each of these individual rat cytochromes P-450 were prepared as described elsewhere [13]. All other chemicals and solvents were of reagent grade or better.

Animal treatment. Eight-week-old male C57BL/6J and DBA/2J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were fed standard laboratory rodent chow (Prolab^R RMH 1000 diet, Agway Inc., Syracuse, NY) and tap water *ad lib.* Mice were maintained on a 12-hr light–dark cycle for 1 week before use. Each mouse received a single intraperitoneal injection of either TCDD in an olive oil vehicle or vehicle alone (0.1 ml/20 g of body weight). For the dose–response study, C57BL/6J mice were given doses of 0.15, 0.6, 3, 15, 45, 150, or 300 μg of TCDD/kg of body weight. DBA/2J mice received doses of 1.5, 6, 30, 150, 300, 600, or 900 μg of TCDD/kg. Mice were killed by decapitation 7 days after treatment. For the time–course study, TCDD treatment consisted of single intraperitoneal injections at doses of 3 or 150 μg/kg for C57BL/6J mice and 30 or 600 μg/kg for DBA/2J mice. Mice were killed by decapitation 1, 3, or 7 days after treatment.

Microsome preparation. Hepatic microsomal fractions were prepared according to the method of Anders [14] except that 0.15 M potassium chloride solution was used instead of sucrose solution. Microsomal pellets were washed by resuspension in 0.15 M potassium chloride solution and recentrifugation for 30 min at 105,000 *g*. Washed microsomal pellets were resuspended in 0.15 M potassium chloride solution at a protein concentration ranging from 10 to 15 mg/ml. A portion of each freshly prepared microsomal suspension was used to measure cytochrome P-450 concentration, EROD, NADPH–cytochrome *c* reductase, and type 1 UDP-glucuronosyltransferase activities. The remainder of each microsomal suspension was centrifuged for 30 min at 105,000 *g* at 4°C. The supernatant fractions were discarded and the intact pellets were stored at −70° in 2 ml of 0.25 M sucrose solution containing 1 mM EDTA and 20% glycerol for up to 1 week before measurement of aminopyrine *N*-demethylase activity and benzo[*a*]pyrene metabolism or for up to 3 months before quantification of cytochrome P-450 isozymes.

Enzyme assays. The N-demethylation of aminopyrine was performed according to the method of Sladek and Mannering [15]. Substrate was omitted from the blank reactions. Formaldehyde production was determined spectrophotometrically according to the method of Nash [16].

EROD activity was measured by the spectroflurometric method of Prough *et al.* [17]. The initial reaction rate was recorded and, after a 2-min reaction time, each assay was calibrated by the addition of 100 pmol of resorufin standard.

Benzo[*a*]pyrene metabolism was quantitated radiometrically by measuring the production of polar [^{3}H]benzo[*a*]pyrene metabolites following their separation by reverse-phase thin-layer chromatography [18].

Microsomal NADPH–cytochrome *c* reductase was measured spectrophotometrically according to the method of Phillips and Langdon [19]. The reaction rate was calculated using a molar extinction coefficient of 21/mM/cm.

Type 1 UDP-glucuronosyltransferase activity using *p*-nitrophenol as the aglycone was measured for microsomal suspensions by the spectro-

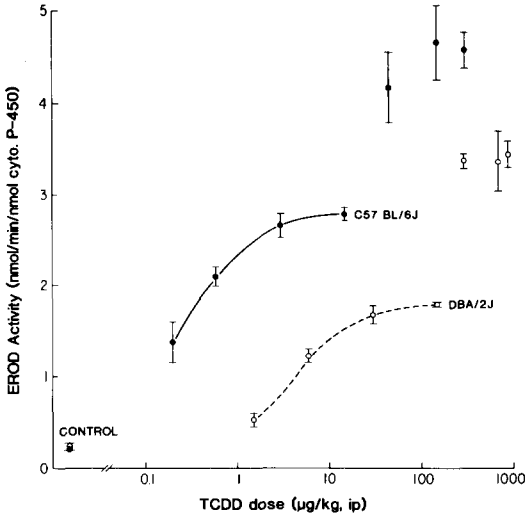

Fig. 1. Log dose–response relationship for the induction of hepatic microsomal EROD activity by TCDD in C57BL/6J and DBA/2J mice. EROD activities were measured in hepatic microsomal suspensions prepared from C57BL/6J (●) and DBA/2J (○) mice 7 days following a single i.p. dose of TCDD in olive oil. Each data point is the mean ± SD of three to six animals per group.

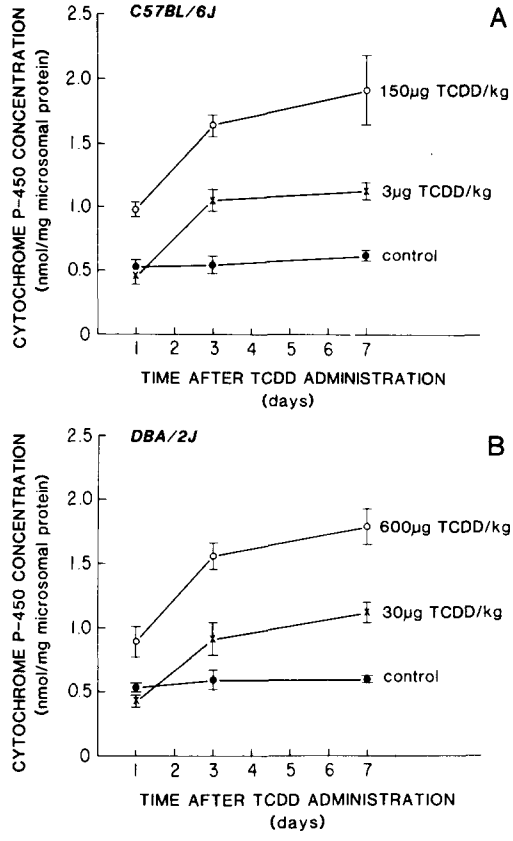

Fig. 2. Effect of TCDD on hepatic microsomal cytochrome P-450 concentration in C57BL/6J and DBA/2J mice. TCDD in olive oil was given as a single i.p. injection. Cytochrome P-450 concentration was determined by spectral analysis as described in Methods. Each data point is the mean ± SD of four animals per group.

photometric method of Bock *et al.* [20]. The amount of unmetabolized *p*-nitrophenol remaining in the reaction mixtures was calculated using an extinction coefficient of 18/mM/cm.

Other assay methods. Microsomal protein concentrations were determined by the method of Lowry *et al.* [21] using bovine serum albumin as the standard. Hepatic microsomal cytochrome P-450 concentrations were determined by the method of Omura and Sato [22]. The amount of cytochrome P-450 present in microsomal suspensions was calculated from the reduced-carbon monoxide versus reduced difference spectrum ($A_{450-490}$) using a molar extinction coefficient of 91/mM/cm.

Gel electrophoresis and immunoblotting conditions. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed essentially according to the method of Laemmli [23]. The samples were prepared in 62.5 mM Tris–HCl buffer (pH 6.8) containing 1% (w/v) sodium dodecyl sulfate, 10% (v/v) glycerol, 5% (w/v) β-mercaptoethanol, and 0.001% (w/v) bromphenol blue or pyronin Y as the tracking dye and heated to >90° for 5 min. The molecular weight standards (with corresponding M_r values) were phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500). After electrophoresis, the gel was either (1) fixed in an aqueous solution containing 25% (v/v) isopropanol and 10% (v/v) glacial acetic acid, stained with Coomassie Blue R250 [0.05% (w/v) in 25% isopropanol/10% methanol/65% water], and destained with an aqueous solution containing 10% (v/v) isopropanol and 10% (v/v) glacial acetic acid, or (2) electrophoretically transferred (Western blotting; Hoeffer TE Series model) to a nitrocellulose membrane (0.45 μm pore size) according to the method of Towbin *et al.* [24] at a constant voltage of 50 V for 4 hr. The transferred proteins were visualized on the nitrocellulose blot by amido black staining [1% (w/v) in 45% methanol/10% glacial acetic acid/45% water] followed by destaining with an aqueous solution containing 10% (v/v) isopropanol and 10% (v/v) glacial acetic acid.

Cytochromes P_1-450 and P_3-450 were quantitated by a modification of the immunological methods employed by Guengerich *et al.* [25]. To saturate nonspecific protein binding sites, nitrocellulose blots containing the transferred proteins were incubated at 21° with shaking in 100 ml of 10 mM sodium phosphate buffered 0.9% (w/v) saline (PBS, pH 7.4) containing 3% (w/v) instant non-fat dry milk for 1 hr with one change of an equal volume of this PBS/milk solution. The blots were washed four times with 150 ml of PBS containing 1% (v/v) Tween 20 (15 min/wash at 21° with gentle shaking). Each blot was incubated with the appropriate rabbit antiserum diluted in PBS/milk solution for 1 hr at 21° on an orbital mixer and then stored at 4° for 14–16 hr. Antiserum to cytochrome P_1-450 (anti-rat P-450_{BNF-B}, which is also known as anti-rat P-450c) was diluted 30-fold, whereas antiserum to cytochrome P_3-450 (anti-rat P-$450_{BNF/ISF-G}$, which is also known

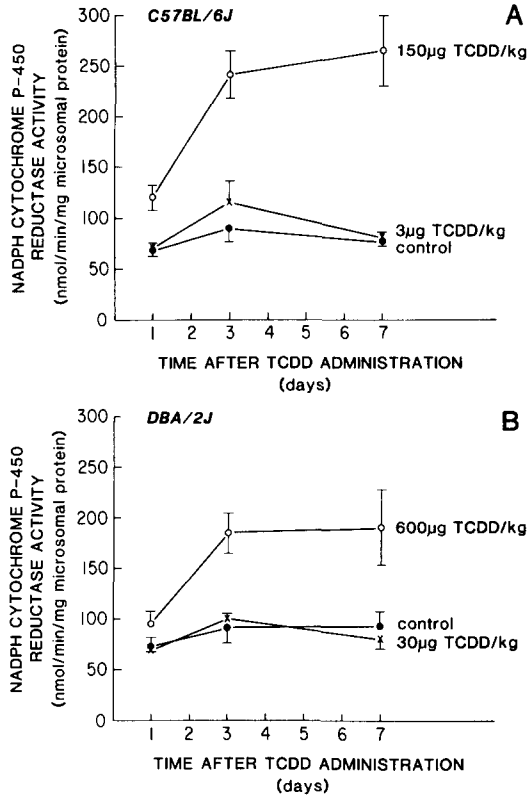

Fig. 3. Effect of TCDD on hepatic microsomal NADPH–cytochrome *c* reductase activity in C57BL/6J and DBA/2J mice. TCDD in olive oil was given as a single i.p. injection. Each data point is the mean ± SD of four animals per group.

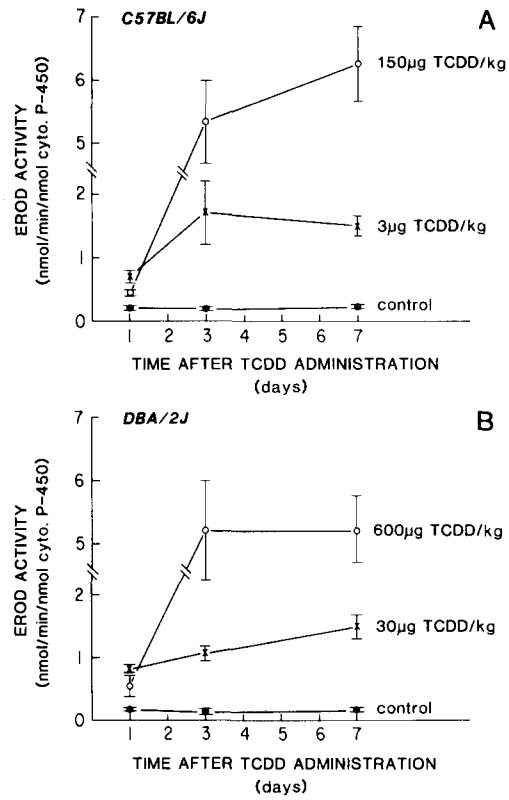

Fig. 4. Effect of TCDD on hepatic microsomal EROD activity in C57BL/6J and DBA/2J mice. TCDD in olive oil was given as a single i.p. injection. Each data point is the mean ± SD of four animals per group.

as anti-rat P-450d) was diluted 75-fold. Blots were washed four times with PBS containing 1% (v/v) Tween 20 to remove unbound antibody in the same manner as stated above. Washed blots were incubated at 21° in PBS/milk solution containing 0.1% (v/v) goat anti-rabbit IgG alkaline phosphatase conjugate for 2 hr with shaking. After the blots were washed four times with PBS/Tween 20 solution, the immunoreactive cytochromes P-450 were visualized using an alkaline phosphatase reagent kit (Vector Laboratories, Inc.). After a 30-min incubation at 21°, the reaction was stopped by washing the blot with water.

The intensity of immunostaining was measured by laser densitometry using an LKB Ultroscan XL (model 2222-010, LKB Diagnostics, Inc., Gaithersburg, MD) and absorption peaks were plotted by a line printer. The area of each peak was estimated by gravimetric methods. Known amounts of purified rat hepatic cytochromes P-450 orthologous to mouse P_1-450 (rat cytochrome P-450_{BNF-B}, which is also termed P-450c [26]) and P_3-450 (rat cytochrome P-$450_{BNF/ISF-G}$, which is also termed P-450d [26]) were applied to each gel and transferred onto nitrocellulose to produce standard curves because of differences in staining intensity between blots.

Photographs of gels and blots were taken over a fluorescent light box using Kodak technical panchromatic film 2415 and D11 developer (6 min; Eastman Kodak Co., Rochester, NY). A yellow filter (Kodak Wratten gelatin filter No. 2) was used to photograph gels and amido black stained blots to enhance the protein staining bands.

Statistical analysis. Data were statistically evaluated by one-way analysis of variance. Significant differences between group means was assessed by Duncan's multiple-range test using a chosen significance level of $P < 0.05$.

RESULTS

The log dose–response curves for the induction of hepatic microsomal EROD activity by TCDD in C57BL/6J and DBA/2J mice are depicted in Fig. 1. Seven days after TCDD administration, EROD activity appeared to reach a plateau at 3 μg/kg for C57BL/6J mice and at 30 μg/kg for DBA/2J mice. The apparent ED_{50} values for these responses were 0.2 μg/kg for C57BL/6J mice and 3.5 μg/kg for DBA/2J mice. However, at higher doses of TCDD the hepatic EROD activity increased further, exhibiting an unusual biphasic dose–response relationship. EROD activity was increased by almost 2-fold from these apparent maximal rates at doses of 45 μg of TCDD/kg for C57BL/6J mice and 300 μg of TCDD/kg for DBA/2J mice. EROD activity remained at these elevated rates in both C57BL/6J and DBA/2J mice for doses approaching and exceeding the respective LD_{50} values for each strain.

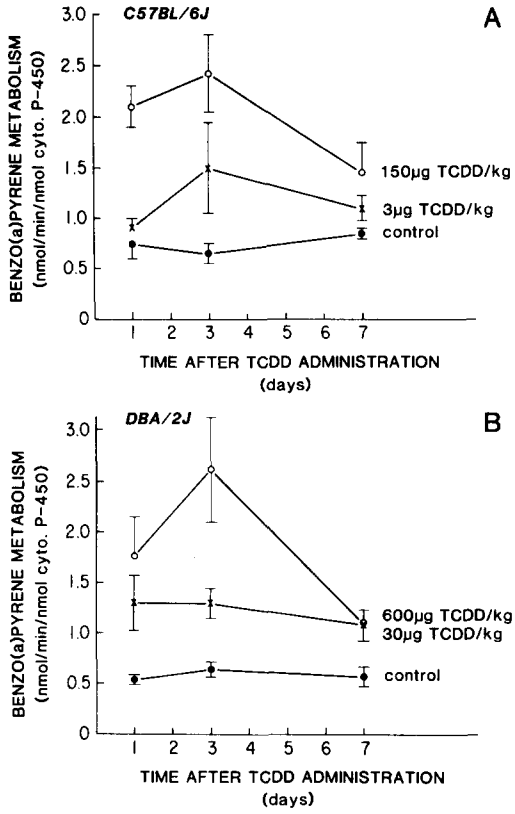

Fig. 5. Effect of TCDD on hepatic microsomal benzo[*a*]pyrene metabolism in C57BL/6J and DBA/2J mice. TCDD in olive oil was given as a single i.p. injection. Each data point is the mean ± SD of four animals per group.

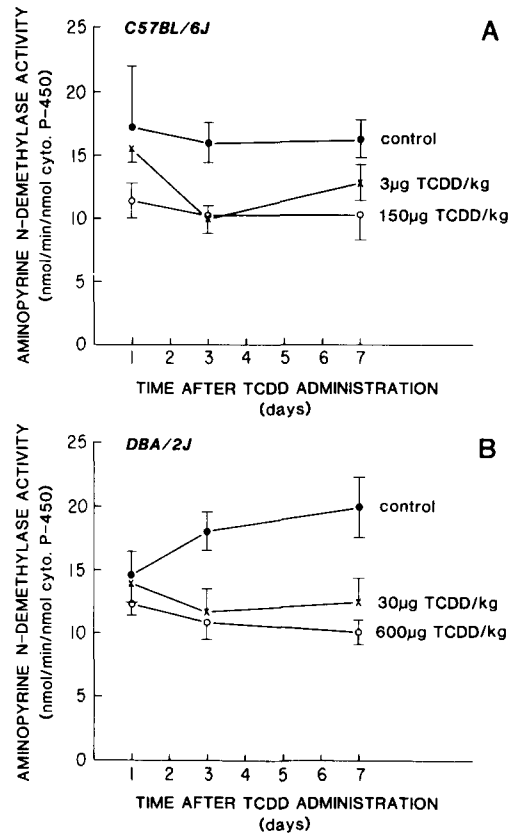

Fig. 6. Effect of TCDD on hepatic microsomal aminopyrine *N*-demethylase activity in C57BL/6J and DBA/2J mice. TCDD in olive oil was given as a single i.p. injection. Each data point is the mean ± SD of four animals per group.

In all treatment groups, no overt signs of toxicity were observed during the 7 days following exposure to TCDD. This was due to the characteristic pattern of delayed toxicity following acute exposure to TCDD [2, 10, 27].

Temporal changes in several variables of hepatic drug-metabolizing systems were measured in C57BL/6J mice treated with 3 or 150 μg of TCDD/kg and in DBA/2J mice treated with 30 or 600 μg of TCDD/kg to characterize the mechanism(s) involved in this biphasic induction phenomenon. The lower doses are generally considered to be maximally inducing for AHH activity [8] and the higher doses are similar to the reported LD_{50} values of 132 and 620 μg/kg for C57BL/6J and DBA/2J mice respectively [27]. The patterns of induction of total hepatic microsomal cytochrome P-450 concentration were similar for C57BL/6J and DBA/2J mice (Fig. 2). The lower doses of TCDD resulted in increases in cytochrome P-450 concentrations ranging from 53 to 92% in C57BL/6J and DBA/2J mice. Administration of LD_{50} doses of the toxin resulted in further elevations in cytochrome P-450 content (57–83% increase from the low dose values) that could be measured as early as 1 day after treatment. The greatest effects were detected 3 and 7 days post-treatment. Hepatic NADPH–cytochrome *c* reductase activity was unaffected in both C57BL/6J and DBA/2J mice by the lower doses of TCDD; however, pretreatment with LD_{50} doses resulted in a 2-fold increase from control values (Fig. 3). These increases were detected on days 3 and 7 post-treatment.

Seven days after treatment, 3 μg of TCDD/kg caused a 7-fold increase in hepatic EROD activity for C57BL/6J mice, while 150 μg/kg resulted in a 28-fold induction compared to control values (Fig. 4A). EROD activity was increased 8- and 29-fold in DBA/2J mice 7 days after receiving 30 and 600 μg of TCDD/kg respectively (Fig. 4B). Regardless of the dose, an increase in EROD activity compared to controls could be detected for both strains on day 1 post-treatment with the highest rate detected on day 3 or 7 following exposure to TCDD. The pattern and degree of induction of hepatic microsomal benzo[*a*]pyrene metabolism by TCDD (Fig. 5) were different from those for EROD activity. The greatest degree of induction elicited by the LD_{50} doses was detected 3 days after treatment for both C57BL/6J (3.7-fold) and DBA/2J (4.1-fold) mice. Seven days post-treatment, the degree of induction produced by the LD_{50} doses was similar to that produced by the lower doses for both strains (1.5-fold). For C57BL/6J mice, the induction of benzo[*a*]pyrene metabolism by 3 μg of TCDD/kg was greatest on day 3. In contrast, maximum induction occurred by day 1 in

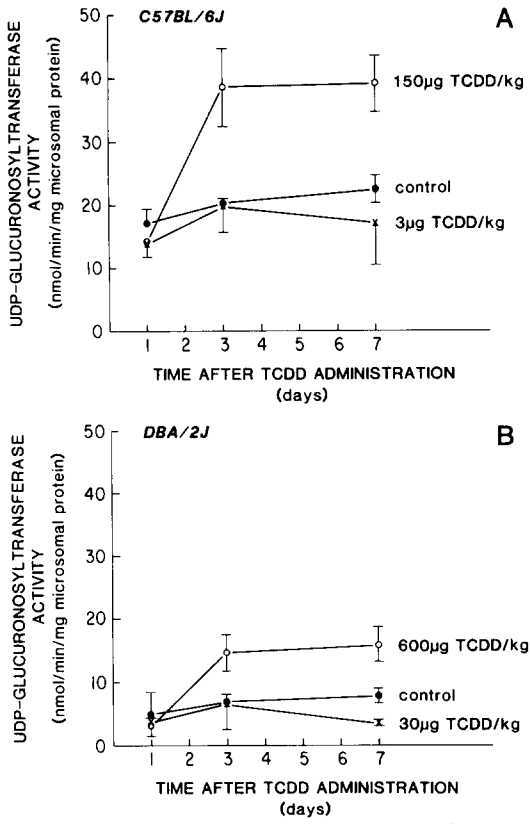

Fig. 7. Effect of TCDD on hepatic microsomal $UDPGT_1$ activity in C57BL/6J and DBA/2J mice. TCDD in olive oil was given as a single i.p. injection. *p*-Nitrophenol was used as the aglycone. Each data point is the mean ± SD of four animals per group.

DBA/2J mice given 30 μg of TCDD/kg and remained elevated at day 7 post-treatment.

TCDD treatment with either the low or high doses generally resulted in a decrease in aminopyrine *N*-demethylase activity for both C57BL/6J and DBA/2J mice (Fig. 6). Compared to the hepatic microsomes from control DBA/2J mice, microsomes from control C57BL/6J mice had about three times greater activity in the glucuronidation of *p*-nitrophenol, a measure of $UDPGT_1$ activity (Fig. 7). The low doses of TCDD had no effect on *p*-nitrophenol glucuronidation for either C57BL/6J or DBA/2J mice. For both strains, treatment with the respective LD_{50} doses of TCDD resulted in almost a 2-fold increase in $UDPGT_1$ activity on days 3 and 7 post-treatment.

The hepatic microsomal contents of cytochromes P_1-450 and P_3-450 were examined in the same samples used for the enzyme analyses. The SDS–PAGE electrophoretic migration profiles for hepatic microsomal proteins from control and TCDD-pretreated mice are depicted in Fig. 8A. The M_r values for protein staining bands induced by TCDD in the mouse samples, i.e. P_1-450 (56,000) and P_3-450 (54,000), were similar to those of the orthologous rat P-450 standards, i.e. $P\text{-}450_{BNF\text{-}B}$ and $P\text{-}450_{BNF/ISF\text{-}G}$ respectively. The amido black staining of microsomal proteins on a Western blot indicates that these cytochromes P-450 can be transferred to the nitrocellulose membrane (Fig. 8B). Immunostaining of blots revealed the apparent selective cross-reactivity of rabbit anti-rat $P\text{-}450_{BNF\text{-}B}$ and anti-rat $P\text{-}450_{BNF/ISF\text{-}G}$ with the murine forms of cytochrome P-450; however, evidence for this cross-reactivity was difficult to photographically reproduce (Fig. 8, C and D). Although each antiserum was cross-reactive with both major TCDD-inducible mouse cytochromes P-450, the 56,000 M_r protein was more intensely stained after incubation with anti-rat $P\text{-}450_{BNF\text{-}B}$, whereas the 54,000 M_r protein was more intensely stained after incubation with anti-rat $P\text{-}450_{BNF/ISF\text{-}G}$.

The hepatic cytochrome P_1-450 and P_3-450 concentrations of TCDD-treated C57BL/6J and DBA/2J mice were estimated by Western immunoblot using antibodies to rat P-450 isozymes and are shown in Table 1. Integration of the peak areas of the protein bands indicated that the relationship between staining intensity and quantity of purified rat cytochrome P-450 was linear for the working range of the standard curve (1–4 pmol of cytochrome P-450; data not shown). For both strains and at all doses used, TCDD treatment resulted in an increase in cytochrome P_1-450 and P_3-450 concentrations compared to control values. C57BL/6J and DBA/2J mice treated with 3 and 30 μg of TCDD/kg, respectively, had similar induced P_1-450 contents with maximum induction occurring 7 days after exposure. These same doses of TCDD also increased P_3-450 content in C57BL/6J and DBA/2J mice with the peak amounts detected by 3 days post-treatment. For C57BL/6J mice, the maximum cytochrome P_3-450 concentration was 73% greater than the maximum cytochrome P_1-450 content detected after treatment with 3 μg of TCDD/kg. The time–course and degree of cytochrome P_3-450 induction were similar in DBA/2J mice given 30 μg of TCDD/kg. In both mouse strains, the respective LD_{50} dose of TCDD induced cytochromes P_1-450 and P_3-450 to a greater extent than that observed at the respective lower dose. By comparing the maximum amounts of cytochrome P-450 produced during the time–course study, treatment with the LD_{50} doses resulted in 46 and 73% increases in P_1-450 content for C57BL/6J and DBA/2J mice, respectively, compared to the effects mediated by the lower doses (3 μg/kg for C57BL/6J and 30 μg/kg for DBA/2J). In an analogous comparison, P_3-450 was increased by 53% for C57BL/6J mice and by 62% for DBA/2J mice. The time–course of induction was slightly different between the two strains. The greatest amounts of each cytochrome P-450 isozyme were generally detected 3 days after treatment for C57BL/6J mice and 7 days after treatment for DBA/2J mice.

DISCUSSION

In the present study, C57BL/6J and DBA/2J mice exposed to a wide range of doses of TCDD exhibited an unusual biphasic dose–response relationship for the induction of the hepatic microsomal cytochrome P-450 monooxygenase system (Fig. 1). EROD activity was measured as an index of Ah responsiveness to TCDD for the dose–response study [1, 28]. This cytochrome P-450-catalyzed activity

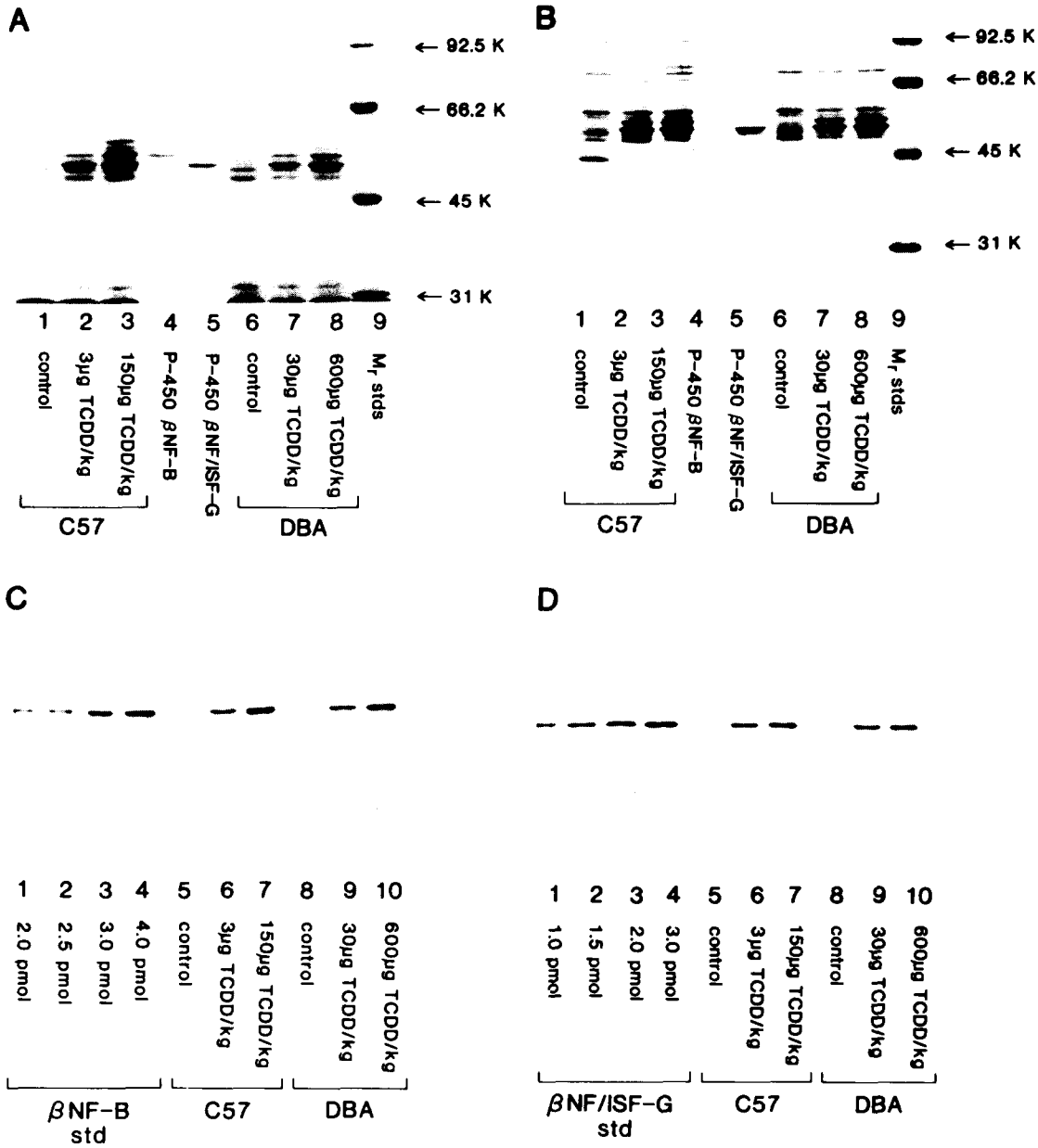

Fig. 8. SDS–PAGE and immunoblotting of hepatic microsomal cytochrome P_1-450 and P_3-450 from control and TCDD-pretreated C57BL/6J and DBA/2J mice. TCDD in olive oil was given as a single i.p. injection 3 days prior to preparation of hepatic microsomes. In part A, 5 μg of microsomal protein (lanes 1–3 and 6–8) and 10 pmol (lane 4), 5 pmol (lane 5), or 6 μg (lane 9) of purified protein were electrophoresed; and the gel was stained with Coomassie Brilliant Blue R-250. In part B, proteins were transferred from the gel (using the same protein concentrations in part A) to a sheet of nitrocellulose, which was subsequently stained with Amido Black. In part C, 2–4 pmol of purified protein (lanes 1–4) and 5 μg (lanes 5 and 8) or 4 μg (lanes 6, 7, 9 and 10) of microsomal protein were electrophoresed. The proteins were transferred from the gel to a sheet of nitro-cellulose, which was subsequently probed with rat anti-$P\text{-}450_{BNF\text{-}B}$. In part D, 1–3 pmol of purified protein (lanes 1–4) and 5 μg (lanes 5 and 8) or 0.8 μg (lanes 6, 7, 9 and 10) of microsomal protein were electrophoresed. The proteins were transferred from the gel to a sheet of nitrocellulose, which was subsequently probed with anti-rat-$P\text{-}450_{BNF/ISF\text{-}G}$.

appeared to be maximally induced (reaching a plateau) in hepatic microsomes isolated from C57BL/6J and DBA/2J mice treated with 3 and 30 μg of TCDD/kg respectively. Similarly, Poland and Glover [8] found that TCDD maximally induces hepatic cytochrome P_1-450-associated AHH activity at doses of 3 μg/kg for C57BL/6J and 30 μg/kg for DBA/2J mice. The ED_{50} values for the apparent maximal induction of AHH and EROD activities by TCDD are also similar for C57BL/6J (AHH activity: 0.4 to 0.5 μg/kg [12]; EROD activity: 0.2 μg/kg) and DBA/2J (AHH activity: 4.4 to 5.2 μg/kg [12];

Table 1. Immunoquantitation of hepatic microsomal cytochromes P_1-450 and P_3-450 from control and TCDD-pretreated C57BL/6J and DBA/2J mice*

Strain	Dose (μg/kg)	P_1-450 (nmol/mg protein) Day 1	Day 3	Day 7	P_3-450 (nmol/mg protein) Day 1	Day 3	Day 7
C57BL/6J	None	ND	<0.20	ND	ND	<0.13	ND
	3	0.61 ± 0.06	0.63 ± 0.22	0.98 ± 0.07	0.35 ± 0.10	1.69 ± 0.37	1.42 ± 0.10
	150	0.80 ± 0.01	1.43 ± 0.31	1.18 ± 0.11	0.97 ± 0.30	2.60 ± 0.13	2.05 ± 0.18
DBA/2J	None	ND	<0.20	ND	ND	<0.13	ND
	30	0.61 ± 0.04	0.63 ± 0.13	1.11 ± 0.15	0.65 ± 0.03	1.64 ± 0.28 8.	1.80 ± 0.17
	600	0.74 ± 0.03	1.05 ± 0.29	1.93 ± 0.51	1.22 ± 0.02	1.79 ± 0.23	2.91 ± 0.32

* TCDD in olive oil was given as a single i.p. injection 3 days prior to preparation of hepatic microsomes. See Methods for description of immunoquantitation procedures. Data are the means ± SD of three animals per group. ND: not determined.

EROD activity: 3.5 μg/kg) mice. However, the highest doses of TCDD used in the Poland and Glover study (13 μg/kg for C57BL/6J and 130 μg/kg for DBA/2J mice) are at least five times lower than the respective LD_{50} values for each strain. At higher doses of TCDD (at least 45 μg/kg for C57BL/6J and at least 300 μg/kg for DBA/2J mice), we found that EROD activity was further increased from the apparent maximal (plateau) levels for both mouse strains (Fig. 1). The induction of EROD activity by high doses of TCDD appears to be associated, in part, with the Ah locus, since two other enzyme activities that are coordinately regulated by the Ah locus, i.e. AHH activity (benzo[*a*]pyrene metabolism) and $UDPGT_1$ activity, were also increased by respective LD_{50} doses of TCDD in both strains (Figs 5 and 7). In addition, aminopyrine *N*-demethylase activity, an inducible cytochrome P-450 monooxygenase activity not associated with the Ah locus [1], was not induced by any dose of TCDD in either strain (Fig. 6).

For both strains, the extended, second phase of induction of EROD activity at high doses of TCDD was associated with concomitant increases in total cytochrome P-450 concentration, specifically cytochromes P_1-450 and P_3-450, and NADPH–cytochrome *c* reductase activity. Although EROD activity has been correlated with AHH activity [29], a cytochrome P_1-450 associated activity, the form of cytochrome P-450 responsible for EROD activity in the mouse has not been firmly established. The biphasic induction phenomenon observed in the current study was not clearly associated with the increase in one specific form of cytochrome P-450. TCDD-mediated induction of cytochrome P_1-450 and P_3-450 content as measured by radioimmunoassay has been reported previously [30]. In agreement with an earlier report by Negishi *et al.* [30], we observed that cytochrome P_3-450 represents the major polycyclic hydrocarbon-inducible form of cytochrome P-450. The induction of both cytochrome P_1-450 and P_3-450 has been shown to be due to specific transcriptional activation of the corresponding genes and mRNA stabilization [31, 32]. Recently, Whitlock [33] reviewed the field of cytochrome P-450 gene expression and its regulation. He proposes that the inducer–receptor complex binds to a genomic domain upstream of the promoter and stimulates P_1-450 gene transcription. A hypothetical, cycloheximide-sensitive labile repressor binds to a second upstream genomic domain and inhibits transcription. In the present study, the biphasic induction of P_1-450 and associated activities may represent an alteration in the balance between positive and negative regulatory factors at high doses of TCDD, which are also associated with acute toxicity.

Furthermore, it is possible that stabilization, i.e. decreased degradation, of the cytochromes may also be occurring at the high doses of TCDD which are associated with the extended second phase of induction of hepatic EROD activity. Goldstein and Hardwick [34] suggest that differences in the half-lives of the proteins or the mRNA can contribute to differences in the ratios of isozymes of cytochrome P-450 induced by TCDD and other agents.

The induction of NADPH–cytochrome *c* reductase activity appears to play a role in the second phase of induction of EROD activity. While EROD activity generally paralleled the increase in cytochrome P-450, the extended, second phase of induction was detected only at those time points and high doses of TCDD that yielded an increase in reductase activity. The induction of reductase activity by TCDD is noteworthy since this enzyme activity is not regulated by the Ah locus in mice [1]. Lang and coworkers [35] found that 3-methylcholanthrene pretreatment had no effect on hepatic microsomal NADPH–cytochrome *c* reductase activity for Ah-responsive heterogeneic stock (HS) mice. To our knowledge the induction of hepatic NADPH–cytochrome *c* (P-450) reductase activity by TCDD has not been reported previously for the mouse. In contrast, TCDD pretreatment of rats (at doses that approach and exceed the LD_{50} value) has been reported to either increase (by 24–30%) or have no effect on hepatic microsomal reductase activity [36–38]. The mechanism responsible for the induction of reductase activity by TCDD is not clear; however, a nonspecific induction due to TCDD-mediated proliferation of smooth endoplasmic reticulum does not appear to be responsible since aminopyrine *N*-demethylase activity was not induced regardless of the pretreatment dose of TCDD.

It is significant to note that the extended second phase of induction of hepatic EROD activity occurred at doses of TCDD (C57BL/6J: 45, 150, and 300 μg/kg; DBA/2J: 300, 600, and 900 μg/kg) which have been associated with signs of delayed acute toxicity and, at the highest doses, lethality [9, 10, 27]. The altered expression of the cytochrome P-450 monooxygenase system at high doses of TCDD may result in significant changes in the metabolism of numerous endogenous compounds. This, in turn, can result in the expression of various delayed toxic responses associated with acute exposure to TCDD. Further studies are required to determine the consequences of this biphasic induction phenomenon in relationship to TCDD-mediated toxicity.

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