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Differential Time–Course and Dose–Response Relationships of TCDD-Induced CYP1B1, CYP1A1, and CYP1A2 Proteins in Rats

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This study examined the relationship between dose- and time-dependent hepatic localization of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and expression of CYP1B1, CYP1A1 and CYP1A2 proteins. A dose-dependent increase in hepatic TCDD in female Sprague-Dawley rats treated with 0.01-30.0 μg TCDD/kg was observed. TCDD induced CYP1A1 protein in rats treated with 0.3 μg TCDD/kg or higher. TCDD induced CYP1A2 and CYP1B1 proteins in rats treated with 1.0 μg TCDD/kg or higher. The *in vivo* ED_{50} (μg TCDD/kg) for TCDD-induced CYP1A1, CYP1A2 and CYP1B1 proteins were 0.22, 0.40 and 5.19, respectively. Hepatic accumulation of TCDD reached a maximum at 8 hours post dosing with a $t_{1/2}$ of approximately 10 days. TCDD-induced CYP1A1/CYP1A2 protein expression was increased time-dependently, reaching a maximum at 3 days after dosing and remaining elevated for 35 days. In contrast, TCDD-induced CYP1B1 protein showed significant expression at 3 days after dosing and decreased to basal concentrations by 35 days. This study demonstrates that TCDD exhibits differential dose-response and time-course relationships on hepatic localization and cytochrome P-450 protein expression.

The best characterized aryl hydrocarbon (Ah) receptor (AhR)-mediated biochemical response elicited by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the induction of cytochrome P-450 gene expression through interactions of ligand:AhR complexes with xenobiotic response elements (XREs) located within regulatory regions [1]. These phase I cytochrome P-450s induced by AhR ligands include CYP1A1, CYP1A2 and CYP1B1. However, differences in the dose-response relationships of CYP1A1 and CYP1B1 mRNA expression by TCDD and related compounds has been observed [2-4]. For example, in the MCF-7 human breast cancer-derived cell line, CYP1B1 mRNA is highly induced by TCDD compared to CYP1A1 mRNA [3]. In contrast, recent dose-response studies in mice suggested that CYP1B1 mRNA is less inducible by TCDD than CYP1A1 mRNA [4]. Due to the the differences observed in the dose-response relationships of CYP1A1 and CYP1B1 mRNA observed after both *in vivo* and *in vitro* exposure to TCDD, and the lack of hepatic TCDD localization associated with these differences, this study focused on correlating the relative dose- and time-dependent expression of functional TCDD-induced hepatic CYP1B1, CYP1A1 and CYP1A2 proteins with hepatic localization of TCDD in female Sprague-Dawley rats. The results suggest that TCDD exhibits a differential time- and dose-dependent effect on CYP1A1, CYP1A2 and CYP1B1 protein expression.

MATERIALS AND METHODS

Chemicals, animals, treatment, and hepatic TCDD. 2,3,7,8-tetrachloro[1,6-^{3}H]dibenzo-*p*-dioxin (34.7 Ci/mmol) was obtained from Chemsyn Science Laboratory (Lenexa, KS) and radiochemical purity (≥99%) was verified as described [5]. TCDD was purchased (≥98% chemical purity) from Radian Corp. (Austin, TX). Female Sprague-Dawley rats (8 weeks old, 225-250g) were received from Charles River Laboratories (Raleigh, NC), randomly assigned, and acclimated for 1 week under controlled conditions with free access to food/water. Rats received a single oral dose of either a corn oil solution containing 10 μg TCDD/kg body weight (bw) or corn oil vehicle alone

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at 5 ml/kg bw. At 30 min, 1, 3, 8 or 24 hr, or 7, 14 or 35 days after dosing, rats were euthanized by CO_2 asphyxiation. In a separate experiment, rats received a single oral dose of 0.0 (corn oil), 0.01, 0.1, 0.3, 1.0, 10.0 or 30.0 μg TCDD/kg at 5 ml/kg and euthanized as noted above 3 days after dosing. The hepatic concentration of TCDD was determined by sample oxidation [5]. Hepatic $t_{1/2}$ of TCDD (with 95% confidence intervals) were calculated using the slope of a linear regression analysis of the log concentration of ng TCDD/g tissue versus time.

CYP1A1/CYP1A2/CYP1B1 proteins. Hepatic microsomal proteins were prepared [5] and quantified [6] using BSA as the standard. Protein concentrations in the linear range of the assay (0.5-20 μg) were resolved by SDS-PAGE using a 10% acrylamide resolving gel and a 4% stacking gel [7] and transferred to a 0.2 μm nitrocellulose membrane at 200 mA (1 hr) using a Trans-Blot SD Semi Dry Transfer Cell (Biorad Laboratories Inc., Hercules, CA) [8]. Membranes were blocked for 1 hr at 22°C or overnight at 4°C in Tris-buffered-saline pH 7.5 with 0.05 % Tween (TBST), containing 5-10% non-fat milk. Membranes were probed with a 1:5000 dilution of a rabbit polyclonal antibody against CYP1A1 and CYP1A2 (Human Biological, Phoenix, AZ) or rabbit polyclonal antibody against CYP1B1 in TBST [9] for 2 hr at 22°C or overnight at 4°C. Membranes were probed with a 1:1000 dilution of a secondary goat anti rabbit IgG (H+L)-(human adsorbed) alkaline phosphatase conjugate (Gibco BRL, Gaithersburg, MD) for 1 hr at 22°C. CYP1A1, CYP1A2 and CYP1B1 proteins were visualized by an alkaline phosphatase reaction for 5-15 min and quantified as optical density units/μg protein with a Masterscan densitometer (Billerica, MA). Molecular weights of CYP1A1, CYP1A2 or CYP1B1 immunostained proteins were determined from protein standards (Biorad). The statistical intergroup comparisons were determined using a one-way analysis of variance followed by Fisher's protected least significant difference (PLSD) with $p<0.05$. All data are represented as the mean $\pm$ standard deviation using a $\log_{10}$ transformation of the data. The Sigmoid E-max function [10] was used to determine ED_{50} values for the dose-reponse induction of TCDD-induced CYP1A1, CYP1A2 and CYP1B1 protein expression [11]. For the purpose of presentation only, the immunoblots were translated into TIFF formatted files using a Umax Super Vista S-12 scanner (Umax-Data Systems, Inc., Hsinchu, Taiwan) at 300 dots per inch (dpi) and printed using Adobe photoshop 4.0 (Adobe Systems Inc., Mountain View, CA) at 300 dpi (see Figures 1b and 2b).

RESULTS AND DISCUSSION

Acute exposure (3 days) of female Sprague-Dawley rats with a single oral dose of 0.01, 0.1, 0.3, 1.0 , 10.0 or 30.0 μg [^{3}H]TCDD/kg bw resulted in a linear (r^2= 0.993) dose-dependent accumulation in hepatic TCDD concentration (Figure 1a), which correlates with previous studies showing dose-dependent hepatic localization of TCDD with increasing dose in acutely-exposed rats [12]. Figure 1b shows a representative scanned immunoblot for hepatic CYP1A1, CYP1A2 and CYP1B1 protein expression in female Sprague-Dawley rats treated with TCDD. For accurate quantitation of CYP1A1, CYP1A2 and CYP1B1 proteins, a higher concentration of microsomal protein was loaded to determine both constitutive and TCDD-induced protein expression (see Materials and Methods). A significant ($p< 0.05$) increase in CYP1A1 protein (~56 kDa) expression compared to control animals was observed in female Sprague-Dawley rats treated with 0.3 μg TCDD/kg (1.75$\pm$0.30 ng TCDD/g liver) or higher (Figures 1a & 1b). In contrast, a significant ($p< 0.05$) increase in hepatic CYP1A2 protein (~54 kDa) expression compared to controls was observed in female Sprague-Dawley rats treated with 1.0 μg TCDD/kg (6.06$\pm$0.85 ng TCDD/g liver) or higher (Figures 1a), which may be related to the higher constitutive expression of CYP1A2 compared to CYP1A1 [13]. TCDD-induced maximal hepatic CYP1A1 protein expression at 1.0 μg TCDD/kg which corresponded to a hepatic concentration of 6.06$\pm$0.85 ng TCDD/g liver. In contrast, TCDD-induced maximal hepatic CYP1A2 protein expression at 10.0 μg TCDD/kg which corresponded to a hepatic concentration of 71.64$\pm$12.13 ng TCDD/g liver. The calculated ED_{50} for TCDD-induced CYP1A1 and CYP1A2 protein expression using a Sigmoid E-max function were 0.22$\pm$0.05 and 0.40$\pm$0.16 μg TCDD/kg, respectively. These data correlate with previous studies showing similar dose-response curves for CYP1A1 and CYP1A2 protein expression in TCDD-treated male Fisher rats [13]. In contrast to hepatic CYP1A1 and CYP1A2 protein expression, hepatic CYP1B1 protein expression was undetected in control and rats treated with 0.3 μg TCDD/kg or lower (Figure 1a). However, a low concentration of hepatic CYP1B1 (~56 kDa) was observed in female Sprague-Dawley rats treated with 1.0 μg TCDD/kg. Previous single dose studies have illustrated an approximate 10-fold lower concentration of CYP1B1 present in the liver compared to the adrenals [9], which was also observed in these dose-response studies (data not shown). In contrast, to the lack of TCDD-induced CYP1B1 expression in the adrenals (data not shown), a significant ($p<0.05$) dose-dependent increase in TCDD-induced hepatic CYP1B1 protein expression was observed in female Sprague-Dawley rats treated with 10.0 μg TCDD/kg (Figures 1a and 1b), which corresponded to a hepatic concentration of 71.64$\pm$12.13 ng TCDD/g liver. The calculated ED_{50} for TCDD-induced CYP1B1 protein expression using a Sigmoid E-max function was 5.19$\pm$3.68 μg TCDD/kg, respectively. The ED_{50} data correlates with recent mRNA dose-response studies in mice suggesting that CYP1B1 is less inducible by TCDD than CYP1A1 [4]. However, this is the first *in vivo* study correlating the hepatic concentration of TCDD with TCDD-induced CYP1A1, CYP1A2 and CYP1B1 protein expression and demonstrating that TCDD elicits a distinctly different profile on the relative dose-dependent expression of hepatic CYP1B1 protein expression in acutely-exposed female Sprague-Dawley rats compared to CYP1A1 and CYP1A2 proteins.

After oral administration of 10 μg [^{3}H]TCDD/kg, the time-dependent hepatic distribution of TCDD was also examined. Hepatic accumulation of TCDD reached a maximum 8 hours post-dosing (Figure 2a). This correlates with early time point studies of hepatic TCDD localization in male rats [14] suggesting a similar time-dependent hepatic accumulation in male and female

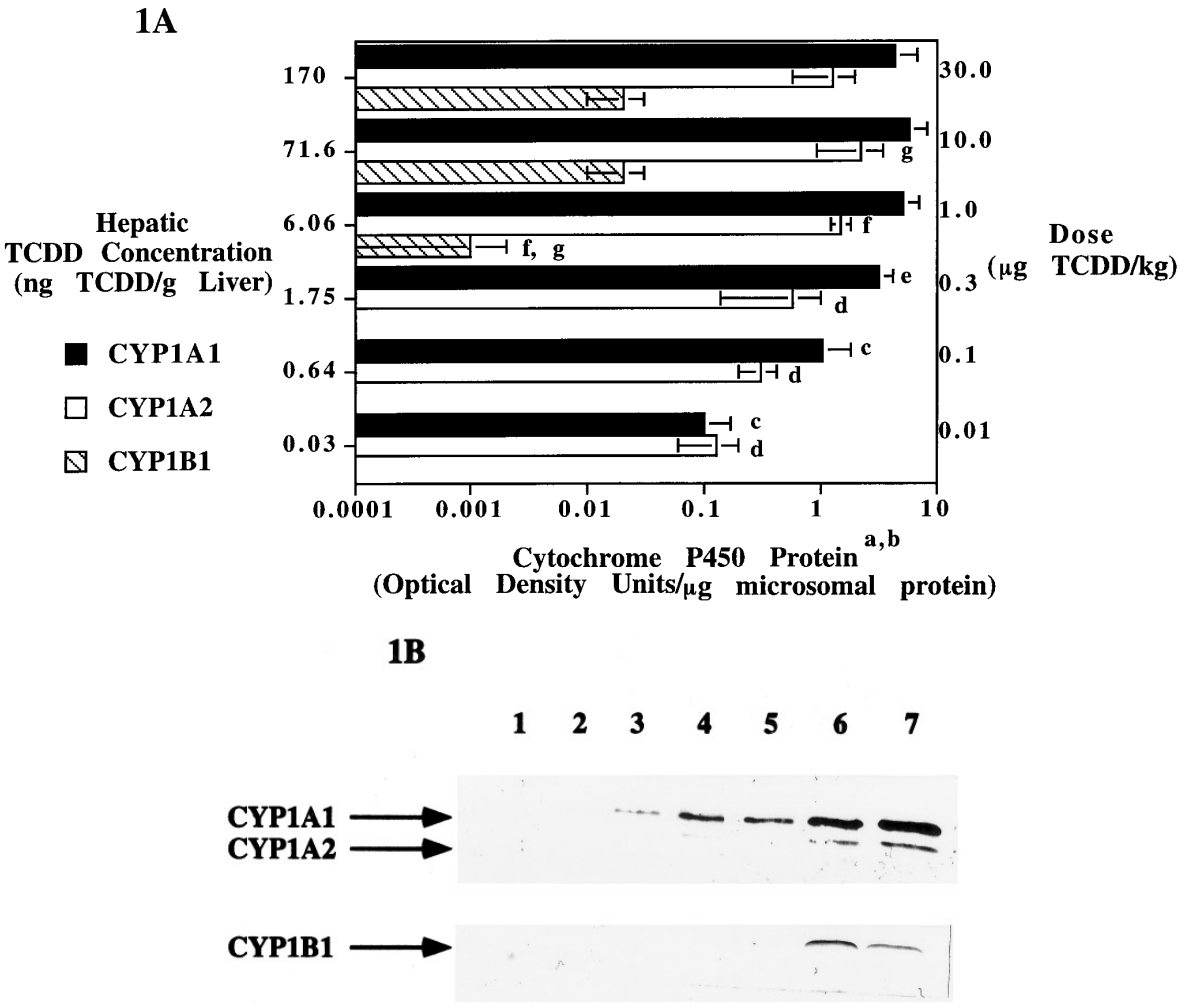

FIG. 1. Dose-Dependent Hepatic Localization of TCDD and Induction of CYP1A1, CYP1A2 and CYP1B1(A). Hepatic TCDD concentration (ng TCDD/ g liver) was determined as described in the Materials and Methods section. Cytochrome P-450 protein was determined by Western blot analysis as described in the Materials and Methods section. [a]All protein data are represented as the mean ± standard deviation (n=3-4) using a $\log_{10}$ transformation of optical density/ μg microsomal protein. [b]CYP1A1 contol value = 0.21± 0.25, CYP1A2 control value = 0.08 ± 0.33, CYP1B1 control value = non detectable. The statistical intergroup comparisons were determined using a one-way analysis of variance followed by Fisher's protected least significant difference (PLSD) with $p<0.05$. [c]statistically different than the 0.3, 1.0, 10.0 and 30.0 μg TCDD/kg treatment groups. [d]statistically different than the 1.0, 10.0 and 30.0 μg TCDD/kg treatment groups. [e]statistically different than the 1 and 10.0 μg TCDD/kg treatment groups. [f]statistically different than the 10.0 μg TCDD/kg treatment group. [g]statistically different than the 30.0 μg TCDD/kg treatment group. Representative Western Blots of the Dose-Dependent Effects of TCDD on Hepatic CYP1A1, CYP1A2 and CYP1B1 Protein Expression in Female Sprague-Dawley Rats (B). Rats were treated with corn oil (lane 1), 0.01 (lane 2), 0.1 (lane 3), 0.3 (lane 4), 1.0 (lane 5), 10.0 (lane 6) or 30.0 (lane 7) μg TCDD/kg as described in the Materials and Methods section. Hepatic microsomal proteins were prepared as described in the Materials and Methods section. Protein loadings for both control and TCDD-treated rats were 0.5 or 10.0 μg microsomal protein/lane for CYP1A1/CYP1A2 and CYP1B1 proteins, respectively. Cytochrome P-450 proteins were determined by Western blot analysis as described in the Materials and Methods section. Immunoblots were scanned and printed as described in the Materials and Methods section.

Sprague-Dawley rats. The accumulation of TCDD was followed by a time-dependent decrease in TCDD with a $t_{1/2}$ of approximately 10 days (8.9-11.4 days), which correlates with previous studies by Abraham and coworkers (1988). In addition, the hepatic $t_{1/2}$ value obtained in these studies are similar to the hepatic $t_{1/2}$ value (15 days) reported for TCDD clearance in female Sprague-Dawley rats treated with 5.6 μg TCDD/kg iv [14,15]. Figure 2b shows a representative scanned immunoblot for hepatic CYP1A1, CYP1A2 and CYP1B1 protein expression in female Sprague-Dawley rats treated with TCDD. For accurate quantitation of CYP1A1, CYP1A2 and CYP1B1 proteins, a higher concentration of microsomal protein was loaded to determine both constitutive and TCDD-induced protein expression (see Materials and Methods). Significant ($p<0.05$) expression of TCDD-induced hepatic CYP1A1 or CYP1A2 proteins compared to control animals was

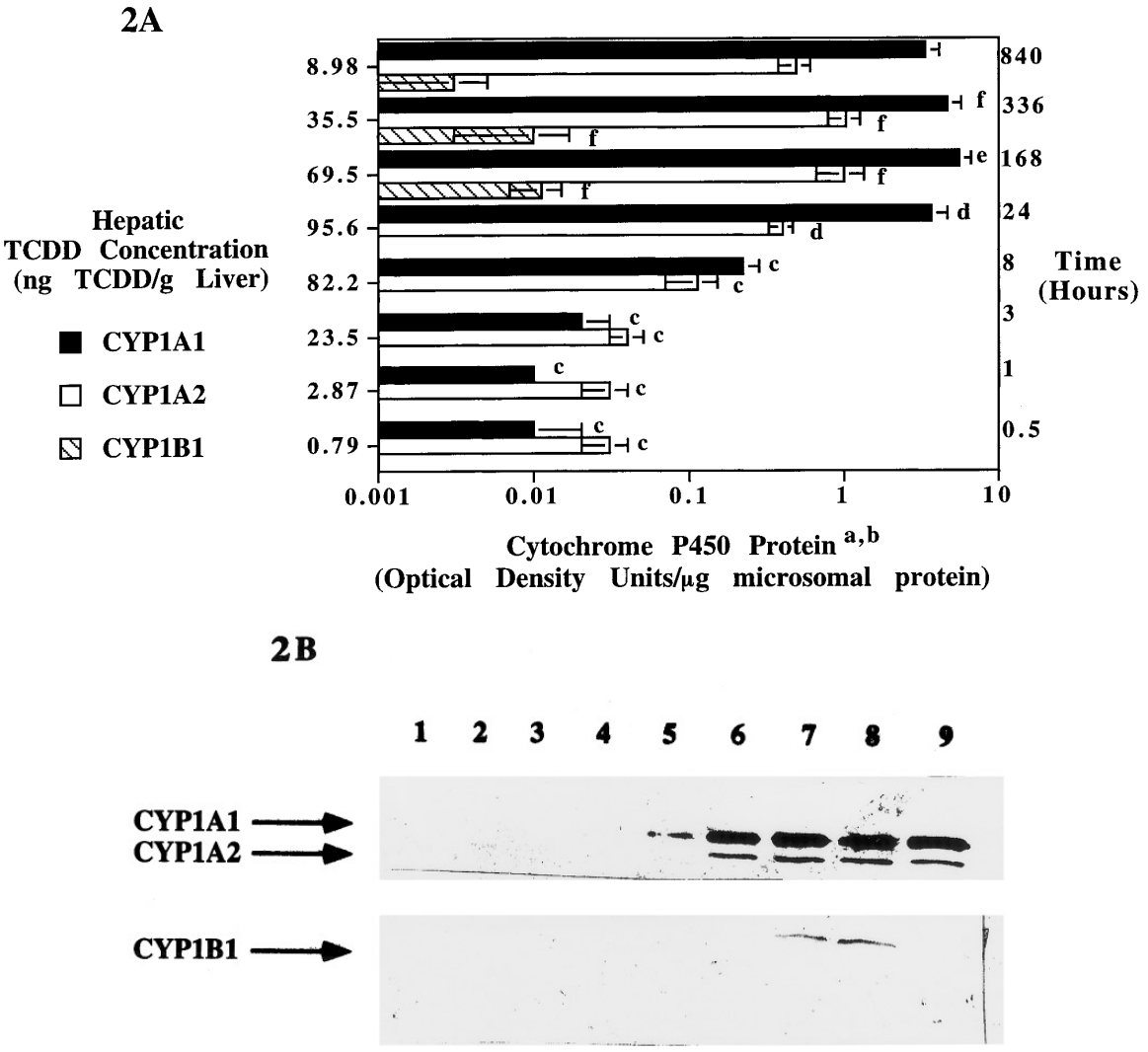

FIG. 2. Time-Dependent Hepatic Localization of TCDD and Induction of CYP1A1, CYP1A2 and CYP1B1 (A). Hepatic TCDD concentration (ng TCDD/ g liver) was determined as described in the Materials and Methods section. Cytochrome P-450 protein was determined by Western blot analysis as described in the Materials and Methods section. [a]All protein data are represented as the mean ± standard deviation (n=4-5) using a $\log_{10}$ transformation of optical density/ μg microsomal protein. [b]CYP1A1 contol value = 0.02 ± 0.00, CYP1A2 control value =0.03 ± 0.00, CYP1B1 control value = non detectable. The statistical intergroup comparisons were determined using a one-way analysis of variance followed by Fisher's protected least significant difference (PLSD) with $p<0.05$. [c]statistically different vesus 24, 168, 336 and 840 hr time points within same P-450. [d]statistically different vesus 168 and 336 hr time points within same P-450. [e]statistically different vesus 336 and 840 hr time points by within same P-450. [f]statistically different vesus 840 hr time point by within same P-450. Representative Western Blots of the Time-Dependent Effects of TCDD on Hepatic CYP1A1, CYP1A2 and CYP1B1 Protein Expression in Female Sprague-Dawley Rats (B). Hepatic CYP1A1, CYP1A2 and CYP1B1 proteins were determined from control (lane 1), 30 min (lane 2), 1 hr (lane 3), 3 hr (lane 4), 8 hr (lane 5), 24 hr (lane 6), 7 day (lane 7), 14 day (lane 8) or 35 day (lane 9) treatment groups as described in the Materials and Methods section. Protein loadings for both control and TCDD-treated rats were 0.5 or 10.0 μg microsomal protein/lane for CYP1A1/ CYP1A2 and CYP1B1 proteins, respectively. Cytochrome P-450 proteins were determined by Western blot analysis as described in the Materials and Methods section. Immunoblots were scanned and printed as described in the Materials and Methods section.

observed 24 hr post TCDD-administration, which corresponded to a hepatic localization of 95.62±23.69 ng TCDD/g liver (Figures 2a & 2b). A decrease in hepatic TCDD concentration (35.48±5.80 ng TCDD/g liver) was observed at 14 days (336 hr) post treatment with maximal TCDD-induced CYP1A1 and CYP1A2 protein expression being unchanged (Figures 2a & 2b). Thirty-five days (840 hr) after TCDD administration, a slight but significant ($p<0.05$) decrease in TCDD-induced hepatic CYP1A1/CYP1A2 protein expression was observed, which was associated with a hepatic concentration of 8.98±1.50 ng TCDD/g liver (Figures 2a & 2b). In contrast, TCDD-induced hepatic CYP1B1 protein expression was first detected at 3 days post TCDD-administration (Figures 1a and 1b, lane 6), which remained elevated for 14 days post treatment (Figures

2a & 2b). In contrast to the persistence of TCDD-induced CYP1A1 and CYP1A2, TCDD-induced hepatic CYP1B1 protein expression was not significantly induced 35 days (840 hr) post TCDD-treatment (Figures 2a & 2b). One reason for the dramatic decrease in TCDD-induced CYP1B1 protein expression may be related to the rapid degradation of CYP1B1 ($t_{1/2}$=4 hr in C3H/10$T_{1/2}$ cells) [2]. In contrast, the *in vivo* $t_{1/2}$ values for rat hepatic induced CYP1A1 and CYP1A2 are approximately 20-37 days [16-18]. However, this is the first *in vivo* study showing that TCDD elicits two distinctly different profiles on the relative time-dependent increase and loss of hepatic CYP1A1/CYP1A2 vs CYP1B1 proteins in acutely-exposed female Sprague-Dawley rats.

This is the first evidence that TCDD exposure results in a differential time- and dose-dependent effect on hepatic CYP1A1, CYP1A2 and CYP1B1 protein expression. These studies are comparable with previous *in vivo* data showing that the dose-response relationships and induction kinetics for TCDD-induced aldehyde dehydrogenase and CYP1A1 expression are different [19]. Recently, a ligand-specific effect on CYP1A1 and CYP1B1 mRNA expression was demonstrated in a mouse embyro fibroblast cell line treated with TCDD and benz[a]anthracene (BA) [2]. In addition, BA-treated cells resulted in a stabilization of CYP1B1-dependent activity, which was not observed in TCDD-treated cells. These studies and others have suggested that TCDD modulation of aldehyde dehydrogenase, CYP1A1, CYP1A2 and CYP1B1 gene regulation may be mediated through both AhR- and non AhR-mediated pathways involving multiple factors and/or pathways [1,2,19]. In addition, the location and number of XREs, as well as the potential binding sites for other factors within the cytochrome P-450 regulatory regions, are variable [1,2,19]. Thus, the differential mechanism(s) of cytochrome P-450 gene expression and subsequent transcriptional activation may be responsible for the differences observed in time- and dose-response relationships observed in hepatic CYP1B1, CYP1A1 and CYP1A2 protein expression. CYP1B1 has been proposed in the metabolic activation of estradiol to reactive intermediates which may be involved in the carcinogenic process [20]. Therefore, differences in the dose-response relationships of cytochrome P-450s may have implications for risk estimates in low-dose exposure to TCDD.

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