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Effect of Trichloroethylene on Cytochrome P450 Enzymes in the Rat Liver

N. Hanioka,[1] E. Omae,[2] R. Yoda,[2] H. Jinno,[1] T. Nishimura,[1] M. Ando[2]

[1]Division of Environmental Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokoyo 158, Japan
[2]Department of Chemistry, Kyoritsu College of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105, Japan

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Trichloroethylene (TCE) is extensively used in industry, primarily as a dry cleaning solvent and a metal decreasing agent. It is no longer applied as a grain fumigant and is only occasionally used in anesthesia. The most significant uptake of TCE in humans is through inhalation of the vapour, but uptake can also occur through the skin or via the gastrointestinal tract (World Health Organization 1985). Exposure to TCE causes a variety of disorders, including central nervous system depression, hepatotoxicity and nephrotoxicity (World Health Organization 1985). Also, it has been reported that TCE or its metabolite is mutagenic and carcinogenic to some mammalian species (National Cancer Institute 1976). The major urinary metabolizes of TCE in vivo are trichloroethanol, trichloroacetic acid and chloral hydrate (World Health Organization 1985). The enzyme system that catalyzes the primary oxidation of TCE to chloral hydrate appears to be the hepatic microsomal cytochrome P450 (P450) system (Costa and Ivanetich 1980). TCE is metabolized to reactive intermediates that irreversibly bind to nucleic acid and proteins, and TCE may be the metabolize responsible for this binding (World Health Organization 1985).

Nakajima et al. (1992) have demonstrated the unique capability of a monoclinal antibody to define the P450 isozyme responsible for TCE metabolism in rats. CYP2E1 biotransforms TCE more effectively than CYP2C11/6 in control microsomes. CYP2B1/2 and CYP1A1/2 are also involved i n the biotransformation of TCE in microsomes from rats exposed to phenobarbital and 3-methylcholanthrene, respectively. Furthermore, TCE binds to phenobarbital inducible P450 and it chemically alters the heme moiety of the P450 in the rat liver (Miller and Guengerich 1983). These reports suggest that the hepatic P450 system is closely associated with the metabolism and toxicity of TCE. However, very little has been reported about the induction of P450 enzymes by TCE. To clarify the roles of microsomal P450 isozymes in the metabolism and metabolic activation of TCE, we examined the effects of TCE on P450-dependent monooxygenase activities and P450 protein levels in the rat liver.

MATERIALS AND METHODS

TCE (purity, >99.8%) was obtained from Wako Pure Chemical Ind. (Osaka, Japan). Resorufin, 7-ethoxyresorufin, 6β -hydroxytestosterone, erythromycin and lauric acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 7-Methoxyresorufin and 7-benzyloxyresorufin were obtained from Molecular Probes, Inc. (Eugene, OR, USA). 7-Ethoxycoumarin and 12-hydroxylauric acid were from Aldrich Chemical Co. (Milwaukee, WI, USA). 7α -Hydroxytestosterone was obtained

Correspondence to: N. Hanioka

from Steraloids, Inc. (Wilton, NH, USA.). Benzphetamine and 2α–hydroxytestosterone were kindly donated by Dr. Y. Nakahara (National Institute of Health Sciences, Tokyo, Japan) and Dr. M. Matsui (Kyoritsu College of Pharmacy, Tokyo, Japan), respectively. Goat anti-rat CYP1A1/2, CYP2B1/2, CYP2C11/6, CYP2E1, CYP3A2/1 and CYP4A1 antibodies were from Daiichi Pure Chemical Co. (Tokyo, Japan). Peroxidase labeled rabbit anti-goat IgG was obtained from Zymed Laboratories, Inc. (San Francisco, CA, USA). All other reagents and solvents were of the highest quality commercially available.

Male Wistar rats (280-320 g) obtained from Charles River Japan, Inc. (Yokohama, Japan), were fed with laboratory chow and water ad libitum. They were housed in plastic cages at a constant temperature (22-26°C) and humidity (50-60%) under a 12 hr light/dark cycle (light: 6:00 a.m. to 6:00 p.m.). The animals were given TCE i.p. at a dose of 400, 800 or 1200 mg/kg/5.0 ml corn oil for 4 consecutive days, then sacrificed 24 hr after the last injection of TCE. Control animals received only corn oil. In this study, 4 dose levels were used because the highest dose of the test compound TCE produced an optimum response without producing over toxic effects.

The livers were promptly removed, perfused with cold physiological saline and homogenized in 50 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose, 25 mM KCl and 5 mM $MgCl_2$ using a glass-teflon homogenizer. The homogenates were centrifuged at 9000 x g for 20 min and the supernatant was further centrifuged at 105000 x g for 60 min. The microsomal pellets were suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M KCl and centrifuged again. The final microsomal pellets were resuspended in 50 mM Tris-HC1 buffer (pH 7.5) containing 20% glycerol and stored at -80°C. The protein concentration was determined as described by of Lowry et al. (195 1).

The hepatic P450 and cytochrome b_5 (b5) contents were measured according to Omura and Sato (1964). NADPH cytochrome c reductase (fp2) and NADH - ferricyanide reductase (fp1) activities were measured as described (Phillips **and** Langdon 1962; Takesue and Omura 1970). The **activities of 7-ethoxyresorufin O-**deethylase (EROD), 7-methoxyresorufin O-demethylase (MROD) and 7-benzyloxyresorufin O-debenzylase (BROD) were determined fluorometrically from the amount of resorufin produced (Pohl and Fouts 1980; Nerurkar et al. 1993). 7-Ethoxycoumarin O-deethylase (ECOD) was measured by means of the fluorometric determination of 7-hydroxycoumarin (Aitio 1978). Benzphetamine N-demethylase (BZND), aminopyrine N-demethylase (APND) and erythromycin N-demethylase (EMND) activities were determined by measuring the formaldehyde concentration (Nash 1953). p-Nitrophenol hydroxylase (PNPH) and ϖ-lauric acid hydroxylase (LAOH) were measured by the formation of p-nitrocatechol and 12-hydroxylauric acid, respectively (Aoyama and Sato 1988; Tassaneeyakul et al. 1993). Testosterone 2α-hydroxylase (TS2AH), testosterone 6β-hydroxylase (TS6BH) and testosterone 7α-hydroxylase (TS7AH) were measured by the formation of 2α-, 6β and 7α–hydroxytestosterone, respectively (Yoshihara et al. 1982).

Rat liver microsomal proteins were separated electrophoretically on the SDS-polyacrylamide gel using 7.5% polyacrylamide (Laemmli 1970). Following electrophoresis, the protein was transferred onto nitrocellulose membranes according to the procedure described by Towbin et al. (1979), then probed with goat anti-rat CYP1A1/2, CYP2B1/2, CYP2C11/6, CYP2E1, CYP3A2/1 and CYP4A1 antibodies. P450 antibodies bound to microsomal proteins were detected by the incubating the membranes with peroxidase labeled rabbit anti-goat IgG, followed by a calorimetric determination with 4-chloro-1-naphthol and hydrogen peroxide. The intensity of

the stained bands was determined by scanning with a CS-9300PC densitometer (Shimadzu Co., Kyoto, Japan).

Mean values were statistically evaluated using Student's t-test, and were considered significant y different at $P<0.05$ or $P<0.01$ as denoted.

RESULTS AND DISCUSSION

Rats treated with TCE, as well as control rats, remained healthy in appearance and normal in behavior during the study. Table 1 summarizes the increased body and organ weights. The body weight increases in rats dosed with 800 and 1200 mg/kg of TCE were significantly different from the control. However, the relative weights of the liver, spleen and thymus were not significantly different from the control in any of the TCE-treated groups. Highly toxic chlorinated compounds such as polychlorinated dibenzo-p-dioxins cause a loss of body weight, liver hypertrophy, and spleen and thymus atrophy (Mason et al. 1986). Thus, the cause of growth suppression by TCE may be different from that of other chlorinated compounds. The effect of TCE on hepatic microsomal protein and mixed function oxidase components is shown in Table 2. Rats given TCE at doses of 800 and 1200 mg/kg for 4 days had significantly increased P450 and fp2 levels (from 1.1 -to 1.3-fold), although the liver microsomal protein content was not changed by the TCE. On the other hand, b5 and fp1 levels were only increased by TCE at 800 mg/kg. However, there was no apparent change in the protein and mixed function components at a dose of 400 mg/kg.

Table 1. Body and organ weights of control and TCE-treated rats

	TCE (mg/kg)			
	0	400	800	1200
Δ Body weight[a)]	9.82 ± 1.04	9.18 ± 1.17	6.72 ± 1.49*	0.44 ± 1.52†
Organ weight				
Liver[b)]	4.55 ± 0.30	4.94 ± 0.26	4.96 ± 0.25	4.71 ± 0.15
Spleen[b)]	0.29 ± 0.02	0.27 ± 0.01	0.26 ± 0.02	0.25 ± 0.02
Thymus[b)]	0.17 ± 0.03	0.15 ± 0.01	0.14 ± 0.02	0.14 ± 0.02

Rats were treated, i.p., for 4 consecutive days with 0, 400, 800 or 1200 mg TCE/kg and were killed on the following day. Each value represents the mean ± SD of 4 animals.
[a)]%.
[b)]g/100 g body weight.
*Significantly different from the control ($P<0.05$).
†Significantly different from the control ($P<0.01$).

Table 3 shows the effect of TCE administration on various P450-dependent monooxygenase activities in the rat liver microsomes. LAOH in rat liver microsomes indicate CYP4A1 (Tamburini et al. 1984). Here, the level of this activity induced by TCE in rats was the highest among the P450-dependent monooxygenase activities tested (from 2.9- to 3.0-fold). Furthermore, immunoblotting showed that TCE induces the levels of CYP4A1 protein in liver microsomes from rats (Figure 1 and Table 4). This profile differed from those of other P450 isozymes, with the apparent maximum being induced by a dose of 400 mg/kg TCE. Zanelli et al. (1996) have reported that trichloroacetic acid induces LAOH and CYP4A1 protein levels in the rat liver,

although to a lesser extent than clofibrate. Because trichloroacetic acid is a TCE metabolite in rats (World Health Organization 1985), the CYP4A1 induced in this study may be due to its formation. On the other hand, PNPH and TS6BH in rat liver microsomes are catalyzed by CYP2E1 and CYP3A2, respectively (Nagata et al. 1990; Tsutsumi et al. 1993). These activities were dose-dependently increased by TCE and the induction of the P450 protein levels was also detected by immunoblotting (Figure 1, Tables 3 and 4).

Table 2. Protein, P450, b5, fp2 and fp1 levels in liver microsomes from control and TCE-treated rats

	TCE (mg/kg)			
	0	400	800	1200
Protein[a)]	15.2 ± 1.8	15.2 ± 0.8	16.0 ± 2.1	17.1 ± 1.3
P450[b)]	0.68 ± 0.03	0.72 ± 0.04	0.78 ± 0.06*	0.76 ± 0.06*
b5[b)]	0.32 ± 0.02	0.34 ± 0.04	0.37 ± 0.01*	0.33 ± 0.01
fp2[c)]	0.23 ± 0.01	0.23 ± 0.01	0.29 ± 0.01†	0.26 ± 0.01*
fp1[c)]	8.69 ± 0.66	9.47 ± 1.32	9.94 ± 0.74*	9.19 ± 1.16

Experimental conditions were the same as those given Table 1. Each value represents the mean ± SD of 4 animals.
[a)]mg/g liver.
[b)]nmol/mg protein.
[c)]μmol/min/mg protein.
*Significantly different from the control ($P<0.05$).
†Significantly different from the control ($P<0.01$).

BROD and BZND activities in rat liver microsomes associated with CYP2B1/2 (Ryan and Levin 1990, Nerurkar et al. 1993), were slightly increased by TCE at doses of 800 and 1200 mg/kg (Table 3). The band intensity of immunoblotted CYP2B1/2 was increased approximately 2-fold by TCE at doses of 800 and 1200 mg/kg and the ratio of induction was higher than those of P450-dependent monooxygenase activities such as BROD and BZND (Figure 1 and Table 4). This finding indicates that TCE selectively induces CYP2B2 rather than CYP2B1. EROD and ECOD are generally regarded as having CYP1A1 activity, whereas MROD is catalyzed by CYP1A2 (Ryan and Levin 1990; Nerurkar et al. 1993). Moreover, it has been reported that substrate specificity of ECOD also exists for CYP2B1/2 and even CYP2A2 (Ryan and Levin 1990). In this study, EROD and ECOD activities were significantly increased by TCE at dose of 800 or 1200 mg/kg (Table 3). However, immunoblotting did not detect CYP1A1 and the immunoreactive CYP1A2 level was not affected by TCE at any dose (Figure 1 and Table 4). Therefore, the increased EROD and ECOD activities may be due to other P450 isozymes than the CYP1A subfamily.

CYP2C11 is expressed in only adult male rats and TS2AH activity is an important marker for the P450 isozyme (Waxman 1984; Ryan and Levin 1990). TCE significantly decreased the TS2AH activity and CYP2C11 protein level at a dose of 1200 mg/kg (Figure 1, Tables 3 and 4). It is thought that the CYP2C11 is not induced by typical P450 inducers such as 3-methylcholanthrene, phenobarbital and pregnenolon-16α-carbonitrile (Ryan and Levin 1990). However, CYP2B2, CYP2E1, CYP3A2 and CYP4A1 are markedly induced by phenobarbital, ethanol, pregnenolon-16α-carbonitrile and clofibrate, respectively, and these isozymes are also constitutive

as well as CYP2C11 (Tamburini et al. 1984; Nagata et al. 1990; Ryan and Levin 1990; Tsutsumi et al. 1993). Therefore, the decrease in the CYP2C11 protein level caused by TCE may be a result of the induction of other constitutive P450 isozymes. APND, EMND and TS7AH activities which are catalyzed by CYP2D1, CYP3A4 and CYP2A1 (Wrighton et al. 1985; Ryan and Levin 1990), were not affected at any dose of TCE (Table 3). Thus, although the constitutive P450 isozymes such as CYP2B2, CYP2C11, CYP2E1, CYP3A2 and CYP4A1 involved in metabolism or regulation of endogenous substances were generally affected by TCE in the rat liver, the results suggested that a metabolic disturbance of constitutive P450 isozymes caused TCE toxicity.

Table 3. P450-dependent monooxygenase activities in liver microsomes from control and TCE-treated rats

	TCE (mg/kg)			
	0	400	800	1200
EROD[a)]	43.0 ± 8.1	45.7 ± 2.7	57.3 ± 9.5	64.4 ± 3.4*
MROD[a)]	45.4 ± 7.2	44.8 ± 6.0	50.4 ± 11.9	49.6 ± 11.1
ECOD[b)]	0.34 ± 0.03	0.35 ± 0.02	0.45 ± 0.04†	0.48 ± 0.06*
BROD[a)]	18.8 ± 3.2	23.1 ± 4.6	29.8 ± 6.9*	27.9 ± 6.1*
BZND[b)]	3.82 ± 0.50	4.29 ± 0.25	4.71 ± 0.22*	4.50 ± 0.20*
APND[b)]	4.02 ± 0.51	4.27 ± 0.15	4.80 ± 0.43	4.91 ± 0.67
PNPH[b)]	0.32 ± 0.03	0.39 ± 0.05*	0.47 ± 0.05†	0.55 ± 0.03†
EMND[b)]	7.26 ± 1.10	8.13 ± 0.70	9.41 ± 1.15*	8.40 ± 0.31
LAOH[b)]	0.44 ± 0.05	1.34 ± 0.43†	1.28 ± 0.32†	1.31 ± 0.06†
TS2AH[b)]	0.60 ± 0.17	0.68 ± 0.12	0.56 ± 0.08	0.35 ± 0.08*
TS6BH[b)]	0.88 ± 0.16	1.08 ± 0.26	1.27 ± 0.10†	1.56 ± 0.33†
TS7AH[b)]	0.33 ± 0.08	0.34 ± 0.04	0.31 ± 0.05	0.32 ± 0.01

Experimental conditions were the same as those given Table 1. Each value represents the mean ± SD of 4 animals.
[a)]pmol/min/mg protein.
[b)]nmol/min/mg protein.
*Significantly different from the control ($P<0.05$).
†Significantly different from the control ($P<0.01$).

It has been suggested that the formation of intermediate epoxides by the P450 system is the initial step in metabolic transformation of chlorinated ethylenes such as 1,1-dichloroethylene, trans-1,2-dichloroethylene, TCE and tetrachloroethylene in mammals (Miller and Guengerich 1983). These epoxides can react with cellular nucleophiles such as DNA, proteins or glutathione by alkylation or they may undergo intramolecular rearrangement forming acid chlorides or chlorinated aldehydes (Allemand et al. 1978). Asymmetrical chlorinated ethylenes such as 1,1-dichloroethylene and TCE indicate only weak mutagenicity, whereas trans-1,2-dichloroethylene and tetrachloroethylene have no effect (Greim et al. 1975). Furthermore, it has been reported that 1,1-dichloroethylene and TCE degrade the heme of P450 in vitro and in vivo, whereas tetrachloroethylene has no effect (Costa and Ivanetich 1980). Also, it is suggested that tetrachloroethylene is metabolized by only phenobarbital-inducible P450 (Costa and Ivanetich 1980), whereas TCE is mainly metabolized by CYP2E1 or CYP2B1 /2 in the mouse and rat in vitro (Nakajima et al. 1992). On the other hand, we reported that tetrachloroethylene markedly induces CYP2B1/2 in the rat liver (Hanioka et al. 1995). This study indicated that TCE

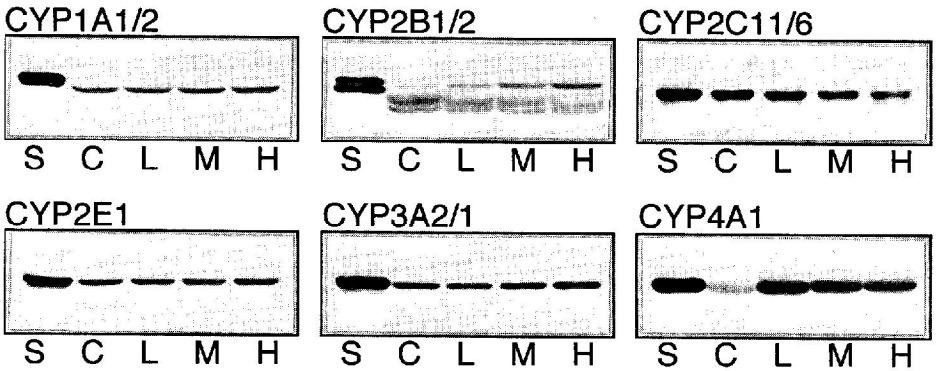

Figure 1. Immunoblotting of liver microsomes from control and TCE-treated rats with anti-rat CYP1A1/2, CYP2B1/2, CYP2C11/6, CYP2E1, CYP3A2/1 and CYP4A1 antibodies. Liver microsomes from rats given 3-methylcholanthrene (20 mg/kg for 3 days, i.p.) for anti-CYP1A1/2 antibody, phenobarbital (80 mg/kg for 3 days, i.p.) for anti-CYP2B1/2 antibody, nothing for anti-CYP2C11/6 antibody, pyridine (200 mg/kg for 3 days, i.p.) for anti-CYP2El antibody, dexamethasone (100 mg/kg for 3 days, i.p.) for anti-CYP3A2/1 antibody and clofibrate (400 mg/kg for 3 days, i.p.) for anti-CYP4A1 antibody were used as standards. Lanes: S, standard; C, control; L, 400 mg/kg TCE; M, 800 mg/kg TCE and H, 1200 mg/kg TCE. Microsomal protein levels: lane S, 2.0, 2.0, 8 .0, 6.0, 6.0 and 6.0 μg; lanes L, M and H, 32.0, 24.0, 8.0, 12.0, 8.0 and 24.0 μg for anti-rat CYP1A1, CYP2B1/2, CYP2C11/6, CYP2E1, CYP3A2/1 and CYP4A1 antibodies, respectively.

Table 4. P450 protein levels in liver microsomes from control and TCE-treated rats

	TCE (mg/kg)			
	0	400	800	1200
CYP1A1/2	1.00 ± 0.14	0.97 ± 0.19	1.09 ± 0.18	1.07 ± 0.12
CYP2B1/2	1.00 ± 0.33	1.07 ± 0.28	1.91 ± 0.53*	2.49 ± 0.36†
CYP2C11/6	1.00 ± 0.10	1.09 ± 0.04	0.90 ± 0.07	0.65 ± 0.04†
CYP2E1	1.00 ± 0.14	1.03 ± 0.10	1.20 ± 0.05*	1.32 ± 0.13*
CYP3A2/1	1.00 ± 0.17	1.02 ± 0.16	1.28 ± 0.07*	1.31 ± 0.13*
CYP4A1	1.00 ± 0.10	2.14 ± 0.43†	2.07 ± 0.42†	2.01 ± 0.16†

Experimental conditions were the same as those given Table 1. The immunoblotting results were normalized to those of control rats. Each value represents the mean ± SD of 4 animals.
*Significantly different from the control ($P<0.05$).
†Significantly different from the control ($P<0.01$).

induces the constitutive P450 isozymes, CYP2B2, CYP2E1, CYP3A2 and CYP4A1, but not the inducible P450 isozymes. Therefore, the difference in toxicities such as mutagenicity between TCE and tetrachloroethylene may be due to induced P450 isozymes or to interaction with the heme of P450 by chlorinated ethylenes.

In conclusion, this study showed that TCE induces the constitutive P450 isozymes such as CYP2B2, CYP2E1, CYP2C11, CYP3A2 and CYP4A1, but not inducible P450 isozymes such as CYP1A1 and CYP2B1 in rats. CYP4A1 was induced at a

low dose of TCE. The induction profile of P450 enzymes by TCE differed from that of tetrachloroethylene and an imbalance among constitutive P450 isozymes in the liver may be key to elucidating the toxicities of TCE and of asymmetrical chlorinated ethylenes.

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