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Comparative Study on the Effect of Trichloroethylene on the Expression of P450 Isoforms in Rat Lung and Liver

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Trichloroethylene (TCE) is a highly toxic compound which belongs to the class of volatile halogenated hydrocarbons, which are well-known pollutants of drinking water and the atmosphere. The effects of moderately toxic concentration of TCE on the levels of cytochrome P450 (CYP) isoforms and on their phenobarbital (PB) induction in rats were studied. Monooxygenase activities associated with individual CYP isoforms, *i.e.*, CYP1A, 2B, 2E1 and 3A, were measured in microsomal fractions prepared from both lungs and livers of male and female Wistar rats (7-weeks old). Differences in constitutive levels of CYP isoforms were observed between liver and lung of mock-treated rat; microsomal CYP2B activity was solely detected in lung while the activities of CYP1A, 2E1 and 3A, but not 2B were detected in liver. Among these, the pulmonary CYP2B and hepatic CYP2E1 activities were reduced by TCE-treatment. PB-treatment resulted in the detection of increased levels of hepatic CYP1A and 2E1 and of pulmonary CYP2B activities, and also the appearance of hepatic CYP2B activity. Coadministration of TCE was suppressive against the activities of CYP isoforms except for hepatic CYP2E1 in PB-treated rats. The lowered CYP2B activity in the presence of TCE was accompanied by the reduction in the amount of CYP2B apoprotein.

Key words —— trichloroethylene, cytochrome P450 isoform, organ specificity, rat, lung, liver

INTRODUCTION

Recently, lung cancer incidence has been increasing, due to not only smoking but also to pollution of the environment. Trichloroethylene (TCE), widely used in industrial fields as a metal-degreasing solvent and in dry-cleaning laundries, is known to be a toxic and carcinogenic environmental-pollutant, accounting one of the risk factors for lung cancer.[1,2]

Volatile halogenated hydrocarbons (VHH), represented by TCE, increased carcinogenic risks in liver and lymphohematopoietic tissues for workers exposed to these solvents.[3] Primary pneumatosis cystoides intestinalis occurred more frequently in women than in men who were engaged in the degreasing of manufactured products with TCE.[4] TCE can also cause autoimmune disorders such as systemic sclerosis.[5] In studies using rats, TCE caused hepatic hypertrophy and an increase in the transaminase activity in serum. Thus, the toxicity of TCE to various organs has been reported, as well as toxicity to lung, the primary organ exposed to TCE uptaken by the respiratory system.

TCE is first metabolized by CYP isoforms to such compounds as TCE oxide and chloral hydrate (phase I metabolism), and these compounds have the potential to bind to cellular DNA, RNA, proteins and lipids, resulting in toxicity and carcinogenicity. CYP2E1, 2C11/6, 2B1/2 and 1A1/2 were shown to be involved in the phase I metabolism of inhaled TCE in rat liver by using antibodies against CYP isoforms.[6] By coincidence, inducers of certain CYP isoforms, *e.g.*, phenobarbital (PB) and ethanol, were shown to enhance the toxicity of TCE.[7] In general, metabolites produced by phase I enzymes are detoxicated by phase II enzymes and secreted into urine. In the case of VHH, however, the toxicity of the primary metabolites are known to be further enhanced by the phase II enzyme, glutathione *S*-transferase.[8] The divergent expression profiles of CYP isoforms and phase II enzymes among various organs[9] may be determi-

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nants for organ specific VHH toxicities. Likewise, the sexual difference in TCE toxicity could be partly explained. Furthermore, the host defense mechanism could be mobilized as a countermeasure to TCE toxicity, by modulating the expression of metabolizing enzymes such as CYP isoforms.

We are exposed simultaneously to many environmental chemicals, interacting with each other in their metabolic pathways. As previously referred to, in rat liver PB could induce certain CYP isoforms, which could in turn hasten the metabolism of TCE to far more toxic compounds.[7] Therefore, we are also interested in the effect of PB on the toxicity of TCE and the expression profile of CYP isoforms when both are concomitantly administered.

MATERIALS AND METHODS

Reagents——Resorufin, 7-ethoxyresorufin, 7-pentoxyresorufin and erythromycin were purchased from Sigma Chemical Company (U.S.A.). *p*-Nitrophenol and 4-nitrocatechol are products of Wako Pure Chemical Ind. (Osaka). Goat anti-rat CYP2B1 and 2E1 sera and peroxidase-labeled anti-goat IgG serum were obtained from Daiichi Chemical Co. (Tokyo). The other reagents were all purchased from Wako Pure Chemical Ind. (Osaka).

Animals and Treatments——Wistar rats (Nihon Clea, 7 weeks old) were divided into four groups, each consisting of 5—6 animals. The TCE-treated, PB-treated, TCE and PB-treated and control groups were served with i.p. injection of TCE (1 g/kg body weight/d), PB (80 mg/kg/d), TCE (1 g/kg body weight/d) and PB (80 mg/kg body weight/d), and corn oil, respectively, for 3 d.

Preparation of Microsomes——Lungs and livers were removed from rats 24 h after the last treatment, and the wet weights of these organs were measured. Both lung and liver were homogenized with 4 volumes of cold 1.15% KCl–50 mM Tris–HCl (pH 7.4)–1 mM EDTA. The homogenates were centrifuged at 900 × *g* for 5 min and then 9000 × *g* for 15 min at 4℃. Microsomal pellets were obtained from the 9000 × *g*-supernatants with centrifugation at 105000 × *g* for 60 min at 4℃. The microsomal pellets were homogenized in a cold mixture containing 1.15% KCl–50 mM Tris–HCl (pH 7.4)–1 mM EDTA and 20% glycerol. The microsomes were kept at −80℃ until use. The measurement of protein was carried out according to Miller's modification of the Lowry method.[10)]

Enzyme Assays——Ethoxyresorufin *O*-deethylase (EROD):[11)] Reaction mixture consisted of 50 mM phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$, microsomal suspension and 0.3 mM 7-ethoxyresorufin in a final volume of 2.95 ml. Reaction was started by the addition of 50 μl of 7.5 mM NADPH in a fluorimeter cuvette at 25℃. The fluorescence intensity was recorded with spectrophotofluorimeter (Hitachi, F-2000; excitation wavelength, 530 nm; emission wavelength, 585 nm) and the initial rate of resorufin formation was calculated.

Pentoxyresorufin *O*-dealkylase (PROD):[12)] The PROD activity was measured under the same conditions as the EROD activity except that 0.3 mM 7-pentoxyresorufin was used as a substrate instead of 7-ethoxyresorufin.

p-Nitrophenol hydroxylase (PNPH):[13)] The reaction mixture containing 100 mM phosphate buffer(pH 6.8), microsomal suspension and 10 mM *p*-nitrophenol in a final volume of 1.90 ml was preincubated at 30℃ for 3 min, and then the reaction was started by the addition of 100 μl of 20 mM NADPH. After 10 min in the case of hepatic microsomes or 20 min for pulmonary microsomes at the same temperature, the reaction was terminated by the addition of 1 ml of 10 N perchloric acid. After centrifugation at 1200 × *g* for 5 min, 0.2 ml of 10 N NaOH was added to the supernatant and the absorbance at 546 nm was measured with a spectrophotometer (Hitachi, 100-20).

Erythromycin *N*-demethylase (EMND):[14)] The 0.95 ml of reaction mixture containing 50 mM phosphate buffer (pH 7.4), microsomal suspension and 1 mM erythromycin (stock solution, 200 mM in DMSO) was preincubated at 37℃ for 3 min, the reaction was started by the addition of 50 μl of 7.5 mM NADPH. After being incubated at 37℃ for 10 min, the reaction was ended by the addition of 17% perchloric acid. The whole was centrifuged at 1200 × *g* for 5 min and Nash's reagent was added to the supernatant for the coloration of formed formaldehyde to proceed by heating at 70℃ for 20 min. The absorbance was measured at 412 nm.

Western Immunoblot——Immunoblot analysis of microsomes was performed for the detection of CYP isoform apoproteins. SDS-PAGE was carried out with 10% acrylamide according to the method of Laemmli.[15)] CYP apoproteins were transferred electrophoretically with the help of a blotting system (BioRad, Transblot-SD) from the gel to the nitrocellulose sheet. The sheet was treated with goat anti-rat CYP isoform sera and peroxidase-labeled anti-goat IgG. Color

development was performed by using HRP Conjugate Substrate Kit (BioRad). The protein band-images read by the image scanner were analyzed by NIH-image software.

RESULTS

Effect of TCE on Whole Body and Organ Weights

The changes in the whole body weights and organ wet weights of rats treated with TCE and/or PB are shown in Table 1, as well as those of the control animals. In contrast to the gain of body weight in the control group, decreased body weight was observed in the TCE-treated and TCE and PB-coadministered groups. The most significant toxicity in terms of body weight loss was observed in female rats treated with TCE and PB in combination. In addition, the highest hepatic toxicity, reflected in hepatic hypertrophy, was observed in male rats treated with a combination of TCE and PB (+31%).

Effect of TCE on CYP Isoform-specific Monooxygenase Activities

Male and female rats were treated with TCE and/or PB for 3 d, whereas the animals in the control group were treated with corn oil vehicle. We then measured which CYP isoform-specific monooxygenase activities, EROD, PROD, PNPH and EMND, basically correspond to the functions of CYP1A, 2B, 2E1 and 3A, respectively. The results are shown in Table 2 comparatively between liver and lung, and male and female.

A marked contrast in the expression profiles of constitutive CYP isoforms (control group) was shown between liver and lung. The PROD activity was exclusively detected in pulmonary microsomes and the other three enzyme activities, EROD, PNPH and EMND, were detected in hepatic microsomes without being accompanied by the PROD activity over the detection limit. The constitutive expression of pulmonary PROD activity and those of hepatic PNPH and EMND activities were higher in male compared with female rats.

The pulmonary PROD activity in the control group was reduced by *ca.* 50% in the TCE-treated group for both male and female rats. In contrast, the responses of hepatic enzymes to TCE-treatment were diverse; the PNPH activity was moderately reduced, especially in female rats.

When treated with PB, the hepatic EROD and PNPH activities were enhanced over the respective constitutive levels. Although the PROD activity was constitutive and marginally enhanced by the PB-treatment in pulmonary

Table 1. Effect of TCE and/or PB Treatment to Body Weight and Organ Wet Weight

	Body weight (g)			Liver wet weight (g/100g body wt.)
	Before dosing	After dosing	Rate(%)	
Control[a]				
Male	216±12	235±12	8.8±1.2	4.81±0.21(100)
Female	156±8	163± 7	4.8±0.9	4.97±0.17(100)
PB-Treatment				
Male	235±3	244±3	3.6±0.3 *	5.28±0.07(110)
Female	144±3	152±3	5.3±0.6	5.64±0.05(114) *
TCE-treatment				
Male	245±22	237±19	-2.8±1.4 ***	4.66±0.21(97)
Female	173±10	168± 8	-2.5±1.3 *	4.89±0.12(98)
TCE+PB-treatment				
Male	220±9	208±13	-5.5±3.1*	6.31±0.23(131)**
Female	172±9	157± 8	-8.6±1.3**	5.31±0.18 (107)

Rats were treated with a daily i.p. injection of trichloroethylene (1 g/kg) and/or phenobarbital (80 mg/kg) for 3 d. Data are shown as the mean±S.E. for 4—6 individual rats with statistically significant differences compared to the control group at $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***). Figures in parentheses are the percentage of control.

a) Corn oil dosing.

Table 2. Effects of TCE and/or PB Treatment on P450 Dependent-Monooxygenase Activities of the Liver and Lung in the Rat

Enzyme activity		Liver male	Liver female	Lung male	Lung female
Ethoxyresorufin *O*-deethylase (pmol/min per mg protein)	Control	36.2± 4.2[a]	33.9 ± 4.4	n.d.	n.d.
	PB	182.7± 14.6***	210.3 ± 6.6***	n.d.	n.d.
	TCE	65.8± 18.7	62.1 ± 9.0	n.d.	n.d.
	TCE+PB	148.4± 16.4**	77.6 ± 16.2*	n.d.	n.d.
Pentoxyresorufin *O*-dealkylase (pmol/min per mg protein)	Control	n.d.	n.d.	34.6 ± 2.0	23.9 ± 3.0
	PB	633.5 ± 51.8	241.9 ± 6.9	42.7 ± 1.3**	29.0 ± 3.3
	TCE	n.d.	n.d.	16.1 ± 0.7***	13.2 ± 1.6*
	TCE+PB	279.8 ± 16.5	172.6 ± 26.1	3.4 ± 2.3***	6.9 ± 1.7**
p-Nitrophenol hydroxylase (nmol/min per mg protein)	Control	0.37± 0.01	0.25± 0.01	n.d.	n.d.
	PB	0.71± 0.03***	0.77± 0.14*	n.d.	n.d.
	TCE	0.31± 0.03	0.16± 0.01**	n.d.	n.d.
	TCE+PB	0.74± 0.02***	0.74± 0.08**	n.d.	n.d.
Erythromycin *N*-demethylase (nmol/min per mg protein)	Control	0.64± 0.03	0.18± 0.01	n.d.	n.d.
	PB	0.77± 0.03*	0.24± 0.01**	n.d.	n.d.
	TCE	0.75± 0.06	0.16± 0.01	n.d.	n.d.
	TCE+PB	0.72± 0.03	0.28± 0.03*	n.d.	n.d.

Rats were treated with a daily i.p. injection of trichloroethylene (1 g/kg) and/or phenobarbital (80 mg/kg) for 3 d. *a)* Data are shown as the mean±S.E. for 4—6 individual rats with statistically significant differences compared to the control group at $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***). n.d.: not detected. (EROD, PROD <1 pmol/min, PNPH, EMND <0.1 nmol/min)

microsomes, the detection of the same enzyme in hepatic microsomes required PB-induction. The coadministered TCE suppressed the PB-induced hepatic EROD and PROD activities by 25% and 50% in male rats, and 60% and 30% in female rats, respectively. The effect of TCE on the activity of hepatic PNPH in the PB-treated rats was trivial as in the case of the PB-untreated rats, even though the enzyme plays a major role in the metabolism of TCE. Pulmonary PROD activity in the PB-treated rats was suppressed much more than that in the PB-untreated rats.

The CYP isoform-apoproteins detected by immunoblot under various experimental conditions are shown in Figs. 1 and 2. The levels of CYP2B-apoprotein expression were in good correlation to the PROD activities, irrespective of sex, organ and PB-induction. However, the accumulation of CYP2E1 apoprotein does not seem to result in enhanced PNPH activity. TCE did not affect the activity of CYP3A, which was not involved in TCE metabolism, both in the absence and presence of PB.

DISCUSSION

In this study, the experimental animals were treated with TCE under sub-lethal conditions, i.p. injection at 1 g/kg body weight/d for 3 d, in order to discover the existence of biological defense mechanisms controlled by CYP isoforms against the pulmonary and hepatic toxicities. In industrial fields, workers are supposedly exposed to TCE *via* respiration. In this study, however, the animals were loaded with TCE intraperitoneally because the i.p. -injected TCE is excreted from pulmonary blood into gaseous compartment in an unchanged form. Furthermore, these procedures are convenient to control the dose of TCE to the animal, although the organic distribution of loaded TCE remains an uncertainty.

In the previous publications, it was suggested that the pulmonary toxicity of TCE could be potentiated metabolically by cellular enzymes including CYP isoforms. Forkert *et al.*[16] reported that the administration of TCE (2.5 g/kg body weight) caused injury in Clara cells of bronchiolar epithelium and alveolar Type II cells of parenchyma in the lung of mouse, and the total microsomal P450 content and arylhydrocarbon

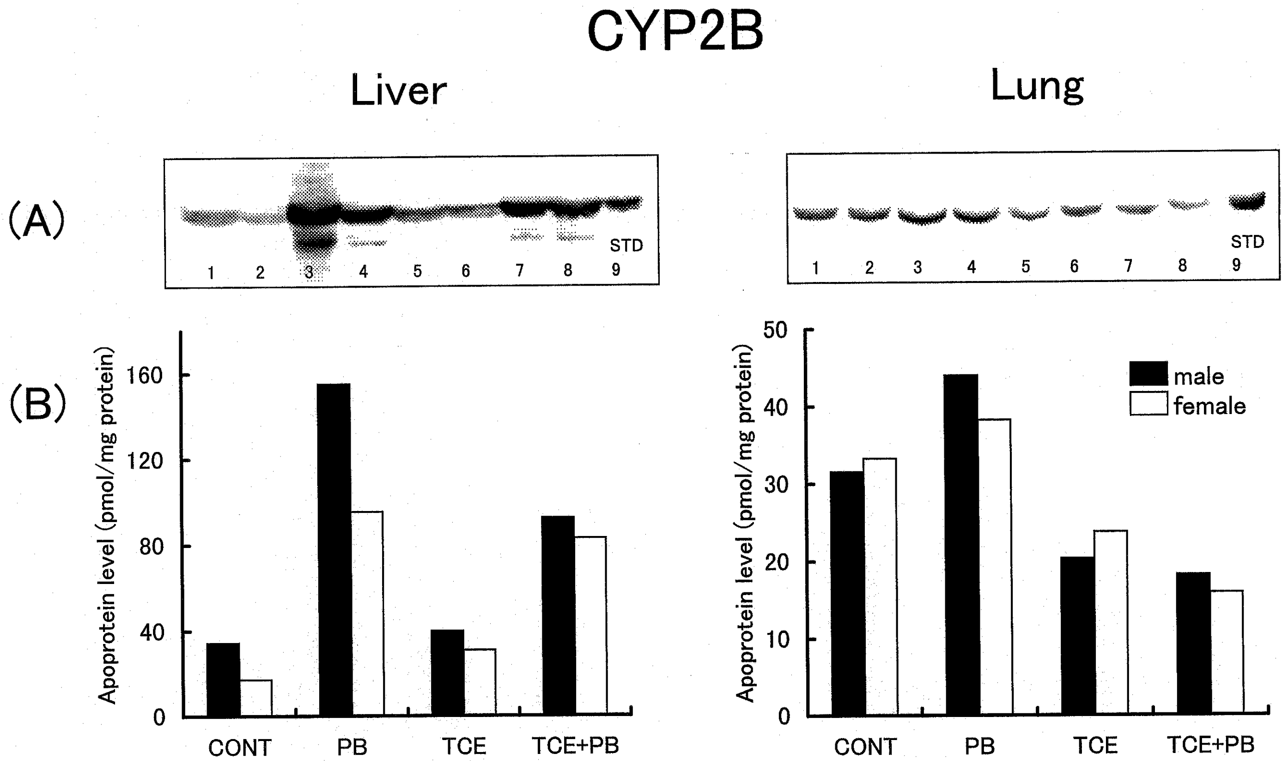

Fig. 1. Comparison of CYP2B-apoprotein Expression between Lung and Liver

(A) Western immunoblots. Each lane was loaded with a mixture of microsomal samples prepared from 5 individual animals (50 μg as protein). Lane 1, male control; lane 2, female control; lane 3, male PB-treatment; lane 4, female PB-treatment; lane 5, male TCE-treatment; lane 6, female TCE-treatment; lane 7, male TCE and PB-treatment; lane 8, female TCE and PB-treatment; lane 9, standard microsome. (B) Readings from Western blots. CONT, corn oil-treated control; PB, PB-treatment; TCE, TCE-treatment; TCE+PB, TCE and PB-treatment.

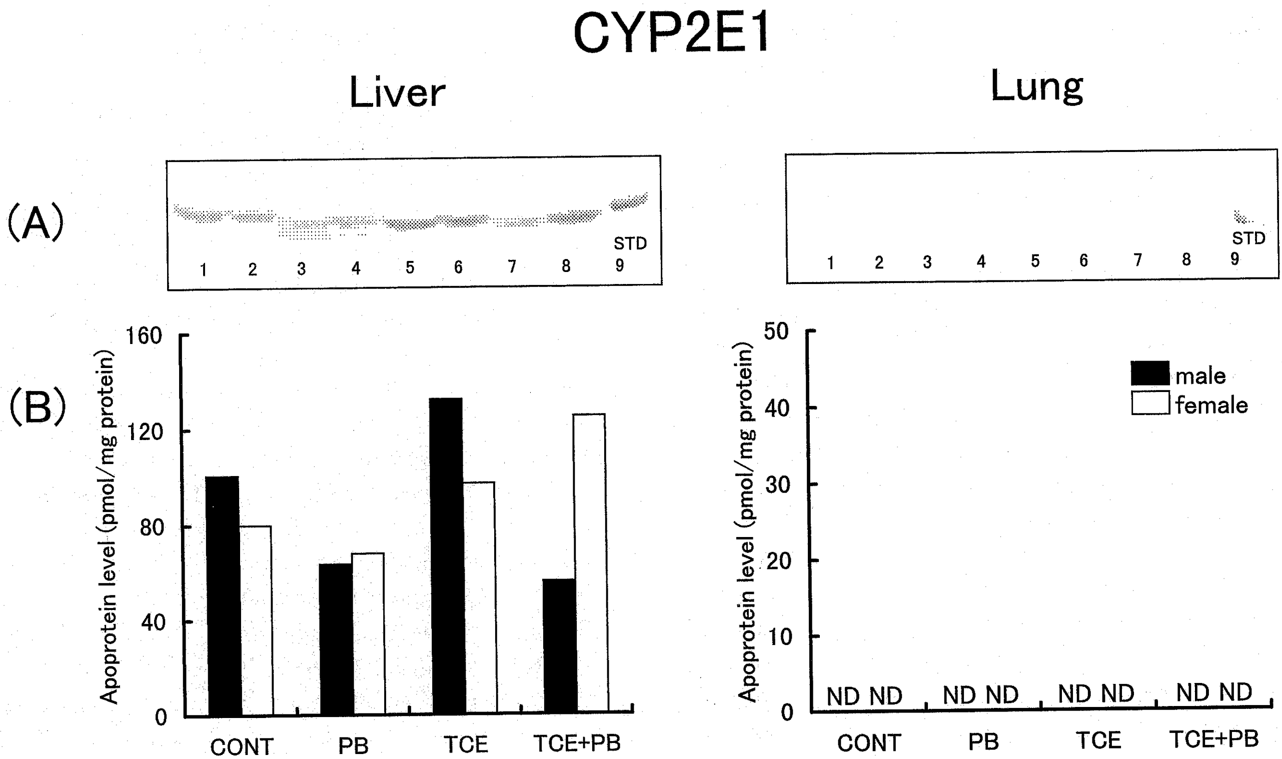

Fig. 2. Comparison of CYP2E-apoprotein Expression between Lung and Liver

(A) Western immunoblots. Each lane was loaded with a mixture of microsomal samples prepared from 5 individual animals (50 μg as protein). Lane 1, male control; lane 2, female control; lane 3, male PB-treatment; lane 4, female PB-treatment; lane 5, male TCE-treatment; lane 6, female TCE-treatment; lane 7, male TCE and PB-treatment; lane 8, female TCE and PB-treatment; lane 9, standard microsome. (B) Readings from Western blots. CONT, corn oil-treated control; PB, PB-treatment; TCE, TCE-treatment; TCE+PB, TCE and PB-treatment; ND, not detected.

hydroxylase activity in lung were decreased markedly. Nichols *et al.*[17] showed selective binding of TCE to pulmonary epithelial cells, causing pneumotoxicity. Mazzullo *et al.*[18] showed that TCE was covalently bound *in vivo* to DNA in rat lung. However, none of these studies were concerned with specification of the pulmonary CYP isoforms responsible for TCE activation.

Although the CYP2B apoproteins could be detected constitutively in both hepatic and pulmonary microsomal fractions, PROD activity could be detected in the lung, but not the liver (Table 2). CYP2B consists of the immunologically crossreacting components, CYP2B1 and CYP2B2. Among them, the former is mainly responsible for the PROD activity, being presumed to be a main component of pulmonary CYP2B. In contrast, CYP2B2 might be predominant in liver.

The suppression of pulmonary CYP2B activity was marked in the TCE-treated rats in accordance with the decreased level of CYP2B apoprotein. Lacking CYP2B activity, CYP2E1 plays a dominant role in TCE-metabolism among the phase I enzymes in rat liver. Because CYP2E1 could not be detected in the rat pulmonary microsomal fraction, CYP2B in lung might take the place of CYP2E1 in liver. By lowering CYP2B activity, which would potentiate the TCE toxicity, animals could avoid the fatal pulmonary toxicity of TCE.

Although the mechanism is not yet understood, the activated metabolites of TCE may adversely affect the expression of CYP2B in a process where pituitary hormones or interleukins may participate.[19,20]

When compared with the rats treated with corn oil, the TCE-treated animals showed different hepatic CYP isoform-profiles. The hepatic CYP2B activity, which was only detectable in PB-treated rats, was suppressed by TCE in accord with the decreased level of apoprotein, as was the case with the pulmonary counterpart. In accordance with the results of Imaoka *et al.*,[21] the activity of pulmonary CYP2B was not affected by PB. The activity of hepatic CYP1A was enhanced by individual treatment with TCE or PB ; however, TCE and PB were antagonistic to each other in a combination usage in terms of the CYP1A activity. This could be explained as follows. TCE could enhance the CYP1A activity by itself, while it might interfere somewhere in the process leading to potentiation of the same enzyme by PB.

The activation of TCE by some CYP isoforms, specifically CYP2E1, was proven by the fact that TCE toxicity was enhanced in the presence of PB or ethanol.[7] On the other hand, TCE is known to work as a suicide substrate for its major metabolizing enzyme CYP2E1 in mouse liver by covalently binding to the enzyme.[5,22] Although the amount of CYP2E1 apoprotein seemed to be increased by TCE, there appeared to be a decrease in the enzyme activity (control group *vs.* TCE-treated group) probably due to inactivation of CYP2E1 by formation of a covalent adduct. The same was true in the presence of PB (PB-treated group *vs.* TCE and PB-treated group).

The observations with TCE for the expression of CYP isoforms were not non-specific events, because there was no effect on CYP3A1, which is not involved in TCE metabolism. CYP2B might be a pulmonary counterpart of hepatic CYP2E1, playing a major TCE-metabolizing role. In liver, CYP2B is a typical inducible enzyme. However, both pulmonary and hepatic CYP2B activities were highly susceptible to TCE-inhibition at the steps up to translation. The reduction in CYP2B activity might be considered to be a part of a biological defense mechanism against the toxicity of TCE. In contrast, the effect of TCE on hepatic CYP2E1 activity seems to be complicated by its bidirectional functions and remains to be further studied.

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