Comparative Study on the Mode of Action of Chlorinated Ethylenes on the Expression of Rat CYP Forms

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Many environmental chemicals (xenobiotics), including polychlorinated ethylenes (CEs), are known to be metabolically intoxicated *via* the formation of hazardous intermediates (referred to as biological activation), while the expression of metabolic enzymes might be interfered with by xenobiotics at various stages in a system to minimize their adverse biological effects on the host animals. Three CEs, *i.e.*, tetrachloroethylene (PCE), trichloroethylene (TCE) and 1,1-dichloroethylene (1,1-DCE), were comparatively studied for their effects on the *in vivo* expression of CYP forms, which are responsible for their metabolic activation, as well as organospecificities between the liver and lung. Furthermore, their effects on the expression of CYP forms were studied in the animals simultaneously administered with phenobarbital (PB), known as an inducer of CYP2B. Individual CEs were administered intraperitoneally at 0.5 g/kg alone or simultaneously with PB (80 mg/kg/d) to 7-week-old male Wistar rats weighing about 200 g. The testosterone hydroxylase activities, CYP2B- and 2E1-mRNA, and CYP2B- and 2E1-proteins were measured 24 hr after the treatment. Testosterone 2β-hydroxylase (2βTSH) and 16βTSH activities are attributable to the functions of CYP3A2, and CYP2B plus CYP3A2, respectively. The induction of hepatic CYP2B in the PB-treated animals might be masked by the suppressive effect of CYP3A2 in terms of the 16βTSH activity, while the pulmonary 16βTSH activity was confined to the function of CYP2B, which was insensitive to the inducing effect of PB. The inhibition of hepatic 16βTSH activity was observed exclusively in the presence of 1,1-DCE, especially in PB-coadministered animals, suggesting the preferable suppression of CYP2B. In the lung, however, PCE suppressed the 16βTSH activity in the absence of PB. The expression levels of hepatic CYP2B mRNA and protein were significantly lowered by 1,1-DCE in the presence of PB. Concerning hepatic CYP2E1, no alternation was observed in the levels of mRNA and protein by any of the CEs in the absence or presence of PB, except for a marked decrease in the amount of mRNA when the rats were treated with 1,1-DCE in combination with PB. In the lung, both mRNA and protein were not detected under the given assay conditions, as in our previous studies. Based on these results, it is suggested that 1,1-DCE suppresses the induction of hepatic CYP2B and 2E1 in advance of the transcriptional stage. The expression of pulmonary CYP2B was obstructed by PCE posttranslationally in the absence of PB.

Key words —— tetrachloroethylene, trichloroethylene, 1,1-dichloroethylene, CYP mRNA, CYP protein, testosterone hydroxylase

INTRODUCTION

Cytochrome P450 (CYP) is a principal monooxygenase superfamily that is involved in the phase I metabolism of a variety of endogenous and exogenous substrates. A wide range of substrates is covered by individual CYP forms that are regulated independently. The induction of CYP1A1 by polycyclic aromatic hydrocarbons is known to be mediated by an aromatic hydrocarbon receptor (AhR).[1] The nuclear receptor, CAR, was recently found to be a putative mediator of the PB-dependent CYP2B1 induction,[2] and interaction with the pregnane X receptor (PXR) was required for the induction of CYP3A1 by glucocorticoid.[3] The induction of CYP2E1 by organic solvents, such as ethanol and pyridine, seems to be under translational regulation.[4]

When the metabolism of xenobiotics, generally toward intoxicification, is accompanied by the formation of toxic intermediates, the host defense systems should be mobilized to maintain homeostasis by either inducing or suppressing the enzyme activities. In addition to the well-characterized xenobiotic-elicited induction, the regulatory mecha-

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nisms involved in the control of the steady-state activity of CYP1A1 were elucidated by Morel *et al*. as follows.[5] CYP1A1, like many monooxygenases, can produce reactive oxygen species, which in turn repress the CYP1A1 gene promoter activity in a negative-feedback fashion. This mechanism limits the potentially toxic CYP1A1 activity within the cell. At much higher concentrations, xenobiotics with a potential to be metabolically activated suppressed the function of CYP1A1 in the culture cells.[6]

Previously, we observed the suppressive effects of tetrachloroethylene (PCE), trichloroethylene (TCE) and 1,1-dichloroethylene (1,1-DCE) on the activities of the CYP forms, by which the polychlorinated ethylenes (CEs) were metabolized to their active epoxides.[7] Furthermore, the behavior of the CYP forms was found to be organospecific, as shown by the fact that the constitutive expression of pulmonary CYP2B was insensitive to the inducing effect of phenobarbital (PB), while the hepatic counterpart was characterized as a typical inducible-type enzyme.[8,9]

The *in vivo* mode of action of the CEs on the expression of the CYP forms of both constitutive and inducible types, especially CYP2B and CYP2E1, is reported, with emphasis on the organospecificities.

MATERIALS AND METHODS

Reagents —— PCE and TCE were purchased from Wako Pure Chemical Ind. (Osaka, Japan). 1,1-DCE was a product of Aldrich Chemical Co. (U.S.A.). Goat anti-rat 2B1 and 2E1 sera and peroxidase-labeled anti-goat IgG serum were obtained from Daiichi Chemical Co. (Tokyo, Japan). First-Strand Beads, PCR Beads and Hybond-N^+ are products of Amersham Pharmacia Biotech, U.K. G3PDH primers and G3PDH cDNA control probe were purchased from Clontech Laboratories. BcaBEST (Takara, Japan) and [α-P32]dCTP were used for the labeling of the probes.

Animals and Treatment —— Seven-week-old male Wistar rats (Clea Japan) were divided into 8 groups, each consisting of 3–5 animals; PCE-treated, TCE-treated, 1,1-DCE-treated groups, cotreated groups with individual CEs plus PB, control group and PB-treated group. The rats were i.p. injected with the individual CEs (0.5 g/kg body weight) alone or simultaneously with corn oil or PB (80 mg/kg body weight). The lungs and livers were removed from the animals 24 hr after the treatment.

Microsomal Preparation —— The preparation of the microsomes has been described previously.[9] Briefly, the homogenates of the lungs and livers were centrifuged at 900 *g* for 5 min and then at 9000 *g* for 15 min at 4°C. The microsomes were obtained from the 9000 *g*-supernatants by centrifugation at 105000 *g* for 60 min at 4°C. The microsomal protein content was determined using Lowry's method.[10]

Analysis of Testosterone Hydroxylation —— A mixture of the microsomes (0.5–2.0 mg protein), 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 0.3 mM testosterone, and NADPH-generating system consisting of 0.8 mM NADP, 8.0 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase, and 6.0 mM magnesium chloride in a final volume of 1.0 ml was incubated for 10 min at 37°C. After cortisol acetate was added as an internal standard, the reaction mixture was extracted with dichloromethane and the solvent was removed under a N_2 stream. The metabolites of testosterone were analyzed as follows[11]: HPLC (Waters Co., Waters 600E) equipped with ODS column (3.9 × 20 mm) was developed with a linear gradient of 40–45% aqueous acetonitril for 12 min at a flow rate of 1.0 ml/min. The effluents were monitored at 240 nm.

Western Immunoblot —— An immunoblot analysis of the microsomes was performed to determine the CYP form apoproteins. An SDS-PAGE was carried out with 10% acrylamide, according to Laemmli.[12] The CYP apoproteins were transferred electrophoretically with the help of a blotting apparatus (BioRad, Transblot-SD), from the gel to a nitrocellulose sheet. The sheet was treated with goat anti-rat CYP form sera and peroxidase-labeled anti-goat IgG. Chemiluminescence was achieved using the Western blotting detection reagent, ECL Plus. The protein band-images were read by Storm TM830 (Amersham Pharmacia Biotech, U.K.) and analyzed with Image Quant software.

Total RNA Preparation and RT-PCR —— Total RNA was prepared by the RNeasy kit (Quiagen, Germany) from the liver and lung tissues. First-Strand Beads were used to obtain to cDNA from the total RNA. PCR Beads were used to perform the PCR reaction of the CYP mRNA. The integrity of the mRNA was assessed by ethidum bromide staining of 5% agarose gels. The mRNA band-images were read using Fluor Imager (Amersham Pharmacia Biotech, U.K.) and analyzed with Image Quant software. The primers used were as follows: 5′-GAGTTCTTCTCTGGGTTCCTG-3′, 5′-

Table 1. The Effects of CEs and/or PB on Testosterone Hydroxylase Activity in the Microsomes from Rat Livers and Lungs

Enzyme activity		Liver	Lung
Single-administration			
Testosterone 16β-hydroxylase	Control	1.44 ± 0.14 (100)[a)]	0.075 ± 0.009 (100)
(nmol/min per mg-protein)	PCE	1.30 ± 0.06 (90)	0.035 ± 0.004 (46)**
	TCE	1.25 ± 0.04 (87)	0.055 ± 0.012 (73)
	1,1-DEC	0.84 ± 0.14 (59)*	0.060 ± 0.005 (80)
Testosterone 2β-hydroxylase	Control	1.07 ± 0.10 (100)	n.d.
(nmol/min per mg-protein)	PCE	0.94 ± 0.13 (88)	n.d.
	TCE	0.97 ± 0.07 (91)	n.d.
	1,1-DCE	0.69 ± 0.10 (65)*	n.d.
Co-administration			
Testosterone 16β-hydroxylase	PB	1.14 ± 0.14 (79)	0.054 ± 0.005 (72)
(nmol/min per mg-protein)	PB+PCE	1.14 ± 0.04 (79)	0.053 ± 0.008 (70)
	PB+TCE	1.08 ± 0.04 (75)	0.055 ± 0.007 (73)
	PB+1,1-DCE	0.30 ± 0.02 (21)**	0.046 ± 0.012 (61)
Testosterone 2β-hydroxylase	PB	0.44 ± 0.12 (41)**	n.d.
(nmol/min per mg-protein)	PB+PCE	0.61 ± 0.09 (58)*	n.d.
	PB+TCE	0.38 ± 0.04 (36)**	n.d.
	PB+1,1-DCE	0.20 ± 0.06 (18)**,†	n.d.

The rats were treated with an i.p. injection of trichloroethylene (0.5 g/kg) and/or phenobarbital (80 mg/kg). *a*) The data are shown as the mean ± S.E. for 4–6 individual rats with statistically significant differences compared to the control group or PB-trated group at $p < 0.05$(*) and $p < 0.01$(**), (†). The figures in parentheses are the percentage of control group. n.d.: not detected.

ACTGTGGGTCATGGAG-AGCTG-3′ for the CYP2B mRNA; 5′-CTCCTCGTCATATCCATCTG-3′, 5′-GCAGCCAATCAG-AAATGTGC-3′ for the CYP2E1 mRNA.

Preparation of Probes —— The amplified products obtained from the CYP mRNAs by RT-PCR were cloned into a pBluescript II(Stratagene) within the SmaI recognition site. The plasmid DNA amplified in the XL1-Blue cells was digested with Pst I and BamH I to obtain the CYP cDNA fragments, which were labeled with [α-^{32}P]dCTP, using a BcaBEST labeling kit (Takara, Japan), as well as a human G3PDH cDNA control probe (Clontech, U.S.A.).

Northern Blotting —— Total RNA (30 μg) was separated electrophoretically on the denatured 1.2% agarose/2.2M formaldehyde gel and stained with ethidium bromide for the analysis of mRNA. The gel was equilibrated in 20 × SSPE (3M NaCl, 200 mM sodium phosphate, 20 mM EDTA, pH 7.4), and the RNA was transferred on to Hybond-N$^+$ using a capillary blotting unit (Scotlab, U.K.). The membrane was prehybridized for 3 hr at 42°C, and hybridized for 20 hr with ^{32}P-labeled cDNA pobe at 42°C. The membrane was exposed to Imaging Plate and analyzed by Storm TM830.

Statistics ——Statistical significance was determined using a Student's *t*-test. The 0.05 level of probability was adopted as a criterion of significance.

RESULTS

Effects of CEs on Testosterone Hydroxylase Activities

The rats were treated with individual CEs, either with or without PB, while those in the control group were given a corn oil vehicle or PB. Using microsomal fractions prepared from the lung and liver removed from each animal, the testosterone hydroxylase activities were measured. The testosterone 16β- and 2β- hydroxylations were attributable to the functions of CYP2B plus CYP3A2, and CYP3A2, respectively, and the results are shown in Table 1.

The decrease in the hepatic testosterone 16β-hydroxylase (16βTSH) activity was obvious in the 1,1-DCE-treated group, and a grater decrease was observed in combination with PB, which alone had no significant effect on the 16βTSH activity. The

marked suppression in the pulmonary 16βTSH was exclusively shown by the PCE-treatment in the absence of PB. The decrease in the hepatic 2βTSH activity was shown by 1,1-DCE additively with PB, which also suppressed hepatic 2βTSH. The same activity was not observed in the pulmonary samples, irrespective of the existence of PB.

Effects of CEs on the Expression of CYP Apoproteins

The determination of CYP 2B and 2E1 apoproteins was carried out by immunoblotting and the results are shown in Figs. 1 and 2.

The amount of hepatic CYP2B apoprotein was not significantly affected by the treatment with the individual CEs, however, about a 4-fold increase, resulting from the PB-treatment, was completely reversed by the 1,1-DCE. In contrast, the pulmonary CYP2B apoprotein was tended to decrease when the animals were treated with CEs in the absence of PB.

Both PCE and TCE showed the enhancing effect on the amount of hepatic CYP2E1 apoprotein in the absence of PB, which alone increased the amount of CYP2E1 apoprotein. The amount of pulmonary CYP2E1 apoprotein was lower than the detection limit for this protein.

Comparison of the Expression of CYP2B mRNA between Liver and Lung

The expression of CYP2B mRNA in the liver and lung was monitored by RT-PCR in the presence or absence of PB (Fig. 3). The clearly separated twin bands of the CYP2B mRNA were detected in the liver of the control rat. The upper and lower bands correspond to the PCR-products from the CYP2B2 mRNA (573 bp) and CYP2B1 mRNA (549 bp), respectively, based on their molecular weights, although CYP2B1/2 could not be detected in terms of the PROD activity.[9] In the lung, the selective expression of CYP2B1 mRNA was confirmed, with only the lower band being detected.

Effects of CEs on the Expression of CYP2B mRNA

The levels of CYP2B mRNA in the liver and lung were measured by Northern blot analysis. The results, normalized by G3PDH mRNA, are presented in Fig. 4. The constitutive expression level of CYP2B mRNA in the lung was more than 4-fold of that in liver. The expression of CYP2B mRNA was suppressed by about 50% by the addition of 1,1-DCE in both the liver and lung, while PCE and TCE were devoid of any notable effects on the expression of CYP2B mRNA.

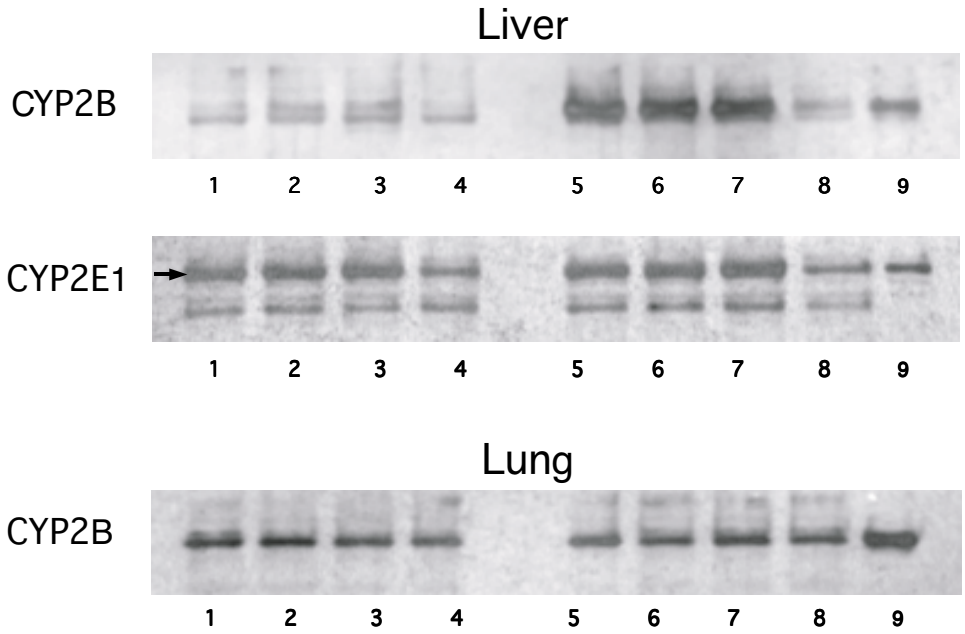

Fig. 1. Immunoblots of the Liver and Lung Microsomes from the Chlorinated Ethylene-Treated Rats Stained with Anti-Rat CYP2B1and CYP2E1 Antibodies

The liver microsomes from the rats treated with phenobarbital and pyridine were used as the standards for CYP2B and 2E1, respectively. CE-monotreatment (left side): lane 1, control; lane 2, PCE; lane 3, TCE; lane 4, 1,1-DCE. Co-treatment with PB (right side): lane 5, PB; lane 6, PCE; lane 7, TCE; lane 8, 1,1-DCE; lane 9, standard.

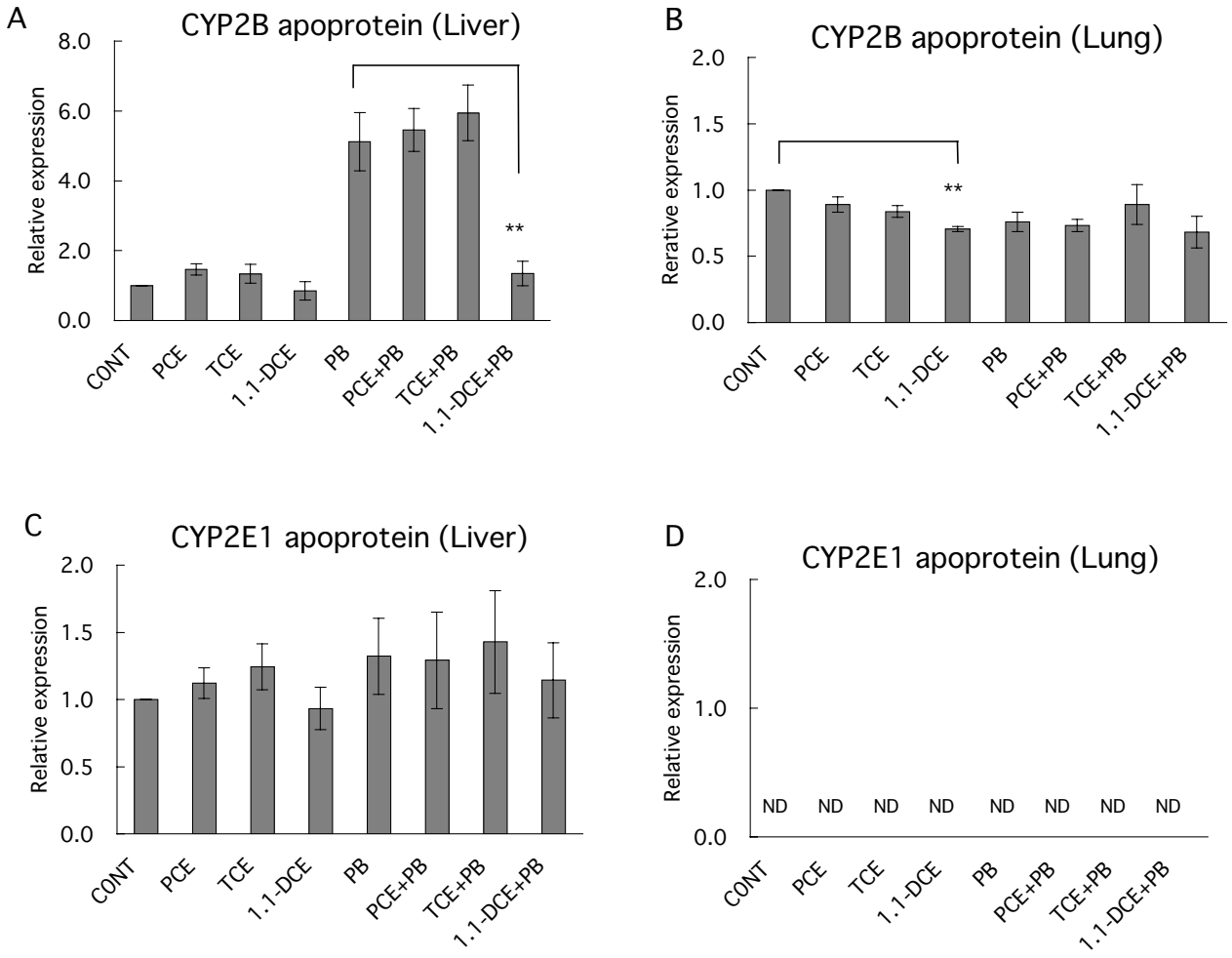

Fig. 2. The effects of CEs on the Normalized Expression Levels of CYP Apoproteins

The readings from the Western blots are shown in Fig. 1. Abbreviations: CONT, corn oil-treatment; PCE, PCE-treatment; TCE, TCE-treatment; 1,1-DCE, 1,1-DCE- treatment; PB, PB-treatment; PCE+PB, PCE and PB-cotreatment; TCE+PB, PCE and PB-cotreatment; 1,1-DCE +PB, 1,1-DCE and PB-cotreatment.

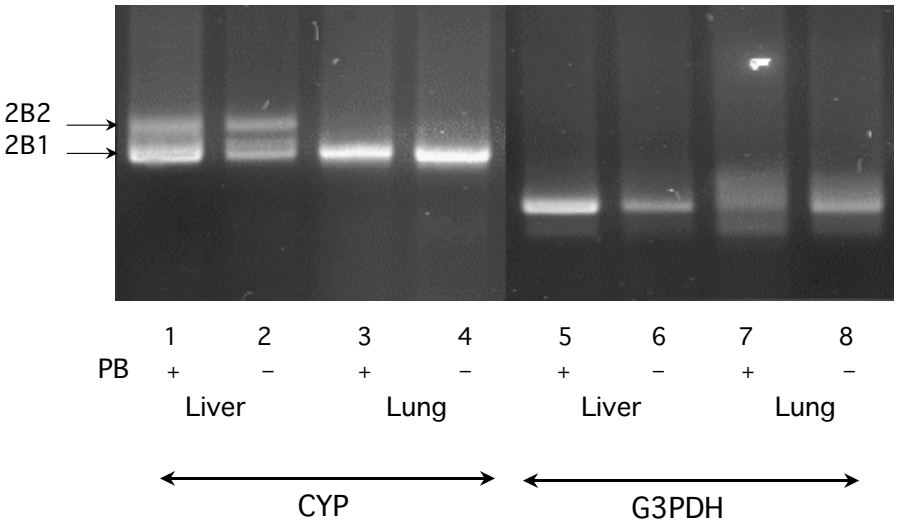

Fig. 3. The comparison of Expression Profiles of CYP2B mRNA Subfamilies Detected by RT-PCR between the Lung and Liver

Lanes 1 and 5, PB-treated liver; lanes 2 and 6, untreated liver; lanes 3 and 7, PB-treated lung; lanes 4 and 8, control lung.

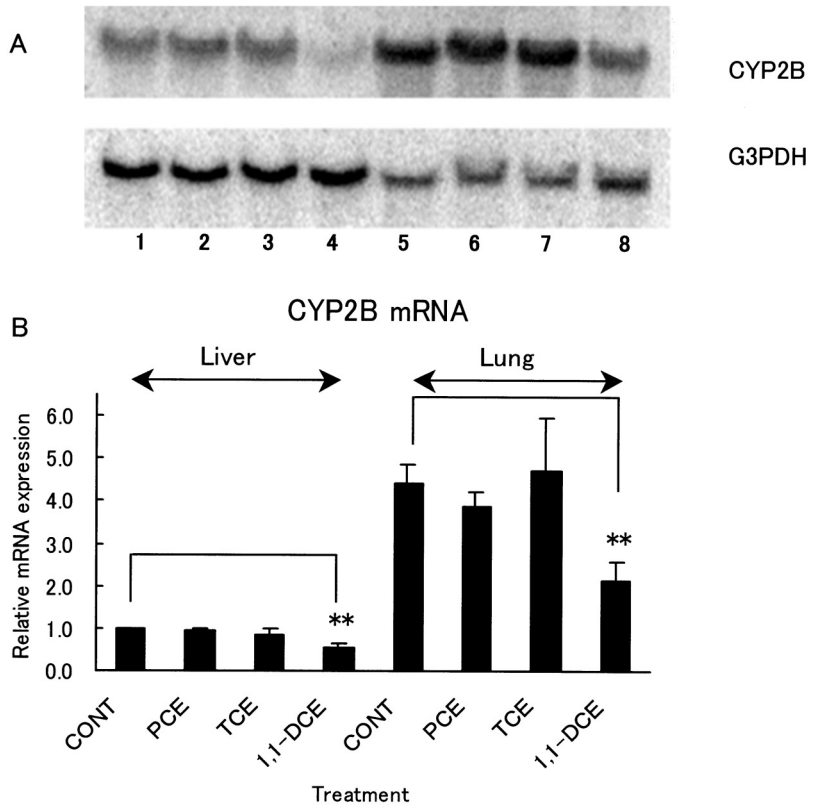

Fig. 4. The effects of CEs on the Normalized Expression Levels of CYP2B mRNA in the Liver and Lung

(A) Northern blots. Each lane was loaded with total RNA samples prepared from 5 individual animals (30 μg as RNA). Lane 1, control liver; lane 2, PCE-treated liver; lane 3, TCE-treated liver; lane 4, 1,1-DCE-treated liver; Lane 5, control lung; Lane 6, PCE-treated lung; Lane 7, PCE-treated lung; Lane 8, 1,1-DCE -treated lung. (B) Readings from Northern blots. Abbreviations: CONT, corn oil-treatment; PCE, PCE-treatment; TCE, TCE-treatment; 1,1-DCE, 1,1-DCE- treatment.

A marked induction of hepatic CYP2B mRNA was observed by the PB-treatment (Fig. 5). However, more than 95% inhibition was shown by 1,1-DCE in marked contrast to the negligible effects of PCE and TCE. The expression of pulmonary CYP2B mRNA, free from the effects of PB, also decreased by the 1,1-DCE-treatment to a much lesser extent.

Effects of CEs on the Expression of CYP2E1 mRNA

The expression of CYP2E1 mRNA was observed in the hepatic, but not pulmonary, preparation of the vehicle-inoculated rat. The decrease in the hepatic CYP2E1 mRNA level was observed after the 1,1-DCE-treatment (Fig. 6).

Comparing the band intensities of the hepatic CYP2E1 mRNA, relative to those of the G3PDH mRNA (Figs. 6 and 7), PB alone was found to be an inducer of mRNA as well as apoprotein (Fig. 2). As with CYP2B, 1,1-DCE showed the same potent inhibitory effects on the expression of CYP2E1 mRNA in the livers of PB-treated rats, accompanied by the same, but much reduced response, in the apoprotein level (Figs. 2 and 7).

DISCUSSION

The expression of CYP2B and 2E1 in the liver and lung of rats treated with CEs alone, or in combination with PB, have been discussed in our previous papers at the levels of apoprotein expression and enzyme activity.[7–9] Briefly, the PROD activity conferred by CYP2B was missing in the liver, but remarkable activity was observed under the PB-treatment. In contrast, the constitutively-expressed pulmonary PROD activity may not be affected by PB. In the case of CYP2E1, the *p*-nitrophenol hydroxylase (PNPH) activity was only observed in the liver with 2 to 3-fold increases being observed when treated with PB. 1,1-DCE generally suppressed the expression of CYP forms, including CYP2B and CYP2E1, especially in combination with PB in terms of the levels of both apoprotein and enzyme activi-

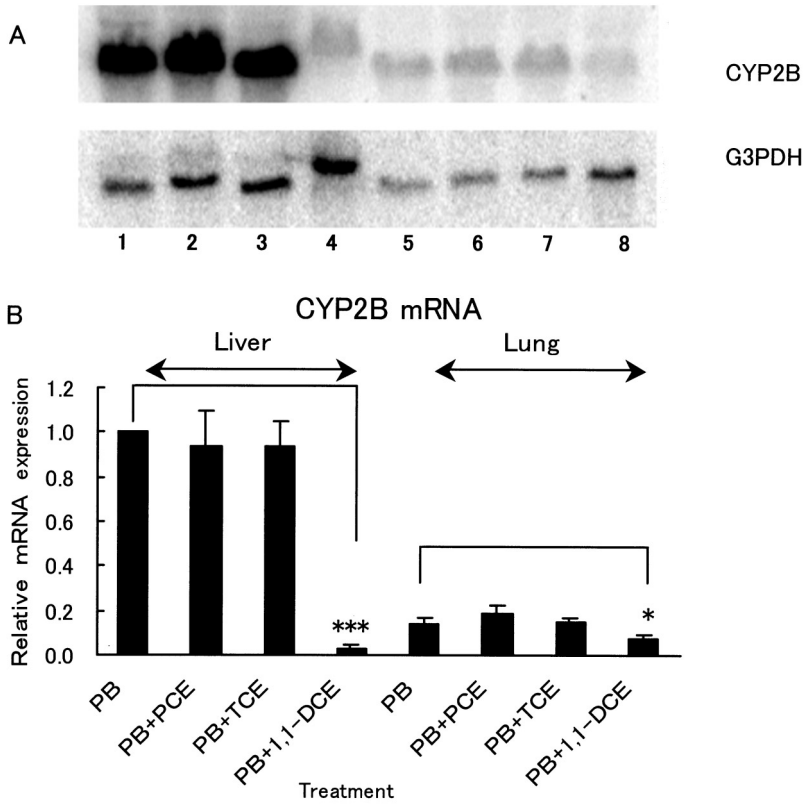

Fig. 5. The effects of Cotreatment with CEs and PB on Normalized Expression Levels of CYP2B mRNA in the Liver and Lung

(A) Northern blots. Each lane was loaded with total RNA samples prepared from 5 individual animals (30 μg as RNA). Lane 1, PB-treated liver; lane 2, PCE and PB-cotreated liver; lane 3, TCE and PB-cotreated liver; lane 4, 1,1-DCE and PB-cotreated liver; lane 5, PB-treated lung; lane 6, PCE and PB-cotreated lung; lane 7, TCE and PB-cotreated lung; lane 8, 1,1-DCE and PB-cotreated lung. (B) Readings from Northern blots. Abbreviations: PB, PB-treatment; PCE +PB, PCE and PB-cotreatment; TCE+PB, PCE and PB-cotreatment; 1,1-DCE +PB, 1,1-DCE and PB-cotreatment.

ties. In order to understand the mode of action of the CEs on the expression of CYP forms, the mRNAs and apoproteins of CYP2B and 2E1 were determined in this study, as well as some testosterone hydroxylase activities, in the absence or presence of PB.

1,1-DCE suppressed the expression of CYP2B and 2E1 in both the liver and lung, although detection of the enzyme activity in the hepatic CYP2B remained to be induced by PB. It is obvious that 1,1-DCE was inhibitory on the constitutive expression of the CYP forms transcriptionally. Regarding the effect on the expression of the PB-inducible type CYP forms, the interference of 1,1-DCE might take place in advance of the transcriptional stages, somewhere in a signal transduction step triggered by PB, as almost complete inhibition of the expression of mRNA of CYP forms was observed (Figs. 2 and 5).

The 16βTSH activity in the pulmonary microsomes, which was exclusively attributable to the function of CYP2B due to the lack of 2βTSH activity, was adversely affected with PCE, without being accompanied by any effect on the expression levels of mRNA and apoprotein. Furthermore, the suppressive effects of PCE on the 16βTSH activity was nullified by the PB-treatment (Table 1).

Recently, Negishi *et al.* (1998) have proposed that the binding of the heterodimer of the nuclear orphan receptor CAR and retinoid X receptor (RXR) to the NR1 site, which was located in the phenobarbital-responsive enhancer module (PBREM) found in PB-inducible *CYP2B* genes, was requisite for the response to PB. A CAR-RXR heterodimer has been characterized as a trans-acting factor for the PB-dependent induction of the *CYP2B* gene.[2] Kawamoto *et al.* (1999) have shown the cytoplasmic location of CAR, in the livers or primary hepatocytes originating from untreated mice, indicating that the nuclear translocation of CAR is critical for the nuclear events triggered by the PB-treatment.[13]

In the case of human hepatoma HepG2 cells, the green fluorescent protein (GFP)-CAR was found to be localized in the nuclei of transfected cells, suggesting the spontaneous translocation of CAR into the nuclei and the PB-irresponsiveness of this cul-

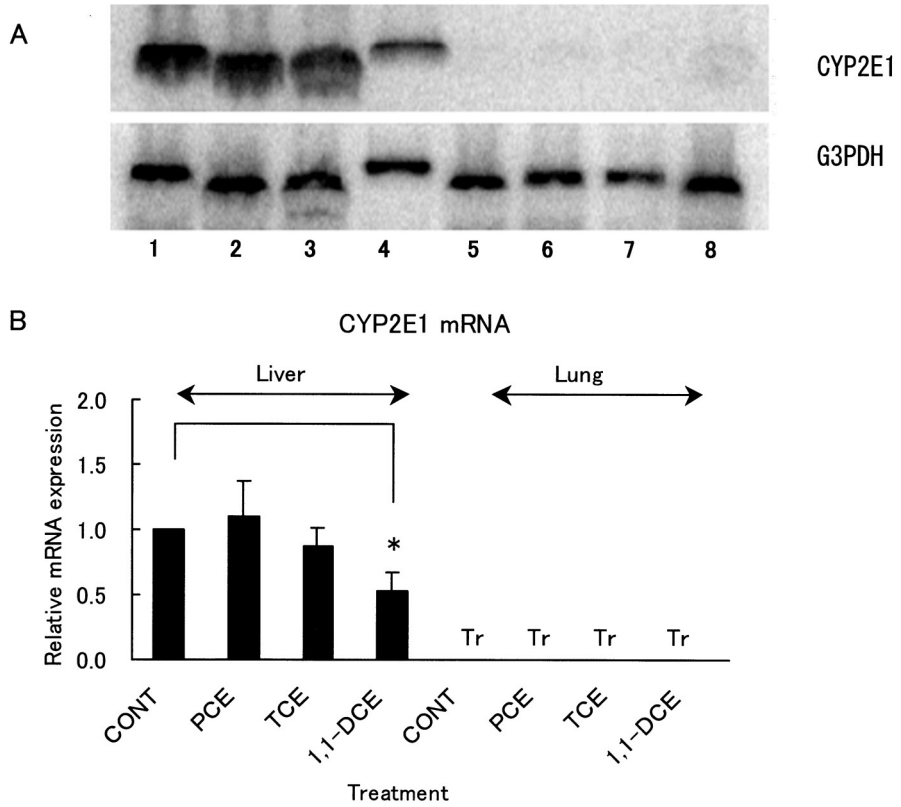

Fig. 6. The Effects of CEs on Normalized Expression Levels of CYP2E1 mRNA in the Liver and Lung
Data are expressed as described in the legend to Fig. 4.

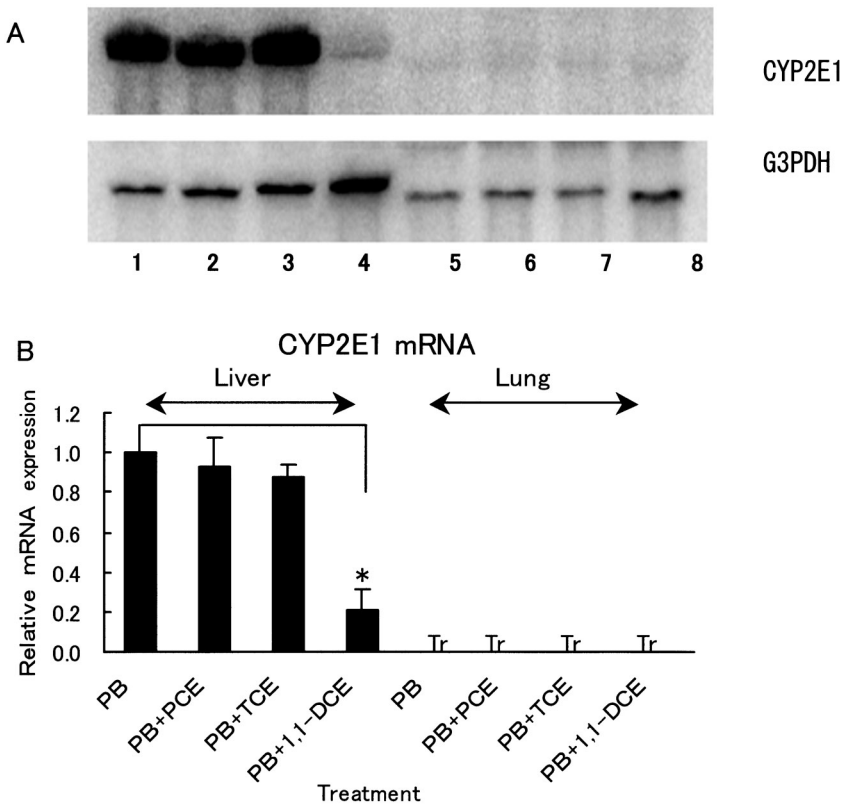

Fig. 7. The Effects of Cotreatment with CEs and PB on Normalized Expression Levels of CYP2E1 mRNA in the Liver and Lung
Data are expressed as described in the legend to Fig. 5.

ture cell line. Therefore, the suppression of the hepatic *CYP2B* gene expression by 1,1-DCE could be partly accounted for by the obstructed nuclear translocation of CAR. The hypothesis that CAR was spontaneously localized in the nuclei of the lung cells might be rational, as the expression of the pulmonary *CYP2B* gene was constitutive and irresponsive to the PB-treatment.

We have previously reported that the constitutive expression of PNPH activity conferred by CYP2E1 was observed in the liver, but not in the lung.[9] A similar type of organospecificity was observed in this study for the expression of CYP2E1 mRNA, implying a transcriptional basis for the organospecific expression of CYP2E1.

1,1-DCE was found to be a potent inhibitor of the signal transduction for the PB-dependent transcriptiptional activation of the *CYP2E1* gene, based on the differential effects of 1,1-DCE on the expression of CYP2E1 mRNA in the presence or absence of PB (Fig. 6 and 7). By comparing the magnitude of expression of the CYP2E1 apoprotein with previous results on the PNPH activity, the posttranslational effects of 1,1-DCE could also be proposed. Oesch-Bartlomowicz *et al*. (1998) have also reported that the expression of CYP2E1 is controlled posttranslationally by the phosphorylation of serine-129 in the protein kinase A (PKA) recognition sequence motif in addition to the transcriptional control.[4] The reduction of CYP2E1 enzymatic activity has been reported in the presence of db-cAMP, although this cAMP-related down-regulation was not accompanied by a decrease in the CYP2E1 apoprotein content. In addition to the effects on the PKA pathway, the formation of 1,1-DCE-adducts might be an alternative cue for the posttranslational effects of 1,1-DCE.

The induction of CYP forms by CEs is generally recognized in the *in vitro* assay system, using primary hepatocyte culture (Nakahama *et al*., unpublished data). In this study, however, CEs were found to suppress the expression of CYP forms *in vivo*. CEs are metabolically activated by CYP forms to show their toxicities,[14] to which various host defense systems were mobilized to reverse the toxicities of CEs. The expressions of CYP forms are reportedly down-regulated by interleukins,[15] insulin,[16] active oxygen species,[5] glucocorticoide[17] *etc*. Therefore, these extracellular factors might be responsible for the marked differences in the responsive behaviors between the *in vitro* and *in vivo* results.

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