PULMONARY BIOACTIVATION OF TRICHLOROETHYLENE TO
CHLORAL HYDRATE: RELATIVE CONTRIBUTIONS OF CYP2E1,
CYP2F AND CYP2B1

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Abbreviations used are: CH, chloral hydrate; DCA, dichloroacetic acid; PNP, p-nitrophenol; rCYP2E1, recombinant CYP2E1; rCYP2F4, recombinant CYP2F4; rCYP2B1, recombinant CYP2B1; SDS-PAGE, sodium dodecyl sulphate gel electrophoresis; TCA, trichloroacetic acid; TCE, trichloroethylene; TCOH, trichloroethanol.
ABSTRACT:

Pulmonary cytotoxicity induced by trichloroethylene (TCE) is associated with cytochrome P450-dependent bioactivation to reactive metabolites. In this investigation, studies were undertaken to test the hypothesis that TCE metabolism to chloral hydrate (CH) is mediated by cytochrome P450 enzymes including CYP2E1, CYP2F and CYP2B1. Recombinant rat CYP2E1 catalyzed TCE metabolism to CH with greater affinity than did the recombinant P450 enzymes, rat CYP2F4, mouse CYP2F2, rat CYP2B1 and human CYP2E1. The catalytic efficiencies of recombinant rat CYP2E1 ($V_{max}/K_m = 0.79$) for generating CH was greater than those of recombinant CYP2F4 ($V_{max}/K_m = 0.27$), recombinant mouse CYP2F2 ($V_{max}/K_m = 0.11$), recombinant rat CYP2B1 ($V_{max}/K_m = 0.07$) or recombinant human CYP2E1 ($V_{max}/K_m = 0.02$). Decreases in lung microsomal immunoreactive CYP2E1, CYP2F2 and CYP2B1 were manifested at varying time-points after TCE treatment. The loss of immunoreactive CYP2F2 occurred prior to those of immunoreactive CYP2E1 and CYP2B1. These protein decreases coincided with marked reduction of lung microsomal $p$-nitrophenol hydroxylation and pentoxyresorufin $O$-dealkylation. Rates of CH formation in the microsomal incubations were time-dependent and were incremental from 5 to 45 min. The production of CH was also determined in human lung microsomal incubations: the rates were low and were detected in only three out of eight subjects. These results showed that, although CYP2E1, CYP2F and CYP2B1 are all capable of generating CH, TCE metabolism is mediated with greater affinity by recombinant rat CYP2E1 than by recombinant CYP2F, CYP2B1 or human CYP2E1. Moreover, the rates of CH production were substantially higher in murine than in human lung.
Trichloroethylene (C₂HCl₃; TCE) is a volatile and lipophilic chemical used commonly as an industrial solvent for cold and vapor cleaning of metal parts. TCE has also been used as an extractant in food processing, and as a lubricant and a dry cleaning agent. Production of TCE increased from about 260,000 pounds in 1982 to 320 million pounds in 1991 (Pastino et al, 2000). The National Toxicology Program has estimated that 3.5 million workers are exposed annually to TCE in the United States (NTP, 1990). Although the current occupational exposure limit for TCE is 50 ppm (ACGIH, 2004), workers are still exposed to TCE under conditions where adequate protective measures are not in place (NIOSH, 1978; Forkert et al., 2003). As a result of its versatility and widespread use, TCE has become a prevalent soil and groundwater contaminant (Davidson and Beliles, 1991).

The major target organs of TCE exposure are the liver, kidney and lung (for review, see Lash et al., 2000). Substantial data have accrued to affirm that an obligate step in the toxicities induced by TCE is its bioactivation to reactive metabolites capable of binding covalently to tissue constituents including proteins. The metabolism of TCE is mediated through conjugative and oxidative pathways. Although P450 enzymes are present in the kidney, the nephrotoxic effects of TCE are mediated via the glutathione conjugation pathway. Initial conjugation with glutathione, which takes place mainly in the liver, produces S-(1,2-dichlorovinyl)glutathione, and subsequent metabolic processes including transport and biotransformation lead to formation of S-(1,2-dichlorovinyl)-L-cysteine, a metabolite that has been shown to be nephrotoxic in rats (Elfarra and Anders, 1984; Elfarra et al., 1986; Dekant et al., 1990). However, the predominant pathway of TCE metabolism is oxidation via the cytochrome P450 system, mainly by CYP2E1, although
other P450 enzymes including CYP1A1/2, CYP2B1/2 and CYP2C11/6 have also been implicated (Guengerich et al., 1991; Nakajima et al., 1990, 1992).

Oxidative metabolism of TCE yields the primary metabolites chloral, TCE oxide and dichloroacetyl chloride (Fig. 1). Chloral, a predominant TCE metabolite, is rapidly converted to chloral hydrate (CH) that then undergoes oxidation and reduction by aldehyde dehydrogenase and alcohol dehydrogenase to form trichloroacetic acid (TCA) and trichloroethanol (TCOH), respectively (Green and Prout, 1985; Dekant et al., 1986). Dichloroacetyl chloride subsequently undergoes decomposition to form dichloroacetic acid (DCA), which can also be formed from dechlorination of TCA (for review, see Lash et al., 2000). Thus, exposure to TCE and its metabolism results in the generation of several metabolites including chloral, CH, dichloroacetyl chloride, TCA, TCOH and DCA. It has been proposed that TCA mediates the hepatotoxicity of TCE (Bull, 2000), while TCA and DCA are responsible for its hepatocarcinogenic effects (Larson and Bull, 1992). Although it has been difficult to identify the reactive species responsible for a specific toxic effect, there is general agreement that TCA, TCOH and DCA mediate the toxic and/or carcinogenic effects of TCE (Pastino et al., 2000).

Although the liver is the primary tissue in which TCE oxidation occurs, oxidative metabolism also takes place in other tissues such as the lung where injury is selectively manifested in Clara cells of the bronchiolar epithelium (Forkert et al., 1985; Forkert and Birch, 1989). The pneumotoxic lesion is associated with TCE metabolism to reactive intermediates that bind covalently to lung proteins. In this investigation, we have undertaken studies to test the hypothesis that cytochrome P450 enzymes including CYP2E1, CYP2F2 and CYP2B1 catalyzed the bioactivation of TCE. Kinetic studies
were performed to determine the relative affinities for TCE metabolism to CH, using incubations of TCE with recombinant rat and human CYP2E1 (rCYP2E1), recombinant rat CYP2B1 (rCYP2B1), recombinant rat CYP2F4 (rCYP2F4) or recombinant mouse CYP2F2 (rCYP2F2). Unlike CYP2E1 and CYP2B1, which are present in liver at high levels, the CYP2F enzyme resides primarily in lung tissue and is expressed as only a single member in each of the species studied: human (2F1) (Nhamburo et al., 1989), mouse (2F2) (Ritter et al., 1991), goat (2F3) (Wang et al., 1998) and rat (2F4) (Baldwin et al., 2005). Alterations of immunoreactive CYP2E1, CYP2F2 and CYP2B1 proteins and associated enzyme catalytic activities were evaluated in lung microsomes from TCE-treated mice. Formation of CH was also determined in incubations of lung microsomes from mice and humans. Our results demonstrated that rat and human rCYP2E1, rCYP2F and rCYP2B1 were all capable of mediating TCE metabolism to CH, although rat rCYP2E1 exhibited greater affinity than rCYP2F, rCYP2B1 or human rCYP2E1. Moreover, markedly higher rates of CH production were observed in murine than human lung.
MATERIALS AND METHODS

Chemicals and Reagents. Chemicals were purchased from suppliers as follows: trichloroethylene (purity 99.5%) and ethyl acetate (Aldrich, Montreal, QC, Canada); goat anti-rabbit horseradish peroxidase, acrylamide/BIS, polyvinylidene difluoride membrane, chemiluminescence reagents (Immuno-star HRP Substrate kit) and protein assay dye (BioRad, Hercules, CA); goat anti-mouse horseradish peroxidase (BD Biosciences, Mississauga, ON); p-nitrophenol, 4-nitrocatechol, bovine serum albumin, rabbit anti-goat horseradish peroxidase, Ponceau S stain, 1,3-dibromopropane, resorufin and pentoxyresorufin (Sigma Chemical Co., St. Louis, MO); recombinant rat CYP2B1 and recombinant human CYP2E1 (BD Biosciences Discovery Labware, Bedford, MA); human CYP2F1-expressed β-lymphoblastoid microsomes (BD Gentest, Woburn, MA). The CYP2E1 polyclonal antibody was obtained from Oxford Biomedical (Hornby, ON, Canada). The CYP2F1 antibody was donated by Dr. Garold S. Yost and has been shown in previous studies to cross-react with CYP2F2 (Simmonds et al., 2004a). The CYP2B1 monoclonal antibody (mAb 2-66-3) was donated by Dr. S. S. Park. Recombinant rat CYP2F4, mouse CYP2F2 and rat NADPH-cytochrome P450 reductase were donated by Dr. Alan R. Buckpitt.

Animal Treatment. Male CD-1 mice, weighing 25-28 g, were purchased from Charles River Canada (St. Constant, QC, Canada). The animals were housed in an accredited (Canadian Council on Animal Care) facility with a 12 h light/dark cycle and free access to water and food (Mouse Diet 5015, PMI Nutrition International, Inc., Brentwood, MO). Mice were acclimatized to laboratory conditions for at least one week before being used in an experiment. For immunoblotting and enzyme catalytic activity
studies, mice were treated with 750 mg/kg (i.p.) TCE in corn oil, and were sacrificed at 5, 15 and 30 min or 1, 2 and 4 h after treatment. Control mice were treated with equivalent volumes of the vehicle.

**Incubation of TCE with Recombinant P450 Enzymes.** Incubations with TCE were performed with recombinant P450 enzymes that were co-expressed with NADPH-cytochrome P450 reductase, with the exception of rCYP2F4 and rCYP2F2. Reaction mixtures in a final volume of 250 µl of 50 mM Tris-HCl buffer, pH 7.4, contained rat rCYP2E1 (10 pmol), human rCYP2E1 (15 pmol) or rat rCYP2B1 (10 pmol) and TCE (0.05 - 5.0 mM) in acetonitrile (0.4%, v/v) and were preincubated at 37°C for 3 min. The reaction was initiated by addition of NADPH (1.0 mM), and the incubations were continued for an additional 20 min. The reaction was terminated by freezing in liquid nitrogen. Reaction mixtures containing rCYP2F4 (5 pmol) or rCYP2F2 (5 pmol), NADPH-cytochrome P450 reductase (10 units/pmol P450) and 2 mM 3-[(−cholamidopropyl)dimethyl-ammino]−1-propanesulfonate in 0.1 M Na₂HPO₄, pH 7.4, were preincubated at 4°C for 2 h. NADPH-cytochrome oxidoreductase was added to the incubations containing rCYP2F4 or rCYP2F2 because the recombinant enzymes were not co-expressed with the reductase. The reductase was purified from rat liver and specific activity determined using procedures described previously (Shultz et al., 2001).

Following preincubation, an NADPH-generating system (2 U of glucose-6-phosphate dehydrogenase, 15 mM glucose-6-phosphate, 2 mM NADP, and 1 mM MgCl₂) was added and brought to a final volume of 250 µl. TCE concentrations of 0.25-3.0 mM in acetonitrile (0.4% v/v), were added and the incubation was carried out for 20 min at 37°C. Reactions were terminated by freezing in liquid nitrogen.
**Preparation of Microsomes.** Human lung tissue (10-50 g) was obtained from Kingston General Hospital (Kingston, ON, Canada) from consenting patients undergoing surgical lobectomies. The protocol for the studies in human lung was approved by the Human Ethics Committee of Queen’s University. Tissue distant from the primary lesions was surgically excised, placed on ice, transferred immediately to a biohazard facility, sectioned into smaller pieces, frozen in liquid nitrogen and stored at -80°C. Human lung microsomes were prepared by differential centrifugation using procedures described previously (Forkert *et al.*, 2001). Murine lung microsomes were prepared as described in previous studies (Simmonds *et al.*, 2004b), with minor modifications. Lung tissues from 10 mice were pooled for each microsomal sample, while human lung tissue was processed as individual samples. Lung tissue was minced and homogenized in 4 volumes of cold phosphate-buffered KCl (139 mM KCl, 100 mM K2HPO4, 1.5 mM EDTA, pH 7.4). The homogenate was subjected to centrifugation at 12,500g for 20 min at 2°C. The supernatant was then centrifuged at 105,000g for 60 min. The pellet was resuspended in buffer with approximately three strokes of the pestle and centrifuged at 105,000g for 60 min. The final pellet was resuspended in buffer. Aliquots of lung microsomes were frozen in liquid nitrogen and stored at -70°C. Microsomal protein concentrations were determined by the Bradford method (Bradford, 1976), using bovine serum albumin as the standard.

**Protein Immunoblotting.** Protein blots were prepared using a modification of procedures described previously (Forkert, 1995). Microsomal proteins were separated by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE), using an 8.5% gel, and transferred to a polyvinylidene difluoride membrane. Low-range SDS-PAGE standards
(BioRad, Hercules, CA) were included on each gel. Microsomal proteins from human β-lymphoblastoid cells expressing CYP2F1 were included as a positive control for murine CYP2F2 protein. Membranes were stained with Ponceau S to confirm uniform protein loading and membrane transfer. Membranes were then incubated overnight in a blocking solution consisting of 10% non-fat milk powder in 20 mM Tris-HCl containing 500 mM NaCl, pH 7.5. After rinsing in Tween 20/Tris-buffered saline, the membrane was incubated for 2 h with a CYP2E1, CYP2F1 or CYP2B1 antibody. The CYP2F1 antibody was generated against a cyclic peptide of 23 amino acids (Nichols et al., 2003) and was affinity purified using a SulfoLink Coupling gel kit (Pierce Chemical, Rockford, IL).

The blots were incubated with a horseradish-peroxidase conjugated secondary antibody for 2 h as follows: CYP2E1 with rabbit anti-goat IgG (1:5 000), CYP2F1 with goat anti-rabbit-IgG (1:18 000) and CYP2B1 with a goat anti-rabbit IgG (1:5 000). The protein bands were visualized using chemiluminescence reagents (Immuno-Star™ HRP Substrate Kit, BioRad, Hercules, CA). Densitometric analyses of the Ponceau S stained protein bands and radiographs were performed using a Gel Logic 200 Imaging System and Kodak 1D Image Analysis Software (Eastman Kodak CO., New Haven, CT).

**p-Nitrophenol Hydroxylase and Pentoxyresorufin O-Dealkylase Activities.** PNP hydroxylation was used as a catalytic marker for CYP2E1 and CYP2F2, while pentoxyresorufin O-dealkylation was used as a marker for the CYP2B enzymes (Schultz et al., 1999; Simmonds et al., 2004a). For determination of PNP hydroxylase activity, reaction mixtures contained lung microsomal protein (0.5 mg) from TCE-treated mice and NADPH (1.5 mM). A total volume of 250 µl 100 mM potassium phosphate buffer containing 100 mM ascorbic acid, pH 6.8, was used. After preincubation for 3 min at
37°C, PNP (1 mM) in dimethyl sulfoxide was added, and the incubations were continued for an additional 10 min. Microsomal proteins were precipitated with perchloric acid (70%, 20 µl) and removed by centrifugation. Quantification of 4-nitrocatechol in the supernatant fraction was determined by HPLC analysis as described previously (Duescher and Elfarra, 1993). Briefly, samples (100 µl) were analyzed using a Beckman System Gold Programmable Solvent Module 126 HPLC, a reverse phase C\textsubscript{18} column (5 µm, 4.6 x 250 mm; Beckman Ultrasphere ODS) and a Beckman System Gold Module 168 UV detector. The isocratic mobile phase was 25% acetonitrile: 75% H₂O:0.1% trifluoroacetic acid with a flow rate of 1.5 ml/min. The column effluent was monitored at 345 nm. Levels of PNP hydroxylase activity were assessed by formation of 4-nitrocatechol, which eluted from the column at 5.5 min, and quantified by relating peak area to a standard calibration curve of known amounts of 4-nitrocatechol.

Lung microsomes from TCE-treated mice were used for measurement of pentoxyresorufin O-dealkylase activity. Reaction mixtures in a total volume of 200 µl potassium phosphate buffer, pH 7.4, contained microsomal protein (0.1 mg), NADPH (1.0 mM) and pentoxyresorufin (2.0 mM), and were incubated for 15 min at 37°C. The reaction was terminated by cooling on ice. A volume of 200 µl of each sample was loaded into a 96-well microplate and read on a fluorescence plate reader (Spectra MAX Gemini XS fluorescent plate reader/Softmax\textsuperscript{®} PRO software), using an excitation wavelength of 535 nm and an emission wavelength of 580 nm. Pentoxyresorufin O-dealkylase activity was determined by the formation of resorufin, and measured by reference to a standard curve containing known amounts of resorufin.
Formation of Chloral Hydrate in Murine and Human Lung Microsomal Incubations. Reaction mixtures in a total volume of 500 µl of 50 mM Tris-HCl buffer, pH 7.4, consisted of lung microsomal protein (0.25 mg) from untreated mice, NADPH (1.0 mM) and TCE (1.0 mM) in acetonitrile (0.4%, v/v). The incubations were performed at 37°C for 0, 20, 30, 45 and 60 min. Reactions were terminated by freezing in liquid nitrogen. Reaction mixtures in the human lung metabolism study contained microsomal protein (1.25 mg), NADPH (1.0 mM) and TCE (4.0 mM) in acetonitrile (0.4%, v/v) in a total volume of 250 µl of 50 mM Tris-HCl buffer, pH 7.4. Incubations were maintained for 20 min at 37°C. All reactions were terminated by addition of 1,3-dibromopropane (6.2 nmol) in 250 µl ethyl acetate.

Detection of Chloral Hydrate by Gas Chromatography. Quantitation of CH in murine and human lung microsomal incubations was performed using the methods described previously (Cummings et al., 2001). Briefly, microsomal proteins from the murine (500 µl) and human (250 µl) lung microsomal incubations were thawed and extracted with ethyl acetate (0.5 ml) after which 1,3-dibromopropane (10 nmol) was added as an internal standard. Analysis for TCE and CH was performed by gas chromatography with electron capture detection, using a Perkin-Elmer PE-210 capillary column (30 m x 0.25 mm I.D. x 0.5 µm film thickness) and a Perkin-Elmer AutoSystem XL GC system. Injector temperature was 200°C, detector temperature was 300°C, and He was the carrier gas at a flow rate of 24.8 ml/min at 150°C. The oven ramp method used for sample elution consisted 11 min at 35°C, a linear gradient from 35 to 120°C from 11 to 19 min, and holding at 120°C from 19 to 38 min. Retention times for TCE and CH
were 3.90 and 6.15 min, respectively. Limits of detection (in pmol/mg protein) were 0.2 and 0.05 for TCE and CH, respectively.

**Statistical Analysis.** The kinetics of CH formation was analyzed using Michaelis-Menten kinetics and GraphPad Prism (Version 4, GraphPad Software, Inc., San Diego, CA).
Results

Kinetic Analysis of CH Formation by Recombinant P450 Enzymes. To establish linearity of reactions of TCE with individual recombinant P450 enzymes, incubations were performed with enzyme concentrations ranging from 2.5 to 25 pmol and incubation times from 0 to 60 min. Controls comprising of incubations carried out in the absence of NADPH or TCE confirmed that formation of CH was absent or minimal. The results of these studies showed that the amounts of CH generated from incubating TCE with the recombinant P450 enzymes were all dependent on enzyme concentrations and incubation times. On the basis of the data obtained, incubations with TCE were performed at 37°C for 20 min, using the following concentrations of recombinant enzymes: rat rCYP2E1 (10 pmol), human rCYP2E1 (15 pmol), rCYP2B1 (10 pmol), rCYP2F4 (5 pmol) or rCYP2F2 (5 pmol). Rates of CH formation by the recombinant enzymes were highly correlated with TCE concentrations (0.05 - 5.0 mM) used in the incubations ($R^2 = 0.8861 – 0.9541$) (Fig. 2). Kinetic constants obtained are summarized in Table 1. Rat rCYP2E1 had the lowest $K_m$ value ($K_m = 14 \pm 3 \mu M$) with rCYP2F4, rCYP2F2, rCYP2B1 and human rCYP2E1 having $K_m$ values that were 5-, 8-, 9- and 14-fold higher, respectively. The calculated ratio of $V_{max}/K_m$ for rat rCYP2E1 was approximately 3-, 7-, 11- and 40-fold higher than the ratios derived for rCYP2F4, rCYP2F2, rCYP2B1 and human rCYP2E1, respectively. Hence, the $K_m$ values and calculated ratios for $V_{max}/K_m$ were consistent with catalytic efficiencies with the following rank order: rat rCYP2E1 > rCYP2F4 > rCYP2F2 > rCYP2B1 > human rCYP2E1.

Protein Immunoblotting. Immunoblots of lung microsomal proteins using the anti-CYP2E1 antibody yielded a single immunoreactive band with an apparent molecular
weight of 51 kDa (Fig. 3A), similar to the molecular mass reported previously (Simmonds et al., 2004a). The amount of immundetectable CYP2E1 was unchanged in lung microsomes from mice sacrificed from 5 to 60 min after TCE treatment. However, 70 and 35% decreases in immunoreactive CYP2E1 protein were observed at 2 and 4 h, respectively (Fig. 3A and 3B). Protein blots probed with the CYP2F1 antibody demonstrated cross-reactivity with murine lung CYP2F2, as reported previously (Simmonds et al., 2004a). A protein band of about 56 kDa was detected and is similar to the molecular mass reported previously for this protein (Simmonds et al., 2004a). Recombinant CYP2F1 baculosomes used as a positive control were recognized by the human anti-CYP2F1 antibody as a single protein band that migrated at a slightly slower rate than the one containing microsomal proteins (data not shown), and is in agreement with the result obtained in previous studies (Simmonds et al., 2004a). Densitometric scanning revealed that, compared to controls, CYP2F2 protein content was decreased by about 75% at 15 min after TCE, was abolished at 30 min and partially recovered at 60 min when CYP2F2 levels amounted to about 60% of the control (Fig. 3D). The anti-CYP2B1 antibody detected a protein band of about 55 kDa (Fig. 3E). Densitometric scanning showed no changes in CYP2B1 protein at 5 to 15 min after TCE treatment (Fig. 3F). However, decreases of 37 to 57% in CYP2B1 protein contents were detected 1 to 4 h after TCE treatment.

**p-Nitrophenol Hydroxylase and Pentoxyresorufin O-Dealkylase Activities.**

Time-course studies revealed marked decreases in lung PNP hydroxylase activity as a result of treatment of mice with TCE. A 30% loss of lung microsomal PNP hydroxylase activity was detected as early as 15 min after TCE treatment, and this was exacerbated to
a 75% reduction by 30 min (Fig. 4A). The nadir was reached at 2 to 4 h when levels of residual PNP hydroxylase activity comprised only about 7-10% of the control level. Thus, there is virtual elimination of hydroxylase activity observed 2 to 4 h after TCE treatment. Treatment of mice with TCE also produced significant decreases in pentoxyresorufin O-dealkylase activity (Fig. 4B). A loss of 33% of dealkylase activity was detected at 5 min after TCE treatment. Further decreases were observed at 15 min to 2 h after treatment, and at 4 h, the levels were reduced to 36% of the control level. Thus, TCE treatment caused decreases of lung microsomal PNP hydroxylase activity that were more pronounced than were observed for pentoxyresorufin O-dealkylase activity.

Formation of Chloral Hydrate in Murine and Human Lung Microsomal Incubations. Using incubations of lung microsomes from untreated mice, the generation of CH from TCE was found to increase from 5 to 45 min, with rates ranging from 3.7 ± 0.6 to 6.7 ± 0.8 nmol/min/mg protein (Fig. 5). No further increase in CH production was observed at 60 min but rather the rates declined. The ability of human lung microsomal proteins to catalyze the formation of CH from TCE was extremely low, with rates ranging from 0.44 to 0.58 pmol/min/mg protein, and was detectable in only three of the eight subjects (Table 2). Formation of CH was not detected in control incubations, which were performed in the absence of NADPH or TCE.
Discussion

Previous studies reported that at least three forms of P450 enzymes were involved in TCE metabolism to CH (Nakajima et al., 1990). A high affinity enzyme present in liver microsomes from control and ethanol-treated rats catalyzed CH formation from TCE. An additional P450 isoform with low affinity for TCE metabolism was induced by phenobarbital, while a form with intermediate affinity was induced by 3-methylcholanthrene. Subsequent studies using inhibitory monoclonal antibodies and rat liver microsomes yielded findings implicating CYP2E1, CYP2B1/2, CYP2C11/6 and CYP1A1/2 in TCE metabolism to CH (Nakajima et al., 1992). However, the inhibitory effects of the antibodies were most pronounced for CYP2E1 and CYP2B1, suggesting relevant contributions of these P450 isoforms to TCE metabolism in rat liver. More recent studies found that formation of chloral and TCE oxide was greater with liver microsomes from rats treated with phenobarbital to induce CYP2B1 than from rats treated with isoniazid to induce CYP2E1 (Cai and Guengerich, 2000). Findings from other studies confirmed that human liver CYP2E1 was also capable of metabolizing TCE, albeit at a diminished level (Guengerich et al., 1991). Although studies of TCE metabolism have been mainly conducted in the liver, similar studies have also been carried out in the lung. The results of studies in the lung showed that mice were more susceptible to TCE-induced toxicity than rats (Odum et al., 1992), and further that this effect was proposed to be due, in part, to higher rates of chloral formation in the former than in the latter (Green et al., 1997). The enhanced rates of chloral formation were attributed to the higher CYP2E1 levels found in mice vs. rats. Relevant in this context are recent findings showing that CYP2F is also present at higher levels in the lungs of
mice than rats (Baldwin et al., 2004). However, the P450 enzymes responsible for lung metabolism of TCE have not been identified and characterized.

An objective of this study was to identify the P450 enzymes involved in TCE metabolism in murine and human lung. An initial approach was to select candidate lung P450 enzymes to determine their relative affinities for TCE metabolism. The enzymes CYP2E1, CYP2F2 and CYP2B1 are all present constitutively in lung tissue and are preferentially localized in the bronchiolar Clara cells (Buckpitt et al., 1995; Forkert, 1995), a cell type that is a selective target of TCE-induced lung cytotoxicity (Forkert et al., 1985). Incubations were performed with rat and human rCYP2E1, rCYP2F2, rCYP2F4 and rCYP2B1 to assess their relative capabilities for TCE metabolism to CH. These studies yielded data to demonstrate that all the recombinant P450 enzymes catalyzed TCE metabolism to CH, with highly positive correlations between TCE concentrations and CH formation (Fig. 2). Kinetic analysis revealed that rat rCYP2E1 exhibited the lowest $K_m$ value and the highest $V_{max}/K_m$ ratio for TCE metabolism to CH (Table 1), indicating that this P450 catalyzed TCE metabolism to CH with relatively high affinity and catalytic efficiency. Our results also showed that rCYP2F4 catalyzed TCE metabolism with greater affinity and catalytic efficiency than did rCYP2F2, rCYP2B1 and human rCYP2E1, which had the highest $K_m$ value and lowest $V_{max}/K_m$ ratio. These findings demonstrated that all the recombinant enzymes metabolized TCE to CH, albeit at differing catalytic affinities and efficiencies.

Although the present studies have clearly demonstrated the abilities of the recombinant enzymes CYP2E1, CYP2F4, CYP2F2 and CYP2B1 to metabolize TCE, the relative contributions of each isoform to in vivo lung metabolism of TCE remain
unknown. In order to address this issue, immunoblotting studies were performed to determine the involvement of CYP2E1, CYP2F2 and CYP2B1 in TCE metabolism in murine lung. The rationale for this approach was based on findings from previous studies indicating that TCE metabolism produces reactive intermediates capable of inactivating P450 enzymes responsible for its bioactivation (Cai and Guengerich, 2001). Time-course experiments were carried out with lung microsomes from mice treated with TCE (750 mg/kg, i.p.) for time periods ranging from 5 min to 4 h. The loss of immunoreactive CYP2E1, CYP2F2 and CYP2B1 proteins were all detected (Fig. 3). The protein content of CYP2F2 was decreased at 15 min after TCE treatment and was abolished by 30 min (Fig. 3C and 3D). On the other hand, the loss of CYP2B1 was manifested at 1 to 4 h (Fig. 3E and 3F), while decreases of the CYP2E1 protein were detected at a later time at 2 to 4 h (Figs. 3A and 3B). The differing time-points at which P450 proteins are lost and recovered precludes the possibility that the decreases are due to death and sloughing of Clara cells, leading to diminished recovery of lung microsomal proteins. These findings suggested that modification of the apoprotein moieties of lung CYP2F2, CYP2E1 and CYP2B1 takes place as a result of TCE treatment. The loss of immunoreactive CYP2E1, CYP2F2 and CYP2B1 proteins (Fig. 3) suggested that these P450 enzymes are inactivated by TCE metabolites at their site of formation. To verify that this inactivation occurs, PNP hydroxylation and pentoxyresorufin O-dealkylation were measured in lung microsomes from TCE-treated mice. Significant decreases in hydroxylase activity were observed from 15 min to 4 h (Fig. 4A). Residual hydroxylase activity detected at 2 to 4 h comprised only about 7-10% of the control level (Fig. 4A). Pentoxyresorufin O-dealkylase activity was reduced from 5 min to 4 h after TCE treatment, with the level
remaining at 4 h comprising 36% of the control. The findings showed that the maximal loss of enzyme activity was higher for PNP hydroxylation (93% of control) than for pentoxyresorufin dealkylation (64% of control). These results are consistent with the greater affinity of rCYP2E1 and rCYP2F for TCE metabolism than rCYP2B1 (Table 1). The lack of concordance between the loss of enzyme catalytic activities and loss of immunoreactive proteins are in agreement with previous studies showing that catalytic deactivation in the case of CYP2E1 preceded a decrease in immunoreactive protein (Ronis et al., 1991). It should additionally be noted that previous studies have reported loss of heme in incubations of TCE with rat liver microsomes (Miller and Guengerich, 1983). Whether the decreased catalytic activity in the lung may be ascribed also to the effect of TCE on P450 heme remains to be investigated. Taken together, the findings of this study supported the view that lung metabolism of TCE causes P450 inactivation, resulting in loss of immunoreactive P450 proteins including CYP2E1, CYP2F2 and CYP2B1 and associated catalytic activities.

In this study, generation of CH in lung microsomal incubations was used as an index of TCE metabolism. Formation of CH was time-dependent, with rates that were incremental from 5 to 45 min and saturation at 60 min (Fig. 5). The maximal rates of CH produced by murine lung microsomes (7.9 ± 0.9 nmol/min/mg protein) were orders of magnitude greater than the rates found for human lung microsomes, which ranged from 0.44 to 0.58 pmol/min/mg protein from three subjects (Table 1). These data are consistent with the kinetic constants demonstrating the markedly lower \( K_m \) and higher \( V_{max}/K_m \) ratios for rat rCYP2E1 vs. human rCYP2E1 (Table 1). The low rate of CH production by human lung microsomes in the three subjects coupled with the lack of
detectability in five other subjects suggested that TCE metabolism in human lung is not an important event. These findings are in agreement with those of previous studies that reported an absence of chloral formation in human lung microsomal incubations (Green et al., 1997). These data suggested that human lung is unlikely to generate a sufficient level of TCE metabolites to elicit a toxic response. However, further studies involving a larger population is required to fully establish the validity of this tentative conclusion.

It is of interest that in previous studies with 1,1-dichloroethylene, a structural analog of TCE that also causes Clara cell toxicity, CYP2E1 and CYP2F2 mediated its metabolism to an epoxide and are both inactivated in the process (Simmonds et al., 2004, a and b). However, it is not known whether CYP2B1 is involved also in the metabolism of 1,1-dichloroethylene, as was found with TCE in this study. Nevertheless, dichloroethylene and TCE may have common pathways of metabolism in lung due to their structural similarities. It is also of interest to compare the disposition of TCE in lung and liver; the findings indicated that the identities of the P450 enzymes responsible for TCE metabolism are not identical in the two tissues. Whereas CYP2E1 and CYP2B1 have important roles in TCE metabolism in the liver (Cai and Guengerich, 2001), as was also found for the lung in this study, CYP2F2, a P450 expressed mainly in the lung, is additionally involved and appears to have a more important role than CYP2B1, as suggested by the results from experiments with the recombinant P450 enzymes. In summary, the results of this investigation have provided evidence to support a pertinent role for CYP2E1, CYP2F2 and CYP2B1 in the metabolism of TCE in murine lung. Moreover, the findings suggested that CYP2E1 may have a more prominent role than either CYP2F2 or CYP2B1.
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ACGIH (2004) Threshold Limit Values and Biological Exposure Indices for Chemical Substances and Physical Agents, American Conference of Governmental Industrial Hygienists, Cincinnati, Ohio.


Footnotes

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FIGURE LEGENDS

FIG. 1. Proposed scheme of TCE metabolism. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase.

FIG. 2. Kinetic analysis of the rates of CH formation in incubations of TCE with rat rCYP2E1 (A), human rCYP2E1 (B), rat rCYP2F4 (C), mouse rCYP2F2 (D) or rat rCYP2B1 (E). Reaction mixtures contained rat rCYP2E1 (10 pmol), human rCYP2E1 (15 pmol), rCYP2F4 (5 pmol), rCYP2F2 (5 pmol) or rat rCYP2B1 (10 pmol), NADPH (2.0 mM) and TCE (0.025 - 5.0 mM), and the incubations were carried out for 20 min at 37°C.

FIG. 3. Immunoblot analysis for CYP2E1 (A), CYP2F2 (C) and CYP2B1 (E) in lung microsomes from mice treated with TCE. The histograms represent relative protein levels of CYP2E1 (B), CYP2F2 (D) and CYP2B1 (F), as assessed by densitometric analysis of the protein bands standardized to Ponceau S staining. Mice were treated with TCE (750 mg/kg, i.p.) and sacrificed at the indicated time-points for preparation of lung microsomes. For each time-point, the first lane contains proteins from control mice and the second lane contains proteins from TCE-treated mice. All lanes were loaded with 40 µg of microsomal proteins.

FIG. 4. Time-dependent effects on rates of PNP hydroxylation (A) and pentoxyresorufin O-dealkylation (B) in lung microsomes from TCE-treated mice. Mice were treated with TCE (750 mg/kg, i.p.) and sacrificed at 5, 15, 30, 60, 120 and 240 min after treatment. PNP hydroxylase activity was determined by HPLC analysis as described in Materials and Methods. Pentoxyresorufin O-dealkylase activity was determined with a 96-well microplate and a fluorescence plate reader, using the method...
described in *Materials and Methods*. Data are expressed as mean ± S.D. of quadruplicate determinations.

**FIG. 5.** Time-dependent formation of CH in incubations of TCE with lung microsomes from untreated mice. Reaction mixtures in a total volume of 500 µl of 50 mM Tris-HCl buffer, pH 7.4, contained microsomal proteins (0.25 mg), NADPH (1.0 mM) and TCE (1.0 mM) in acetonitrile (0.4%, v/v). The incubations were performed at 37°C for 0 to 60 min. Rates of CH formation were determined by gas chromatography using the method described in *Materials and Methods*. Data are expressed as mean ± S.D. of quadruplicate determinations.
TABLE 1

*Kinetic Analysis of Rates of Chloral Hydrate Formation in Incubations of TCE with Recombinant Rat or Human CYP2E1, Recombinant Rat CYP2F4 or Recombinant Rat CYP2B1*

Reaction mixtures contained rat rCYP2E1 (10 pmol), human rCYP2E1 (15 pmol), rat rCYP2B1 (10 pmol), rat rCYP2F4 (5 pmol) or mouse rCYP2F2 (5 pmol), 2.0 mM NADPH and TCE concentrations ranging from 0.25 to 5.0 mM. The incubations consisting of rCYP2F4 or rCYP2F2 additionally contained NADPH-cytochrome P450 reductase (10 units/pmol P450). The incubations in a total volume of 250 µl of 50 mM Tris-HCl buffer, pH 7.4, were carried out at 37°C for 20 min. Control incubations were performed in the absence of NADPH or TCE. Rates of CH formation were determined by gas chromatography as described in *Materials and Methods*. Data (mean ± S.E.M.) were derived from Michaelis-Menten kinetic analysis of quadruplicate determinations.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min/pmol P450)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat rCYP2E1</td>
<td>14 ± 3</td>
<td>11 ± 0.3</td>
<td>0.79</td>
</tr>
<tr>
<td>Human rCYP2E1</td>
<td>196 ± 40</td>
<td>4 ± 0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Mouse rCYP2F2</td>
<td>114 ± 17</td>
<td>13 ± 0.4</td>
<td>0.11</td>
</tr>
<tr>
<td>Rat rCYP2F4</td>
<td>64 ± 9</td>
<td>17 ± 0.5</td>
<td>0.27</td>
</tr>
<tr>
<td>Rat rCYP2B1</td>
<td>131 ± 36</td>
<td>9 ± 0.5</td>
<td>0.07</td>
</tr>
</tbody>
</table>
TABLE 2

*Rates of Formation of Chloral Hydrate in Incubations of Human Lung Microsomes with TCE*

Reaction mixtures in a total volume of 250 µl of 50 mM Tris-HCl buffer, pH 7.4, contained human lung microsomal proteins (1.25 mg), TCE (4.0 mM) and NADPH (1.0 mM), and were incubated for 20 min at 37°C. Rates of CH formation were determined by gas chromatography as described in *Materials and Methods*. Values are expressed as mean ± S.D. of triplicate determinations.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Chloral hydrate (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-55</td>
<td>N.D.</td>
</tr>
<tr>
<td>HP-40</td>
<td>0.44 ± 0.22</td>
</tr>
<tr>
<td>HP-48</td>
<td>0.51 ± 0.24</td>
</tr>
<tr>
<td>HP-54</td>
<td>0.58 ± 0.24</td>
</tr>
<tr>
<td>HP-35</td>
<td>N.D.</td>
</tr>
<tr>
<td>HP-36</td>
<td>N.D.</td>
</tr>
<tr>
<td>HP-69</td>
<td>N.D.</td>
</tr>
<tr>
<td>HP-37</td>
<td>N.D.</td>
</tr>
</tbody>
</table>
Figure 1
Rates of CH Formation (pmol/min/pmol rat 2E1) $R^2 = 0.9541$

Rates of CH Formation (pmol/min/pmol human 2E1) $R^2 = 0.9231$

Rates of CH Formation (pmol/min/pmol rat 2F4) $R^2 = 0.9037$

Rates of CH Formation (pmol/min/pmol mouse 2F2) $R^2 = 0.9481$

Rates of CH Formation (pmol/min/pmol rat 2B1) $R^2 = 0.8861$

Figure 2
Figure 3

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Figure 4
Figure 5