TOXICOLOGY AND APPLIED PHARMACOLOGY **142,** 311–318 (1997)
ARTICLE NO. TO968040

Cytochrome P450-Dependent Metabolism of Trichloroethylene: Interindividual Differences in Humans

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Received July 5, 1996; accepted October 7, 1996

Cytochrome P450-Dependent Metabolism of Trichloroethylene: Interindividual Differences in Humans. LIPSCOMB, J. C., GARRETT, C. M., AND SNAWDER, J. E. (1997). *Toxicol. Appl. Pharmacol.* **142, 311–318.**

Trichloroethylene (TRI) is an industrial solvent with a history of use in anesthesia, and is a common groundwater contaminant. Cytochrome P450 (CYP)-dependent metabolism of TRI produces chloral hydrate (CH) and is rate limiting in the ultimate production of trichloro- and/or dichloroacetic acid from TRI. Exposure of rodents to TRI results in lung and liver tumors (mice) and nephrotoxicity (rats). The toxicity is exacerbated by pretreatment of mice with CYP inducers. We report significant variability in TRI metabolism in a sample of 23 human hepatic microsomal samples and demonstrate the dependence of TRI metabolism on CYP2E1. K_m values in this limited sample population are not normally distributed. We have correlated microsomal CH formation with the activity toward routine CYP2E1 substrates and with immunologically detectable CYP2E1 protein. Further, TRI metabolism in microsomes from lymphoblastoid cell lines expressing CYP2E1, CYP1A1, CYP1A2, or CYP3A4 indicated minimal involvement of the latter forms, with CYP2E1 catalyzing more than 60% of total microsomal TRI metabolism. These results indicate that humans are not uniform in their capacity for CYP-dependent metabolism of TRI and increased CYP2E1 activity may increase susceptibility to TRI-induced toxicity in the human.

The widespread use of trichloroethylene (TRI) as a solvent and degreaser in industrial as well as commercial products has led to the appearance of measurable amounts of this agent in air and ground and surface waters. Because of this high potential for exposure, TRI was evaluated in the rodent bioassay for cancer. TRI exposure has been demonstrated to produce lung and liver tumors in mice (Forkert *et al.*, 1985; Fukuda *et al.*, 1983; NCI, 1976; NTP, 1983) and renal toxicity/carcinoma in rats (Maltoni *et al.*, 1988; NTP, 1983, 1987). The classification of TRI as a carcinogen is currently being revised by the U.S. Environmental Protection Agency.

The toxicity of TRI is dependent upon metabolism and the induction of cytochrome P450 (CYP) increases toxicity (Carlson, 1974; Cornish and Adefuin, 1966; Moslen *et al.*, 1977; Nakajima *et al.*, 1988; Okino *et al.*, 1991). TRI is metabolized through chloral hydrate (CH) to compounds including trichloroacetic acid (TCA) and dichloroacetic acid (DCA), which alter intercellular communication (Klaunig *et al.*, 1989), induce peroxisome proliferation (DeAngelo *et al.*, 1989; Nelson *et al.*, 1989), and promote tumor production (Daniel *et al.*, 1992; Herren-Freund *et al.*, 1987). This step is catalyzed by CYP2E1 (and other forms) in rodents (Nakajima *et al.*, 1990, 1993) and is rate-limiting in the further production of TCA (Ikeda *et al.*, 1980). Because differences in the rates of metabolism of another CYP2E1 substrate, benzene (Seaton *et al.*, 1994), influence its toxicity (Kenyon *et al.*, 1996; Seaton *et al.*, 1995), we have evaluated the degree to which this step varies in a sample set of 23 human hepatic microsomal fractions.

These data indicate that CYP2E1 is the predominant form responsible for TRI metabolism in the human, as it is in the rodent species; this finding will aid in the identification of populations of humans who may be at increased risk of toxicity following exposure to TRI (Barton *et al.*, 1996; Raucy, 1995). In addition, we report that CYP1A and CYP3A forms may be involved in TRI metabolism in the human. Because these data further detail the capacity and variability of human biotransformation of TRI, they can be used in the reevaluation of the human health risk assessment for TRI.

MATERIALS AND METHODS

Chemicals. All chemicals were at least reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI) unless otherwise noted. Alkoxyresorufins were obtained from Molecular Probes (Eugene, OR). Glucose-6-phosphate and $NADP^+$ were obtained from Boehringer-Mannheim (Indianapolis, IN), and ketoconazole was purchased from Janssen Biotech (Olen, Belgium).

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0041-008X/97 $25.00

Microsome preparation and metabolism of TRI. Human microsomes obtained from a commercial supplier (International Institute for the Advancement of Medicine, Exton, PA) were prepared via the method of Guengerich (1989). Microsomal protein content was determined by the BCA method with bovine serum albumin as a standard. Total CYP was determined using the differential spectrophotometric method of Omura and Sato (1964). Some incubations contained CZX (100 μM) and 0.9 mM NADPH. Additionally, microsomes prepared from human lymphoblastoid cell lines selectively expressing CYP1A1, CYP1A2, CYP2E1, and CYP3A4 were incubated at concentrations of 42, 42, 22, and 96 pmol CYP/ml, respectively (see Shimada *et al.*, 1994), with TRI and NADPH-regenerating system.

Metabolism of TRI. Incubations were carried out with microsomal protein, NADPH-regenerating system, and TRI in 0.1 M TRIS, 0.005 M $MgCl_2$, pH 7.4, the optimal pH for the reaction (Ikeda *et al.*, 1980; Leibman, 1965). TRI was dissolved in acetone to final concentrations which allowed the introduction of acetone vehicle into microsomal incubations at concentrations of acetone up to 0.1% final volume. Previous experiments demonstrated that this concentration of acetone did not inhibit DMN metabolism (not shown). CH and TCOH in samples were extracted with 4 vol ethyl acetate and 1-μl aliquots were analyzed by GC-ECD. Ethyl acetate extract was injected by a Hewlett–Packard 7673 liquid autosampler onto a Hewlett–Packard Model 5890 GC equipped with a Vocol capillary column (0.53 mm $\times$ 30 m) and interfaced with a ^{63}Ni ECD. Gas (95/5 argon/methane) flow was 46 ml/min. Authentic TCOH and CH were dissolved in ethyl acetate at known concentrations and served as external standards, against which experimental samples were quantified. Extraction efficiency was determined to be 100% for CH and 95% for TCOH and was not concentration-dependent.

CYP form-specific activities. CYP form-specific activities of CYP1A1/1A2, CYP3A, and CYP2B1/2B2 were estimated by determining the O-dealkylation of alkoxyresorufin substrates (ethoxy-EROD, benzoxy-BROD, and pentoxy-PROD, respectively) by the method of Burke *et al.* (1994). Alkoxy resorufin assays were conducted at substrate concentrations of 2.0 μM. CYP2E1-dependent, *p*-nitrophenol hydroxylase (PNP-OH, 100 μM; Reinke and Moyer, 1985) or *N*-dimethylnitrosamine *N*-demethylase (DMN, 1.0 mM; Matsubara *et al.*, 1977) activities, CYP1A2-dependent phenacetin *O*-deethylase (PAD, 1.0 mM) and CYP3A-dependent erythromycin *N*-demethylase (ERY, 1.0 mM) were assayed as previously described (Snawder *et al.*, 1994). Chlorzoxazone (CZX) metabolism was assayed by the method of Peter *et al.* (1990). All reactions were initiated by the addition of an NADPH-regenerating system (in 0.1 M potassium phosphate buffer) containing 14 mmol of glucose-6-phosphate, 0.66 mmol of $NADP^+$, and 3 units of glucose-6-phosphate dehydrogenase.

Electrophoresis of proteins and Western blot analyses. Human liver microsomal fractions were separated by electrophoresis on 12% SDS–polyacrylamide gels as described by Laemmli (1970) and electroblotted onto nitrocellulose membranes. Conditions of Western blot analysis were as described by Towbin *et al.* (1979). The immunodetection of protein bands on the nitrocellulose membranes was carried out as described previously using the appropriate primary antibodies to different CYP forms and secondary antibodies conjugated to alkaline phosphatase. The relative band intensities on the immunoblots were scanned and quantified using a Scanmaster 3 reflecting densitometer and Master Scan Version 3.1 analysis software (Scananalytics, Billerica, MA).

Enzyme kinetics and statistical analysis. Michaelis–Menten kinetic data of the enzyme activities were analyzed using EnzFitter (Elsevier Biosoft, Ferguson, MO) on an IBM personal computer. Data were evaluated by Student's *t* test and analysis of variance with post hoc evaluation of differences by Student–Newman–Keuls test ($p \leq 0.05$). The tests were performed using a SAS General Linear Models program (SAS Institute, Cary, NC) or by SigmaStat (Jandel Scientific, San Rafael, CA) on an IBM personal computer.

RESULTS

Microsomal incubations containing 0.5 mg microsomal protein and 3.9 to 125 μM TRI yielded hyperbolical plots consistent with Michaelis–Menten kinetics. The distribution of kinetic parameters, especially K_m values (Table 1), appeared skewed. Statistical analysis indicated that the K_m values were

TABLE 1
Kinetic Parameters for Microsomal TRI Metabolism in 23 Human Samples

Sample	Cigarettes (ppd)	Ethanol	K_m (μM TRI)	V_{max} (pmol/min/mg)
46 C F	0.5	Social	16.1	714
35 C F	nr	nr	15.2	909
55 C F	nr	nr	18.8	3309
55 A F	nr	nr	12.6	490
63 P F	nr	nr	16.6	908
50 C F	nr	nr	17.7	1113
22 H F	nr	nr	13.3	1724
52 C M	2.5	Heavy	17.3	1039
43 C M	nr	Heavy	19.6	1432
36 C M	nr	nr	19.67	825
		Mean:	16.7[A]	1246[D]
		SD:	2.45	805
50 C F	Yes	nr	28.6	1422
46 H M	1	Moderate	29.9	1746
60 C F	nr	nr	33.37	1004
25 C M	nr	Yes	27.9	943
38 C M	2	nr	31.2	1627
24 H M	nr	Social	35.0	1416
43 H M	nr	Previous	28.92	2353
51 C M	nr	Yes	26.95	890
24 C M	nr	nr	36.28	1584
		Mean:	30.9[B]	1442[D]
		SD:	3.33	464
47 C M	1	Social	55.7	2078
60 C M	2.5	Occasional	52.1	2623
55 C F	nr	nr	46.7	2936
40 C M	nr	Yes	50.0	3455
		Mean:	51.1[C]	2773[E]
		SD:	3.77	577
All Data		Mean:	28.3	1589
		SD:	12.9	840
		Normality test:	Fail	Pass

Note. Samples are identified by age in years, ethnic background, sex, where C, Caucasian; A, Asian; P, Filipino; H, Hispanic; M, Male; F, Female. Cigarette smoking is reported in packs per day (ppd) when quantified. Ethanol consumption was subjectively reported; nr, a negative response was given for cigarettes and/or ethanol. Kinetic data were derived from analysis of Michaelis–Menten kinetics of TRI metabolism from individual human liver microsomal samples performed at six substrate concentrations (3.9 to 125 μM). Saturation was evident in all analyses. Means of K_m and V_{max} values for each group were compared by ANOVA; means with the same letter are not significantly ($p \leq 0.05$) different.

not normally distributed. Data were subjectively divided into three populations (K_m 12–20 μM; K_m 26–37 μM; and $K_m > 46$ μM TRI) and analysis of variance (ANOVA) was performed. Results revealed significant differences between the three populations (Table 1). Further, K_m values for TRI metabolism were significantly lower (21.9 ± 3.5 μM, $n = 10$) in females than in males (33.1 ± 3.5 μM, $n = 13$). Although V_{max} values generally correlated well with increasing K_m, analysis of V_{max} data revealed a normal distribution ($p = 0.053$). The four samples comprising the high-K_m group (group 3) displayed significantly higher V_{max} values than either of the other two groups. There was no significant difference in V_{max} values for TRI (1452 ± 300 pmol/min/mg for females vs 1693 ± 213 pmol/min/mg for males, mean ± SEM).

To determine whether the differences in TRI metabolism in our sample of human microsomes could be dependent upon total CYP content of microsomal samples, cytochrome CYP content of samples within each group was assessed. Analysis of variance revealed no significant differences between CYP content of the groups. However, TRI metabolism (pmol/min/nmol CYP) significantly differed between the groups (Table 2). These data suggest that differences in TRI metabolism in the human should be attributed to differences in some factor beyond variations in total microsomal CYP content. V_{max} values for TRI metabolism were not significantly different between males and females, but the mean K_m value in females (21.9 ± 3.5 μM) was significantly lower than that in males (33.1 ± 3.5 μM, mean ± SEM).

To assess dependence of TRI metabolism on individual CYP forms, we performed a regression analysis of TRI metabolism against results from the incubation of these samples with standard substrates. Figure 1 depicts the relationships between TRI metabolism and activity of CYP1A, CYP2E1, and CYP3A4. Correlations of TRI metabolism with EROD activity (CYP1A), PAD activity (CYP1A2), DMN *N*-demethylase and CZX activities (CYP2E1), and ERY activity (CYP3A4) are given in the figure legend.

To confirm and extend the correlation of TRI metabolism with CYP2E1 and other CYPs, microsomal samples were subjected to polyacrylamide gel electrophoresis and Western blotting using antibodies selective for several CYP forms. The correlation of TRI V_{max} values and absorbance intensities of CYP1A, CYP2E1, and CYP3A4 forms was assessed by linear regression. Results in Fig. 2 demonstrate a high degree of correlation between TRI metabolism and CYP2E1 intensity, but not CYP1A intensity.

To demonstrate the dependence of TRI metabolism on CYP2E1 in the human, we evaluated the impact of CZX on TRI metabolism. Human microsomes ($n = 3$) from the low K_m group incubated with CZX (100 μM) and TRI (30 μM) exhibited activity which was 54% (± 25% SD) of control activity. Analysis of Michaelis–Menten parameters of microsomes from the low-K_m group ($n = 3$) revealed that 100 μM CZX significantly reduced V_{max} values from 1123 (±304 SD) to 646 (±205 SD) pmol/min/mg ($p = 0.016$). No significant change in K_m values was observed. Neither ketoconazole nor α-napthoflavone inhibited TRI metabolism (30 μM TRI) in low-K_m samples. However, the incubation of a mid-K_m microsomal sample with ketoconazole or α-napthaflavone decreased V_{max} from 1422 to 1166 and to 992 pmol/min/mg, respectively.

To determine the contribution of specific CYP forms to TRI metabolism, incubations were constructed to include 1.0 mg microsomal protein or the equivalent content of specific forms (42 pmol 1A forms, 22 pmol 2E1, and 96 pmol 3A4) based on data presented in Shimada *et al.* (1994). Results presented in Fig. 3 indicate that CYP2E1 accounts for 81% of TRI metabolism (in low-K_m human microsomes) when assessed at 16 μM TRI, and that this contribution is reduced to 63% when assessed at 100 μM TRI. A separate analysis of TRI metabolism by microsomes from a lymphoblastoid cell line specifically expressing CYP2E1 indicated a K_m of approximately 12 μM TRI and a V_{max} of approximately 540 pmol/min/22pmol CYP2E1.

To determine the variability of CYP2E1-dependent activity in a larger sample of human microsomes, CZX activity was plotted against total CYP content of microsomes. Re-

TABLE 2
Cytochrome P450 Content and TRI Metabolism in Human Microsomes

	Cytochrome P450 content[a]			TRI metabolism[b]		
Group	Range	Mean	SD	Range	Mean	SD
Low K_m (10)	0.21–0.71	0.408[A]	0.140	1567–7695	3370[A]	2070
Mid K_m (9)	0.26–0.73	0.463[A]	0.173	1731–5867	3435[A]	1445
High K_m (4)	0.32–0.45	0.383[A]	0.064	6703–7678	7216[B]	449
All samples (23)	0.21–0.73	0.420	0.147	1567–7695	4064	2174

[a] Cytochrome P450 content is presented as nmol CYP/mg microsomal protein as determined by the method of Omura and Sato (1964).

[b] TRI metabolism is presented as maximal rates, pmol TRI/min/nmol total CYP, (*n*) per group. Groups were assigned based on data presented in Fig. 1 and Table 1. Means with the same letter are not significantly different by ANOVA.

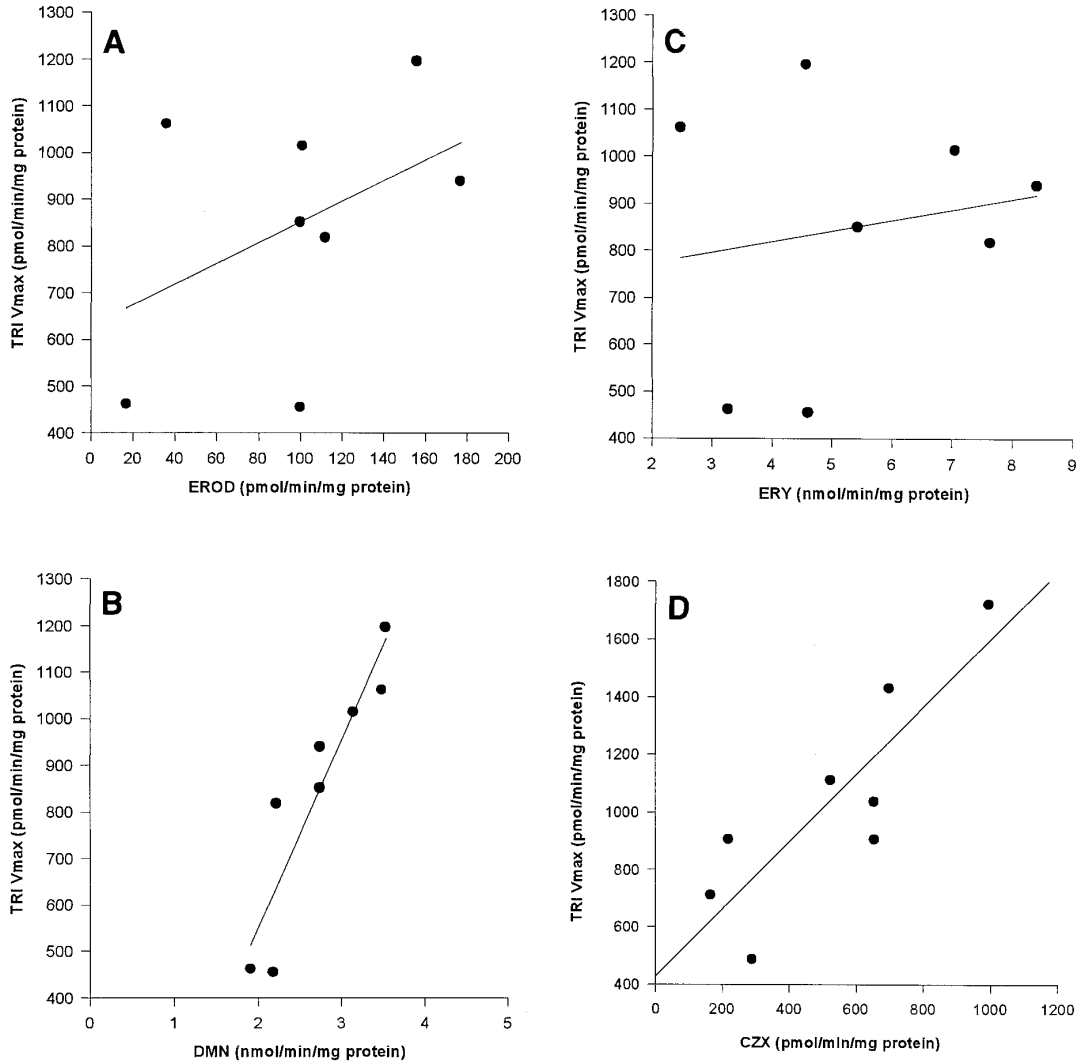
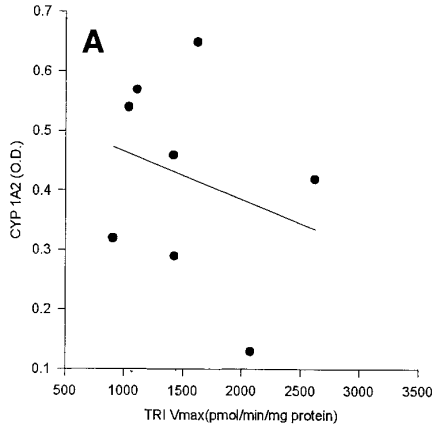


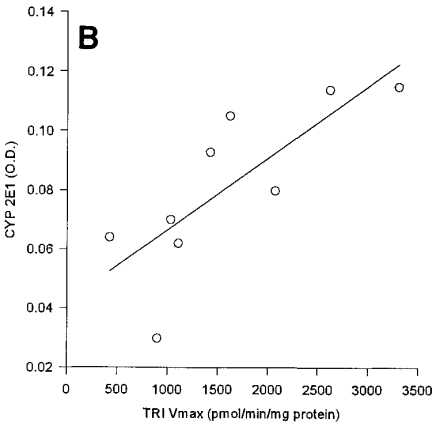
FIG. 1. Simple linear regression of CYP form-selective activities against TRI metabolism. (A) CYP1A2 activity toward ethoxyresorufin (r^2 = 0.19), (B) CYP2E1 activity toward dimethylnitrosamine (r^2 = 0.84*), (C) CYP3A4 activity toward erythromycin (r^2 = 0.03), (D) CYP2E1 activity toward chlorzoxazone (r^2 = 0.51*). Additional data (not shown) indicated that TRI metabolism was significantly correlated (r^2 = 0.46* and 0.71*) with CYP1A2 activity toward phenacetin and CYP2E1 activity toward *p*-nitrophenol, respectively. *Significant correlation ($p \leq 0.05$).

sults indicate an approximate 10-fold variability in CYP content/mg microsomal protein and CZX activity (pmol/min/mg microsomal protein). To determine whether microsomal total CYP content variation could account for the range of CZX activities we expressed CZX metabolism in microsomal samples (n = 54) per unit microsomal protein and per unit CYP content. Activity per unit protein was 0.291 to 2.743 nmol CZX/min/mg microsomal protein, spanning a 9.4-fold range, and activity per unit CYP was 0.65 to 11.0 nmol CZX/min/nmol CYP, spanning a 16.9-fold range. These data indicate that CYP content alone cannot be responsible for differences in CZX metabolism. When data were grouped (n = 3 or 4) according to CYP content (nmol CYP/mg microsomal protein), it was demonstrated that the average variability of CZX activity (pmol/min/mg microsomal protein) was more than 2.5-fold for a given microsomal CYP content (Table 3). Individual regression analysis of CZX activity/nmol CYP and CZX activity/mg microsomal protein against CYP content revealed correlation coefficients of 0.052 and 0.015, respectively, indicating that total CYP content (0.30 to 0.51 nmol/mg) is not related to CZX metabolism.

DISCUSSION

We have demonstrated the contribution of CYP2E1 to TRI metabolism in the human. Although limited by sample size, we demonstrated that the activity of CYP2E1 varies





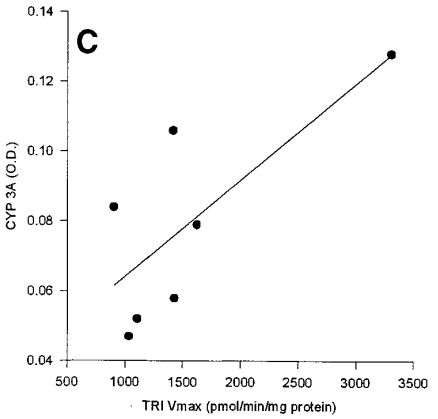


FIG. 2. Simple linear regression of absorbance units from Western blot analysis against microsomal TRI metabolism. (A) Immunodetectible CYP1A2 ($r^2 = 0.07$), (B) immunodetectible CYP2E1 ($r^2 = 0.61$*), and (C) immunodetectible CYP3A ($r^2 = 0.56$*). *Significant correlation ($p \leq 0.05$).

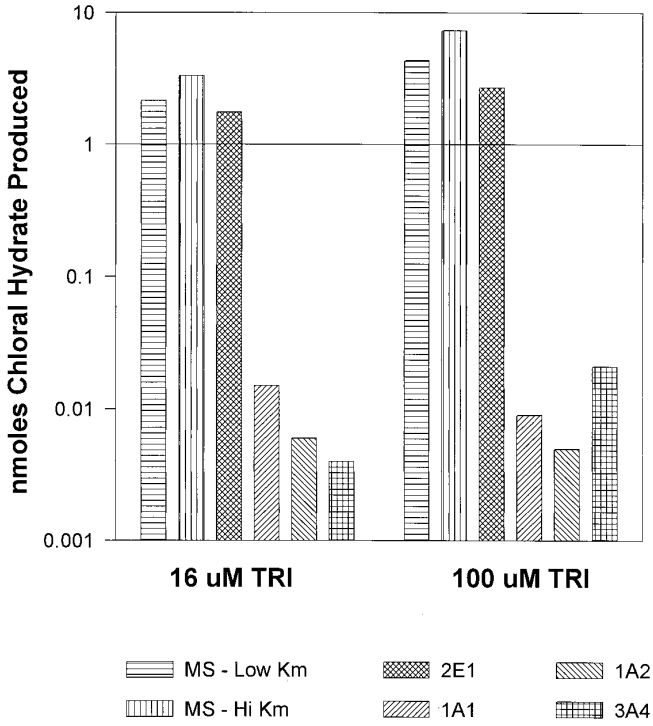


FIG. 3. Metabolism of TRI by human microsomes and genetically expressed CYP forms. Assays contained 1.0 mg microsomal protein/ml or the equivalent content of genetically expressed protein based on reported content (Shimada *et al.*, 1994).

less than 10-fold among our 23 human samples. We observed no significant sex-dependent differences in activities toward the CYP2E1 substrate, CZX, but TRI K_m values were significantly higher in males. The further analysis of K_m values for TRI indicates that these samples can be easily divided into three groups, although the statistical power of the analysis is low. The demonstration of statistically different K_m and V_{max} values between the low-K_m and high-K_m groups indicates that humans vary appreciably in their capacity for TRI metabolism, but this variability is well within 10-fold. We have shown that while K_m values can be separated into three statistically distinct populations, and that there are significant differences in V_{max} values among these groups, there is no difference in spectrally determined total microsomal cytochrome P450 content among the groups. Samples expressing identical spectrally determined total microsomal cytochrome P450 contents varied more than 3-fold in CZX activity. The relationship between mean K_m and V_{max} values among the three groups invites consideration. The partition coefficient for TRI (human blood:air value of 8.1; Gargas *et al.*, 1989) predicts a blood concentration of 16 μM at equilibrium with an atmosphere containing TRI at the TLV (50 ppm). The application of this concentration to determine rates in the low- and high-K_m group predicts rates which differ by only 10%. Although the human samples vary con-

TABLE 3
Variation of Chlorzoxazone Metabolism within Eight Groups of Human Microsomes [a]

CYP content [b]	nmol/min/mg protein		nmol/min/nmol CYP		Magnitude of difference [c] (fold)
	Mean	Range	Mean	Range	
0.30	0.987	0.781–1.659	3.29	1.74–5.53	3.18
0.31	0.751	0.468–1.359	2.42	1.37–4.38	3.20
0.33	0.697	0.419–0.921	2.11	1.27–2.79	2.20
0.35	0.255	0.649–1.644	4.70	1.85–4.70	2.54
0.42 (n = 4)	1.141	0.656–1.600	2.72	1.56–3.81	2.25
0.43	0.736	0.512–0.996	1.71	1.19–2.32	1.95
0.45	1.234	0.656–1.946	2.72	1.46–4.32	2.96
0.51 (n = 4)	0.627	0.335–1.031	1.32	0.65–2.02	3.11
				Mean:	2.67
				SD:	0.50

[a] Microsomes were selected based on total CYP content. Each sample within the group had exactly the same CYP content. Three samples per group (unless n, specified otherwise).
[b] CYP content is expressed as nmoles of total cytochrome CYP/mg microsomal protein.
[c] Magnitude of difference is expressed as maximal activity divided by minimal activity.

siderably in V_{max} assessed *in vitro,* it is unlikely that these individuals would express significantly different rates of TRI metabolism during an occupational exposure, even at TLV concentrations.

Our assessment of genetically engineered human CYP2E1 indicates that this form accounts for more than 60% of TRI metabolism, even under saturating conditions. The apparent K_m for CYP2E1 is in agreement with the average K_m of the low-K_m human population. These results confirm the significant contribution of CYP2E1 to TRI metabolism in the human. Because of the substrate specificity of CYP2E1, the modulation of CYP2E1 content and activity by hereditary and environmental factors, and the frequency of encountering TRI, the conditions which modulate CYP2E1 activity should be considered as modifying factors in TRI toxicity. Like other CYP2E1 substrates such as acrylonitrile, acetaminophen, and carbon tetrachloride, susceptibility to TRI-induced toxicity may also be increased by modulating CYP2E1 activity (Prasad *et al.*, 1985; Sipes *et al.*, 1973; Subramanian and Ahmed, 1995).

While a positive correlation was observed for PAD activity and TRI metabolism; these studies failed to correlate TRI metabolism with CYP1A2 protein or EROD activity in human liver microsomes. It is likely that the correlation of TRI metabolism with CYP1A2 protein and EROD activity in microsomes from human liver are overshadowed by the significant involvement of CYP2E1 in the metabolism. Together, we interpret these data to indicate that CYP1A2 metabolizes TRI, but the overall contribution is low.

The involvement of CYP3A4 is also unclear. Ketoconazole, a substrate for CYP3A4, reduced V_{max} appreciably in a sample from the mid-K_m group, and the correlation of TRI metabolism with immunological detection of CYP3A4 is significant. Genetically engineered microsomes expressing CYP3A4 did form chloral hydrate, but at rates much lower than by CYP2E1. These rates increased approximately fivefold when the TRI concentration was increased from 16 to 100 μM, a finding consistent with the CYP3A4 being a low-affinity enzyme for TRI metabolism.

Because of the apparent dependence of TRI-associated toxicity on metabolites arising from CYP-dependent CH formation (e.g., TCA and DCA), metabolism of TRI by CYP should be regarded as a crucial step in TRI bioactivation. The assessment of this reaction in a sample of human microsomes has revealed statistically significant differences, especially with respect to K_m values. Our data indicate that maximal rates of TRI metabolism by human microsomes *in vitro* vary less than 10-fold. Because of the consistency in metabolite pathway and CYP form involvement between rodents and humans, it is possible that interindividual and interspecies differences in CYP2E1 content and activity may influence TRI's toxicity. This report has clearly demonstrated that differences in the capacity of human microsomes to metabolize TRI include differences in affinity as well as V_{max}. The overriding factor seems to be the degree of expression of CYP2E1. For these reasons, factors which increase the expression/activity of CYP2E1 should be included in an evaluation of TRI toxicity.

Although genetic screening of individuals for chemical risk may seem a distant option, the assessment of enzymatic activity in biopsy samples may provide a useful diagnostic tool. Quantifiable species-dependent differences in bioactivation of TRI and the identification of factors contributing to interindividual differences in TRI metabolism will aid the explanation and the extrapolation of rodent toxicity data to the human and thereby refine the human health risk assessments for TRI.

ACKNOWLEDGMENTS

This work was supported in part by the Strategic Environmental Research and Development Program, and work was conducted in part through a contract with GEO-Centers, Inc. This study was made possible and greatly enhanced by the efforts of U.S. Air Force Trichloroethylene Project Director Jay Kidney and Senior Staff Scientist Jeff Fisher. Preliminary findings were presented at the Society of Toxicology Meetings in 1995 and 1996.

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