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COMPARATIVE METABOLISM OF BIS(2-METHOXYETHYL) ETHER BY RAT AND HUMAN HEPATIC MICROSOMES: FORMATION OF 2-METHOXYETHANOL*

M. A. TIRMENSTEIN

Department of Health and Human Services, Public Health Service, Centers for Disease Control, National Institute for Occupational Safety and Health, Division of Biomedical and Behavioral Science, Cellular Toxicology Section, 4676 Columbia Parkway, Cincinnati, OH 45226, USA

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Abstract—Rat hepatic microsomes catalysed the NADPH-dependent cleavage of the central ether linkage of bis(2-methoxyethyl) ether (diglyme) yielding 2-methoxyethanol (2ME). Microsomes isolated from phenobarbital- or ethanol-pretreated rats exhibited an increased capacity to cleave diglyme to 2ME. This ethanol-induced increase in 2ME formation was not observed if incubations contained the cytochrome *P*450IIE1 inhibitor isoniazid. Pretreatment of rats with diglyme significantly increased microsomal *P*-450 levels, *P*-450-associated enzyme activities and the conversion of diglyme to 2ME. Following the diglyme pretreatment, an almost 30-fold increase in pentoxyresorufin dealkylase activity (*P*450IIB1/2) was evident in rat hepatic microsomes. Human hepatic microsomes also catalysed the NADPH-dependent cleavage of diglyme to 2ME. The formation of 2ME from diglyme correlated with the aniline hydroxylase activity (*P*450IIE1) levels measured in human hepatic microsomes. Studies using microsomes isolated from a cell line transfected with specific human *P*-450 cDNAs indicate that human CYP2E1 catalyses the conversion of diglyme to 2ME. These results suggest that the central ether linkage of diglyme is cleaved by rat and human *P*-450 and the specific involvement of hepatic *P*450IIE1 in this process is implicated.

INTRODUCTION

Bis(2-methoxyethyl) ether (CAS Reg. No. 111-96-6; diethylene glycol dimethyl ether; diglyme) is a member of the glycol ether class of chemicals. These compounds are used extensively in industry as solvents and emulsifiers. In addition to these uses, diglyme has industrial applications as an anhydrous reaction medium for organometallic syntheses. Several studies have demonstrated that diglyme is toxic to laboratory animals. Testicular (Cheever *et al.*, 1989b; Lee *et al.*, 1989) and developmental (Daniel *et al.*, 1991; Price *et al.*, 1987) toxicity has been reported following the administration of diglyme to rodents. Daily oral administration of diglyme to rats at a dose of 684 mg/kg body weight produced primary and secondary spermatocyte degeneration after 6–8 days (Cheever *et al.*, 1989b). In studies conducted with pregnant CD-1 mice (Price *et al.*, 1987), oral administration of diglyme at a dose of 500 mg/kg body weight over a period of 10 days was associated with a post-implantation loss of 50% per litter and a 96% incidence of malformed live foetuses per litter. In addition, there is recent evidence that diglyme may be a developmental toxin in humans. Exposure to diglyme has been linked to an increased rate of miscarriages in women employed in the computer chip industry (*Environmental Health Letter*, 1992).

In a previous study from this laboratory, Cheever *et al.* (1988) demonstrated that diglyme was metabolized to 2-methoxyethanol (2ME) and 2-methoxyacetic acid (2MAA) following oral administration to rats. These products originated from the cleavage of the central ether linkage of diglyme and were excreted in the urine. Once 2ME is formed, it can be further metabolized to 2MAA by the enzymes alcohol and aldehyde dehydrogenase. The total amount of 2ME and 2MAA excreted accounted for approximately 8% of the administered dose of diglyme after 96 hr. Studies also indicate that the percentage of diglyme metabolized to 2ME and 2MAA could be increased by prior treatment of rats with phenobarbital (PB) or diglyme (Cheever *et al.*, 1989a).

2ME has been demonstrated to be an immunotoxicant (Smialowicz *et al.*, 1991), a teratogen (Sleet *et al.*, 1988) and a testicular toxicant (Moss *et al.*, 1985) in laboratory animals. In each of these studies, evidence suggests that 2ME must be oxidized to 2MAA in order for toxicity to occur. Evidence also suggests that 2ME formation and its subsequent

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Abbreviations: β-NF = β-naphthoflavone; cyt = cytochrome; diglyme = bis(2-methoxyethyl) ether; EDTA = ethylenediaminetetraacetic acid; 2MAA = 2-methoxyacetic acid; 2ME = 2-methoxyethanol; PB = phenobarbital; *P*-450 = cytochrome *P*-450.

metabolism to 2MAA may account for the toxicity of diglyme (Cheever *et al.*, 1988; Daniel *et al.*, 1991).

The present study was conducted to characterize further the metabolism of diglyme to 2ME and to gain more information on the potential risks of this compound to humans. Microsomes isolated from animals pretreated with cytochrome *P*-450 inducers were used in addition to inhibitors of specific *P*-450 isozymes in an attempt to identify which isozymes are involved in 2ME formation. In addition, human hepatic microsomes and microsomes isolated from cell lines transfected with human *P*-450 genes were assessed for their capacity to cleave the central ether linkage of diglyme and yield the product 2ME.

MATERIALS AND METHODS

Chemicals. Diglyme and 2ME were purchased from Fluka Chemical Corp. (Ronkonkoma, NY, USA). Radiolabelled diglyme [1,2-*ethylene*-^{14}C]bis (2-methoxyethyl) ether with a specific activity of 29.8 mCi/mmol was acquired from Chemsyn Science Laboratories (Lenexa, KS, USA). 2-Ethoxyethanol and 2-(2-methoxyethoxy)ethanol were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Heptafluorobutyrylimidazole was obtained from Pierce Chemical Co. (Rockford, IL, USA), and pentoxyresorufin was purchased from Molecular Probes Inc. (Eugene, OR, USA). Triacetyloleandomycin (TAO) was a generous gift from Dr John Ostrosky of Roerig, a division of Pfizer Inc. (New York, NY, USA). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Animals. Male Sprague–Dawley rats were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA, USA). PB was administered by ip injection at a dose of 100 mg/kg/day for 3 days before they were killed. β-Naphthoflavone (β-NF), suspended in corn oil, was injected ip for 3 days at a dose of 80 mg/kg/day. Rats were given drinking water containing 15% (v/v) ethanol for 3 days before the isolation of microsomes. Diglyme pretreatment consisted of providing animals with drinking water containing 0.6% (w/v) diglyme for 4 days. Based on average consumption of the diglyme solution, rats were given a daily dose of diglyme of about 700 mg/kg body weight during the course of the induction. All diglyme and ethanol drinking water solutions were prepared fresh daily and provided *ad lib.* Animals used in these experiments weighed between 160 and 230 g when they were killed.

Microsomes. Rats were asphyxiated with carbon dioxide. Livers were immediately excised, rinsed and then homogenized in ice-cold 0.25 M sucrose containing 0.125 M Tris–HCl (pH 7.4) and 1 mM ethylenediaminetetraacetic acid (EDTA). Homogenates were centrifuged at 15,000 *g* for 15 min at 4°C, and microsomes were pelleted from the resulting supernatant by centrifuging at 100,000 *g* for 1 hr. Microsomes were washed to remove cytosolic contamination by resuspending the pellet in 0.05 M potassium phosphate buffer (pH 7.4) and centrifuging at 100,000 *g*. Human hepatic microsomes were acquired from Human Biologics, Inc. (Phoenix, AZ, USA). Microsomes isolated from the human B-lymphoblastoid AHH-1 cell line and expressing human *P*-450s were purchased from Gentest Corp. (Woburn, MA, USA). These microsomal preparations were assayed for specific *P*-450-catalysed enzyme activities, and in most cases, the measured specific activities are within the range expressed by human hepatic microsomal preparations. All microsomes were stored at −80°C before use. Microsomal incubations were conducted at 37°C in 0.05 M potassium phosphate (pH 7.4), 5 mM $MgCl_2$ buffer and contained 1 mM diglyme. The NADPH-generating system consisted of the following: 10 mM glucose-6-phosphate, 1.3 U glucose-6-phosphate dehydrogenase and 0.5 mM $NADP^+$. Blanks contained no $NADP^+$. When radiolabelled diglyme was added to the incubations, the final specific activity was diluted to 6 mCi/mmol. Unless otherwise indicated, all diglyme incubations contained 1 mg microsomal protein and were terminated after 30 min by the addition of phosphoric acid to a final concentration of 55 mM.

Biochemical assays. Pentoxyresorufin dealkylase activity was measured according to the procedures of Lubet *et al.* (1985). NADPH-cytochrome (cyt) *c* (*P*-450) reductase activity was measured as described previously (Williams and Kamin, 1962) except that 0.1 mM EDTA was added to the assay buffer. Incubations of aniline hydroxylase activity and ethoxycoumarin deethylase activity were performed in 0.05 M potassium phosphate (pH 7.4), 5.0 mM $MgCl_2$ buffer and contained the NADPH-generating system described for diglyme incubations. The 30-min aniline hydroxylase incubations contained 5.0 mM aniline. The product of aniline hydroxylase activity, *p*-aminophenol, was measured as described by Ko *et al.* (1987). Ethoxycoumarin deethylase activity was assayed according to the procedures of Greenlee and Poland (1978) as modified by Guengerich (1978). *P*-450 content was determined spectrophotometrically (Omura and Sato, 1964). Protein levels were measured according to Lowry *et al.* (1951) as modified by Peterson (1977), with bovine serum albumin used as a standard.

Analytical methods. Metabolites of radiolabeled diglyme were analysed by a previously described HPLC method (Cheever *et al.*, 1988). Following addition of phosphoric acid, samples were centrifuged for 10 min at 14,000 *g*. The supernatant was filtered and injected on an HPLC equipped with a TRACE II radioactivity monitor (Packard Instrument Co.). The identity of metabolites was determined by comparing their retention times to that of authentic standards generated in a previous study (Cheever *et al.*, 1988).

2ME levels were determined by spiking incubation mixtures with 2 nmol 2-ethoxyethanol (internal

CH_3-O-CH_2-CH_2-O-CH_2-CH_2-O-CH_3
(a)

CH_3-O-CH_2-CH_2-O-CH_2-CH_2-OH
(b)

CH_3-O-CH_2-CH_2-OH
(c)

Fig. 1. Structures of (a) diglyme and its rat hepatic microsomal metabolites (b) 2-(2-methoxyethoxy)ethanol and (c) 2-methoxyethanol (2ME).

standard) and extracting incubations three times with two volumes of ethyl acetate. The ethyl acetate was dried over anhydrous $MgSO_4$, evaporated under N_2 to near dryness, and then derivatized with 10 μl heptafluorobutyrylimidazole for 15 min at room temperature. Reactions were terminated by the addition of 100 μl water. Derivatives were extracted with 200 μl benzene and aliquots of the benzene layer were injected on a gas chromatograph with an electron capture detector and a SPB-5 capillary column (30 m × 0.53 mm i.d.; 1.5 μm film thickness) (Supelco Inc., Bellefonte, PA, USA). Helium was used as a carrier gas and P-5 was used as a detector make-up gas. Samples were injected using the splitless mode of injection on a column maintained at 65°C. After 10 min, the temperature was increased at a rate of 2°/min to 80°C and then raised at a rate of 15°C/min to 250°C. The identity of the heptafluorobutyryl ester of 2ME was confirmed by gas chromatography–mass spectrometry performed on a VG MicroMass 7070HS mass spectrometer. Electron impact mass spectra were acquired at an electron energy of 70 eV and at a source temperature of 220°C.

Statistical analysis. One-way analysis of variance was performed using the Statgraphics, version 5 (STSC, Inc., Rockville, MD, USA) statistical package. Differences between untreated controls and specific treatments were determined using Scheffe's test for multiple comparisons. Least square linear regression and the Student's t-test were performed to evaluate the strength of association between two variables.

RESULTS

NADPH-dependent metabolism of diglyme by rat hepatic microsomes

Diglyme metabolites were determined using radiolabelled diglyme and HPLC analysis. Three major metabolites were detected in rat hepatic microsomal incubations containing an NADPH-generating system. Two of the metabolites were identified as 2ME and 2-(2-methoxyethoxy)ethanol (Fig. 1). The identity of the third metabolite was not determined. Analysis of incubations containing rat hepatic microsomes from untreated rats, an NADPH-generating system and 1 mM diglyme indicate that less than 2% of the added diglyme was metabolized after 30 min.

Effects of P-450 inducers on 2-methoxyethanol formation

The effects of several standard *P*-450 inducers on rat hepatic microsomal *P*-450 reductase and *P*-450-associated enzyme activities are shown in Table 1. As expected, aniline hydroxylase activity was increased over two-fold following ethanol pretreatment. Previous studies have shown that aniline hydroxylase activity is associated with *P*450IIE1 (Lucas *et al.*, 1990), the major ethanol-inducible form of *P*-450. Pretreatment of animals with PB significantly increased the activities of NADPH-cyt *c* reductase, ethoxycoumarin deethylase and pentoxyresorufin dealkylase. Pentoxyresorufin dealkylase activity is a marker for *P*450IIB1/IIB2 (Lubet *et al.*, 1985). β-NF pretreatment produced a nine-fold increase in ethoxycoumarin deethylase activity but did not significantly increase the other *P*-450 associated enzyme activities measured. Guengerich *et al.* (1982) also found large increases in ethoxycoumarin deethylase activity in rat hepatic microsomes following β-NF induction. The results reported in Table 1 confirm that the standard induction protocols used were effective in inducing characteristic *P*-450 enzyme activities.

Treatment of rats with drinking water containing diglyme before they were killed significantly increased microsomal levels of *P*-450 ($P < 0.05$). *P*-450 levels increased by 70% above control values to 1.79 ± 0.09

Table 1. Effects of diglyme and *P*-450 inducers on NADPH-cyt *c* reductase and *P*-450-associated enzyme activities in rat hepatic microsomes†

Treatment	Enzyme activity (nmol product formed/min/nmol *P*450)			
	NADPH-cyt *c* reductase	Aniline hydroxylase	Ethoxycoumarin deethylase	Pentoxyresorufin dealkylase
Untreated	43.1 ± 1.9	0.54 ± 0.03	0.44 ± 0.05	0.014 ± 0.002
Ethanol	43.6 ± 2.0	1.28 ± 0.06*	1.56 ± 0.04*	0.010 ± 0.001
Phenobarbital	56.7 ± 2.7*	0.50 ± 0.02	1.49 ± 0.10*	0.376 ± 0.029*
β-Naphthoflavone	28.8 ± 2.3*	0.46 ± 0.02	3.95 ± 0.23*	0.009 ± 0.001
Diglyme	36.6 ± 1.3	0.41 ± 0.01	1.12 ± 0.04*	0.426 ± 0.033*

†Rats were pretreated with the indicated inducers before the hepatic microsomes were isolated and assayed for enzymatic activity.

Values represent the mean ± SEM of six determinations, and those marked with asterisks differ significantly (one-way analysis of variance) from the controls (*$P < 0.05$)

Table 2. Effects of *P*-450 inducers on the metabolism of diglyme to 2-methoxyethanol (2ME) by rat hepatic microsomes†

Treatment	2ME formed per 30-min incubation period	
	nmol 2ME/mg protein	nmol 2ME/nmol *P*-450
Untreated	2.8 ± 0.2	2.7 ± 0.1
Ethanol	11.5 ± 0.3*	11.2 ± 0.5*
Phenobarbital	13.2 ± 0.9*	6.7 ± 0.3*
β-Naphthoflavone	3.3 ± 0.1	1.9 ± 0.1
Diglyme	7.2 ± 0.9*	4.1 ± 0.6

†Rats were pretreated with the indicated inducers before the microsomes were isolated and assayed for diglyme cleavage to 2ME. Incubations contained 1 mM diglyme, 1 mg protein and an NADPH-generating system. Incubations were conducted at 37°C.

Values represent the mean ± SEM of six determinations, and those marked with asterisks differ significantly (one-way analysis of variance) from the controls (*$P < 0.05$).

(mean ± SEM, n = 6) nmol *P*-450/mg protein. Diglyme pretreatment also significantly increased *P*-450-associated enzyme activities (Table 1). Statistically significant increases were noted in the specific activity levels of ethoxycoumarin deethylase and pentoxyresorufin dealkylase following the diglyme pretreatment. A comparison of the treatment groups in Table 1 suggests that the pattern of induction of *P*-450 associated enzyme activities produced by diglyme pretreatment most resembles that produced by PB. Only the diglyme and PB pretreatments gave dramatic increases in pentoxyresorufin dealkylase activity levels.

Pretreatment of animals with either ethanol or PB augmented the metabolism of diglyme to 2ME catalysed by rat hepatic microsomes (Table 2). Both the ethanol- and PB-induced increases were determined to be significant regardless of whether the amount of 2ME formed was expressed per mg protein or per nmol *P*-450. β-NF induction did not significantly affect the metabolism of diglyme to 2ME.

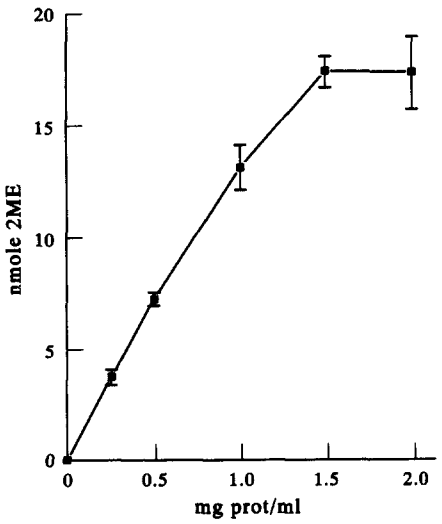

Fig. 2. Protein-dependent formation of 2ME. Phenobarbital-induced rat hepatic microsomes were incubated for 30 min with 1 mM diglyme and an NADPH-generating system at 37°C. Each point is the mean of three determinations ± SEM.

Pretreatment of animals with diglyme also significantly ($P < 0.05$) increased the capacity of microsomes to convert diglyme to 2ME. Following the diglyme induction protocol, microsomes incubated for 30 min produced 7.2 nmol 2ME/mg of protein (Table 2). This represents a 157% increase above that seen with control microsomes from untreated rats. However, the increase in 2ME formation observed with these microsomes was not judged to be significant when values were normalized per nmol *P*-450 because of the diglyme-induced increases in *P*-450 levels.

Time- and protein-dependent formation of 2-methoxyethanol

The effects of incubation time on the cleavage of diglyme to 2ME by PB-induced rat hepatic

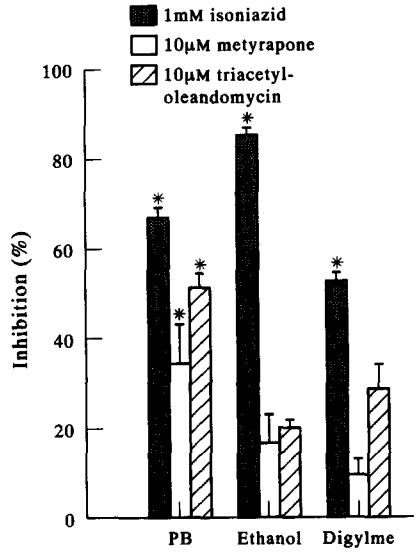

Fig. 3. Effects of *P*-450 isozyme inhibitors on the cleavage of diglyme to 2ME. Animals were pretreated with phenobarbital (PB), ethanol or diglyme. Following pretreatments, animals were killed and microsomes were isolated. Microsomes were assayed for NADPH-dependent cleavage of diglyme to 2ME. The indicated inhibitors were preincubated with microsomes at 37°C for 5 min. Values are expressed as the percentage inhibition produced by each inhibitor with the indicated induced microsomes. *Statistically significant ($P < 0.05$; one-way analysis of variance).

Table 3. Aniline hydroxylase activity in human hepatic microsomes

Subject no.	Age, sex, history	Cause of death	*P*-450 content (nmol/mg protein)	Aniline hydroxylase activity*
1	57 yr, F	Stroke	0.38	0.29
2	53 yr, F, smoker, diuretics	Stroke	0.72	1.06
3	33 yr, M, heavy alcohol use, cocaine	Gunshot	0.31	0.88
4	41 yr, M	Gunshot	0.73	0.66
5	42 yr, M	Head injury	0.28	0.45
6	51 yr, F, smoker, darvocet, voltaren	Stroke	0.47	0.48
7	45 yr, M, smoker, heavy alcohol use	Head injury	0.29	0.60
8	50 yr, F, heavy smoker	Stroke	0.84	0.37

*Values expressed as nmol *p*-aminophenol formed/min/nmol *P*-450. All values are the mean of duplicate determinations (SEM was 10% or less in all cases). Mean value ± SEM = 0.60 ± 0.09 (n = 8) for pooled results from all subjects.

microsomes were examined. 2ME formation was time dependent, and measured rates of formation for 2ME were similar over the first 30 min of the experiment (about 0.5 nmol/min). After 30 min, rates of 2ME formation appeared to diminish slightly with increasing incubation times.

The amount of diglyme cleaved to 2ME during 30-min incubations with PB-induced microsomes was also dependent on the concentration of microsomal protein present in the incubation media (Fig. 2). Increasing concentrations of microsomal protein produced proportionally greater amounts of 2ME up to about 1 mg protein/ml. Additions of microsomal protein above 1.5 mg/ml did not increase the amount of 2ME formed during the 30-min incubations.

The effects of P-450 inhibitors on 2-methoxyethanol formation

Figure 3 shows the effects of inhibitors of *P*-450 isozymes on diglyme cleavage to 2ME. Metyrapone is an inhibitor of PB-induced *P*-450s. Lubet *et al.* (1985) demonstrated that about 80% of pentoxyresorufin dealkylase activity assayed in rat hepatic microsomes from Aroclor-1254-induced animals was inhibited by 10 μM metyrapone. TAO is a potent and specific inhibitor of *P*450IIIA catalysed activities and forms an inactive substrate enzyme complex with this enzyme (Wrighton *et al.*, 1985). A concentration of 10 μM TAO was used by Sinclair *et al.* (1991) to inhibit microsomal *P*450IIIA- catalysed propoxycoumarin depropylase. Isoniazid is purported to be a type II inhibitor of *P*450IIE1 (Persson *et al.*, 1990). In their study, it was determined that 1 mM isoniazid reduced NADPH oxidization by purified rat *P*450IIE1 by 70%.

All three inhibitors significantly reduced the cleavage of diglyme to 2ME catalysed by PB-induced microsomes. Isoniazid produced a 67% inhibition of 2ME formation in PB-induced microsomes, while metyrapone and TAO produced inhibitions of 34 and 51%, respectively. Only isoniazid significantly reduced the cleavage of diglyme to 2ME catalysed by microsomes isolated from ethanol- or diglyme-pretreated rats. Isoniazid inhibited 2ME formation by 85% with ethanol-induced microsomes and by 53% with microsomes isolated from diglyme-pretreated rats.

Metabolism of diglyme by human hepatic microsomes

Human hepatic microsomes obtained from eight subjects were assayed for aniline hydroxylase activity (Table 3). The mean value for the amount of aniline metabolized/min/nmol *P*-450 for humans was 0.60 (Table 3). Heavy alcohol use was reported in the case histories of two of the subjects listed in Table 3 (nos 3 and 7). The aniline hydroxylase activities measured in microsomes isolated from these subjects were equal to or greater than the mean but were not substantially elevated over the other measured values.

Human hepatic microsomes catalysed the NADPH-dependent cleavage of diglyme to 2ME (Table 4). In addition to 2ME, 2-(2-methoxyethoxy)ethanol and an unknown metabolite were also identified in human microsomal incubations containing radiolabelled diglyme. Formation of these products was demonstrated to be NADPH-dependent. As was shown previously, these same products were also identified with rat hepatic microsomes containing an NADPH-generating system. A mean value of 19.7 nmol 2ME/min/nmol *P*-450 was determined for the 30-min incubations with human microsomes (Table 4).

Table 4. Metabolism of diglyme to 2-methoxyethanol (2ME) by human hepatic microsomes*

	2ME formed per 30-min incubation period	
Subject no.	nmol 2ME/mg protein	nmol 2ME/nmol *P*-450
1	7.7	20.3
2	27.0	37.6
3	5.4	17.3
4	9.6	13.1
5	6.8	24.4
6	7.6	16.2
7	6.1	20.9
8	6.5	7.7

*Microsomal incubations contained 1 mg protein, 1 mM diglyme and an NADPH-generating system. Incubations were conducted at 37°C.

All values are the mean of duplicate determinations (SEM was 12% or less in all cases). Mean values ± SEM are 9.6 ± 2.5 mol/min/mg protein and 19.7 ± 3.1 nmol/min/nmol *P*-450 for pooled results from all subjects.

A test of the association between the aniline hydroxylase activity (nmol/min/mg protein) of human microsomes and the amount of 2ME (nmol/mg protein) formed by these microsomes during a 30-min incubation yielded a correlation coefficient of 0.8658. In a similar manner, the combined aniline hydroxylase and the 2ME results from untreated and ethanol-, PB-, diglyme- and β-NF-pretreated rats yielded a correlation coefficient of 0.7127. The association between these two variables for human and rat hepatic microsomes was statistically significant ($P < 0.01$).

Human P-450 isozyme involvement in 2-methoxyethanol formation

Microsomes isolated from a human B-lymphoblastoid cell line transfected with various human *P*-450 cDNAs were screened for their capacity to convert diglyme to 2ME. Microsomes isolated from cells transfected with cDNAs and encoding human CYP1A1, 1A2, 2A6, 2B6 or 3A4 failed to cleave diglyme to 2ME in 2-hr incubations containing an NADPH-generating system (less than 0.1 nmol 2ME/mg protein). However, microsomes isolated from cells transfected with human CYP2E1 cDNA catalysed the NADPH-dependent cleavage of diglyme to 2ME. Following a 2-hr incubation, 2ME levels were measured at 0.9 ± 0.0 nmol/mg protein (mean ± SEM). This corresponds to a value of about 90 nmol 2ME formed/nmol CYP2E1.

DISCUSSION

The results of the present study implicate *P*-450 in the cleavage of the central ether linkage of diglyme and the formation of 2ME. Several lines of evidence support this conclusion. Metabolism of diglyme to 2ME by microsomes was NADPH-dependent and the pretreatment of rats with the *P*-450 inducers PB and ethanol significantly increased the microsomal metabolism of diglyme to 2ME. In addition, experiments indicate that the formation of 2ME from diglyme is dependent on the concentration of microsomal protein present in the incubation media and the duration of the incubation. Increasing concentrations of microsomal protein, up to about 1 mg protein/ml, resulted in a linear increase in the amount of 2ME formed. This relationship is consistent with a microsomal protein, such as *P*-450, catalysing the cleavage of the central ether linkage of diglyme to 2ME. Finally, pretreatment of microsomes with *P*-450 inhibitors decreased the amount of 2ME formed.

Ethanol is known to induce *P*450IIE1 levels in both humans and laboratory animals (Koop, 1992). In recent years, numerous low molecular weight solvents have been identified as substrates for *P*450IIE1. Benzene, aniline, ethanol, chloroform, acetone and diethyl ether have all been reported to be substrates for this *P*-450 isozyme (Guengerich and Shimada, 1991; Koop, 1992). Aniline hydroxylase activity has been used as a marker for *P*450IIE1 levels (Lauriault *et al.*, 1992). In the present study, ethanol pretreatment yielded a greater than two-fold increase in aniline hydroxylase activity. This same ethanol induction of rats also significantly increased the cleavage of diglyme to 2ME by hepatic microsomes isolated from these animals. Isoniazid, an inhibitor of *P*450IIE1 (Persson *et al.*, 1990), reduced the levels of 2ME formed by ethanol-induced microsomes to below those catalysed by control microsomes. In addition, microsomes from cells transfected with human CYP2E1 cDNA catalysed the formation of 2ME from diglyme. Of the *P*-450 isozymes tested, only CYP2E1 catalysed the formation of measurable amounts of 2ME. Correlation coefficients indicate that a significant positive correlation exists between aniline hydroxylase activity and 2ME formation for rat and human hepatic microsomes. These experiments suggest that the central ether linkage of diglyme is cleaved by both the human and rat orthologues of *P*450IIE1 and that agents which induce *P*450IIE1 levels can increase the cleavage of diglyme to 2ME.

In addition to ethanol, pretreatment of animals with both diglyme and PB was found to significantly increase the NADPH-dependent cleavage of diglyme to 2ME. These findings agree with those obtained *in vivo* by Cheever *et al.* (1989b). In this study, pretreatment of rats with PB or diglyme nearly doubled the total urinary excretion of 2ME and 2MAA (the product of 2ME oxidation by alcohol and aldehyde dehydrogenases). PB is known to induce a number of *P*-450 isozymes in the rat including *P*450IIB1/2 and *P*450IIIA (Gonzales *et al.*, 1986). *P*450IIB1/2 is the major *P*-450 isozyme induced by phenobarbital in the rat and is inhibited by metyrapone (Lubet *et al.*, 1985). Results indicate that metyrapone inhibited the 2ME formation by PB-induced microsomes by 34%. TAO, a specific inhibitor of *P*450IIIA (Wrighton *et al.*, 1985), reduced 2ME formation by 51% in PB-induced microsomal incubations. Isoniazid significantly inhibited the cleavage of diglyme to 2ME in microsomes isolated from diglyme- and PB-treated animals. Since PB induction is known to repress *P*450IIE1 (Johansson *et al.*, 1988), these results are consistent with isoniazid, at the concentrations used in this experiment, inhibiting other *P*-450 isozymes in addition to *P*450IIE1. Collectively, the inhibition experiments suggest that other isozymes of *P*-450 in addition to *P*450IIE1 may be involved in the conversion of diglyme to 2ME especially with PB-induced rat hepatic microsomes.

The addition of diglyme to the drinking water of rats yielded significant increases in hepatic microsomal *P*-450 levels. Significant increases were also observed in the specific activity levels (per mg protein) of *P*-450-associated enzyme activities. Pentoxyresorufin dealkylase activity, a marker for *P*450IIB1/2 (Lubet *et al.*, 1985), was increased about 30-fold following the diglyme pretreatment. This large increase was not seen with any of the other inducers

except PB. These findings indicate that diglyme, or some metabolite of diglyme, is a *P*-450 inducer and, like PB, is a strong inducer of *P*450IIB1/2. The diglyme induction results are strikingly similar to those found for other ether compounds which are cleaved by *P*-450. The ether linkage of diethyl ether (Brady *et al.*, 1988) and methyl *tert*-butyl ether (Brady *et al.*, 1990) have been shown to be cleaved by *P*450IIE1, but pretreatment with diethyl ether yielded only small increases in *P*450IIE1 or no change in the amount of this isozyme in the case of methyl *tert*-butyl ether. However, both compounds were determined to be strong inducers of *P*450IIB1 and produced large increases in pentoxyresorufin dealkylase activity.

The results of the present study indicate that human hepatic microsomes metabolize diglyme to 2ME. Although the results with human microsomes displayed more variability than that found with rats, there was evidence that human hepatic microsomes were more effective in catalysing the conversion of diglyme to 2ME than rat hepatic microsomes. The mean value for 2ME formation with human microsomes was seven times that observed with microsomes from untreated rats and nearly twice the value from ethanol pretreated animals (nmol/nmol *P*-450). This disparity was not found when rat and human hepatic microsomal aniline hydroxylase activity levels were compared. The mean value for aniline hydroxylase activity in human hepatic microsomes was similar to that measured for untreated control rats.

In summary, the results from this study suggest that the central ether linkage of diglyme is cleaved by ethanol, PB and diglyme inducible forms of *P*-450 in the rat. In particular, the involvement of *P*450IIE1 rat and human orthologues in the conversion of diglyme to 2ME is implicated. These findings may be relevant in assessing the health risks associated with diglyme exposures in the workplace. Prior exposure of humans to ethanol, industrial solvents or other inducers of CYP2E1 may increase the metabolism of diglyme to 2ME and thereby enhance the toxicity of diglyme. In addition, further research is needed to determine if diglyme or one of its metabolites is a *P*-450 inducer in humans as it appears to be in rats.

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