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SHORT COMMUNICATION

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Role of cytochromes P450 in the metabolism of methyl *tert*-butyl ether in human livers

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Abstract Methyl *tert*-butyl ether (MTBE) is widely used as a gasoline oxygenate for more complete combustion in order to reduce the air pollution caused by motor vehicle exhaust. The possible adverse effects of MTBE on human health is a major public concern. However, information on the metabolism of MTBE in human tissues is lacking. The present study demonstrates that human liver is active in metabolizing MTBE to *tert*-butyl alcohol (TBA), a major circulating metabolite and a marker for exposure to MTBE. The activity is localized in the microsomal fraction (125 ± 11 pmol TBA/min per mg protein, $n = 8$) but not in the cytosol. This activity level in human liver microsomes is approximately one-half of the value in rat and mouse liver microsomes. Formation of TBA in human liver microsomes is NADPH-dependent, and is significantly inhibited by carbon monoxide (CO), an inhibitor of cytochrome P450 (CYP) enzymes, suggesting that CYP enzymes play a critical role in the metabolism of MTBE in human livers. Both CYP2A6 and 2E1 are known to be constitutively expressed in human livers. To examine their involvement in MTBE metabolism, human CYP2A6 and 2E1 cDNAs were individually co-expressed with human cytochrome P450 reductase by a baculovirus expression system and the expressed enzymes were used for MTBE metabolism. The turnover number for CYP2A6 and 2E1 was 6.1 and 0.7 nmol TBA/min per nmol P450, respectively. The heterologously expressed human CYP2A6 was also more active than 2E1 in the metabolism of two other gasoline ethers, ethyl *tert*-butyl ether (ETBE) and *tert*-amyl methyl ether (TAME). Although the contributions of other human CYP forms to MTBE metabolism remain to be determined, these results strongly suggest that CYP enzymes play an important role in the metabolism of MTBE in human livers.

Abbreviations *MTBE* Methyl *tert*-butyl ether · *ETBE* ethyl *tert*-butyl ether · *TAME* *tert*-amyl methyl ether · *TBA* *tert*-butyl alcohol · *TAA* *tert*-amyl alcohol · *CYP* cytochromes P450 · *GC* gas chromatography · *CO* carbon monoxide

Key words Methyl *tert*-butyl ether · Metabolism · Human liver microsomes · Cytochromes P450

Introduction

To reduce the production of carbon monoxide (CO) and other pollutants in motor vehicle exhaust during wintertime, methyl *tert*-butyl ether (MTBE) and other ethers, such as ethyl *tert*-butyl ether (ETBE) and *tert*-amyl methyl ether (TAME), are added to gasoline as oxygenates for more complete combustion. Among them, MTBE is the most widely used. The use of MTBE has significantly increased since 1992 in areas with severe air pollution. Currently, approx. 20% of the gasoline sold in the United States contains 2 to 15% MTBE, and the use of MTBE and other ethers as oxygenates is expected to increase over the next decade (Costantini 1993).

The possible toxic effects of MTBE exposure in humans have received increasing attention. In November 1992, shortly after the introduction of MTBE in wintertime into oxygenated fuel in Fairbanks, Alaska, reports of illnesses attributed to exposure to the oxygenated fuel started. The major symptoms reported included headache, nausea or vomiting, burning sensation of the nose or mouth, coughing, dizziness, disorientation, and eye irritation (Middaugh 1992, 1993). Subsequent studies by the Centers for Disease Control and Prevention (CDC) and White et al. showed that in both Alaska and Stamford, Connecticut, an area also par-

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ticipating in the wintertime oxygenated fuel program, persons with higher blood levels of MTBE had more complaints of symptoms (CDC 1993a; White et al. 1995). Although the currently available information does not provide sufficient evidence to demonstrate that exposure to MTBE results in an increase in symptoms, there may be subpopulations with greater susceptibility to MTBE.

The blood levels in exposed people of MTBE and *tert*-butyl alcohol (TBA), a major circulating metabolite and a suitable marker for MTBE exposure, as well as the pharmacokinetics data have been reported (Clayton Environmental Consultants 1991; Prah et al. 1994; Johanson et al. 1995; Cain et al. 1996). Previously, Brady et al. from this group reported the metabolism of MTBE by rat liver microsomes in which the involvement of cytochrome P450 (CYP) enzymes was implicated (Brady et al. 1990). However, the metabolism of MTBE in human tissues and the enzymes involved are unknown. This information is important in our understanding of the health effects of MTBE in humans, and is critical in assessing the human relevance of the pharmacokinetics and toxicity data obtained from animal studies. In the present paper, we examined the metabolism of MTBE to TBA in human liver microsomes and by human CYP2A6 and 2E1.

Materials and methods

MTBE (99.8% pure) and TBA (99.5% pure) were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and $NADP^+$ were from Sigma Chemical Co. (St. Louis, Mo.). All other chemical used were of reagent grade and obtained from standard supplies. Human liver samples were from liver cancer patients (37 to 80 years old) and provided by the Cancer Institute of New Jersey affiliated Tissue Retrieval and Distribution Service (New Brunswick, N.J.). Prior to cryosurgery, these patients signed a consent form permitting the research use of their resected tissues. The tissue collection procedures were pre-approved by the Institutional Review Board. The samples were noncancerous neighboring tissues of the liver tumors and were of normal morphology. Samples were snap frozen in liquid nitrogen within 30 min of surgical removal to assure freshness, transferred to our laboratory in liquid nitrogen, and stored at −80 °C prior to use.

Male Sprague-Dawley rats at 10 weeks of age were obtained from Taconic Farms (Germantown, N.Y.) and female A/J mice at 7 weeks of age were from Jackson Laboratory (Bar Harbor, Me.). After sacrifice, the livers of these untreated animals were immediately removed and stored at −80 °C prior to the preparation of cytosol and microsomes. Liver microsomes and cytosolic fractions were prepared from the human and animal samples by differential centrifugation (Hong and Yang 1985). The protein content was determined by the method of Lowry et al. (1951).

Human CYP2A6 and 2E1 enzymes were obtained by expressing the corresponding cDNAs in a baculovirus expression system as previously described (Pattern and Koch 1995). For co-expression of the CYP enzymes and the human cytochrome P450 reductase, Sf9 insect cells were infected simultaneously either with the CYP2A6 and the reductase recombinant viruses, or with the CYP2E1 and the reductase recombinant viruses. Microsomes from the infected Sf9 cells were prepared by a brief sonication followed by a centrifugation at 40 000 rpm. P450 content and the P450 reductase activity were determined as previously described (Patten and Koch 1995).

Incubations for MTBE metabolism were performed according to our previous report (Brady et al. 1990). The incubation mixture (0.4 ml final volume) contained 50 mM TRIS-HCl (pH 7.4), 10 mM $MgCl_2$, 150 mM KCl, an NADPH-generating system (0.4 mM $NADP^+$, 10 mM glucose 6-phosphate, 0.2 Unit glucose-6-phosphate dehydrogenase), liver microsomes or cytosol, or the microsomes prepared from the infected Sf9 cells, and 1 mM MTBE. The amount of sample protein included in the assay was 600 μg (microsomes) or 1.5 mg (cytosol). Incubation was carried out in a sealed headspace vial and the reaction was initiated by injecting MTBE into the solution. After a 30 min incubation, the reaction was terminated with 25% $ZnSO_4$ followed by saturated $Ba(OH)_2$. Formation of TBA was determined by a headspace gas chromatography (GC) method as previously described (Brady et al. 1990) with slight modifications. A Perkin-Elmer model 8500 gas chromatograph was used with a Carbopack B/5% Carbowax 20M stainless steel column ($1/8'' \times 6'$) and a HS-101 headspace autoinjector. The carrier gas was helium and the flow rate was 20 ml/min. The injector and flame ionization detector were at 160 °C, while the oven temperature was 60 °C. The same incubation and GC conditions were used for analyzing the metabolism of ETBE and TAME by human CYP2A6 and 2E1, in which the formation of TBA (also a metabolite of ETBE) and of *tert*-amyl alcohol (TAA, a metabolite of TAME) was determined respectively.

Results and discussion

A representative gas chromatogram is shown in Fig. 1. Under our analytical conditions, the retention time of MTBE and TBA was 5.21 and 6.77 min, respectively. All of the eight human liver microsomal samples were found to be active in metabolizing MTBE to TBA: the activities (pmol/min per mg protein) ranged from 86 to 175 with an average activity of 125 (Table 1). In collaboration with Dr Li-Dong Wang (Henan Medical University, China), we obtained two noncancerous resected human livers from China and determined the TBA formation activity in the liver microsomes. The activity level (167 and 221 pmol/min per mg protein) was similar to that of the American samples. In com-

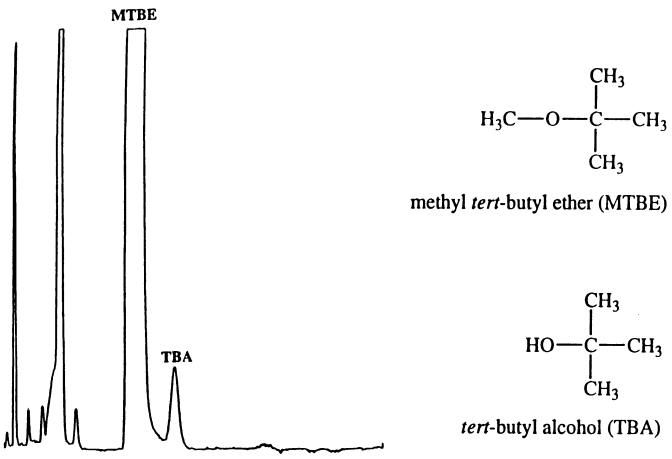

Fig. 1 Headspace GC analysis of MTBE metabolism and the structures of MTBE and TBA. Human liver microsomes (600 μg protein) were incubated with 1 mM MTBE at 37 °C for 30 min. Formation of TBA was determined by headspace GC as described in the Materials and methods

parison to human liver microsomes, the level of MTBE metabolizing activity in rat and mouse liver microsomes is approx. twofold higher (Table 1). TBA is not a substrate of alcohol dehydrogenase and has been thought of as a 'non-metabolizable' alcohol, although some reports indicated the production of various products from TBA metabolism (Costantini 1993). Incubation of rat liver microsomes with 0.1 mM TBA did not result in detectable loss of TBA during the incubation period (Brady et al. 1990).

In contrast to the human liver microsomes, there was little TBA formation when the human liver cytosol fractions were incubated with MTBE, although a 2.5-fold higher protein concentration was used in the incubation (Table 1). This subcellular localization of the activity in metabolizing MTBE to TBA was also demonstrated in the rat and mouse livers, suggesting that CYP enzymes could be responsible for the catalyzed reaction. Encoded by different genes, CYP enzymes are a family of hemoproteins, which play a vital role in the biotransformation of a variety of xenobiotics and endogenous compounds (Nelson et al. 1996). In the liver cells, CYP enzymes are mainly localized in the microsomal fraction, specifically the endoplasmic reticulum. It is well documented that the CYP-catalyzed reactions require NADPH and are susceptible to CO inhibition (Lu and West 1980). To further establish the role of CYP enzymes in the metabolism of MTBE, we selected three human liver microsome samples for determining the effects of NADPH and CO on TBA formation. When the incubation was carried out in the absence of an NADPH-generating system, the microsomal activity in metabolizing MTBE to TBA was not detectable. Bubbling the incubation mixture with 95% CO for 3 min prior to initiation of the reaction with MTBE caused an 80.5% reduction in TBA formation (23 ± 8 vs 116 ± 26 pmol/min per mg protein). Together these results provide strong evidence to support our hypothesis that the metabolism of MTBE in human liver is catalyzed by CYP enzymes.

We next examined the roles of two individual human CYP enzymes, 2A6 and 2E1, in the metabolism of MTBE, using the enzymes obtained from a baculovirus expression system. Both CYP2A6 and 2E1 are constitutively expressed in human livers and catalyze the biotransformation of many important environmental chemicals and drugs (Pelkonen and Raunio 1995; Yang et al. 1990). CYP2E1 is involved in the metabolism of low molecular weight organic molecules, including diethyl ether (Brady et al. 1988; Yang et al. 1990). In rat liver microsomes, an immunoinhibition study indicated that CYP2E1 partially contributed to the metabolism of MTBE (Brady et al. 1990). The present study showed that with the co-expressed P450 reductase, both human CYP2A6 and 2E1 were found to be active in metabolizing MTBE to TBA, as well as in the metabolism of two other gasoline ethers, ETBE and TAME. However, the activities for CYP2A6 were always much higher than those for CYP2E1 (Table 2). Further studies, including enzyme kinetics and immunoinhibition, are needed to substantiate the roles of CYP2A6 and 2E1 as well as other forms of CYP enzymes in the metabolism of MTBE in human livers.

The presence of human subpopulations, which are reported to be sensitive to MTBE, has raised great public concern on the safety of MTBE. One possible explanation for the reported increased sensitivity could be due to the differences in an individual's ability to metabolize MTBE. Genetic polymorphism of human CYP enzymes, including CYP2A6 and 2E1, has been documented (Yamano et al. 1990; Watanabe et al. 1990; Hayashi et al. 1991; Daly et al. 1994). The polymorphism is believed to be an important factor in determining an individual's sensitivity to environmental chemicals via alteration of the expression level and function of CYP enzymes (Daly et al. 1994; Hong and Yang 1996). Considerable inter-individual variations were observed in the blood and urine levels of TBA in subjects exposed to MTBE (Johanson et al. 1995; White et al. 1995; Health Effects Institute 1996). In the present study the maximal difference in the MTBE metabolizing activity, as measured by TBA formation in eight human liver samples was of approx. two fold however, a larger difference might be observed if more samples were analyzed. Further studies are needed to elucidate the relationship between individual sensitivity to MTBE and the genetic polymorphism of human CYP enzymes responsible for MTBE metabolism.

Table 1 Metabolism of MTBE in liver microsomes and cytosol: formation of TBA assayed for MTBE-metabolizing activity. Values are mean ± SE. Each rat or mouse sample was pooled from 3 animals. (*n.d.* Not detectable.)

Species	Microsomes	Cytosol
	(pmol TBA/min per mg protein)	
Human ($n = 8$)	124.9 ± 11.3	$1.3 \pm 0.5^*$
Rat ($n = 5$)	284 ± 14	n.d.
Mouse ($n = 4$)	288 ± 29	n.d.

*Significantly different from microsomes ($P < 0.05$)

Table 2 Metabolism of MTBE and other gasoline ethers[a] by human CYP2A6 and 2E1. Human CYP2A6 and 2E1 were individually co-expressed with the human cytochrome P450 reductase via a baculovirus expression system. The incubation mixture contained 0.04 nmol of CYP2A6 or 2E1 and 1 mM of substrate

	MTBE	ETBE	TAME
		(nmol metabolite/min per nmol P450)	
CYP2A6	6.1	13.6	37
CYP2E1	0.7	0.8	0.4

[a]TBA formation was assayed for MTBE- and ETBE metabolizing activities and TAA formation was assayed for TAME metabolizing activity. The differences between the duplicate incubations, or between repeat assays were <10%

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