Sex Differences in the Metabolism of (+)- and (−)-Limonene Enantiomers to Carveol and Perillyl Alcohol Derivatives by Cytochrome P450 Enzymes in Rat Liver Microsomes

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(+)-Limonene is reported to cause nephropathy in male rats, but not in female rats and other species of animals including mice, rabbits, guinea pigs, and dogs. Male rats contain high levels of α2u-globulin in kidneys, and it has been shown that limonene and/or its metabolites are able to bind noncovalently to α2u-globulin, resulting in an accumulation of protein droplets in the renal tubules. In this study, we investigated whether (+)- and (−)-limonene enantiomers are differentially metabolized by liver microsomes of male and female rats. (+)- and (−)-limonene enantiomers were found to be oxidized to their respective *trans*-carveol (6-hydroxylation) and perillyl alcohol (7-hydroxylation) derivatives in greater amounts by liver microsomes of male rats than those of female rats. The limonene hydroxylation activities were not detected in liver microsomes of rat fetuses and were increased developmentally after birth, only in male rats. Treatment of male rats with phenobarbital significantly increased liver microsomal 6-hydroxylation activities with both enantiomers whereas *β*-naphthoflavone, isosafrole, and pregnenolone 16α-carbonitrile did not cause such effects. Anti-P450 2C9 which cross-reacts with rat P450 2C11 inhibited limonene hydroxylations catalyzed by liver microsomes of untreated male rats, and it was also found that anti-P450 2B1 suppressed the activities catalyzed by liver microsomes of phenobarbital-treated rats. Possible roles of P450 2C11 and P450 2B1 in the limonene hydroxylation activities were supported by the experiments with purified rat liver P450s in reconstitution systems and with recombinant rat P450s in *Trichoplusia ni*. Our present results showing that there are sex-related differences in the oxidative metabolism of limonene enantiomers by liver microsomes may provide useful information on the basis of limonene-induced toxicities in different animal species.

Introduction

(+)- and (−)-limonene enantiomers, the monocyclic monoterpenes found in orange peel and other plants, are the most commonly utilized natural terpenes which are used as fragrances in household products and components of artificial essential oils (*1*). Concentrations of limonene in essential oils of citrus fruits, vegetables, herbs, and other species have been reported to vary from ∼1% (palmarosa) to ∼95% (lemon, orange, and mandarin) (*2*). (+)-Limonene isomeric form has been found to be most abundantly present in these essential oils, followed by the racemic mixture and (−)-isomeric form (*3*). A number of microorganisms and some plants have been shown to convert limonene to several alcohols, acids, and ketones such as terpineol, perillic acid, and carvone (*3*−*8*). We have recently reported that the larvae of the common cutworm (*Spodoptera litera*) converts limonene to uroterpenol and perillic acid (*9*).

(+)-Limonene, when administered in vivo to animals at relatively high doses, has been shown to cause nephrotoxicity and kidney tumors; however, these cytotoxic effects have not been observed in female rats and other species of animals including mice, rabbits, guinea pigs, and dogs (*10*−*12*). Mechanisms underlying specific induction of nephrotoxicity in male rats have been shown to be related to the high level of expression of an α2u-globulin in the liver of male rats (*11*, *13*, *14*). This protein is rapidly secreted from the liver into other organs such as kidneys, submaxillary, lachrymal, prepuital, and mammary glands, and anterior pituitary. It was reported that the concentration of the α2u-globulin in kidneys is more than 100-times higher in male rats than female rats (*14*, *15*).

It has also been reported that several diverse chemicals such as unleaded gasoline, *tert*-butyl alcohol, 1,4-dichlorobenzene, dimethyl methylphosphonate, hexachloroethane, isophorone, (+)-limonene, pentachloroethane, 2,2,4-trimethylpentane, and tetrachloroethylene induce nephrotoxicity by binding reversibly with α2u-globulin in the kidney, thus preventing protein degradation (hydrolysis)

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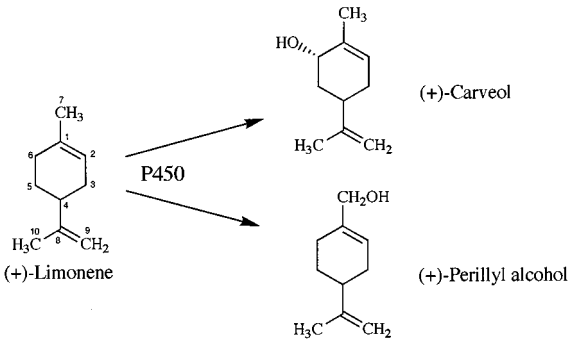

Figure 1. Metabolism of (+)-limonene by rat P450 enzymes. It was suggested that (−)-limonene is also metabolized through a similar fashion by P450 enzymes in rats.

that causes accumulation of protein droplets in the renal tubules (*11*, *14*). Several metabolites of these chemicals including *d*-limonene have been reported to be more active than the parent compounds in interacting with α2u-globulin in male rats (*13*, *14*, *16*). (+)-Limonene has been shown to be metabolized to several oxidation and conjugating products in vivo, and species-related differences in the formation of these metabolites have been reported in rats, mice, rabbits, guinea pigs, dogs, and humans (*17*−*21*). Incubations with liver microsomes from these animal species are able to convert (+)-limonene to several oxidation products such as 1,2- and 8,9-epoxides, carveol (6-hydroxylation), and perillyl alcohol (7-hydroxylation) (Figure 1) (*20*, *22*, *23*).

In this study, we examined the metabolism of (+)- and (−)-enantiomers of limonene using liver microsomes from male and female rats, and the products formed were analyzed with GC-MS. Liver microsomes of male rats treated with PB,[1] BNF, ISF, and PCN were used to examine which P450 enzymes are more active in catalyzing (+)- and (−)-limonene enantiomers. Purified P450s isolated from rat liver microsomes or recombinant rat P450s coexpressing NADPH−P450 reductase in *Trichoplusia ni* cells were also used to determine which P450s are the principal enzymes in the oxidation of limonene enantiomers.[2]

Experimental Procedures

Chemicals. (+)- and (−)-limonene enantiomers were purchased from Wako Pure Chemical Co. (Osaka) and were used without further purification; the purities of these compounds were judged to be >99% and >95%, respectively, on analysis with GC-MS. (+)- and (−)-carvone derivatives, whose purities were >99%, were purchased from Fluka Co. (Tokyo, Japan). $NADP^+$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium borohydride were obtained from Wako Pure Chemical Co. Other reagents and chemicals used were obtained from sources as described previously or of the highest qualities commercially available (*24*−*27*).

GC/MS Analysis. A Hewlett-Packard model 5890A gas chromatograph (Atlanta, GA) equipped with a split injector was combined with direct coupling to a Hewlett-Packard 5972 mass spectrometer. The metabolites were separated by a TC-WAX FFS (GL Sciences, Tokyo, Japan) silica capillary column (0.25 mm × ∼60 m) using helium (at 1 mL/min) as a carrier gas. The column temperature was increased from 80 to 180 °C at the rate of 4 °C/min and then held at 180 °C for 10 min. The injector temperature was at 270 °C. The detector interface temperature was at 280 °C with the actual temperature in the MS source reaching approximately 180 °C and the ionization voltage of 70 eV. The electron impact (EI) mode was used. A GC/MS system equipped with a Wiley 138 K Mass Spectral Database software was used for identification of the products.

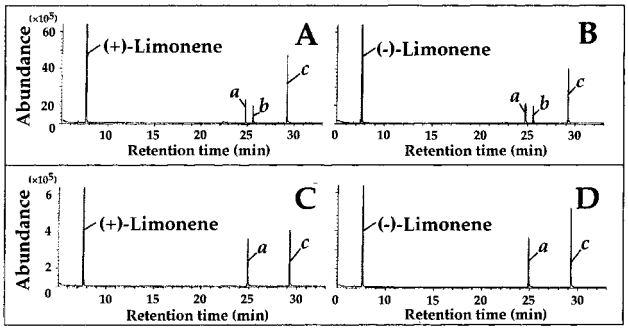

Figure 2. GC-MS analysis of (+)- and (−)-limonene enantiomers and their oxidation products. In (A) and (B), chromatographic peaks of synthetic products of *trans*- (*a*) and *cis*-carveol (*b*) and (+)- and (−)-perillyl alcohol (*c*) as well as (+)- and (−)-limonene enantiomers are shown. In (C) and (D), chromatographic peaks of products formed through metabolism of (+)- and (−)-limonene, respectively, by liver microsomes of untreated (male) rats in the presence of an NADPH-generating system are shown.

Synthesis of Carveol Isomers. (+)- and (−)-carvone (2.0 mM) dissolved in ethanol were treated with $NaBH_4$ (3.0 mM) and 10% NaOH in ethanol at room temperature. After mixing vigorously for 2 h, the reaction mixture was evaporated to dryness under the reduced pressure. The residue was extracted with diethyl ether and then washed with 10% HCl. The organic layer was evaporated under reduced pressure and transferred to an insert for analysis with GC-MS. The retention times of GC-MS for compounds *a*, *b*, and *c* were 24.8, 25.6, and 29.2 min, respectively (Figure 2). These compounds (*a*, *b*, and *c*) were compared with their mass spectra with Wiley 138 K Mass Spectral Database software. Peak *a* was suggested to be *trans*-carveol with MS *m*/*z* of 152 (M^+, 6), 137 (9), 119 (14), 109 (100), 95 (20), 91 (34), 84 (68), 69 (33), 56 (24), 55 (50), and 41 (64). Peak *b* corresponded to *cis*-carveol with MS *m*/*z* of 152 (M^+, 1), 134 (37), 119 (25), 109 (54), 95 (25), 91 (37), 84 (100), 69 (48), 56 (29), 55 (64), and 41 (80). Peak *c* was suggested to be perillyl alcohol with MS *m*/*z* of 150 (M^+, 7), 134 (8), 121 (43), 109 (22), 93 (68), 79 (100), 68 (98), 55 (65), and 41 (80).

Enzymes. Male and female Sprague−Dawley rats (at ages of 7 weeks and 8 weeks, respectively; weighing about 200 g) were obtained from Nihon Clea Co. (Osaka, Japan). Male rats were treated with PB (80 mg/kg, daily for 3 days), BNF and ISF (50 mg/kg, daily for 3 days), and PCN (100 mg/kg, daily for 3 days) (*24*, *28*, *29*). Rats were starved overnight before killing. Liver microsomes were prepared as described and suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v) (*30*, *31*).

P450s 1A1, 1A2, 2B1, 2C11, and 2C12 were purified from rat liver microsomes as described previously (*30*, *32*). Recombinant P450s 1A1, 1A2, 2B1, 2C11, 2C12, 3A1, and 3A2 expressed in *T. ni* cells infected with a baculovirus containing rat P450 and NADPH−P450 reductase cDNA inserts were obtained from Gentest Co. (Woburn, MA); the P450 contents in these systems were used as described in the data sheets provided by the manufacturer.

Rabbit antiserum raised against purified human liver P450 2C9 was prepared as described previously (*33*). Anti-P450 2B1 antibodies were prepared as described (*30*).

Oxidation of (+)- and (−)-Limonene by P450 Enzymes in Rat Liver Microsomes. Oxidations of (+)- and (−)-limonene enantiomers by liver microsomes of male and female rats and by rat P450 enzymes were determined as follows. Standard

[1] Abbreviations: P450, cytochrome P450; PB, phenobarbital; BNF, β-naphthoflavone; ISF, isosafrole; PCN, pregenolone 16α-carbonitrile; DLPC, L-α-dilauroyl-*sn*-glycero-3-phosphocholine.

[2] Miyazawa, M., and Shindo, M., unpublished results.

Table 1. Oxidation of (+)- and (−)-Limonenes by Liver Microsomes of Male and Female Rats and Effects of Treatment of Male Rats with Chemical Inducers[a]

	(+)-limonene metabolism [nmol of products min^{-1} (mg of protein)$^{-1}$]		(−)-limonene metabolism [nmol of products min^{-1} (mg of protein)$^{-1}$]	
treatment	(+)-carveol	(+)-perillyl alcohol	(−)-carveol	(−)-perillyl alcohol
female rats				
untreated	<0.1	0.04 ± 0.01	<0.1	0.05 ± 0.01
male rats				
untreated	0.87 ± 0.21	1.23 ± 0.25	0.61 ± 0.17	1.03 ± 0.28
PB	1.98 ± 0.14	0.71 ± 0.15	1.94 ± 0.09	0.29 ± 0.10
BNF	0.18 ± 0.03	0.12 ± 0.02	0.17 ± 0.03	0.05 ± 0.02
ISF	0.20 ± 0.02	0.72 ± 0.08	0.41 ± 0.04	0.34 ± 0.03
PCN	0.41 ± 0.09	0.14 ± 0.04	0.53 ± 0.11	0.12 ± 0.04

[a] Oxidation of (+)- and (−)-limonene enantiomers by rat liver microsomes was determined by the methods described. Data are means from three animals ± SD. Procedures for the treatment of rats with chemical inducers were described under Experimental Procedures.

reaction mixture contained liver microsomes (0.05 mg of protein/mL) or recombinant P450 (3 pmol/mL) and 200 μM (+)- or (−)-limonene in a final volume of 0.50 mL of 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 0.5 unit of glucose-6-phosphate dehydrogenase/mL) (*30*, *33*). For the reconstitution studies, purified rat liver P450 enzymes were mixed with 2-fold molar excess of rabbit liver NADPH−P450 reductase and 5 μg of DLPC/mL and were mixed with other components (*34*, *35*). Incubations were carried out at 37 °C for 30 min and terminated by adding 1.0 mL of dichloromethane. The extracts (organic layer) were collected by centrifugation at 3000 rpm for 5 min and were used for analysis with GC-MS for identification of the metabolites.

Other Assays. P450 and protein contents were estimated by the methods described elsewhere (*36*, *37*).

Statistical Analysis. Kinetic parameters for (+)- and (−)-limonene oxidations by rat P450 enzymes were estimated using a computer program (KaleidaGraph from Synergy Software, Reading, PA) designed for nonlinear regression analysis (*38*). Statistical analysis was done using Student's *t*-test.

Results

Identification of Metabolites of (+)- and (−)-Limonene Enantiomers by Rat Liver Microsomes. We first examined the metabolism of (+)- and (−)-limonene enantiomers by liver microsomes of untreated (male) rats in the presence of an NADPH-generating system, and the metabolites formed were analyzed with GC-MS as described under Experimental Procedures (Figure 2). Liver microsomes from male rats catalyzed conversion of (+)- and (−)-limonene enantiomers to respective metabolites, and these were identified to be respective (+)- and (−)-*trans*-carveol (6-hydroxylation) and (+)- and (−)-perillyl alcohol (7-hydroxylation).

Oxidation of (+)- and (−)-Limonene Enantiomers by Rat Liver Microsomes. Liver microsomes of male rats converted (+)- and (−)-limonene enantiomers to corresponding (+)- and (−)-*trans*-carveol and (+)- and (−)-perillyl alcohol (Table 1). Female rats had very low limonene oxidation activities by liver microsomes (Table 1). Treatment of male rats with PB caused increases in the formation of carveol metabolites, whereas perillyl alcohol metabolites were not induced by PB (Table 1). Other chemical inducers such as BNF, ISF, and PCN did not cause any significant increases in (+)- and (−)-limonene hydroxylation activities.

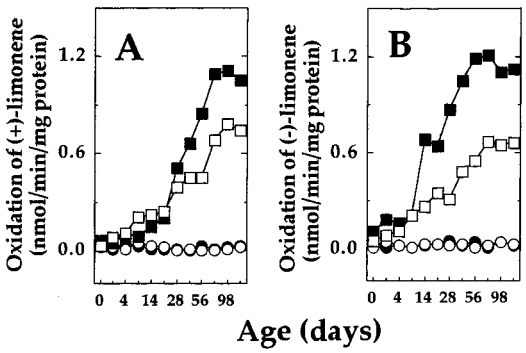

Figure 3. Developmental changes in the formation of carveol (□ and ○) and perillyl alcohol (■ and ●) by liver microsomes of male rats (□ and ■) and female rats (○ and ●) when (+)-limonene (in panel A) and (−)-limonene (in panel B) were used as substrates. Data are means of duplicate determinations of combined microsomes obtained from 3−6 animals.

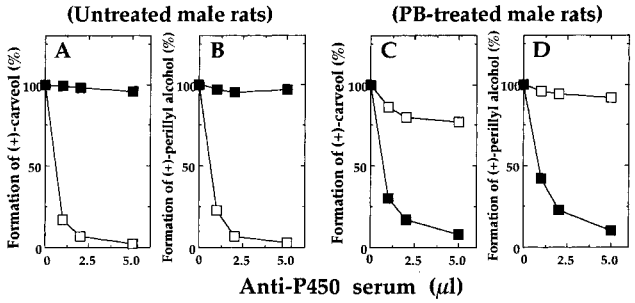

Figure 4. Effects of anti-P450 2C9 (□) and anti-P450 2B1 (■) on the conversion of (+)-limonene to (+)-carveol (A and C) and (+)-perillyl alcohol (B and D) by liver microsomes of untreated rats (A and B) and rats treated with PB (C and D). Activities are represented as percent controls (in the absence of antisera); control activities in the absence of antisera were very similar to those presented in Table 1. Preimmune antisera did not inhibit significantly the limonene hydroxylation activities by rat liver microsomes (data not shown). Data are means of duplicate determinations.

Developmental changes in rats in the oxidation of (+)- and (−)-limonene enantiomers by liver microsomes were examined (Figure 3). Fetal liver microsomes contained very low or undetectable rates for limonene hydroxylation activities. After their birth, limonene hydroxylation activities were developmentally increased only in male, but not female, rats, and the formations of (+)- and (−)-perillyl alcohol were found to be more rapidly increased than those of carveol metabolites (Figure 3). The maximal activities in the oxidation of (+)- and (−)-limonene hydroxylations were achieved at ages of 7−8 weeks.

Effects of anti-human P450 2C9 antibodies [which react with rat P450 2C11 and 2C12 as well as P450 2C9 (*33*)] and anti-rat P450 2B1 antibodies on (+)-limonene hydroxylation activities were examined in liver microsomes of untreated (male) rats and of rats treated with PB (Figure 4). Anti-P450 2C9 inhibited formation of carveol and perillyl alcohol of (+)-limonene by liver microsomes of untreated rats, but did not affect the limonene oxidation activities by liver microsomes of PB-treated rats. Conversely, anti-P450 2B1 inhibited limonene hydroxylation activities only in liver microsomes of PB-treated rats. We also determined the effects of anti-P450 2C9 and anti-P450−2B1 antibodies on (+)-limonene oxidation activities by liver microsomes of untreated male rats and rats treated with PB. The results showed that anti-P450 2C9 significantly inhibited activities of liver

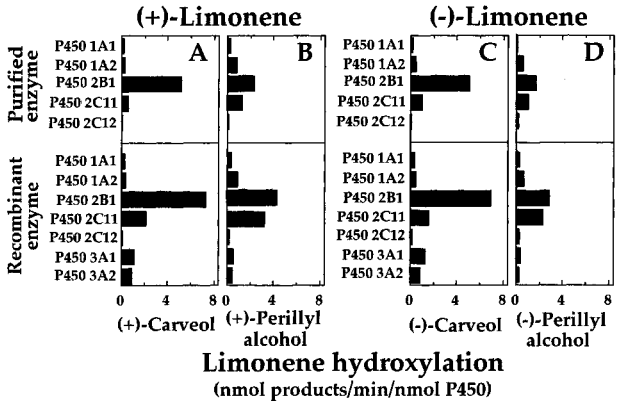

Figure 5. Formation of carveol (A and C) and perillyl alcohol (B and D) by P450s 1A1, 1A2, 2B1, 2C11, and 2C12 in reconstituted systems and by P450 1A1, 1A2, 2B1, 2C11, 2C12, 3A1, and 3A2 in recombinant (*T. ni* cell) systems. Data are means of triplicate determinations.

microsomes of untreated male rats and anti-P450 2B1 caused decreases in the activities of liver microsomes of PB-treated rats (data not shown).

Oxidation of (+)- and (−)-Limonenes by Reconstituted Systems Containing Purified Rat P450 Enzymes and by Recombinant (*T. ni* Cells) Rat P450 Enzymes. Purified rat liver P450s 1A1, 1A2, 2B1, 2C11, and 2C12 were reconstituted with rat liver NADPH−P450 reductase on the DLPC membranes to examine which P450s are the principal enzymes in catalyzing oxidations of limonene enantiomers (Figure 5). P450 2B1 was the highest in converting (+)- and (−)-limonenes to form the corresponding carveol and perillyl alcohol followed by P450 2C11 and P450 1A2. P450 1A1 and P450 2C12 had very low rates in limonene hydroxylation activities.

We also determined limonene hydroxylation activities by recombinant systems in *T. ni* cells expressing different forms of rat P450 and NADPH−P450 reductase (Figure 5). Again, P450 2B1 was the highest in catalyzing 6-hydroxylation of limonene enantiomers, followed by P450 2C11 (Figure 5). The rates of formation of perillyl alcohol were similar in P450 2B1 and P450 2C11 toward oxidation of (+)- and (−)-limonene enantiomers. Other recombinant P450s including P450 1A1, 1A2, 2C12, 3A1, and 3A2 had relatively low catalytic rates for limonene hydroxylations.

Kinetic analyses of limonene hydroxylations by liver microsomes of male rats and rats treated with PB and by recombinant systems containing P450 2C11 and 2B1 were examined (Table 2). K_m values for hydroxylations of (+)- and (−)-limonenes by rat liver microsomes and recombinant P450 2C11 and 2B1 systems were not very different, except that P450 2C11 had slightly lower rates in the formation of perillyl alcohol in the recombinant systems. V_{max} values for the formation of carveol were higher in liver microsomes of PB-treated rats than those of untreated rats and were also higher in P450 2B1 than P450 2C11 in the recombinant systems (Table 2). V_{max} values for the formation of perillyl alcohol were slightly higher in PB-treated rats and recombinant P450 2B1 than those of untreated rats and recombinant P450 2C11, respectively. The V_{max}/K_m ratios (enzyme efficiencies) for the formation of carveol were greater in liver microsomes of PB-treated rats and in the recombinant P450 2B1 system. Interestingly, enzyme efficiencies toward formation of perillyl alcohol were similar in P450 2B1 and P450 2C11 with (+)-limonene and were higher in P450 2C11 than in P450 2B1 with (−)-limonene as substrate.

Discussion

The present results showed that both (+)- and (−)-limonene enantiomers were converted to their respective carveol and perillyl alcohol by P450 2C11 in liver microsomes of untreated male rats. Female-specific P450 2C12 was suggested to be inactive in catalyzing limonene hydroxylations, since liver microsomes of female rats had very low or undetectable rates for the hydroxylations of limonene enantiomers as compared with those catalyzed by liver microsomes of male rats. Recombinant P450 2C12 was found to have very low activities in oxidizing limonenes. Developmental studies showed that limonene hydroxylation activities (formation of carveol and perillyl alcohol) were increased with age only in male, but not female, rats by liver microsomes; the patterns of increases in limonene hydroxylation activities are suggested to be closely related to the developmental increases in P450 2C11 in male rats (*39*). Possible roles of P450 2C11 in the limonene hydroxylations by liver microsomes of untreated rats were confirmed by the experiments with purified rat P450 enzymes in the reconstitution system and with recombinant rat P450s in *T. ni* cells.

Treatment of rats with PB was found to cause increases in the conversion of limonene enantiomers to their respective carveol metabolites by liver microsomes, while the formations of perillyl alcohols were not in-

Table 2. Kinetic Analysis of Oxidation of (+)- and (−)-Limonene Enantiomers by Liver Microsomes of Untreated Rats and Rats Treated with PB and by Recombinant Systems Containing P450 2C11 and P450 2B1

	K_m (μM)		V_{max} [nmol min^{-1} (nmol of P450)$^{-1}$]		V_{max}/K_m (nM^{-1}min^{-1})	
	carveol	perillyl alcohol	carveol	perillyl alcohol	carveol	perillyl alcohol
			(+)-Limonene Hydroxylation			
liver microsomes						
untreated rats	680	580	1.1	1.5	1.6	2.6
PB-treated rats	780	510	15	4.5	16	7.6
recombinant P450						
P450 2C11	490	400	3.2	4.7	6.5	12
P450 2B1	750	490	21	5.8	28	12
			(−)-Limonene Hydroxylation			
liver microsomes						
untreated rats	670	771	0.9	1.5	1.3	1.9
PB-treated rats	710	874	8.9	3.9	10	4.4
recombinant P450						
P450 2C11	400	180	2.2	2.7	5.5	15
P450 2B1	830	820	12	4.7	15	5.7

creased, but rather decreased, by PB. Anti-P450 2B1 antibody, however, inhibited both activities forming carveol and perillyl alcohol derivatives from limonene enantiomers by liver microsomes of PB-treated rats, but not those of untreated male rats. Conversely, anti-P450 2C9, which reacts with rat P450 2C11 as well as human P450 2C9 (*33*), significantly inhibited limonene hydroxylation activities catalyzed by microsomes from untreated male rats specifically. These results suggest that P450 2C11 is a principal enzyme in the oxidation of limonene enantiomers in rats at the physiological state and P450 2B1 is involved in the reactions when rats are treated with P450 2B inducers such as PB and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*40*). Kinetic analysis with P450 2C11 and 2B1 in reconstituted and recombinant (*T. ni* cells) systems suggests that turnover rates for the formation of carveol by P450 2B1 are higher than those by P450 2C11, whereas both enzymes have similar rates for the formation of perillyl alcohol in rats (Table 2).

Previous studies have suggested that (+)-limonene induces nephropathy only in male rats, but not in female rats and other species of animals including mice, rabbits, guinea pigs, and dogs (*11*, *13*, *14*). The mechanism in male-specific toxicities caused by (+)-limonene has been shown to be related to the high concentration of α2u-globulin in the kidneys of male rats (*13*, *15*); limonene and its metabolites have been shown to bind noncovalently to α2u-globulin, causing accumulation of protein droplets in the tubules of kidneys in the male rats (*13*, *14*). The results of Dietrich and Swenberg (*41*) using NCI-Black-Reiter male rats are of interest, since this strain of rats has been shown to fail to synthesize α2u-globulin and is shown to be resistant to nephropathy by several compounds including (+)-limonene. It is also interesting to note the results of Lehman-McKeeman and Caudill (*42*), who showed that (+)-limonene induces nephropathy in α2u-globulin transgenic mice.

(+)-Limonene-1,2-epoxide has been shown to be more active than the parent compound in interacting with α2u-globulin in male rats (*13*, *14*). Rat P450 enzymes have been reported to convert limonene to 1,2- and 8,9-epoxide metabolites, carveol (6-hydroxylation), perillyl alcohol (7-hydroxylation), and others (*18*, *20*). Our present results showed that liver microsomes of untreated male rats convert (+)- and (−)-limonene enantiomers to their respective carveol and perillyl alcohol as major oxidation products on analysis with GC-MS. It is, however, not known at present whether these two metabolites are more active than the parent compounds in interacting with α2u-globulin and whether the sex-related differences in limonene-induced toxicities are related to the different catalytic specificities of liver microsomes of male and female rats toward oxidation of limonene. It also remains unclear whether treatment of rats with PB causes an increase in limonene-induced toxicities in vivo by increasing the formation of limonene metabolites by P450 2B1. Further work will be required to solve this problem.

A number of studies have suggested that (+)-limonene has chemopreventive activities in experimental animal models although its mechanisms underlying inhibition of chemically induced cancers remain unclear (*1*, *43*). Crowell et al. (*44*) have reported that some of the hydroxylated metabolites of (+)-limonene, such as sobrerol, carveol, and uroterpenol, are more potent than the parent compound in preventing mammary tumors caused by 7,12-dimethyldibenz[*a*]anthracene. (+)-Limonene is shown to be inactive in inducing P450 enzymes, but is able to induce phase II enzymes such as glutathione *S*-transferase and UDP-glucuronyltransferase enzymes (*43*, *45*). These latter findings are of interest, since the phase II enzymes have been shown to detoxify the reactive metabolites of chemical carcinogens formed through metabolism by P450 and other enzymes (*46*). To better understand the basis of mechanisms of chemopreventive action of limonene, it is required to examine whether metabolism of limonene in various animal species affects the chemoprevention activities.

In summary, the present results showed that (+)- and (−)-limonene enantiomers were converted to their respective carveol and perillyl alcohol by P450 2C11 in liver microsomes of untreated male rats and by P450 2B1 in liver microsomes of PB-treated rats. Female-specific P450 2C12 was found to be inactive in catalyzing limonene enantiomers. These data may provide new information in understanding the basis of sex-related difference in susceptibilities toward actions of limonene enantiomers in rats.

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References

(1) Crowell, P. L., and Gould, M. N. (1994) Chemoprevention of mammary carcinogenesis by hydroxylated derivatives of *d*-limonene. *Crit. Rev. Oncog.* **5**, 1−22.

(2) Lis-Balchin, M., Ochocka, R. J., Deans, S., Asztemborska, M., and Hart, S. (1996) Bioactivity of the enantiomers of limonene. *Med. Sci. Res.* **24**, 309−310.

(3) Haudenschild, C., Schalk, M., Karp, F., and Croteau, R. (2000) Functional expression of regiospecific cytochrome P450 limonene hydroxylases from mint (*Mentha* spp.) in *Escherichia coli* and *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **379**, 127−136.

(4) Lupien, S., Karp, F., Wildung, M., and Croteau, R. (1999) Regiospecific cytochrome P450 limonene hydroxylases from mint (*Mentha*) species: cDNA isolation, characterization, and functional expression of (−)-4*S*-limonene-3-hydroxylase and (−)-4*S*-limonene-6-hydroxylase. *Arch. Biochem. Biophys.* **368**, 181−192.

(5) Vanek, T., Valterova, I., and Vaisar, T. (1999) Biotransformation of (*S*)-(−)- and (*R*)-(+)-limonene using *Solanum aviculare* and *Dioscorea deltoidea* plant cells. *Phytochemistry* **50**, 1347−1351.

(6) Tan, Q., and Day, D. F. (1998) Bioconversion of limonene to terpineol by immobilized *Penicillium digitatum*. *Appl. Microbiol. Biotechnol.* **49**, 96−101.

(7) Rensburg, E. V., Moleleki, N., Walt, J. P. V. D., Botes, P. J., and Dyk, M. S. V. (1997) Biotransformation of (+)-limonene and (−)-piperitone by yeasts and yeast-like fungi. *Biotechnol. Lett.* **19**, 779−782.

(8) Noma, Y., Yamasaki, S., and Asakawa, Y. (1992) Biotransformation of limonene and related compounds by *Aspergillus cellulosae*. *Phytochemistry* **31**, 2725−2727.

(9) Miyazawa, M., Wada, T., and Kameoka, H. (1998) Biotransformation of (+)- and (−)-limonene by the larvae of common cutworm (*Spodoptera litura*). *J. Agric. Food Chem.* **46**, 300−303.

(10) Lehman-Mckeeman, L. D., Caudill, D., Robert, E., and Young, J. A. (1990) Comparative disposition of *d*-limonene in rats and mice: relevance to male-rat-specific nephrotoxicity. *Toxicol. Lett.* **53**, 193−195.

(11) Hard, G. C. (1998) Mechanisms of chemically induced renal carcinogenesis in the laboratory rodent. *Toxicol. Pathol.* **26**, 104−112.

(12) Kanerva, R. L., and Alden, C. L. (1987) Review of kidney sections from a subchronic *d*-limonene oral dosing study conducted by the National Cancer institute. *Food Chem. Toxicol.* **25**, 355−358.

(13) Lehman-Mckeeman, L. D., Rodriguez, P. A., Takigiku, R., Caudill, D., and Fey, M. L. (1989) *d*-Limonene-induced male rat-specific nephrotoxocity: evaluation of the association between *d*-limonene and α2u-globulin. *Toxicol. Appl. Pharmacol.* **99**, 250−259.

(14) Borghoff, S. J., Short, B. G., and Swenberg, J. A. (1990) Biochemical mechanisms and pathobiology of α2u-globulin nephropathy. *Annu. Rev. Pharmacol. Toxicol.* **30**, 349−367.
(15) Daniel, R., and Swenberg, J. A. (1991) The presence of α2u-globulin is necessary for *d*-limonene promotion of male rat kidney tumors. *Cancer Res.* **51**, 3512−3521.
(16) Lock, E. A., Chabonneau, M., Strasser, J., Swenberg, J. A., and Bus, J. S. (1987) 2,2,2-Trimethylpentane-induced nephrotoxicity II. The reversible binding of a TMP metabolite to a renal protein fraction containing α2u-globulin. *Toxicol. Appl. Pharmacol.* **91**, 182−192.
(17) Igimi, H., Nishimura, M., Kodama, R., and Ide, H. (1974) Studies on the metabolism of *d*-limonene (*p*-mentha-1,8-diene) I. The absorption, distribution and excretion of *d*-limonene in rats. *Xenobiotica* **4**, 77−84.
(18) Regan, J. W., and Bjeldanes, L. F. (1976) Metabolism of (+)-limonene in rats. *J. Agric. Food Chem.* **24**, 377−380.
(19) Kodama, R., Yano, T., Furukawa, K., Noda, K., and Ide, H. (1976) Studies on the metabolism of *d*-limonene (*p*-mentha-1,8-diene). IV. Isolation and characterization of new metabolites and species differences in metabolism. *Xenobiotica* **6**, 377−389.
(20) Watabe, T., Hiratsuka, A., Ozawa, N., and Isobe, M. (1981) A comparative study on the metabolism of *d*-limonene and 4-vinylcyclohex-1-ene by hepatic microsomes. *Xenobiotica* **11**, 333−344.
(21) Kodama, R., Noda, K., and Ide, H. (1974) Studies on the metabolism of *d*-limonene (*p*-mentha-1,8-diene) II. The metabolic fate of *d*-limonene in rabbits. *Xenobiotica* **4**, 85−95.
(22) Jager, W., Mayer, M., Platzer, P., Rezicek, G., Dietrich, H., and Buchbauer, G. (1999) Stereoselective metabolism of the monoterpene carvone by rat and human liver microsomes. *J. Pharm. Pharmacol.* **52**, 191−197.
(23) Watabe, T., Hiratsuka, A., Isobe, M., and Ozawa, N. (1980) Metabolism of *d*-limonene by hepatic microsomes to nonmutagenic epoxides toward *Salmonella typhimurium*. *Biochem. Pharmacol.* **29**, 1068−1071.
(24) Yamazaki, H., and Shimada, T. (1998) Formation in vitro of an inhibitory cytochrome P450·Fe^{2+}-metabolite complex with roxithromycin and its decladinosyl, *O*-dealkyl, and *N*-demethyl metabolites in rat liver microsomes. *Xenobiotica* **28**, 995−1004.
(25) Miyazawa, M., Shindo, M., and Shimada, T. (2001) Oxidation of 1,8-cineole, the monoterpene cyclic ether originated from *Eucalyptus polybractea*, by cytochrome P450 3A enzymes in rat and human liver microsomes. *Drug Metab. Dispos.* **29**, 200−205.
(26) Shimada, T., Tsumura, F., Yamazaki, H., Guengerich, F. P., and Inoue, K. (2001) Characterization of (±)-bufuralol hydroxylation activities in liver microsomes of Japanese and Caucasian subjects genotyped for *CYP2D6*. *Pharmacogenetics* **11**, 143−156.
(27) Shimada, T., Tsumura, F., and Yamazaki, H. (1999) Prediction of human liver microsomal oxidations of 7-ethoxycoumarin and chlorzoxazone using kinetic parameters of recombinant cytochrome P450 enzymes. *Drug Metab. Dispos.* **27**, 1274−1280.
(28) Mimura, M., Yamazaki, H., Sugahara, C., Hiroi, T., Funae, Y., and Shimada, T. (1994) Differential roles of cytochromes P450 2D1, 2C11, and 1A1/2 in the hydroxylation of bufuralol by rat liver microsomes. *Biochem. Pharmacol.* **47**, 1957−1963.
(29) Yamazaki, H., Hiroki, S., Urano, T., Inoue, K., and Shimada, T. (1996) Effects of roxithromycin, erythromycin and troleandomycin on their *N*-demethylation by rat and human cytochrome P450 enzymes. *Xenobiotica* **26**, 1143−1153.
(30) Shimada, T., and Nakamura, S. (1987) Cytochrome P-450-mediated activation of procarcinogens and promutagens to DNA-damaging products by measuring expression of *umu* gene in *Salmonella typhimurium* TA1535/pSK1002. *Biochem. Pharmacol.* **36**, 1979−1987.
(31) Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F. P. (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J. Pharmacol. Exp. Ther.* **270**, 414−423.
(32) Shimada, T., Nakamura, S., Imaoka, S., and Funae, Y. (1987) Genotoxic and mutagenic activation of aflatoxin B_1 by consitutive forms of cytochrome P-450 in rat liver microsomes. *Toxicol. Appl. Pharmacol.* **91**, 13−21.
(33) Shimada, T., Misono, K. S., and Guengerich, F. P. (1986) Human liver microsomal cytochrome P-450 mephenytoin 4-hydroxylase, a prototype of genetic polymorphism in oxidative drug metabolism. Purification and characterization of two similar forms involved in the reaction. *J. Biol. Chem.* **261**, 909−921.
(34) Yamazaki, H., Gillam, E. M. J., Dong, M.-S., Johnson, W. W., Guengerich, F. P., and Shimada, T. (1997) Reconstitution of recombinant cytochrome P450 2C10 (2C9) and comparison with cytochrome P450 3A4 and other forms: Effects of cytochrome P450−P450 and cytochrome P450−b_5 interactions. *Arch. Biochem. Biophys.* **342**, 329−337.
(35) Shimada, T., and Yamazaki, H. (1998) Cytochrome P450 reconstitution systems. In *Methods in Molecular Biology* (Phillips, I. R., and Shephard, E. A., Eds.) pp 85−93, Humana Press, Totowa, NJ.
(36) Omura, T., and Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**, 2370−2378.
(37) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265−275.
(38) Komatsu, K., Ito, K., Nakajima, Y., Kanamitsu, S., Imaoka, S., Funae, Y., Green, C. E., Tyson, C. A., Shimada, N., and Sugiyama, Y. (2000) Prediction of in vivo drug−drug interactions between tolbutamide and various sulfonamides in humans based on in vitro experiments. *Drug Metab. Dispos.* **28**, 475−481.
(39) Imaoka, S., Fujita, S., and Funae, Y. (1991) Age-dependent expression of cytochrome P-450s in rat liver. *Biochim. Biophys. Acta* **1097**, 187−192.
(40) Ryan, D. E., and Levin, W. (1990) Purification and characterization of hepatic microsomal cytochrome P-450. *Pharmacol. Ther.* **45**, 153−239.
(41) Dietrich, D. R., and Swenberg, J. A. (1991) NCI-Black-Reiter (NBR) male rats fail to develop renal disease following exposure to agents that induce α2u-globulin (α2u) nephropathy. *Fundam. Appl. Toxicol.* **16**, 749−762.
(42) Lehman-Mckeeman, L. D., and Caudill, D. (1994) *d*-Limonene induced Hyaline droplet nephropathy in α2u-globulin transgenic mice. *Fundam. Appl. Toxicol.* **23**, 562−568.
(43) EI−Bayoumy, K. (1994) Evaluation of chemopreventive agents against breast cancer and proposed strategies for future clinical intervention trials. *Carcinogenesis* **15**, 2395−2420.
(44) Crowell, P. L., Kennan, W. S., Haag, J. D., Ahmad, S., Vedejs, E., and Gould, M. N. (1992) Chemoprevention of mammary carcinogenesis by hydroxylated derivatives of *d*-limonene. *Carcinogenesis* **13**, 1261−1264.
(45) Ariyoshi, T., Araki, M., Ideguchi, K., and Ishizuka, Y. (1975) Studies on the metabolism of *d*-Limonene (*p*-Mentha-1,8-diene). III. Effects of *d*-limonene on the lipids and drug-metabolizing enzymes in rat livers. *Xenobiotica* **5**, 33−38.
(46) Guengerich, F. P., and Liebler, D. C. (1985) Enzymatic activation of chemicals to toxic metabolites. *CRC Crit. Rev. Toxicol.* **14**, 259−307.

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