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Roles of CYP2A6 and CYP2B6 in nicotine C-oxidation by human liver microsomes

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Abstract Nicotine C-oxidation by recombinant human cytochrome P450 (P450 or CYP) enzymes and by human liver microsomes was investigated using a convenient high-performance liquid chromatographic method. Experiments with recombinant human P450 enzymes in baculovirus systems, which co-express human nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH)-P450 reductase, revealed that CYP2A6 had the highest nicotine C-oxidation activities followed by CYP2B6 and CYP2D6; the K_m values by these three P450 enzymes were determined to be 11.0, 105, and 132 μM, respectively, and the V_{max} values to be 11.0, 8.2, and 8.6 nmol/min per nmol P450, respectively. CYP2E1, 2C19, 1A2, 2C8, 3A4, 2C9, and 1A1 catalysed nicotine C-oxidation only at high (500 μM) substrate concentration. CYP1B1, 2C18, 3A5, and 4A11 had no measurable activities even at 500 μM nicotine. In liver microsomes of 16 human samples, nicotine C-oxidation activities were correlated with CYP2A6 contents at 10 μM substrate concentration, whereas such correlation coefficients were decreased when the substrate concentration was increased to 500 μM. Contribution of CYP2B6 (as well as CYP2A6) was demonstrated by experiments with the effects of orphenadrine (and also coumarin and anti-CYP2A6) on the nicotine C-oxidation activities by human liver microsomes at 500 μM nicotine. CYP2D6 was found to have minor roles since quinidine did not inhibit microsomal nicotine C-oxidation at both 10 and 500 μM substrate concentrations. These results support the view that CYP2A6 has major roles for nicotine C-oxidation at lower substrate concentration and both CYP2A6 and 2B6 play roles at higher substrate concentrations in human liver microsomes.

Key words Nicotine · CYP2A6 · CYP2B6 · Human P450 · Liver microsomes

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Introduction

Nicotine has been shown to be one of the major constituents in tobacco smoke and to be oxidized by cytochrome P450 (P450 or CYP) enzymes to a nicotine-1′(5′)-iminium ion; this intermediate is further oxidized by cytosolic aldehyde oxidase to form cotinine (Flammang et al. 1992; Benowitz et al. 1996; Messina et al. 1997). Flammang et al. (1992) have reported that of 12 human P450 enzymes expressed in cultured human hepatoma cells (HepG2), CYP2B6 is the most active in catalyzing nicotine C-oxidation activities. These authors also showed that several other forms including CYP2A6, 2C8, 2C9, 2E1, 2F12, and 4B1 have considerable activities, although lower than CYP2B6, for nicotine C-oxidation. Very recently, Messina et al. (1997) have found that CYP2A6 must be the most important enzyme in catalyzing nicotine C-oxidation by human liver microsomes, based on the correlation with activities of coumarin 7-hydroxylation in different human liver microsomal preparations and on the chemical- and immuno-inhibition of the activities catalysed by liver microsomes. However, it is still unclear whether or not other P450 enzymes are significantly involved in the oxidation of nicotine by liver microsomes.

In this study, we further examined the roles of P450 enzymes in the oxidation of nicotine to cotinine by liver microsomes using liver cytosol fraction as a source of aldehyde oxidase activity. The assay method for the detection of cotinine as reported by Messina et al. (1997) was modified. Fourteen forms of recombinant human P450 enzymes in baculovirus systems, which co-express

human nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH)-P450 reductase, were also used to determine which P450 enzymes are very important in the oxidation reaction. Kinetic analysis for the oxidation of nicotine by liver microsomes and by recombinant P450 enzymes and the effects of chemical P450 inhibitors and anti-CYP2A6 antibodies on liver microsomal nicotine C-oxidation activities were also examined.

Materials and methods

Reagents

Nicotine, cotinine, and ketamine were purchased from Sigma Chemical Co. (St. Louis, Mo., USA) and were used without further purification. Other chemicals and reagents used in this study were obtained from sources described previously or were of the highest quality commercially available (Shimada et al. 1997).

Enzyme preparation

Recombinant CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5 and 4A11 were expressed in microsomes of *Trichoplusia ni* cells infected with a baculovirus containing human P450 and NADPH-P450 reductase cDNA inserts. These were obtained from Gentest Co. (Woburn, MA Mass., USA); the P450 contents in these systems were used as described in the data sheets provided by the manufacturer. Human liver samples were obtained from organ donors or patients undergoing liver resection as described previously (Yamazaki et al. 1997; Inoue et al. 1997). For preparation of microsomal and cytosol fractions, the livers were homogenized with 4 vol of 0.1 M TRIS-HCl buffer, pH 7.4, containing 0.1 M KCl and 1 mM ethylenediaminetetraacetic acid (EDTA) in a Potter-type homogenizer. The homogenate was centrifuged at 9000 *g* for 15 min, and the microsomal and cytosol fractions were obtained from the resultant supernatant by centrifugation at 78 000 *g* for 90 min. The microsomal pellet was washed once with the same buffer and finally suspended in 0.01 M TRIS-HCl buffer, pH 7.4 containing 1 mM EDTA and 20% glycerol.

CYP2A6 was purified to electrophoretic homogeneity from human liver microsomes as described (Yun et al. 1991) and rabbit anti-CYP2A6 antibodies were prepared as described (Kaminsky et al. 1981). Rabbit anti-monkey CYP2B17 (Ohmori et al. 1993) and rat CYP2D1 (Imaoka et al. 1991) antibodies were the generous gifts from Dr Shigeru Ohmori of Chiba University Hospital and Dr Yoshihoko Funae of Osaka City University Medical School, respectively.

Enzyme assays

Nicotine C-oxidation activities were determined by the method of Messina et al. (1997) with slight modifications. The standard incubation mixture (final volume of 0.25 ml) contained liver microsomes (0.5 mg protein/ml) or recombinant human P450 (20 pmol/ml), liver cytosol (0.4 mg/ml), 50 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system consisting of 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, 0.5 Unit of glucose 6-phosphate dehydrogenase/ml, and nicotine (10–500 μM). Incubations were carried out at 37 °C for 30 min and terminated by adding 1.5 ml of CH_2Cl_2 and 25 μl of 2 M NaCl containing 1 M Na_2CO_3. Ketamine was added as an internal standard to this solution at final concentration of 40 μM. The organic phase was taken to dryness under a nitrogen stream at 37 °C. The residue was dissolved in 200 μl of 0.01 N HCl. Product formation was determined by high-performance liquid chromatography (HPLC) with a 4.6 × 150 mm Nucleosil octylsilyl (C_8) reverse-phase column (Chemco Scientific, Osaka). The elution was conducted with a mixture of 15% CH_3CN (v/v) containing 20 mM sodium phosphate buffer, pH 4.0 and 1 mM octane sulfonic acid at a flow rate of 0.6 ml/min; detection was by UV absorbance at 259 nm. Coumarin 7-hydroxylation activities were determined as described previously (Yun et al. 1991; Yamazaki et al. 1994).

Other assays

Concentrations of P450 and protein were estimated by the methods of Omura and Sato (1964) and Lowry et al. (1951), respectively. The contents of human P450 proteins in liver microsomes were determined by coupled sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunochemical development (Western blotting; Guengerich et al. 1982). Kinetic parameters for substrate oxidations by recombinant P450 enzymes and human liver microsomes were estimated using a computer program (KaleidaGraph program from Synergy Software, Reading, Pa; USA) designed for nonlinear regression analysis.

Results

HPLC analysis for the detection of nicotine oxidation by human liver microsomes and recombinant human P450 enzymes

Nicotine was incubated with human liver microsomes or by recombinant CYP2A6 in the presence of a liver cytosol fraction (as a source of aldehyde oxidase activity). The product cotinine was separated from nicotine and an internal standard ketamine by HPLC (Fig. 1). Addition of liver cytosol fraction to the incubation mixture

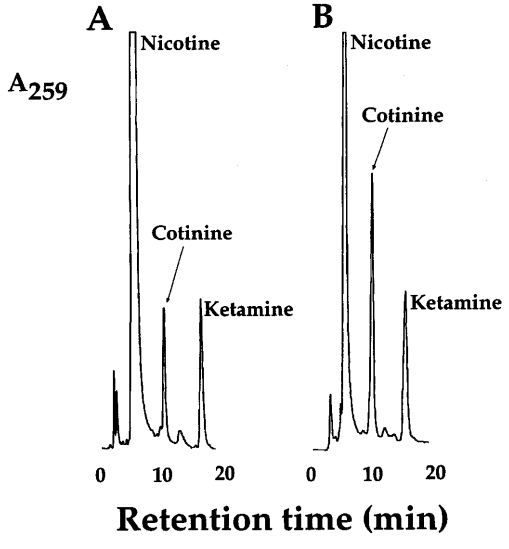

Fig. 1 High-performance liquid chromatography (HPLC) analysis of nicotine C-oxidation by liver microsomes of human sample HL-18 (**A**) and recombinant CYP2A6 (**B**). Standard incubation mixtures containing liver microsomes (0.5 mg protein/ml) or recombinant CYP2A6 (20 pmol/ml) in the presence of liver cytosol (0.4 mg/ml) were used as described in the Materials and methods. Incubations were done at 37 °C for 30 min and terminated by adding 1.5 ml of CH_2Cl_2 containing 33 mM NaCl and 16 mM Na_2CO_3. Ketamine was used as an internal standard by adding final concentration of 40 μM. Other details were as described in the Materials and methods

containing human liver microsomes caused an increase in the formation of cotinine in a concentration dependent manner. The maximal cotinine formation was attained when liver cytosol was added at 0.4 mg protein/ml incubation mixture (Fig. 2). Cotinine formation from nicotine in standard reaction mixture containing human liver microsomes and cytosol increased linearly with time up to 60 min and with microsomal protein concentration up to 0.12 mg/ml (Fig. 3).

Characterization of nicotine C-oxidation by recombinant human P450 enzymes and liver microsomes

Fourteen forms of recombinant human P450 enzymes expressed in baculovirus system co-expressing human NADPH-P450 reductase were used to compare which P450 forms are more active in catalyzing nicotine C-oxidation at substrate concentrations of 50 and 500 μM (Fig. 4). Since Messina et al. (1997) have reported that the apparent K_m value for nicotine C-oxidation by human liver microsomes (n = 31) is 64.9 ± 32.7 μM, we selected the substrate concentration of 50 μM and the tenfold higher concentration of 500 μM in this experiment. At 50 μM nicotine, CYP2A6 should the highest activity in converting nicotine to cotinine, followed by CYP2D6 and 2B6. Other forms of human P450 enzymes gave very low activities at 50 μM nicotine. When the substrate concentration was increased to 500 μM, several P450 enzymes including CYP1A1, 1A2, 2C8, 2C9, 2C19, 2E1, and 3A4 as well as CYP2A6, 2B6, and 2D6, but not CYP1B1, 2C18, 3A5, and 4A11, catalyzed nicotine C-oxidation to varying extents.

The above results suggested that major P450 enzymes involved in nicotine C-oxidation may be CYP2A6, 2B6, and 2D6. Correlations between the contents of CYP2A6, CYP2B6, and CYP2D6 and activities of nicotine C-oxidation by liver microsomes of 16 human samples were examined (Fig. 5). Two substrate concentrations, 10 μM and 500 μM, were used. At low substrate concentration, nicotine C-oxidation activities were correlated (r = 0.75) with the contents of CYP2A6. At 500 μM nicotine, the correlation coefficient between nicotine oxidation and CYP2A6 content was decreased (r = 0.60). None of the CYP2B6 and 2D6 contents showed good correlations with nicotine C-oxidations activities in these liver microsomes at either of the 10 μM and 500 μM concentrations. Kinetic analysis of the nicotine C-oxidation by recombinant CYP2A6, 2B6, and 2D6 showed that K_m values for these enzymes were 11, 105, and 132 μM. V_{max} values were 11, 8.2, and 8.6 nmol/min per nmol P450, respectively (Table 1). Liver microsomes of human sample HL-18 gave two K_m values of 15 and 125 μM for nicotine C-oxidation.

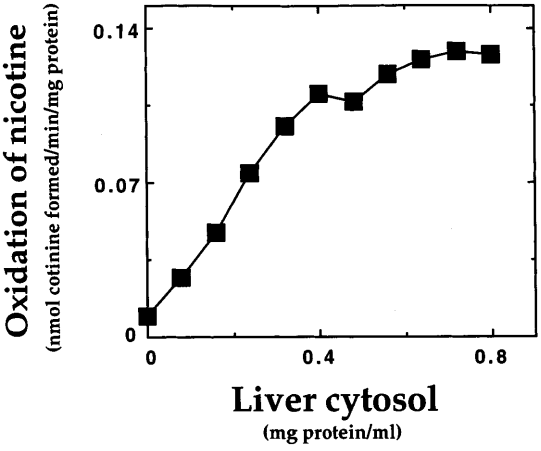

Fig. 2 Effects of liver cytosol on C-oxidation of nicotine by human liver microsomes (human sample HL-18). Standard incubation mixture was used except that liver cytosol proteins ranged from 0 to 0.8 mg protein/ml

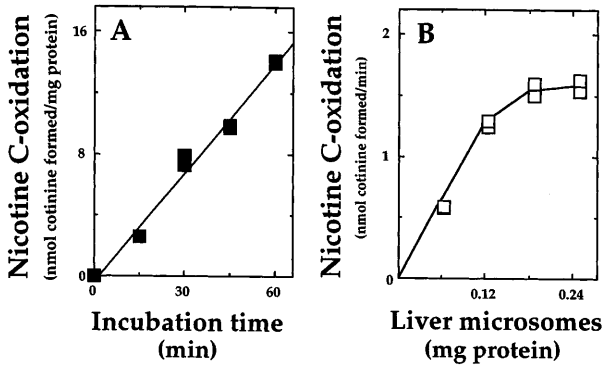

Fig. 3 Effects of incubation time (**A**) and concentrations of microsomal proteins (**B**) on nicotine C-oxidation by human liver microsomes (human sample HL-18) in the presence of liver cytosol fraction (0.4 mg protein/ml). Microsomal protein concentration in **A** was 0.5 mg/ml and incubation time in **B** was 30 min. Data are shown of duplicate determinations

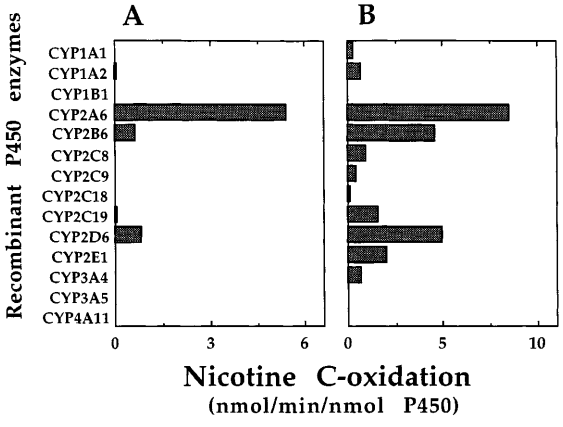

Fig. 4A, B Effects of substrate concentrations on nicotine C-oxidation by recombinant human P450 enzymes. Standard incubation mixtures containing recombinant P450 enzymes (20 pmol/ml) and liver cytosol (0.4 mg/ml) were used except that two nicotine concentrations of 50 μM (**A**) and 500 μM (**B**) were used. Data are means of duplicate determinations

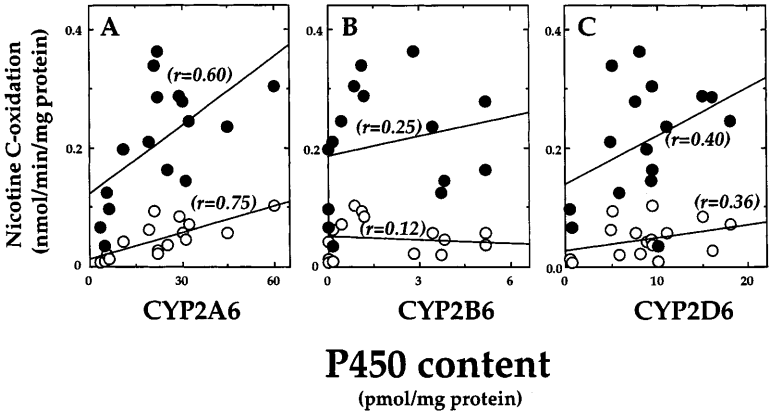

Fig. 5 Relationship between nicotine C-oxidation activities and CYP2A6 (**A**), CYP2B6 (**B**), and CYP2D6 (**C**) contents in liver microsomes of 16 human samples. Nicotine C-oxidation was determined at 10 μM (*open circles*) and 500 μM (*closed circles*) substrate concentrations. Correlation coefficients (*r*) are given

Effects of chemical P450 inhibitors and anti-CYP2A6 antibodies on nicotine C-oxidation by human liver microsomes

Effects of coumarin, orphenadrine, and quinidine, and anti-CYP2A6 immunoglobulin G (IgG) on nicotine C-oxidation activities catalysed by liver microsomes of HL-13 and HL-18 were determined at substrate concentrations of 10 μM and 500 μM nicotine (Table 2). At low substrate concentration, coumarin and anti-CYP2A6 IgG were very effective in inhibiting nicotine C-oxidation by liver microsomes. Both orphenadrine and quinidine did not inhibit nicotine metabolism at 10 μM substrate concentration. However, when 500 μM nicotine was used, orphenadrine and coumarin inhibited nicotine C-oxidation by 52 and 33%, respectively, in sample HL-13 and by 56 and 59%, respectively, in sample HL18. Quinidine did not inhibit the activities even at 500 μM nicotine. Anti-CYP2A6 IgG inhibited the microsomal nicotine C-oxidation activities at 500 μM although the inhibitory effects were less than those seen at 10 μM nicotine.

Discussion

Several carcinogenic chemicals have been identified in tobacco smoke and these carcinogens have been suggested to be one of the major causes of respiratory diseases including lung cancer in humans (Hecht et al. 1981; Hecht 1994, 1996). Tobacco-related nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and N′-nitrosonornicotine (NNN), have been reported to be the constituents in tobacco smoke causing human cancers (Hoffmann and Hecht, 1985; Hecht and Hoffmann 1988; Hecht 1996). These chemicals require metabolic activation by P450 enzymes in order to evoke their carcinogenic potentials in mammalian cells; CYP2A6, CYP2E1, and CYP2D6 have been reported to be involved in the activation process (Yamazaki et al. 1992; Hecht 1994, 1996; Crespi et al. 1991; Tiano et al. 1993, 1994). Since these P450 enzymes have been reported to show genetic polymorphisms in humans, it is believed that the genetic defects in P450 genes may influence the susceptibilities of individuals towards various types of cancers.

Nicotine has also been shown to be present in tobacco smoke and to have pharmacological and physiological effects involving the central and peripheral nervous systems (Hoffmann and Hecht 1985; Messina et al. 1997; Pianezza et al. 1998). This chemical has been reported to be oxidized by P450 enzymes to form nicotine-1′(5′) imminium ion and this intermediate metabolite is further oxidized by a cytosolic aldehyde oxidase to form the stable metabolite cotinine in mammals (Williams et al. 1990a, b). In this study, we used liver cytosol fraction as a source of aldehyde oxidase activity and found that 0.4 mg of cytosol protein was required to convert the nicotine-1′(5′) imminium ion to cotinine. The slightly modified HPLC method for the detection of nicotine and cotinine reported by Messina et al. (1997) (C8 column instead of C6 column, detection at 259 nm instead of 210 nm, and 15 instead of 20% CH_3CN for the elution) gave good separation using this method. The limit of detection in this assay system was defined to be 100 pmol cotinine/ml of incubation mixture and the efficiency of extraction of the cotinine was ~90%.

The present results supported the view that CYP2A6 is one of the major enzymes involved in nicotine C-oxidation catalyzed by human liver microsomes according to the following lines of evidence. Of fourteen forms of

Table 1 Kinetic analysis of nicotine C-oxidation by recombinant CYP2A6, 2B6, and 2D6 enzymes and liver microsomes sample of HL-18. Nicotine oxidation activities were determined at different nicotine concentrations between 3.2 and 500 μM. Data presented are means ± SE

	K_m (μM)	V_{max} (nmol/min per nmol P450)
CYP2A6	11 ± 2	11.0 ± 2.1
CYP2B6	105 ± 25	8.2 ± 2.1
CYP2C6	132 ± 31	8.6 ± 3.3
HL-18	15 ± 3	0.19 ± 0.1
	125 ± 22	0.29 ± 0.3

Table 2 Effects of coumarin, orphenadrine, and a quinidine, and anti-CYP2A6 immunoglobulin G (IgG) on nicotine oxidation catalyzed by human liver microsomes at substrate concentrations of 10 μM (*upper panel*) and 500 μM (*lower panel*). Nicotine C-oxidation activities were determined at nicotine concentrations of 10 μM or 500 μM. Data are means of triplicate determinations ± SD

Addition	Concentration	Nicotine C-oxidation	
		HL-13	HL-18
	(μM)	(nmol/min per mg protein)	
None	–	0.055 ± 0.005	0.084 ± 0.008
Coumarin	25	0.013 ± 0.003*	0.011 ± 0.002*
Orphenadrine	300	0.045 ± 0.005	0.078 ± 0.009
Quinidine	5	0.057 ± 0.006	0.082 ± 0.010
	(mg IgG/nmol P450)		
Preimmune IgG	2	0.055 ± 0.002	0.085 ± 0.003
Anti-CYP2A6	1	0.015 ± 0.002*	0.025 ± 0.003*
Anti-CYP2A6	2	0.009 ± 0.002*	0.012 ± 0.002*
	(μM)	(nmol/min per mg protein)	
None	–	0.171 ± 0.022	0.224 ± 0.028
Coumarin	25	0.114 ± 0.025*	0.091 ± 0.012*
Orphenadrine	300	0.082 ± 0.011*	0.098 ± 0.019*
Quinidine	5	0.167 ± 0.026	0.242 ± 0.030
	(mg IgG/nmol P450)		
Preimmune IgG	2	0.181 ± 0.023	0.232 ± 0.033
Anti-CYP2A6	1	0.120 ± 0.012*	0.105 ± 0.013*
Anti-CYP2A6	2	0.096 ± 0.022*	0.092 ± 0.032*

* $P < 0.01$, as compared with control (None group)

recombinant human P450 enzymes examined, CYP2A6 was found to be the most active in catalyzing nicotine C-oxidation at low substrate concentration. CYP2B6 and 2D6 were also active in nicotine C-oxidation, but had lower activity than CYP2A6. Kinetic analysis showed that CYP2A6 gave the lowest K_m value (11 μM) compared with CYP2B6 (105 μM) and CYP2D6 (132 μM) for nicotine C-oxidation activities. Using liver microsomes from 16 human samples, contents of CYP2A6, but not CYP2B6 and 2D6, were found to be well correlated with nicotine C-oxidation activities by liver microsomes at 10 μM nicotine concentration. In addition, nicotine C-oxidation activities catalyzed by human liver microsomes were inhibited by coumarin and anti-CYP2A6 antibodies very significantly at low substrate concentration.

The results presented in this study also support the suggestion that CYP2B6 as well as CYP2A6 is involved in nicotine C-oxidation by human liver microsomes particularly at high substrate concentrations. The K_m value (125 μM) for the low-affinity component in human liver microsomes (HL-18) was similar to that (105 μM) of recombinant CYP2B6 (Table 1). The inhibition in human liver microsomes of nicotine C-oxidation by orphenadrine, a relatively selective inhibitor of CYP2B6 (Reidy et al. 1989; Stevens et al. 1997), supports the above suggestion. At different substrate concentrations in recombinant P450 enzyme systems, CYP2D6 was also found to have nicotine C-oxidation activities, having K_m and V_{max} values of 132 μM and 8.6 nmol/min per nmol P450; these values were very similar to those catalyzed by recombinant CYP2B6. However, contribution of CYP2D6 to nicotine oxidation may be minor, because quinidine, a potent inhibitor of CYP2D6 (Guengerich and Shimada 1991; Ching et al. 1995; Strobl et al. 1993), did not inhibit the activities catalyzed by human liver microsomes at both 10 and 500 μM nicotine concentrations. Recent in vivo studies by Benowitz et al. (1996) have also shown that CYP2D6 is not a major P450 form involved in the metabolism of nicotine and cotinine.

In reconstituted systems containing 15 forms of rat P450 enzymes, CYP2B1 has been reported to have the highest activities for nicotine oxidation, followed by CYP2C11, CYP1A2, CYP2B2, and CYP2D1 (Nakayama et al. 1993). The authors also found that neither CYP2A1 nor 2A2 catalyzed conversion of nicotine to cotinine in reconstituted systems. Marked species-related differences in testosterone 7α-hydroxylation and coumarin 7-hydroxylation activities by CYP2A enzymes in rat and man have been reported in several laboratories (Yamazaki et al. 1994; Pearce et al. 1992; Fernandez-Salguero and Gonzalez 1995). Nicotine C-oxidation is presumably another case for species-related differences in the catalytic roles of CYP2A enzymes in rat and human liver microsomes. It is interesting to note in this context that rabbit CYP2A enzymes catalyze nicotine oxidation in nasal and lung tissues (Williams et al. 1990a, b).

In conclusion, the present results support the suggestion that CYP2A6 is a major enzyme involved in nicotine C-oxidation catalyzed by human liver microsomes. Recombinant CYP2B6 was also found to be involved in nicotine C-oxidation, particularly at high substrate concentration. While recombinant CYP2D6 catalyzed nicotine C-oxidation at significant levels, the contribution of this enzyme may be minor because a potent inhibitor of CYP2D6, quinidine, did not affect the microsomal nicotine C-oxidation activities. CYP2A6 and 2B6 are found to be expressed in respiratory organs as well as in liver and these two P450 forms may have roles for tobacco-related biological effects in humans.

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