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Purification and Characterization of the Rat Liver CYP2D1 and Utilization of Reconstituted CYP2D1 in Caffeine Metabolism

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***ABSTRACT** : In order to assess the possibility whether CYP2D is involved in caffeine metabolism, we have purified and characterized the rat liver microsomal cytochrome P4502D1 (CYP2D1), equivalent to CYP2D6 in human liver, and have utilized the reconstituted CYP2D1 in the metabolism of 4 primary caffeine (1,3,7-trimethylxanthine) metabolites such as paraxanthine (1,7-dimethylxanthine), 1,3,7-trimethylurate, theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine). Rat liver CYP 2D1 has been purified to a specific content of 8.98 nmole/mg protein (13.4 fold purification, 1.5% yield) using ω-aminooctylagarose, hydroxlapatite, and DE52 columns in a sequential manner. As judged from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the purified CYP2D1 was apparently homogeneous. Molecular weight of the purified CYP2D1 was found to be 51,000 Da. Catalytic activity of the purified and then reconstituted CYP2D1 was confirmed by using bufuralol, a known substrate of CYP2D1. The reconstituted CYP2D1 was found to produce to 1-hydroxylbufuralol at a rate of 1.43±0.13 nmol/min/nmol P450. The kinetic analysis of bufuralol hydroxylation indicated that Km and Vmax values were 7.32 μM and 1.64 nmol/min/nmol P450, respectively. The reconstituted CYP2D1 could catalyze the 7-demethylation of PX to 1-methylxanthine at a rate of 12.5 pmol/min/pmol, and also the 7- and 3- demethylations of 1,3,7-trimethylurate to 1,3-dimethylurate and 1,7-dimethylurate at 6.5 and 12.8 pmol/min/pmol CYP2D1, respectively. The reconstituted CYP2D1 could also 3-demethylate theophylline to 1-methylxanthine at 5 pmol/min/pmol and hydroxylate the theophylline to 1,3-dimethylurate at 21.8 pmol/min/pmol CYP2D1. The reconstituted CYP2D1, however, did not metabolize TB at all (detection limits were 0.03 pmol/min/pmol). This study indicated that CYP2D1 is involved in 3- and 7-demethylations of paraxanthine and theophylline and suggested that CYP2D6 (equivalent to CYP 2D1 in rat liver) present in human liver may be involved in the secondary metabolism of the primary metabolites of caffeine.*

***Key Words** : Purified CYP2D1, Caffeine metabolism*

I. INTRODUCTION

Drug biotransformation catalyzed by many hepatic drug metabolizing enzymes is a major factor in determining the therapeutic or toxic outcome for many clinically important drugs. In human, several of these hepatic drug metabolizing enzymes are known to have genetic polymorphism and to have differential influences on metabolism, half-life, therapeutic, toxic and carcinogenic effect of drugs (Gonzalez and Idle, 1994).

Among the many drug oxidizing enzymes, cytochrome P450 (CYP) is known to be one of the most important enzymes involved in the oxidative drug biotransformation and is a super-family of multi-isoenzymes (Nelson *et al.*, 1993). CYP2D6 isoform is one of the P450 enzymes which are expressed polymorphically in human and is involved in catalyzing more than 30 important clinically useful drugs (Mahgoub *et al.*, 1977). The phenotypic polymorphism of CYP2D6 revealed that it is responsible for the highly variable activities in metabolizing sparteine and debrisoquine among different individuals. In the white European and Caucasian populations, about 5~10% of individuals show poor metabolic activities and the remainders are phenotyped to be extensive metabolizers for these drugs (Agundez *et al.*, 1995). In

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contrast, only less than 2% of the Oriental citizenry are phenotyped as poor metabolizers (Bertilsson *et al.*, 1992).

For studies regarding differential CYP2D6 activities in Koreans, it is necessary to determine the variable hepatic expressions of CYP2D6 among individuals, and it is necessary to utilize the antibody which has cross-reactivity against the human liver CYP2D6. However, it was not possible to obtain large quantities of human liver tissues from which we could purify CYP2D6 and utilize the purified protein in raising antibodies. Thus, alternatively, we sought to purify the rat liver CYP2D 1, an orthologous isoform with a 79% of amino acid sequence homology (Matsunaga *et al.*, 1989) with that of human liver CYP2D6, and use the purified CYP2D1 to raise its antibody by injection into rabbit. The harvested CYP2D1 antibody could then be used as the probe for detection of CYP2D6 expression in Koreans in order to assess the ability of Koreans in metabolizing many clinically useful drugs.

Thus, we have purified and characterized the rat liver CYP2D1 as the initial step for eventual genetic study on CYP2D6 in Koreans. In this study, we report that the purified and reconstituted CYP2D1 can catalyze the metabolism of primary caffeine metabolites such as theophylline (TP), paraxanthine (PX), and 1,3,7-trimethylurate (137U), which are produced from caffeine. These primary metabolites of caffeine are both demethylated and hydroxylated. These secondary metabolic products are formed at a rate of 5~21 pmol/min/pmol CYP2D1. This is the first report indicating that CYP2D isoform could be involved in further metabolism of caffeine.

II. MATERIALS AND METHODS

1. Chemicals and Materials

(±)-Bufuralol and 1-hydroxybufuralol used as the substrate of CYP2D1 and its metabolite standard were purchased from Gentest (Woburn, MA, USA). The purified rat liver NADPH-P450 reductase utilized in the reconstitution of functional CYP2D1 was obtained from Oxoford Biomed. Res. (Oxoford, MI, USA). The column materials like ω-aminooctylagarose, phenylmethylsulfonyl fluoride (PMSF), and l-α-dialuroyl-sys-glycero-3-phosphocholine were obtained from Sigma (St. Louis, MO, USA), hydroxlapatite was from Calibiochem-Behring Corp. (La Jolla, CA, USA), and DE52 was from Whatman (Springfield, ME, USA). Spectro/por membrane (MWCO: 25,000) was purchased from Spectrum (Houston, TX, USA), Diaflo ultrafiltration membrane (PM30) from Amicon (Beverly, MA, USA), 4~20% gradient tris-glycine gel from Novex (San Diego, CA, USA), and prestained protein molecular weight standard from Gibco BRL (New York, NY, USA).

2. Purification of CYP2D1

The purification of CYP2D1 from rat liver microsomes was conducted as described by Gut *et al.*, (1986) with slight modifications. Unless otherwise mentioned, all procedures for the purification of CYP2D1 were carried out at 4°C.

1) Preparation of Microsomes

Livers were taken from male Sprague-Dawley rats (250 g) and homogenized in ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 0.15 M KCl and 1 mM EDTA. Microsomes were prepared by an initial centrifugation at 9,000 g for 30 min at 4°C, with subsequent centrifugation of the supernatant at 100,000 g for 60 min. The microsomal pellet was washed and recentrifuged in the homogenization buffer and resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA and then stored at -70°C until used.

2) Solubilization of Microsomes

Rat liver microsome (5000 mg) was solubilized into 2 mg/ml with a stepwise addition of sodium cholate solution (finally 0.6%) in 0.1M potassium phosphate buffer containing 1 mM EDTA, 0.4 mM PMSF and 20% glycerol. Solubilized microsomes were stirred for 30 min and centrifuged at 100,000 g for 1hr to collect supernatant which was used for the next purification step.

3) ω-aminooctylagarose Step

About 200g of ω-aminooctylagarose (AOA) resin was preequilibrated overnight with the *AOA equilibrium buffer* (0.1 M potassium phosphate, pH 7.25, containing 1 mM EDTA, 20% glycerol and 0.6% sodium cholate) and packed. The solubilized microsomes (2500 mg×2) were applied to two prepacked AOA columns (2.6×40 cm each, 212 cm^3) at a flow rate of 60 ml/hr. Subsequently, the column was washed initially with 250 ml of the *AOA washing buffer* (AOA equilibrium buffer containing 0.42% sodium cholate) at the flow rate of 50 ml/hr and was then eluted with 830 ml of the *AOA P450 elution buffer* (AOA washing buffer containing 0.33% sodium cholate and 0.06% Lubrol) at the flow rate of 43 ml/hr. The column was eluted finally with 930 ml of the *AOA P4502D1 elution buffer* (AOA washing buffer containing 0.33% sodium cholate and 0.5% Lubrol) at a flow rate of 40 ml/hr and 13 ml fractions were collected. UV absorbance was monitored at 280 nm for the eluted protein and at 417 nm for P450. As we followed the purification method of Gut *et al.* (1986), the fractions containing P450 were combined into three portions and their aliquots were assayed for bufuralol 1-hydroxylation, to identify the portion containing CYP2D1.

4) Hydroxylapatite Step

The combined fraction containing CYP2D1 was then dialyzed with 1 *l* of HAP equilibrium buffer (5 mM potassium phosphate, pH 7.25, containing 1 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol, 0.2% sodium cholate and 0.2% Lubrol) at 4°C for 18 hr. The dialyzed CYP2D1 containing portion was then loaded to the prepacked HAP column (1.6×20 cm, 40 cm^3) at a flow rate of 25 ml/hr. The hydroxylapatite column was prepared with 12 g of hydroxylapatite (HAP) resin which had been preequilibrated overnight with 250 ml of the HAP equilibrium buffer. Subsequently, the column was washed with 50 ml of the HAP equilibrium buffer at the flow rate of 25 ml/hr and the CYP2D1 was eluted with a linear gradient of potassium phosphate concentrations ranging from 5 to 180 mM in a total volume of 300 ml of the HAP equilibrium buffer and finally with another 280 ml of 180 mM potassium phosphate buffer at a flow rate of 60 ml/hr. The collected fractions were monitored for their protein and P450 contents as well as the bufuralol hydroxylation activity as described above. Again the factions containing CYP2D1 were combined.

5) DE52 Step

The combined CYP2D1 fraction obtained from HAP column was dialyzed again with 1 *l* of DE52 equilibrium buffer (5 mM potassium phosphate, pH 7.8, containing 1 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol, 0.2% sodium cholate and 0.2% Lubrol) at 4°C for 18 hr. The dialyzed CYP2D1 was then loaded to the DE52 column (1×20 cm, 15 cm^3) at a flow rate of 25 ml/hr. The DE52 column was prepared with 15 g of diethylaminoethyl 52 cellulose (DE52) resin which had been preequilibrated overnight with 80 ml of the DE52 equilibrium buffer. The column was then washed with 75 ml of the DE52 equilibrium buffer at a flow rate of 25 ml/hr and the CYP2D1 was eluted with a linear gradient of potassium chloride concentrations ranging from 0 to 0.25 M in a total volume of 400 ml of the DE52 equilibrium buffer and finally with another 100 ml of 0.25 M potassium chloride in DE52 equilibrium buffer at a flow rate of 25 ml/hr. The collected fractions were monitored for their protein and P450 contents as described above.

3. SDS-PAGE of the CYP2D1

To determine the molecular weight of the purified CYP2D1, 10 μg of the purified protein was loaded onto 4~20% gradient Tris-glycine gel and separated by electrophoresis under the constant power supply (200 V). Prestained protein molecular weight standards were used to identify the molecular weight of the purified CYP2D1 and the CYP2D1 protein on the gel was stained using coomassie brilliant blue staining solution.

4. Enzyme Assay Methods

Metabolism of primary caffeine metabolites such as theobromine (TB), TP, PX, and 137U by the purified CYP2D1 was conducted with reconstituted P 450 system which was composed with 10~50 pmol

of the purified CYP2D1, 50 pmol of the purchased P450 reductase, 10 μmol of L-α-dialuroyl-sys-glycero-3-phosphocholine, 1 mM NADPH in 0.1 M phosphate buffer (pH 7.4). Metabolic incubations were carried out with 10 mM TB, TP, PX, or 137U at 37°C for 30 min. After termination of the metabolic reaction in ice-cold waterbath, proteins were removed by 30 min centrifugation at 20,000 g.

The supernatants obtained from centrifugation were injected into HPLC and analyzed to determine the amounts of the secondary metabolites produced from various substrates employed. The HPLC system consisted of Waters 996 photo diode array detector and a Nova-pak C-18 column (3.9× 300 mm, 4 μm). The mobile phase for the elution of HPLC column was 5% methanol containing 0.05% acetic acid (A) and 70% methanol (B). Metabolites were separated by an initial 8-min isocratic elution with solution A and a subsequent 4-min linear gradient from 0% to 30% of solution B and continued with an 18-min isocratic elution with solution B. The flow rate was set at 0.6 ml/min. The eluate was screened at 200 to 300 nm for qualitative analyses and then monitored at 280 nm for quantitative analyses of the eluted metabolites.

Bufuralol 1-hydroxylase activity catalyzed by the purified CYP2D1 was determined as described by Mimura *et al.* (1994). Protein determination was carried out as described by Lowry *et al.* (1951) and P450 content was determined by the method of Omura and Sato (1964).

III. RESULTS

1. Purification of CYP2D1

The result of purification of the rat liver CYP2D1 is summarized in Table 1. Because P450 is a membrane-anchored protein, it needs to be solubilized for the purification. After 5 g of rat liver microsome is solubilized in 0.6% cholate detergent solution, only 7% of the total amount of P450 was lost but the specific P450 content remained constant. The nature of protein purification using AOA resin depends on the hydrophobicity of the target protein and the hydrophobic proteins are strongly adsorbed to the AOA resin. As the loaded AOA column is eluted with increasing detergent gradient, hydrophilic proteins are eluted earlier and hydrophobic proteins are eluted later. CYP2D1 is very hydrophobic protein and thus, it is collected at the last pool of P450 fractions. Their absorbencies were monitored at 417nm and they showed a high bufuralol 1-hydroxylase activity, a CYP2D1 specific enzyme activity (Fig. 1). The recovered CYP2D1 fraction represented 19% of the total microsomal P450 content and the specific content of P450 after the AOA column was 1.86 nmol/mg protein.

The next strategy we employed for the purification was to utilize the HAP resin. The resin is composed of $CaPO_4$ to which proteins bind avidly. The combined CYP2D1 containing protein obtained from the AOA column was applied to HAP

Table 1. The summary of purification of the rat liver microsomal CYP2D1

Step	Protein (mg)	Specific P450 (nmol/mg)	Total P450 (nmol)	Yield (%)
Microsomes	5044	0.67	3379	100
Sol. Microsomes	4700	0.68	3196	93
ω-aminooctylagarose	348	1.86	647	19
Hydroxylapatite	57	3.52	200	6
DE-52	6	8.98	53	1.5

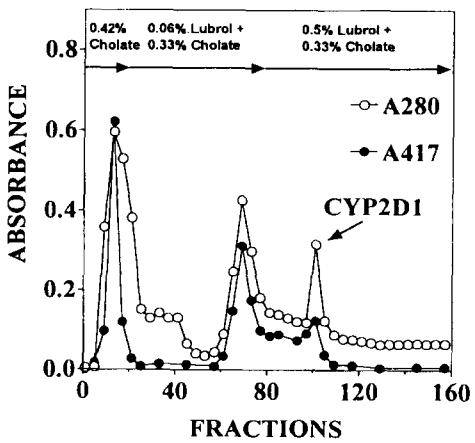

Fig. 1. Elution profile from the ω-aminooctylagarose (AOA) column employed for the purification of the rat liver microsomal CYP2D1.
AOA washing buffer was used to elute fractions 1 to 20. AOA P450 elution buffer was used to elute fractions 21~85 and AOA P450 2D1 buffer for elution of fractions 86~158. Each collected fraction was 12 ml. UV absorbance was monitored at 280 nm to estimate the protein contents and at 417 nm for P450 contents. Fractions 99~103 were combined for further purification of CYP2D1.

resin column and the resin bound P450 protein was eluted in a gradient of phosphate buffer ranging from 5 to 180 mM. The recovered CYP2D1 fraction yielded 5.9% of the total micosomal P450 content and the specific content of P450 after the HAP column was 3.52 nmol/mg protein and this represented 5.9 fold purification from the microsome state (Fig. 2).

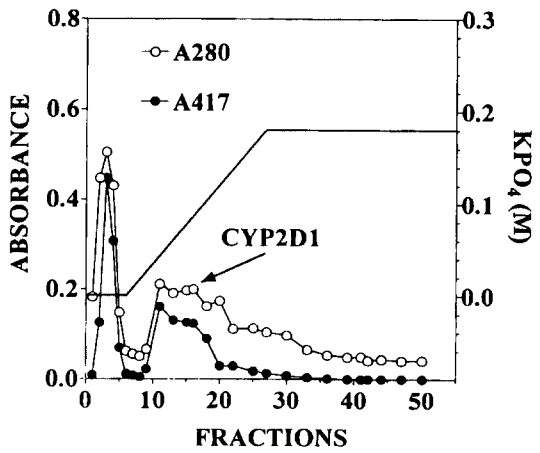

Fig. 2. Elution profile from the hydroxylapatite (HAP) column.
HAP equilibrium buffer was used to elute fractions 1 to 6. A linear potassium phosphate gradient ranging from 5 to 180 mM in a volume of 300 m*l* was started at fraction 7. The volume of each collected fraction was 12 m*l*. Fractions 9~21 were combined for further purification of CYP2D1.

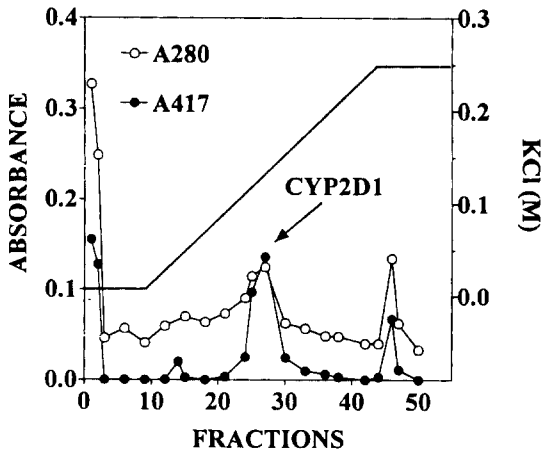

Fig. 3. Elution profile from the Diethylaminoethyl cellulose 52 (DE52) column.
DE equilibrium buffer was used to elute from fractions 1 to 8. A linear potassium chloride gradient ranging from 0 to 0.25 M in a volume of 400 m*l* was started at fraction 9. The volume of each collected fraction was 12 m*l*. Fractions 24~35 were combined and considered as purified CYP2D1.

The final step utilized for purification was the use of DE52 resin, an anion exchanger. Thus, the strength of P450 protein binding to DE52 resin is determined by its (-) charge and the elution of CYP 2D1 bound to DE52 resin is accomplished with KCl gradient ranging from 0 to 0.25 M. The CYP 2D1 fraction was eluted at around 0.12 M potassium chloride. Finally, the rat liver CYP2D1 has been purified to a specific content of 8.98 nmole/mg protein with 13.4 fold purification and 1.5% yield (Fig. 3).

2. Purity and Molecular Weight of CYP2D1

The purified CYP2D1 was apparently homogeneous as judged from SDS-PAGE. The molecular weight of purified CYP2D1 was found to be 51,000 Da upon comparing its mobility on the SDS-PAGE with those of standard molecular weight markers (Fig. 4).

3. Catalytic Activity of CYP2D1

In order to confirm the subsistence of catalytic activity in the purified CYP2D1, the catalytic activity of the reconstituted CYP2D1 was determined by measuring the hydroxylation of (±)-bufuralol, a

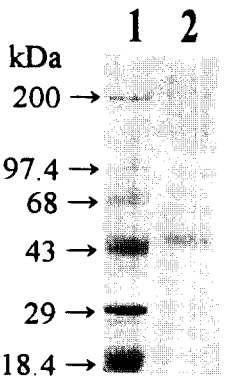

Fig. 4. SDS-PAGE of the purified CYP2D1.
Polyacrylamide concentration gradient ranged from 4 to 20%. In lane 1, the prestained protein molecular weight standard was applied. In lane 2, 15 μg of the purified CYP2D1 obtained from the DE52 column was applied. The prestained protein molecular weight standards employed were myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and β-lactoglobulin (18.4 kDa). The molecular weight of the purified CYP2D1 was found to be 51 kDa.

Table 2. Metabolism of paraxanthine, theophylline, 1,3, 7,-trimethylurate and theobromine by reconstituted CYP 2D1

Substrate / Product	Parax-anthine (17X)	Theo-phylline (13X)	1,3,7,-tri-methy-lurate (137U)	Theo-bromine (37X)
1-methylxanthine (1X)	12.5	5.0	–	–
1,3-dimethylurate (13U)	–	21.8	6.5	–
1,7-dimethylurate (17U)	ND	–	12.8	–
3-methylxanthine (3X)	–	ND	–	ND
	ND	–	–	ND

Reactions were duplicated and the detailed procedure is illustrated in materials and methods.
Unit: pmol/min/pmol CYP2D1.
ND: not detectable (detection limits: at least 0.03 pmol/min/pmol CYP2D1).
–: not only 'not detectable' but also 'not expected from either demethylation or hydroxylation of the employed substrate'.

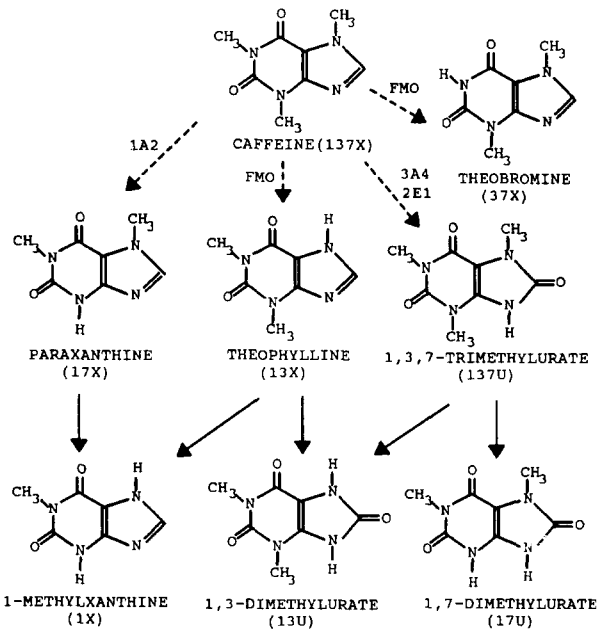

Fig. 5. Secondary metabolism of the primary caffeine metabolites by reconstituted CYP2D1.
Broken arrows indicate the primary metabolic pathways of caffeine showing the involved enzyme which have been already studied. Solid arrows indicate the secondary metabolic pathways in which the involvement of CYP2D1 was found in this study.

known substrate of CYP2D1. Reconstituted CYP 2D1 catalyzed bufuralol to 1-hydroxylbufuralol at a rate of 1.43±0.13 nmol/min/nmol CYP2D1. In kinetic analysis of bufuralol hydroxylation catalyzed by the reconstituted CYP2D1, Km and Vmax values were found to be 7.32 μM and 1.64 nmol/min/nmol CYP2D1, respectively.

The reconstituted CYP2D1 system was employed in metabolic studies utilizing the primary metabolites of caffeine as substrates to substantiate the involvement of CYP2D1 in the secondary further metabolism of caffeine. The reconstituted CYP2D1 was found to catalyze the N3-demethylations of 137U and TP, and also the N7-demethylations of PX and 137U (Table 2). Also the reconstituted CYP 2D1 hydroxylated TP at C-8 position. Surprisingly, the reconstituted CYP2D1 could not metabolize the TB to any appreciable degree. Based on these results, potential involvement of CYP2D1 in the secondary further metabolism of the primary metabolites of caffeine has been summarized in Fig. 5.

IV. DISCUSSION

Caffeine (1,3,7-trimethylxanthine), contained in coffee, undergoes metabolic oxidation by N-demethylation at each of the 3-tertiary amine nitrogen atoms and is known to be metabolized to TB, PX and TP by the hepatic microsomal enzymes. In addition to the demethylation, the microsomal oxidase is also capable of producing 137U by C-8 hydroxylation of caffeine. When caffeine is ingested, it undergoes extensive biotransformations and generates at least 17 metabolites which are detectable in human urine (Rost and Roots, 1994). Among these 17 urinary metabolites of caffeine, PX is the most abundant metabolites and the production of PX is known to be catalyzed by CYP1A2. Thus, the rate of PX production from caffeine has been used as a convenient probing method to phenotype humans for their CYP1A2 activities (Nakajima *et al.*, 1994; Kalow and Tang, 1991).

Attempts are being made by several laboratories including this one to identify the various enzymes involved in the production of these caffeine metabolites for the purpose of non-invasive phenotyping of humans. In this connection, Zang and Kaminsky (1995) indicated that CYP2D6 is potentially involved in the secondary metabolism of TP, the primary metabolite of caffeine. As CYP2D6

is known to be one of the most abundant CYPs present in human liver which is expressed polymorphically (Mahgoub *et al.*, 1977), and is also responsible for the differential metabolism of more than 30 clinically useful drugs among different individuals, we wished eventually to study their genetic polymorphism in Korean people.

As an initial step for eventual genetic study of CYP2D6 in Koreans, we wanted to purify the Korean CYP2D6 and obtain antibody that demonstrates cross-reactivity against CYP2D6. However, due to limited availability of human liver tissues required for purification of CYP2D6, as an alternative source, we decided to utilize the rat liver tissue and purify the CYP2D1 instead. The rat liver CYP2D1 is known to have 79% homology in its amino acid sequence with that of human liver CYP2D6 (Matsunaga *et al.*, 1989), and thus, if purified in quantity, the CYP2D1 will become useful in raising functional antibody to CYP2D6 to detect its expression in Koreans.

The purified CYP2D1 was apparently homogeneous as judged from SDS-PAGE. The molecular weight of purified CYP2D1 was found to be 51,000 Da based on its mobility in SDS-PAGE. Gut *et al.*, (1986) reported that the molecular weight of the purified CYP2D1 was 50,000 Da. SDS-PAGE has been the traditional convenient method utilized for assessment of the molecular weight of the purified P450s. This estimation, however, produces some variations up to 3000 Da. Thus, differences of 1000 Da in the estimated molecular weight of the purified CYP2D1 is not a critical factor and furthermore, because the reconstituted CYP2D1 had catalytic activity towards bufuralol, a known substrate of CYP2D, the purified P450 was functional.

Ingested caffeine is known to undergoes extensive biotransformations in human resulting in the generation of at least 17 different metabolites detectable in urine (Rost and Roots, 1994). Enzymes known to be involved in caffeine metabolism are CYP1A2, 2A6, 2E1, 3A4, 3A5, N-acetyltransferase2 and xanthine oxidase (Rostami-Hodjegan *et al.*, 1996) and flavin-containing monooxygenase (Chung *et al.*, 1997). Studies for identification of the CYP enzymes which could be responsible for conversion of caffeine to TB, PX, TP and 137U have been attempted in several laboratories including this one (Berthou *et al.*, 1991; Butler *et al*, 1989; Chung *et al*, 1997; Fuhr *et al*, 1992; Kalow and Tang, 1991; Tassaneeyakul *et al*, 1994). However, attempts to identify the CYP enzymes involved in the secondary metabolism of these primary caffeine metabolites is very rare, except for the metabolic study on TP (Zhang and Kaminsky, 1995) which is not only a primary metabolite of caffeine but also a drug used clinically for the control of asthma. In the metabolic transformation of TP to 1,3-dimethylurate, CYP2E1, 2D6 and 1A2 are known to be involved and the catalytic activity of CYP2D6 has been found to be 5 times more potent than that of CYP1A2 (Zhang and Kaminsky, 1995).

In an indirect effort to determine if CYP2D6 is also involved in the secondary further metabolism of the primary caffeine metabolites, we have endeavored to determine the involvement of CYP2D1 in the metabolism of primary caffeine metabolites using PX, TP, 137U and TB. After the conversion of caffeine to PX by CYP1A2 in human, PX is known to be hydroxylated at its C-8 position to produce 1,7-dimethylurate as the major metabolite by CYP1A2. The purified and reconstituted CYP2D1 did demethylate the PX at its N-7 position to produce 1-methylxanthine but did not hydroxylate the PX to 1,7-dimethylurate. As expected from the study of Zhang and Kaminsky (1995), the reconstituted CYP2D1 hydroxylated TP to 1,3-dimethylurate at a high rate. This fact implied that not only CYP1A2 but also other CYP isozymes like 2E1 and 2D6 may contribute to caffeine metabolism in human and suggested the possibility that specific secondary pathways of caffeine metabolisms may also be utilized as probes to determine the phenotypic activities for other enzymes like CTP2D6. Thus, the value of caffeine as a non-invasive probe to phenotype humans for their drug metabolizing enzymes could be more significant than expected.

Several researchers have used the value of (PX+ 1,7-dimethylurate)/(caffeine) in urine as the index of CYP1A2 activity (Butler *et al.*, 1992; Nakajima *et al.*, 1994; Chung *et al.*, 1997). As shown by the result in Table 2, the reconstituted CYP2D1 metabolized 137U to 1,7-dimethylurate at a considerable rate. While 137U is found to be one the

major metabolite produced upon incubation of caffeine with human liver microsomes, it is found only in minor amounts in human urine after taking caffeine (Nakajima *et al.*, 1994). Therefore, it is possible that the 1,7-dimethylurate detected in significant amounts in human urine may have been generated from the 137U by CYP2D6 action rather than from the PX by CYP1A2. This may help explain the reason that the urinary ratio of (PX+1,7-dimethylurate)/(caffeine) used as the index of CYP 1A2 activity, although the index is highly correlated, is not consummate in representing the CYP1A2 activity.

TB (3,7-dimethylxanthine) has a similar structure to those of PX (1,7-dimethylxanthine) and TP (1,3-dimethylxanthine). However, reconstituted CYP2D1 did not demethylate or hydroxylate TB. The underlying reasons for the inability of CYP2D1 to metabolize TB remain elusive at this time.

In conclusion, CYP2D1 has been purified from rat liver microsomes and characterized. The purified and reconstituted CYP2D1 demethylated primary caffeine metabolites such as PX, TP and 137U and also hydroxylated TP. This study suggested that CYP2D6 as well as CYP1A2 in human may be involved in the secondary further metabolism of the initial primary metabolites of caffeine (Fig. 5). The purified CYP2D1 will be utilized to raise antibody and the antibody will be used as probes to detect the expression of CYP2D6 and will be used in the eventual genetic studies dealing with its polymorphic expression in Korean people.

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