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H. R. Ha · J. Chen · S. Krähenbühl · F. Follath

Biotransformation of caffeine by cDNA-expressed human cytochromes P-450

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Abstract. *Objectives*: The biotransformation of caffeine has been studied in vitro using human cytochrome P-450 isoenzymes (CYPs) expressed in human B-lymphoblastoid cell lines, namely CYP1A1, 1A2, 2A6, 2B6, 2D6-Val, 2E1 and 3A4, and microsomal epoxide hydroxylase (EH). In addition, CYP 2D6-Met was also studied, in which a valine in the wild type (CYP2D6-Val) has been replaced by a methionine due to a G to A mutation in position 112.
Results: At caffeine 3 mmol · l^{-1}, five CYPs (1A1, 1A2, 2D6-Met, 2E1 and 3A4) catalysed the biotransformation of caffeine. Among the enzymes studied, CYP1A2, which predominantly catalysed paraxanthine formation, had the highest intrinsic clearance (160 l · h^{-1} · $mmol^{-1}$ CYP). Together with its high abundance in liver, it should be considered, therefore, to be the most important isoenzyme in caffeine metabolism. The affinity of caffeine for CYP1A1 was comparable to that of its homologue 1A2. CYP2D6-Met, which catalysed caffeine metabolism by demethylation and 8-hydroxylation, also had a relatively high intrinsic clearance (3.0 l · h^{-1} $mmol^{-1}$ CYP), in particular for theophylline and paraxanthine formation, with k_M values between 9–16 mmol · l^{-1}. In contrast, the wild type, CYP2D6-Val, had no detectable activity. In comparison, CYP2E1 played a less important role in in vitro caffeine metabolism. CYP3A4 predominantly catalysed 8-hydroxylation with a k_M value of 46 mmol · l^{-1} and an intrinsic clearance of 0.60 l · h^{-1} · $mmol^{-1}$ CYP. Due to its high abundance in human liver, the latter CYP may contribute significantly to the in vivo formation of TMU.
Conclusion: The findings of this study indicate that i) microsomes from transfected human B-lymphoblastoid cell lines give results close to those obtained with microsomes isolated from human liver, ii) at least four CYP isoforms are involved in caffeine metabolism, iii) at a substrate concentration < 0.1 mmol · l^{-1}, CYP1A2 and 1A1 are the most important isoenzymes, iv) at higher concentrations the participation of other isoenzymes, in particular CYP3A4, 2E1 and possibly also CYP2D6-Met, are important in caffeine metabolism, and v) the nucleotide composition at position 1120 of CYP2D6 determines the activity of this isoenzyme in caffeine metabolism.

Key words Caffeine, Biotransformation; CYP1A2, CYP1A1, CYP2D6-Met CYP2D6-Val, CYP2E1, cDNA-expressed microsomes

Abbreviations *AFMU* 5-acetylamino-6-formylamino-3-methyluracil; *CYP* human cytochrome P-450; *PAH* polycyclic aromatic hydrocarbon; *17X* paraxanthine; *37X* theobromine; *13X* theophylline; *137U* trimethyluric acid.

Introduction

Caffeine is one of the most frequently ingested chemicals. For the populations of Europe and North America the daily consumption of caffeine is estimated to be 3–7 mg · kg^{-1}, leading to average blood concentrations of 20–40 μmol · l^{-1} (Lelo et al. 1986). In man, caffeine is principally biotransformed by hepatic cytochrome P-450 isoenzyme(s) (CYPs) via N-demethylation to paraxanthine (17X), theobromine (37X) and theophylline (13X), and by hydroxylation to

H. R. Ha (✉) · F. Follath
Cardiovascular Therapy Research Unit, Cardiology Division, University Hospital Zuerich, CH-8091 Zurich, Switzerland
J. Chen
Huadong Hospital, Shanghai, P. R. China
S. Krähenbühl
Division of Clinical Pharmacology and Toxicology, Department of Internal Medicine, University Hospital Zurich, CH-8091 Zurich, Switzerland

trimethyluric acid (137U). After administration of caffeine to healthy patients, the area under the plasma drug concentration-time curves of paraxanthine, theobromine or theophylline represented 42, 9 and 8%, respectively, of that of unchanged caffeine (Bonati et al. 1982). These primary caffeine metabolites may be further biotransformed by CYP(s) and other enzymes, such as xanthine oxidase, and then eliminated by renal excretion as monomethylxanthine or uric acid derivatives. About 85% of an ingested dose of caffeine is recovered in the urine within 48 h. The finding that the main metabolites of caffeine in urine were 1-methyluric acid, 1-methylxanthine, 1,7 dimethyluric acid, 7-methylxanthine and 1,7 dimethylxanthine related to the predominance of paraxanthine in plasma (Grant et al. 1983, Ullrich et al. 1992). An interesting, secondary elimination pathway of caffeine is scission of the C8-N9 bond of the paraxanthine molecule, which leads to the formation of 5-acetylamino-6-formylamino-3-methyluracil (AFMU). The production of AFMU is subject to genetic polymorphism in man (Grant et al. 1983). More details about the metabolism and pharmacology of caffeine can be found in a recent review (Sawynok et al. 1993).

It has been shown that caffeine is mainly metabolised by polycyclic aromatic hydrocarbon (PAH)-inducible CYP(s) (Campell et al. 1987). This CYP family contains two CYP isoforms, namely 1A1 and 1A2. In man, CYP1A2 undergoes maturational changes, and is fully developed approximately four weeks after birth (Ratanasavanh et al. 1991), whereas CYP1A1 is mainly expressed extrahepatically (Shimada et al. 1992) and cannot be detected in all human liver samples (McKinnon et al. 1991). One of the characteristics of CYP1A1 is that this isoform is inducible, e.g. by tobacco smoking. Previous studies led to the conclusion that CYP1A2 is the main CYP isoform involved in caffeine metabolism. However, in vitro investigations performed with human liver microsomes have indicated that more than one CYP may be involved in caffeine biotransformation (Grant et al. 1987, Campell et al. 1987, Berthou et al. 1991, Tassaneeyakul et al. 1992, 1994). Studies using cDNA-expressed CYP isoforms have demonstrated that CYPs 1A2, 2E1 and 3A are involved in caffeine metabolism (Gu et al. 1992; Fuhr et al. 1992, Eugster et al. 1993, Tassaneeyakul et al. 1992, 1994) and that the activity of the expressed CYPs depends on the expression system (Table 1). In the studies of Tassaneeyakul et al. (1994) the apparent k_M values of CYP1A2 expressed in COS-7 cells were close to those measured in human liver microsomes. However, although cDNA-expressed CYP2E1 was found to be involved both in caffeine demethylation and hydroxylation at high caffeine concentrations, no close correlation between the CYP2E1 content and the activities of caffeine 3-demethylase and 8-hydroxylase in human liver microsomes was found. Moreover, inhibition experiments performed with diethyldithiocarbamate demonstrated that the isoform of CYP2E1 could only be partly reponsible for the formation of 37X and 13X in human liver microsomes at high caffeine concentrations. The results suggest that more than one CYP is responsible for caffeine metabolism at a high substrate concentration.

In order to test this hypothesis, microsomes obtained from human B-lymphoblastoid cell lines stably transfected with different human CYPs or epoxide hydroxylase were used to characterise CYP isoforms involved in caffeine metabolism.

Materials and methods

Chemicals and biological materials

Caffeine, paraxanthine, theobromine, theophylline, trimethyluric acid and the internal standard 7-(β-hydroxypropyl)-theophylline were obtained from Sigma Chemical Co. (St. Louis, MO 63178 USA). All other chemicals and solvents were of analytical grade.

Microsomal preparations from human B-lymphoblastoid cell lines transfected with specific human CYPs (1A1, 1A2, 2A6, 2B6, 2D6-Val, 2E1, 3A4) and epoxide hydroxylase were purchased from Gentest Co. (Woburn, MA 01801 USA). In addition, CYP2D6-Met, which contains a G to A mutation at position 1120, with a methionine instead of the valine found in the wild type, was also investigated (obtained from Gentest). The microsomal preparations were delivered frozen and were stored at −75 °C. The protein concentration was 10 mg · ml^{-1}. The individual CYPs are characterised in Table 2. Their CYP contents were measured by the method of Omura and Sato (1964). The specific activity of cDNA-expressed CYPs was assayed as recommended by Gentest (Gentest Information Sheet 1994) and the values obtained were close to those provided by the manufacturer.

Purification of caffeine by liquid chromatography

Commercially available caffeine (about 0.1 g) was dissolved in 5 ml dichloromethane. The solution was injected onto a column packed with silica (24 × 1 cm 40–63 µm, Merck, 8953 Dietikon, Switzerland) and eluted with dichloromethane:methanol (90:10 v/v). The fractions free of impurities (as assayed by analytical HPLC) were collected and dried in a rotary evaporator at 40 °C.

HPLC assay for caffeine and its metabolites

Caffeine metabolites were assayed by a modification of the HPLC method of Sved and Wilson (1977). Briefly, the assay was carried out using a silica HPLC column (Spherisorb S5W 25 × 4.6 cm, Kontron Co., CH-6952 Schlieren, Switzerland). The mobile phase consisted of dichloromethane:solution of 0.02 g ammonium formiate and 0.017 ml 97% formic acid in 100 ml methanol:n-hexane (89.98:0.02:10 v:v:v). The flow rate was 1.5 ml · min^{-1} and the column was kept thermostated at 37 °C. Xanthines were detected at 280 nm with an UV detector. Quantification was accomplished by adding the internal standard (7-(β-hydroxypropyl)-theophylline) to the incubation reaction just before extraction.

Caffeine biotransformation assay

In a 1.5 ml polypropylene test tube, caffeine (0.05–60 mmol · l^{-1}) was incubated in 0.1 mol · l^{-1} sodium phosphate buffer, pH 7.4, in the presence of a NADPH generating system (0.5 IU isocitrate dehy-

Table 1 Biotransformation of caffeine by human CYP isoforms in different expression systems

Expression system	Caffeine concentration (mmol · l^{-1})	Product formed				Reference
		37X	13X	17X	137U	
HepG2 cells	0.125–2.0	1A2(0.93)[a] (+++)[b]	1A2(2.44)[a] (++)[b]	1A2(1.08)[a] (++++)[b]	1A2(1.52)[a] (+)[b]	Gu et al. (1992)
	1	2E1 (++)	2E1 (+++)	—	2E1 (++++)	
	1	—	—	—	3A	
V79 Chinese amster ovary cells	4	1A2 (++++)	1A2 (+)	1A2 (++++)	1A2 (++)	Fuhr et al. (1992)
Yeast (Saccharomyces cerevisiae)	1.5	1A2 (+++)	—	1A2 (++++)	—	Eugster et al. (1993)
	1.5	1A1 (++)	—	1A1 (+++)	1A1 (++++)	
COS-7 cells	0.1	1A1 (+++)	—	1A1 (++++)	—	Tassaneeyakul et al. (1992)
COS-7 cells	0.025–1.0	1A2(0.28) (+++)	1A2(0.24) (++)	1A2(0.24) (++++)	—	Tassaneeyakul et al. (1994)
	7–60	2E1(28) (+)	2E1(43) (+++)	2E1(106) (++)	2E1(48) (++++)	

[a] CYP isoform studied (k_M [mM]).
[b] relative V_{max}: (++++) > (+++) > (++) > (+)

drogenase, 3 mmol · l^{-1} NADP, 5 mmol · l^{-1} sodium isocitrate and 5 mmol · l^{-1} $MgCl_2$) in a final volume of 0.5 ml. After a preincubation of 2 min at 37 °C, the reaction was started by addition of 0.25 mg microsomal protein previously maintained at 0°C. After an incubation at 37°C for 2 h air atmosphere, the reaction was stopped by adding 50 µl 2 mol · l^{-1} HCl and 7 ml dichloromethane:isopropyl alcohol (80:20 v/v). 500 ng internal standard (7-(β-hydroxypropyl)-theophylline) dissolved in 0.1 ml water and 0.5 g ammonium sulphate were added to the reaction mixture. The tube was capped and shaken at 300 agitations per minute for 10 min (Labshaker, Basel, Switzerland). After centrifugation at 1,000 × g for 5 min, the organic phase was transferred into a conical test tube and evaporated to dryness under a stream of nitrogen, at 37 °C. The residue was dissolved in 150 µl mobile phase, and 120 µl of the solution was injected onto the HPLC column. In the assay performed with CYP2A6, the phosphate buffer was replaced by Tris buffer 0.1 mol · l^{-1}, pH 7.4, because phosphate buffer inhibits the activity of this CYP (information from Gentest).

Michaelis-Menten parameters were determined in duplicate incubations performed on the same day using 8 caffeine concentrations (0.05 to 2 mmol · l^{-1}) for CYP1A1, 6 concentrations (0.05 to 2 mmol · l^{-1}) for CYP1A2, 11 concentrations (0.2 to 60 mmol · l^{-1}) for CYP2D6-Met, 6 concentrations (0.05 to 10 mmol · l^{-1}) for CYP2E1 and 12 concentrations (0.5 to 40 mmol · l^{-1}) for CYP3A4.

Data analysis

The reaction rates were fitted to the corresponding substrate concentrations using Michaelis-Menten kinetics and nonlinear regression. The apparent affinity constant k_M and the maximal rate V_{max} were estimated directly using the Profit software package (QuantumSoft, 8023 Zurich, Switzerland).

Using the calculated enzyme parameters, the amount of each caffeine metabolite produced per h and mol of the individual CYP at caffeine concentrations ranging from 0.01 to 100 mmol · l^{-1} was calculated. In order to determine the contribution of individual CYPs to the formation of a given metabolite, the calculations were normalised for the relative abundance of each CYP in human liver, as determined by Shimada et al. (1994); the average specific contents of CYPs 3A4, 1A2, 2E1, 2D6-Met were assumed to be 30, 13, 7, 2%, respectively, of the total hepatic CYP content. Since the specific content of CYP1A1 was not reported in Shimada's work, it was set arbitrarily at 1%.

Table 2 Expression level and catalytic activities of cDNA-expressed human CYP isoforms in human B-lymphoblastoid cell lines

CYP	Lot Nr.	CYP content pmol · mg^{-1} protein	Substrate[a] (conc.used)	Activity[c] pmol · min^{-1} · mg^{-1} protein
1A1	9	25	EROD (1 mM)	120
1A2	17	40	EROD (1 mM)	69
2A6	14	55	Coumarin (0.4 mM)	769
2B6	10	60	EFC (0.1 mM)	150
2D6-Met[b]	29	160	Bufuralol (0.1 mM)	870
2E1[b]	13	40	p-nitrophenol (0.5 mM)	760
3A4[b]	1	20	Testosterone (1 nM)	1100
Epoxide hydrolase	4	—	BZP (4 µM)	150

[a] BZP: benzo(a)pyrene, EFC: 7-ethoxy-4-trifluoromethylcoumarin, EROD: 7-ethoxyresorufin
[b] with reductase
[c] The experimental conditions recommanded by Gentest were used

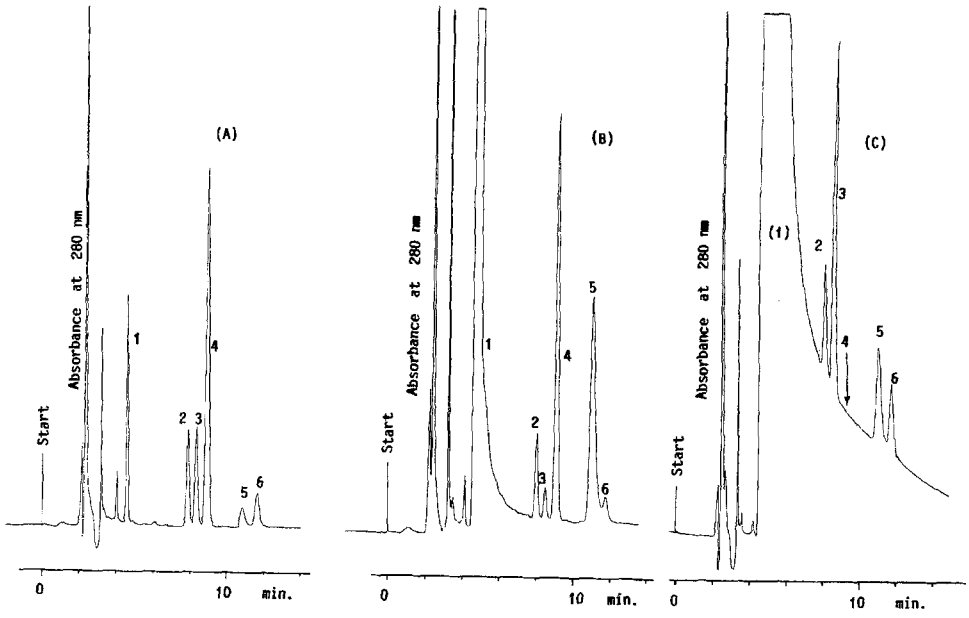

Fig. 1A–C Analysis of caffeine and its primary metabolites by HPLC. Separation of standard substances (**A**). Separation of caffeine metabolites from an incubate containing human CYP1A2 (caffeine concentration 1 mmol · l^{-1}) (**B**) and from an incubate containing human CYP2D6-Met (caffeine concentration 3 mmol · l^{-1}) (**C**) Peaks 1) caffeine 2) theobromine 3) theophylline 4) internal standard (7-(β-hydroxy-propyl)-theophylline), 5) paraxanthine, 6) trimethyluric acid (137U). The retention times were 4.8, 8.0, 8.7, 9.1, 11, 12 min, respectively

Results

The HPLC assay used in the study was a modification of the method of Sved et al. (1977). In our hands, caffeine and its related compounds were well separated (Fig. 1A). The limit of detection was 4 ng · ml^{-1} for 37X and 13X, and 8 ng · ml^{-1} for TMU and 17X. The coefficients of variation of the between-day and within-day determinations for each caffeine metabolite were less than 7% in the concentration range 50–1'000 ng · ml^{-1} and less than 15% at concentrations below 50 ng · ml^{-1}. The rate of caffeine biotransformation was linear with the amount of microsomal protein used (range 0.10–0.50 mg per incubation) over a period of more than 3 hr. Accordingly, 0.25 mg protein per assay and an incubation time of 2 h were used in all experiments.

At a substrate concentration of 3 mM, CYP1A1, 1A2, 2D6-Met, 2E1 and 3A4 catalysed caffeine biotransformation to demethylated and 8-hydroxylated metabolites, whereas the production of metabolites in incubations containing CYPs 2A6, 2B6, 2D6-Val or microsomal EH was below the detection limit. A representative chromatogram of an incubation containing CYP1A2 and a caffeine concentration of 1 mM is shown in Fig 1B. The catalytic activity of CYP2D6-Met had to be investigated at higher caffeine concentrations (Fig. 1C). At caffeine 3 mM, the caffeine signal (peak 1) was large and it was found that CYP2D6-Met catalysed the biotransformation of caffeine to 13X, 17X, 37X and 137U.

The Michaelis-Menten parameters (SD) of caffeine metabolism calculated for each CYP isoform are shown in Table 3. The apparent k_M values of CYP1A2 were

Table 3 In vitro biotransformation of caffeine by cDNA-expressed human CYP isoforms

	Parameters	Cytochrome P-450 isoform (CYP) 1A1	1A2	2D6-Met	2E1	3A4
Theobromine	k_M[a]	0.41 (0.31)	0.16 (0.02)	15.9 (3.8)	1.44 (0.56)	—[d]
37X	V_{max}[b]	0.82 (0.13)	3.00 (0.12)	16.7 (1.6)	0.48 (0.05)	
	CL_{int}[c]	2.0	18.7	1.05	0.33	
Theophylline	k_M[a]	—[d]	0.25 (0.04)	12.5 (2.6)	0.84 (0.40)	—[d]
13X	V_{max}[b]		1.12 (0.06)	37.8 (3.0)	0.36 (0.04)	
	CL_{int}[c]		4.50	3.02	0.43	
Paraxanthine	k_M[a]	0.59 (0.18)	0.19 (0.02)	11.0 (3.2)	—[d]	—[d]
17 X	V_{max}[b]	2.69 (0.18)	30.5 (0.80)	33.5 (3.6)		
	CL_{int}[c]	4.56	161	3.04		
Trimethyluric acid	k_M[a]	0.26 (0.08)	0.27 (0.06)	9.13 (4.18)	1.04 (0.52)	46 (19)
137U	V_{max}[b]	3.78 (0.24)	1.95 (0.14)	12.4 (1.9)	2.95 (0.40)	27.5 (7.2)
	CL_{int}[c]	14.6	7.22	1.36	2.83	0.60

[a] k_M (±90% confidence interval) (mmol · l^{-1})
[b] V_{max} (mol · h^{-1} · mol^{-1} CYP)
[c] Intrinsic clearance = V_{max}/k_M (l · h^{-1} · $mmol^{-1}$ CYP)
[d] below detection limit

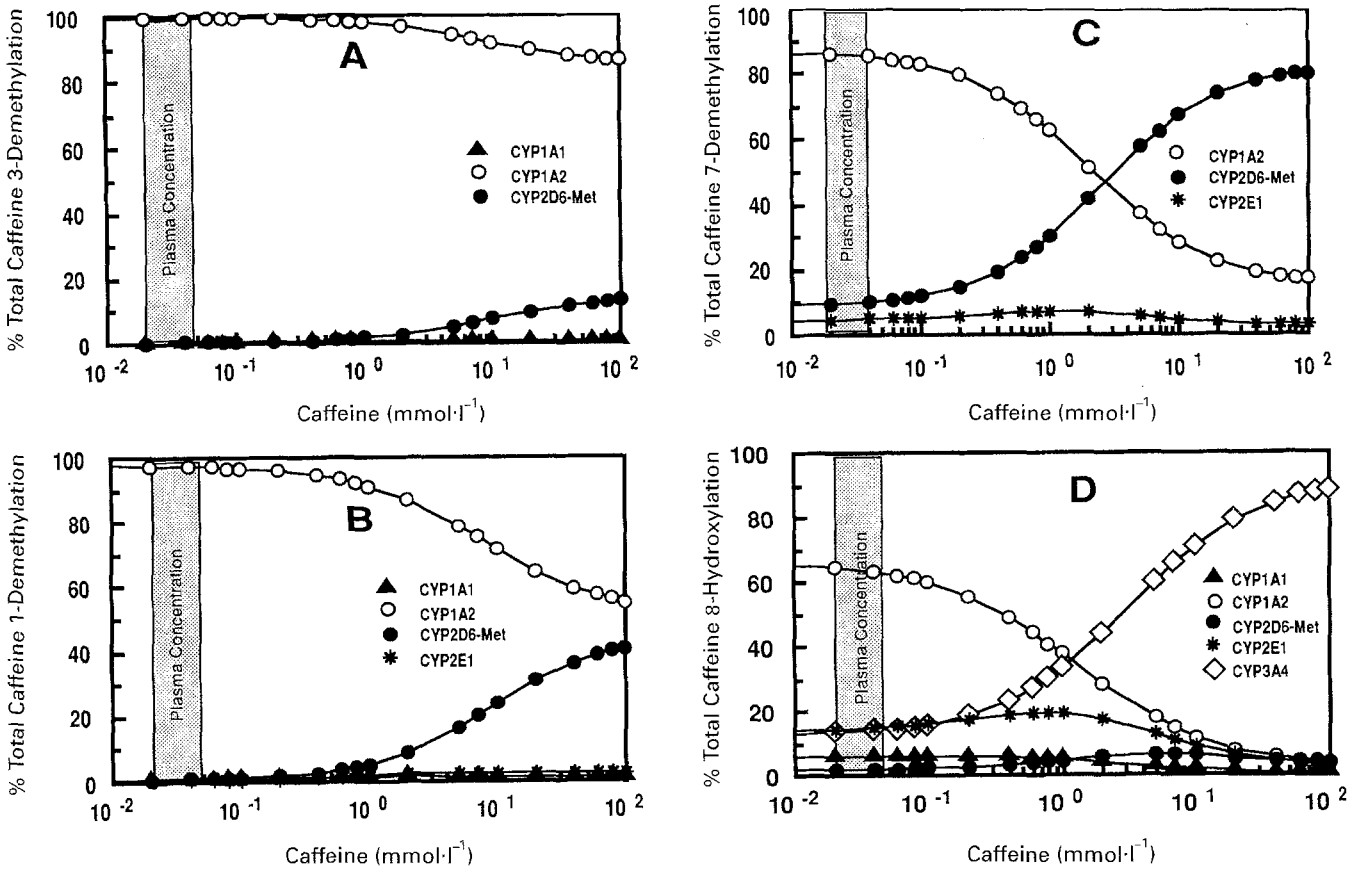

Fig. 2A–D Participitation of individual CYPs in metabolism of caffeine in vivo at a caffeine concentration < 0.1 mmol · l^{-1}; demethylation of caffeine is catalysed mainly by CYP1A2 (**A–C**). At concentration > 0.1 mmol · l^{-1} the activity of CYP2D6- Met is significantly increased in caffeine N7-demethylation (**C**). Caffeine 8-hydroxylation is catalyzed by CYPs 1A1,1A2,2D6-Met, 2E1 and 3A4 at caffeine concentrations below 0.1 mmol · l^{-1}, but at a higher range CYP3A4 is more active (**D**)

in the range of 0.16–0.27 mmol · l^{-1} and were close to those reported for CYP1A2 obtained from COS-7 cells (Tassaneeyakul et al. 1994), and for human liver microsomes (Tassaneeyakul et al. 1992). In agreement with the data of Eugster et al. (1993), CYP1A1 did not catalyse the formation of 13X, whereas it strongly catalyzed the 8-hydroxylation of caffeine, as shown by the high intrinsic clearance for the formation of trimethyluric acid (14.6 l · h^{-1} · $mmol^{-1}$ CYP).

In agreement with earlier studies, CYP2E1 catalysed the formation of 37X, 13X and 137U but not 17X (Gu et al. 1992, Tassaneeyakul et al. 1994). The k_M value was between 0.84 and 1.44 mmol · l^{-1}, which is clearly lower than the 28–108 mmol · l^{-1} reported by Tassaneeyakul et al. (1994) using cDNA-expressed CYP2E1 in COS cells. These findings suggest that the kinetic parameters of a given CYP may depend on the expression system (assuming that cDNAs used in different laboratories are identical) .

For CYP2D6, the wild type (CYP2D6-Val) and a mutant (CYP2D6-Met) were investigated. In the presence of CYP2D6-Met, caffeine was metabolized to 37X, 13X, 17X and 137U (Figure 1C, Table 3), whereas no catalytic activity was detectable in the presence of the wild type, CYP2D6-Val. The apparent k_M of CYP2D6-Met ranged from 9 to 16 mmol · l^{-1}, which is higher than the apparent k_M of CYP1A2, 1A1 and 2E1, but close to the 19–30 mmol · l^{-1} determined for low-affinity enzyme(s) in human liver microsomes by Tassaneeyakul et al. (1992). In incubations containing CYP2D6-Met and 100 µmol · l^{-1} quinidine, an efficient inhibitor of CYP2D6, no catalytic activity was detectable, demonstrating the specificity of the reaction catalyzed by CYP2D6-Met.

In comparison to the other CYPs investigated, CYP3A4 catalysed caffeine metabolism solely to 137U and had a high apparent k_M and a low intrinsic clearance (0.6 l · h^{-1} $mmol^{-1}$ CYP) for this reaction.

Using data on the relative abundance of CYPs in human liver (Shimada et al. 1994), it is possible to estimate the participation of the individual CYP isoforms in hepatic caffeine metabolism. These calculations predict that at caffeine concentrations lower than 0.1 mmol · l^{-1}, CYP1A2 is the main isoenzyme responsible for caffeine demethylation, and partly also for 8-hydroxylation, (Fig. 2A-D). However, in the higher concentration range, the reactions are catalysed by more than one CYP. The data in Fig. 2 suggest also that in vivo, with caffeine plasma concentrations in the range of 20–40 µmol · l^{-1}, the participation of the

low-affinity high-capacity CYP isoenzymes in caffeine demethylation is negligible. However, in interpretation of these calculations it is necessary to consider that some CYP families, such as CYP2C (approximately 18% of the total hepatic CYP content), were not investigated, and that Shimada's data represent an average of 60 human samples in which induction and/or inhibition of different CYP isoforms had not been taken into consideration.

Discussion

The metabolic pathway of caffeine is complex, because the metabolites formed in the first step may be substrates of the same or other enzymes in subsequent steps. A possible way to determine the participation of a specific CYP isoform in these reactions therefore, is to investigate its role under controlled conditions in vitro. Today, experiments of this kind have become possible, because CYP isoforms can be expressed in and isolated from transfected cell lines. However, as illustrated in Table 1, when caffeine metabolism is studied in vitro, the catalytic activity of the CYPs used may depend on the expression system (Table 1). The current studies show that human CYPs expressed in human B-lymphoblastoid cell lines give results which are consistent with previous in vitro and in vivo data.

In agreement with earlier studies (Gu et al. 1992; Tassaneeyakul et al. 1992, 1994), our data demonstrate that 17X is a major metabolites of caffeine. With the exception of 137U formation, the reactions leading to primary caffeine metabolites had the highest intrinsic clearance in the presence of CYP1A2. The combination of this finding with the relative abundance of CYP1A2 in human liver underscores the dominant role of CYP1A2 in caffeine metabolism. For CYP1A1, apparent K_m values in the same range as for CYP1A2 were obtained, suggesting that this isoform may contribute to extrahepatic caffeine metabolism in vivo, in particular when it is induced, e.g by tobacco smoking.

As shown in Fig. 2, at caffeine concentrations higher than 0.1 mmol $\cdot$ l^{-1}, in addition to CYP1A2 other CYPs become important in caffeine metabolism. One of them is CYP2D6-Met, whereas CYP2D6-Val had no activity. Since similar results were obtained for the metabolism of theophylline (Ha et al. 1995), the replacement of a valine by a methionine due to the G to A mutation in position 1120 must be considered functionally important, at least for the metabolism of xanthines. Since, to the best of our knowledge at the present time, the expression of both CYP2D6-Val and CYP2D6-Met in human liver cannot be excluded, this mutation may also have some importance in vivo. Our results show that the apparent k_M values for CYP2D6-Met were comparable to those measured in human liver microsomes (Tassaneeyakul et al. 1992), suggesting that CYP2D6-Met may correspond to one of the low-affinity high-capacity CYP isoenzymes involved in caffeine metabolism. Alternatively, other CYPs, e.g. CYP2C, may also contribute to caffeine metabolism at a high caffeine concentration. The participation of different CYPs in caffeine metabolism at a high concentration could explain the poor correlation between the theophylline formation rate and the amount of immunoreactive CYP1A2 found in the studies of Berthou (1991). In addition, the finding that both 1-demethylation and 7-demethylation, but not 3-demethylation, are the most important reactions of caffeine metabolism in newborns (Berthou et al. 1988) can be explained by the lack of CYP1A2 expression in the first four weeks after birth (Ratanasavanh et al. 1991) and consequently the participation of other CYPs in caffeine metabolism, such as CYP2E1 and possibly also CYP6D2-Met.

In comparison to CYP2D6-Met, the intrinsic clearance of caffeine demethylation by the CYP2E1 isoform was lower, suggesting that the contribution of this isoform to caffeine metabolism in vitro and possibly also in vivo is of little importance. This finding contrasts with the role of CYP2E1 in the metabolism of theophylline, where CYP2E1 represents an important low-affinity high-capacity enzyme (Ha et al. 1995). Since only CYP2E1 but not CYP2D6 is inhibited by diethyldithiocarbamate, the incomplete inhibition of the formation of 37X and 13X by diethyldithiocarbamate at high caffeine concentrations in the investigations of Tassaneeyakul et al. (1994) also suggests the participation of low affinity high capacity CYPs different from CYP2E1 in caffeine metabolism.

Consistent with the previous studies of Gu et al. (1992) using cDNA expressed microsomes, and those of Tassaneeyakul et al. (1992, 1994) and Cazeneuve et al. (1994) using human liver microsomes, cDNA-expressed CYP3A4 in human B-lymphoblastoid cell lines biotransformed caffeine solely to TMU. Owing to its high content in the liver (Shimada et al. 1994), and its relatively high k_M (46 mmol $\cdot$ l^{-1}), the participitation of this CYP isoform in TMU formation may be significant but only at a high substrate concentration (Fig. 2D). The data in Fig. 2D would also predict that the CYP3A content in human liver microsomes would correlate better with TMU formation at a high rather than a low caffeine concentration (Tassaneeyakul et al. 1992, 1994).

In summary, our data show that caffeine metabolism can be reliably studied by using human CYPs isolated from transfected B-lymphoblastoid cell lines, and that at least four CYPs participate in the human metabolism of caffeine. The data confirm the principal role of CYP1A2 among these isoforms and reveal that CYPs 2E1, 3A4 and 2D6-Met, too, may act as low-affinity, high-capacity isoforms in the demethylation and 8-hydroxylation of caffeine.

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