Pharmacogenetics (1992) 2, 73–77

Original article

Biotransformation of caffeine, paraxanthine, theobromine and theophylline by cDNA-expressed human CYP1A2 and CYP2E1

Lie Gu[1], Frank J. Gonzalez[2], Werner Kalow[1] and Bing K. Tang[1]*

[1]*Department of Pharmacology, University of Toronto, Medical Sciences Building, Toronto, Canada M5S 1A8*
[2]*Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institute of Health, Bethesda, MD 20892 USA*

Received 22 November 1991 and accepted 27 December 1991

Six human cytochrome P450s expressed in HepG2 cells using vaccinia virus cDNA-directed expression, were used to study the biotransformation of caffeine and its metabolites. CYP1A2 alone was responsible for caffeine 3-demethylation and paraxanthine 7-demethylation; in addition, 1A2 catalysed virtually all reactions related to caffeine and its metabolites. The metabolic profile of caffeine biotransformation by CYP1A2 averaged 81.5% for paraxanthine, 10.8% for theobromine and 5.4% for theophylline formation. It remained quite uniform when caffeine concentrations were varied. The most striking finding was that CYP2E1 (the ethanol-inducible form) had major influences upon caffeine metabolism: in particular, it catalysed the formation of theophylline and theobromine from caffeine. Thus, the *in vivo* metabolite profiling of caffeine may reveal CYP2E1 activities in addition to the previously documented activities of CYP1A2, polymorphic N-acetyltransferase and xanthine oxidase.

Introduction

Caffeine (137X, see Fig. 1) is almost completely metabolized in the body (Somani & Gupta, 1988). The demethylated xanthines and ring hydroxylated urates together with the acetylated uracil account for the major portion of caffeine metabolism in humans (Arnaud & Welsch, 1980; Tang *et al.*, 1983; Tang-Liu *et al.*, 1983). Hydroxylation of caffeine to give 1,3,7-trimethyluric acid (137U) is only important for *in vitro* studies (Fig. 1). It has been established by Butler *et al.* (1989) that the major route of caffeine 3-demethylation in humans is catalysed by CYP1A2. Berthou *et al.* (1991) confirmed this observation and suggested that the formation of other demethylated metabolites (e.g. theobromine, 37X, and theophylline, 13X) is mediated, at least partly, by other P450 enzymes.

Since the urinary metabolite ratio which was derived from the molar fraction of 7-demethylation of paraxanthine (17X) after ingestion of caffeine correlated well with caffeine 3-demethylation, it was concluded that 7-demethylation of paraxanthine was catalysed mostly or completely by the same caffeine metabolizing enzyme (Campbell *et al.*, 1987; Kalow & Tang, 1991). Subsequently, the urinary metabolite ratio was proposed as the CYP1A2 index (Kalow & Tang, 1991). The objectives of this study are to investigate the role of CYP1A2 in the pathways of caffeine metabolism and to explore whether any of the six available P450s are involved in the biotransformation of caffeine and its metabolites; the human P450s were produced in human hepatoma-derived HepG2 cells through vaccinia virus cDNA expression.

Materials and methods

Chemicals

Caffeine, paraxanthine, theobromine and theophylline were obtained from Sigma Chemical Co. (St Louis, USA). The purity of the chemicals was checked by HPLC and they were found to be free of interfering substances except for paraxanthine, which was received approximately 95% pure and was further purified by HPLC before use to reach a purity of higher than 99.99%.

*To whom correspondence should be addressed.

0960 314X

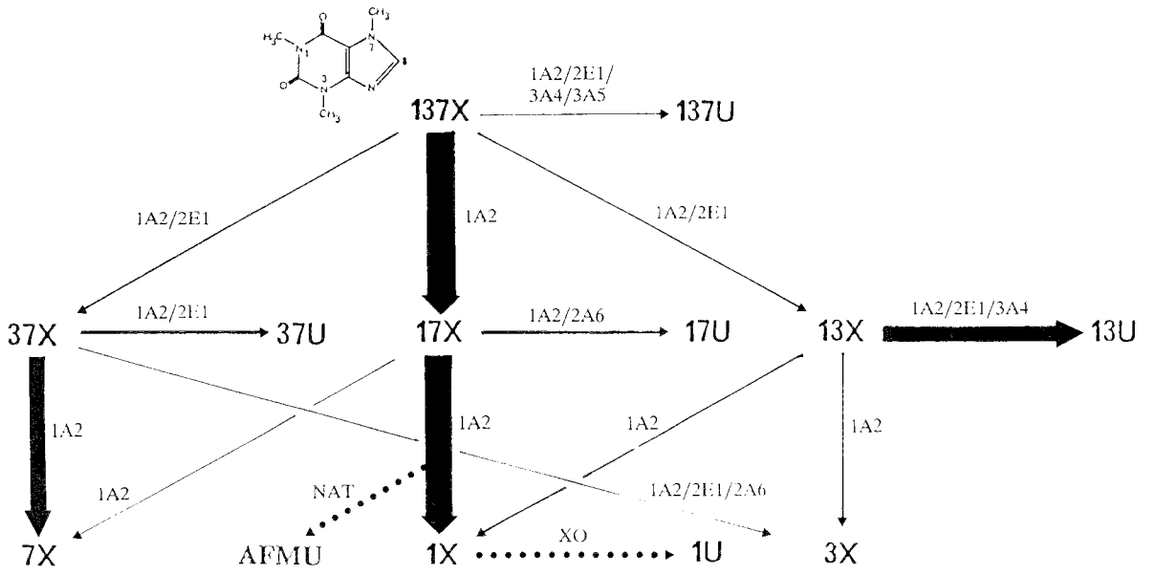

Fig. 1. Involvement of human cytochrome P450 isozymes from HepG2 cells through vaccinia virus cDNA expression in the biotransformation of caffeine and metabolites. Heavy arrows indicate the relative proportions of the pathway via CYP1A2. 137X: caffeine; 137U: 1,3,7-trimethyluric acid; 17X: paraxanthine; 37X: theobromine; 13X: theophylline. 2A3 is now known as 2A6.

Human cytochrome P450 isozymes

Six human vaccinia virus-expressed P450s were used in this study. They were CYP1A2, 2A6, 2B6, 2E1, 3A4 and 3A5. The preparation of these P450s has been described previously (Gonzalez *et al.*, 1991). Briefly, corresponding human P450 cDNAs were inserted into vaccinia viruses and the recombinant viruses were used to infect HepG2 cells. Twenty-four hours after infection, the intact cells were collected and stored as a cell pellet at −70 C. Prior to use, the cell pellets were thawed on ice, sonicated briefly and diluted with phosphate buffer solution (0.2 M, pH = 7.4) so that the protein concentration was 10 mg/ml as estimated by the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL).

Caffeine, paraxanthine, theobromine and theophylline assays

Methylxanthine assays were based on the methods of Grant *et al.* (1983) and Campbell *et al.* (1987). The incubate mixture of 0.4 ml contained 0.1 ml of caffeine or dimethylxanthine as substrate, 0.1 ml of phosphate buffer solution (0.2 M, pH = 7.4), 0.1 ml of 1.15% KCl, 0.1 ml of 0.95% $MgCl_2$ and 0.5 mg NADPH. The mixture was pre-incubated for 2 min at 37 C and the reaction was started by the addition of 1 mg cell protein which had been kept at 4 C. After incubation at 37 C for 60 min, the reaction was terminated by the addition of 0.05 ml of 1.5 M HCl. Then 0.02 ml of *N*-acetyl-4-aminophenol (1 mg 100 ml^{-1} H_2O) as internal standard and 150 mg of ammonium sulfate was added. The mixture was extracted with 8 ml of chloroform:isopropanol (85:15, v:v), vortexed for 30 s and centrifuged for 5 min at 2500 rpm. The organic phase was then dried under nitrogen. The residue was redissolved in 0.15 ml of the mobile phase (11% methanol in 0.05% acetic acid, v:v) and 0.1 ml was injected onto the Ultrasphere ODS column (Beckman, 5 μm, 25 cm × 4.6 mm I.D.). The xanthines and urates were monitored by ultraviolet spectrophotometer at 280 nm.

Various concentrations of caffeine and paraxanthine, ranging from 0.125 to 2.0 mM, were used with CYP1A2 as catalyst for the estimation of Michaelis-Menten parameters using the program ENZFITTER (Elsevier-Biosoft Co., 1987).

Results

Caffeine

CYP1A2 catalysed both 1-, 3- and 7-demethylations and ring hydroxylation of caffeine (Fig. 1). The paraxanthine (17X) formation represented about 79% of total biotransformation via 1A2 (Table 1). Theobromine (37X), theophylline (13X) and 1,3,7-trimethylurate (137U) constituted 10.9%, 6.2% and 3.7%, respectively. The overall rate of caffeine biotransformation was about three times higher

Table 1. Biotransformation of caffeine, paraxanthine theobromine and theophylline by cDNA expressed human cytochrome P450 isozymes*

P450	1A2	2A6	2B6	2E1	3A4	3A5
137X						
13X	1.8 (6.2%)	–	–	2.1 (33.1%)	–	–
17X	22.6 (79.2%)	–	–	–	–	–
37X	3.1 (10.9%)	–	–	0.9 (13.8%)	–	–
137U	1.1 (3.7%)	–	–	3.4 (53.1%)	3.5	1.1
Sum	28.6 (100%)			6.4 (100%)		
17X						
1X	7.0 (75.8%)	–	–	–	–	–
7X	–	–	–	–	–	–
17U	2.2 (24.2%)	2.8	–	–	–	–
Sum	9.2 (100%)					
37X						
3X	0.7 (6.7%)	2.1	–	0.6 (19.1%)	–	–
7X	6.3 (61.4%)	–	–	–	–	–
37U	3.3 (31.8%)	–	–	2.6 (80.9%)	–	–
Sum	10.2 (100%)			3.2 (100%)		
13X						
1X	2.4 (23.0%)	–	–	–	–	–
3X	2.4 (23.0%)	–	–	–	–	–
13U	5.5 (54.1%)	–	–	4.6 (100%)	1.4	–
Sum	10.2 (100%)					

*All substrate concentrations were 1 mM, incubated with 1 mg cell protein for 60 min.
Values were averaged velocity of metabolite formation from two measurements (pmol min^{-1} mg^{-1} protein with normalized rate % in brackets).
– indicated below level of detection (detection limit = 20 nM).

than the biotransformation rate of any of the dimethylxanthines.

Interestingly, CYP2E1 (the ethanol-inducible form of cytochrome P450) was the only other P450 that showed *N*-demethylation activity with distinct metabolite profiles. While there was no detectable amount of paraxanthine, theophylline (33%) and theobromine (13.8%) represented the major *N*-demethylation products of caffeine via 2E1 (Table 1, Fig. 2).

In addition, 8-hydroxylation of caffeine was manifest after incubation with both 2E1 and 3A4, which showed higher activity than 3A5. *CYP2B6* did not metabolize caffeine under the present experimental conditions.

Table 2 shows the biotransformation of caffeine and paraxanthine via 1A2. The Km for caffeine metabolites ranged from 0.93 to 2.44 mM which is not significantly different from that of 1-methylxanthine formation from paraxanthine (2.5 mM).

It should be noted that the conversion percentage of each metabolite of caffeine is virtually constant when the concentration of caffeine is varied from 0.125 to 2 mM, with an average of 81.5% for paraxanthine, 10.8% for theobromine and 5.4% for theophylline (Table 2, Fig. 2).

Paraxanthine

It was demonstrated that 1A2 could catalyse the 7-demethylation of paraxanthine (Table 1) to give 1-methylxanthine (75.9%). Furthermore, 1A2 was

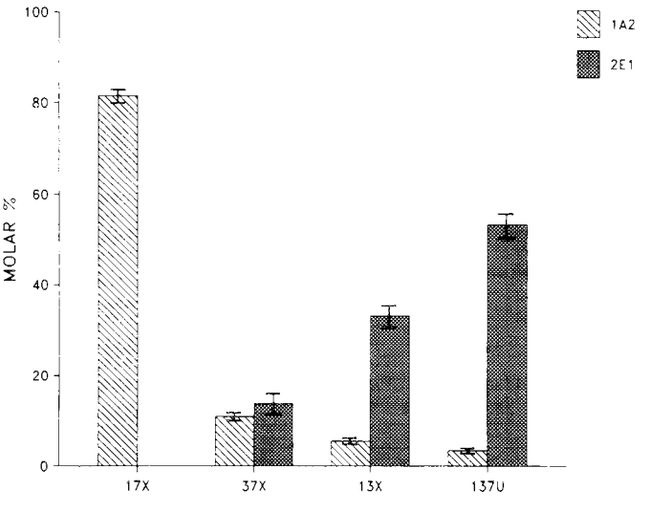

Fig. 2. Distinct metabolite profiles of caffeine catalysed by CYP1A2 and CYP2E1. Standard deviations are marked at the top of each bar for CYP1A2 and ranges for CYP2E1.

Table 2. Biotransformation of various concentrations of caffeine and paraxanthine by CYP1A2 to form products as indicated

Caffeine (mM)	pmol min^{-1} mg^{-1} (normalized rate %)				Paraxanthine (mM)	pmol min^{-1} mg^{-1} (normalized rate %)		
	17X	37X	13X	137U		1X	7X	17U
0.125	4.8 (85.8)	0.6 (11.2)	–	0.2 (2.9)	0.125			
0.25	10.3 (82.4)	1.3 (10.3)	0.5 (4.2)	0.4 (3.1)	0.25	1.7 (80.2)	–	0.4 (19.8)
0.5	15.7 (80.3)	2.3 (11.6)	1.0 (4.9)	0.6 (3.3)	0.5	3.5 (77.5)	–	1.0 (22.5)
1	22.6 (79.2)	3.1 (10.9)	1.8 (6.2)	1.1 (3.7)	1	7.0 (75.8)	–	2.2 (24.2)
2	32.4 (79.6)	4.2 (10.3)	2.6 (6.4)	1.5 (3.7)	2	9.9 (80.6)	0.4 (3.0)	2.0 (16.4)
Km*	1.08 ± 0.15	0.93 ± 0.10	2.44 ± 0.45	1.52 ± 0.15	Km	2.5 ± 0.9		
Kcat	49.2 ± 3.47	6.1 ± 0.30	5.9 ± 0.68	2.6 ± 0.14	Kcat	22.7 ± 5.4		

*Km (mM) and Kcat (pmol min^{-1} mg^{-1}) were estimated from the program ENZFITTER.

the only P450 which catalysed the demethylation of paraxanthine (2E1 could not metabolize paraxanthine). 7-methylxanthine was detected only at a concentration higher than 1 mM of paraxanthine (Table 2). However, 8-hydroxylation of paraxanthine could be mediated by both 1A2 and 2A6.

Theobromine

7-methylxanthine (61.4%), the major metabolite of theobromine, was formed only by 1A2. The second major metabolite, 3,7-dimethylurate (31.8%) could be formed by P450s 1A2 and 2E1 (Table 1).

The metabolite profile of theobromine was quite different when 2E1 was used as a catalyst for incubation. The major metabolite was 37U (80.9%) and there was no 7X formation.

Theophylline

Theophylline was N-demethylated and hydroxylated by 1A2. In contrast, 1,3-dimethylurate (13U) was the only product detected after incubation with 2E1.

Discussion

Results from cDNA-expressed CYP1A2 gave direct evidence that 1A2 is the primary P450 responsible for caffeine 3-demethylation and paraxanthine 7-demethylation. This study confirms our use of the urinary caffeine ratio, which is based on the molar fraction of 7-demethylation of paraxanthine after ingestion of caffeine, as the index of CYP1A2 activity (Campbell *et al.*, 1987; Kalow & Tang, 1991); indeed, CYP1A2 catalysed virtually all reactions related to caffeine and metabolites, although other P450 enzymes also contribute to the biotransformation of caffeine (Fig. 1 and Table 1).

The relative rates of paraxanthine, theobromine and theophylline formation via CYP1A2 remained unchanged as caffeine concentrations were varied. Equivalent measurements in preparations of human liver microsomes showed more formation of theophylline and of theobromine than produced by CYP1A2 (Table 3). This is as expected since microsomes must contain CYP2E1. Differences of CYP2E1 content in the microsomes from different livers may account for the larger standard deviations in the studies with microsomes compared to those with CYP1A2.

CYP2E1, the ethanol-inducible P450 form, is known to metabolically-activate small molecular weight nitrosamines and many small solvent molecules (Koop & Tierney, 1990; Lieber, 1990; Yang *et al.*, 1990; Guengerich *et al.*, 1991). The present results suggest that the contribution of 2E1 to the 7- and 1-demethylation of caffeine can be estimated because of the distinct patterns of dimethylxanthines

Table 3. Mean (±SD) of relative rate ratios (% of sum) for the formations of three dimethylxanthines from caffeine

	[137X]	17X	37X	13X
CYP1A2*	0.125–2 mM	84.3 (2.3)	11.2 (0.5)	5.6 (1.0)
Microsomes**	0.012 mM	70.0 (7.5)	17.4 (5.4)	12.6 (2.9)

*Average of normalized rates from Table 2 at various concentrations of caffeine. Data from this study.

**Average of normalized rates of 5 human microsome preparations from Table 4 of Berthou *et al.* (1989).

formation via 2E1 and 1A2. Since 3-demethylation of caffeine was catalysed only by CYP1A2, paraxanthine formation may serve as the basis for the *in vivo* estimation of the expected contribution of 1A2 to the theophylline and theobromine pathways. Subtracting these expected contribution from the observed rates should yield the contributions of 2E1 to the 7- and 1-demethylation pathways. However, the exact involvement of 2E1 in caffeine metabolism and estimation of 2E1 activity through the shifting of the 3-demethylation to the 1- and 7-demethylation pathways need to be validated. Thus caffeine, which can be used as an index of activity of three enzymes, the carcinogen-activating CYP1A2, the polymorphic *N*-acetyltransferase, and xanthine oxidase (Tang *et al.*, 1991; Kalow & Tang, 1991), may also be useful in estimating the activity of CYP2E1, the ethanol-inducible cytochrome P450.

1,3,7-trimethylurate formation represents a minor metabolic pathway via 1A2 and in the *in vivo* situation. In contrast, it is a major pathway in *in vitro* metabolism by human microsomes (Campbell *et al.*, 1987; Berthou *et al.*, 1989). This discrepancy has yet to be explained.

Since in our previous studies, 8-hydroxylation activity correlated poorly with the demethylation activity in microsomes, it was suggested that hydroxylation and demethylation functions are mediated by different enzymes (Kalow & Campbell, 1988). This is confirmed by the evidence of this study that the formation of all four urates was catalysed by more than a single cytochrome: CYP2E1 exhibited high activity for the formation of all urates except for 17U formation (Table 1, Fig. 1). Since the CYP3A P450s, which are among the most abundantly expressed in humans they may contribute to *in vivo* production of 1,3,7-trimethylurate.

In summary, six human cDNA-expressed P450s were tested in a study of the biotransformation of caffeine and three dimethylxanthines. It was demonstrated that CYP1A2 participated in all the demethylations and ring hydroxylations of caffeine and of the dimethylxanthine metabolites. The second major P450 involved in caffeine demethylation was CYP2E1 which exhibited major influences in theophylline and theobromine formation. Moreover, CYP2E1 demonstrated high activity for the 8-hydroxylations of caffeine, theobromine and theophylline. Of the other six cytochrome P450s only 2A6 showed demethylation activity in the 7-demethylation of theobromine. CYP2A6 was also involved in the 8-hydroxylation of paraxanthine. While CYP3A4 could catalyse the 8-hydroxylation of both caffeine and theophylline, CYP3A5 could only catalyse caffeine 8-hydroxylation. CYP2B6 did not show any activity in the biotransformation of caffeine or its metabolites.

References

Arnaud MJ, Welsch C. Caffeine metabolism in human subjects. *Ninth International Colloquium on Science and Technology of Caffeine*, London, 1980: 385–395.

Berthou F, Ratanasavanh D, Riche C, Picart D, Voirin T, Guillouzo A. Comparison of caffeine metabolism by slices, microsomes and hepatocyte culture from adult human liver. *Xenobiotica* 1989; **19**, 401–417.

Berthou F, Finois JP, Ratanasavanh D, Beaune P, Riche C, Guillouzo A. Evidence for the involvement of several cytochromes P-450 in the first steps of caffeine metabolism by human liver microsomes. *Drug Metab Disposition* 1991; **19**, 561–567.

Butler MA, Iwasaki M, Guengerich FP, Kadlubar FF. Human cytochrome P-450pa (P-450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Biochemistry* 1989; **86**, 7696–7700.

Campbell ME, Grant DM, Inaba T, Kalow W. Biotransformation of caffeine, paraxanthine, theophylline, and theobromine by polycyclic aromatic hydrocarbon-inducible cytochrome(s) P-450 in human liver microsomes. *Drug Metab Disposition* 1987; **15**, 237–249.

Gonzalez FJ, Aoyama T, Gelboin HV. Expression of mammalian cytochrome P450 using vaccinia virus. *Methods Enzymol* 1991; **206**, 85–92.

Guengerich FP, Kim DH, Iwasaki M. Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* 1991; **4**, 168–179.

Grant DM, Tang BK, Kalow W. Variability in caffeine metabolism. *Clin Pharmac Ther* 1983; **33**, 591–602.

Kalow W, Campbell M. Biotransformation of caffeine by microsomes. In ISI Atlas of Science: Pharmacology. Philadelphia: Institute for Scientific Information Inc, 1988: 381–386.

Kalow W, Tang BK. Use of caffeine metabolite ratios to explore CYP1A2 and xanthine oxidase. *Clin Pharmac Ther* 1991; **50**, 508–519.

Koop DR, Tierney DJ. Multiple mechanisms in the regulation of ethanol-inducible cytochrome P450IIE1. *BioEssays* 1990; **12**, 429–435.

Lieber CS. Mechanism of ethanol induced hepatic injury. *Pharmacol Ther* 1990; **46**, 1–41.

Somani SM, Gupta P. Caffeine: a new look at an age-old drug. *Int J Clin Pharmac Ther Toxicol* 1988; **26**, 521–533.

Tang BK, Grant DM, Kalow W. Isolation of identification of 5-acetylamino-6-formylamino-3-methyluracil as a major metabolite of caffeine in man. *Drug Metab Disposition* 1983; **11**, 218–220.

Tang BK, Kadar D, Qian L, Iriah J, Yip J, Kalow W. Caffeine as a metabolic probe: Validation of its use for acetylator phenotyping. *Clin Pharmac Ther* 1991; **49**, 648–657.

Tang-Liu DD, Williams RL, Riegelman S. Disposition of caffeine and its metabolites in man. *J Pharmac Exp Ther* 1983; **224**, 180–185.

Yang CS, Yoo JH, Ishizaki H, Hong J. Cytochrome P450IIE1: roles in nitrosamine metabolism and mechanisms of regulation. *Drug Metab Rev* 1990; **22**, 147–159.