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CAFFEINE METABOLISM BY HUMAN HEPATIC CYTOCHROMES P450: CONTRIBUTIONS OF 1A2, 2E1 AND 3A ISOFORMS

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Abstract—Caffeine (CA) *N*1-, *N*3- and *N*7-demethylase, CA 8-hydroxylase and phenacetin *O*-deethylase activities were measured in microsomes from 18 separate human livers which had been characterized previously for a range of cytochrome P450 (CYP) isoform-specific activities and immunoreactive CYP protein contents. Correlations between the high affinity components of the three separate CA N-demethylations were highly significant ($r = 0.77$–0.91, $P < 0.001$) and each of the three high affinity CA N-demethylations correlated significantly ($r = 0.64$–0.93, $P < 0.05$–0.001) with the high affinity phenacetin *O*-deethylase, 2-acetylaminofluorene N-hydroxylation and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) mutagenicity (all predominantly CYP1A2-mediated reactions). Consistent with these observations, cDNA-expressed human CYP1A2 catalyzed the N1-, N3- and N7-demethylation of CA and apparent K_m values were similar (0.24–0.28 mM) for all three reactions and comparable to those observed previously with human liver microsomes. The low affinity components of CA N1- and N7-demethylation correlated significantly ($r = 0.55$–0.85, $P < 0.05$–0.001) with immunoreactive CYP2E1 content and the CYP2E1-specific activities 4-nitrophenol and chlorzoxazone hydroxylation. Diethyldithiocarbamate, a selective inhibitor of CYP2E1, inhibited the low affinity CA N1- and N7-demethylation, with IC_{50} values of 23 μM and 11 μM, respectively. The apparent K_m values for CA N1- and N7-demethylation by cDNA-expressed CYP2E1 (namely 28 and 43 mM, respectively) were of a similar order to those calculated for the low affinity microsomal activities. Significant correlations ($r = 0.87$–0.97, $P < 0.001$) were observed between CA 8-hydroxylation and immunoreactive CYP3A content and the CYP3A-mediated reactions benzo(*a*)pyrene hydroxylation, omeprazole sulfoxidation and aflatoxin B1 mutagenesis. Effects of α-naphthoflavone, erythromycin, troleandomycin and nifedipine on microsomal CA 8-hydroxylation were generally consistent with CYP3A involvement. Taken together with previous data, the results indicate a major involvement of CYP1A2 in the high affinity component of all three human hepatic CA N-demethylations. In contrast, CYP2E1 appears to be the main enzyme involved in the low affinity components of CA N1- and N7-demethylation while CA 8-hydroxylation is catalysed predominantly by a CYP3A isoform(s).

Key words: caffeine; cytochrome P450; drug metabolism; human liver; substrate probe

CA¶ (1,3,7-trimethylxanthine) undergoes extensive biotransformation in humans and other mammalian species. Since CA is one of the most commonly consumed dietary chemicals, there has been widespread interest in recent years in the development of this compound as a substrate probe for a number of xenobiotic metabolizing enzymes. In particular, it has been proposed that CA N3-demethylation (see below) reflects the activity of cytochrome P4501A2 (CYP1A2), an enzyme responsible for the activation of numerous promutagens and procarcinogens [1–3].

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¶ Abbreviations: AAF, 2-acetylaminofluorene; CA, caffeine; CYP, cytochrome P450; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; PX, paraxanthine; TB, theobromine; TP, theophylline; TMU, trimethyluric acid.

The primary metabolic pathways of CA in humans are illustrated in Fig. 1. N3-Demethylation to form PX (1,7-dimethylxanthine), N1-demethylation to form TB (3,7-dimethylxanthine) and N7-demethylation to form TP (1,3-dimethylxanthine) account for 80, 11 and 4%, respectively, of CA metabolism in humans *in vivo* [4]. 8-Hydroxylation to give TMU, C8–N9 bond scission to form 6-amino-5-[*N*-formylmethylamino]-1,3-dimethyluric acid, and renal elimination of unchanged drug account for the remainder of caffeine clearance in man [4]. PX, TB and TP are all subject to extensive biotransformation once formed *in vivo*, but further metabolism of the primary CA metabolites does not occur in incubations of CA with human liver microsomes or cDNA-expressed cytochromes P450 [5].

In vitro, human liver CA N1-, N3-, and N7-demethylation exhibit biphasic kinetics [5]. Mean apparent K_m values for the high- and low-affinity

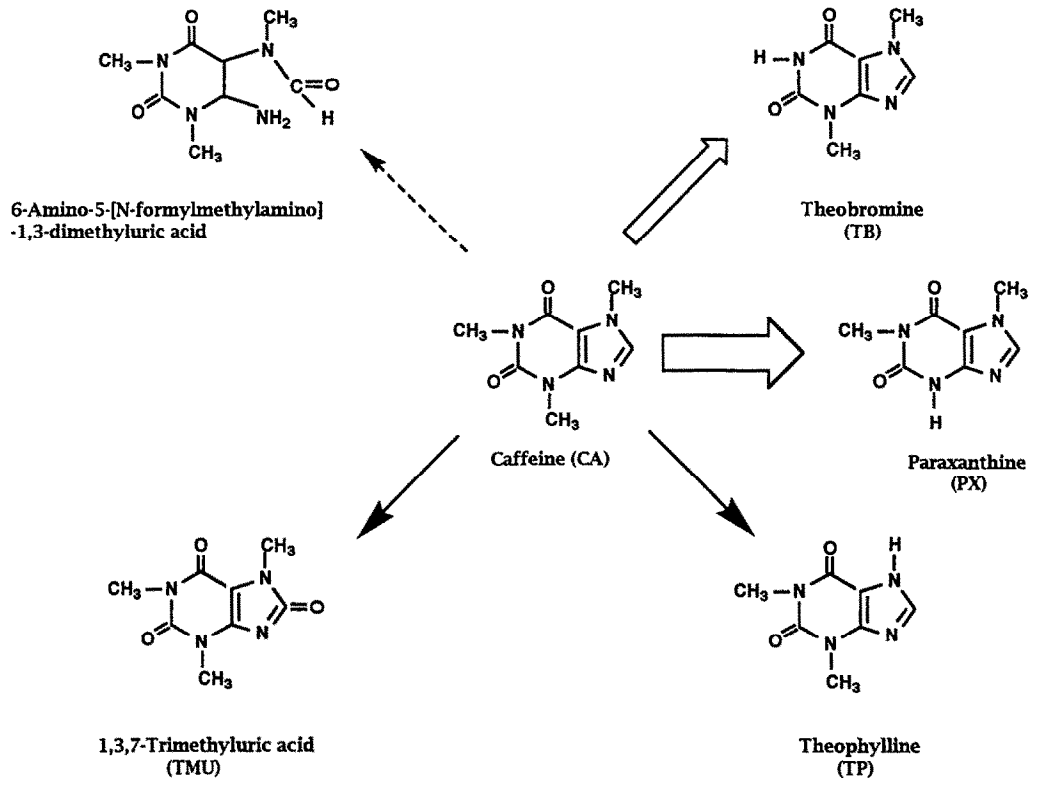

Fig. 1. Primary metabolic pathways of caffeine in humans.

components of the demethylations range from 0.13–0.31 and 19.2–30.0 mM, respectively. While the ratios of the V_{max} values for the high affinity components of N3-, N1- and N7-demethylation are similar to the relative partial metabolic clearances observed for the respective pathways *in vivo* (i.e. approximately 20:3:1), this was not the case for the mean V_{max} values of the low affinity components of CA N-demethylation where ratios varied less than 3-fold [5]. As *in vivo*, CA 8-hydroxylation to form TMU also occurs in human liver microsomes.

Evidence to support CYP1A2 as the enzyme responsible for the high affinity component of CA N3-demethylation (i.e. PX formation) in uninduced human liver is now overwhelming [5–10]. Recent work in this and other laboratories using cDNA-expressed CYP1A2 and human liver microsomal kinetic and inhibitor techniques has provided some evidence that CYP1A2 is also most likely responsible for the high affinity components of CA N1- and N7-demethylation (i.e. TB and TP formation) [5, 7, 11]. Other studies, however, have concluded CYP isoforms other than 1A2 may catalyse these two reactions [8, 11, 12]. The limited data concerning the identity of the CYP isoform(s) mediating CA 8-hydroxylation are similarly conflicting [5, 12] and, in contrast to the high affinity CA N-demethylations, little attention has been given to identifying the enzyme(s) responsible for the low affinity components of CA N-demethylation.

The studies described here aimed to characterize the CYP isoforms responsible for the high affinity components of TB and TP formation and for the low affinity components of PX, TB and TP formation. TMU formation exhibits atypical kinetics in human liver microsomes so the isoform(s) involved in this reaction was studied at low and high CA concentrations. Methods used included correlations between rates of formation of PX, TB, TP and TMU and a range of isoform-specific activities in human liver microsomes. CA metabolism studies with cDNA-expressed CYP 1A2 and 2E1 and the use of CYP isoform-specific inhibitors were used to complement the correlation data.

MATERIALS AND METHODS

Chemicals and reagents. Chemicals and reagents required for the measurement of 2-acetylaminofluorene (AAF) N-hydroxylation, benzo(*a*)pyrene hydroxylation, CA demethylation and hydroxylation, 4-nitrophenol hydroxylation, phenacetin O-deethylation, omeprazole hydroxylation and sulfoxidation, phenytoin hydroxylation, tolbutamide hydroxylation, the mutagenicity of IQ and PhIP, and the immunoquantitation of CYP2E1 and CYP3A have all been reported previously [3, 5, 13–16]. 2-[^{14}C]-CA was purified as described by Tassaneeyakul *et al.* [5], except that ^{14}C-labelled compound was co-purified with unlabelled CA to

give a final specific activity of 28 mCi/mmol. α-Naphthoflavone, troleandomycin and diethyldithiocarbamate were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A). Erythromycin was obtained from FH Faulding Co. (Adelaide, Australia) and nifedipine was provided by Bayer Pharmaceutical (Sydney, Australia).

Human liver samples and cDNAs. Human livers were obtained from renal transplant donors with the approval of the next-of-kin and, where required, the Adelaide Coroner's Office. Relevant details of the donors of the 18 livers used in the present study (namely H5–H15, H17–H23) have been described previously [3]. All liver samples were obtained within 30–60 min of removal of life support systems. Hepatic microsomes were prepared by differential centrifugation [17] and stored in aliquots at −70° in 0.1 M phosphate buffer, pH 7.4 containing 20% (v/v) glycerol. Microsomal protein concentrations were determined by the method of Lowry *et al.* [18], using bovine serum albumin as the standard.

CYP1A2 and CYP2E1 cDNAs were isolated and transfected into COS-7 cells as described previously [5, 14, 16]. Cells transfected with expression vector alone served as negative controls.

Enzyme assays and immunoquantitation of human hepatic CYP isoform content. CA N-demethylation and 8-hydroxylation activities were determined according to the procedure of Tassaneeyakul *et al.* [5]. Human liver microsomal CA N-demethylation and 8-hydroxylation activities were measured at substrate concentrations of 0.1 and 20 mM, which differentiate the high- and low-affinity components of the CA N-demethylations (see Discussion). CA N-demethylation kinetics by cDNA-expressed CYP1A2 were determined over the substrate concentration range of 0.025–1 mM, utilizing 2-^{14}C-CA (28 mCi/mmol, see "chemicals and reagents"), while CA N-demethylation and 8-hydroxylation kinetics by cDNA-expressed CYP2E1 were determined over a substrate range of 7.0–60.0 mM with unlabelled compound. A substrate concentration of 10 μM was utilized for the measurement of the high affinity component of human liver microsomal phenacetin *O*-deethylase activity [16]. The mutagenicity of aflatoxin B1 was determined using the preincubation procedure essentially as outlined by McManus and McKinnon [19]. Each incubation contained 6.4 μM aflatoxin B1, 1×10^8 TA 98 bacterial cells, 15 μg of human liver microsomes, 0.34 μM of NADPH and the incubation time was 20 min.

Data for all other enzyme activities and hepatic CYP isoform contents were taken from previously reported studies performed in this department which utilized livers H5–H15 and H17–H123 (i.e. the same livers in which CA and phenacetin metabolic activities were measured). All metabolic activities using these livers were determined in a period of less than 2 years. Substrate concentrations and methods employed for other human liver microsomal enzyme activities were: AAF, 10 μM [3]; benzo(*a*)-pyrene, 40 μM [3]; IQ, 1 μM [3]; PhIP, 22 μM [3]; 4-nitrophenol, 200 μM [14]; chlorzoxazone, 400 μM [14]; omeprazole 5 μM [15]; tolbutamide, 2 mM; and phenytoin, 150 μM [13].

Table 1. Relationships between the rates of formation of paraxanthine, theobromine, theophylline and trimethyluric acid and certain cytochrome P450 isoform activities and contents in microsomes from 18 human livers (or §N = 17)

	TBH	PXH	TPH	TMU0.1	TBL	PXL	TPL	TMU20	POD	IQ	PhIP	AAF	AFB1§	BP§	OMS	OMH	TBOH	HPPH	4NPOH	CZOH	2E1	3A§
TBH	1.00	0.91**	0.77**	0.22	0.59*	0.68*	0.26	0.23	0.84**	0.80**	0.79**	0.63*	0.30	0.16	0.03	0.39	−0.18	0.05	0.14	0.36	0.38	0.24
PXH		1.00	0.81**	0.07	0.44	0.56*	0.15	0.14	0.93**	0.83**	0.83**	0.76**	0.28	0.09	−0.05	0.39	−0.08	0.08	−0.05	0.17	0.31	0.12
TPH			1.00	0.13	0.42	0.52	0.38	0.10	0.81**	0.80**	0.81**	0.81**	0.22	0.10	−0.09	0.42	0.08	0.20	0.25	0.35	0.49	−0.01
TMU0.1				1.00	0.26	0.60	−0.19	0.78	−0.03	0.06	0.06	0.10	0.69*	0.76**	0.73**	0.48	0.02	0.12	0.18	0.06	−0.19	0.73**
TBL					1.00	0.74**	0.65*	0.38	0.37	0.19	0.21	0.11	0.34	0.20	0.23	0.29	−0.02	0.24	0.55*	0.72**	0.61*	0.33
PXL						1.00	0.18	0.78**	0.51	0.39	0.39	0.34	0.77**	0.63*	0.61*	0.72**	−0.10	0.06	0.28	0.31	0.30	0.74**
TPL							1.00	−0.26	0.08	0.05	0.05	0.04	−0.26	−0.41	−0.25	−0.17	−0.07	0.16	0.70**	0.85**	0.78**	−0.34
TMU20								1.00	0.10	−0.02	−0.03	0.07	0.93**	0.87**	0.91**	0.61*	0.04	0.04	0.05	−0.05	−0.13	0.97**

Values are Spearman rank coefficients. Significance of correlation: *$P < 0.05$, **$P < 0.001$. Abbreviations for activities: TBH, PXH, TPH: high affinity component of CA N1-, N3- and N7-demethylation, respectively; TBL, PXL, TPL: low affinity component of CA N1-, N3- and N7-demethylation, respectively; TMU0.1 and TMU20: CA 8-hydroxylation at substrate concentrations of 0.1 and 20 mM, respectively; TBOH: tolbutamide hydroxylase; HPPH: phenytoin hydroxylase; 4-NPOH: 4-nitrophenol hydroxylase; CZOH: chlorzoxazone hydroxylase; AFB1, IQ and PhIP: aflatoxin B1, IQ and PhIP mutagenicity, respectively; BP: benzo(*a*)pyrene hydroxylase; OMS: omeprazole sulfoxidation; OMH: omeprazole hydroxylase; POD: high affinity phenacetin *O*-deethylase.

Human liver microsomal proteins were separated by SDS–PAGE on 10% (w/v) polyacrylamide gels. Immunoblotting and densitometric quantitation of CYP2E1 and CYP3A was performed as described previously using anti-human 2E1 IgG [14] and anti-human 3A3 IgG [3], respectively.

Inhibition and activation studies. The effects of α-naphthoflavone, diethyldithiocarbamate, erythromycin, troleandomycin and nifedipine on CA metabolism were determined at high (20 mM) and low (0.1 mM) substrate concentrations. Studies with troleandomycin employed a 15 min pre-incubation prior to addition of substrate. α-Naphthoflavone, troleandomycin, erythromycin and nifedipine were added to incubations as solutions in DMSO while diethyldithiocarbamate was dissolved in phosphate buffer. Where DMSO was used as solvent, the final concentration in incubations was 0.5% (v/v); control incubations contained an equivalent concentration of solvent. DMSO at this concentration affected

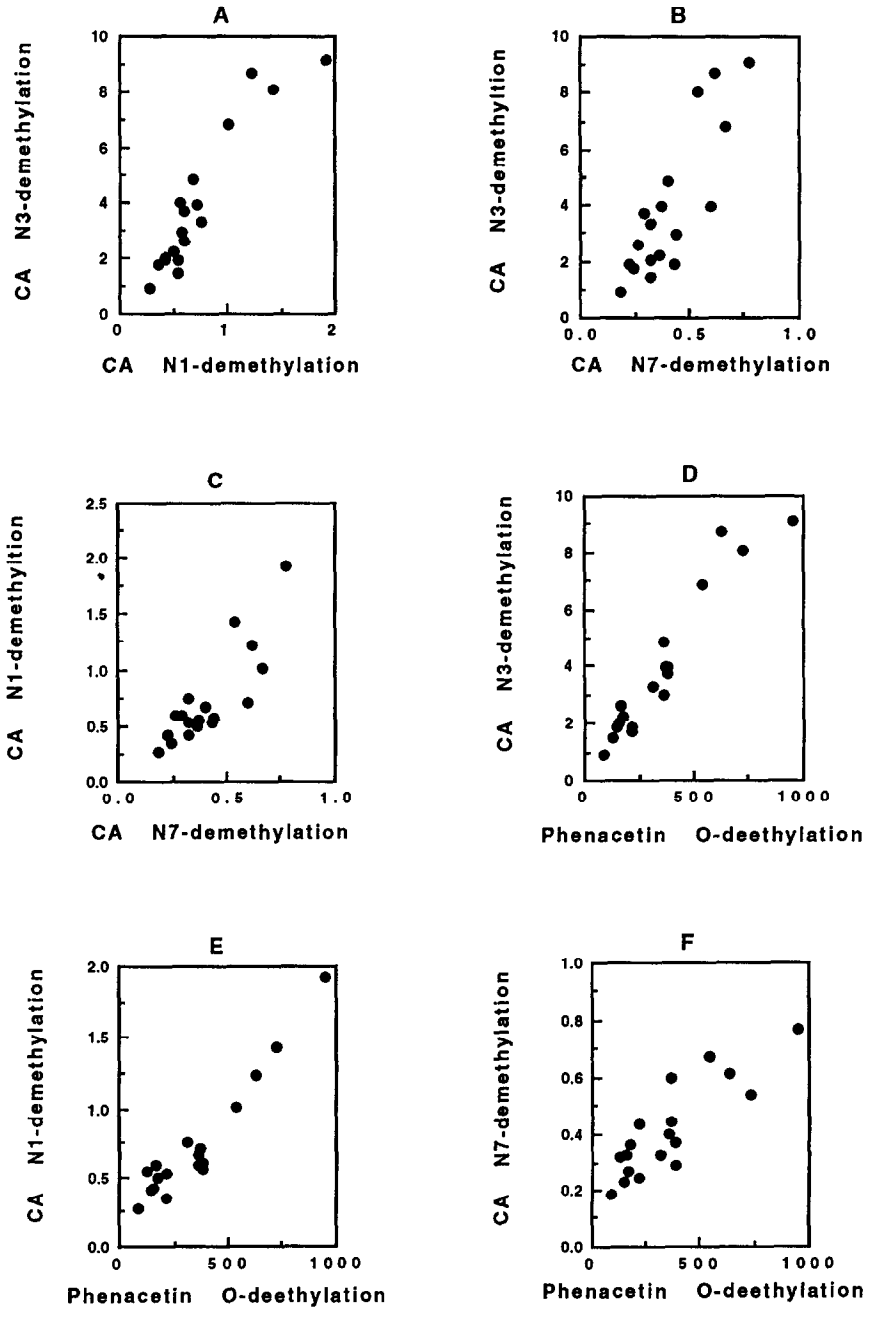

Fig. 2. Relationships between the high affinity components of CA N1-, N3- and N7-demethylation (A–C) and between the high affinity component of phenacetin O-deethylation and the high affinity components of CA N1-, N3- and N7-demethylation (D–F). Comparisons performed with 18 separate human liver microsomal preparations. Activity units: pmol/min/mg microsomal protein.

control activities for the CA demethylations by less than 10% and the 8-hydroxylation by less than 30%. Final concentrations of xenobiotic inhibitors are shown in Fig. 4 and Table 2.

Analysis of results. Results are presented as means ± SD. Correlation coefficients were determined using the Spearman rank test. IC_{50} values (determined from sigmodal plots of data) for diethyldithiocarbamate inhibition of the low affinity components of CA N-demethylation were compared by analysis of variance and Fisher's exact test. The Michaelis–Menten parameters K_m and V_{max} were calculated using MK model, an extended least squares modelling program [20].

RESULTS

High affinity CA N-demethylases

At a substrate (CA) concentration of 0.1 mM, there were significant correlations between the high affinity components of CA N3- and N1-demethylation ($r = 0.91$, $P < 0.001$), N3- and N7-demethylation ($r = 0.81$, $P < 0.001$) and N1- and N7-demethylation ($r = 0.77$, $P < 0.001$) (Table 1 and Fig. 2). All three high affinity CA N-demethylation activities correlated significantly ($P < 0.05$–0.001) with other known CYP1A2 activities, namely the high affinity phenacetin *O*-deethylase ($r = 0.81$–0.93), AAF N-hydroxylation ($r = 0.64$–0.81), IQ mutagenicity ($r = 0.80$–0.83), and PhIP mutagenicity ($r = 0.80$–0.83) (Fig. 2 and Table 1). Correlations between the high affinity CA N-demethylations and tolbutamide hydroxylation, phenytoin 4-hydroxylation, 4-nitrophenol hydroxylation, chlorzoxazone 6-hydroxylation, omeprazole hydroxylation and sulfoxidation, benzo(*a*)pyrene hydroxylation, aflatoxin B1 mutagenicity, and immunoreactive CYP 2E1 and 3A content were all non-significant ($P > 0.05$).

cDNA-expressed CYP1A2 catalyzed the conversion of CA to PX, TB and TP. Over the substrate concentration range 0.025–1 mM, calculated apparent K_m values for the CA N1-, N3- and N7-demethylations were 0.28, 0.24 and 0.24 mM, respectively (Fig. 3). V_{max} values for the CYP1A2-catalysed N1-, N3- and N7-demethylations were 0.74, 3.84 and 0.47 pmol/min/mg. The rank order of these V_{max} values is the same as that observed for the high affinity human liver CA N-demethylations [5].

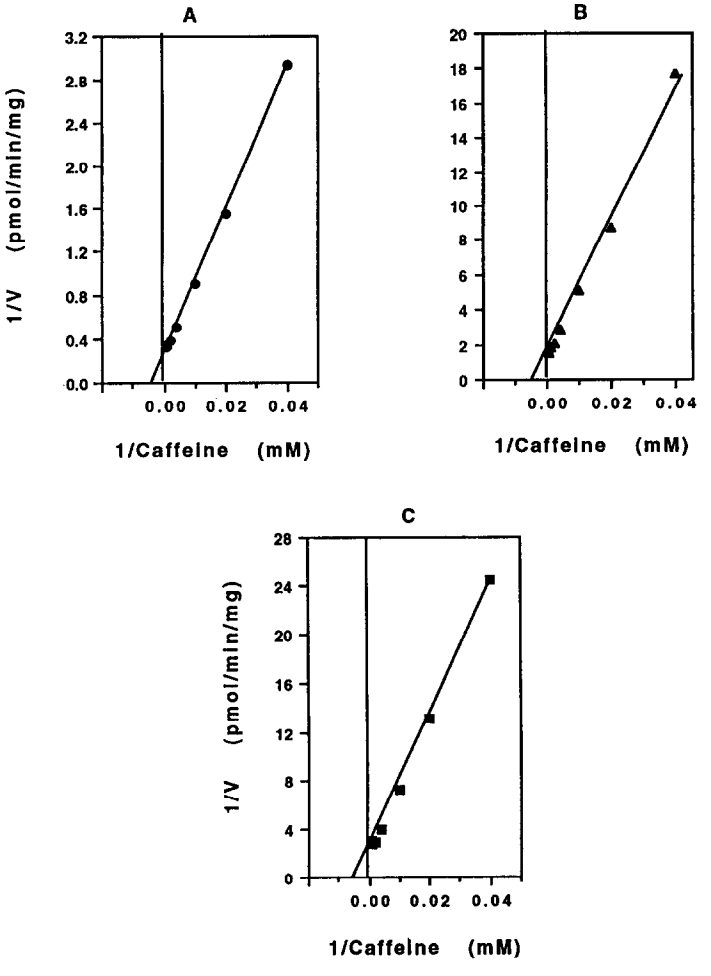

Fig. 3. Lineweaver–Burk plots for CA N3- (A), N1- (B) and N7- (C)-demethylations by cDNA-expressed CYP1A2.

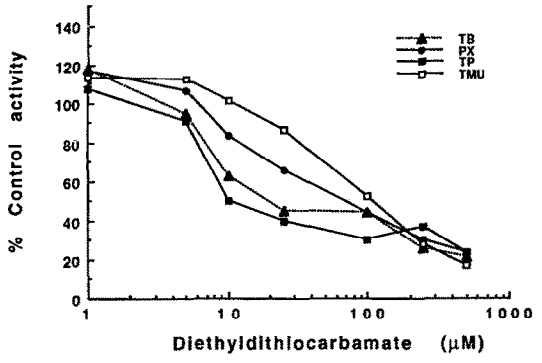

Fig. 4. Effect of diethyldithiocarbamate on the individual CA N-demethylations and CA 8-hydroxylation at a substrate concentration of 20 mM. Results expressed as mean from three livers. (H7, H10 and H15.)

Low affinity CA N-demethylases

There were no significant correlations between the high- and low-affinity components of the individual CA N-demethylations, although the correlation between the high affinity N1-demethylation and the low affinity N3-demethylation was statistically significant ($r = 0.68$, $P < 0.05$) (Table 1). The low affinity CA N1- and N7-demethylations both correlated significantly with the immunoreactive CYP2E1 content ($r = 0.61$ and 0.72, $P < 0.05$ and 0.001) and the chlorzoxazone 6-hydroxylase ($r = 0.72$ and 0.85, $P < 0.001$) and 4-nitrophenol hydroxylase ($r = 0.55$ and 0.70, $P < 0.05$ and 0.001) activities. The low affinity component of CA N3-demethylation correlated significantly with immunoreactive CYP3A content ($r = 0.74$, $P < 0.001$), aflatoxin B1 mutagenesis, benzo(*a*)pyrene hydroxylation, CA 8-hydroxylation and omeprazole sulfoxidation ($r = 0.63$–0.77, $P < 0.05$–0.001) (Table 1).

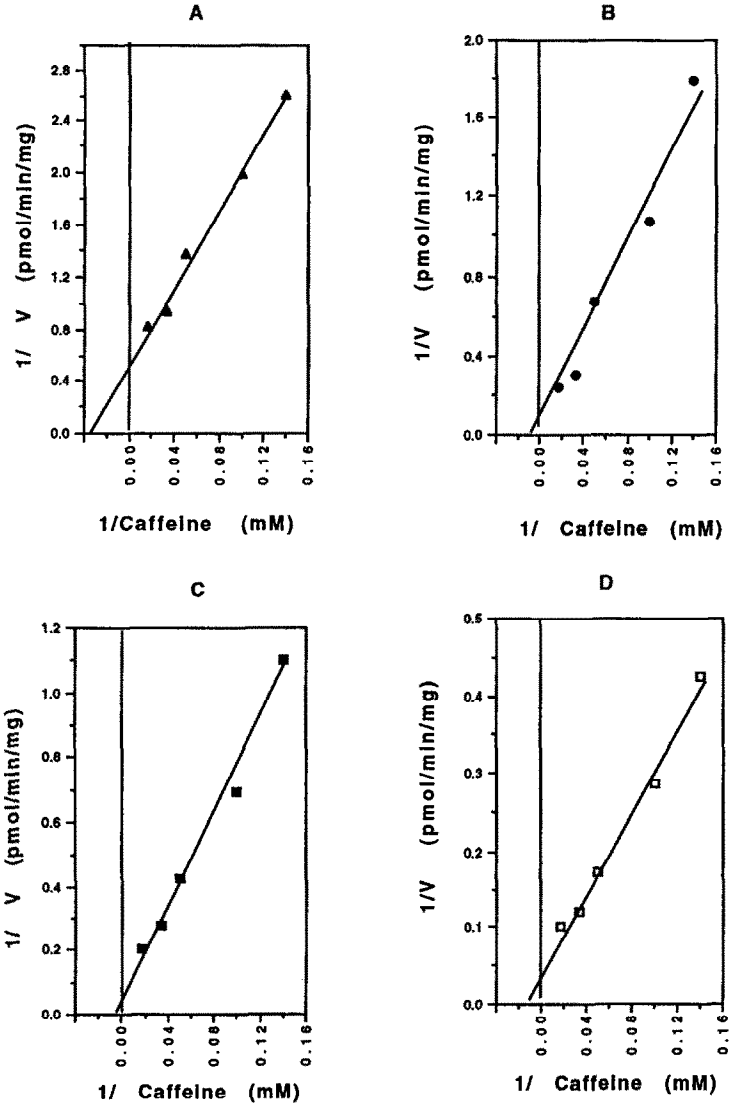

Fig. 5. Lineweaver–Burk plots for CA N1- (A), N3- (B) and N7- (C)-demethylation and CA 8-hydroxylation (D) by cDNA-expressed CYP2E1.

The effects of diethyldithiocarbamate on the low affinity CA N1- and N7-demethylations were investigated to assess further the involvement of CYP2E1 in these reactions. IC_{50} values for diethyldithiocarbamate inhibition of the low affinity N1- and N7-demethylases were 23 and 11 μM, respectively (Fig. 4). While diethyldithiocarbamate additionally inhibited the low affinity CA N3-demethylase and TMU formation, IC_{50} values were >75 μM and significantly different ($P < 0.05$) to the observed values for the low affinity CA N1- and N7-demethylases. The IC_{50} values for diethyldithiocarbamate inhibition of all three high affinity CA demethylases were also >75 μM (data not shown).

cDNA-expressed CYP2E1 catalyzed all three CA N-demethylations as well as CA 8-hydroxylation (Fig. 5). Apparent K_m values for all four reactions were, however, high; 106, 28, 43 and 48 mM for PX, TB, TP and TMU formation, respectively.

CA 8-hydroxylation

At a substrate concentration of 20 mM, human liver microsomal CA 8-hydroxylation (TMU formation) correlated significantly with immunoreactive CYP3A content ($r = 0.97$, $P < 0.001$), aflatoxin B1 mutagenesis ($r = 0.93$, $P < 0.001$), benzo(*a*)pyrene hydroxylation ($r = 0.87$, $P < 0.001$) and omeprazole sulfoxidation ($r = 0.91$, $P < 0.001$) (Table 1, Fig. 6). Consistent with these observations, TMU formation was inhibited by troleandomycin, erythromycin (CYP3A inhibitors) and nifedipine (CYP3A substrate) (Table 2). Moreover, α-naphthoflavone

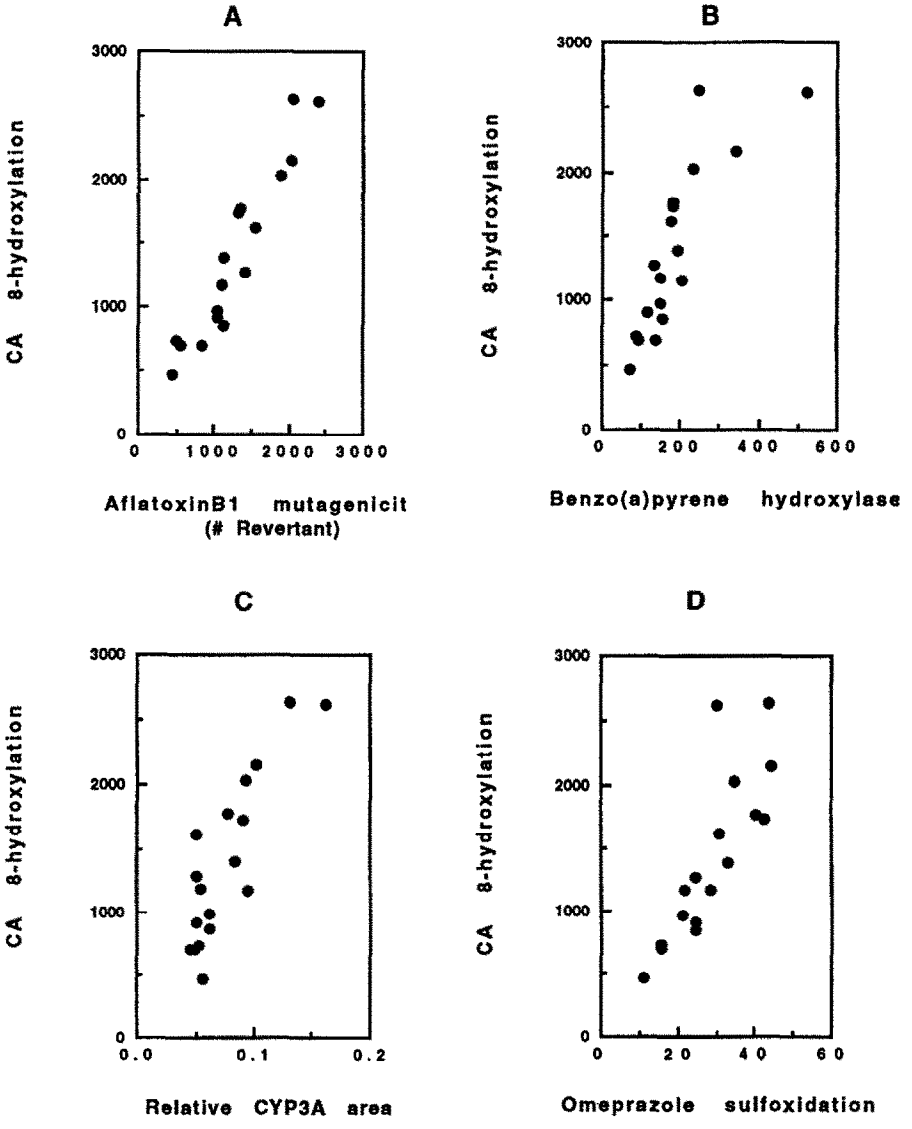

Fig. 6. Relationships between CA 8-hydroxylase activity (at a substrate concentration of 20 mM) and aflatoxin B1 mutagenicity §(A), benzo(*a*)pyrene hydroxylase §(B), immunoreactive CYP3A content §(C) and omeprazole sulfoxidation (D) in microsomes from a panel of 18 human livers (or §N = 17). Activity units as described in Table 1.

Table 2. Effects of xenobiotics on caffeine 8-hydroxylation

Substrate concentration	Per cent control CA 8-hydroxylation activity			
	α-NP (0.25 mM)	TAO (0.05 mM)	ERY (1 mM)	NIF (0.25 mM)
20 mM	300 ± 25	25 ± 3	24 ± 5	53 ± 14
0.1 mM	5644 ± 347	86 ± 15	65 ± 7	230 ± 53

Results expressed as means ± SD of estimations in microsomes from three livers (H7, H10, H15); TMU formation by these livers was in the mid-range of activities observed (Fig. 6). α-NP, α-naphthoflavone; TAO, troleandomycin; ERY, erythromycin; NIF, nifedipine.

concentrations above 10 μM enhanced TMU formation; CA 8-hydroxylation was stimulated 3-fold at an α-naphthoflavone concentration of 250 μM (Table 2).

At a substrate concentration of 0.1 mM, TMU formation also correlated significantly with immunoreactive CYP3A content and CYP3A-mediated activities, although correlation coefficients were lower than those observed at the higher substrate concentration (Table 1). α-Naphthoflavone caused 56-fold activation of CA 8-hydroxylation and nifedipine increased TMU formation 2.3-fold (Table 2). Although erythromycin caused modest inhibition of TMU formation at the lower substrate concentration, troleandomycin was without effect (Table 2).

DISCUSSION

It is now generally accepted that CYP1A2 is the enzyme responsible for the high affinity component of uninduced human liver microsomal CA N3-demethylation, the primary CA metabolic pathway *in vivo*, and results presented here are consistent with that view. In contrast to CA N3-demethylation, the identity of the high affinity CA N1- and N7-demethylation isoform(s) has remained somewhat controversial. Although recent evidence from this and other laboratories suggests that CYP1A2 is responsible for the high affinity components of all three CA N-demethylations [5, 7, 10], a role for non-CYP1A isoforms in these reactions has also been argued [8, 11, 12]. The present data are, however, in agreement with the proposal that the high affinity components of CA N1-, N3- and N7-demethylation are all catalyzed by CYP1A2.

Apparent K_m values for the conversion of CA to PX, TB and TP by cDNA-expressed CYP1A2 were all close in value (0.24–0.28 mM) and similar to the respective apparent K_m values for the high affinity components of the human liver CA N-demethylations reported previously (i.e. 0.13–0.31 mM) [5]. Furthermore, correlations between the separate high affinity CA N-demethylations in microsomes from 18 livers were highly significant, and all three *N*-demethylase activities correlated highly with the known CYP1A2-mediated processes phenacetin O-deethylation, AAF N-hydroxylation, and PhIP and IQ mutagenicity. We have reported previously that IC_{50} values for inhibition of all three CA N-demethylations by α-naphthoflavone were similar for both expressed CYP1A2 and the high affinity human liver microsomal demethylases [5]. Consistent with the predominant involvement of CYP1A2, the high affinity CA N-demethylations and markers for CYP2C9/10 (tolbutamide and phenytoin hydroxylation), CYP2E1 (4-nitrophenol and chlorzoxazone hydroxylation, immunoreactive protein content), CYP3A (benzo(*a*)pyrene hydroxylation, aflatoxin B1 mutagenesis, omeprazole sulfoxidation, immunoreactive protein content), and *S*-mephenytoin hydroxylase (omeprazole hydroxylation) were not significantly correlated.

In performing this work it has been assumed that use of substrate concentrations of 0.1 and 20 mM differentiate the high- and low-affinity components of CA N-demethylation. Based on calculated kinetic parameters [5], the high affinity component of CA N3-demethylation accounts for >90% of total activity at 0.1 mM CA while the low affinity components of the respective N-demethylations account for 80–90% of total activities at a substrate concentration of 20 mM. Similar calculations suggest, however, that the high affinity components of CA N1- and N7-demethylation account for about 50–65% of total activities at the lower substrate concentration but these values are most likely underestimates due to the low activities observed for the N1- and N7-demethylations compared to N3-demethylation, particularly at low CA concentrations [5]. Support for this view is provided by the correlation data presented here and published inhibition data; CYP1A inhibitors such as α-naphthoflavone abolished the vast majority of the high affinity components of all three CA N-demethylation pathways [5].

Michaelis–Menten parameters for cDNA-expressed human CYP1A2 catalysed CA N3-demethylation have been reported previously from this [5] and another [8] laboratory; estimates of apparent K_m differed substantially (0.082 mM vs 1.08 mM). Reasons for the discrepancy are not clear, although the substrate concentration ranges studied (0.025–0.4 mM [5] vs 0.125–2 mM [8]) and expression systems (COS-7 cells [5] vs vaccinia virus-infected HepG2 cells [8]) both differed. In the present study a substrate concentration range of 0.025–1 mM was employed in a reinvestigation of CYP1A2 catalyzed CA N3-demethylation, and use of the higher concentrations additionally allowed determination of apparent K_m values for N1- and N7-demethylation. The three apparent K_m values were similar (0.24–

0.28 mM) and were in good agreement with previously reported apparent K_m values for the high affinity components of human liver microsomal CA N1-, N3- and N7-demethylation [5]. Although somewhat higher than our initial estimate of the apparent K_m for CA N3-demethylation (viz. 0.082 mM), apparent K_m values obtained here remain appreciably lower than those reported by Gu *et al.* [8] for CYP1A2-catalyzed CA N1-, N3- and N7-demethylation (0.93, 1.08 and 2.44 mM, respectively).

While probably of little significance *in vivo*, low affinity (apparent K_m values 19.2–30 mM) CYP isoforms contribute to CA N-demethylation in human liver *in vitro* [5]. The low affinity CA N1- and N7-demethylation activities were found here to correlate significantly with immunoreactive CYP2E1 content and CYP2E1-mediated enzyme activities. The IC_{50} values for diethyldithiocarbamate inhibition of these two reactions were of the order normally associated with an effect on CYP2E1, whereas inhibition of the low affinity CA N3-demethylation and the high affinity CA N-demethylation activities occurred more in the range associated with a non-specific inhibitory effect of diethyldithiocarbamate [14, 21]. The incomplete inhibition observed with diethyldithiocarbamate (Fig. 4A) suggests that up to 60–70% of the low affinity CA N1- and N7-demethylations are due to CYP2E1. cDNA-expressed CYP2E1 was shown to convert CA to TB and TP and the apparent K_m values observed previously for the expressed enzyme (28 and 43 mM) were of a similar order to those previously observed for the low affinity human liver microsomal CA N1- and N7-demethylases (approximately 20 mM) [5]. Although cDNA-expressed CYP2E1 also catalysed the N3-demethylation and 8-hydroxylation of CA (with apparent K_m values of 106 and 48 mM, respectively), the lesser inhibition by diethyldithiocarbamate and poor correlations with CYP2E1 content and activities indicates that this isoform does not contribute to human hepatic PX and TMU formation to any significant extent. It has been reported previously that CYP2E1 was capable of catalysing CA 8-hydroxylation and the N1- and N7-, but not N3-, demethylation of CA [8]; kinetic constants were not determined.

In the present study the low affinity component of CA N3-demethylation was found to correlate with immunoreactive CYP3A content and with CYP3A-mediated activities. Although inhibited to a minor extent (25%) by troleandomycin, this reaction was neither stimulated by α-naphthoflavone nor inhibited by an anti-CYP3A antibody (W. Tassaneeyakul, J.O. Miners and D.J. Birkett, unpublished data and Ref. [5]). In the absence of consistent corroborative data, a major role for a CYP3A isoform in the low affinity component of CA N3-demethylation seems unlikely. Multiple CYP isoforms may be responsible for this reaction.

In a recent publication from this laboratory it was reported that human liver microsomal CA 8-hydroxylation (at both low and high substrate concentration) was inhibited approximately 80% by an anti-CYP3A antibody [5]. In the present study TMU formation was correlated with immunoreactive CYP3A content and the CYP3A mediated benzo(*a*)-pyrene hydroxylation, aflatoxin B1 mutagenesis and omeprazole sulfoxidation. Other data presented here are also consistent with a predominant role of CYP3A in CA 8-hydroxylation, but aspects of the results warrant additional comment. At both high and low substrate concentrations TMU formation was stimulated by α-naphthoflavone, although activation was substantially greater at the low substrate concentration. Nifedipine, a known CYP3A substrate and activator under certain conditions [22], caused modest activation of CA 8-hydroxylation at the low substrate concentration but inhibition at the high substrate concentration. Troleandomycin and erythromycin, known CYP3A inhibitors, both reduced TMU formation by 75% at the high substrate concentration but had a lesser or no effect at the low substrate concentration. As reported previously [5], human liver microsomal CA 8-hydroxylation exhibits atypical kinetics suggestive of substrate activation. The differential effects of compounds such as α-naphthoflavone, nifedipine and troleandomycin at high and low CA concentrations observed in the present work may occur as a result of enzyme activation with increasing CA concentration. Alternatively, separate isoforms of the CYP3A subfamily, which respond differently to inhibitors and activators, may contribute to CA 8-hydroxylation to variable extents at the two substrate concentrations studied here.

Expressed CYP 3A4, 3A5 and 2E1 have been shown to have the capacity to convert CA to TMU (Ref. [8] and present work). Eugster *et al.* [12] reported that expressed CYP1A1 may also 8-hydroxylate CA and suggested that this isoform may be responsible for TMU formation. Both cDNA-expressed CYP1A1 and 1A2 have been found in this laboratory to 8-hydroxylate CA (W. Tassaneeyakul, D.J. Birkett and J.O. Miners, unpublished data), but given the negligible expression of CYP1A1 in human liver [23] and the results of the present work a significant role for this enzyme in TMU formation seems highly unlikely.

In summary, it has been demonstrated that, as with CA N3-demethylation, CYP1A2 is almost certainly the enzyme responsible for the high affinity components of human liver microsomal CA N1- and N7-demethylation. In contrast, CYP2E1 appears to be the major enzyme involved in the low affinity components of CA N1- and N7-demethylation. The low affinity component of CA N3-demethylation to form PX may be carried out by multiple CYP isoforms since no single isoform could be characterized as responsible for this activity. Available evidence suggests that CA 8-hydroxylation to form TMU is mediated largely by an isoform(s) of the CYP3A subfamily. The use of CA as a CYP3A substrate probe *in vivo* warrants further investigation.

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