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METHYLCHOLANTHRENE BUT NOT PHENOBARBITAL ENHANCES CAFFEINE AND THEOPHYLLINE METABOLISM IN CULTURED ADULT HUMAN HEPATOCYTES

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Abstract—Biotransformation of caffeine and theophylline and the effect of two well-known inducers of P-450 isozymes, namely phenobarbital (PB) and methylcholanthrene (3-MC) were studied in cultured hepatocytes from six human adult donors. Hepatocytes co-cultured with rat liver epithelial cells maintained a higher metabolic capacity than pure cultures. PB treatment of cultured hepatocytes for 3 days slightly increased the rate of caffeine metabolism 1.4 ± 0.5-fold (N = 6) vs controls, and theophylline metabolism 1.2 ± 0.4-fold (N = 6), whereas 3-MC treatment increased metabolism markedly 5.8 ± 2.3- and 3.3 ± 1.1-fold (N = 6) vs controls for caffeine and theophylline, respectively. Paraxanthine and theophylline formations from caffeine were the most induced by 3-MC. Their increase was significantly correlated ($r_s = 0.89$, $P < 0.007$) but not with TB formation, suggesting that at least two isozymes of the P-450IA family are involved in the first demethylations of caffeine. In addition, the N-1 demethylation of theophylline (mean increase of 554% vs controls) was not correlated with the N-1 demethylation of caffeine (mean increase 247% vs controls) for the same donor after 3-MC treatment, suggesting that these two demethylations are mediated by a different P-450.

Caffeine (CA) and theophylline (TP)§ which are probably the most widely ingested drugs in beverages and foods are mainly metabolized in human liver by the microsomal cytochrome P-450 (P-450) enzymes [1, 2]. This had led some investigators to study the validity of such compounds as *in-vivo* probes for assessing the liver drug metabolizing enzymes in humans [3]. Numerous *in-vivo* and *in-vitro* experiments have determined the involvement of polycyclic aromatic hydrocarbon (PAH)-inducible P-450 isozyme(s) in metabolism of these two methylxanthines in animals and humans. Indeed 3-methylcholanthrene (3-MC), but not, or to a lesser extent, phenobarbital (PB), was found to stimulate caffeine biotransformation in rats [4–6] and dogs [7]. The two-fold decrease in caffeine half-life in smokers vs non-smokers [8–11] and the highly significant correlation between caffeine demethylation into paraxanthine (PX) and the ethoxyresorufin *O*-deethylase (EROD) activity in human liver microsomes [12] suggest that caffeine metabolism could be used as a probe of the P-450IA family. Similarly, theophylline biotransformation has been shown to be quantitatively modified by PB and 3-MC in rat [13], mouse [14] and man [15].

Until now investigations have been unable to provide a definitive distinction between contributions of P-450IA1 and P-450IA2 isozymes (formerly called P1-450 or P-450c and P3-450 or P-450d in humans and rats, respectively) in the first demethylations of caffeine and theophylline. Another question concerns the number of P-450 isozymes involved in these primary demethylations. In addition to the P-450IA family, unidentified enzyme(s) contribute(s) to the hydroxylation of these methylxanthines [16].

Until recently, most studies in methylxanthine biotransformation were conducted on liver microsomes of pre-treated laboratory animals [4, 13, 17, 18] or humans [1, 2, 12, 19]. Although the phenomenon of induction has long been recognized in man, it has been difficult to study its specificity because of ethical considerations. The use of human hepatocyte cultures has allowed us to characterize methylxanthine metabolism in humans [20] and consequently to investigate the effect of inducers. Indeed other studies have demonstrated that hepatocyte cultures retain P-450 induction capacity [21]; and, specific P-450 isozymes were better maintained in hepatocyte co-cultures (CC) than in pure cultures (PC) [22, 23]. Accordingly the co-culture model was used to study the effects of two well-known inducers

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§ Abbreviations used: Caffeine metabolites = AFMU, 5-acetylamino-6-formylamino-3-methyluracil; TMU, 1,3,7-trimethyluric acid; 1,3,7-DAU, 6-amino-5[*N*-methylformylaminol]1,3-dimethyl uracil; DMX, dimethylxanthines; TP, theophylline or 1,3-dimethylxanthine; TB, theobromine or 3,7-dimethylxanthine; PX, paraxanthine or 1,7-dimethylxanthine; CA, caffeine or 1,3,7-trimethylxanthine. Theophylline metabolites = 1,3-DMU, 1,3-dimethyl uric acid; 3-MX, 3-methylxanthine; 1-MX, 1-methylxanthine; 1-MU, 1-methyl uric acid; P-450, microsomal cytochrome P-450; EROD, 7-ethoxyresorufin *O*-deethylase activity; PB, phenobarbital; 3-MC, 3-methylcholanthrene; DMSO, dimethylsulfoxide; RLEC, rat liver epithelial cells; PC, pure cultures; CC, co-cultures; PAH, polyaromatic hydrocarbon.

Table 1. Liver donors and microsomal cytochrome P-450 activities

Case number	Age (years)	Sex	Total P-450*	EROD†	Metabolic rate of caffeine‡	Acetanilide 4-hydroxylation†
1 (Br017)	26	M	0.59	477	365	2450
2 (Br015)	43	M	0.41	171	114	1260
3 (Br019)§	45	M	0.46	324	246	1980
4A (SB01)§	16	M	nd	nd	nd	nd
4B (SB01)	16	M	nd	nd	nd	nd
5 (Br021)	49	M	0.25	53	78	1420
6 (Br024)	15	F	0.50	111	107	830

* nmol/mg microsomal protein.
† pmol/min/mg microsomal protein.
‡ pmol/min/mg microsomal protein with 1 mM caffeine.
§ Hepatocytes incubated with 0.1 mM caffeine or theophylline; other cases, including 4B were incubated with 1 mM.
nd, not determined.

of P-450 isozymes, namely PB and 3-MC, on the caffeine and theophylline metabolism in human hepatocytes.

MATERIAL AND METHODS

Chemicals. Methylxanthines were purchased from two commercial sources, either Sigma Chemical Co. (St Louis, MO) or Fluka (Buchs, Switzerland). 5-Acetylamino-6-formylamino-3-methyl uracil (AFMU) and 6-amino-5[*N*-methylformylamino]-1,3-dimethyluracil (1,3,7-DAU) were kindly supplied by Dr Arnaud (Nestec, La Tour-du-Peilz, Switzerland). The purity of caffeine and theophylline purchased from Sigma was checked by high performance liquid chromatography (HPLC) as described previously [12] and exceeded 99.9% with respect to the other methylxanthines.

Liver samples. Human livers were obtained from six adult kidney transplantation donors (15–49-years-old) who died from traffic accidents (Table 1). Sampling was made following the recommendations of the French ethical committee. Dietary habits and exposure to environmental chemicals before death were not known.

Cytochrome P-450 activities. Liver microsomes were prepared according to procedure previously described [12]. Total cytochrome P-450 and EROD activity were determined according to the methods referenced in [12]. Acetanilide 4-hydroxylase activity was carried out according to Guenther *et al.* [24].

Cell isolation and culture. Adult human hepatocytes were prepared by the two-step collagenase perfusion method as previously described [25]. The cells were seeded at a density of 2.5×10^6 cells per 28 cm^2 Petri dish in 4 ml of nutrient medium containing 10 μg/ml bovine insulin, 0.2% bovine serum albumin and 10% foetal calf serum [20]. The culture medium was renewed 16 hr later, when co-cultures were set up by adding 2.5×10^6 rat liver epithelial cells (RLEC) [26]. The medium was changed 24 hr later and supplemented with 3×10^{-6} M hydrocortisone hemisuccinate (Roussel-UCLAF, Paris, France).

Cell treatment by PB and 3-MC. When co-culture was established, i.e. 24 hr after RLEC addition, cells were treated with 3.2 mM PB or 5 μM 3-MC for 3 or 5 days according to a procedure previously described [21]. The culture medium was renewed every day. PB was dissolved in phosphate buffer saline (PBS) and 3-MC in dimethylsulfoxide (DMSO) before adding to the medium. The final concentration of DMSO in medium was 0.02% (v/v). Control cultures received the same volume of solvent. No difference in metabolism of caffeine and theophylline was observed between PBS and DMSO controls.

Incubation of hepatocytes with caffeine and theophylline. Control pure culture and co-culture of human hepatocytes and RLEC were incubated with methylxanthines at concentrations of either 10^{-4} M (case numbers 3 and 4A) or 10^{-3} M for 2.5×10^6 hepatocytes. These concentrations were found to be non toxic over a 24 hr incubation [20]. After 3 or 5 days of PB or 3-MC treatment, cultured hepatocytes were incubated in the absence of inducers for 24 hr with methylxanthines dissolved in culture medium according to the same procedure used for control hepatocytes.

Evaluation of overall metabolism. After 24 hr incubation, an aliquot of 4 ml of culture medium saturated with ammonium sulfate was extracted by two volumes of chloroform/isopropranol (85:15; v/v) using a mechanical shaker for 2 hr. Overall biotransformation was expressed as the percentage of substrate transformed into known metabolites relative to the untransformed substrate, and both the parent drug and metabolites were determined only in culture media. The rate of caffeine or theophylline metabolism was expressed as nmole of transformed substrate/10^6 hepatocytes seeded/24 hr. Values reported represent the mean of at least two plates for each experiment.

Quantification of methylxanthine metabolites by HPLC. After extraction metabolites were separated by HPLC using a gradient elution system as described previously [12]. Amounts of each metabolite were expressed as a molar percentage of all metabolites quantified in the sample with respect to their relative UV response factor. Metabolites were identified by their retention times compared with those of the standard.

Table 2. Metabolic rate of theophylline and caffeine in human hepatocyte cultures

Case number	Theophylline		Caffeine	
	CC	PC	CC	PC
1	15.1	13.5	16.8	8.5
2	21.2	11.2	21.1	18
4B	37.1	nd	18.7	nd
5	19.5	nd	22.2	nd
6	74.6	27.7	52.3	15.7
Mean ± SD	33.5 ± 24.4	17.5 ± 8.9	26.2 ± 14.7	14.1 ± 5.0

Results are expressed as nmole of transformed substrate/10^6 hepatocytes/24 hr.
Substrate concentration was 1 mM in both pure cultures (PC: after 1 day of culture) and co-cultures (CC: after 3 days of culture).
nd, not determined.

Detection limit of each metabolite was about 25 ng injected in HPLC (i.e. 0.05 nmol/10^6 hepatocytes/24 hr. Variation coefficients between the plates were about 15–20% in the range 5 to 20 nmol/10^6 hepatocytes/24 hr and about 5–8% in the range above 50 nmol/10^6 hepatocytes/24 hr.

Data analysis. Data are given as mean ± SD, calculated for six subjects or seven observations (hepatocytes of case number 4 were incubated with 0.1 and 1 mM substrates). Correlation coefficients were determined according to two procedures. Firstly, for the correlation between the changes in the overall metabolism of caffeine or theophylline induced by PB or 3-MC the regression curves are given with correlation coefficient r, based on log transformations of the fold increase data. This was done to correct the skewness in the distribution of the data. Secondly, the Spearmann rank correlation method was used as a non parametric test for studying changes induced in metabolic profiles of theophylline or caffeine. Results are given as correlation coefficient r_s; that allows us to ignore the shape of data distribution.

RESULTS

Comparative metabolic rate of methylxanthines in human hepatocyte pure cultures and co-cultures

Metabolic capacity of human hepatocytes was estimated in both pure culture and co-culture. Regardless of the donor or culture duration, it was always higher in the latter culture system (Table 2 and Fig. 1). To verify that this was not due to RLEC, pure cultures of this cell type were assayed for caffeine metabolism. Detectable HPLC peaks of metabolites were obtained only by incubating as many as 10^7 cells in 10 ml medium with 1 mM substrate. Caffeine metabolism did not exceed 0.2 nmol/10^6 cells/24 hr. Treatment by PB or 3-MC did not affect this metabolic rate. Accordingly co-cultures of human hepatocytes were used for studying the effects of these two compounds on caffeine and theophylline metabolism.

Metabolic profiles of caffeine and theophylline in control cultured hepatocytes

Five primary metabolites [three demethylated compounds: TB, PX, TP and two oxidized products: 1,3,7-trimethyluric acid (TMU) and 1,3,7-DAU] out of the sixteen possible caffeine metabolites found in human urine [27] were detected in the medium of untreated cultures (controls). HPLC peaks were more easily detected in media of co-cultures than in those of pure cultures (Fig. 2). However, when the results were expressed as the relative ratio of primary demethylated metabolites, the caffeine metabolic profile was found to be quite similar whatever the culture conditions. TB, PX and TP accounted for 20 (±4), 60 (±11) and 20 (±11) per cent of total 3-DMX (mean ± SD, N = 6 subjects) respectively in co-cultures versus 23 (±4), 58 (±3) and 19 (±5) per cent in pure cultures (mean ± SD, N = 3 subjects, donor numbers 1, 2 and 6).

Using theophylline as the substrate, four metabolites, namely 3-methylxanthine (3-MX) and 1,3-dimethyluric acid (1,3-DMU), CA and 1-methylxanthine (1-MX) were detected (Fig. 2). The minor metabolite 1-MX which was often below the detection limit in pure culture was easily detected in co-culture (Fig. 2). This observation confirms that enzyme(s) involved in theophylline metabolism was (were) better maintained in co-culture than in pure culture. Nevertheless when results were expressed as percentage of metabolites formed, the metabolic profile of theophylline was quite similar in the two culture systems. For example, 1,3-DMU and 3-MX accounted for 73 (±17) and 27 (±17) per cent (mean ± SD, N = 6 subjects) of the total of these two metabolites in co-cultures versus 74 (±16) and 26 (±16) per cent (mean ± SD, N = 3 subjects, donor numbers 1, 2 and 6) in pure cultures. In a previous study this ratio was 70 (±10, SD) and 30 (±5, SD) per cent for pure cultures of six other donors [20].

Effect of PB and 3-MC on the overall metabolism of caffeine and theophylline

Addition of DMSO at a final concentration of 0.02% (v/v) did not significantly affect metabolism of caffeine (16.8 and 21.1 nmol/10^6 hepatocytes/24 hr in DMSO controls vs 15.1 and 20.9 in PBS controls for Br017 and Br015 hepatocytes respectively) and theophylline (13.5 nmol/24 hr/10^6 hepatocytes in DMSO controls vs 12.2 in PBS plates for

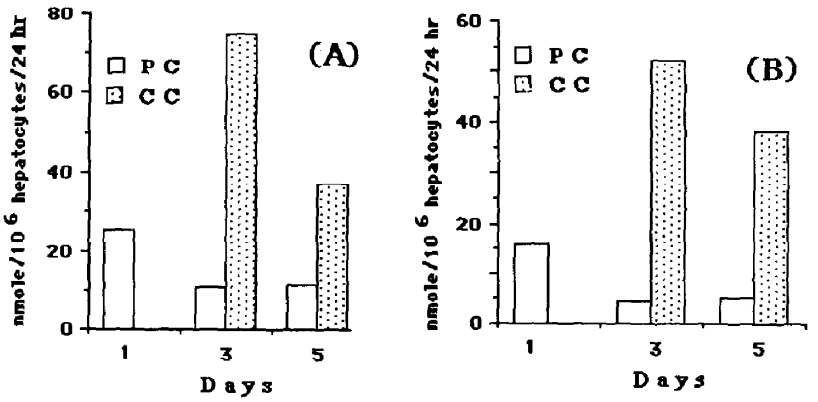

Fig. 1. Overall metabolic rates of theophylline (A) and caffeine (B) in human hepatocytes (case number 6: Br024) cultured either as pure culture (PC) or as co-culture (CC) versus duration of culture. Results are the means of three plates.

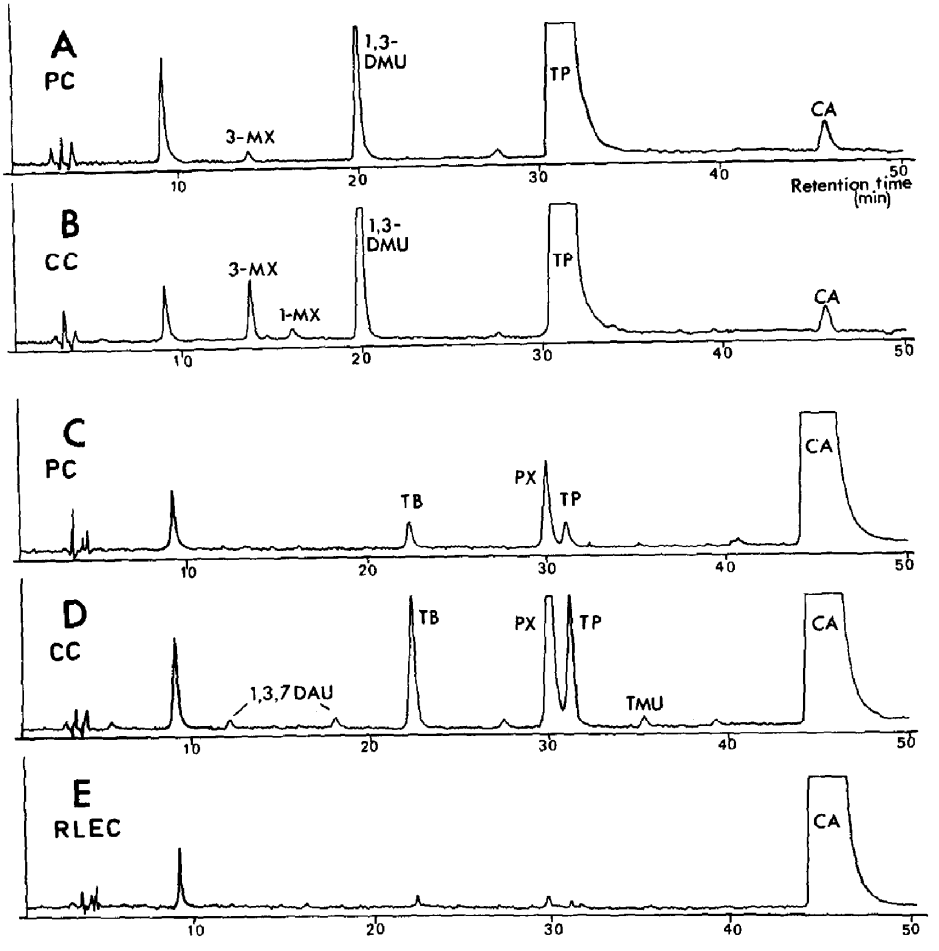

Fig. 2. HPLC profiles of extracts from cultured hepatocyte media (case number 6). A and C: pure culture (PC); B and D: co-culture (CC). A and B: 2.5×10^6 hepatocytes incubated with 1 mM theophylline; C and D: 2.5×10^6 hepatocytes incubated with 1 mM caffeine. E: 10^7 rat liver epithelial cells (RLEC) incubated with 1 mM caffeine. For abbreviations, see footnote.

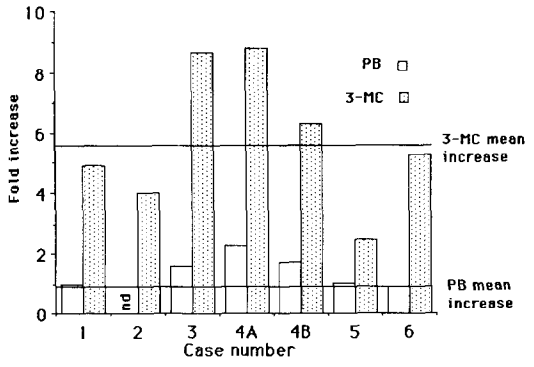

Fig. 3. Effects of PB and 3-MC on the overall metabolism of caffeine in co-cultured human hepatocytes. Results are expressed as fold increase compared to control. The cells were treated for 3 days. Rates of caffeine biotransformation were for controls: case 1 = 16.8; case 2 = 21.1; case 4B = 18.7; case 5 = 22.2 and case 6 = 52.3 nmoles of 1 mM caffeine transformed/10^6 hepatocytes/24 hr; case 3 = 2.9 and case 4A = 4.6 nmoles of 0.1 mM caffeine transformed/10^6 hepatocytes/24 hr. No significant differences were observed between PBS and DMSO controls (see text). nd, not determined.

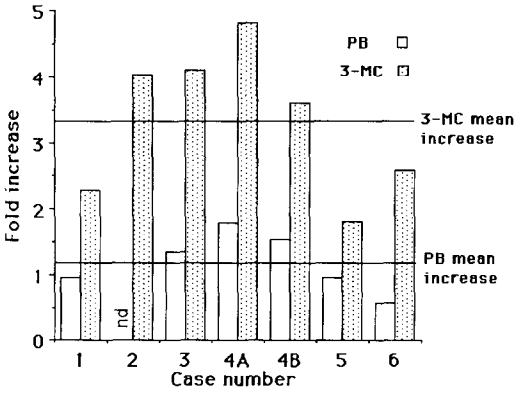

Fig. 4. Effect of PB and 3-MC treatment on the overall metabolism of theophylline in co-cultured human hepatocytes. The cells were treated for 3 days. Results are expressed as fold increase compared to controls. Rates of theophylline biotransformation were for controls: case 1 = 15.1; case 2 = 21.2; case 4B = 37.1; case 5 = 19.5; case 6 = 74.6 nmoles of 1 mM theophylline transformed/10^6 hepatocytes/24 hr; case 3 = 1.3 and case 4A = 4.5 nmoles of 0.1 mM theophylline transformed/10^6 hepatocytes/24 hr. nd, not determined.

Table 3. Metabolic rates of TB, TP and PX formation from caffeine incubated with co-cultured hepatocytes (case number 5: Br021)

	TB	PX	TP	1,3,7-TMU	Total rate
Control*	5.3	15.1	1.8	<0.05	22.2
PB	3.5	17.5	1.7	0.1	22.8
3-MC	7.7	45.1	2.2	0.15	55.15
Control†	1.7	5.5	0.9	<0.05	8.1
PB	2.9	17.5	1.3	0.2	21.9
3-MC	3.1	14.7	157.7	0.2	186.2

* 3-day treatment.
† 5-day treatment.
Results (means of three plates) are expressed as nmol/10^6 hepatocytes/24 hr.

Br017). Accordingly results were compared relatively to the same controls.

A 3-day PB treatment of human hepatocyte cocultures resulted in a slight increase, if any difference, of overall caffeine metabolism: 1.4 ± 0.5-fold (mean ± SD, N = 5 subjects) vs control while 3-MC treatment increased it markedly: 5.8 ± 2.3-fold (mean ± SD, N = 6 subjects) (Fig. 3). No significant additive increase of overall caffeine metabolism was observed after 5 days of treatment with either PB or 3-MC except for case number 5 (Br021) where 3-MC treatment increased the caffeine metabolic rate up to 186 nmol/10^6 hepatocytes/24 hr vs 55 nmol/10^6 hepatocytes/24 hr in 3 days treated cultures (Table 3).

Overall theophylline metabolism was slightly increased after 3 days of PB treatment: 1.2 ± 0.4-fold (mean ± SD, N = 5 subjects) vs controls while 3-MC treatment increased it markedly: 3.3 ± 1.1-fold (mean ± SD, N = 6) (Fig. 4).

Although inter-individual variations were noted, the differences are beyond the magnitude of any experimental error. Furthermore, the change in metabolism of the two methylxanthines by cultured hepatocytes from the same donor was highly correlated whatever the treatment and the test compound (Fig. 5). Thus changes in caffeine and theophylline metabolism were highly correlated after PB (Fig. 5A: $r = 0.92$, $P < 0.01$, $N = 6$) or 3-MC treatment (Fig. 5B: $r = 0.77$, $P < 0.04$). Similarly, changes in caffeine metabolism were highly correlated after PB and 3-MC treatments (Fig. 5C: $r = 0.81$, $P < 0.05$, $N = 6$); the same phenomenon was observed for theophylline (Fig. 5D: $r = 0.83$, $P < 0.04$, $N = 6$).

Effects of PB treatment on caffeine and theophylline metabolic profiles

A 3-day exposure to PB of human hepatocyte cocultures modified the relative ratio of the three DMX formed from caffeine. In all cases studied, such a treatment slightly increased the N-3 demethylation (1.6 ± 0.6-fold) and the N-7 demethylation (1.3 ± 0.6-fold) (mean ± SD, N = 5) whereas the N-1 demethylation was not significantly modified (Fig. 6). Although marked inter-individual variations were observed, a significant correlation between the PX and TP changes was noted ($r_s = 0.88$, $N = 6$, $P < 0.02$). A PB treatment up to 5 days slightly increased caffeine biotransformation into PX in relation to the 3 day-treatment whereas the formation of TP and TB was not modified compared to controls (results not shown).

The treatment by PB had little effect if any on the formation of 3-MX and 1,3-DMU from theophylline. In addition, PB was inefficient on TP methylation

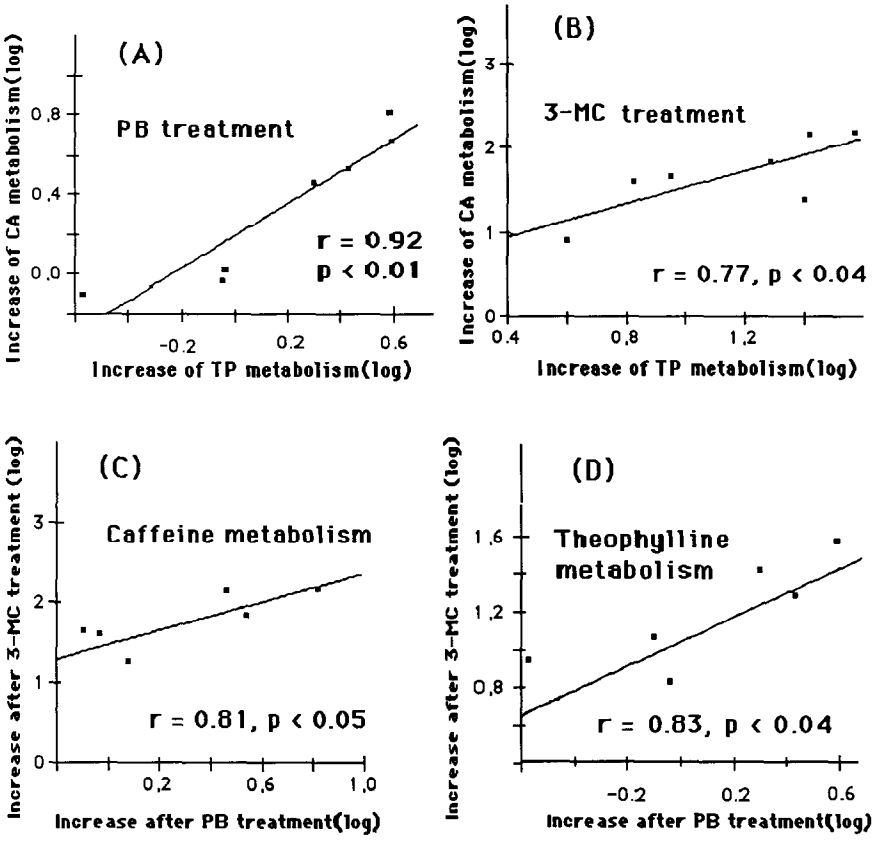

Fig. 5. Relationship between changes of caffeine and theophylline biotransformations induced by PB and 3-MC treatments. Modifications of overall metabolic rates are expressed as fold increase compared to controls. Correlations are reported between changes induced in caffeine and theophylline metabolism by PB (A) or 3-MC (B) treatments and between effects of PB and 3-MC treatments on caffeine (C) or theophylline (D) metabolism.

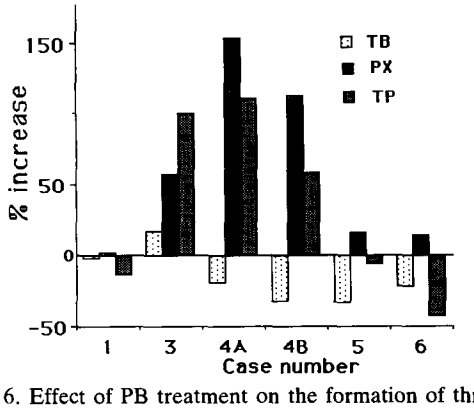

Fig. 6. Effect of PB treatment on the formation of three DMX (TB, PX, TP) from caffeine in co-cultured human hepatocytes. The results are expressed as percentage of controls. The ratios of TB/PX/TP (total three DMX = 100) were in control cultures: case 1 = 14/56/30, case 2 = 27/43/30, case 3 = 20/76/4, case 4A = 12/69/19, case 4B = 20/56/24, case 5 = 24/68/8 and case 6 = 24/54/22 per cent of total three DMX formed.

into CA (Table 4). It was not possible to evaluate the effect of PB on 1-MX formation because the level of this compound in control cultures was too low.

Effect of 3-MC treatment on caffeine and theophylline metabolic profiles

A 3-day 3-MC treatment significantly increased PX formation from caffeine in all cell populations (mean 633%; 198–915% range) (Fig. 7). It also increased TP formation (mean 257%; 22–666% range). Statistical analysis indicated that PX and TP formations were highly correlated ($r_s = 0.89$, $P < 0.007$). On the contrary no correlation was observed between TB formation and the other two DMXs ($r_s = 0.14$ with PX and 0.14 with TP). 3-MC treatment up to 5 days had no additive effect on the formation of the 3 DMXs,except for case number 5 (Br021) where TP was largely increased and became the major metabolite of caffeine (Table 3). In all cases, TMU formation was a minor pathway. For example, the Σ(DMX)/TMU ratio was 55, 83 and 125 (case Br024) 85, 83 and 210 (case Br017) in controls, PB and 3-MC treated cultures respectively. Another minor metabolite of caffeine was detected, namely 1,3,7-DAU (Fig. 2). PB treatment did not modify the formation of this diaminouracil whereas 3-MC significantly increased its formation (9.4-fold in Br024 hepatocytes

Table 4. Theophylline metabolites formed in co-cultured human hepatocytes

Case	Treatment (3 days)	1,3-DMU	3-MX	1-MX	CA
1	None	2.8	1.9	0.1	10.2
	PB	4.0	3.4	<0.05	8.4
	3-MC	11.1	11.0	0.8	11.2
2	None	8.3	1.9	<0.05	10.9
	PB	nd	nd	nd	nd
	3-MC	57.3	19.8	1.25	7.4
3*	None	0.4	0.5	<0.50	0.4
	PB	0.5	0.95	0.1	0.2
	3-MC	2.25	2.45	0.45	0.2
4A*	None	3.0	0.95	<0.50	0.5
	PB	5.3	2.45	<0.05	0.5
	3-MC	15.5	6.05	<0.05	0.6
4B	None	27.6	3.7	<0.05	5.4
	PB	41.5	9.9	0.08	5.3
	3-MC	93.3	36.3	0.6	3.8
5	None	11.7	4.1	1.85	1.8
	PB	10.8	5.2	1.25	1.5
	3-MC	17.0	10.5	6.1	1.75
6	None	53.8	7.7	1.1	11.0
	PB	23.4	7.8	0.5	10.5
	3-MC	134.9	45.8	3.1	8.7

Metabolic rates are expressed as nmol/10^6 hepatocytes/24 hr.

* Theophylline concentration = 0.1 mM; nd, not determined.

1,3-DMU = 1,3-dimethyluric acid; 3-MX = 3-methylxanthine; 1-MX = 1-methylxanthine; CA = caffeine.

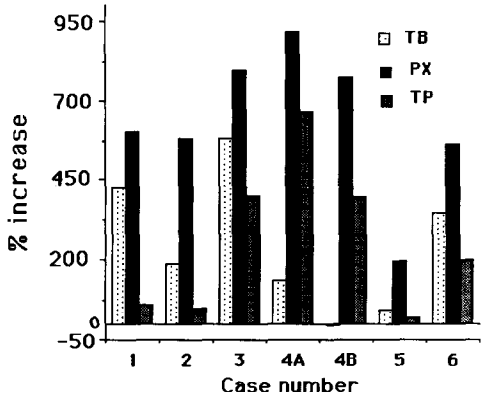

Fig. 7. Effect of 3-MC treatment on the formation of the three DMX (TB, PX, TP) from caffeine in co-cultured human hepatocytes. The results are expressed as percentage of controls. The ratios of TB/PX/TP in controls are indicated in Fig. 6.

for example). In all cases, human hepatocyte cultures failed to produce a detectable amount of the acetylated metabolite of caffeine, namely AFMU.

Like PB, 3-MC-treatment did not affect methylation of theophylline into CA. It enhanced the N-demethylation reaction leading to 3-MX by 554% (156–942% range) and the formation of 1,3-DMU by 314% (45–590% range). The 3-MC induced changes in formation of 1,3-DMU and 3-MX were not correlated ($r_s = 0.46$). 1-MX, which was barely detected in control plates was easily evidenced after 3-MC treatment; and in cases Br021 and Br024, its formation was increased 3.3 and 2.8 times respectively (Table 4). Two additional days of 3-MC treatment did not further alter metabolic profiles of TP compared to a 3-day treatment.

DISCUSSION

The advantages of the hepatocyte culture model, especially from human origin, in toxico-pharmacological studies, was extensively discussed in recent reviews [28–30]. This *in vitro* model correlated with the *in vivo* situation. In this present report, huge inter-individual variations regarding caffeine and theophylline metabolism and the response of cultured human hepatocytes to inducers were observed. The basal values of metabolic rates between six donors varied about 4- and 5-fold for caffeine and theophylline respectively in cultured hepatocytes. Similarly, inter-individual variations of the same magnitude were observed for EROD (9-fold), acetanilide 4-hydroxylation (3-fold) and caffeine biotransformation (5-fold) activities measured in liver microsomes (Table 1). These variations certainly reflect great *in vivo* variability in caffeine and theophylline half-life times [31]. They may be related to numerous causes such as genetic and environmental factors or premedication before death [28].

Although overall caffeine and theophylline metabolism in co-cultured human hepatocytes was higher than that observed in pure culture, the rate of biotransformation was low compared to that known *in vivo*. However, as previously reported [20], this low rate of methylxanthine metabolism in cultured hepatocytes was only apparent. When the results are expressed as nmoles per million hepatocytes per 24 hr a rough correlation between *in vitro* and *in vivo* results can be demonstrated.

Caffeine and theophylline are so extensively transformed in the liver of most species by oxidative N-demethylation and oxidation reactions that only a small amount is excreted unchanged. Many experiments have demonstrated a relationship between PAH-inducible P-450 activity and biotransformation of both caffeine and theophylline. Thus, pretreatment of rat and dog with 3-MC markedly enhanced the metabolic capacity to eliminate caffeine whereas PB has only a small effect [4–7]. In addition the clearance of caffeine was higher in smokers than in non-smokers [8–11]. The highly significant correlation between EROD activity and caffeine transformation in human liver microsomes was also reported [12, 19]. EROD activity was more highly induced in 3-MC-treated than in PB-treated human hepatocytes (1 pmol/min/mg microsomal protein in controls, 21 and 198 in PB-treated and 3-MC-treated cultures respectively, results obtained from case

Br017). Results reported here demonstrate that P-450 isozyme(s) inducible by 3-MC were involved in the metabolism of caffeine and theophylline in human hepatocytes. Our data confirm not only the hypothesis formulated for humans *in vivo* but also show that PB is not a specific inducer of theophylline and caffeine metabolism. PB seems only able to stabilize P-450 activity involved in these biotransformations in hepatocyte culture.

Although the results reported here demonstrate the involvement of 3-MC-inducible P-450 in caffeine and theophylline metabolism, at present the number of PAH-inducible P-450 isozymes involved in the first demethylation reactions of caffeine is still unknown. Our results suggest that at least two isozymes are involved in these reactions. Correlation studies suggest that TB formation is mediated by a P-450 isozyme different from that involved in PX and TP formations. This assertion agrees with the reported distinctive maturation of TB formation compared to TP and PX formation in newborns [32]. Similarly TB formation was modified compared to the other two demethylated metabolites in cirrhotic patients (P. Bechtel, personal communication). Two other results suggest that at least the N-1 demethylation is performed by a P-450 isozyme different from that involved in N-7 demethylation. Firstly, only the N-7 demethylation was detected in newborn hepatocyte cultures [20]. Secondly the N-7 demethylation of caffeine into TP became the major metabolite in Br021 cultured hepatocytes treated by 3-MC for 5 days. This somewhat surprising behaviour could not be explained at this time, but it must be kept in mind that it was previously observed in cultures of untreated human hepatocytes [20]. Further studies are needed to elucidate the physiological significance of this modification in caffeine metabolic profile which has been reported *in vitro* by 3-MC treatment.

PB treatment modified slightly the overall metabolism of theophylline (1.2-fold increase) whereas 3-MC treatment increased it markedly (3.3-fold increase). Our findings clearly show that this increase was basically due to the demethylation reaction into 3-MX (554% mean increase) and the oxidation of theophylline into 1,3-DMU (314% mean increase). The methylation of theophylline into caffeine was not modified by inducers. Although this methylation reaction was believed to be unique to the newborn [33–35], previous results either *in vivo* [36] or *in vitro* using the human hepatocyte culture model [20] have demonstrated it in adults. As it is not PAH-inducible, this methylation is performed by an enzyme different from that involved in demethylations. It is generally assumed that N-1 and N-3 demethylations are predominantly performed by the same P-450 isozyme [2, 19, 37, 38]. The results reported here suggest that two different P-450 isozymes are involved in these two demethylations of theophylline. The formation of 1-MX in cultured hepatocytes was very low whereas 3-MX represented the major demethylated metabolite of theophylline. The small amount of 1-MX detected in culture media is surprising. This could be due to its transformation to 1-MU by hepatic xanthine oxidase. Although the extraction conditions used in this study allowed only about 50% recovery of 1-MU [12], this metabolite was never detected. Moreover Mulder *et al.* [39] using an analytical procedure without an extraction step, also failed to detect 1-MU or 1-MX in isolated rat hepatocytes incubated with theophylline. 3-MC treatment increased more significantly 3-MX formation (554% mean increase; 156–942% range) than 1-MX formation (182 and 229% for case numbers 5 and 6, respectively). Our results agree with previous studies indicating that the C8-hydroxylation of theophylline is performed by a P-450 isozyme different from those involved in demethylations [2, 19, 27]. Although these two types of reactions are induced by 3-MC, it is clear that 3-MX formation was increased more significantly than C8-hydroxylation. Indeed the 1,3-DMU/3-MX ratio was 3.8 ($\pm$2.5) in control plates versus 2.1 ($\pm$0.8) (mean $\pm$ SD, N = 6) in 3-MC induced plates (Table 4). Furthermore, the changes induced by 3-MC regarding their respective formation were not correlated ($r_s = 0.46$).

It has been assumed that methylxanthines including theophylline and caffeine are metabolized by a common methylxanthine group of P-450 isozymes [12]. Our findings suggest that the N-1 demethylations of theophylline into 3-MX and caffeine into TB are not performed by the same isozyme. Indeed, after 3-MC treatment, the 3-MX formation from theophylline increased more significantly (554% mean increase) than the TB formation from caffeine (200% mean increase). Moreover, the 3-MC induced changes in N-1 demethylation of caffeine and theophylline were not at all correlated ($r_s = -0.3$). Similarly, the N-3-demethylation of theophylline into 1-MX and caffeine into PX are performed by a different isozyme. Indeed, 1-MX from theophylline was formed so weakly that it was hardly detected whereas PX was the major metabolite of caffeine in control and in induced hepatocytes.

By using cultured human hepatocytes the induction of theophylline C-8 hydroxylation pathway by 3-MC treatment was clearly demonstrated. When caffeine was used as substrate this C-8 hydroxylation remained a minor pathway. These data raised the question of the nature of P-450 isozyme(s) involved in caffeine and theophylline C-8 hydroxylation. As such an extensive isozymic heterogeneity was not previously recognized in caffeine and theophylline metabolism it must be kept in mind that all results reported here were obtained with relatively high concentrations (1 mM) of these methylxanthines and after cell treatment by PB or 3-MC.

Our results confirm that mainly the P-450IA is involved in primary demethylations of caffeine and theophylline. This PAH-inducible family contains two enzymes, P-450IA1 and P-450IA2. Studies using selective substrates of these isozymes or specific inhibitors were not able to distinguish which isozyme is involved in these biotransformations [16, 19]. This could be due to overlapping specificity of these compounds. However, as P-450IA1 is not expressed in human liver [40] the hypothesis that P-450IA2 plays a major role in oxidative demethylations of methylxanthines may be put forward.

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