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Theophylline Metabolism by Human, Rabbit and Rat Liver Microsomes and by Purified Forms of Cytochrome P450

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Abstract—The capacity of human, rabbit and rat liver microsomes and purified isozymes of cytochrome P450 to metabolize theophylline has been assessed. In all three species the 8-hydroxylation of theophylline to 1,3-dimethyluric acid (1,3-DMU) was the major pathway. In human, control rabbit and rat liver microsomes this metabolite accounted for 59, 77 and 94%, respectively, of the total metabolites formed. In both human and control rabbit liver microsomes the *N*-demethylation of theophylline to 1-methylxanthine (1-MX) accounted for 20% of the total metabolites formed. *N*-demethylation of theophylline to 3-methylxanthine (3-MX) accounted for 21% of theophylline metabolism in human microsomes but was a minor pathway in control rabbit and rat microsomes. Acetone and phenobarbitone pretreatment markedly increased the formation of 1,3-DMU by rabbit liver microsomes. Rifampicin and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) administration caused a slight but significant increase in this pathway. In general the *N*-demethylation pathways in rabbit liver microsomes were refractory to induction. In the rat, the metabolism of theophylline to 1-MX, 3-MX and 1,3-DMU were all significantly increased in Aroclor 1254, dexamethasone, phenobarbitone and 3-methylcholanthrene-treated microsomes. In reconstitution experiments the polycyclic hydrocarbon inducible rabbit cytochrome P450 Forms 4 and 6 and the constitutive Form 3b all metabolized theophylline to its three metabolites. In human liver microsomes from four subjects anti-rabbit cytochrome P450 Form 4 IgG inhibited the metabolism of theophylline to 1-MX, 3-MX and 1,3-DMU by approximately 30%. These data indicate that theophylline is metabolized by multiple forms of cytochrome P450 in human, rabbit and rat liver microsomes.

Theophylline is widely used as a bronchodilator in the treatment of patients with reversible obstructive airways disease. In man theophylline undergoes extensive metabolism, with only 10% of the dose being excreted unchanged. The major metabolic route is 8-oxidation to 1,3-dimethyluric acid (1,3-DMU), which accounts for approximately half of the total theophylline clearance. Theophylline is also *N*-demethylated to 3-methylxanthine (3-MX) and 1-methylxanthine (1-MX), which account for the remaining metabolic clearance. 1-MX undergoes further oxidation by xanthine oxidase to 1-methyluric acid (Grygiel & Birkett 1981; Hendeles & Weinberger 1983; Grygiel et al 1984). Marked interindividual differences in the clearance of theophylline have been observed which have complicated the use of this drug because of its narrow plasma therapeutic range (10–20 $\mu g\ mL^{-1}$) for optimal bronchodilator efficacy (Hendeles & Weinberger 1983). In a recent study employing human liver microsomes and anti-human NADPH-cytochrome P450 reductase IgG, we showed that both the 8-hydroxylation and *N*-demethylations of theophylline were cytochrome P450-mediated (Robson et al 1987, 1988). Further, in-vivo induction and inhibition studies in man suggest that at least two forms of cytochrome P450 are involved in the metabolism of theophylline (Grygiel et al 1984; Robson et al 1984; Miners et al 1985). These observations have also been confirmed at the microsomal level (Robson et al 1988).

Variability in theophylline clearance is thought to reflect differences in the expression of cytochrome P450 isozymes involved in its metabolism. Both human (Grygiel & Birkett 1981; Campbell et al 1987) and animal (Lohmann & Miech 1976; Williams et al 1979; Betlach & Tozer 1980) data are suggestive of a major role of polycyclic hydrocarbon inducible forms of cytochrome P450 in theophylline metabolism. Therefore, to determine the contribution of these forms and other cytochrome P450 isozymes in this process, the metabolism of theophylline was studied in control and induced rabbit and rat liver microsomes and by purified forms of rabbit cytochrome P450. In addition, to gain information on the human isozymes involved in theophylline metabolism, an antibody raised against a TCDD-inducible rabbit cytochrome P450 (Form 4) was tested for its ability to inhibit this process in human liver microsomes.

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Methods

Animals

Male and female White New Zealand rabbits, 3·0–4·5 kg, and male hooded Wistar rats, 200–300 g, were obtained from the Institute of Medical and Veterinary Science (Adelaide, Australia), housed in plastic cages and allowed free access to food and water. Three male animals per group were treated with the following compounds at the indicated dosages: sodium phenobarbitone, 70 $mg\ kg^{-1}$ in 0·15M NaCl administered i.p. once daily for five days; rifampicin, 50 $mg\ kg^{-1}$ in 10 mM sodium phosphate (pH 8·2) containing 0·15M sodium chloride given i.p. once daily for four days; β-naphthoflavone, 40 $mg\ kg^{-1}$ in corn oil i.p. once daily for four days; 3-methylcholanthrene, 40 $mg\ kg^{-1}$ in corn oil i.p. once daily for four days; acetone, 1% v/v in drinking water for nine days; Aroclor 1254, 300 $mg\ kg^{-1}$ injected once in corn oil i.p. three days before death; dexamethasone, 300 $mg\ kg^{-1}$ dissolved in 0·9% NaCl (saline) containing a few drops of Tween 20, by gastric gavage once daily for four days. Four female rabbits were injected once each with 10 $\mu g\ kg^{-1}$ 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) dissolved in dimethylsulphoxide i.p. five days before death. Control animals received

no treatment. Animals were killed by cervical dislocation and liver microsomes were prepared as previously described (McManus et al 1983). The drugs and chemicals used were obtained by McManus, Burgess, Snyderwine & Stupans (to be published).

Enzymes and antibodies

Rabbit liver cytochrome P450 Forms 3b, 4 and 6 were purified from control, β-naphthoflavone- and TCDD-pretreated rabbit liver microsomes, respectively, as previously described (McManus et al 1984). NADPH-cytochrome P450 reductase was purified from phenobarbitone-induced rabbit liver microsomes as previously described (McManus et al 1984). An antibody (IgG) against Form 4 was raised in the goat as described by McManus et al (1987b). The polyclonal antibody raised against rabbit cytochrome P450 Form 6 was a kind gift from Dr E. F. Johnson, Scripps Clinic and Research Foundation, La Jolla, Ca, USA. Immunoquantitation of cytochrome P450 Forms 4 & 6 content of control and induced rabbit liver microsomes was carried out as described by McManus et al (to be published).

Human microsomes

Microsomal fractions of human liver were obtained as described previously from samples of liver from renal transplant donors maintained on life-support systems until the kidneys could be removed (McManus et al 1987a). The use of such tissue in these studies had local Research Ethics Committee approval and where appropriate, Coroner's permission. Samples were stored at −80°C until required.

In-vitro theophylline assay

The in-vitro metabolism of theophylline was measured according to the method of Robson et al (1987). Briefly, 12·5 nmol [8- ^{14}C]theophylline plus unlabelled theophylline to give a final concentration of 200 μM, microsomal protein 4 mg mL^{-1} and 0·1M phosphate buffer pH 7·4 in a final volume of 0·5 mL were incubated for 2 h with a NADPH-generating system. For reconstitution experiments, 0·2 nmol of either cytochrome P450 Forms 3b, 4 or 6, 0·75 units of NADPH-cytochrome P450 reductase and 60 μg of dilauroyl-L-α-lecithin were substituted for the microsomal protein. Reactions were stopped by cooling on ice and by the addition of 50 μL 5M hydrochloric acid. Authentic standards of 3-MX, 1-MX and 1,3-DMU were added to visualize absorbance peaks during chromatography.

The aqueous reaction mixture was saturated with ammonium sulphate and extracted twice with 10 mL of dichloromethane–isopropanol (80:20 v/v). The pooled extracts were evaporated under nitrogen and the residue redissolved in 0·1 mL mobile phase for chromatograhic analysis. Reconstituted sample (85 μL) was injected onto a reversed-phase C-18 column (ICI ODS-2, 4 mm × 15 cm). The initial mobile phase composition was 1% (v/v) methanol–1% (v/v) acetonitrile–98% (v/v) 0·01M acetic acid, pH 3·5. Absorbance was monitored at 280 nm. At 13 min, the mobile phase composition was changed to 7·5% methanol–7·5% acetonitrile–85% 0·01M acetic acid, pH 3·5. The mobile phase flow rate was 2·0 mL min^{-1}. Retention times for 3-MX, 1-MX 1,3-DMU and theophylline under these conditions were 6·75 min, 8 min, 11·5 min and 16·5 min, respectively. Column effluent was collected in aliquots and counted on a liquid scintillation counter (Beckman Model LS 3801). Chromatography was performed using a Beckman 342 gradient liquid chromatograph modular system, consisting of a model 420 system controller, two model 114 solvent delivery modules, a model 340 organiser, and a model 160 selectable wavelength detector.

Results

Table 1 shows the rate of formation of 1-MX, 3-MX and 1,3-DMU in human, and control rabbit and rat liver microsomes. In all three species the 8-hydroxylation of theophylline to 1,3-DMU was the major pathway. In human, rabbit and rat liver microsomes this metabolite accounted for 59, 77 and 94%, respectively, of total metabolites formed. In human and rabbit liver microsomes the *N*-demethylation of theophylline to 1-MX was also a major pathway accounting for 20% of the total metabolites formed in each species. This pathway accounted for less than 4% of the theophylline metabolites formed in rat liver microsomes. The *N*-demethylation of theophylline to 3-MX accounted for 21, 3·3 and 2·9% of total theophylline metabolites formed in human and control rabbit and rat liver microsomes, respectively.

Fig. 1 shows the effect of inducing agents on the metabolism of theophylline by rabbit and rat liver microsomes. Both acetone and phenobarbitone pretreatment of rabbits significantly induced the 8-hydroxylation of theophylline. While rifampicin also caused a significant increase in 1,3-DMU formation in rabbit liver microsomes, the increase was minor compared with the effects of either acetone or phenobarbitone. TCDD caused a statistically significant increase in the *N*-demethylation of theophylline to 3-MX while pretreatment of rabbits with rifampicin caused a significant reduction in this metabolic pathway. The 3-demethylation of theophylline to 1-MX was not induced in any of the pretreated microsomes tested. In the rat statistically significant increases in the rates of formation of 1-MX, 3-MX and 1,3-DMU were observed in microsomes from Aroclor 1254, phenobarbitone, dexamethasone and 3-methylcholanthrene induced animals. Aroclor 1254 was the most potent inducer of both 1,3-DMU and 1-MX formation, whereas the three inducers were approximately equipotent in inducing 3-MX formation.

In reconstitution experiments the polycyclic hydrocarbon inducible rabbit cytochrome P450 Forms 4 & 6 and the constitutive Form 3b all metabolized theophylline (Table 2).

Table 1. Theophylline metabolism in human and control rabbit and rat liver microsomes.

	Theophylline metabolism (pmol mg^{-1} min^{-1})		
Species	1-MX	3-MX	1,3-DMU
Man	1·19±0·53	1·30±0·48	3·62±0·97
Rabbit	2·25±0·49	0·36±0·05	8·50±0·42
Rat	0·27±0·08	0·34±0·03	11·11±0·35

Results represent mean±s.d., n≥3.

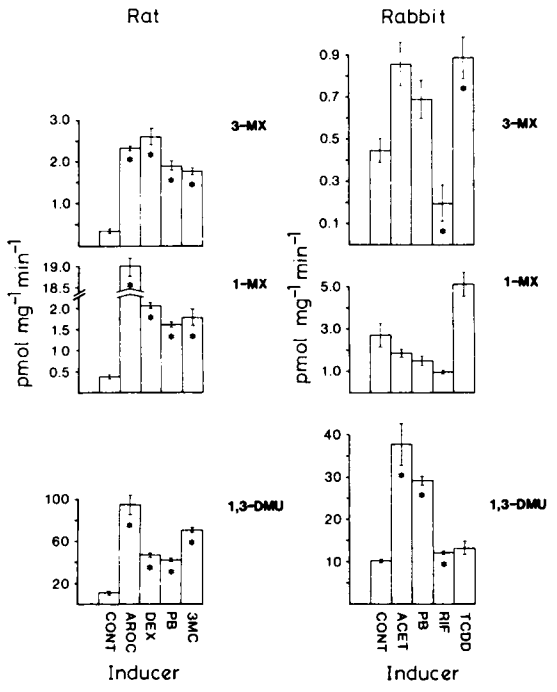

FIG. 1. Effect of induction on the metabolism of theophylline by rat and rabbit liver microsomes. Details of the induction protocols are given under Methods. Symbols represent: cont, control; Aroc, Aroclor 1254; Dex, dexamethasone; PB, phenobarbitone; 3MC, 3-methylcholanthrene; Acet, acetone; Rif, rifampicin and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Results are expressed as the mean ± s.e.m., n = 3. Values significantly different from the control ($P < 0.05$) are indicated by an asterisk.

Table 2. Metabolism of theophylline by purified rabbit liver cytochromes P450.

	Theophylline metabolism (pmol nmol^{-1} P450 min^{-1})		
Cytochrome P450	1-MX	3-MX	1,3-DMU
Form 3b	0·36	0·50	3·38
Form 4	0·83	0·34	6·2
Form 6	0·26	0·34	6·6

Table 3. Inhibition of theophylline metabolism in human liver microsomes by anti-rabbit cytochrome P450 Form 4 IgG.

		Theophylline metabolism (pmol mg^{-1} min^{-1})		
Subject	Treatment	1-MX	3-MX	1,3-DMU
H6	Preimmune	1·88	1·81	4·83
	Anti-form 4	1·19	1·32	3·00
H7	Preimmune	0·63	0·72	2·70
	Anti-form 4	0·47	0·58	1·84
H8	Preimmune	0·97	1·12	2·99
	Anti-form 4	0·64	0·78	2·03
H9	Preimmune	1·27	1·53	3·95
	Anti-form 4	0·84	1·13	2·42

The major metabolite formed in all cases was 1,3-DMU and Form 3b exhibited approximately half of the activity of Forms 4 or 6 in forming this metabolite. The combined amounts of 1-MX and 3-MX constituted 20% or less of the total metabolites formed by all purified forms of cytochrome P450 tested. When the contents of cytochrome P450 Forms 4 & 6 in control and induced rabbit liver microsomes, determined by Western blotting, were correlated with the formation of 1-MX, 3-MX and 1,3-DMU significant positive correlations were obtained only for 1-MX formation. The correlation coefficient between 1-MX: Form 4 was 0·72 and between 1-MX: Form 6 was 0·65 ($P < 0.05$, n = 15). Correlation coefficients between 3-MX: Form 4 and 1,3-DMU: Form 4 were 0·47 and −0·08, respectively. Corresponding values for 3 MX: Form 6 and 1,3-DMU: Form 6 were 0·37 and −0·13, respectively.

Table 3 summarizes the capacity of anti-rabbit cytochrome P450 Form 4 IgG to inhibit theophylline metabolism in human liver microsomes from four subjects. The mean percentage inhibitions of the formation of 1-MX, 3-MX and 1,3-DMU in human liver microsomes by anti-rabbit cytochrome P450 Form 4 IgG were 33 ± 5, 26 ± 5 and 35 ± 4, respectively. The Form 6 antibody used for immunoquantitation of this isozyme was non-inhibitory and consequently no inhibition data are provided.

Discussion

A major goal of these studies was to develop an animal model to identify specific isozymes of cytochrome P450 involved in the metabolism of theophylline. The rationale for developing an animal model is predicated on the demonstration of considerable transpecies homology in cytochrome P450 isozymes between animals and man (Quattrochi et al 1985; Guengerich et al 1986). Indeed, Guengerich et al (1986) have used this approach successfully to identify human liver cytochromes P450 involved in polymorphisms of drug oxidation. In the present study the major metabolite produced by human and control rabbit and rat liver microsomes was 1,3-DMU. The formation of 1-MX was also similar in human and rabbit liver microsomes and comprised approximately 20% of the total metabolites formed. In rat liver microsomes 1-MX formation was a minor pathway (< 4%). 3-MX formation comprised 21% of theophylline metabolites formed in human liver microsomes but was less than 4% of metabolites formed in control rabbit and rat microsomes. These data show that control rabbit liver microsomes, for at least two pathways, resemble the human situation more closely than control rat microsomes.

Rat cytochromes P450 appear to exhibit no specificity towards theophylline as a substrate. For example, pretreatment of rats with 3-methylcholanthrene which induces P450 c and d, dexamethasone (P450p), phenobarbitone (P450b and e), and Aroclor 1254 which induces both 3-methylcholanthrene and phenobarbitone inducible forms (Conney 1986), all increased the microsomal metabolism of theophylline to its three metabolites. These data suggest that theophylline is a substrate for at least five forms of rat cytochrome P450. Further, the results of this study are consistent with previous in-vivo and in-vitro studies that have shown

both 3-methylcholanthrene and phenobarbitone pretreatments to increase the metabolism of theophylline in the rat (Lohmann & Miech 1976; Williams et al 1979).

Various observations in both man and animals suggest that polycyclic hydrocarbon inducible forms of cytochrome P450 are involved in the metabolism of theophylline (Grygiel & Birkett 1981; Campbell et al 1987; Lohmann & Meich 1976; Williams et al 1979; Betlach & Tozer 1980). Definitive proof for this assumption is provided in this study in that both rabbit cytochromes P450 Forms 4 & 6 were capable of mediating the *N*-demethylations and 8-hydroxylation of theophylline. The constitutive Form 3b (Schwab & Johnson 1987) also catalysed these reactions. However, statistically significant correlations were only obtained between 1-MX formation: Form 4 content and 1-MX formation: Form 6 content, and these only accounted for 52 and 42% of the variance, respectively. The above data plus the lack of a correlation between the formation of 1,3-DMU, the major metabolite, and Form 4 and Form 6 content in control and induced microsomes strongly indicates that these forms play only a minor role in the metabolism of theophylline in rabbit liver microsomes. A marked increase in the formation of 1,3-DMU was observed in rabbit liver microsomes following induction with acetone and phenobarbitone. Acetone treatment has been shown to induce cytochrome P450 Form 3a (Koop et al 1985; Schwab & Johnson 1987) and phenobarbitone Forms 2 and 5 in the rabbit (Schwab & Johnson 1987). Thus besides the three purified forms of rabbit cytochrome P450 in this study that have been shown to metabolize theophylline, this compound may also be a substrate for at least three other forms.

The partial inhibition ($\approx$30%) of theophylline metabolism in human liver microsomes by anti-rabbit cytochrome P450 IgG suggest that the human ortholog of Form 4 is involved in this process. Grygiel & Birkett (1981) have previously shown that cigarette smoking caused a 2- and 1·68-fold increase in the clearance of theophylline in-vivo via *N*-demethylation and 8-hydroxylation pathways, respectively. It is tempting to speculate that smoking induces the human ortholog of Form 4 which accounts for the increased clearance of theophylline. However, it is clear from the present data that theophylline is a promiscuous substrate, in that it is metabolized by a number of forms of rabbit cytochrome P450 and its microsomal metabolism is induced by different inducing agents in the rabbit and rat. These animal data are consistent with previous in-vivo and in-vitro studies from this group which have shown that at least two forms of cytochrome P450 are involved in theophylline metabolism in man (Grygiel & Birkett 1981; Grygiel et al 1984; Miners et al 1985).

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