Multiplicity of Cytochrome P-450 Species Involved in Theophylline Metabolism in Mouse Hepatic Microsomes

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To ascertain the multiplicity of the cytochrome P-450 (P-450) species participating in the individual metabolic conversion of theophylline by 8-hydroxylation, 3-demethylation and 1-demethylation in mice, kinetics were studied under various conditions using untreated and inducer-treated mouse hepatic microsomes. Eadie–Hofstee plots of 1-demethylation in untreated microsomes exhibited a straight line, whereas those of 8-hydroxylation and 3-demethylation were curved lines. The biphasic kinetics indicated the contribution of two P-450 populations to the respective metabolic pathways; one characterized by high affinity and low capacity, the other by low affinity and high capacity. The high affinity population was efficiently induced by β -naphthoflavone (β -NF), and was highly susceptible to inhibition by a specific CYP1A inhibitor. The low affinity population was sensitive to induction by phenobarbital (PB), and was markedly inhibited by preferential inhibitors for PB-inducible P-450 species. The present results indicated that two P-450 populations contributed to the theophylline metabolism in mouse hepatic microsomes, and that the high and low affinity populations corresponded, respectively, to CYP1A, and a PB-inducible P-450 species having a much higher capacity than CYP1A.

Key words theophylline metabolism; cytochrome P-450 species; theophylline 8-hydroxylation; theophylline demethylation; CYP1A; mouse hepatic microsome

Theophylline is extensively metabolized by 8-hydroxylation and 1- or 3-demethylation, to 1,3-dimethyluric acid (1,3-DMU), 3-methyl xanthine (3-MX) and 1-methyl xanthine (1-MX), respectively. These oxidative reactions are mediated by the hepatic microsomal cytochrome P-450 (P-450) species.^{1,2)}

A particular characteristic of theophylline metabolism common to many mammals, including humans, is that it is enhanced upon exposure to several polycyclic aromatic hydrocarbon (PAH) constituents.^{3–7)} The intimate involvement of PAH-inducible P-450 species, termed CYP1A, in theophylline oxidation, has been confirmed by enzymological and immunochemical studies.^{8–13)} On the other hand, animal experiments^{4,9,10)} and clinical trials^{14,15)} have demonstrated that theophylline metabolism is induced by some agents, including phenobarbital (PB), which are irrelevant to PAH. Furthermore, it has been found that a considerable number of theophylline-metabolizing enzymes resistant to blockade by polyclonal anti-CYP1A antibody exist in human hepatic microsomes,¹²⁾ and that several P-450 species separately expressed in cell lines are capable of oxidizing theophylline.¹⁶⁾ These findings suggest that P-450 species other than CYP1A also participate in the biotransformation of theophylline, in view of evidence that P-450 consists of multiple forms of essentially distinct species.¹⁷⁾ However, it remains unclear to what degree such species contribute to the overall metabolic conversion of theophylline.

We therefore addressed this issue by means of kinetic analyses of the enzyme activities implicated in theophylline metabolism in mouse hepatic microsomes, since there is a similarity between humans and mouse regarding the quantitative proportion of the three metabolites produced via respective pathways.^{18,19)}

MATERIALS AND METHODS

Materials Theophylline, 1,3-DMU, 1-MX, 3-MX and ellipticine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium salt of PB, β -naphthoflavone (β -NF) diphenhydramine hydrochloride and sodium diethyldithiocarbamate (DEDTC) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). SKF 525-A and ketoconazole were gifts from Smith Kline & French Laboratories (Philadelphia, PA, U.S.A.) and Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), respectively. All other reagents and chemicals were of analytical grade.

Animal Treatment Male ddY strain mice (4–6 weeks old) were used after acclimation for 7 d, and they were allowed free access to food and water. The mice were injected intraperitoneally with PB (80 mg/kg/d) dissolved in propylene glycol once a day for 3 consecutive days, or with β -NF (80 mg/kg/d) suspended in propylene glycol once on the second day and with the vehicle on the first and third day. Control animals received vehicle alone at the same time. The mice were sacrificed by cervical dislocation, 24 h after the last injection.

Preparation of Hepatic Microsomes Livers from untreated mice or those treated as described above were perfused, then homogenized in ice-cold isotonic potassium chloride (1:4, w/v). The homogenate was centrifuged at $10000 \times g$ for 15 min, and supernatant fractions were centrifuged at $105000 \times g$ for 60 min to obtain microsomal pellets. The microsomes were suspended in 0.1 M sodium-potassium phosphate buffer, pH 7.4, to a final concentration of 15 mg protein/ml. The protein concentration was determined colorimetrically²⁰⁾ using bovine serum albumin as a standard. The P-450 content was measured by the method of Omura and Sato.²¹⁾

Enzyme Assay The enzyme assay mixture contained 70 mM sodium-potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, an NADPH-generating system (0.5 mM

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NADP, 5 mM glucose-6-phosphate, 5 mM MgCl_2 , and 2 units of glucose-6-phosphate dehydrogenase), microsomal protein (1.5 mg/ml), and theophylline (0.1–15 mM) in a final volume of 1.0 ml. The reaction was started by adding an NADPH-generating system which had been incubated for 3 min. The incubations were carried out at 37 °C with shaking for 30 min, and terminated with 100 μl of 15% trichloroacetic acid. If necessary, the reactions were performed in the presence of an inhibitor. SKF 525-A, diphenhydramine and DEDTC were added to the incubation mixture at a final concentration ranging from 0–500 μM . Ellipticine and ketoconazole were added in 1 μl dimethyl sulfoxide (DMSO) to give a final concentration of 0–40 μM . An equivalent volume of DMSO was added to all incubation mixtures for comparison. The 8-hydroxylase, 3-demethylase and 1-demethylase activities for theophylline were assayed by simultaneously determining the quantities of 1,3-DMU, 1-MX and 3-MX formed, according to the isocratic HPLC method following solid phase extraction, as described.²²⁾ The enzyme reaction proceeded with a requirement for NADPH, and the three metabolites were produced in a linear fashion with respect to the incubation period and microsomal protein, at least up to the levels adopted here.

Determination of Kinetic Parameters The values of the Michaelis constant (K_m) and maximum reaction velocity per unit protein ($V_{\max}$) were determined according to the following Michaelis–Menten equation: $V = \sum (V_{\max} \cdot S / (K_m + S))$, where V is the reaction velocity and S is the substrate concentration. The parameters were determined employing an extended least-squares nonlinear curve-fitting program²³⁾ run on a microcomputer (NEC, Model PC-9801VM2), using initial parameter estimates obtained from graphic analysis of Lineweaver–Burk plots of the data. Each data point was weighted so as to be numerically equal, and convergence was defined as a relative change of less than 10^{-2} in the residual sum of the squares. The values of maximum reaction velocity per unit P-450 ($V'_{\max}$) were determined by dividing $V_{\max}$ by the P-450 content.

Statistical Analysis The K_m , $V_{\max}$ and $V'_{\max}$ values for formation of the three metabolites, following exposure to the inducers, were compared by the analysis of variance followed by Fisher's multiple comparison of means. A probability value of 0.05 or less was considered to be significant.

RESULTS

Kinetic Analysis of Theophylline Metabolism Typical Eadie–Hofstee plots for theophylline 8-hydroxylation producing 1,3-DMU, and for 3- and 1-demethylation producing 1-MX and 3-MX, respectively, are shown in Fig. 1. The plot for 1-demethylation fitted a single line, whereas those for 8-hydroxylation and 3-demethylation exhibited a biphasic nature, indicating a composite of two saturable components. Two apparent K_m values (K_{m1} and K_{m2} , for high and low affinity phases, respectively) and two corresponding $V_{\max}$ values ($V_{\max1}$ and $V_{\max2}$) were obtained for 8-hydroxylation and 3-demethylation. The values of K_{m1} and K_{m2} differed by one order of magnitude, and there was also an 8–10 fold disparity between the two $V_{\max}$ values. The order of the K_m value for 1-demethylation was in agreement with that of the K_{m2} values for 8-hydroxylation and 3-demethylation.

Inducibility of Theophylline-Metabolizing Enzymes by β -NF and PB The enzyme activity of the three individual metabolic pathways was measured at varied theophylline concentrations using microsomes induced with β -NF and PB, which represent the two major classes of mono-oxygenase inducers.²⁴⁾ As shown in Fig. 2, the enzyme activities were enhanced in a different manner, depending upon the inducers and the substrate concentration. β -NF markedly enhanced 8-hydroxylation and 3-demethylation, as compared with PB at a lower substrate region, whereas PB enhancement exceeded that of β -NF at substrate concentrations over 0.5–1 mM. The 1-demethylation was enhanced only by PB.

The changes in kinetic parameters following exposure to these inducers are presented in Table 1. The values of $V_{\max1}$ for 8-hydroxylation and 3-demethylation were significantly increased by β -NF, but not by PB. In contrast, a significant increase in the $V_{\max2}$ values in all metabolic pathways were caused only by PB. There was no significant change in the K_{m1} values, irrespective of exposure to both inducers, although PB tended to reduce the K_{m2} values. The coincident changes in $V'_{\max}$ values caused by the inducers are shown in Table 2. β -NF led to a pronounced increase in the $V'_{\max1}$ values without altering the $V'_{\max2}$ values, in the same way as the $V_{\max}$. In contrast, PB tended to decrease the $V'_{\max}$ values.

The Effect of P-450 Inhibitors on Theophylline Metabo-

Fig. 1. Typical Eadie–Hofstee Plots for the 8-Hydroxylation (A), 3-Demethylation (B) and 1-Demethylation (C) of Theophylline in Hepatic Microsomes

Each point was experimentally obtained and the solid line is the computer-generated fitting curve. S , substrate concentration (mM). V , reaction velocity (pmol/mg/min).

Fig. 2. Profiles of the Enzyme Activities of 8-Hydroxylation (A), 3-Demethylation (B) and 1-Demethylation (C) of Theophylline after Exposure to Inducers

Each activity represents the mean $\pm$ S.D. of experiments with five animals given vehicle alone ($\circ$), β -NF ($\bullet$) or PB ($\blacktriangle$).

Table 1. Changes in the Kinetic Parameters for the Individual Metabolic Conversion of Theophylline for High Affinity (A) and Low Affinity Phases (B) in Mouse Hepatic Microsomes Caused by β -NF and PB

Inducer	8-Hydroxylation		3-Demethylation		1-Demethylation	
	$K_{m1}^a)$	$V_{max1}^b)$	$K_{m1}^a)$	$V_{max1}^b)$	$K_{m1}^a)$	$V_{max1}^b)$
Control	0.24 ± 0.05	117 ± 28	0.26 ± 0.09	38 ± 11	— ^{c)}	— ^{c)}
β -NF	0.18 ± 0.07	$440 \pm 211^d)$	0.17 ± 0.05	$197 \pm 92^d)$	— ^{c)}	— ^{c)}
PB	0.19 ± 0.04	131 ± 22	0.25 ± 0.12	33 ± 11	— ^{c)}	— ^{c)}

Inducer	8-Hydroxylation		3-Demethylation		1-Demethylation	
	$K_{m2}^a)$	$V_{max2}^b)$	$K_{m2}^a)$	$V_{max2}^b)$	$K_{m2}^a)$	$V_{max2}^b)$
Control	2.92 ± 0.77	1270 ± 190	3.84 ± 0.92	416 ± 77	3.02 ± 0.70	152 ± 20
β -NF	3.90 ± 1.29	1460 ± 320	4.95 ± 0.89	469 ± 124	2.38 ± 0.55	149 ± 35
PB	1.85 ± 0.47	$2020 \pm 350^d)$	$2.10 \pm 0.95^d)$	$763 \pm 154^d)$	2.58 ± 0.43	$405 \pm 81^d)$

The values of K_m and V_{max} were determined according to the Michaelis-Menten equation described in the text. Based on the profiles of Eadie-Hofstee plots, kinetic analyses were performed on the assumption of $r=1$ for 1-demethylation, and $r=2$ for 8-hydroxylation and 3-demethylation. Each value represents the mean $\pm$ S.D. from five animals. Control mice received vehicle alone. The vehicle had no significant effect on the kinetic parameters. a) mM. b) pmol/mg/min. c) Not detected. d) Significantly different from control ($p < 0.05$).

Table 2. Changes in the Individual Metabolic Capacity per Unit P-450 Content Following Exposure to β -NF and PB

Inducer	8-Hydroxylation		3-Demethylation		1-Demethylation	
	$V'_{max1}^a)$	$V'_{max2}^a)$	$V'_{max1}^a)$	$V'_{max2}^a)$	$V'_{max1}^a)$	$V'_{max2}^a)$
Control	115 ± 22	1270 ± 260	37 ± 7	419 ± 93	— ^{b)}	156 ± 27
β -NF	$403 \pm 219^c)$	1380 ± 220	$169 \pm 54^c)$	397 ± 106	— ^{b)}	141 ± 50
PB	63 ± 25	$918 \pm 205^c)$	14 ± 6	340 ± 41	— ^{b)}	180 ± 21

The values of V'_{max1} and V'_{max2} for each metabolic pathway were designated as maximum reaction velocity per nmol P-450 content for high and low affinity phases, respectively. a) pmol/nmol/min. b) Not detected. c) Significantly different from control ($p < 0.05$).

lism The inhibitor concentrations required to reduce the enzyme activities of 8-hydroxylation and 3-demethylation by 50% (apparent IC_{50} values) in untreated microsomes were determined at 0.2 and 5 mM theophylline (named $IC_{50(H)}$ and $IC_{50(L)}$, respectively). The probes were ellipticine,²⁵⁾ a potent and specific inhibitor of CYP1A,

SKF 525-A^{26,27)} and diphenhydramine,²⁸⁾ which preferentially inhibit CYP2B and/or other minor PB-inducible species, and ketoconazole^{29,30)} and DEDTC,³⁰⁾ inhibitors for CYP2C and CYP2A/2E, respectively.

The results are presented in Table 3, along with the ratios of $IC_{50(L)}$ to $IC_{50(H)}$. These ratios were considered

Table 3. Effects of Enzyme Inhibitors on 8-Hydroxylation and 3-Demethylation at Theophylline Concentrations of 0.2 and 5 mM

Inhibitor	8-Hydroxylation			3-Demethylation		
	IC ₅₀ (μM)		IC _{50(L)} /IC _{50(H)}	IC ₅₀ (μM)		IC _{50(L)} /IC _{50(H)}
	IC _{50(H)}	IC _{50(L)}		IC _{50(H)}	IC _{50(L)}	
Ellipticine	0.23	15	65.2	0.43	17	39.5
SKF 525-A	220	180	0.8	83	67	0.8
Diphenhydramine	360	290	0.8	270	170	0.6
DEDTC	85	87	1.0	75	100	1.3
Ketoconazole	12	37	3.1	10	14	1.4

The IC_{50(H)} was defined as the IC₅₀ at a substrate concentration of 0.2 mM, and the IC_{50(L)} was defined as that at 5 mM. The control reaction proceeded in the absence of inhibitors. Each value is the mean of experiments with four separate untreated microsomal preparations.

to reflect the relative specificity for inhibition of the two P-450 populations because the proportion of their contribution to each metabolic route alternated between 0.2 and 5 mM theophylline. The IC_{50(L)}/IC_{50(H)} ratios for ellipticine were about two orders of magnitude greater than those for the other agents in both metabolic routes. Conversely, the high affinity phase was refractory to inhibition in the presence of the inhibitors, except for ellipticine, although the IC_{50(L)} values for the two metabolic routes were not necessarily close each other.

DISCUSSION

The mode of individual metabolic conversion of theophylline was clarified by its kinetics. Both 8-hydroxylation and 3-demethylation, which accounted for most theophylline oxidation, proceeded in a biphasic manner. This biphasic nature can be explained by the participation of two or more P-450 species^{8,31)} in the metabolic process. Assuming the involvement of two P-450 populations, kinetic analyses showed that one was characterized by high affinity and low capacity, the other by low affinity and high capacity.

Contrasting behavior of the $V_{\max 1}$ and $V_{\max 2}$ was evidenced following exposure to PB and β -NF, the latter being similar to PAH in qualitative pattern of enzyme induction²⁴⁾ (Fig. 2, Table 1). This is ascribed to the essential difference in the inducibility of the two P-450 populations by these agents. Concomitant elevation of cytochrome c reductase activity by treatment with PB³²⁾ will aid in optimizing P-450 function through the effective transfer of electrons supplied from NADPH. The slight shift in K_{m2} values caused by PB is probably due to some inequality of inductive action on the low affinity population. This phenomenon can be interpreted as meaning that the low affinity population consists of a number of similar P-450 species, and that their activity is estimated as the sum of the individual isoforms. Supposing that the P-450 species for which theophylline was a good substrate were selectively induced, $V'_{\max}$ values would be also increased (Table 2). In this aspect, a strong tendency toward a reduction in $V'_{\max}$ after treatment with PB is suggestive of a co-induction of P-450 species unavailable for efficient metabolization of theophylline. However, PB did not induce a high affinity population, and the low affinity species was unaffected by β -NF. Therefore, the

P-450 populations responsible for 8-hydroxylation and 3-demethylation may be classifiable as PAH-inducible, termed CYP1A,^{30,33)} for the high affinity population and as PB-inducible for the low affinity population. As CYP1A is constitutively expressed at low levels in the liver,³⁰⁾ these results indicate that CYP1A serves as a preferential catalyst of theophylline, although this drug is accepted as an ordinary substrate for PB-inducible P-450 species despite their low affinity.

Judging from the values of the IC_{50(L)}/IC_{50(H)} ratios (Table 3), the susceptibility of the constitutive P-450 populations against inhibition was also distinctive among the probes used. This suggests that the high affinity phase is composed of P-450 species that are predominantly impaired by CYP1A inhibitors,²⁵⁾ and that the low affinity population is an aggregate subject to selective impairment by inhibitors of CYP2B, CYP2C and/or other PB-inducible species.²⁶⁻³⁰⁾ This consequence is broadly supported by the previous report¹⁰⁾ that besides CYP1A, CYP2B/2C played a moderate role in the catabolic step of theophylline in rat, but some disparities in the IC_{50(L)} values between 8-hydroxylation and 1-demethylation indicate that the two pathways depend, at least in part, on separate P-450 species which belong to the PB-inducible population. For the identification of individual P-450 species participating in theophylline oxidation, there may be a need for further detailed study with the use of purified proteins or isoform-specific antibodies. However, the inhibitory response of each population was in keeping with the findings from the induction approach, and this consistency provided further support for differentiation between the two P-450 populations. On the other hand, 1-demethylation of theophylline is regarded as a metabolic route catalyzed by an apparently limited P-450 species, because the reaction proceeded in a monophasic kinetic fashion. In view of its high K_m being nearly compatible with the K_{m2} values for the other two metabolic pathways, and the difference in sensitivity to the inducers, it seems that this reaction is performed exclusively by the PB-inducible species, being practically independent of CYP1A mediation.

In clinical practice, theophylline is susceptible to alteration in its metabolic clearance when given in combination with other drugs.^{14,15,34)} These interactions cannot be fully elucidated by modification of CYP1A activity alone, but are attributable to the multiplicity of P-450 species

involved in theophylline metabolism, as presented in mouse.

In this study, we clarified that two P-450 populations contributed to theophylline metabolism in mouse hepatic microsomes, and that the high affinity population corresponded to CYP1A, and the low affinity isoform was associated with a PB-inducible P-450 species having a much higher capacity than CYP1A.

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