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METABOLISM OF FK506, A POTENT IMMUNOSUPPRESSIVE AGENT, BY CYTOCHROME P450 3A ENZYMES IN RAT, DOG AND HUMAN LIVER MICROSOMES

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Abstract—The oxidative metabolism of FK506 by liver microsomes and purified cytochrome P450 (P450) enzymes from rats, dogs and humans was studied. The major metabolite formed by liver microsomes from all species was 13-demethylated FK506, named M-I. In adult rats, liver microsomal metabolic activity toward FK506 was higher in males than in females and was stimulated by treatment with P450 3A inducers such as dexamethasone and phenobarbital. In a reconstituted monooxygenase system containing various forms of purified P450 3A enzymes, rat P450 3A2, dog P450 DPB-1 (a form of the P450 3A family) and human P450 3A4 catalyzed FK506 oxidation efficiently in the presence of cytochrome b_5, a mixture of phospholipids (dilauroylphosphatidylcholine, dioleoylphosphatidylcholine and phosphatidylserine), and sodium cholate. Rat P450 2C6 and 2D1 and human P450 $2C_{MP}$ also metabolized FK506, with significant lower activity than the P450 3A enzymes, and other rat P450 1A, 2A, 2B, 2C and 2E families including C11 did not show catalytic activities for FK506. Anti-P450 3A2 and anti-P450 3A4 antibodies strongly inhibited FK506 oxidation catalyzed by rat and human liver microsomes, respectively. The formation rate of M-I correlated well with testosterone 2β- and 6β-hydroxylase activities in rat liver microsomes and with immunoquantified P450 3A4 content, nifedipine oxidase activity, and testosterone 6β-hydroxylase activity in human liver microsomes. These *in vitro* findings indicate that the P450 3A enzymes in liver microsomes from various species of animals, including human, play a major role in the first step oxidation of FK506.

Key words: FK506 metabolism; cytochrome P450; P450 3A; liver microsomes

FK506 (Fig. 1), a macrolide antibiotic produced by *Streptomyces tsukubaensis*, is a potent immunosuppressive agent, that is being developed as an immunosuppressant in organ transplantation and autoimmune diseases [1]. FK506 is metabolized extensively in rats, with little excretion of the unchanged drug in the urine, bile, or feces; bilary excretion is the major route of elimination of radioactively labeled drug and metabolites [2]. The biotransformation of FK506 is very complicated since more than 100 metabolites have been found in the bile and feces of rats treated with [^{14}C]-FK506 (unpublished results). We have structurally elucidated four metabolites of FK506 formed by rat liver microsomes, and have identified the major metabolite to be the 13-demethyl derivative, M-I (Fig. 1) [3]. M-I has been shown to retain 10% of the immunosuppressive activity of the parent FK506, whereas M-II, a 31-demethyl derivative, exhibits almost the same activity as FK506 [2].

Cytochrome P450s (P450s)‖ are known to play an important role in the biotransformation of numerous endogenous compounds and xenobiotics, including steroids, fatty acids, drugs and carcinogens [4]. The liver is the major organ involved in P450-mediated metabolism of xenobiotics, and multiple forms of hepatic P450 have been purified and characterized from experimental animals and humans [5]. The role of hepatic P450 in the genetic polymorphism of drug metabolism in the human also has been established. Drug interaction mediated by P450 enzymes is a clinically important problem. The simultaneous administration of multiple drugs that are metabolized by the same P450 may inhibit their metabolism, increase their blood levels, and potentiate their actions and toxicities. After induction of a specific P450 by the administration of an inducer drug, blood concentrations of the coadministered drug, which is metabolized by the induced P450, decrease, and the efficacy of this drug may be shortened or diminished.

A detailed knowledge of the enzyme system involved in the hepatic metabolism of FK506 is required since the drug is used clinically for long periods in combination with other drugs. This information can facilitate the prediction of adverse drug interaction and the avoidance of unwanted side-effects from concomitantly administered therapeutic agents. Recent studies from several laboratories have suggested that P450s belonging to the 3A

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‖ Abbreviation: P450, cytochrome P450.

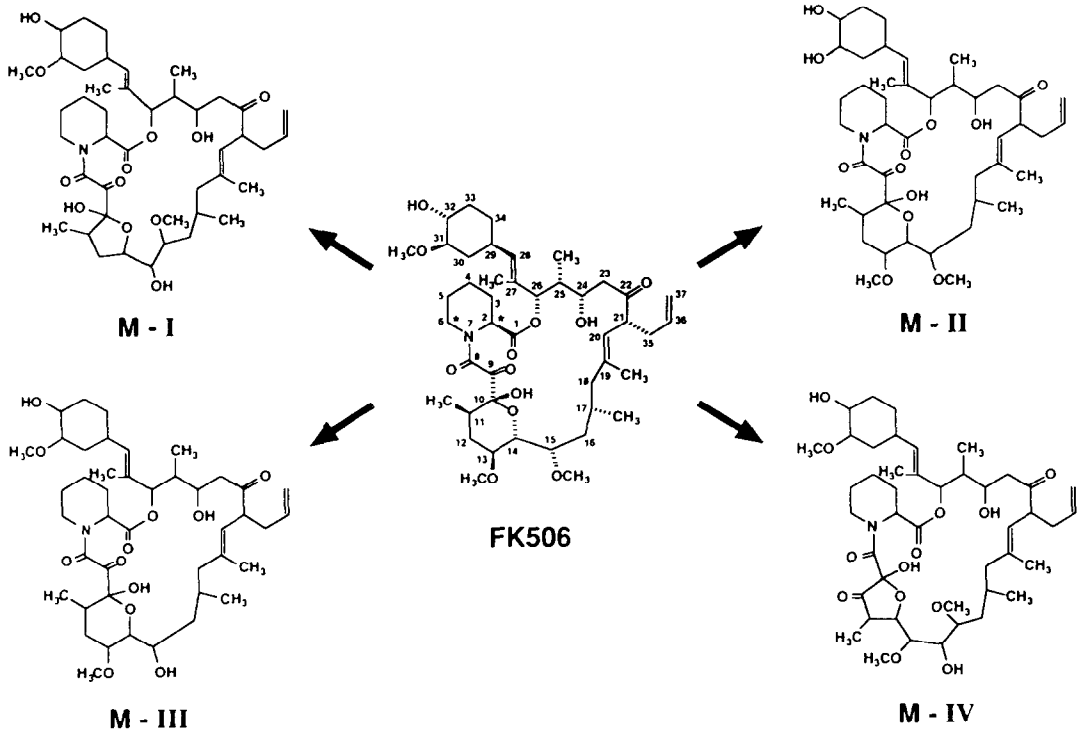

Fig. 1. Structures of FK506 and its oxidative metabolites. M-I, rearranged 13-demethyl derivative; M-II, 31-demethyl derivative; M-III, 15-demethyl derivative; M-IV, rearranged 12-hydroxy derivative. The asterisks donate the position of the ^{14}C label.

subfamily are involved in the oxidative metabolism of FK506 in liver microsomes of various species of animals [6, 7]. However, these results were obtained mainly from a reaction system using liver microsomes and antibody inhibition. Thus, only limited information is available on the possible roles of P450 enzymes, other than P450 3A proteins, in the metabolism of FK506, and on the systematic analysis of its oxidative metabolism using a reconstituted monooxygenase system containing purified P450 enzymes.

In this study, we examined the role of P450 enzymes in the metabolism of FK506. The characteristics of FK506 metabolism by P450 enzymes seen in this study were as follows: (a) in adult rats, FK506 metabolism was higher in males than in females and was induced by P450 3A inducers; (b) unlike nifedipine, a typical substrate for P450 3A2 and 2C11 in the rat [8], FK506 was metabolized only by rat P450 3A2; (c) like the other P450 3A-mediated reactions, oxidative metabolism of FK506 by rat, dog and human P450 3A enzymes required the addition of cytochrome b_5, a phospholipid mixture, and sodium cholate to the reaction system for maximal enzymatic activities; and (d) the rate of M-I formation by human liver microsomes correlated well with immunoquantified P450 3A4 content. These data clearly suggest that P450 proteins belonging to the 3A family are the major enzymes involved in the oxidative metabolism of FK506.

MATERIALS AND METHODS

Chemicals. FK506 and [^{14}C]FK506 were biosynthesized at the Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). [^{14}C]FK506 was prepared by fermentation, using [2,6-^{14}C]pipecolic acid as a precursor. Its specific activity was 13 mCi/mmol, and the radiochemical purity was greater than 97% as shown by TLC and HPLC. The authentic metabolites of FK506, nifedipine and its metabolite (pyridine derivative) were also prepared by the Fujisawa Pharmaceutical Co., Ltd. 2β-Hydroxytestosterone was prepared from 4-androsten-2β,17β-diol-3-one diacetate (Steraloids Inc., Wilton, NH, U.S.A.) by enzymatic hydrolysis. The product was purified by preparative HPLC using a reverse phase column. Dilauroylphosphatidylcholine and dioleoylphosphatidylcholine were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phosphatidylserine (bovine brain) was purchased from Serdary Research Laboratories (London, U.K.). A reverse phase column (Inertsil ODS-2) was obtained from GL Science Inc. (Tokyo, Japan). All other reagents used were of the highest quality commercially available.

Preparation of microsomes and purification of P450. Seven-week-old male and female Sprague–Dawley rats were obtained from Clea Japan Inc. (Tokyo, Japan). The following treatments were used to induce P450 enzymes: phenobarbital, 80 mg/kg/

day for 3 days (i.p., in 0.9% NaCl); β-naphthoflavone, 40 mg/kg/day for 4 days (i.p., in olive oil); clofibrate, 400 mg/kg/day for 3 days (i.p., in olive oil); and dexamethasone, 100 mg/kg/day for 4 days (i.p., in olive oil). Adult male beagle dogs weighing 9–12 kg were obtained from Ichiyanagi Farm (Shizuoka, Japan). Human liver samples were obtained from patients undergoing resection or from organ donors as described previously [9–12]. Hepatic microsomes were prepared by a method described previously [13] and stored at −80° until used. The purification of P450 from rat liver microsomes has been reported elsewhere [13–17]. Dog P450 DPB-1 was purified from a male dog treated with phenobarbital [18] by a method similar to that used in the purification of rat P450 3A2 [17]. Briefly, liver microsomes from a phenobarbital-treated dog were solubilized with sodium cholate, chromatographed on an octylamino–Sepharose 4B column, and subjected to HPLC with a preparative DEAE-5PW column (Tosoh, Tokyo, Japan). A pass-through fraction of the DEAE-5PW column was put on HPLC with an ES-502CP column (CM-type, Asahi Chemical, Tokyo, Japan), and the P450 DPB-1 fraction was purified further by HPLC with a KB-column (hydroxyapatite column, Koken, Tokyo, Japan). The purification of human P450 3A4 and P450 $2C_{MP}$ has been described elsewhere [8, 9]. The P450 nomenclature used in this study is that of Nebert *et al.* [20].

Assay of FK506 metabolism. [^{14}C]FK506 was used to determine the disappearance of FK506 or the formation of its metabolites. The reaction mixture contained 0.025 to 1.0 mg of liver microsomal protein, 0.1 M potassium phosphate buffer (pH 7.4), an NADPH-generating system (5 mM $MgCl_2$, 0.5 mM NADP, 0.5 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase), and 5–100 μM [^{14}C]- FK506 in a final volume of 0.5 mL. The reaction mixture for the reconstituted experiments on the P450 3A enzymes consisted of 0.1 M potassium phosphate buffer (pH 7.4), 0.1 nmol purified P450, 0.3 U NADPH-P450 reductase, 10 μg dilauroylphosphatidylcholine, 0.1 mg sodium cholate, the NADPH-generating system and 10 μM [^{14}C]FK506 in a final volume of 0.5 mL. The modified reconstituted system contained a mixture of 10 μg of phospholipids consisting of dilauroylphosphatidylcholine, diolcoylphosphatidylcholine and phosphatidylserine (1:1:1) instead of dilauroylphosphatidylcholine in the reconstituted system described above [21, 22]. The reaction mixture for the reconstituted experiments on P450s other than that of the P450 3A subfamily consisted of 0.1 M potassium phosphate buffer (pH 7.4), 0.1 nmol purified P450, 0.3 U NADPH-P450 reductase, 5 μg dilauroylphosphatidylcholine, the NADPH-generating system and 10 μM [^{14}C]FK506 in a final volume of 0.5 mL. In some experiments, 0.1 nmol of cytochrome b_5 was added to the reconstituted system. The reaction was started by the addition of the NADPH-generating system and was allowed to incubate for 10 min at 37°. The reaction was stopped by adding 25 μL of 1 N HCl on ice. [^{14}C]- FK506 and its metabolites were extracted with 3.5 mL of ethyl acetate. The ethyl acetate layer (3.0 mL) was then removed and was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 200 μL of methanol and stored at −80° until analysis. The extraction efficiency of radioactivity was greater than 97% in all cases. An aliquot (50 μL) of the dissolved residue was analyzed on an HPLC apparatus (Varian 5000 LC, Varian Associated Inc., Walnut Creek, CA, U.S.A.) with an Inertsil ODS-2 column (5 μm, 4.6 × 250 mm) equipped with an Inertsil ODS-2 guard column (5 μm, 4.6 × 10 mm). The column temperature was set at 50°. The mobile phase used was 0.1% (v/v) phosphoric acid as eluent A and acetonitrile as eluent B; the flow rate was 1.2 mL/min. [^{14}C]FK506 and its metabolites were eluted by the following linear gradient; analysis time 0 min, 50% B; analysis time 1 min, 50% B; analysis time 20 min, 57% B; analysis time 29 min, 100% B; analysis time 31 min, 100% B. The column was subsequently washed with 100% methanol for 2 min at a flow rate of 3 mL/min and allowed to re-equilibrate to the starting conditions. The eluate was collected every 15 sec and was mixed with 4.5 mL of Aquasol-2 (Du Pont NEN Research Products, Boston, MA, U.S.A.) for determination of radioactivity. The radioactivity was measured as described previously [23]. A blank incubation consisted of either zero-time incubation or a 10-min incubation with denaturated microsomes. The total metabolism of [^{14}C]FK506 was calculated from the disappearance of [^{14}C]FK506, which eluted 26–29 min after injection (see Fig. 2A). The formation of M-I was calculated from the total radioactivities eluting in three peaks (indicated by three arrows in Fig. 2A).

Immunoinhibition with antibodies. Liver microsomes from male rats (untreated, 0.3 mg protein; dexamethasone-treated, 0.041 mg protein) were preincubated with rabbit preimmune sera or rabbit anti-P450 serum (antiserum, 0–20 μL; total serum volume, 20 μL) in 0.1 M potassium phosphate buffer (pH 7.4) at room temperature for 30 min. Then [^{14}C]FK506 (10 μM) and the remaining components were added and the reaction was started. Human liver microsomes (HL-4, 0.025 mg protein, 0.022 nmol P450) were preincubated with rabbit preimmune IgG or rabbit anti-P450 3A4 IgG (6.3 mg IgG/nmol P450) in 0.1 M potassium phosphate buffer (pH 7.4) at room temperature for 30 min.

Other methods and materials. The total P450 contents were determined according to the method of Omura and Sato [24]. Rat NADPH-P450 reductase and cytochrome b_5 were purified as described previously [25]. The specific activity of purified reductase was 38 U/mg protein. One unit of reductase is defined as the amount that catalyzes the reduction of cytochrome *c*/min at 37° as assayed by the method of Phillips and Langdon [26]. The specific content of purified cytochrome b_5 was 28 nmol/mg protein when assayed by the method of Omura and Sato [24]. Protein content was determined by conventional methods, using bovine serum albumin as the standard [27]. Polyclonal antibodies raised against the purified P450s used in this study have been characterized elsewhere [10, 28]. P450 3A4 content was estimated by immunoblotting as described previously [29]. The activities for testosterone hydroxylation and nifedipine oxidation

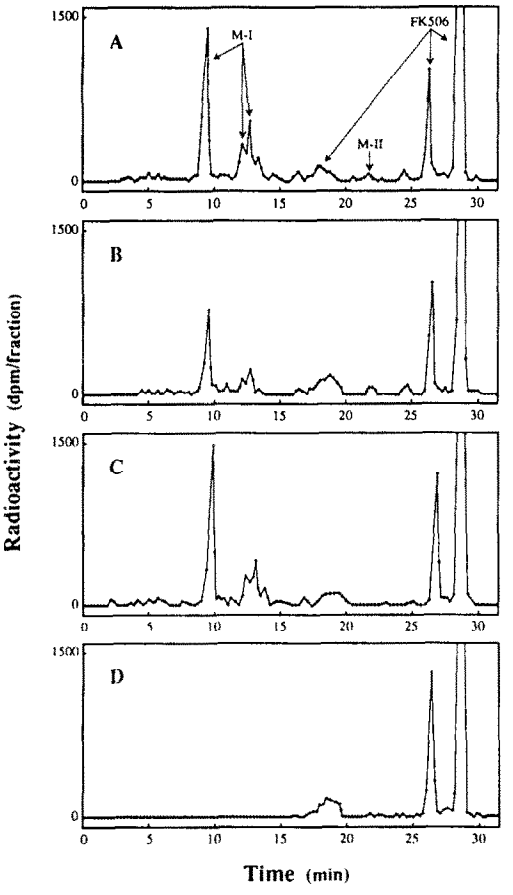

Fig. 2. Typical HPLC radiochromatograms obtained from incubations of [^{14}C]FK506 with liver microsomes. Liver microsomes (untreated male rat, 0.5 mg protein; untreated male dog, 0.5 mg protein; human HL-12, 0.05 mg protein) were incubated for 10 min at 37° with 10 μM [^{14}C]FK506 in the presence of an NADPH-generating system in a final volume of 0.5 mL. [^{14}C]FK506 and its metabolites were extracted and analyzed by linear gradient HPLC. Key: (A) rat; (B) dog; (C) human; and (D) blank incubation with denaturated microsomes. Retention times for major isomer/tautomer of authentic standards were: M-I, 9.1 min; M-II, 22.0 min; M-III, 16.2 min; M-IV, 18.6 min; and FK506, 28.3 min.

were estimated by procedures described elsewhere [8, 30].

RESULTS

Metabolism of FK506 by rat, dog and human liver microsomes. FK506 and some of its metabolites show tautomeric equilibrium resulting from the presence of a hemiketal function. Their corresponding isomers/tautomers can be separated into two or more peaks on HPLC using a reverse phase column. Figure 2 shows representative radiochromatograms of metabolites of [^{14}C]FK506 formed by rat, dog and human liver microsomes. These metabolites were identified by comparing HPLC retention times with those of authentic chemicals. The major metabolite was M-I in all microsomal incubations used. The formation of M-II was detected with liver microsomes from rat and dog, but not with human microsomes. On the other hand, no significant formation of M-III or M-IV was detected under the incubation conditions used. These metabolites were detectable upon long-term incubation with high substrate and protein concentrations (data not shown). Table 1 shows the catalytic activities for FK506 in liver microsomes from untreated male rats and dogs. The mean rate of M-I formation was 0.13 and 0.07 nmol/min/mg protein in rat and dog liver microsomes, respectively. Liver microsomes from rat and dog catalyzed M-II formation, but their rates were less than one-tenth of M-I formation in both species. Table 2 shows the P450 content and the rate of FK506 oxidation in liver microsomes from untreated and P450 inducer-treated rats. M-I was a major metabolite in all incubations. A clear sex-related difference was observed in the oxidative metabolism of FK506 in rats. Pretreatment of rats with dexamethasone caused a 7-fold increase in the total metabolism of FK506 (data not shown), and 3- and 6-fold increases in M-I and M-II formation, respectively. M-I formation was not induced drastically by dexamethasone, because the M-I formed seemed to be metabolized further. Phenobarbital and clofibrate treatment of rats caused a 2-fold stimulation of M-I formation, whereas pretreatment with β-naphthoflavone was essentially without effect. These results suggest that FK506 oxidation is catalyzed by male-dominant and dexamethasone-, phenobarbital- and clofibrate-inducible P450 isozymes in rat liver microsomes.

The rate of M-I formation by human liver microsomes varied ~200-fold with a mean activity of 0.18 (±0.22, SD) nmol/min/mg protein, determined at a substrate concentration of 10 μM, while total P450 content varied 8-fold with a mean value of 0.39 (± 0.19, SD) nmol/mg protein (see Fig. 5). The rate of M-I formation by human liver microsomes was 1.4 and 2.6 times higher than those of rat and dog, based on the mg of microsomal protein, and was 3.5 and 1.4 times higher than those of rat and dog, based on the nmol of P450, respectively.

Kinetic parameters for M-I formation. Michaelis–Menten parameters were determined using liver microsomes from untreated male rats and humans (HL-7). The initial FK506 concentration was varied over the range of 5–100 μM. The K_m and V_{max} values for M-I formation, which were calculated from Hanes–Woolf plots, were 6.7 μM and 0.18 nmol/min/mg protein, respectively, in rats, and 6.2 μM and 0.38 nmol/min/mg protein in humans. These kinetic parameters with human liver microsomes did not differ substantially from those reported previously [7].

Metabolism of FK506 by purified P450. The FK506 metabolic activities of purified hepatic P450 from rats, dogs and humans are shown in Tables 3 and 4. The P450 3A form, including rat P450 3A2, dog P450 DPB-1 and human P450 3A4, could catalyze the formation of M-I in a reconstituted monooxygenase system containing sodium cholate and dilauroyl-

Table 1. Metabolism of FK506 by rat and dog liver microsomes

Microsomal sample	P450 content (nmol/mg protein)	Activity (nmol/min/mg protein)	
		M-I formation	M-II formation
Untreated male rat	1.25 ± 0.05	0.130 ± 0.020	0.005 ± 0.001
Untreated male dog	0.27 ± 0.05	0.071 ± 0.006	0.007 ± 0.003

Catalytic activities were determined with 10 μM [^{14}C]FK506. Results are expressed as means ± SD of 3 animals.

Table 2. Metabolism of FK506 by rat liver microsomes

Treatment	Sex	P450 content (nmol/mg protein)	Activity (nmol/10 min/mg protein)	
			M-I formation	M-II formation
Untreated	Male	0.81	1.66 (1.0)	0.083 (1.0)
Untreated	Female	0.61	0.28 (0.2)	0.030 (0.4)
Phenobarbital	Male	1.24	3.29 (2.0)	0.418 (5.0)
Clofibrate	Male	1.19	2.93 (1.8)	0.215 (2.6)
β-Naphthoflavone	Male	1.29	1.52 (0.9)	0.120 (1.4)
Dexamethasone	Male	1.54	4.57 (2.8)	0.493 (5.9)

A reaction mixture containing 1.0 mg of microsomal protein, [^{14}C]FK506 (50 μM) and an NADPH-generating system was incubated for 10 min. Values were determined with microsomes pooled from 5 rat livers. Numbers in parentheses indicate relative activity compared with untreated male rats.

Table 3. Metabolism of FK506 by purified P450 3A enzymes in a reconstituted system

Reconstituted system	M-I formation (nmol/min/nmol P450)	
	$-b_5$	$+b_5$
P450 3A2		
DLPC	<0.005	0.053
Mix	0.007	0.117
P450 DPB-1		
DLPC	0.193	0.711
Mix	0.333	0.778
P450 3A4		
DLPC	0.016	0.183
Mix	0.045	0.158

DLPC: a reaction mixture containing purified P450 (0.1 nmol), rat NADPH-P450 reductase (0.3 U), sodium cholate (0.1 mg), [^{14}C]FK506 (10 μM), dilauroylphosphatidylcholine (10 μg), and an NADPH-generating system. Mix: a reaction mixture containing purified P450 (0.1 nmol), rat NADPH-P450 reductase (0.3 U), sodium cholate (0.1 mg), [^{14}C]FK506 (10 μM), an NADPH-generating system and a mixture of phospholipids (10 μg), dilauroylphosphatidylcholine, phosphatidylserine and dioleoylphosphatidylcholine (1:1:1). $+b_5$: addition of rat cytochrome b_5 (0.1 nmol) to the reconstituted system.

Table 4. Metabolism of FK506 by purified P450s other than the P450 3A form

Cytochrome P450	M-I formation (nmol/min/nmol P450)	
	$-b_5$	$+b_5$
1A1	—	—
1A2	—	—
2A1	—	—
2A2	—	—
2B1	—	—
2B2	—	—
2C6	—	0.005
2C7	—	—
2C11	—	—
2C12	—	—
2C13	—	—
2D1	0.007	0.006
2E1	—	—
$2C_{MP}$	—	0.005

Catalytic activities were determined with 10 μM [^{14}C]-FK506. b_5, cytochrome b_5. Activities less than 0.005 nmol/min/nmol P450 are expressed as "—". The disappearance of [^{14}C]FK506 was not significant in any preparation (<0.03 nmol/min/nmol P450).

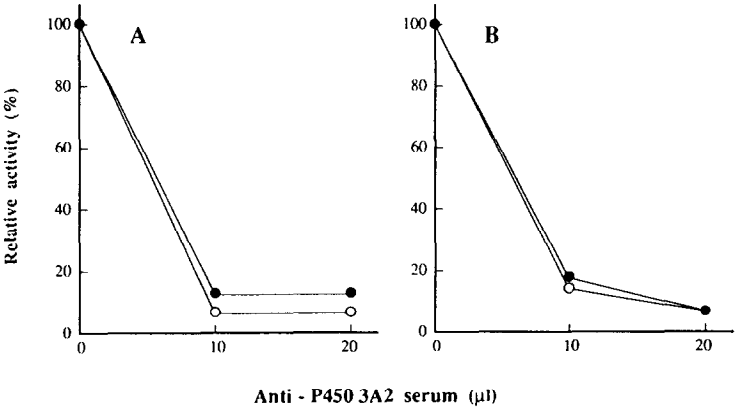

Fig. 3. Inhibition of FK506 metabolism in liver microsomes from untreated (A) and dexamethasone-treated (B) male rats by anti-P450 3A2. Liver microsomes (untreated, 0.3 mg protein; dexamethasone-treated, 0.041 mg protein) were incubated with various amounts of preimmune serum or anti-P450 3A2 serum for 30 min at room temperature, and then [^{14}C]FK506 (10 μM) and the remaining components were added. Total metabolism (●) and M-I formation (○) were measured. Activities are expressed as a relative percentage of the control experiments. The 100% values for untreated male rats were 0.25 and 0.15 nmol/min/mg protein of total metabolism and M-I formation, respectively. The 100% values for dexamethasone-treated rats were 3.5 and 1.5 nmol/min/mg protein of total metabolism and M-I formation, respectively.

phosphatidylcholine or a phospholipid mixture. The addition of cytochrome b_5 to the reconstituted system enhanced catalytic activity by more than 2.5-fold in all cases. Human P450 $2C_{MP}$ and rat 2C6 and 2D1 catalyzed the formation of M-I, but with a much lower activity than the P450 3A forms. The rate of M-I formation by P450 DPB-1 was 7 and 5 times higher than those by P450 3A2 and 3A4, respectively, in the modified reconstituted system including cytochrome b_5. Other rat P450 species, including the 1A, 2A, 2B, 2C and 2E family, did not show catalytic activities for FK506. The formation of M-II, M-III or M-IV was not detected in any preparation in the reconstituted system.

Inhibition of FK506 metabolic activity by anti-P450 3A2 and anti-P450 3A4 in rat and human liver microsomes. In the reconstituted study on FK506 metabolism, the P450 3A form was shown to catalyze it. To determine the participation of the P450 3A form in the metabolism of FK506 by microsomes, immunoinhibition experiments were carried out using various anti-P450 antibodies. Antibodies raised against rat P450 3A2 strongly inhibited the total metabolism of FK506 and M-I formation catalyzed by liver microsomes of untreated male rats (Fig. 3). Similarly, anti-P450 3A2 antibodies almost completely inhibited FK506 metabolism by liver microsomal activities in dexamathesone-treated rats. Thus, the P450 isozymes responsible for the metabolism of FK506 were not different in untreated and dexamethasone-treated male rats. In human liver microsomes, anti-P450 3A4 IgG inhibited 77% of the total metabolism and 97% of M-I formation, at IgG levels of 6.3 mg protein/nmol P450. Antibodies raised against rat P450 1A1, 2B1, 2C11 and 2D1 did not show any significant inhibition of FK506 metabolism by liver microsomes of untreated male rats (data not shown). These data suggested that the P450 3A forms are the major catalyst for the metabolism of FK56 in rat and human hepatic microsomes.

Correlation of FK506 M-I formation with P450 contents, nifedipine oxidation and testosterone hydroxylation. P450 3A forms have been shown previously to be the major catalyst for testosterone 2β- and 6β-hydroxylation in rat and human liver microsomes [8, 17, 22]. P450 3A4 has also been demonstrated to be a major form responsible for the oxidation of nifedipine in human liver microsomes [8, 21]. Good correlation was noted between the level of M-I formation and the catalytic activities for both testosterone 2β-hydroxylation ($r = 1.00$, $P < 0.001$) and testosterone 6β-hydroxylation ($r = 0.98$, $P < 0.001$) in rat liver microsomes (Fig. 4). In contrast, M-I formation activity did not correlate with the levels of testosterone 2α-, 7α-, 16α-, or 16β-hydroxylation (data not shown). The correlations of FK506 M-I formation activity with P450 3A4 contents and P450 3A-dependent monooxygenase activities in 18 different human liver samples were compared (Fig. 5). M-I formation correlated with total P450 content ($r = 0.90$, $P < 0.001$), P450 3A4 content ($r = 0.82$, $P < 0.001$), nifedipine oxidation activity ($r = 0.95$, $P < 0.001$) and testosterone 6β-hydroxylation activity ($r = 0.95$, $P < 0.001$). These results provide further evidence that P450 3A forms are the major catalyst of FK506 oxidation in rat and human liver microsomes.

DISCUSSION

This study demonstrated that the P450 proteins belonging to the 3A gene family are the major enzymes responsible for the initial oxidation of FK506 using purified P450 preparations from human, rat and dog liver. This conclusion is based on the following lines of evidence. In the reconstituted monooxygenase system, P450 3A enzymes including

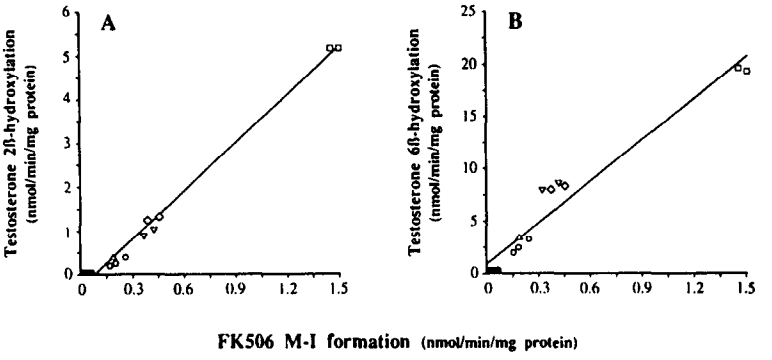

Fig. 4. Correlation of FK506 M-I formation activity with testosterone 2β-hydroxylation activity (A) and testosterone 6β-hydroxylation activity (B) in rat liver microsomes. Catalytic activities were measured with 100 μM [^{14}C]FK506 and 1 mM testosterone. Open and closed symbols indicated the values of individual male and female rats, respectively. Symbols indicate the values from untreated (○), phenobarbital- (◇), clofibrate- (▽), β-naphthoflavone- (△) and dexamethasone- (□) treated rats.

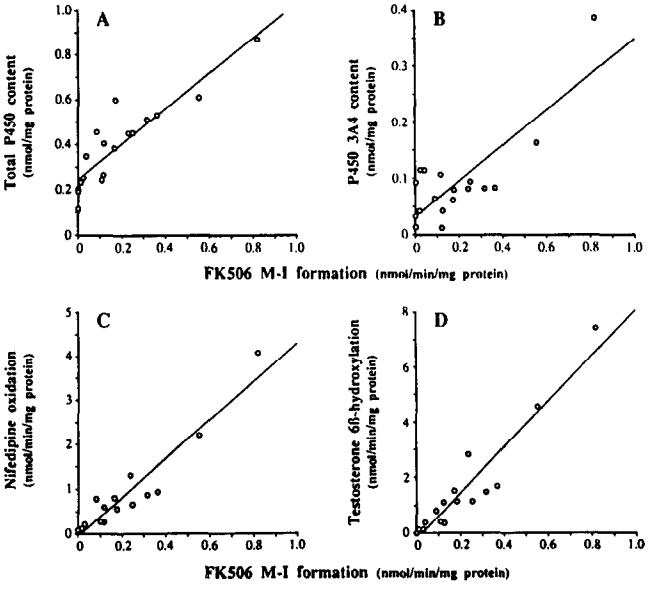

Fig. 5. Correlation of FK506 M-I formation activity with total P450 content (A), immunoquantified P450 3A4 content (B), nifedipine oxidation activity (C) and testosterone 6β-hydroxylation activity (D) in 18 different human liver microsomes.

human 3A4, rat 3A2 and dog DPB-1 gave higher turnover numbers for the oxidation of FK506. Other P450 forms including P450 1A, 2B, 2C, 2D and 2E were found to be poor or inert catalysts for FK506 in the reconstituted system. Moreover, in human liver microsomes from different human samples, a significant correlation between the rate of FK506 metabolism and the immunochemically determined levels of P450 3A and P450 3A-dependent monooxygenase activities (testosterone 6β-hydroxylation and nifedipine oxidation) was observed. In addition, antibodies raised against P450 3A4 strongly inhibited FK506 metabolism catalyzed by human liver microsomes. The rate of M-I formation correlated well with P450 3A-dependent activities, namely testosterone 2β- and 6β-hydroxylation in rat liver microsomes. Metabolism of FK506 by liver microsomes was higher in adult male rats than in adult females and was induced by treatment with P450 3A inducers. Metabolism of FK506 by rat liver microsomes was found to be inhibited markedly by anti-P450 3A2 antibodies, although antibodies against P450 1A1, 2B1, 2C11 and 2D1 had no such effect. Thus FK506 metabolism would be catalyzed exclusively by P450 3A enzymes in liver microsomes from humans and rats, and probably those from dogs. The present results agree with the results of experiments using liver microsomes and antibodies [6, 7].

In the reconstituted reaction system of purified P450, P450 3A enzymes exhibited different component requirements when compared with other forms of P450 [21, 22]. P450 3A needed a mixture of phospholipids, cytochrome b_5 and sodium cholate for maximal catalytic activities. The metabolism of FK506 by P450 was also found to have properties similar to those reported for other P450 3A substrates. Interestingly, nifedipine was metabolized by both P450 3A2 and 2C11 [8] but FK506 was only metabolized by P450 3A2.

P450 3A4 has been reported to be one of the major forms in liver microsomes of adult humans and is known to have a role in the metabolism of a wide variety of chemicals including clinically important drugs and several carcinogens [31, 32]. Expression of P450 3A4 has been reported to be highly variable among human liver specimens [8, 21, 33], and P450 3A4 has been induced by barbiturates, antibodies and dexamethasone [34–36]. FK506 is administered chronically to patients in combination with other drugs, and the therapeutic index of this drug is low [1]. Any co-administered drugs that can inhibit or induce P450 3A4 and related protein(s) may alter the metabolism of FK506, and may lead to changes in the blood levels of FK506 in patients. Also, it can be anticipated that any drugs metabolized by P450 3A4 and related protein(s) would cause metabolic drug interactions with FK506 when given in association. Accordingly, caution must be used when administering FK506 concomitantly with drugs known to be inhibitors, inducers and substrates of P450 3A enzymes. Large interindividual variation was detected in the oxidation of FK506 by human liver microsomes. This variability could be responsible in part for the variations of the pharmacokinetic parameters of FK506 in patients with normally functioning livers and kidneys [1]. Therefore, clinical level monitoring of FK506 is required to optimize the therapy with this novel and potent immunosuppressant.

M-I was the major metabolite in the initial oxidative metabolism of FK506 in liver microsomes of various animal species including humans. When [^{14}C]FK506 was administered to rats, a very small amount of M-I was excreted in the urine and bile (less than 0.5% of the dose within 48 hr), indicating that further metabolism of M-I may occur (unpublished results). In fact, M-I has been shown to be further biotransformed to several metabolites by human, rat and dog liver microsomes. These activities were inhibited extensively by anti-P450 3A2 and were induced drastically by treatment with dexamethasone in rats (unpublished results). We are currently making efforts to characterize, in detail, the structures of these metabolites, and our findings will be reported in another paper.

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