A NOVEL CYTOCHROME P450 ENZYME RESPONSIBLE FOR THE METABOLISM OF EBASTINE IN MONKEY SMALL INTESTINE

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ABSTRACT:

Small intestinal microsomes of cynomolgus monkeys were found to catalyze hydroxylation and dealkylation of an H1-antihistamine prodrug, ebastine. To identify the main enzyme responsible for ebastine hydroxylation, which has been hitherto unknown, we purified two cytochrome P450 isoforms, named P450 MI-2 and P450 MI-3, from the intestinal microsomes on the basis of the hydroxylation activity. P450 MI-2 and P450 MI-3 showed the respective apparent molecular weights of 56,000 and 53,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The internal amino acid sequence of P450 MI-2 had high similarity with those of human CYP4F2, CYP4F3, and CYP4F8. The first 27 amino acid residues of P450 MI-3 were highly homologous with those of monkey CYP3A8 and human CYP3A4/5/7. Furthermore, P450 MI-2 and P450 MI-3 were recognized by anti-CYP4F and anti-CYP3A antibodies, respectively, in immunoblot analysis and catalyzed leukotriene B4 ω-hydroxylation and testosterone 6β-hydroxylation, which are known to be mediated by CYP4F and CYP3A, respectively. Although both enzymes had ebastine hydroxylation activity, the Vmax value of P450 MI-2 was much higher than that of P450 MI-3 (37.0 versus 0.406 nmol/min/nmol of P450), and the former K M was smaller than the latter K M (5.1 μM) was smaller than the latter (10 μM). Anti-CYP4F antibody inhibited the hydroxylation in small intestinal microsomes strongly (70%), but anti-CYP3A antibody did not. These results indicate that P450 MI-2 belongs to the CYP4F subfamily and is mainly responsible for hydroxylation of ebastine in monkey small intestinal microsomes. This suggests that the small intestinal CYP4F enzyme, P450 MI-2, can play an important role in the metabolism of drugs given orally.

Ebastine [4′-tert-buty1-4-[4-(diphenylmethoxy)piperidino]butyrophenone] is a new generation, nonsedative, H1-antihistamine. After oral administration to experimental animals and humans, ebastine, which per se possesses scarce antihistamine activity, is almost completely metabolized to the pharmacologically active principle, carebastine (a carbboxylylated metabolite of ebastine). To identify this enzyme using small intestinal microsomes of cynomolgus monkeys seems to be an unknown P450 isoform. In this study, we tried to identify this enzyme using small intestinal microsomes of cynomolgus monkeys, which have been poorly characterized, in contrast to hepatic drug-metabolizing enzymes. We found that a major enzyme responsible for the hydroxylation of ebastine has not been identified (Hashizume et al., 1998).

Our previous metabolism studies in rats using intravenous-intra-portal infusion techniques in situ indicated that the small intestine quite actively converted the orally administered ebastine to carebastine (i.e., via hydroxylated ebastine) and the dealkylated metabolite (Fujii et al., 1997). In fact, for the orally administered ebastine, the contribution of the intestine, the first organ exposed to the drug, in the first-pass metabolism was estimated to be around 90% relative to that of liver, the second organ, which was around 10%. Therefore, it seemed that the small intestine played an important role in the first-pass metabolism of this drug and that the enzymes responsible for the ebastine metabolism exist there. Recent investigations have demonstrated that extrahepatic tissues contribute to the drug metabolism, and the small intestine has been recognized as the important first-pass metabolism organ of therapeutic drugs given orally (Wu et al., 1995; Thummel et al., 1996; Lin et al., 1999). However, only a limited number of P4501 enzymes of small intestine have been purified and characterized, in contrast to hepatic drug-metabolizing enzymes (Nelson et al., 1996). At present, P450 enzymes in four P450 subfamilies have been purified only from rabbits: the CYP1A, CYP2C, CYP2J, and CYP4A isoforms (Ichihara et al., 1983; Koike et al., 1997; Shimizu et al., 1997). Ebastine hydroxylase in the small intestine, as well as in the liver, seems to be an unknown P450 isoform. In this study, we tried to identify this enzyme using small intestinal microsomes of cynomolgus monkeys, which have been poorly characterized, in contrast to hepatic drug-metabolizing enzymes.
monkeys because of experimental convenience and possible similarity to humans. We found a novel intestinal P450 molecule involved in the therapeutically important metabolism of ebastine.

**Experimental Procedures**

**Chemicals.**  
[14C]Ebastine [4′-tert-butyl-4-{4-[(ring-U-14C)diphenylmethoxy]piperidino}butyrophenone] was synthesized by using the method described previously (Fujii et al., 1994), with a specific activity of 1.08 MBq/mg and radiochemical purity of 99%. Authentic metabolites [hydroxy-ebastine, 4′-(2-hydroxy-1,1-dimethylethyl)-4-[[4-(diphenylmethoxy)piperidino]butyrophenone; desalkyl-ebastine, 4-(diphenylmethoxy) piperidine; and carebasine] were supplied by Almirall-Prodesfarma S.A. (Barcelona, Spain). Emulgen 911 was a generous gift from Kao-Atlas Co. (Tokyo, Japan). Dithiothreitol (DTT), dilauroyl-L-3-phosphatidylcholine (DLPC), glucose 6-phosphate dehydrogenase, and NADP were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). LTB4, 20-hydroxylleukotriene B4, and 20-carboxyleukotriene B4 were purchased from Cayman Chemical Company (Ann Arbor, MI). Other chemicals used were of the highest grade commercially available.

**Preparation of Microsomes from Monkey Small Intestine.** Small intestines of 10 male cynomolgus monkeys weighing about 4 kg were provided by Toyo Roshi, Tokyo, Japan, diluted 10-fold with 20 mM Tris acetate buffer (pH 7.5) containing 20% (v/v) glycerol, and then applied to a DEAE-5PW anion exchange column (2.15 cm, 15 cm, Bio-Rad Laboratories, Hercules, CA) equilibrated with buffer C [20 mM Tris-acetate buffer (pH 7.5) containing around 120 mM sodium acetate]. Other P450-containing fractions eluted with the buffer containing sodium acetate of low concentration (0–30 mM) were pooled, concentrated, and applied to an ES-502C cation-exchange column (2.5 cm, 15 cm, Tosoh Corp., Tokyo, Japan) previously equilibrated with buffer B [100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, and 0.2% (w/v) Emulgen 911]. P450 was eluted with a linear gradient of sodium acetate (0–500 mM over 60 min) in the buffer D at a flow rate of 1.0 ml/min. Finally, P450 was recovered in the eluate with 400 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, and the fractions containing P450 were pooled. For removal of the detergent to monitor ebastine hydroxylation activity, a part of the pooled fraction was applied to a hydroxyapatite column (Biogel-HT, 0.8 cm, Tosoh Corp., Tokyo, Japan), diluted 10-fold with 20 mM Tris acetate buffer (pH 7.5) containing 20% (v/v) glycerol, and then applied to a DEAE-5PW anion exchange column (2.15 cm, 15 cm, Bio-Rad Laboratories, Hercules, CA) equilibrated with buffer A [100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM EDTA, 0.1 mM DTT, and 0.4% (w/v) sodium cholate]. After washing the column with the buffer A alone, P450 was eluted with the buffer A containing 0.2% (w/v) Emulgen 911. The eluted P450 was monitored for heme absorption at 417 nm, the peak fraction was assayed for P450 content, and the fractions containing P450 were pooled. For further purification to determine the amino acid sequence, each P450 (150 pmol) of the pooled fraction for further purification was concentrated with an ultrafiltration membrane (UK-50, Toyo Roshi, Tokyo, Japan). LTB4, 20-hydroxylleukotriene B4, and 20-carboxyleukotriene B4 were purchased from Cayman Chemical Company (Ann Arbor, MI). Other chemicals used were of the highest grade commercially available.

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**Purification of Intestinal P450.** Small intestinal microsomes were solubilized in 0.8% (w/v) sodium cholate for 1 h at 4°C and centrifuged at 105,000 g for 20 min. The supernatant was applied to an α-aminoacyl Sepharose 4B column (2.5 cm, 30 cm) that had been equilibrated with buffer A [100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM EDTA, 0.1 mM DTT, and 0.4% (w/v) sodium cholate]. After washing the column with the buffer A alone, P450 was eluted with the buffer A containing 0.2% (w/v) Emulgen 911. The eluted P450 was monitored for heme absorption at 417 nm, the peak fraction was assayed for P450 content, and the fractions containing P450 were pooled. For removal of the detergent to monitor ebastine hydroxylation activity, a part of the pooled fraction was applied to a hydroxyapatite column (Biogel-HT, 0.8 cm, Bio-Rad Laboratories, Hercules, CA) equilibrated with buffer B [100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol], and the column was washed with buffer B. After absorption of Emulgen 911 disappeared at 280 nm, P450 was recovered in the eluate with 400 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol. Ebastine hydroxylation activity was assayed using the reconstituted system. On the other hand, the rest of the pooled fraction for further purification was concentrated with an ultrafiltration membrane (UK-50, Toyo Roshi, Tokyo, Japan), diluted 10-fold with 20 mM Tris acetate buffer (pH 7.5) containing 20% (v/v) glycerol, and then applied to a DEAE-5PW anion exchange column (2.15 cm, 15 cm, Tosoh Corp., Tokyo, Japan) previously equilibrated with buffer C [20 mM Tris-acetate buffer (pH 7.5) containing 20% (v/v) glycerol, and then applied to a DEAE-5PW anion exchange column (2.15 cm, 15 cm, Tosoh Corp., Tokyo, Japan) previously equilibrated with buffer B [100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, and 0.4% (w/v) Emulgen 911]. P450 was eluted with a linear gradient of sodium acetate (0–200 mM over 180 min) in buffer C at a flow rate of 2.0 ml/min as described previously (Komori et al., 1988). The P450 adsorbed on the DEAE-5PW column was eluted in two peak fractions (MI-1 and MI-2) with buffer C containing around 120 mM sodium acetate. Other P450-containing fractions eluted with the buffer containing sodium acetate of low concentration (0–30 mM) were pooled, concentrated, and applied to an ES-502C cation-exchange column (0.76 cm, 10 cm, Showa Denko K.K., Tokyo, Japan) equilibrated with buffer D [20 mM sodium phosphate buffer (pH 6.5) containing 20% (v/v) glycerol and 0.4% (w/v) Emulgen 911]. The P450 was eluted in one peak fraction (MI-3) using a linear gradient of sodium acetate (0–500 mM over 60 min) in the buffer D at a flow rate of 1.0 ml/min. Finally, to remove Emulgen 911, the respective P450 fraction was applied to a hydroxyapatite column as described above.
purified by the above chromatography method was subjected to SDS-PAGE with 7.5% polyacrylamide gel as described previously (Laemmli, 1970) and electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories) at 2.0 mA/cm² of membrane for 90 min with a horizontal blotting system (Atto, Tokyo, Japan) in 100 mM Tris-HCl buffer (pH 8.3) containing 192 mM glycine and 20% methanol by the method described previously (Towbin et al., 1979). For homogeneity of P450 enzyme to be analyzed, the protein band stained with Coomassie Brilliant Blue R-250 was selected based on its molecular weight location of around 50,000 (that of P450 molecules), excised out from the membrane, and then destained. NH₂-Terminal amino acid sequences of P450 MI-2 and P450 MI-3 were directly analyzed by automated Edman degradation using an HP G1005A N-terminal Protein Sequencing System (Hewlett Packard Co., Palo Alto, CA). For P450 MI-2, the internal amino acid sequence was analyzed by the following procedure because determination of its NH₂-terminal amino acid sequence was not successful, possibly due to modification or deletion of the terminus. The P450 MI-2 (600 pmol) separated was digested with 33 pmol of lysylendopeptidase (Wako Pure Chemical Industries, Osaka, Japan) in 0.1 ml of 100 mM Tris-HCl buffer (pH 9.0) containing 1% (w/v) trifluoroacetic acid solution at a flow rate of 0.2 ml/min. The absorbance was monitored at 214 nm to collect the peptide fractions, which were then analyzed in the peptide sequence analyzer.

**Purification of Recombinant Human CYP4F2.** CYP4F2 cDNA was isolated from human liver cDNA library (CLONTECH, Palo Alto, CA) with PCR and expressed in yeast (Saccharomyces cerevisiae) as described (Hashizume et al., 2001). Recombinant yeast was cultured as described previously (Murakami et al., 1986; Sakaki et al., 1991), and the microsomal preparation was carried out according to the previous method (Oeda et al., 1985). Microsomes of 250 mg of protein (169 nmol of P450) obtained were solubilized in 100 mM potassium phosphate buffer (pH 7.2) containing 20% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 0.6% (w/v) sodium cholate for 1 h at 4°C. The solution, after centrifugation at 15,000g for 20 min, was applied to an a-aminoeyctyl Sepharose 4B column (2 x 4 cm) equilibrated with buffer E [100 mM potassium phosphate buffer (pH 7.2) containing 20% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 0.5% (w/v) sodium cholate]. The column was washed with the buffer E, and the P450 was eluted by the buffer E containing 0.2% (w/v) Emulgen 911. The eluted P450 fraction (20 ml) was dialyzed overnight against 20 mM Tris-acetate buffer (pH 7.2) containing 20% glycerol, concentrated to 5 ml, and applied to HPLC using a DEAE-5PW column (7.5 x 75 mm, Tosoh Corp.), which had been equilibrated with the buffer C. CYP4F2 protein was eluted with a linear gradient of sodium acetate (0–200 mM over 60 min) in the buffer C. The fraction was applied to HPLC with a hydroxyapatite column (KB-column, Koken, Tokyo, Japan). P450 was eluted with a linear gradient of 10 to 350 mM sodium phosphate buffer (pH 7.2) containing 20% (v/v) glycerol, 0.2% (w/v) Emulgen 911, and 0.2% (w/v) sodium cholate for 50 min at a flow rate of 0.7 ml/min. The eluted P450 fraction was diluted 5-fold with 10 mM sodium phosphate buffer (pH 7.2) containing 20% glycerol and applied to a hydroxyapatite column to remove Emulgen 911 as described before. The purified CYP4F2 gave a single band on SDS-PAGE, and its specific content was 16.8 nmol of P450/mg of protein.

**Preparation of Polyclonal Anti-CYP4F Antibody.** Polyclonal antibody raised against recombinant CYP4F2 was produced in two male Japanese White rabbits (about 2 kg). Purified P450 (80 μg/mouse) was mixed with Freund’s complete adjuvant and injected intradermally. Additionally, P450 (40 μg) was injected to a rabbit three times on every 3 weeks and finally bled 3 weeks after the last injection. The cross-reactivity of antibody was tested by immunoblot analysis and immunoinhibition study as described elsewhere (Imaoka et al., 1989; Jin et al., 1998). Anti-CYP4F2 antibody could cross-react with recombinant human CYP4F2 (Hashizume et al., 2001) but not with following recombinant P450s: CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11 (GENTEST Corp., Woburn, MA) (data not shown). In the immunoinhibition study, the anti-CYP4F2 antibody could inhibit LTB₄ ω-hydroxylation mediated by recombinant CYP4F2 and by human liver microsomes (80%) (data not shown).

**Immunoblot Analysis Using Antibodies against CYP4F and CYP3A.** Microsomes or purified P450s were separated on 7.5% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane (Bio-Rad Laboratories) as described previously (Imaoka et al., 1989). The membrane was treated with the polyclonal anti-CYP4F2 antibody as prepared above or anti-CYP3A antibody purchased from Daiichi Pure Chemicals (Tokyo, Japan) and goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad Laboratories), and stained with the use of an Amplified alkaline phosphatase kit (Bio-Rad Laboratories).

**Measurement of Monoxygenase Activities.** The reaction mixture for the microsomal metabolism of ebastine consisted of intestinal microsomes (31 pmol of P450/mg; 0.31 mg of protein/ml), [¹⁴C]ebastine (kinetic study: 1.25, 5, 10, 20, and 50 μM; others: 20 μM), an NADPH-generating system (0.8 mM NADPH, 6 mM MgCl₂, 8 mM glucose-6-phosphate, and 1 unit/ml glucose-6-phosphate dehydrogenase) in a final volume of 0.5 ml of 50 mM potassium phosphate buffer (pH 7.4). For the reconstituted system, P450 MI-2 (kinetic study: 3.3 pmol; others: 25 pmol), P450 MI-3 (25 pmol), or other P450-containing fractions (50 pmol), human NADPH-P450 reductase (0.5 unit), DLPC (5 μg), and human cytochrome b₅ (0–125 pmol) were used in place of above microsomes. The reaction was started by the addition of the NADPH-generating system and stopped after incubation at 37°C for 30 min by the addition of acetonitrile (3 ml). After centrifugation at 800g for 10 min, an aliquot of the supernatant was evaporated to dryness in a centrifugal concentrator, and the residue was dissolved in 100 μl of methanol and subjected to HPLC as described previously (Hashizume et al., 1998). For inhibition studies using antibodies, preincubation of microsomes with antisemur or preimmune serum was done at room temperature for 15 min prior to starting the reaction. The antiserum used were polyclonal antibodies against NADPH-P450 reductase, CYP1A2, CYP2C13, CYP2D6, CYP3A4 (Daiichi Pure Chemicals Co.), and polyclonal antibody against CYP4F2 prepared in the present study. Chemical inhibition study of CYP3A was performed in the presence of 0.5 μM ketoconazole (a selective CYP3A inhibitor at low concentration) in the microsomal or reconstituted systems as previously reported (Hashizume et al., 1998). LTB₄ ω-hydroxylation activity was determined by the method described previously (Jin et al., 1998) with minor modifications: the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 5 or 50 μM LTBD₄, the NADPH-generating system, and either microsomes (2 mg/ml) or the reconstituted system containing P450 MI-2 (50 pmol) in a final volume of 0.2 ml. Testosterone 6β-hydroxylation activity was determined as described previously (Komori et al., 1988).

**Other Methods.** The protein concentration was determined as described previously (Lowry et al., 1951) using bovine serum albumin as the standard. The P450 content was measured using the method described previously (Omura and Sato, 1964). SDS-PAGE was conducted as described previously (Laemmli, 1970) with an acrylamide concentration of 7.5%. The standard proteins used to estimate molecular weights were as follows: phosphorylase b (97,400), bovine serum albumin (66,000), aldolase (42,400), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100). Proteins were stained with Coomassie Brilliant Blue R-250. The protein sequences were analyzed by BLAST at NCBI (http://www.ncbi.nlm.nih.gov). Determination of kinetic parameters (Vₘₚ₅ and Kₘ) was accomplished with a curve-fitting software (GraFit version 3.0, Erithacus software, Staines, UK).

**Results**

**Metabolism of Ebastine by Monkey Small Intestinal Microsomes.** Small intestinal microsomes of cynomolgus monkeys were found to metabolize ebastine to two major metabolites, hydroxy- and dealkyl-ebastine, in the presence of NADPH. Hydroxylation and dealkylation followed Michaelis-Menten kinetics at 1.25 to 50 μM ebastine. The Vₘₚ₅ value for hydroxylation was about 3-fold higher than that for dealkylation. The Kₘ values for hydroxylation and dealkylation were 2.5 and 9.6 μM, respectively. Parameters are given in Table 2.

Figure 2 shows the effects of polyclonal antibodies against NADPH-P450 reductase, CYP1A, CYP2C, CYP2D, and CYP3A on...
hydroxylation and dealkylation of ebastine by monkey intestinal microsomes. Marked inhibition by anti-NADPH-P450 reductase antibody indicates that the two reactions were mediated by the P450-dependent monooxygenase system. Anti-CYP3A antibody completely inhibited the dealkylation, while none of these anti-P450 antibodies inhibited the hydroxylation. The results indicate that, similar to the human liver microsomes (Hashizume et al., 1998), the dealkylation was mediated by CYP3A, whereas the hydroxylation was mediated mainly by unidentified P450 enzyme(s) other than CYP1A, CYP2C, CYP2D, and CYP3A.

**Purification of Intestinal P450.** To identify the main enzyme(s) responsible for ebastine hydroxylation in monkey intestinal microsomes, we carried out their purification with monitoring of the heme absorbance at 417 nm, subsequent confirmation of P450 by CO-difference spectrometry, and determination of ebastine hydroxylation activity as described under Experimental Procedures. Solubilized microsomes were applied to an ω-aminooctyl Sepharose 4B column, and then P450 was eluted and pooled. Further purification was conducted by HPLC with a DEAE-5PW anion exchange column. Figure 3A shows the elution profile of the P450 from the DEAE-5PW column. P450 isoforms were eluted in four fractions. The first and second peak fractions (PT-1 and PT-2), which might be leaked out, were combined. Isoforms in two peak fractions (MI-1 and MI-2) were found later at around 120 mM sodium acetate. The PT-1 and PT-2 mixture was applied to an ES-502C cation-exchange column, which yielded one peak fraction containing P450 (MI-3) with a gradient of sodium acetate (Fig. 3B).

Table 1 contains a summary of the purification of three P450 isoforms from monkey small intestinal microsomes. The specific contents of P450 MI-1, P450 MI-2, and P450 MI-3 were 5.0, 3.3, and 6.8 nmol of P450/mg of protein, respectively. The apparent molecular weights of P450 MI-1, P450 MI-2, and P450 MI-3 were estimated to be 56,000, 56,000, and 53,000, respectively, as a major protein on the SDS-PAGE (data not shown). The oxidized forms of P450 MI-1, P450 MI-2, and P450 MI-3 had Soret peaks at 417, 418, and 417 nm (data not shown), respectively, suggesting that these P450 isoforms are in a low spin state. Typical CO-difference spectra with absorption maxima at 450, 450, and 449 nm were observed with P450 MI-1, P450 MI-2, and P450 MI-3, respectively (data not shown). Ebastine hydroxylation activity are given in the next section in Table 2.

**Metabolism of Ebastine by the Reconstituted P450.** Hydroxylation and dealkylation of ebastine by the purified P450 MI-1, P450 MI-2, and P450 MI-3 were examined in the reconstituted system (Table 2). P450 MI-1 did not mediate to metabolize ebastine. P450 MI-2 catalyzed ebastine hydroxylation, and P450 MI-3 catalyzed not only the hydroxylation but also the dealkylation. The hydroxylation
and dealkylation activities of P450 MI-3 of ebastine at 20 μM were enhanced to about 3- and 12-fold, respectively, by the addition of cytochrome b₅ to the reconstituted system, whereas the cytochrome b₅ did not affect the P450 MI-2-mediated hydroxylation (data not shown). To evaluate the relative importance of P450 MI-2 and P450 MI-3 in microsomal metabolism of ebastine, we studied the kinetics of hydroxylation and dealkylation in the reconstituted system including cytochrome b₅ (2 pmol/pmol of P450). Table 2 also shows the kinetic parameters for hydroxylation and dealkylation by P450 MI-2 and P450 MI-3. The Vₘₐₓ value for hydroxylation of P450 MI-2 was much higher than that of P450 MI-3, and the Kₘ value of P450 MI-2 was lower than that of P450 MI-3. Therefore, it appears that P450 MI-2 plays a more important role in ebastine hydroxylation than does P450 MI-3.

Amino Acid Sequence Analysis of P450 MI-2 and P450 MI-3.

The amino acid sequence in the internal region of P450 MI-2 was analyzed as follows: P450 MI-2 separated on the SDS-PAGE was digested with lysylendopeptidase, and the resulting peptides were separated by reverse phase HPLC and sequenced. The amino acid sequences of the peptides were compared with all of those available from the internal regions of P450 isoforms of various animal species including monkeys using the BLAST search program. As shown in FIG. 3.

TABLE 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein</th>
<th>Total P450</th>
<th>Specific Content</th>
<th>Recovery</th>
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<td>295</td>
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<td>100</td>
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<tr>
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<td>6.8</td>
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</tbody>
</table>

⁺ Detergent-removed fraction.

TABLE 2

Metabolism of ebastine by the purified P450MI-1, P450MI-2, and P450MI-3 and monkey small intestinal microsomes

The reaction mixture consisted of 1 to 50 μM [¹⁴C]ebastine, an NADPH-generating system, and small intestinal microsomes (155 μg of protein) or a reconstituted system containing P450 (P450 MI-2: 3.3 pmol of P450, P450 MI-3: 25 pmol of P450), NADPH-P450 reductase (0.5 unit), DLPC (5 μg), and cytochrome b₅ (2 pmol/pmol of P450) in a final volume of 0.5 ml of 50 mM potassium phosphate buffer (pH 7.4). The reaction was started by the addition of the NADPH-generating system and stopped after incubation at 37°C for 30 min by the addition of acetonitrile. Results were obtained from two determinations. Values in parentheses indicate activities expressed as nmol/min/mg protein.

<table>
<thead>
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<th>Ebastine Hydroxylation</th>
<th>Ebastine Dealkylation</th>
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<tr>
<td></td>
<td>Kₘ (μM)</td>
<td>Vₘₐₓ (nmol/min/nmol P450)</td>
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<td>Microsomes</td>
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<td>3.15 (0.316)</td>
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<td>P450 MI-1</td>
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<td>N.D.</td>
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<td>P450 MI-2</td>
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<td>0.406 (2.76)</td>
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<td>P450 MI-3</td>
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</table>

N.D., not determinable due to lack of the reaction.
A NOVEL CYTOCHROME P450 IN MONKEY SMALL INTESTINE

Fig. 4, the sequences of all five peptides were similar to the deduced amino acid sequences of CYP4F2, CYP4F3, and CYP4F8 cDNAs, which were cloned from human liver, leukocytes, and seminal vesicle, respectively (Kikuta et al., 1993, 1994; Bylund et al., 1999). The NH2-terminal amino acid sequence of P450 MI-3 was determined as described under Experimental Procedures and compared with that of monkey and human P450 isoforms (Fig. 5). The NH2-terminal amino acid sequence of the first 27-amino acid residues of P450 MI-3 had 88.9, 88.9, 96.3, and 92.6% identity with that of CYP3A8 (Ohta et al., 1989; Komori et al., 1992), CYP3A4 (Gonzalez et al., 1988), CYP3A5 (Aoyama et al., 1989), and CYP3A7 (Komori et al., 1989), respectively. The amino acid sequence analyses indicated that P450 MI-2 and P450 MI-3 can belong to CYP4F subfamily.

Further Characterization of P450 MI-2 and P450 MI-3.

In addition to the contributions made by CYP4F2 and CYP3A4 (Bylund et al., 1999), P450 MI-2 and P450 MI-3 were recognized by anti-CYP4F antibody and anti-CYP3A antibody, respectively, suggesting that P450 MI-2 was immunochemically related to CYP4F subfamily and that P450 MI-3 was related to CYP3A subfamily. Each antibody also recognized a protein at the same molecular weight in small intestinal microsomes. P450 MI-2 and P450 MI-3 can belong to CYP4F and CYP3A subfamily, respectively.

Further Characterization of P450 MI-2 and P450 MI-3.

In the immunoblot analysis of P450 MI-2 and P450 MI-3, the immunoblot analysis of P450 MI-2 and P450 MI-3 was performed using polyclonal antibodies against CYP4F2 and CYP3A4 (Fig. 6). P450 MI-2 and P450 MI-3 were recognized by anti-CYP4F antibody and anti-CYP3A antibody, respectively, suggesting that P450 MI-2 was immunochromically related to CYP4F subfamily and that P450 MI-3 was related to CYP3A subfamily. Each antibody also recognized a protein at the same molecular weight in small intestinal microsomes.

Further Characterization of P450 MI-2 and P450 MI-3.

In addition, we also examined the ability of P450 MI-2 and P450 MI-3 to catalyze LTB4 ω-hydroxylation and testosterone 6β-hydroxylation, which are known to be mediated by the CYP4F (Kikuta et al., 1993; Kikuta et al., 1994) and CYP3A (Guengerich, 1995) isoforms, respectively. The reconstituted P450 MI-2 catalyzed LTB4 ω-hydroxylation (5.2 pmol/min/nmol of P450), but this activity was found much lower than the recombinant CYP4F2 expressed in yeast microsomes (319 pmol/min/nmol of P450) and monkey small intestinal microsomes (142 pmol/min/nmol of P450). On the other hand, the testosterone 6β-hydroxylation activity of P450 MI-3 (0.4 nmol/min/nmol of P450) was seemingly similar to that of CYP3A8 (1.1 nmol/min/nmol of P450) reported previously (Ohta et al., 1989). The activity mediated by P450 MI-3 was strongly inhibited (83%) by 0.5 μM ketoconazole, a selective CYP3A inhibitor at a low concentration.

Inhibition of Ebastine Hydroxylation in Small Intestinal Microsomes by Anti-CYP4F Antibody. To evaluate the contribution of P450 MI-2 to ebastine hydroxylation in monkey small intestinal microsomes, an immunoinhibition study was performed using polyclonal antibody against the purified recombinant CYP4F2 as described under Experimental Procedures. The results are shown in Fig. 7. A concentration-dependent inhibition of microsomal ebastine hydroxylation was observed (70%), indicating that P450 MI-2 regarded as a CYP4F enzyme is mainly responsible for ebastine hydroxylation in monkey small intestinal microsomes.

Discussion

Small intestinal microsomes of cynomolgus monkeys, similarly to human liver microsomes (Hashizume et al., 1998), were found to catalyze the hydroxylation and dealkylation of an H1-antihistamine prodrug, ebastine. Also similarly to human liver microsomes, anti-CYP3A antibody completely inhibited the intestinal microsome-mediated dealkylation in the inhibition study using antibodies against CYP1A, CYP2C, CYP2D, and CYP3A, none of which showed an inhibitory effect on the hydroxylation. These qualitative findings in monkey intestinal microsomes are just the same as those in previous human liver microsomes, thereby indicating that the enzyme responsible for ebastine hydroxylation can be an unidentified molecular species.

As discussed below, on the basis of the hydroxylation activity, we obtained the two purified P450 isoforms, P450 MI-2 and P450 MI-3, from the intestinal microsomes and identified the main enzyme responsible for ebastine hydroxylation. In the purification steps, we determined the apparent molecular weights of P450 MI-2 and P450 MI-3, which were also found to be present in a low spin state and to give typical CO difference spectra.

In the reconstituted system, both P450 MI-2 and P450 MI-3 catalyzed hydroxylation of ebastine. However, the Vmax value of P450 MI-3 (0.406 nmol/min/nmol of P450) was far lower than that of P450 MI-2 (37.0 nmol/min/nmol of P450), and P450 MI-2 had a lower Km value (5.1 μM) than did P450 MI-3 (10 μM). The Km value of P450 MI-2 was close to that found in the intestinal microsomes (2.5 μM), and at a basis of milligrams of protein, the Vmax of P450 MI-2 was much higher (300-fold) than that of the microsomes. Therefore, the conclusion is that P450 MI-2 is the principal enzyme responsible for hydroxylation of ebastine while P450 MI-3, which belongs to the CYP3A subfamily as discussed later, is not essential for ebastine hydroxylation in the small intestine. To support this, anti-CYP3A antibody did not inhibit the microsomal hydroxylation.

Internal amino acid sequences of five peptides derived from the purified P450 MI-2 exhibited high homology with those of human CYP4F2 (Kikuta et al., 1994), CYP4F3 (Kikuta et al., 1993), and CYP4F8 (Bylund et al., 1999). P450 MI-2 was also immunochemically recognized by polyclonal antibody against the purified recom-
binant CYP4F2. Although the reconstituted P450 MI-2 catalyzed LTB4 ω-hydroxylation, which is a typical reaction mediated by the CYP4F subfamily, its catalytic activity was much lower than that by the CYP4F2 expressed in yeast cells and by the monkey intestinal microsomes. P450 MI-2 can thus be a novel CYP4F isoform that differs in substrate specificity for endogenous and foreign compounds from CYP4F isoforms (CYP4F2 or CYP4F3) reported before.

The above conclusions can be thoroughly supported by the immuno-inhibition study using anti-CYP4F antibody. We prepared polyclonal antibody against CYP4F and examined its inhibitory effect on the hydroxylation activity. Anti-CYP4F antibody was found to strongly inhibit ebastine hydroxylation by the intestinal microsomes (Fig. 7), in contrast to the lack of inhibition by other anti-P450 antibodies including that against CYP3A (Fig. 2). This result demonstrates that P450 MI-2, which is a CYP4F4 enzyme, is the main molecule responsible for ebastine hydroxylation in the small intestine. It is thus obvious, as described in the introductory remarks of this article, that the CYP3A subfamily is very important in human drug metabolism (Guengerich, 1995). Among members of the CYP3A subfamily, CYP3A4 is the predominant form of P450 expressed in the small intestine and liver of normal adult humans (Guengerich, 1995; Kivisto et al., 1996). In cynomolgus monkeys, CYP3A8 is the only reported CYP3A isoform and has been shown to be the predominant form of the CYP3A subfamily expressed in the normal liver (Ohta et al., 1989; Komori et al., 1992; Ohmori et al., 1993). However, the NH2-terminal amino acid sequence of P450 MI-3 was not identical with that of CYP3A8. P450 MI-3 thus appears to be a cynomolgus monkey CYP3A isoform, rather than CYP3A8, and also appears to play an important role in the first-pass metabolism (dealkylation) of ebastine in the small intestine. Further study is needed to elucidate the significance of its hydroxylation activity.

FIG. 5. Comparison of the amino acid sequence of P450 MI-3 with the sequences of CYP3A isoforms.

The NH2-terminal amino acid sequence of P450 MI-3 is shown here aligned with those of monkey CYP3A8, human CYP3A4, CYP3A5, and CYP3A7. Amino acid sequences were obtained from the sources in parentheses: CYP3A8 (Ohta et al., 1989; Komori et al., 1992), CYP3A4 (Gonzalez et al., 1988), CYP3A5 (Aoyama et al., 1989), CYP3A7 (Komori et al., 1989). The yields of the amino acids (pmol) per cycle for P450 MI-3 were as follows in their order: 42, 36, 34, 26, 28, 30, 35, 24, 14, 28, 35, 23, 25, 4, 23, 18, 24, 25, 19, 26, 16, and 7.

FIG. 6. Immunoblot analysis of P450 M-2 and P450 MI-3 using anti-CYP4F and anti-CYP3A antibodies.

Microsomes or purified P450s were separated on 7.5% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane as described previously (Imaoka et al., 1989). The membrane was treated with polyclonal anti-CYP4F2 antibody or anti-CYP3A antibody and goat anti-rabbit IgG conjugated to alkaline phosphatase, and stained with the use of an amplified alkaline phosphatase kit as described under Experimental Procedures. A, each lane contained 40 μg of monkey small intestinal microsomes or 1 μg of purified P450 M-2. B, each lane contained 20 μg of monkey small intestinal microsomes or 0.3 μg of purified P450 MI-3.

FIG. 7. Effects of anti-CYP4F antibody on ebastine hydroxylation by monkey small intestinal microsomes.

Small intestinal microsomes (0.31 mg/ml) were preincubated with various amounts of antiserum or preimmune serum for 15 min at room temperature and then incubated with 20 μM ebastine in the presence of an NADPH-generating system. Hydroxylation activity with preimmune serum (100% as the control) was 0.311 pmol/min/mg of protein. Closed circles indicate hydroxylation activity of ebastine. The values represent the average of duplicate determinations.
necessary to ascertain whether P450 MI-3 is also a predominant form of monkey small intestinal cytochrome P450.

In summary, this is the first report of P450 enzymes purified from monkey small intestinal microsomes (named P450 MI-2 and P450 MI-3) belonging to the CYP4F and CYP3A subfamilies, respectively. We showed that P450 MI-2 but not P450 MI-3 is mainly responsible for the hydroxylation of the antihistamine produg, ebastine, in the small intestine. This suggests that the small intestinal CYP4F enzyme, together with CYP3A, can play an important role in the first-pass metabolism of therapeutic drugs given orally, including the present ebastine. Therefore, our findings provide a basic understanding that will aid further studies of drug metabolism in the intestine.

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References


