Involvement of CYP2J2 and CYP4F12 in the Metabolism of Ebastine in Human Intestinal Microsomes

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ABSTRACT

The purpose of the study was to elucidate human intestinal cytochrome P450 isoform(s) involved in the metabolism of an antihistamine, ebastine, having two major pathways of hydroxylation and N-dealkylation. The ebastine dealkylase in human intestinal microsomes was CYP3A4, based on the inhibition studies with antibodies against CYP1A, CYP2A, CYP2C, CYP2D, CYP2E, and CYP3A isoforms and their selective inhibitors. However, ebastine hydroxylase could not be identified. We then examined the inhibitory effects of anti-CYP4F antibody and 17-octadecynoic acid, an inhibitor of the CYP4 family, on ebastine hydroxylation in intestinal microsomes, since CYP4F was recently found to be the predominant ebastine hydroxylase in monkey intestine; and a novel CYP4F isoform (CYP4F12), also capable of hydroxylation of ebastine, was found to exist in human intestine. However, the inhibitory effects were only partial (about 20%) and thus it was thought that, although human CYP4F was involved in ebastine hydroxylation, another predominant enzyme exists. Further screening showed that the hydroxylation was inhibited by arachidonic acid. CYP2J2 was selected as a candidate expressed in the intestine and closely related to arachidonic acid metabolism. The catalytic activity of recombinant CYP2J2 was much higher than that of CYP4F12. Anti-CYP2J antibody inhibited the hydroxylation to about 70% in human intestinal microsomes. These results demonstrate that CYP2J2 is the predominant ebastine hydroxylase in human intestinal microsomes. Thus, the present paper for the first time indicates that, in human intestinal microsomes, both CYP2J and CYP4F subfamilies not only metabolize endogenous substrates but also are involved in the drug metabolism.

Ebastine is a potent nonsedative H1-receptor antagonist (Fig. 1), and after oral administration to experimental animals and humans, the agent is almost completely metabolized to the pharmacologically active principle, the carboxylated metabolite (carebastine), and other inactive metabolites (Fuji et al., 1994; Matsuda et al., 1994; Yamaguchi et al., 1994). Carebastine alone was the major metabolite detectable in the blood. Our previous in situ studies using rats indicated that the small intestine extensively converted the orally given ebastine to carebastine via hydroxylated ebastine and the dealkylated metabolite (Fuji et al., 1997). Therefore, it seemed that small intestine plays an important role in the first-pass metabolism of this drug, and the enzymes responsible for its metabolism exist there.

We reported that ebastine was primarily metabolized by human liver microsomes to two metabolites, hydroxy- and desalkyl-ebastine (Hashizume et al., 1998). N-Dealkylation to desalkyl-ebastine was mediated by CYP3A4, whereas hydroxylation to hydroxy-ebastine, the most important intermediate metabolite yielding carebastine, was mediated by unidentified P450(s) other than CYP3A4. Our recent studies revealed that two novel CYP4F isoforms (P450 MI-2 and CYP4F12) obtained from monkey and human small intestine, respectively, were involved in the ebastine hydroxylation (Hashizume et al., 2001a,b). Results obtained in an inhibition study using anti-CYP4F antibody indicated the involvement of CYP4F isoform (P450 MI-2) in the drug metabolism in monkey intestinal microsomes, although this subfamily had been recognized to be connected with the endobiologic metabolism.

Based on these findings, we attempted to elucidate P450 isoform(s) involved in the metabolism of ebastine in human small intestine. We found that CYP2J and CYP4F were involved in the metabolism of ebastine. The present finding suggests that CYP2J and CYP4F play an important role in the overall first-pass metabolism of drugs in humans.

Experimental Procedures

Materials. [14C]Ebashine [4'-tert-butyl-4-[(ring-U-14C)diphenylmethoxy)piperidino]butyrophenone] was synthesized by using the method described previously (Fuji et al., 1994), with a specific

ABBREVIATIONS: P450, cytochrome P450; 17-ODYA, 17-octadecynoic acid; HPLC, high-performance liquid chromatography.
NADPH and dilauroyl-L-3-phosphatidylcholine were purchased from [4-(diphenylmethoxy)piperidino]butyrophenone; desalkyl-ebastine, -(2-hydroxy-1,1-dimethylethyl)-4-metabolites [hydroxy-ebastine, 4-metabolites (recycling)] were synthesized with a radiochemical purity of 99%. Authentic activity of 1.08 MBq/mg and radiochemical purity of 99%. Authentic authentic metabolites [hydroxy-ebastine, 4′-(2-hydroxy-1,1-dimethylethyl)-4-[(diphenylmethoxy)piperidino]butyrophenone; desalkyl-ebastine, 4′-(diphenylmethoxy)piperidine; and carebastine, 4′-(2-carboxy-1,1-dimethylethyl)-4′-[(diphenylmethoxy)piperidino]butyrophenone] were supplied by Almirall-Prodesfarma S. A. (Barcelona, Spain). NADPH and dilauroyl-1,3-phosphatidylcholine were purchased from Wako Pure Chemical Industries (Osaka, Japan). 17-Octadecynoic acid (17-ODYA), leukotriene B4, and arachidonic acid were purchased from Cayman Chemical (Ann Arbor, MI). Furafylline, sulfaphenazole, and acid (17-ODYA), leukotriene B4, and arachidonic acid were purchased from Sumitomo Chemical Co. (Hyogo, Japan). The resulting plasmid was used for transformation of the Saccharomyces cerevisiae AH22 strain (Murakami et al., 1990). Yeast was cultured as described (Murakami et al., 1986; Sakaki et al., 1991) and the microsome was prepared as described previously (Oeda et al., 1995).

Preparation of Antibodies. Polyclonal antibodies against CYP1A1, CYP2C, CYP2D, and CYP3A were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Polyclonal anti-CYP2E1 antibody and monoclonal anti-CYP2A6 antibody were obtained from Gentest. Anti-CYP2J2 and CYP4F antibodies were prepared as described previously (Imaoka et al., 1990; Hashizume et al., 2001b). The anti-CYP2J2 antibody could cross-react with recombinant human CYP2J2 but not with the following recombinant 450 isoforms: CYP1A1, CYP1A2, CYP1B1, CYP1A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11 (data not shown). Anti-CYP2J2 and CYP4F IgG and control IgG were purified from rabbit sera using protein A Sepharose CL-4B (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK).

Incubations. The reaction mixture for the microsomal metabolism consisted of [14C]ebastine (kinetic study: 1.25, 5, 10, 20, and 50 µM; others 5 or 20 µM), microsomes (human intestine, 0.04–0.2 mg; human liver, 0.08 mg; yeast cells expressing CYP4F12, 95 pmol; CYP4F2, 107 pmol; and CYP2J2, 0.07–1.0 pmol), and 0.8 mM NADPH in a final volume of 0.5 ml of 50 mM potassium phosphate buffer (pH 7.4). For the reconstituted system, 22 pmol of native CYP2J3 (originally named P450 3b) purified from rat liver previously (Imaoka et al., 1990), human NADPH-P450 reductase (0.5 unit), and dilauroyl-1,3-phosphatidylcholine (5 µg) were used in place of the above microsomes. Assay conditions were such that metabolite formation and parent drug consumption were linear with respect to incubation time and protein concentration. For the kinetic study, protein concentrations of individual microsomes were adjusted to avoid further metabolism of hydroxy-ebastine, and the initial velocities of ebastine hydroxylation and N-dealkylation were measured. The reaction was started by the addition of NADPH and stopped after incubation at 37°C for 30 min by the addition of acetonitrile (2 ml). After centrifugation at 800g for 10 min, an aliquot of the supernatant was evaporated to dryness with a centrifugal concentrator. The residue was dissolved in 100 µl of methanol and 30 µl was injected onto a 250 × 4.6 mm Inertsil ODS-3 reverse-phase column (GL Science, Tokyo, Japan) maintained at 40°C (Hashizume et al., 1998). The mobile phase consisted of 12 mM ammonium acetate buffer (pH 4.5) and acetonitrile, at a flow rate of 1.0 ml/min. The proportion of acetonitrile was maintained at 35% from 0 to 3 min and then increased to reach 85% at 25 min. The elution profile of the radioactive compounds was monitored by a FLO-ONE β radioactivity flow detector (Packard Instrument Co., Meriden, CT).

Chemical Inhibition. The following P450 isoform-selective inhibitors were used: 50 µM furafylline (an inhibitor for CYP1A2), 500 µM coumarin (for CYP2A6), 50 µM orphenadrine (for CYP2B6), 10 µM sulfaphenazole (for CYP2C), 20 µM tranylcypromine (for CYP2C19), 10 µM quinidine (for CYP2D6), 50 µM diethyldithiocarbamate (for CYP2E1), 1 µM ketocazole (for CYP3A), and 100 µM lauric acid (for CYP4A). The concentrations of inhibitors described above were decided based on their published IC50, Ki, or Km values for P450 isoform-specific reactions. 17-ODYA (a mechanism-based inhibitor for the CYP4 family; 5, 25, and 100 µM), leukotriene B4 (a CYP4F substrate; 10, 50, and 100 µM), and arachidonic acid (a CYP4F substrate; 10, 50, and 100 µM) were also used (Shak et al., 1985; Rikuta et al., 1994; Zou et al., 1994; Powell et al., 1998).

![Fig. 1. Metabolic pathways of ebastine.](image-url)
Inhibitors except furafylline and 17-ODYA were incubated at 37°C for 30 min in a final volume of 0.5 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 0.2 mg of pooled human intestinal microsomes \((n = 3)\), 0.8 mM NADPH, and 5 \(\mu\)M \(^{14}\)Cebastine. The substrate concentration selected was 2 times the \(K_m\) value for ebastine hydroxylation in human intestinal microsomes. Furafylline and 17-ODYA were preincubated with human intestinal microsomes and NADPH for 20 min in the absence of \(^{14}\)Cebastine. The assay was carried out as described under Incubations.

**Immunoinhibition.** The anti-P450 antibodies, preimmune sera (as control for antibodies against CYP1A, CYP2C, CYP2D, CYP2E, and CYP3A), 25 mM Tris-HCl buffer (pH 7.4) (as control for anti-CYP2A6 antibody), or preimmune IgG (as control for antibodies against CYP4F and CYP2J) were preincubated with intestinal microsomes (0.2 mg), liver microsomes (0.08 mg), and yeast microsomes expressing CYP2F2 (0.2 mg) at room temperature for 30 min. The reaction was started by the addition of \(^{14}\)Cebastine (final concentration, 5 or 20 \(\mu\)M) and was stopped with 2 ml of acetonitrile. The assay was carried out as described under Incubations.

**Data Analysis.** The values represent the average of duplicate or triplicate determinations. Kinetic parameters \((V_{\text{max}}\) and \(K_m\)) were obtained by fitting data to the following Michaelis-Menten equation using the curve-fitting software GraFit version 3.0 (Erithacus Software, Staines, UK):

\[
V = \frac{V_{\text{max}} \times S}{K_m + S}
\]

where \(V\) is reaction velocity; \(S\) substrate concentration; \(V_{\text{max}}\), maximum reaction velocity; and \(K_m\), Michaelis-Menten constant. The kinetic parameters for hydroxylation and \(N\)-dealkylation of ebastine were compared using a paired \(t\) test with the SAS statistics program (SAS Institute, Cary, NC). The value of \(p < 0.05\) was regarded as statistically significant.

**Results**

**Metabolism of Ebastine by Human Intestinal Microsomes.** Figure 2 shows a typical reverse-phase HPLC radiochromatogram obtained after incubation of \(^{14}\)Cebastine with human intestinal microsomes. In the presence of NADPH, human intestinal microsomes metabolized ebastine to two major metabolites, hydroxy- and desalkyl-ebastine. Carebastine, the active principle, was also partly formed from hydroxy-ebastine. Hydroxylation and \(N\)-dealkylation of ebastine followed simple Michaelis-Menten kinetics in human intestinal microsomes under the condition in which carebastine was not formed (Fig. 3). The kinetic parameters of five individual microsomes are summarized in Table 1. Data demonstrated large interindividual variability in the formation of the two major metabolites, and the \(K_m\) values were significantly different \((p < 0.03)\) between the hydroxylation \((2.6 \pm 2.3 \mu\text{M})\) and the \(N\)-dealkylation \((39 \pm 24 \mu\text{M})\). There are no significant differences in the \(V_{\text{max}}\) and \(V_{\text{max}}/K_m\) values between the hydroxylation and the \(N\)-dealkylation \((p > 0.05)\). Both reactions were inhibited almost completely by SKF-525A and anti-NADPH-P450 reductase antibody, suggesting that these reactions were mediated by P450 (data not shown).

**First Screening for Ebastine Hydroxylase in Human Intestinal Microsomes.** Effects of P450 isoform-selective inhibitors on ebastine hydroxylation and \(N\)-dealkylation by human intestinal microsomes are shown in Fig. 4. Ebastine \(N\)-dealkylation was almost completely inhibited by ketoconazole (selective for CYP3A), whereas the hydroxylation was not affected by all inhibitors examined: furafylline (for CYP1A2), coumarin (for CYP2A6), orphenadrine (for CYP2B6), sulfaphenazole (for CYP2C), tranylcypromine (for CYP2C19), quinidine (for CYP2D6), diethylthiocarbamate (for CYP2E1), ketoconazole (for CYP3A), and lauric acid (for CYP4A11). Figure 5 shows the effects of polyclonal antibodies against CYP1A, CYP2A, CYP2C, CYP2D, CYP2E, and CYP3A on ebastine hydroxylation and \(N\)-dealkylation by human intestinal microsomes. Similar to chemical inhibitors, none of the anti-P450 antibodies inhibited ebastine hydroxylation and only anti-CYP3A antibody inhibited the \(N\)-dealkylation completely. These results indicate that CYP3A was the major ebastine dealkylase, whereas P450(s) other than the ordinary drug-metabolizing P450 was involved in hydroxylation.

**Role of CYP4F in Ebastine Hydroxylation.** We determined the kinetic parameters for ebastine hydroxylation by the recombinant human CYP4F12 and CYP4F2, which were
and 0.354 nmol/min/nmol P450, respectively. Kinetic parameters for hydroxylation and N-dealkylation of ebastine by human intestinal microsomes were incubated with [14C]ebastine (1.25, 5, 10, 20, or 50 μM) at 37°C for 30 min in the presence of 0.8 mM NADPH.

Table 1: Kinetic parameters for hydroxylation and N-dealkylation of ebastine by human intestinal microsomes

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Reactions</th>
<th>Human Intestinal Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>$K_m$ (μM)</td>
<td>Hydroxylation</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>N-Dealkylation</td>
<td>30</td>
</tr>
<tr>
<td>$V_{max}$ (nmol/min/mg)</td>
<td>Hydroxylation</td>
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</tr>
<tr>
<td></td>
<td>N-Dealkylation</td>
<td>0.045</td>
</tr>
<tr>
<td>$V_{max}/K_m$ (μl/min/mg)</td>
<td>Hydroxylation</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>N-Dealkylation</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*p < 0.05 (paired t test) as compared with the $K_m$ values for N-dealkylation of ebastine.

Identification of Major Ebastine Hydroxylase in Human Intestinal Microsomes. Results obtained above suggest that the ebastine hydroxylase belongs to P450 isoform(s) that have not been recognized to be ordinary drug-metabolizing P450s and, instead, seemingly possesses the substrate specificity to endogenous compounds. Thereby, to identify the predominant ebastine hydroxylase(s) in human intestinal microsomes, leukotriene B₄ and arachidonic acid (both are CYP4F substrates) were used as the inhibitory probes. Leukotriene B₄ inhibited ebastine hydroxylation by human intestinal microsomes to 85% of control (Fig. 8A) and arachidonic acid, to 2% of control (Fig. 8B), suggesting that arachidonic acid more strongly interacts with the predominant ebastine hydroxylase.

Based on the results, CYP2J2 was selected out of P450 isoforms as a candidate expressed in the intestine (Zeldin et al., 1997) and closely related to arachidonic acid metabolism (Rifkind et al., 1995). We cloned CYP2J2 cDNA from the human small intestine cDNA library and expressed this P450 in yeast cells as described, and the kinetics for ebastine hydroxylation by the recombinant CYP2J2 were examined (Table 2). The $V_{max}$ value of ebastine hydroxylation by CYP2J2 was much higher than that by CYP4F12 (40.6 versus 0.354 nmol/min/nmol P450, respectively). The $K_m$ value of CYP2J2 was half that of CYP4F12. The $V_{max}/K_m$ value of CYP2J2 was 250-fold higher than that of CYP4F12. To assess the involvement of the CYP2J2 subfamily in ebastine hydroxylation, we measured ebastine hydroxylase activity of purified native CYP2J3 (Imaoka et al., 1990). The reconstituted CYP2J3 protein also catalyzed ebastine hydroxylation (6.4 nmol/min/nmol P450, at 10 μM ebastine). At higher concentrations of CYP2J2 and CYP2J3, ebastine was rapidly oxidized to the carboxylic acid metabolite, carebastine, via hydroxy-ebastine and then disappeared completely.

To confirm the role of CYP2J2 as the main catalyst of ebastine hydroxylation in human intestinal microsomes, immunoinhibition experiments were performed using polyclonal anti-CYP2J2 antibody. As shown in Fig. 9, anti-CYP2J2 antibody inhibited ebastine hydroxylation by recombinant CYP2J2 (Fig. 9A) and human intestinal microsomes (Fig. 9B). In addition, anti-CYP2J antibody had the inhibitory effect on ebastine hydroxylase activity in human liver microsomes (25% inhibition at 10 mg IgG/mg microsomal protein). This is consistent with reports that CYP2J2 is also expressed in human liver (Wu et al., 1996; Gu et al., 2000). These results indicate that CYP2J2 is the predominant ebastine hydroxylase in human intestinal microsomes.
Discussion

Recently, clinical investigations have demonstrated that the small intestine participates in the “first-pass” metabolism of orally administered drugs like cyclosporine, midazolam, tacrolimus, and verapamil (Wu et al., 1995; Thummel et al., 1996; Lin et al., 1999). All of these drugs were substrates of CYP3A4; therefore, most studies of intestinal “first-pass” metabolism have focused on CYP3A and little is known about the involvement of other intestinal P450 isoforms. Therefore, the drug that is metabolized by P450(s) other than CYP3A in human small intestine appears to provide new findings for understanding intestinal drug metabolism in humans.

In the present study, we attempted to elucidate P450 isoform(s) involved in the small intestinal metabolism of an antihistamine, ebastine, metabolism of which has been characterized as follows. 1) Rat small intestine in situ almost completely converted the orally given ebastine to carebastine (via hydroxyl-ebastine) and desalkyl-ebastine (Fujii et al., 1997). 2) Ebastine was primarily metabolized by human liver microsomes to desalkyl- and hydroxy-metabolites (Hashizume et al., 1998). The former was shown to be produced by CYP3A4, while the latter, the intermediate metabolite leading to carebastine, was mediated by unidentified P450(s). 3) Very recently, CYP4F was found to be the predominant ebastine hydroxylase in monkey intestinal microsomes (Hashizume et al., 2001b), and a novel human CYP4F isoform (named CYP4F12), being capable of hydroxylating ebastine, was also found to exist in the small intestine (Hashizume et al., 2001a).

Human intestinal microsomes, similar to human liver microsomes, metabolized ebastine to desalkyl-ebastine and hydroxy-ebastine. Inhibition experiments using nine P450 isoform-selective inhibitors and six anti-P450 antibodies demonstrated that CYP3A4 was the main ebastine dealkylase, whereas unidentified P450(s) was involved in the hydroxylation.

We recently purified an ebastine hydroxylase belonging to the CYP4F subfamily from monkey intestinal microsomes (Hashizume et al., 2001b). The reconstituted P450 showed ebastine hydroxylase activity ($V_{\text{max}}$, 37.0 nmol/min/nmol
The recombinants CYP2J2, CYP4F12, and CYP4F2 were incubated with $[^{14}C]$ebastine (0.354 nmol/min/nmol P450) and CYP3A4 (0.023 nmol/min/nmol P450; Hashizume et al., 1998). The value was as high as the $V_{max}$ of the CYP4F isoform purified from monkey intestinal microsomes (37 nmol/min/nmol P450; Hashizume et al., 2001b). In addition, anti-CYP2J antibody inhibited ebastine hydroxylation to about 70% of control by human intestinal microsomes. Results obtained in the kinetic analysis and immunoinhibition experiments indicated that CYP2J2 was the predominant ebastine hydroxylase in human intestinal microsomes. The immunoblotting data reported by Zeldin et al. (1997) suggest that CYP2J2 content in human intestinal microsomes was not high. However, the present studies demonstrated that ebastine hydroxylase activity in human intestinal microsomes was attributable to CYP2J2 for the most part. This finding is supported by our preliminary unpublished immunofunctionalization result (T. Hashizume and S. Imaoke), using antibody raised against the recombinant CYP2J2, that the high correlation ($r = 0.924, p < 0.003$) existed between the relative CYP2J2 content and ebastine hydroxylase activities in seven human intestinal microsomes.

It would be interesting to consider the enzymes responsible for metabolism of another antihistamine, terfenadine, which possesses certain chemical structural similarities to ebastine, as follows: the presence of a terminal tertiary butyl group, two aromatic rings, and a protonatable nitrogen. Terfenadine also has two similar major metabolic pathways of hydroxylation and N-dealkylation, and the carboxylated metabolite formed from the former is the active principle. However, unlike ebastine, both hydroxylation and N-dealkylation of terfenadine are reported to be catalyzed predominantly by CYP3A4 (Yun et al., 1993; Jurin-Romet et al., 1994; Ling et al., 1995; Rodrigues et al., 1995). Recently, a complete computer-assisted conformational and electronic characterization study demonstrated that the preferred three-dimensional spatial orientations were different in the molecular locations of the highest occupied and lowest unoccupied molecular orbitals of the two agents (Segarra et al., 1999). Furthermore, it was reported that, for terfenadine, addi-
tional points of interaction with macromolecules as a hydro-
gen bond donor were found, whereas noncardiotoxic antihis-
tamines including ebastine were lacking. Therefore, the three-dimensional structural and electronic features of these two compounds seem to be related to the difference in the contribution of CYP3A to their microsomal metabolism.

In conclusion, the present paper for the first time indicates that, in human intestinal microsomes, both CYP2J and CYP4F subfamilies not only metabolize endogenous substrates such as arachidonic acid and leukotriene B$_2$, but also are involved in the drug metabolism. To date, actual participation in the intestinal first-pass metabolism in humans has been elucidated for CYP3A4 alone, and in contrast to hepatic metabolism, only a limited number of P450 isoforms of small intestine have been characterized. The results here clearly revealed a potential role of CYP2J and CYP4F subfamilies in the xenobiotic metabolism in small intestinal microsomes and thus will serve for further understanding of the intestinal first-pass metabolism.

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References


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