Poor Correlation between Intestinal and Hepatic Metabolic Rates of CYP3A4 Substrates in Rats

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Purpose. To clarify the contribution of the intestinal first-pass metabolism to the drug bioavailability, the correlation between the intestinal and hepatic metabolism of human CYP3A4 substrates was investigated in rats.

Methods. The metabolic rates of four compounds (lidocaine, quinidine, nifedidpine, and rifabutin) were examined with excised intestinal tissues and liver microsomes. The intestinal and hepatic expression of CYP3A1/23 and CYP3A2 was evaluated by Western blot analysis.

Results. Rifabutin was metabolized fastest, and lidocaine was metabolized slowest in excised intestinal tissues. By contrast, lidocaine was metabolized fastest and rifabutin was the slowest in liver microsomes. The hepatic metabolism of lidocaine was inhibited by a CYP2D6 substrate desipramine, not by a CYP3A4 inhibitor ketoconazole. In addition, members of the CYP3A subfamily expressed in the intestine were different from those expressed in the liver.

Conclusions. Poor correlation between the intestinal and hepatic metabolism of human CYP3A4 substrates in rats may be caused by the contribution of the CYP2D subfamily to the drug metabolisms in the liver and also by the unique expression of the CYP3A subfamily in the intestine.

KEY WORDS: CYP3A4; CYP3A1; CYP3A2; intestinal metabolism; substrate specificity.

INTRODUCTION

Cytochrome P450 (CYP) 3A4 is a drug oxidation enzyme that is expressed predominantly in human liver. Because CYP3A4 recognizes various compounds as substrate, it is involved in the hepatic metabolism of many medicines and affects their blood concentrations and therapeutic effects (1). Recently, the intestinal expression of CYP3A4 was reported (2), and some therapeutic compounds, such as midazolam (3), were shown to be metabolized in the human intestine. This implies that CYP3A4 substrates are subject to first pass metabolism both in intestine and in liver when administered orally. Therefore, it is necessary to examine the intestinal drug metabolism to understand changes in bioavailability and/or drug–drug interactions of CYP3A4 substrates in detail.

To date, various studies on drug metabolism in intestine have been performed in rats, and several compounds, such as midazolam and rifabutin, have been shown to be metabolized extensively in intestine (4,5). Previously, we demonstrated that tacrolimus was also metabolized in rat intestine and revealed that the intestinal first pass metabolism of tacrolimus is significant in rats (6). However, it remains unclear whether all CYP3A4 substrates are metabolized in rat intestine and to what extent the intestinal and hepatic drug metabolisms contribute to first pass metabolism.

In this study, we examined the metabolic rates of CYP3A4 substrates, lidocaine (7), quinidine (8), nifedipine (9), and rifabutin (10) in excised intestinal tissues and liver microsomes from rats and revealed that there was a poor correlation between the intestinal and hepatic metabolisms of CYP3A4 substrates. Then, we investigated some of the mechanisms responsible for the difference between the intestinal and hepatic metabolism of CYP3A4 substrates.

MATERIALS AND METHODS

Materials

Lidocaine was obtained from Nacalai Tesque (Kyoto, Japan). Nifedipine and quinidine were purchased from Sigma (St. Louis, MO, USA). Rifabutin was from Research Diagnostic (Flanders, NJ, USA). Monoethylglycinexylidide (MEGX) was synthesized and purified more than 99.5%. The polyclonal antibodies against CYP3A1 and CYP3A2 were obtained from Chemicon International (Temecula, CA, USA). They distinguish CYP3A1 and CYP3A2 by the amino acid sequence at the C-terminus (11). Because CYP3A1 may be an allelic variant of CYP3A23, we refer to these two enzymes together as CYP3A1/23 in this report. The CYP3A1 and CYP3A2 standard proteins were purchased from Daiichi Pure Chemicals (Tokyo, Japan). All other chemicals were of the finest grade available.

Animals

Male Wistar rats (250–300 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan). All animal experiments were performed using in accordance with the guidelines for animal experiments of Toyama Medical and Pharmaceutical University.

Intestinal Drug Metabolism

The intestinal metabolism of lidocaine, quinidine, nifedipine, and rifabutin in rats was examined as described previously (12). Each compound was used at a concentration of 2 μM to evaluate the intrinsic intestinal metabolic rate. It was incubated with excised intestinal tissues in an isotonic sodium phosphate buffer (pH 7.4) containing 0.3% glucose. After a 5-min pre-incubation period, the metabolic reaction was started by adding 1 mL of the drug solution to the incubation mixture. The incubation was performed for 60 min at 37°C, and then the incubation mixture was collected to determine the drug concentration. The intestinal tissues were retrieved, weighed and homogenized with 9 volumes of the phosphate buffer, and the amount of drug in the tissues was determined. The intestinal metabolic rate was calculated from the drug recovery after the incubation.

Hepatic Drug Metabolism

The hepatic drug metabolism in rats was evaluated with liver microsomes prepared conventionally by differential cen-

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trifugation. The incubation was carried out in an isotonic phosphate buffer (pH 7.4) with 25–800 μg of microsomal protein. The final drug concentration was set at 2 μM to evaluate the intrinsic hepatic metabolic rate. After a 5-min preincubation period, the metabolic reaction was initiated by adding a NADPH solution to the incubation mixture. The NADPH concentration was 1 mM. The incubation was conducted for 10–20 min at 37°C, during which time each compound was metabolized by about 10% of the initial amount applied. The metabolic reaction was terminated by adding 1 mL of 1 N NaOH, and the amount of drug in the incubation mixture was determined. The metabolic rate was calculated from the drug recovery after the incubation.

To evaluate the contribution of the CYP1A, 2C, 2D, and 3A subfamily to the hepatic metabolism of lidocaine, we tried to inhibit the metabolism in rat liver microsomes with theophylline, tolbutamide, desipramine, ketoconazole, or erythromycin. These compounds were used at a concentration of 500 μM. The lidocaine concentration was set at 500 μM to measure the rate at which MEGX formed as well as lidocaine disappeared.

Analytical Method

Lidocaine, MEGX, quinidine, nifedipine, and rifabutin were measured by high-performance liquid chromatography with an octadecyl silica column (3-μM particle, 4.5 mm × 150 mm) at a wavelength of 254 nm or 280 nm. Diethyl ether (5 mL) was added to the alkalized incubation mixture or tissue homogenate for extraction. Then, the organic phase was collected and evaporated to dryness. The residue was dissolved and subjected to high-performance liquid chromatography. The mobile phase was prepared with 10 mM phosphate buffer (pH 2.5) and 20–65% methanol.

Immunoblotting Analysis

The expression of CYP3A1/23 and CYP3A2 in intestinal and hepatic microsomes was evaluated by Western blot analysis. The intestinal microsomes were obtained as follows. The excised intestinal tissue described above was immediately soaked in ice-cold buffer, containing phenylmethylsulfonyl fluoride (40 μg/mL). Then, it was cut lengthwise and food debris was gently washed off. The tissue was flattened on an ice-cold petri dish, and the mucosal layer was stripped off with plate glass. The mucosa was collected and homogenized by a Teflon-potter homogenizer, and the homogenate was centrifuged at 9,000 *g* for 30 min. Then, the supernatant was centrifuged at 100,000*g* for 60 min. The pellet was collected, and the obtained intestinal microsomes were suspended in distilled water. The liver microsomes were prepared as described above. These microsomal proteins (2–20 μg) were resolved by sodium dodecyl sulfate -polyacrylamide (10%) gel electrophoresis, and transferred to a nitrocellulose membrane. The membrane was incubated with the antibody, and the migration pattern was visualized with a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA).

Data Analysis

Data are shown as the mean ± SE. Significant differences were evaluated by analysis of variance followed by Dunnett's test.

RESULTS AND DISCUSSION

The correlation between the intestinal and hepatic metabolic rates of four therapeutic compounds, all of which are metabolized primarily by CYP3A4 in humans, was investigated in rats. As shown in Fig. 1, rifabutin was metabolized the fastest in excised intestinal tissues, followed by nifedipine, quinidine, and lidocaine. By contrast, lidocaine was metabolized the fastest, but rifabutin was metabolized the slowest, in liver microsomes. It was obvious that there was no positive correlation between the intestinal and hepatic metabolism of CYP3A4 substrates in rats (Fig. 1), indicating that the drug metabolic rates in rat intestine were not related proportionally to those in rat liver. Subsequent experiments were conducted to clarify the mechanisms responsible for the poor correlation of drug metabolism in rats.

We first hypothesized that not all CYP3A4 substrates in humans are metabolized by the CYP3A subfamily in rats and that some are metabolized in rat liver by others in the cytochrome P450 subfamily, which are little expressed in rat intestine. In fact, it was known that CYP2D3 participates in lidocaine N-deethylation in rats (13), although CYP2D6 is scarcely involved in lidocaine metabolism in humans (7). Therefore, to evaluate the contribution of the CYP1A, 2C, 2D, and 3A subfamilies to the metabolism of lidocaine in rat liver, we measured the metabolic rates of lidocaine in rat liver microsomes with or without various substrates/inhibitors of cytochrome P450 enzymes. As shown in Fig. 2, theophylline and tolbutamide, which are reported to be metabolized in humans by the CYP1A and the CYP2C subfamily respectively, seemed to have no inhibitory effects. Desipramine, which is metabolized by CYP2D6 in humans, suppressed substantially both the disappearance of lidocaine and the formation of MEGX, which is the primary lidocaine metabolite in humans. However, ketoconazole, which is known to inhibit

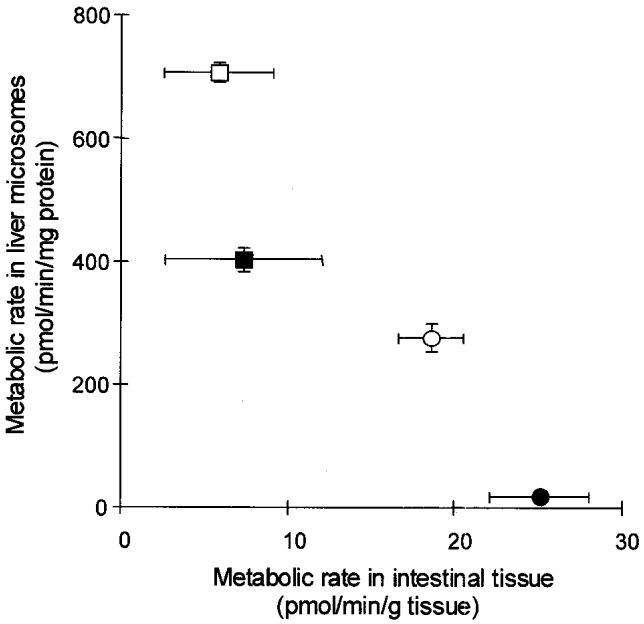

Fig. 1. Relationship between intestinal and hepatic metabolic rates of four CYP3A4 substrates in rats. Data are shown as the mean ± SE for three to six experiments. Keys: (□); lidocaine, (■); quinidine, (○); nifedipine, (●); rifabutin.

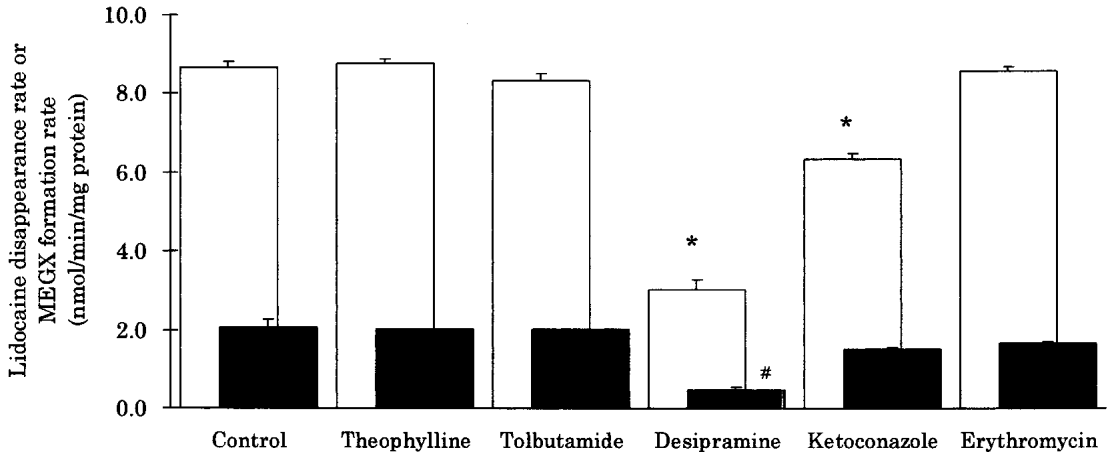

Fig. 2. Inhibitory effects of various compounds on lidocaine elimination (open columns) and monoethylglycinexylidide formation (closed columns) in rat liver microsomes. Data are shown as the mean ± SE for three to four experiments. *$p < 0.01$, #$p < 0.01$; significantly different from the corresponding control group.

drug metabolism mediated by the CYP3A subfamily in rats as well as in humans (14), reduced the rate of disappearance of lidocaine slightly but did not affect the rate of formation of MEGX. A CYP3A4 substrate erythromycin showed no inhibitory effects on the lidocaine metabolism. These results indicated that the CYP2D subfamily is largely involved in the lidocaine metabolism in rat liver. In addition, in conjunction with these results, Fig. 1 suggested that members of the CYP2D subfamily are expressed little in rat intestine. In fact, we have demonstrated that a CYP2D substrate ajmaline that undergoes extensive hepatic metabolism was not metabolized in rat intestine (12).

It was clearly demonstrated that rifabutin was metabolized much faster in intestinal microsomes than liver microsomes in rats (5), and our results on the rifabutin metabolism were consistent with this report. As for the rifabutin metabolism in intestine, a contribution of cytosolic enzyme may not be excluded (10). It is likely that some CYP3A4 substrates are metabolized by another member of the CYP3A subfamily, which is expressed in the intestine much more than in liver (5). It is known that CYP3A1 and CYP3A2 are expressed dominantly in rat liver. However, their expression in rat intestine is still controversial. Recently, it was reported that a novel member of the CYP3A subfamily resembling CYP3A9 is expressed in rat intestine (15). Therefore, we examined the expression of CYP3A1/23 and CYP3A2 in rat intestine and liver by Western blot analysis. In this study, we used polyclonal antibodies against CYP3A1 and CYP3A2, which distinguish between the two based on the amino acid sequence at their C-terminus (11). Because there is a considerable difference between CYP3A1/23 and CYP3A2 in their C-termini (Ile-Ile-Thr-Gly-Ser-COOH in CYP3A1/23 vs. Val-Ile-Asn-Gly-Ala-COOH in CYP3A2), it was expected that the polyclonal antibody against CYP3A1 would distinguish clearly CYP3A1/23 from CYP3A2 and *vice versa*, though slight cross-reactivity might be observed. In addition, since the C-terminus of CYP3A9 (Thr-Val-Asn-Gly-Ala-COOH) is similar to that of CYP3A2, the polyclonal antibody against CYP3A2 was expected to cross-react with CYP3A9 or another unidentified member of the rat CYP3A subfamily resembling CYP3A9.

The intestinal and hepatic microsomal proteins were resolved by SDS-polyacrylamide gel electrophoresis, and then they were visualized with the anti-CYP3A1 antibody (Fig. 3A–C) or the anti-CYP3A2 antibody (Fig. 3D–F). As shown in Fig. 3A–C, CYP3A1/23 was expressed in rat liver but not in rat intestine. The cross-reactivity of the anti-CYP3A1 antibody with CYP3A2 was shown to be negligible. By contrast, as shown in Fig. 3D–F, the cross-reactivity of the anti-CYP3A2 antibody with CYP3A1 was detectable, and the standard protein for CYP3A1 was immunostained and visualized in all three panels. Both CYP3A1/23 and CYP3A2 were clearly expressed in rat liver, with CYP3A1/23 migrating slower than CYP3A2. As for the intestinal expression of the CYP3A sub-

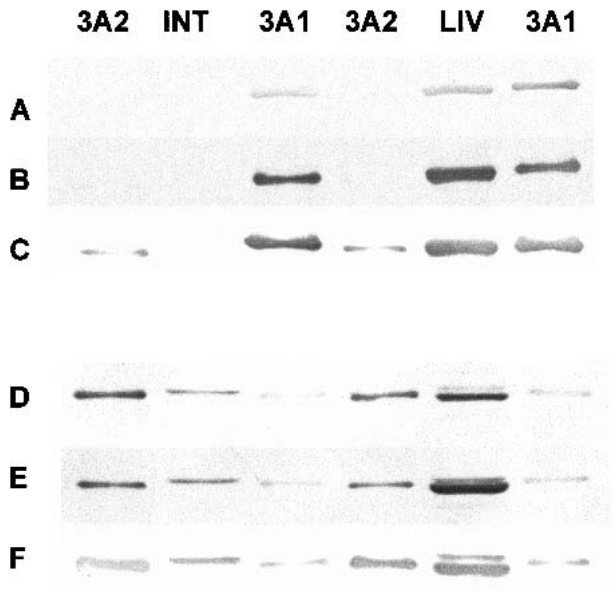

Fig. 3. Evaluation of the expression of the CYP3A subfamily in rat liver and intestine. The protein was detected by the antibody against CYP3A1 in A, B, and C, and by the antibody against CYP3A2 in D, E, and F. After samples were applied in each panel: A and D, CYP3A1 standard (3A1); 50 fmol, CYP3A2 standard (3A2); 50 fmol, the intestinal microsome (INT); 10 μg protein, the liver microsomes (LIV); 2 μg protein. B and E, 3A1; 50 fmol, 3A2; 50 fmol, INT; 10 μg, LIV; 5 μg. In panels C and F; 3A1;100 fmol, 3A2; 100 fmol, INT; 20 μg, LIV; 5 μg. Proteins migrated downward in all panels.

family, we obtained a band signal in rat intestinal microsomes (Fig. 3D–F). This band signal differed slightly from the band signal of CYP3A2 in migration distance. In addition, it was obvious that the anti-CYP3A2 antibody cross-reacted more with this unidentified protein than with CYP3A1. These findings indicated that neither CYP3A1/23 nor CYP3A2 was expressed in rat intestine, and suggested that CYP3A9 or another member of the CYP3A subfamily resembling CYP3A9 was expressed in rat intestine. Expressing a different member of the CYP3A subfamily, the rat intestine may play an individual role in metabolizing xenobiotics, and may contribute to first pass metabolism in a manner different from liver.

In conclusion, we examined the metabolism of CYP3A4 substrates in rats with excised intestinal tissues and liver microsomes. It was shown that metabolic rates in intestine poorly correlated with those in liver. A reason for this poor correlation is probably that the contribution of the CYP3A subfamily to the metabolisms of CYP3A4 substrates is not always significant in rat liver. In addition, we demonstrated that a member of the CYP3A subfamily expressed in rat intestine is different from those expressed in rat liver. The difference in enzyme expression seems to be another reason for the poor correlation in the metabolism of CYP3A4 substrates in rats. Our findings will provide useful information to use animal models for the evaluation of first-pass metabolism of CYP3A4 substrates.

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