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Characterization of cytochrome P450 enzymes involved in drug oxidations in mouse intestinal microsomes

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1. Cytochrome P450 (P450, CYP) enzymes involved in drug oxidations in mouse intestines were characterized for their role in the first-pass metabolism of xenobiotics.

2. Preparation of mouse intestinal microsomes using a buffer containing glycerol and protease inhibitors including (*p*-amidinophenyl) methanesulphonyl fluoride, EDTA, soybean trypsin inhibitor, aprotinin, bestatin and leupeptine gave the highest testosterone 6β-hydroxylase activity among several preparation buffers tested in this study. Testosterone 6β-hydroxylase activity catalysed by mouse intestinal microsomes subjected to freezing and thawing was lower than that catalysed by unfrozen intestinal microsomes.

3. Low but significant catalytic activities of nifedipine oxidation, midazolam 1′- and 4-hydroxylation, chlorzoxazone 6-hydroxylation, bufuralol 1′- and 6-hydroxylations and tolbutamide methylhydroxylation were observed in mouse intestinal microsomes. Testosterone 6β-hydroxylation, chlorzoxazone 6-hydroxylation, and bufuralol 1′- and 6-hydroxylations were inhibited by ketoconazole, diethyldithiocarbamate and quinine respectively.

4. Immunoblot analysis using anti-rat CYP3A antibodies demonstrated two immunoreactive bands showing similar migration in mouse intestinal and hepatic microsomes, although studies using anti-CYP1A, anti-CYP2C, anti-CYP2D and anti-CYP2E1 antibodies did not detect any band in mouse intestinal microsomes.

5. The results suggest that mouse intestinal microsomes should be prepared with glycerol and several protease inhibitors and that Cyp3a enzymes probably play an important role in drug oxidations catalysed by mouse intestine.

Introduction

Cytochrome P450 (P450, CYP) consists of a superfamily of haem-containing monooxygenases associated with the metabolism of substrates, such as drugs, environmental pollutants and endogenous substrates with broad overlapping specificities (Guengerich 1991). P450s are microsomal enzymes that exist mainly in liver as well as in extrahepatic tissues such as intestines, lungs and kidneys (Krishna and Klotz 1994). Recently, the intestine has been recognized as a potential site for drug oxidations because of early exposure to orally administered xenobiotics. The upper part of the small intestine serves as the major site for intestinal CYP3A-mediated first-pass metabolism in man (Paine *et al*. 1997). There have been reports on the metabolism of midazolam by CYP3A4 in human intestine (Paine *et al*. 1996, 1997). The CYP3A subfamily accounts for 30% of the total P450 content in the adult human liver and for the majority of P450 in the human intestine (Watkins *et al*.

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1987, Shimada *et al.* 1994). CYP3A enzymes are known to have a broad substrate specificity and about half the drugs on the market are substrates for CYP3A4 (Guengerich 1995).

Recently, considerable progress has been made in the identification and characterization of intestinal P450 enzymes. Several species of experimental animals have been used in early toxicology and metabolism studies by the pharmaceutical industry. Intestinal microsomes from experimental animals and humans have been prepared by various methods in different laboratories. In rat and non-rodent species including dog and monkey, characterization of small intestinal P450s has been reported (Prueksaritanont *et al.* 1996, Zhang *et al.* 1996). However, few studies have been conducted so far in mouse. Furthermore, little attention has been paid to the use of protease inhibitors and glycerol in the preparation of intestinal microsomes. It should also be mentioned that little is known about changes in the stability of intestinal microsomes by freezing and thawing, in contrast to a large number of studies on human liver microsomes (Pearce *et al.* 1996, Yamazaki *et al.* 1997). Therefore, it was planned to investigate the methods for preparation of mouse intestinal microsomes and to characterize the intestinal drug-metabolizing enzymes.

In this study, methods for preparing intestinal microsomes from the upper part of the mouse small intestine, particularly the effects of protease inhibitors and glycerol, were investigated. The oxidation of testosterone, nifedipine, midazolam, chlorzoxazone, bufuralol and tolbutamide were measured in mouse intestinal microsomes and the inhibitory effects of P450 inhibitors were examined. Immunochemical detection of Cyp3a enzymes in mouse intestinal microsomes was also performed.

Materials and methods

Chemicals

Testosterone and 6β-hydroxytestosterone were from Steraloids (Wilton, NH, USA). Midazolam and 1′- and 4-hydroxymidazolam were kindly provided by Yamanouchi Pharmaceuticals (Tokyo, Japan). Chlorzoxazone, aprotinin, bestatin, leupeptin and trypsin inhibitor (Type II-S: soybean) were from Sigma (St Louis, MO, USA). (±)-Bufuralol, tolbutamide and their metabolites and 6-hydroxychlorzoxazone were from Ultrafine Chemicals (Manchester, UK). (*p*-Amidinophenyl) methanesulphonyl fluoride (APMSF) was from Wako Pure Chemicals (Osaka, Japan). Other reagents were of the highest grade commercially available.

Preparation of intestinal and hepatic microsomes

Male C57BL/6CrSlc mice (8 weeks old, ~ 30 g) were from Japan SLC, Inc. (Hamamatsu, Japan). Microsomes from mouse small intestines were prepared according to Iatsimirskaia *et al.* (1997) with slight modifications. Immediately after excision of the small intestine, a 10-cm segment of the upper part of the small intestine was placed on an ice-cold stainless dish and cut longitudinally, then washed with ice-cold washing solution (0.9% NaCl, 1 mM APMSF, 1 mM EDTA) by gentle swirling for 1 min. The mucosal cells were gently scraped off with a micro-cover glass. The scraped sample was dipped in 3 vols ice-cold buffer A (50 mM Tris-HCl buffer, pH 7.4) containing 150 mM KCl, 1 mM APMSF, 1 mM EDTA, 1 mg/ml trypsin inhibitor, 10 μM leupeptin, 0.04 unit/ml aprotinin, 1 μM bestatin, and 20% (v/v) glycerol, and was homogenized using a motor-driven Teflon-tipped pestle (three strokes). The homogenates were centrifuged at 9000 *g* for 20 min at 4 °C and the resultant supernatant centrifuged at 105000 *g* for 60 min at 4 °C. The fatty layer on the surface was removed carefully and the microsomal pellets were resuspended in ice-cold buffer A.

To investigate the effects of protease inhibitors and glycerol, the following buffers were used instead of buffer A: B, Tris-HCl buffer (pH 7.4) containing KCl, APMSF, EDTA, trypsin inhibitor, aprotinin, bestatin and leupeptin; C, Tris-HCl buffer (pH 7.4) containing KCl, glycerol, APMSF, EDTA and trypsin inhibitor; D, Tris-HCl buffer (pH 7.4) containing KCl, glycerol, APMSF and EDTA at the above concentrations.

Hepatic microsomes were prepared by the same method above except for the use of 0.9% NaCl instead of the washing solution described above.

The effects of freezing and thawing on testosterone 6β-hydroxylase activities in mouse intestinal and hepatic microsomes were examined with microsomes frozen in liquid nitrogen and thawed in water at room temperature within 1 day.

Protein concentrations were determined according to Lowry *et al.* (1951) using bovine serum albumin as the standard.

Enzyme assays

Testosterone 6β-hydroxylation, nifedipine oxidation, midazolam 1′- and 4-hydroxylation, bufuralol 1′- and 6-hydroxylation, tolbutamide methylhydroxylation, and chlorzoxazone 6-hydroxylation were determined as described (Kronbach *et al.* 1987, 1989, Brian *et al.* 1989, Peter *et al.* 1990, Yamazaki *et al.* 1994, Yamazaki and Shimada 1997). The standard incubation mixture (final volume 0.25 ml) contained 0.3 mg protein mouse intestinal microsomes, 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system consisting of 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, 0.5 unit/ml glucose 6-phosphate dehydrogenase and 100 μM substrates except for tolbutamide methylhydroxylation. The incubation mixture for tolbutamide methylhydroxylation contained 0.3 mg protein mouse intestinal microsomes, 50 mM potassium phosphate buffer (pH 7.4), the NADPH-generating system and 250 μM tolbutamide. In the case of hepatic microsomes, 0.1 mg mouse hepatic microsomes was used. The reaction mixtures were incubated at 37 °C for 10 min for testosterone 6β-hydroxylation, nifedipine oxidation, midazolam 1′- and 4-hydroxylation, and chlorzoxazone 6-hydroxylation and for 15 min for bufuralol hydroxylation and tolbutamide methylhydroxylation. The reactions with testosterone, nifedipine, chlorzoxazone and tolbutamide were terminated by the addition of 1.5 ml dichloromethane and 0.3 M NaCl for testosterone 6β-hydroxylation, 0.2 M NaCl–0.1 M sodium carbonate for nifedipine oxidation, 4.3% phosphoric acid for chlorzoxazone hydroxylation, and 0.1 N HCl for tolbutamide methylhydroxylation. After mixing vigorously and centrifugation at 900 *g* for 10 min, the organic phase was evaporated at 40 °C under a gentle nitrogen stream. The residue was dissolved in 200 μl HPLC mobile phase, and a 100-μl portion was subjected to HPLC. The reactions of midazolam and bufuralol were terminated by the addition of 0.25 ml methanol and 10 μl 60% percloric acid respectively. After mixing vigorously, the mixtures were centrifuged at 900 *g* for 10 min. The supernatants (100 μl) were subjected to HPLC analysis.

The substrates and P450 inhibitors (dissolved in methanol) were added simultaneously to the microsomal preparations. The final concentration of solvent in the incubation mixture was $< 1\%$.

HPLC analyses

HPLC analyses were performed using an LC-6A pump, SIL-6B autosampler, SPD-6A UV detector (all Shimadzu, Kyoto, Japan), FP-920 II fluorescence detector (Jasco, Tokyo, Japan) and C-R4A integrator (Shimadzu) equipped with a C_{18} 5-μm analytical column (Mightysil, 150 × 4.6 mm i.d.; Kanto Chemicals, Tokyo, Japan) except for the case of chlorzoxazone hydroxylation (C_8 5-μm analytical column, Mightysil, 150 × 4.6 mm i.d.; Kanto). The column temperature was set at 35 °C in a CTO-6A column oven (Shimadzu). The elution of both testosterone and nifedipine metabolites was conducted with a 64% CH_3OH (v/v) at 1 (testosterone) or 1.5 ml/min (nifedipine). The eluate was monitored at 240 and 254 nm respectively. The elution of midazolam metabolites was conducted with 24% methanol/33% acetonitrile/43% 10 mM potassium phosphate buffer (pH 7.4) (v/v) at 1 ml/min. The eluate was monitored at 220 nm. The elution of bufuralol metabolites was conducted with 33% acetonitrile/67% 20 mM sodium perchloric acid (pH 2.5) (v/v) at 1.5 ml/min. The eluate was monitored fluorometrically (excitation 252 nm, emission 302 nm). The elution of tolbutamide metabolites was conducted with 30% acetonitrile containing 0.04% phosphoric acid (v/v) at 1.5 ml/min. The eluate was monitored at 230 nm. The elution of chlorzoxazone metabolites was conducted with a mixture of 27% acetonitrile containing 0.5% phosphoric acid (v/v) at 1.5 ml/min. The eluate was monitored at 287 nm.

Immunoblot analyses

Polyclonal goat anti-rat CYP1A1, CYP2C6, CYP2E1 and CYP3A2 antisera were from Daiichi Pure Chemicals (Tokyo, Japan). Polyclonal rabbit anti-rat CYP2D1 antiserum was from Funakoshi (Tokyo, Japan). Immunoblot analysis was performed according to Laemmli (1970) with slight modifications. The microsomal proteins were separated on 7.5% polyacrylamide gel electrophoresis and transferred electrophoretically to a nitrocellulose membrane. The secondary antibodies used were anti-goat IgG (anti-rabbit IgG for CYP2D1) and detection was carried out with diaminobenzidine staining.

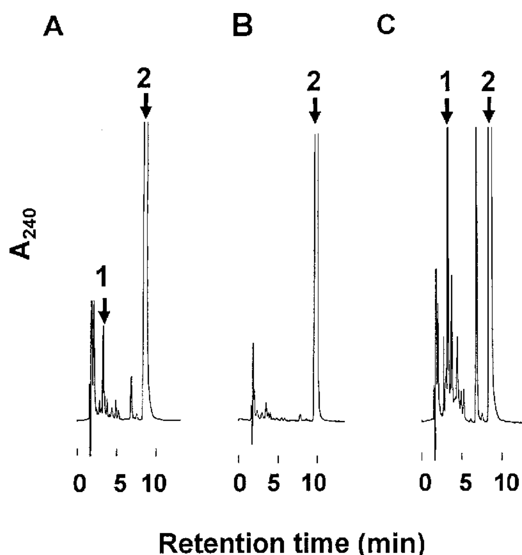

Figure 1. Representative HPLC chromatograms of testosterone metabolites in mouse intestinal and hepatic microsomes. (A) Mouse intestinal microsomes prepared with buffer A; (B) mouse intestinal microsomes prepared with buffer A excluding protease inhibitors and glycerol; (C) mouse hepatic microsomes prepared with buffer A. Peak: 1, 6β-hydroxytestosterone; 2, testosterone. Precision was < 10% and inaccuracy was < 10% with control samples containing 50 and 250 pmol 6β-hydroxytestosterone (n = 5). Detection limit was < 1 pmol/min/mg intestinal microsomal protein.

Statistical analysis

Statistical analysis was performed by a Student's t-test using an InStat computer program (Graphpad Software, San Diego, CA, USA).

Results

Effects of protease inhibitors and glycerol in the preparation buffers and of freezing and thawing on testosterone 6β-hydroxylase activity in intestinal and hepatic microsomes from the mouse

Testosterone 6β-hydroxylase activity was determined in mouse intestinal and hepatic microsomes (figure 1). 6β-Hydroxytestosterone was observed when intestinal microsomes prepared in buffer A were used as an enzyme source (figure 1A). However, when glycerol and these protease inhibitors were omitted from buffer A, testosterone 6β-hydroxylase activity could not be detected (figure 1B). On the other hand, the formation of 6β-hydroxytestosterone catalysed by mouse hepatic microsomes was similar in these two preparation buffers.

The effects of combinations of protease inhibitors and of glycerol in buffer A on testosterone 6β-hydroxylase activity of mouse intestinal and hepatic microsomes were investigated (figure 2). Intestinal microsomes from mouse prepared with buffer A gave the highest activities among those prepared with the buffer systems used in this study (figure 2(1A)). Therefore, intestinal microsomes from mouse were prepared using buffer A thereafter and used as an enzyme source. The effects of freezing and thawing of mouse intestinal microsomes on testosterone 6β-hydroxylase activities were investigated. Testosterone 6β-hydroxylase activities of mouse

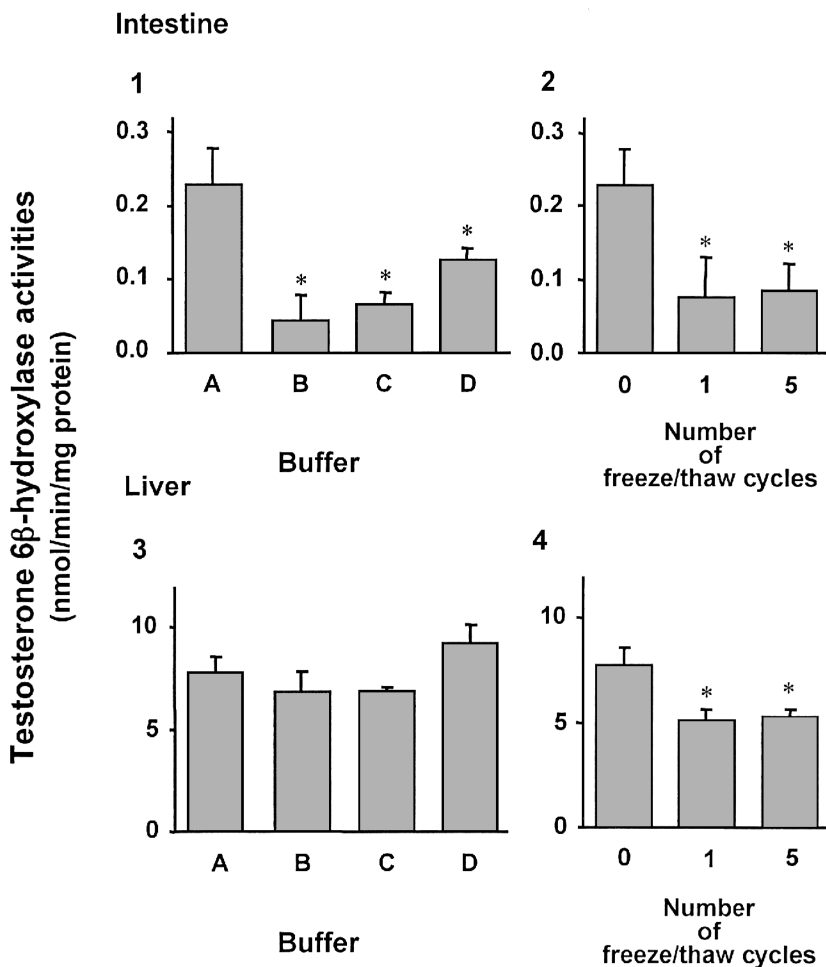

Figure 2. Effects of protease inhibitors and glycerol in preparations of microsomes and of freezing and thawing of microsomes on the testosterone 6β-hydroxylase activity. Intestinal (1) and hepatic (3) microsomes from mice were prepared using buffers A–D and catalytic activities determined in an unfrozen condition according to the method described in the Materials and methods. Both intestinal (2) and hepatic (4) microsomes prepared with buffer A were subjected to one or five cycles of freezing and thawing. * Significantly different from activities of unfrozen intestinal (1 and 2) and hepatic microsomes (3 and 4) prepared with buffer A from mice ($p < 0.05$). Each column represents the mean ± SD from three mice.

intestinal microsomes were decreased to 30% by only one cycle of freezing and thawing (figure 2(2)). Five cycles of freezing and thawing did not further affect the activities decreased by one cycle.

In contrast, the effects of combinations of protease inhibitors and of glycerol on the testosterone 6β-hydroxylase activities of hepatic microsomes were not significant (figure 2(3)). One cycle of freezing and thawing resulted in a 40% decrease of the catalytic activities in mouse hepatic microsomes (figure 2(4)). The degree of decrease of testosterone 6β-hydroxylase activities in mouse hepatic microsomes by freezing and thawing was smaller than that of mouse intestinal microsomes.

Table 1. Drug oxidation activities in intestinal and hepatic microsomes from mouse.

Drug oxidation	Microsomes: Intestine (pmol/min/mg protein)	Microsomes: Liver (pmol/min/mg protein)
Testosterone 6β-hydroxylation	230 ± 50	7800 ± 51
Nifedipine oxidation	88 ± 51	1400 ± 140
Midazolam 1′-hydroxylation	24 ± 5	260 ± 28
Midazolam 4-hydroxylation	21 ± 5	240 ± 23
Chlorzoxazone 6-hydroxylation	67 ± 54	4100 ± 910
Bufuralol 1′-hydroxylation	55 ± 11	3200 ± 220
Bufuralol 6-hydroxylation	0.3 ± 0.1	8.5 ± 0.7
Tolbutamide methylhydroxylation	8 ± 3	670 ± 240

Microsomes were prepared with buffer A and were used for assays without freezing.
Data are the mean ± SD from three mice.

Comparison of drug oxidation activities in intestinal and hepatic microsomes from the mouse

Drug oxidation activities of intestinal and hepatic microsomes from mouse are summarized in table 1. Low but significant activities of nifedipine oxidation, midazolam 1′- and 4-hydroxylation, chlorzoxazone 6-hydroxylation, bufuralol 1′- and 6-hydroxylation, and tolbutamide methylhydroxylation were observed in mouse intestinal microsomes. The maximal turnover number was observed for testosterone 6β-hydroxylation in both intestinal and hepatic microsomes. The ratios of midazolam 1′- and 4-hydroxylation activities in intestinal and hepatic microsomes from mouse were similar.

Effects of P450 inhibitors on oxidation activities in mouse intestinal and hepatic microsomes

The effects of P450 inhibitors on testosterone 6β-hydroxylase, chlorzoxazone 6-hydroxylase, and bufuralol 1′- and 6-hydroxylase activities in intestinal and hepatic microsomes from mouse were investigated (figure 3). Testosterone 6β-hydroxylase activities in both intestinal and hepatic microsomes were extensively inhibited by ketoconazole (figure 3A) and were similar in intestinal microsomes freshly prepared and subjected once to freezing (data not shown). Chlorzoxazone 6-hydroxylase activities in intestinal and hepatic microsomes were inhibited to 68 and 58% respectively by diethyldithiocarbamate (figure 3B). Bufuralol 1′-hydroxylase activities in both intestinal and hepatic microsomes were extensively inhibited by quinine rather than by quinidine (figure 3C). Quinine, quinidine and α-naphthoflavone inhibited bufuralol 6-hydroxylase activities in intestinal microsomes in a similar manner (figure 3D). Bufuralol 6-hydroxylase activity in hepatic microsomes was inhibited to 16% by α-naphthoflavone.

Immunoblot analyses of mouse P450s in intestinal microsomes

Two immunoreactive bands could be observed in intestinal and hepatic microsomes from mouse, showing similar migration detected by anti-rat CYP3A2

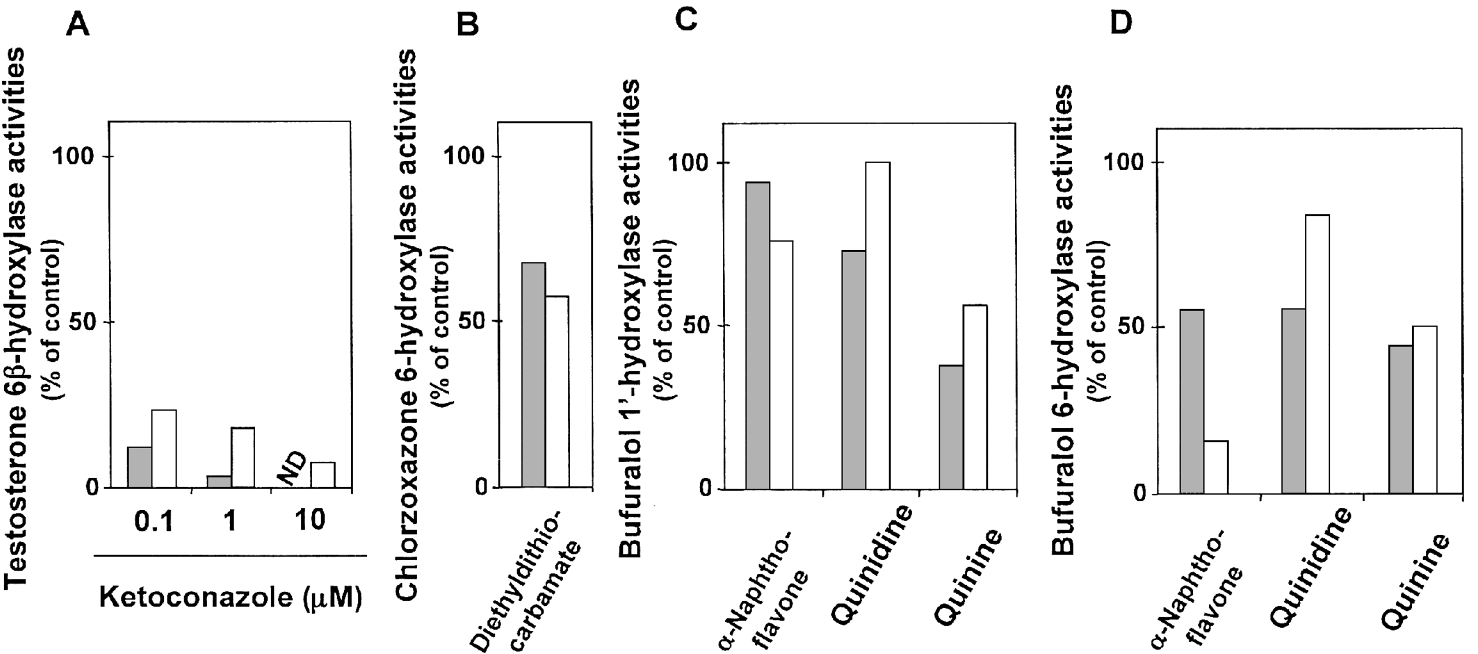

Figure 3. Effects of P450 inhibitors on oxidation activities in intestinal and hepatic microsomes from mice. The concentration of substrates and inhibitors was 0.1 mM except that of ketoconazole (as indicated). Drug oxidation activities in intestinal (close column) and hepatic (open column) microsomes subjected once to freezing were measured. (A) Control activity (without inhibitors) of testosterone 6β-hydroxylation by intestinal and hepatic microsomes was 131 and 7040 pmol/min/mg protein respectively. (B) Control activity of chlorzoxazone 6-hydroxylation by intestinal and hepatic microsomes was 48 and 3080 pmol/min/mg protein respectively. (C) Control activity of bufuralol 1′-hydroxylation by intestinal and hepatic microsomes was 43 and 2950 pmol/min/mg protein respectively. (D), Control activity of bufuralol 6-hydroxylation by intestinal and hepatic microsomes was 0.19 and 7.8 pmol/min/mg protein respectively. Each column represents the average of duplicate determinations. ND, not detected.

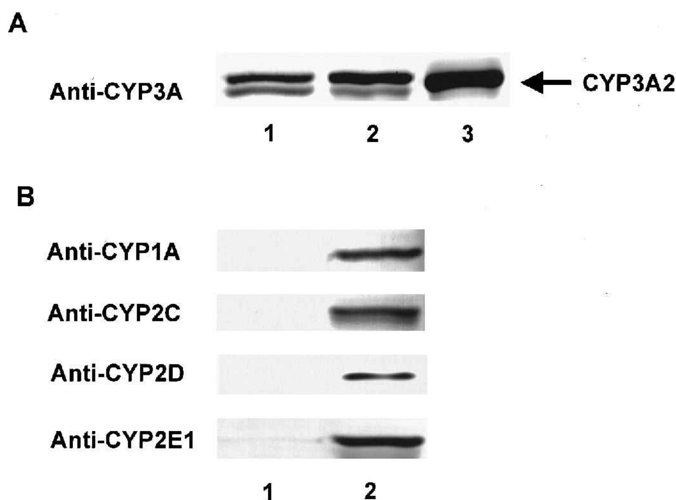

Figure 4. Immunoblot analyses of mouse intestinal and hepatic microsomes. (A) Immunoblot analyses of mouse intestinal and hepatic microsomes were performed using anti-rat CYP3A2 antibodies. Lanes 1, intestinal microsomes (30 μg) from upper part of mouse small intestine; 2, mouse hepatic microsomes (10 μg); 3, rat hepatic microsomes for standard (corresponding to 0.23 pmol CYP3A2). (B) Immunoblot analyses of mouse intestinal and hepatic microsomes were performed using anti-rat CYP1A1, anti-rat CYP2C6, anti-rat CYP2D1 and anti-rat CYP2E1 antibodies. Lanes 1 and 2 were the same as those in (A).

antibodies (Figure 4A). Rat hepatic microsomes showed a single band. Compared with intestinal samples of mouse, the density of the upper band in hepatic microsomes was higher than the lower band. On the other hand, anti-rat CYP1A1, anti-rat CYP2C6, anti-rat CYP2D1 and anti-rat CYP2E1 recognized mouse hepatic P450 enzymes; however, no immunoreactive bands in mouse intestinal microsomes could be detected (figure 4B).

Discussion

In general, the preparation of intestinal microsomes is relatively difficult compared with hepatic microsomes because intestinal microsomes are exposed to an abundance of proteases during preparation. In the preparation of intestinal microsomes from human, monkey and rodent, different laboratories have used different combinations of protease inhibitors. One popular combination of protease inhibitors is phenyl methanesulphonyl fluoride (PMSF) and EDTA, and they have been occasionally used in combination with a trypsin inhibitor or leupeptine (Rosenberg and Mankowski 1994, Kashfi *et al.* 1995). Shimizu *et al.* (1998) reported that PMSF was effective in the preparation of intestinal microsomes from human, although PMSF was not effective in those from monkey. Komura *et al.* (1998) employed a combination of a trypsin inhibitor and EDTA without PMSF in the preparation of intestinal microsomes from dog. In the preparation of recombinant human P450 enzymes from bacterial membranes, protease inhibitors such as PMSF, leupeptine, bestatin, aprotinin and EDTA have been used (Guengerich *et al.* 1998). Therefore, the methods for the preparation of intestinal microsomes from each type of animal should be considered carefully.

In the present study, combinations of protease inhibitors as well as glycerol (buffer A) were necessary to obtain good testosterone 6β-hydroxylase activities in mouse intestinal microsomes (figure 2(1)). In our preliminary experiments, testosterone 6β-hydroxylase activities in intestinal microsomes based on total protein were 4.1-fold higher than those of 9000 *g* supernatant of intestine, although the catalytic activities of 9000 *g* supernatant from mouse intestines were not dependent on the constituents of the preparation buffer (data not shown). These results suggested that P450 enzymes in intestinal microsomes would lose the activities during the ultracentrifugation of 9000 *g* supernatant and/or resuspension process in the absence of protease inhibitors and glycerol. The preparation of intestinal microsomes without glycerol caused a significant loss of catalytic activities in an unfrozen condition compared with those prepared with glycerol in spite of the addition of protease inhibitors (figure 2(1)). Therefore, glycerol could play an important role in the preparation of catalytically active intestinal microsomes (figure 2(1)) in a similar manner as reported in liver microsomes (Guengerich 1994). Intestinal microsomes from rat prepared with buffer A showed lower testosterone 6β-hydroxylase activities (41 ± 9 pmol/min/mg protein, mean $\pm$ SD, $n = 3$) than those from mice in unfrozen condition (data not shown). These results also suggested that there would be species differences in the effects of protease inhibitors for the maintenance of catalytic activities in microsomal preparations.

Several drug oxidation activities were demonstrated in the upper part of small intestine from mouse (table 1). The inhibitory effects of typical P450 inhibitors on testosterone 6β-hydroxylase, chlorzoxazone 6-hydroxylase, and bufuralol 1′- and 6-hydroxylase activities in intestinal and hepatic microsomes from mouse were observed (figure 3). These drug oxidation activities in mouse intestine should be catalysed by P450 enzymes. However, immunoreactive bands of Cyp1a, Cyp2c, Cyp2d and Cyp2e1 enzymes were not detected in mouse intestine, and only Cyp3a proteins were clearly detected using anti-rat CYP3A2 antibodies (figure 4). These results suggested that catalytic analysis might have higher sensitivity than immunoblot analysis to characterize microsomal P450 enzymes in mouse intestine.

The density of two immunoreactive bands detected using anti-rat CYP3A2 antibodies was different in intestinal and hepatic microsomes (figure 4A). Sakuma *et al.* (2000) reported that both Cyp3a11 and Cyp3a13 were the major Cyp3a enzymes in mouse small intestine and liver. In livers of the male mouse, Cyp3a11 predominated over Cyp3a13 (Yanagimoto *et al.* 1997). The molecular weights of mouse Cyp3a11 (SwissProt accession no. Q64459), Cyp3a13 (Q64464) and rat CYP3A2 (P05183) have been reported to be 57.9, 57.5 and 57.7 kDa respectively (SWISS-PROT protein database). Although there was an organ difference with respect to the expression of Cyp3a enzymes, the upper and lower bands observed immunochemically are very likely Cyp3a11 and Cyp3a13 respectively.

In conclusion, proposed here is an appropriate method for the preparation of intestinal microsomes from mouse using glycerol and a combination of protease inhibitors. Freezing of mouse intestinal microsomes should be avoided as much as possible for the analysis of catalytic activities. The present authors are currently examining the metabolic profiles of drugs in the intestine using intestinal microsomes from other animals and humans.

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