ABSTRACT:

Diazepam was metabolized to three primary metabolites, 3-hydroxy-diazepam, N-desmethyl-diazepam, and p-hydroxy-diazepam. Our previous studies reported metabolic position-specific inter- or intrastrain differences in diazepam metabolism among Sprague-Dawley, Brown Norway, Dark Agouti, and Wistar rats. Especially, there were marked (~300 fold) inter- or intrastrain differences in diazepam p-hydroxylation activity at low concentration of substrate. In this study, we investigated the enzyme that catalyzes diazepam p-hydroxylation. The activity toward diazepam p-hydroxylation was inhibited by anti-cytochrome P450 2D (CYP2D) antibody, suggesting that this activity was catalyzed by CYP2D isoforms. Comparing the expression levels of the CYP2D subfamily in liver microsomes from various strains of rats using anti-CYP2D antibody, we found that there was a band of protein that was consistent with the phenotype of diazepam p-hydroxylation. N-terminal amino acid sequences of the specific protein exactly corresponded to those of CYP2D3, indicating that CYP2D3 might be involved in diazepam p-hydroxylation. Moreover, using rat CYP2D isoforms expressed in yeast, we tested CYP2D3s to catalyze diazepam p-hydroxylation. CYP2D1 and CYP2D2 practically did not participate in diazepam metabolism. On the other hand, diazepam p-hydroxylation was catalyzed by CYP2D3. CYP2D4 had high activity toward diazepam N-desmethylation, but not p-hydroxylation. In conclusion, the polymorphic expression of CYP2D3 caused the inter- or intrastrain differences in diazepam p-hydroxylation among rat strains or individuals.

IMPACTANCE OF CYP2D3 IN POLYMORPHISM OF DIAZEPAM P-HYDROXYLATION IN RATS

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Diazepam is one of the benzodiazepines that are widely used in the treatment of anxiety disorders, depression, and insomnia. Neville et al. (1993) reported that diazepam was metabolized to three primary metabolites, 3-hydroxy-diazepam, N-desmethyl-diazepam, and p-hydroxy-diazepam, in liver microsomes of adult male Wistar rats. These metabolic pathways were reported to be catalyzed by isoforms of cytochrome P450 (P450), CYP3A2, CYP2C11, and CYP2D1, respectively (Fig. 1). We found the existence of extensive metabolizers (EMs) and poor metabolizers (PMs) of diazepam metabolism in liver microsomes from Wistar rats at low concentration of substrate (Saito et al., 2004a). EMs from Wistar rats (EM-W) had markedly higher activity toward diazepam p-hydroxylation than did PMs from Wistar rats (PM-W). In addition, we recently reported strain differences in diazepam p-hydroxylation in SD, BN, and DA rats (Saito et al., 2004b). SD and BN rats had 300-fold higher diazepam p-hydroxylation activity than DA rats at a low concentration of substrate (Table 1). As a result of diazepam p-hydroxylation activity, the major metabolic pathways of diazepam differed among rat strains. Thus, we classified SD, BN, and EM-W rats as extensive metabolizers (pEM), and DA and PM-W rats as poor metabolizers (pPM), respectively, in diazepam p-hydroxylation. The reaction of p-hydroxylation was suggested to be catalyzed by CYP2D1 (Neville et al., 1993). However, we demonstrated that there was no significant difference in the expression levels of CYP2D1 in liver microsomes between pEM and pPM (Saito et al., 2004b). Moreover, the patterns of debrisoquine 4-hydroxylation activity, which is a CYP2D2-dependent activity, did not coincide with those of diazepam p-hydroxylation activity in the liver of four rat strains. Then, we concluded that diazepam p-hydroxylation was catalyzed by neither CYP2D1 nor CYP2D2, which contributed to the metabolism of debrisoquine, bunitrolol, and bufuralol (Boobis et al., 1986; Gonzalez et al., 1987; Suzuki et al., 1992; Yamamoto et al., 1996, 1998). The enzyme that catalyzes diazepam p-hydroxylation is still not clear.

It is reported that antibody against CYP2D1 or CYP2D2 isoforms inhibited the activity of diazepam p-hydroxylation in rat liver microsomes (Neville et al., 1993; Saito et al., 2004b). In total, six CYP2D genes are isolated in rats and identified, namely, CYP2D1, CYP2D2, CYP2D3, CYP2D4, CYP2D5, and CYP2D18. CYP2D18 is believed to be the rat brain variant of CYP2D4. Since the rat CYP2D subfamily conserves a high similarity of amino acid sequence (>70%) among its members, the antibody against the full length of CYP2D2 isomorph might inhibit drug-metabolizing activities catalyzed by any form of

ABBREVIATIONS: P450, cytochrome P450; SD, Sprague-Dawley; BN, Brown Norway; DA, Dark Agouti; EM, extensive metabolizer; PM, poor metabolizer; EM-W, EM from Wistar rats; PM-W, PM from Wistar rats; pEM, EM in diazepam p-hydroxylation; pPM, PM in diazepam p-hydroxylation; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.
Adult male SD, DA, and Wistar rats (9 weeks old) were obtained from Japan 1992; Nakamura et al., 1995; Yamamoto et al., 1996). Anti-CYP2D4 peptide analytical grade. purchased from Oriental Yeast Co. (Tokyo, Japan). The other reagents were of Glucose 6-phosphate, NADPH, and glucose-6-phosphate dehydrogenase were were gifts from Japan Hoffman La Roche Pharmaceutical Co. (Tokyo, Japan). CYP2D isoforms. Therefore, the polymorphism of diazepam ɛ-hydroxylation among rat strains was revealed to be due to the differences in expression levels of CYP2D3 in these strains of rats. In addition, this is the first report indicating that CYP2D3 has catalytic activity toward xenobiotic metabolism.

CYP2D without discrimination. In addition, antibodies generated against the purified P450 enzymes are often not specific. Despite the structural similarities among CYP2D isoforms, significant differences in the ability to metabolize drugs have been observed among these CYP2D isoforms (Wan et al., 1997; Chow et al., 1999; Hiroi et al., 2002).

In this study, we identified the enzyme involved in diazepam ɛ-hydroxylation to be CYP2D3 using yeast recombinant CYP2D isoforms. Therefore, the polymorphism of diazepam ɛ-hydroxylation among rat strains was revealed to be due to the differences in expression levels of CYP2D3 in these strains of rats. In addition, this is the first report indicating that CYP2D3 has catalytic activity toward xenobiotic metabolism.

**Materials and Methods**

**Materials and Animals.** Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one) and the internal standard, nitrazepam (1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one), were purchased from Wako Pure Chemicals (Osaka, Japan). The three primary diazepam metabolites, p-hydroxy-diazepam, 3-hydroxy-diazepam, and N-desmethyl-diazepam, were gifts from Japan Hoffman La Roche Pharmaceutical Co. (Tokyo, Japan). Glucose 6-phosphate, NADPH, and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co. (Tokyo, Japan). The other reagents were of analytical grade.

Anti-CYP2D2 antibody was prepared as described previously (Suzuki et al., 1992; Nakamura et al., 1995; Yamamoto et al., 1996). Anti-CYP2D4 peptide antibody was purchased from AFFINITI Research Products Ltd. (Exeter, UK).

**Preparation of Liver Microsomes.** Liver microsomes were prepared according to the method of Omura and Sato (1964). The samples were homogenized with 3 volumes of ice-cold 1.15% KCl. The homogenates were centrifuged at 9000g at 4°C for 20 min, and then ultracentrifuged two times at 105,000g at 4°C for 70 min. The pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4), frozen in liquid nitrogen, and stored in the freezer at −80°C until use.

**Immunoprecipitation and Immunoblotting of the Microsomal Protein.** Immunoprecipitation was carried out using an immunoprecipitation kit (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer. The proteins were concentrated and immunooaffinity-purified with anti-CYP2D2 antibody. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970), using a 12% polyacrylamide gel. Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane and immunostained with dianinobenzidine as substrate. The relative intensities of the immunoblotting were analyzed using NIH Image v. 1.63 (Lemnard, 1990).

**Analysis of Amino Acid Sequence.** Liver microsomes were subjected to SDS-PAGE and electrophoretically transferred to a polyvinyliden fluoride (PVDF) membrane. The blotted PVDF membrane was stained with 0.1% Coomassie Brilliant Blue R-250 containing 1% acetic acid and 40% methanol. After washing with 50% methanol, the band that was specifically expressed in liver microsomes of pEM was cut off from the membrane. The N-terminal sequence of the protein on the PVDF membrane was determined using a Procise 492 Protein Sequencer (PerkinElmer Life and Analytical Sciences, Boston, MA).

**Measurement of Enzyme Activities.** Diazepam metabolites were detected using high performance liquid chromatography according to the method described previously (Saito et al., 2004b), with some modifications. The microsomal fraction containing CYP2D1, CYP2D2, CYP2D3, and CYP2D4 prepared from yeast cells was used as the recombinant enzyme. The reaction mixture (total volume, 0.2 ml) contained 50 µM diazepam, 3 mM MgCl2, 5 mM glucose 6-phosphate, and 1 mg ml−1 for yeast microsomes. The reaction was started by adding 1 mM NADPH and 1 enzyme unit of glucose-6-phosphate dehydrogenase after preincubation at 37°C for 5 min. Incubation was carried out at 37°C for 20 min. The reaction was terminated by the addition of 1.5 ml of ethyl acetate.

**Other Methods.** The protein concentrations were determined by the method of Lowry et al. (1951). The amounts of P450 were estimated by the method of Omura and Sato (1964).

**Results**

**Isolation of the Specific Protein That Was Expressed in pEM.** We have shown that anti-CYP2D2 antibody inhibited diazepam p-
hydrolase activity in rat liver microsomes (Saito et al., 2004b). Moreover, both CYP2D1 and CYP2D2 did not catalyze diazepam p-hydroxylation. Thus, we attempted to isolate the protein that might contribute to p-hydroxylation of diazepam. After immunoprecipitation of liver microsomes of four rat strains using anti-CYP2D2 antibody, we separated proteins in low-bis SDS-PAGE and compared expression levels of the CYP2D subfamily using anti-CYP2D2 antibody (Fig. 2). We detected a band of protein that was expressed in all pEM rats (SD, BN, and EM-W), but not in pPM rats (DA and PM-W).

Comparison of N-Terminal Amino Acid Sequences of the Specific Protein with Those of the CYP2D Subfamily. To analyze the amino acid sequence of the specific band to pEM, we cut PVDF membrane corresponding to the specific band. N-terminal 20-amino acid sequences of the specific band were analyzed with peptide sequencer. We searched the sequence similarity between N-terminal amino acid sequences of the specific band and those of a protein database using FASTA (GenomeNet, Kyoto University). As a result of the comparison, N-terminal amino acid sequences of the specific protein exactly corresponded to those of CYP2D3 (Table 2). We thought that CYP2D3 might be involved in diazepam p-hydroxylation.

Western Blotting Analyses of Liver Microsome and CYP2D Isozymes Expressed in Yeast with Anti-CYP2D2 Antibody. To make sure anti-CYP2D2 antibody can recognize CYP2D3, we carried out Western blotting analyses of liver microsome from SD rats and pure CYP2D isozymes expressed in yeast using anti-CYP2D2 antibody. Microsomal protein (10 μg) or CYP2D2 and CYP2D3 (each 10 μg) expressed in yeast was applied to each lane. Other experimental details are described in the legend of Fig. 2.

Diazepam-Metabolizing Activities of the CYP2D Subfamily. To confirm the above hypothesis, assay of diazepam metabolism in the reconstituted system was performed by using rat CYP2D3 as well as other CYP2D subfamilies expressed in yeast as recombinant enzymes (Table 3). CYP2D3 had a high diazepam p-hydroxylation activity, but none of the other CYP2D isoforms have the activity. Also, CYP2D3 possessed moderate activity toward diazepam N-desmethylation. Interestingly, CYP2D4 exhibited a high diazepam N-desmethylation activity. The activity of CYP2D1 was low toward diazepam N-desmethylation. CYP2D2 had no activity toward three metabolic pathways. None of the CYP2D isoforms had diazepam 3-hydroxylation activity. These results support our earlier studies (Saito et al., 2004a,b) and our expectation that CYP2D3, but not CYP2D1 and CYP2D2, catalyzes diazepam p-hydroxylation.

Expression Level of CYP2D4 in Liver Microsomes among the Four Strains. Since we found that CYP2D4 possessed a high diazepam N-desmethylation activity, which is catalyzed by CYP2C11 or, partially, by CYP3A2, we examined the expression level of CYP2D4 in liver microsomes among the four strains. Western blotting analysis showed that DA rat liver microsomes had a high expression level of CYP2D4 protein (Fig. 4). The expression levels of other strains except for DA rats had no differences. This result was in good agreement with the previous study (Schulz-Utermoehl et al., 1999). Although we revealed that DA rats had a high diazepam N-desmethylation activity and attributed this to high levels of CYP3A2 (Saito et al., 2004b), the high expression of CYP2D4, which had a high diazepam N-desmethylation activity, in DA rats also supported the observation.

Discussion

In this study, we aimed to identify the cytochrome P450 isoform involved in diazepam p-hydroxylation in rats. Until recently, diazepam p-hydroxylation was thought to be catalyzed by CYP2D1 (Neville et al., 1993). However, our previous studies indicated that diazepam p-hydroxylation was not catalyzed by CYP2D1 or CYP2D2 (Saito et al., 2004a,b).

We managed to separate the specific protein expressed in liver microsomes of SD, BN, and EM-W rats, which was not expressed in DA and PM-W rats (Fig. 2). Since the specific protein was observed only in pEM, there is a strong possibility that the protein is diazepam p-hydroxylase. Thus, the amino acid sequence analysis was carried out to clarify the relationships between the specific protein and

**TABLE 2**

<table>
<thead>
<tr>
<th>Protein</th>
<th>N-Terminal Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2D1</td>
<td>MEIAGVEWPAIIFVIFI</td>
</tr>
<tr>
<td>CYP2D2</td>
<td>MEIAGVEWPAIIFVIFI</td>
</tr>
<tr>
<td>CYP2D3</td>
<td>MEIAGVEWPAIIFVIFI</td>
</tr>
<tr>
<td>CYP2D4</td>
<td>N.D.</td>
</tr>
<tr>
<td>CYP2D5</td>
<td>1.19</td>
</tr>
<tr>
<td>CYP2D18</td>
<td>0.29</td>
</tr>
<tr>
<td>CYP2D4</td>
<td>0.89</td>
</tr>
</tbody>
</table>

N.D., not detectable.
diazepam p-hydroxylation. The N-terminal amino acid sequence of the specific protein exactly corresponded to that of CYP2D3 (Table 2).

Among the rat CYP2D subfamily, the mRNA level of CYP2D3 increased with development (Matsunaga and Gonzalez, 1990), whereas similar developmental change was not observed in the mRNA levels of CYP2D1 and CYP2D2 (Chow et al., 1999). Neville et al. (1993) showed that diazepam p-hydroxylation was more active in young adult male rats (>5 weeks) than in neonates that were still in the early stages of development (Neville et al., 1993). Therefore, the mRNA expression pattern of CYP2D3 was highly consistent with developmental changes of diazepam p-hydroxylation. Results from these studies strongly indicate that CYP2D3 is diazepam p-hydroxylase.

To confirm that CYP2D3 has a diazepam p-hydroxylation activity, diazepam metabolism was performed by using the rat CYP2D3 isoforms expressed in yeast as recombinant enzyme (Table 3). As expected, only CYP2D3, but no other isoforms, possessed a high diazepam p-hydroxylation activity. In addition, we carried out the immunoblotting of liver microsome and CYP2D3 expressed in yeast with anti-CYP2D3 antibody. Anti-CYP2D3 antibody recognized CYP2D3, and diazepam p-hydroxylation was the same as CYP2D3 in molecular weight (Fig. 3). This is the first report that diazepam p-hydroxylation is specifically catalyzed by CYP2D3. CYP2D1 had a low diazepam N-desmethylation activity, whereas CYP2D2 had no activity toward diazepam metabolism. Although CYP2D1 and CYP2D2 play an important role in the metabolism of numerous drugs such as debrisoquine (Kobayashi et al., 1989), bunitrolol (Suzuki et al., 1992; Yamamoto et al., 1996, 1998), and propranolol (Fujita et al., 1993), it seems that they do not participate in diazepam metabolism as much.

In previous research, DA rats had the high activities toward diazepam 3-hydroxylation and diazepam N-desmethylation. We showed that the expression level of CYP3A2, which is responsible for diazepam 3-hydroxylation and, partially, diazepam N-desmethylation, was higher in DA rats than in other strains (Saito et al., 2004b). We also showed that the expression levels of CYP2C11, which is mainly responsible for diazepam N-desmethylation, were not different among the four strains. Therefore, it was concluded that the high activity toward diazepam N-desmethylation in DA rats was caused by the high expression of CYP3A2. In the current study, we found that CYP2D4 exhibited a high diazepam N-desmethylation activity, and that DA rats had a higher expression level of CYP2D4 than other strains (Fig. 4). Therefore, not only CYP3A2 but also CYP2D4 may be involved in strain differences in diazepam N-desmethylation among the four strains.

To date, there is little information about the catalytic specificity of CYP2D isoforms, especially CYP2D3 (Chow et al., 1999; Hiroi et al., 2002). In this study, we demonstrated that CYP2D3 was involving in diazepam p-hydroxylation in rats. Moreover, we also showed the polymorphic protein expressions of CYP2D3 between pEM and pPM.

It is concluded that the polymorphic expressions of CYP2D3 are responsible for the significant inter- or intrastrain differences in diazepam p-hydroxylation.

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References


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