Importance of CYP2D3 in polymorphism of diazepam p-hydroxylation in rats

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Running title

Importance of CYP2D3 in polymorphism of diazepam p-hydroxylation

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Abbreviations used in this paper: CYP, cytochrome P450; SD, Sprague-Dawley; BN, Brown
Norway; DA, Dark Agouti; W, Wistar; EM, extensive metabolizers; PM, poor metabolizers; EM-W, EM from Wistar rats; PM-W, PM from Wistar rats; pEM, EM in diazepam p-hydroxylation; pPM, PM in diazepam p-hydroxylation; G-6-P, glucose-6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; HPLC, high performance liquid chromatography
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 intra-strain differences in diazepam metabolism among Sprague-Dawley (SD), Brown Norway (BN), Dark Agouti (DA) and Wistar rats. Especially, there were marked (~300 fold) inter- or intra-strain 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 CYP2D subfamily in liver microsomes from various strains of rats using anti-CYP2D2 antibody, we found that there was a band of protein which was consistent with 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 CYP2Ds 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 intra-strain differences in
diazepam p-hydroxylation among rat strains or individuals.
Introduction

Diazepam is one of the benzodiazepines that are widely used in the treatment of anxiety disorders, depression and insomnia. Neville et al. 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 (Neville et al., 1993). These metabolic pathways were reported to be catalyzed by isoforms of cytochrome P450 (CYP), CYP3A2, CYP2C11 and CYP2D1, respectively (Fig. 1). We found the existence of extensive metabolizers (EM) and poor metabolizers (PM) of diazepam metabolism in liver microsomes from Wistar rats at low concentration of substrate (Saito et al., 2004a). EM from Wistar rats (EM-W) had markedly higher activity toward diazepam p-hydroxylation than PM 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 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), DA and PM-W rats as poor metabolizers (pPM) in diazepam p-hydroxylation, respectively. 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 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 neither catalyzed by 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; Yamamoto et al., 1998). The enzyme which 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). Totally 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 rat CYP2D subfamily conserves a high similarity of amino acid sequence (>70%) among each other and the antibody against the full length of CYP2D isoform might inhibit drug-metabolizing activities catalyzed by any forms of CYP2Ds without discrimination. In addition, antibodies generated against the purified P450 enzymes are often not specific. In spite of 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 \( p \)-hydroxylation to be
CYP2D3 using yeast recombinant CYP2D isoforms. Therefore, the polymorphism of diazepam p-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 towards 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 Co. (Osaka, Japan). The three primary diazepam metabolites, p-hydroxy-diazepam, 3-hydroxy-diazepam, and N-desmethyl-diazepam, were gifts from Japan Hoffman La Rosch Pharmaceutical Co. (Tokyo, Japan). Glucose-6-phosphate (G-6-P), NADPH and glucose-6-phosphate dehydrogenase (G-6-PDH) 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). Adult male SD, DA and Wistar rats (9 weeks old) were obtained from Japan SLC Co. (Shizuoka, Japan). Adult male BN rats (9 weeks old) were obtained from Seac Yoshitomi, Ltd. (Fukuoka, Japan). They were housed under standard laboratory conditions with free access to food and water, and were used for experiments after 1 week of acclimatization. All experiments using animals were performed with the supervision and approval of the Institutional Animal Care and Use Committee of Hokkaido University. CYP2D1, CYP2D2, CYP2D3 and CYP2D4 proteins were expressed in yeast and purified (Wan et al., 1997).
Preparation of liver microsomes

Liver microsomes were prepared according to the method of Omura and Sato (1964). The samples were homogenized with three 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) and 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 Immunoprecipitation Kit (Roche, Mannheim, Germany) as recommended by the manufacture. The proteins were concentrated and immunoaffinity-purified with anti-CYP2D2 antibody. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the methods of Laemmli (1970), using a 12% polyacrylamide gel. Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane and immunostained with diaminobenzidine as substrate. The relative intensities of the immunoblotting were analyzed using the NIH Image v. 1.63 (Lennard 1990).

Analysis of amino acid sequence
Liver microsomes were subjected to SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (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 which was specifically expressed in liver microsomes of pEM was cut off from the membrane. The N-terminal sequence of the protein on PVDF membrane was determined using a Procise 492 Protein Sequencer (PerkinElmer Life Sciences).

**Measurement of enzyme activities**

Diazepam metabolites were detected using high performance liquid chromatography (HPLC) 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 recombinant enzyme. The reaction mixture (total volume, 0.2 ml) contained 50 µM diazepam, 3 mM MgCl₂, 5 mM G-6-P, and 1 mg ml⁻¹ for yeast microsomes. The reaction was started by adding 1 mM NADPH and 1 enzyme unit of G-6-PDH 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. (Lowry et al., 1951). The amounts of P450 were estimated by the method of Omura and Sato (1964).
Results

Isolation of the specific protein which expressed in pEM

We have shown that anti-CYP2D2 antibody inhibited diazepam $p$-hydroxylase 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 which 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 CYP2D subfamily using anti-CYP2D2 antibody (Fig. 2). We detected a band of protein which expressed in all $p$EM rats (SD, BN and EM-W), but not in $p$PM rats (DA and PM-W).

Comparison of N-terminal amino acid sequences of the specific protein with those of CYP2D subfamily

In order to analyze the amino acid sequence of specific band to $p$EM, we cut PVDF membrane corresponding to the specific band. N-terminal 20 amino acid sequences of specific band were analyzed with peptide sequencer. We searched the sequence similarity between N-terminal amino acid sequences of specific band and those of protein database using FASTA (GenomeNet, Kyoto Univ.). 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
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 (CYP2D2 and CYP2D3) expressed in yeast using anti-CYP2D2 antibody (Fig. 3). Anti-CYP2D2 antibody recognized CYP2D3, and CYP2D3 showed the same molecular size as the specific band expressed in SD rats. This result strongly supports our hypothesis that diazepam \( p \)-hydroxylase is CYP2D3.

Diazepam metabolizing activities of CYP2D subfamily

To confirm above hypothesis, assay of diazepam metabolism in 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 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 CYP2D isoforms had diazepam 3-hydroxylation activity.
activity. These results support our earlier studies (Saito et al., 2004a, 2004b) and our expectation that CYP2D3, not CYP2D1 and CYP2D2, catalyzes diazepam p-hydroxylation.

Expression level of CYP2D4 in liver microsomes among four strains

Since we found that CYP2D4 possessed a high diazepam N-desmethylaion activity, which is catalyzed by CYP2C11 or partially CYP3A2, we examined the expression level of CYP2D4 in liver microsomes among 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-desmethylaion activity and attributed this to high levels of CYP3A2 (Saito et al., 2004b), the high expression of CYP2D4, which had a high diazepam N-desmethylaion 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, 2004b).

We managed to separate the specific protein expressed in liver microsomes of SD, BN and EM-W rats, while not expressed in DA and PM-W rats (Fig. 2). Since the specific protein was observed only in $p$EM, 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 diazepam $p$-hydroxylase. 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 et al., 1990), while similar developmental change was not observed in the mRNA levels of CYP2D1 and CYP2D2 (Chow et al., 1999). Neville et al. described in his paper that diazepam $p$-hydroxylation was more active in young adult male rats (>5 weeks) than in neonates that are 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.
In order to confirm that CYP2D3 has a diazepam \( p \)-hydroxylation activity, diazepam metabolism was performed by using the rat CYP2D 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-CYP2D2 antibody. Anti-CYP2D2 antibody recognized CYP2D3, and diazepam \( p \)-hydroxylase 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, while 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; Yamamoto et al., 1998) and propranolol (Fujita et al., 1993), it seems that they do not participate in diazepam metabolism so 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 are responsible for diazepam 3-hydroxylation and partially diazepam N-desmethylation, was higher in DA rats than other strains (Saito et al., 2004b). They also showed that the expression levels of CYP2C11, which is mainly responsible for diazepam N-desmethylation, were not different among 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 current study, we found that CYP2D4 exhibited a high diazepam N-desmethylation activity, and that DA rats had the 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 four strains.

Up to date, there are few 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 intra-strain differences in diazepam p-hydroxylation.
Acknowledgments

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References


Matsunaga E, Gonzalez FJ (1990) Specific cytosine demethylations within the first exons of the rat CYP2D3 and CYP2D5 genes are associated with activation of hepatic gene expression during development. DNA Cell Biol 9:443-452.

antibodies to the P4502D subfamily in rat, dog, and man. Xenobiotica 25:1103-1009.


Footnote:

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Legend

Fig. 1 Metabolic pathways of diazepam in male rat liver microsomes.
Diazepam 3-hydroxylation, N-desmethylation, \( p \)-hydroxylation are mainly catalyzed by CYP3A2, CYP2C11 and CYP2D1, respectively (Neville et al., 1993).

Fig. 2 Western blotting analyses of liver microsomes from four rat strains using anti-CYP2D2 antibody.
Microsomal proteins (10\( \mu \)g) were separated by low bis SDS-PAGE using 12\% polyacrylamide gel. After the transfer of protein onto a PVDF membrane, CYP2D isoforms were reacted with peptide antibody against CYP2D2 and immunostained with diaminobenzidine as a substrate.

Fig. 3 Western blotting analyses of liver microsome from SD rat and pure CYP2D isozymes expressed in yeast using anti-CYP2D2 antibody.
Microsomal protein (10\( \mu \)g), or CYP2D2 and CYP2D3 (each 10\( \mu \)g) expressed in yeast were applied to each lane. Other experimental details are described under legend of Fig. 2.

Fig. 4 Western blotting analyses of liver microsomes from four rat strains using anti-CYP2D4 peptide antibody.
Microsomal proteins (10µg) were separated by SDS-PAGE using 12% polyacrylamide gel. After the transfer of protein onto a nitrocellulose membrane, CYP2D4 was reacted with peptide antibody against CYP2D4 and immunostained with diaminobenzidine as a substrate.
Table 1. Total P450 contents and diazepam metabolic activities in liver microsomes from four rat strains

<table>
<thead>
<tr>
<th></th>
<th>Total P450 contents (nmol/mg protein)</th>
<th>Activities (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p-hydroxylation</td>
</tr>
<tr>
<td>SD</td>
<td>0.80 ± 0.08</td>
<td>171.9 ± 3.0 *</td>
</tr>
<tr>
<td>BN</td>
<td>0.73 ± 0.05</td>
<td>156.4 ± 8.3 *</td>
</tr>
<tr>
<td>DA</td>
<td>0.83 ± 0.03</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
<td>PM-W</td>
<td>0.73 ± 0.08</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>EM-W</td>
<td>0.62 ± 0.05</td>
<td>94.6 ± 16.0 *</td>
</tr>
</tbody>
</table>

The concentration of diazepam was 3.13 μM. Values are expressed as the mean ± SEM. * Significant difference from PM-W rats (p < 0.01). This data was cited from Saito et al. (2004a, 2004b).
Table 2. Comparison of the N-terminal amino acid sequence of the specific protein which expressed in pEM with the sequences of the CYP2D subfamily members.

<table>
<thead>
<tr>
<th>Protein</th>
<th>N-terminal amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DZ p-hydroxylase</td>
<td>MELLAGTGGLWPMAIFTVIFI</td>
</tr>
<tr>
<td>CYP2D1</td>
<td>MELLNGTGGLWSMAIFTVIFI</td>
</tr>
<tr>
<td>CYP2D2</td>
<td>MGLLIGDDLWAVVIFTAILF</td>
</tr>
<tr>
<td>CYP2D3</td>
<td>MELLAGTGGLWPMAIFTVIFI</td>
</tr>
<tr>
<td>CYP2D4</td>
<td>MRMPGTGSELWPIAIFTIIFL</td>
</tr>
<tr>
<td>CYP2D5</td>
<td>MELLNGTGGLWPMAAFVIFI</td>
</tr>
<tr>
<td>CYP2D18</td>
<td>MRMPGTGSELWPIAIFTIIFL</td>
</tr>
</tbody>
</table>
Table 3. Diazepam metabolic activities of CYP2D isoforms

<table>
<thead>
<tr>
<th>Activities (nmol/min/nmol of P450)</th>
<th>p-Hydroxylation</th>
<th>3-Hydroxylation</th>
<th>N-Desmethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2D1</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.02</td>
</tr>
<tr>
<td>CYP2D2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CYP2D3</td>
<td>1.19</td>
<td>N.D.</td>
<td>0.29</td>
</tr>
<tr>
<td>CYP2D4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.89</td>
</tr>
</tbody>
</table>

The concentration of substrate (diazepam) was 50 µM. Other experimental details are described under Materials and Methods. N.D., not detectable.
Figure 1

Diazepam

3-hydroxy-diazepam  p-hydroxy-diazepam  N-desmethyl-diazepam
Figure 4

CYP2D4

PM-W  BN  DA  SD