STRAIN DIFFERENCES IN DIAZEPAM METABOLISM AT ITS THREE METABOLIC SITES IN SPRAGUE-DAWLEY, BROWN NORWAY, DARK AGOUTI, AND WISTAR STRAIN RATS

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ABSTRACT:

Knowledge of strain differences in drug metabolism is important for the selection of animals for pharmacokinetic, pharmacodynamic, and toxicological studies. Hepatic microsomes from Sprague-Dawley (SD) and Brown Norway (BN) rats had 300-fold higher diazepam \( p \)-hydroxylation activity than Dark Agouti (DA) and Wistar (W) rats at a low diazepam concentration \( (3 \, \mu M) \). Kinetic studies indicated that diazepam \( p \)-hydroxylation in SD and BN rats proceeded with lower \( K_m \) and higher \( V_{\text{max}} \) values than in DA and W rats. However, the expression levels of cytochrome P450 CYP2D1, the reported enzyme for diazepam \( p \)-hydroxylation, did not cosegregate with the activity. These results suggest the presence of a new high-affinity diazepam \( p \)-hydroxylation enzyme other than CYP2D1 in SD and BN rats. DA rats showed 3- and 2-fold higher diazepam 3-hydroxylation and 4-desmethylametabolites, respectively, than the other rat strains. In agreement with this, DA rat liver microsomes had a higher expression of CYP3A2, which is responsible for diazepam 3-hydroxylation and partly responsible for N-desmethylation. Values of CLint \( (V_{\text{max}}/K_m) \) indicated that \( p \)-hydroxy-diazepam is the major metabolite in SD and BN rats, whereas 3-hydroxy-diazepam is the major metabolite in DA and W rats. The sum of the CLint in each strain was in the order of DA > SD > BN > W. Strain differences in the pharmacodynamics of diazepam between SD and DA rats may be due to these differences in diazepam metabolism. We found that both the rate of elimination of diazepam and the major metabolic pathways in diazepam metabolism differed among the different rat strains due to polymorphic expression of the two enzymes involved in diazepam metabolism.

Inter- or intrastrain differences in the pharmacodynamics of diazepam have been reported between Sprague-Dawley (SD) and Dark Agouti (DA) rat strains or among outbred Wistar (W) rats (Bert et al., 2001; Mechan et al., 2002). Although they did not study diazepam metabolism, the authors of these studies suggested that inter- or intrastrain variation in diazepam metabolism is the cause of these differences. Generally, variations in drug metabolism among individuals or among strains of animals or different human races are caused by polymorphic expression of one of the enzymes involved in the metabolism. Such polymorphisms in drug metabolism have been reported in the metabolism of debrisoquine, a number of \( \beta \)-blockers, and other drugs metabolized by cytochrome P450. P450 CYP2D6 in humans and CYP2D2 in rats (Marandi et al., 1996; Yamamoto et al., 1998; Schulz-Utermoehl et al., 1999; Vorhees et al., 1999) and in the metabolism of S-mephenytoin and other drugs metabolized by CYP2C19 in humans (Roh et al., 1996; Xie et al., 1996, 1999; Marandi et al., 1997; Adedoyin et al., 1998; Ferguson et al., 1998; Zhou, 2001; He et al., 2002). Inaba et al. (1988) reported interindividual variability of diazepam metabolism. Accumulating reports suggest the possible correlation of diazepam metabolism with the S-mephenytoin hydroxylation phenotype (Bertilsson et al., 1989). These polymorphisms in drug metabolism of a drug, i.e., the existence of extensive and poor metabolizers (EM and PM, respectively), are of clinical importance. Adverse effects typical of overdosage of the drug are the consequence of administration of a “normal” dosage to individuals expressing defective or low levels of metabolizing enzymes (PM).

Neville et al. (1993) reported that \( p \)-hydroxylation, 3-hydroxylation, and N-desmethylation are the pathways of diazepam metabolism in adult male W rat liver microsomes and that they were catalyzed by the respective P450 isoforms of CYP2D1, CYP3A2, and CYP2C11. In our previous report, we found that about 17% of W rats had markedly (more than 200-fold) higher activities of diazepam \( p \)-hydroxylation (EM of diazepam metabolism) than the rest of the W rat strains.

ABBREVIATIONS: SD, Sprague-Dawley; DA, Dark Agouti; W, Wistar; EM, extensive metabolizer(s); PM, poor metabolizer(s); EM W, EM rats from W strain; P450, cytochrome P450; BN, Brown Norway; G-6-P, glucose-6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
population in liver microsomes at low substrate concentrations. The expression levels of CYP2D1 in W rats did not show such dimorphism, but a kinetic study suggested the presence of a high-affinity and high-capacity diazepam \( p \)-hydroxylation enzyme other than CYP2D1 in the liver microsomes of EM rats from W strain (EM W) (Saito et al., 2004).

In this study, we investigated strain differences in diazepam metabolism with particular attention to the metabolic activities of primary pathways of diazepam metabolism at low substrate concentrations using SD, Brown Norway (BN), DA, and W adult male rats. The results showed good agreement with the strain differences in the aforementioned pharmacodynamic studies (Bert et al., 2001; Mechan et al., 2002).

Materials and Methods

Materials. Diazepam, 3,3'-diaminobenzidine tetrahydrochloride dehydrate, and the internal standard nitrazepam were purchased from Wako Pure Chemicals (Osaka, Japan). The three primary diazepam metabolites \( p \)-hydroxy-diazepam, 3-hydroxy-diazepam, and \( N \)-desmethyl-diazepam and the two secondary diazepam metabolites 3-hydroxy-\( N \)-desmethyl-diazepam and \( p \)-hydroxy-\( N \)-desmethyl-diazepam were gifts from Japan Hoffman LaRoche Pharmaceutical Co. (Tokyo, Japan). Glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PDH), and \( \beta \)-NADPHs were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Rabbit anti-rat CYP3A2 and goat anti-rat CYP2C11 antisera were from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Rabbit polyclonal antiserum to rat CYP2D1 was obtained from Cosmo Bio Co., Ltd (Tokyo, Japan). An antibody against CYP2D2, which has an ability to effectively inhibit debrisoquine 4-hydroxylation activity, was prepared as described previously (Suzuki et al., 1992; Masubuchi et al., 1993; Nakamura et al., 1995; Yamamoto et al., 1996). Horseradish peroxidase-labeled anti-goat IgG and anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The other chemicals and reagents used were of analytical and biochemical grade.

Rat Liver Microsomes. Ten-week-old SD, BN, DA (\( n = 4 \) each), and W (\( n = 6 \)) male rats were supplied by Nihon SLC Co. (Shizuoka, Japan). All experiments using animals were performed with the supervision and approval of the Institutional Animal Care and Use Committee of Hokkaido University. Liver microsomes were prepared from the rats according to the method of Omura and Sato (1964). The liver samples were homogenized with three volumes of ice-cold 1.15% KCl. Each homogenate was centrifuged at 9000g at 4°C for 20 min to remove nuclear materials and then ultracentrifuged two times at 105,000g at 4°C for 70 min to attain a mitochondrial-free microsomal pellet. The pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4).

Fig. 1. The rate of disappearance of diazepam in liver microsomes from adult male rats of SD, BN, DA, and W strains. The concentration of the substrate (diazepam) was 3 \( \mu \)M. Other experimental details are described under Materials and Methods. Data represent means \( \pm \) S.E.M. of four animals. **, significant difference from other strains of rats (\( p < 0.01 \)).

Fig. 2. The elution profiles of diazepam and its primary metabolites from the HPLC column after incubation of diazepam with liver microsomes from adult male rats of SD, BN, DA, and W strains. The concentration of the substrate (diazepam) was 3 \( \mu \)M. Other experimental details are described under Materials and Methods. P, \( p \)-hydroxy-diazepam; I, internal standard (nitrazepam); T, 3-hydroxy-diazepam; N, \( N \)-desmethyl-diazepam; DZ, diazepam.
Nitrazepam was added to the mixture as an internal standard, and the reaction was terminated by the addition of 1.5 ml of ethyl acetate. The formation of the three primary metabolites was linear with concentration of substrate (diazepam) was 25 μM. During this period, the formation of the two secondary metabolites 3-hydroxy-N-desmethyl-diazepam and p-hydroxy-N-desmethyl-diazepam was negligible, and the formation of the three primary metabolites was linear with time. The reaction was terminated by the addition of 1.5 ml of ethyl acetate. Nitrazepam was added to the mixture as an internal standard, and it was extracted in the organic phase (ethyl acetate) with diazepam and its metabolites by mixing sufficiently. The mixture was centrifuged at 1200 g for 20 min to separate the organic phase from the aqueous phase. The organic layer was transferred to another tube, evaporated under vacuum, and the residue was dissolved in the HPLC mobile phase to yield the final sample for the HPLC analysis. HPLC was performed using a Shimadzu LC-6AV spectrophotometric detector and data processor (Chromatopac C-R6A) and an Inertosil ODS-2 column (5 μm, 25 cm × 4.6 mm i.d.) (GL Sciences Inc., Tokyo, Japan). The UV absorption intensity was monitored at 210 nm.

**Assay for Diazepam-Metabolizing Activity of Liver Microsomes.** Diazepam metabolism was assayed according to the method described previously, with slight modifications (Fujita et al., 1990b). The reaction mixture (in a final volume of 0.5 ml) contained 3 mM diazepam, 3 mM MgCl₂, 5 mM G-6-P, and 0.25 g of liver microsomes in 0.1 M potassium phosphate buffer (pH 7.4). The reaction was started by adding 1 mM β-NADPH and 1 enzyme unit of G-6-PDH; mixtures were incubated in a shaker bath at 37°C for 5 min. The reaction was stopped with 0.5 ml of 12 N NaOH. After the extraction of debrisoquine and its metabolites with ethyl acetate, glycinexylidide (as the internal standard) and 0.01 N H₂SO₄ were added. After vortex mixing and centrifugation, the upper layer was removed. It was neutralized by 0.01 N NaOH, evaporated to dryness, and dissolved in HPLC mobile phase (10 mM potassium phosphate buffer, pH 3.0; acetonitrile = 85:15 by volume). The sample was applied to an Inertosil ODS column (5 μm, 25 cm × 4.6 mm i.d.) (GL Sciences Inc., Tokyo, Japan). The UV absorption intensity was monitored at 210 nm.

**Immunological Analysis of CYP2D1, CYP3A2, CYP2C11, and CYP2D2.** The expression levels of CYP2D1, CYP3A2, and CYP2C11, which are reported to catalyze diazepam p-hydroxylation, 3-hydroxylation, and N-desmethylation in the liver microsomes of adult male W rats, respectively (Neville et al., 1993), and CYP2D2 were studied using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), with some modifications, and immunoblot technique according to Towbin et al. (1979). Liver microsomal proteins (10 μg of protein/well) from rats were applied to 12% SDS-polycrylamide gels and separated by electrophoresis using a Mini-PROTEAN 2 1-D cell (Bio-Rad, Nijmegen, the Netherlands). The proteins were electrophoretically transferred to nitrocellulose membranes and probed with antibodies against rat CYP2C11 (goat antiseraum), CYP2D1, CYP2D2, and CYP3A2 (rabbit antiseraum). Horseradish peroxidase-labeled anti-goat IgG was used as the secondary antibody for CYP2C11 detection, and horseradish peroxidase-labeled anti-rabbit IgG was used for CYP2D1, CYP2D2, and CYP3A2 detection. Immunoreactive protein bands were revealed colorimetrically by oxidation of 3,3',5,5'-tetramethylbenzidine

**Total P450 Contents and Diazepam Metabolic Activities in Liver Microsomes from Four Rat Strains.**

Total P450 contents and diazepam metabolic activities in liver microsomes from four rat strains were assayed as described previously (Suzuki et al., 1992; Hiroi et al., 2002), with some modifications. The assay mixtures contained 50 μM debrisoquine, 10 mM MgCl₂, 0.5 mM NADPH, 10 mM G-6-P, and 0.1 M potassium phosphate buffer (pH 7.4) for a total volume of 0.5 ml. The reaction was started after 3 min of preincubation at 37°C by adding 1 mM β-NADPH and 1 enzyme unit of G-6-PDH; mixtures were incubated in a shaker bath at 37°C for 5 min. The reaction was stopped with 0.5 ml of 12 N NaOH. After the extraction of debrisoquine and its metabolites with ethyl acetate, glycinexylidide (as the internal standard) and 0.01 N H₂SO₄ were added. After vortex mixing and centrifugation, the upper layer was removed. It was neutralized by 0.01 N NaOH, evaporated to dryness, and dissolved in HPLC mobile phase (10 mM potassium phosphate buffer, pH 3.0; acetonitrile = 85:15 by volume). The sample was applied to an Inertosil ODS column (5 μm, 25 cm × 4.6 mm i.d.) (GL Sciences Inc., Tokyo, Japan). The UV absorption intensity was monitored at 210 nm.

**Immunological Analysis of CYP2D1, CYP3A2, CYP2C11, and CYP2D2.** The expression levels of CYP2D1, CYP3A2, and CYP2C11, which are reported to catalyze diazepam p-hydroxylation, 3-hydroxylation, and N-desmethylation in the liver microsomes of adult male W rats, respectively (Neville et al., 1993), and CYP2D2 were studied using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), with some modifications, and immunoblot technique according to Towbin et al. (1979). Liver microsomal proteins (10 μg of protein/well) from rats were applied to 12% SDS-polycrylamide gels and separated by electrophoresis using a Mini-PROTEAN 2 1-D cell (Bio-Rad, Nijmegen, the Netherlands). The proteins were electrophoretically transferred to nitrocellulose membranes and probed with antibodies against rat CYP2C11 (goat antiseraum), CYP2D1, CYP2D2, and CYP3A2 (rabbit antiseraum). Horseradish peroxidase-labeled anti-goat IgG was used as the secondary antibody for CYP2C11 detection, and horseradish peroxidase-labeled anti-rabbit IgG was used for CYP2D1, CYP2D2, and CYP3A2 detection. Immunoreactive protein bands were revealed colorimetrically by oxidation of 3,3',5,5'-tetramethylbenzidine

**Materials and Methods.** Described under Materials and Methods. Data are expressed as mean ± S.E.M. **Total P450 Contents and Diazepam Metabolic Activities in Liver Microsomes from Four Rat Strains.** The concentration range of substrate (diazepam) was 3 μM. Other experimental details are described under Materials and Methods. Data are expressed as mean ± S.E.M.

<table>
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<tr>
<th>Total P450 Contents</th>
<th>Activities</th>
<th>p-Hydroxylation</th>
<th>3-Hydroxylation</th>
<th>N-Desmethylation</th>
</tr>
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<tr>
<td>ng/mg protein</td>
<td>μmol/min/mg protein</td>
<td>μmol/min/mg protein</td>
<td>μmol/min/mg protein</td>
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<tr>
<td>SD</td>
<td>0.80 ± 0.08</td>
<td>172 ± 3.0**</td>
<td>146 ± 26.1</td>
<td>27.5 ± 0.9</td>
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<td>BN</td>
<td>0.73 ± 0.05</td>
<td>156 ± 8.3**</td>
<td>116 ± 8.3</td>
<td>31.3 ± 1.0</td>
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<tr>
<td>DA</td>
<td>0.83 ± 0.03</td>
<td>0.4 ± 0.0</td>
<td>457 ± 35.7**</td>
<td>67.3 ± 1.8**</td>
</tr>
<tr>
<td>W</td>
<td>0.73 ± 0.08</td>
<td>0.5 ± 0.0</td>
<td>141 ± 19.3</td>
<td>24.8 ± 1.6</td>
</tr>
</tbody>
</table>

**Significant difference from W rats (p < 0.01).**

**FIG. 3.** Effect of substrate concentrations on diazepam metabolism in liver microsomes from four rat strains. Each figure represents typical data from adult male rats of SD, BN, DA, and W strains, respectively. Other experimental details are described under Materials and Methods. Circles, diazepam p-hydroxylation; squares, diazepam 3-hydroxylation; triangles, diazepam N-desmethylation.
tetrahydrochloride dehydrate with hydrogen peroxide and catalyzed by peroxidase in Tris-HCl buffer (pH 7.4). The color intensities of the bands that emerged were measured using NIH Image v. 1.61 (National Institutes of Health, Bethesda, MD) (Lennard, 1990).

Results

Strain Differences in Diazepam Metabolism at a Low Substrate Concentration. Strain differences in the rate of disappearance of diazepam from the microsomal incubation mixtures containing the NADPH generating system, 0.4 to 40 μM diazepam, and liver microsomes from the SD, BN, DA, and W strains of adult male rats are plotted in Fig. 3. The kinetic parameters obtained are listed in Table 2. Kinetic parameters of EM W rats obtained in our previous study (Saito et al., 2004) are also included for comparison. These studies revealed that p-hydroxylation in SD and BN rats proceeds with lower V\textsubscript{max} values than those in DA and W rats, just like the activity observed in EM W. The V\textsubscript{max} of the diazepam 3-hydroxylation in DA rats is 2 times that of other strains. The sum of the CL\textsubscript{int} (V\textsubscript{max}/K\textsubscript{m}), which reflects the metabolic activity at low substrate concentrations in each strain, was in the order of DA > SD > BN > W. The CL\textsubscript{int} also indicates that p-hydroxylation is the preferred pathway of diazepam metabolism in SD and BN rats, whereas 3-hydroxylation is the preferred pathway in DA and W strains of rats at the low substrate concentrations. The sum of V\textsubscript{max} was in the order of DA > SD > BN = W.

Western Blot Analyses Using CYP3A2, 2C11, and 2D1 Antibodies. Western blot analysis revealed that DA rat liver microsomes have a higher expression level of CYP3A2 protein, which is reported

<table>
<thead>
<tr>
<th>Pathway</th>
<th>K\textsubscript{m}</th>
<th>V\textsubscript{max} \text{μmol/min/mg protein}</th>
<th>CL\textsubscript{int} \text{μl/min/mg protein}</th>
<th>Total CL\textsubscript{int}</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>5.5 ± 0.5**</td>
<td>0.5 ± 0.0**</td>
<td>87.0 ± 2.1**</td>
<td>145 ± 11**</td>
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<tr>
<td>BN</td>
<td>71.2 ± 11</td>
<td>3.2 ± 0.3</td>
<td>48.9 ± 9.0</td>
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<tr>
<td>DA</td>
<td>55.9 ± 2**</td>
<td>0.4 ± 0.0**</td>
<td>86.9 ± 6.5**</td>
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<tr>
<td>W</td>
<td>22.2 ± 1</td>
<td>1.6 ± 0.6**</td>
<td>22.4 ± 0.6**</td>
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<td>EM W</td>
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<td>0.2 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>3-Hydroxylation</td>
<td>0.004 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>415 ± 15**</td>
<td></td>
</tr>
<tr>
<td>N-Desmethylation</td>
<td>0.004 ± 0.0</td>
<td>0.2 ± 0.0</td>
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** Significant difference from W rats (p < 0.01).
* Data from Saito et al. (2004).

Kinetic Studies of Diazepam Metabolite Formation. The kinetics of diazepam metabolism in the incubation mixture containing the NADPH generating system, 0.4 to 40 μM diazepam, and liver microsomes from the SD, BN, DA, and W strains of adult male rats are plotted in Fig. 3. The kinetic parameters obtained are listed in Table 2. Kinetic parameters of EM W rats obtained in our previous study (Saito et al., 2004) are also included for comparison. These studies revealed that p-hydroxylation in SD and BN rats proceeds with lower K\textsubscript{m} and higher V\textsubscript{max} values than those in DA and W rats, just like the activity observed in EM W. The V\textsubscript{max} of the diazepam 3-hydroxylation in DA rats is 2 times that of other strains. The sum of the CL\textsubscript{int} (V\textsubscript{max}/K\textsubscript{m}), which reflects the metabolic activity at low substrate concentrations in each strain, was in the order of DA > SD > BN > W. The CL\textsubscript{int} also indicates that p-hydroxylation is the preferred pathway of diazepam metabolism in SD and BN rats, whereas 3-hydroxylation is the preferred pathway in DA and W strains of rats at the low substrate concentrations. The sum of V\textsubscript{max} was in the order of DA > SD > BN = W.

Western Blot Analyses Using CYP3A2, 2C11, and 2D1 Antibodies. Western blot analysis revealed that DA rat liver microsomes have a higher expression level of CYP3A2 protein, which is reported

SD BN DA W

Fig. 4. Western blotting analyses of liver microsomes from adult male rats of SD, BN, DA, and W strains using anti-CYP2D1, CYP3A2, and CYP2C11 antibodies. Microsomal proteins (10 μg) were separated by SDS-PAGE using 12% polyacrylamide gel. After the transfer of proteins onto a nitrocellulose membrane, P450 isoforms were reacted with specific antibodies against CYP2D1, CYP3A2, and CYP2C11 and immunostained with diaminobenzidine as a substrate.
to be responsible for diazepam 3-hydroxylation and partly responsible for diazepam N-desmethylation (Fig. 4). This is in agreement with the high diazepam 3-hydroxylation and N-desmethylation activities in this strain of rats (Tables 1 and 2). The expression levels of CYP2C11, which is responsible for diazepam N-desmethylation activity, are similar in all rat strains tested. In the CYP2D1 expression level, the blot from DA rats was more intense than those from SD, W, and BN rats (DA > SD > W = BN). This result was in agreement with the previous study (Schulz-Utermoehl et al., 1999) but not with the observations of the high diazepam p-hydroxylation activities in SD and BN rats and low diazepam p-hydroxylation activities in DA and W rats (Tables 1 and 2).

Inhibition Study of Diazepam Metabolism Using CYP2D2 Antibody. CYP2D1 has been reported to be responsible for debrisoquine 4-hydroxylation, but later it was corrected to be CYP2D2 (Suzuki et al., 1992; Yamamoto et al., 1996, 1998). Thus, we carried out an inhibition study on diazepam metabolism in the liver microsomes of SD and BN rat strains using an antibody against CYP2D2. An anti-rat CYP2D2 caused about 80% inhibition of the diazepam p-hydroxylation, indicating that the enzyme responsible for diazepam p-hydroxylation cross-reacts with CYP2D2 antibody. It did not inhibit the diazepam 3-hydroxylation and N-desmethylation (Fig. 5).

Debrisoquine 4-Hydroxylation Activity and Expression Level of CYP2D2. If CYP2D2 is the enzyme responsible for diazepam p-hydroxylation, species differences in a known CYP2D2-dependent activity such as debrisoquine 4-hydroxylation and the expression levels of CYP2D2 should cosegregate with that of diazepam p-hydroxylation. We examined debrisoquine 4-hydroxylation activity (Fig. 6) and Western blot analysis for CYP2D2 in the liver microsomes of those rat strains (Fig. 7). Only DA rats showed low debrisoquine 4-hydroxylation activity as expected. We could not find any differences in the activity of debrisoquine 4-hydroxylation among the liver microsomes from SD, BN, and W rats. These results were in agreement with the results of Western blotting analysis for CYP2D2 expression level. The species differences in debrisoquine 4-hydroxylation and CYP2D2 expression level did not cosegregate with that of diazepam p-hydroxylation, indicating that CYP2D2 is not responsible for the high-affinity enzyme activity of diazepam p-hydroxylation.

Discussion

We investigated the strain differences in the kinetics of each metabolic pathway of diazepam among liver microsomes from SD, BN, DA, and W rat strains to elucidate the particular metabolic pathway(s) responsible for the differences. Metabolic activities at low substrate concentrations are estimated by $\text{CL}_{\text{int}} (V_{\text{max}}/K_{\text{m}})$.

As summarized in Table 2, p-hydroxylation is the preferred pathway of diazepam metabolism in SD and BN rats, whereas 3-hydroxylation is the preferred pathway in DA and W rats at low substrate concentrations. DA rats have a higher rate of diazepam metabolism than SD or BN rats (Fig. 1). In spite of the very low activity of diazepam p-hydroxylation in DA rats, the high activities of 3-hydroxylation and N-desmethylation might be sufficient to compensate for the difference in p-hydroxylation activity in DA rats (Table 1). Significant differences were observed in the metabolism of diazepam among these strains of rats at the low substrate concentrations, which is represented by the total $\text{CL}_{\text{int}}$ (DA > SD = BN > W). The results of these studies revealed that the strain difference in diazepam metabolism between DA and other strains of rats (DA > other strains).
CYP2D2 and immunostained with diaminobenzidine as a substrate. The proteins were separated by SDS-PAGE using 12% polyacrylamide gel. After the transfer of proteins onto a nitrocellulose membrane, CYP2D2 was reacted with antibody against CYP2D2 and immunostained with diaminobenzidine as a substrate.

Fig. 7. Western blotting analyses of liver microsomes from adult male rats of SD, BN, DA, and W strains using anti-CYP2D2 antiserum. Microsomal proteins (10 μg) were separated by SDS-PAGE using 12% polyacrylamide gel. After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane and reacted with anti-CYP2D2 antibody. An anti-rat CYP2D2 caused about 80% inhibition of the diazepam p-hydroxylation activity. 62 kDa indicates a higher level of CYP2D2 expression in DA rats than in SD rats.

was mainly due to the differences in diazepam 3-hydroxylation and N-desmethylation activities, whereas the strain differences between SD and W (SD > W) or BN and W rats (BN > W) are due to differences in the activity of diazepam p-hydroxylation. SD and BN rats showed a similar metabolic profile to EM W rats (Saito et al., 2004) in the present study.

Mechan et al. (2002) reported strain differences in the pharmacodynamics of diazepam between SD and DA rat strains (the SD rat is more responsive to diazepam than the DA rat) and indicated that it may be due to the differences in diazepam metabolism between these strains of rats. Here we show that DA rats eliminate diazepam more quickly from the body with a higher Cl\text{min} than SD rats. The quicker metabolic elimination of diazepam in DA rats may result in lower sensitivity to diazepam in these strains of rats than SD. In addition to the above, the fact that the major metabolite in SD rats is p-hydroxy-diazepam, whereas in DA rats it is 3-hydroxy-diazepam, may contribute to the pharmacodynamic difference. Unfortunately, no pharmacodynamic data are available for p-hydroxy-diazepam. If p-hydroxy-diazepam is pharmacodynamically more potent than 3-hydroxy-diazepam, this contributes to the difference. Similar explanations are applicable for the intraspecies pharmacodynamic difference among different outbred W rats (Bert et al., 2001). As has been stated earlier, we found that there is a dimorphic variation in diazepam p-hydroxylation in Wistar rats (Saito et al., 2004).

DA rats had a higher \( V_{\text{max}} \) in diazepam N-desmethylation activity than other strains of rats, whereas the expression levels of CYP2C11 were similar in all four strains. This may be due to the fact that CYP3A2 is partially involved in this reaction and because DA rats express a higher level of CYP3A2, which contributes to diazepam N-desmethylation, and show a higher \( V_{\text{max}} \) in N-desmethylation activity. Interestingly, the expression levels of CYP2D1, supposedly responsible for diazepam p-hydroxylation activity (Neville et al., 1993), did not cosegregate with the \( p \)-hydroxylation activities in these strains of rats. The blots from DA rats were more intense than those from SD, BN, and W rats. Since the original study showing that CYP2D1 is the enzyme responsible for diazepam p-hydroxylation was carried out using only W rats, the presence of the lower \( K_{\text{m}} \) and higher \( V_{\text{max}} \) p-hydroxylation enzyme in SD and BN rats shown in this study was not observed. Therefore, this is the first study to report the presence of this enzyme that should be identified by further study. Previously, we have reported that CYP2D2, but not CYP2D1, contributed to the metabolism of debrisoquine, buprilotol, and bufuralol, which are known as typical CYP2D substrates, in rat liver microsomes (Suzuki et al., 1992; Yamamoto et al., 1996, 1998). Thus, we examined the possibility that CYP2D2 is the catalyst for diazepam p-hydroxylation. We carried out an inhibition study on diazepam metabolism in the liver microsomes of SD and BN rat strains using an antibody against CYP2D2. An anti-rat CYP2D2 caused about 80% inhibition of the diazepam p-hydroxylation, whereas it did not inhibit the diazepam 3-hydroxylation and N-desmethylation (Fig. 5).

fore, we presumed that the rat liver microsomal diazepam p-hydroxylation is mediated largely by CYP2D2 or an enzyme that cross-reacts with anti-CYP2D2 antibody. We also examined CYP2D2-dependent activity, debrisoquine 4-hydroxylation, and Western blotting analysis for CYP2D2 expression levels in the liver microsomes of these rat strains. Debrisoquine 4-hydroxylation activity in DA rats was the lowest among liver microsomes from these strains of rats. We could not find any differences in the activity of debrisoquine 4-hydroxylation among the liver microsomes from SD, BN, and W rats. These results were in agreement with the results of Western blotting analysis for CYP2D2 expression level. Thus, the patterns of debrisoquine 4-hydroxylation activity and CYP2D2 expression coincided with each other as expected, but they did not coincide with diazepam p-hydroxylation activity in the liver of the four rat strains. We concluded that CYP2D2 is not the main contributor to diazepam p-hydroxylation in liver microsomes of adult male rats. But the result of the inhibition study using CYP2D2 antibody suggested that the enzyme that contributed to this pathway in SD and BN rats is closely related to CYP2D2 and may possibly be an isoform of the CYP2D subfamily.

The current study demonstrated that the strain difference in diazepam metabolism is unique in that it is not due to a difference in the expression level of a single enzyme but to two enzymes. The enzymes responsible for the activities of diazepam p-hydroxylation and diazepam 3-hydroxylation are involved in these species differences. Western blot analysis revealed that CYP3A2 is expressed more highly in DA rats than in the other rat strains. Kinetic analysis revealed the presence of a high-affinity, high-capacity diazepam p-hydroxylation enzyme in SD and BN rats. Due to these differences, the major metabolic pathways of diazepam differ among rat strains at low diazepam concentrations. These differences in drug metabolism, in which major metabolites differ depending on the strain of the animal, may cause significant differences not only in the results of pharmacokinetics of the drug, but also in pharmacological or toxicological studies, especially when the metabolites of the test compound have a pharmacological or toxicological effect.

References


Saito ET AL.

- 62 kDa

- 47.5 kDa

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DIFFERENCES IN DIAZEPAM METABOLISM AMONG FOUR RAT STRAINS


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