Short Communication

Genetic Basis of Inter- and Intrastrain Differences in Diazepam p-Hydroxylation in Rats

Received August 30, 2008; accepted October 24, 2008

ABSTRACT:

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one) is widely used as a sedative, hypnotic, and anti-anxiety drug. At low diazepam concentrations, p-hydroxylation is the major metabolic pathway in rat liver microsomes. However, there are marked (−300-fold) inter- and intrastrain differences in the activity among Sprague-Dawley, Brown Norway, Dark Agouti, and Wistar rats. In our previous study, we determined that a deficiency of CYP2D3 protein, not CYP2D2, was responsible for the inter- and intrastrain differences in diazepam p-hydroxylation (Drug Metab Dispos 33:1657–1660, 2005). Quantitative real-time polymerase chain reaction (PCR) did not provide enough evidence to explain the inter- and intrastrain differences in the expression of CYP2D3 protein. Nucleotide sequence analysis revealed the insertion of a thymine in exon 8 of the CYP2D3 gene in the poor diazepam metabolizers. This single nucleotide mutation caused a shift in the reading frame and introduced a premature termination signal. It is noteworthy that the heme binding region, which is essential to maintain proper heme binding and active cytochrome P450 enzymes, was consequently deleted by the premature termination signal. In contrast, no mutation was detected in the CYP2D3 gene of extensive metabolizers. Thus, the truncated CYP2D3 must be a nonfunctional enzyme in poor metabolizers. In addition, we developed a convenient and specific genotyping assay using PCR-restriction, fragment-length polymorphism to distinguish homozygotes from heterozygotes. The genotyping gave results fully consistent with those of the inter- and intrastrain differences in diazepam p-hydroxylation.

A variety of strains of rats have been used as animal models in pharmacological and toxicological studies worldwide. To date, many strains of rats have been established and screened for various purposes, yet little is known about the differences in their drug metabolism characteristics that are ascribable to genetic variation. Currently, information on genetic variation in rat laboratory strains is accumulating as a set of microsatellite markers, simple sequence length polymorphism markers, and a variety of single nucleotide polymorphisms in coding regions (Thomas et al., 2003; Serikawa, 2004). Nevertheless, information on genetic variation in rat strains is still deficient compared with that on humans and mice.

Cytochrome P450 (P450) comprises a superfamily of heme-containing monoxygenases that include enzymes responsible for drug metabolism. Although it occupies only approximately 2% of human liver P450, the CYP2D subfamily encodes for enzymes that are of clinical importance in the metabolism of numerous drugs (Shimada et al., 1996). The CYP2D subfamily has been extensively studied due to the involvement of CYP2D6 in the human debrisoquine/sparteine drug oxidation polymorphism (Eichelbaum and Gross, 1990). The genetic polymorphisms of CYP2D6 are among the most important factors in pharmacogenetics. In rats, six gene homologs to CYP2D6 have been isolated, namely, CYP2D1, CYP2D2, CYP2D3, CYP2D4, CYP2D5, and CYP2D18 (Nelson et al., 1996).

In recent studies, we reported inter- and intrastrain differences in P450-dependent diazepam metabolism in rat liver microsomes (Saito et al., 2004a,b). Sprague-Dawley (SD) and Brown Norway (BN) rats had significantly higher levels of diazepam p-hydroxylation, which is the major metabolic pathway at concentrations of this drug close to the in vivo concentrations used for medication. In contrast, Dark Agouti (DA) rats completely lacked the activity. It is interesting to note that Wistar rats exhibited a phenotype polymorphism, having extensive metabolizers (EM) and poor metabolizers (PM). Thus, we classified EM Wistar rats as EM-W and PM Wistar rats as PM-W, respectively, in tests of diazepam p-hydroxylation. Comparing the expression levels of the CYP2D subfamily in liver microsomes by immunoblotting detected a band of proteins, the N-terminal amino acid sequences of which exactly corresponded to those of CYP2D3, in SD, BN, and EM-W rats. Moreover, using rat CYP2D2B isoforms expressed in yeast, the observation that diazepam p-hydroxylation is catalyzed by CYP2D2 was confirmed. Therefore, the polymorphic expression of CYP2D3 caused the inter- and intrastrain differences in diazepam p-hydroxylation among the four rat strains (Sakai et al., 2005). However, the question of what causes the polymorphic expression of CYP2D3 among the four rat strains remained to be solved.

Clarification of the inter- and intrastrain differences in rats will be useful for predicting variability in human pharmacokinetics. Therefore, it is worthwhile to fully characterize animals used in pharmacokinetic studies.
kinetics studies from the point of view of the genetic expression of metabolic enzymes. In this study, we describe the relationship between a defect in the CYP2D3 gene (genotype) and the inter- and intrastrain differences in diazepam p-hydroxylation (phenotype) among four rat strains.

Materials and Methods

Materials and Animals. All reagents were of analytical grade. Adult male SD, DA, and Wistar rats (9 weeks old) were obtained from Nihon SLC Co. (Shizuoka, Japan). Adult male BN rats (9 weeks old) were obtained from Kyudo Co., Ltd. (Fukuoka, Japan). They were housed under standard laboratory conditions with free access to food and water, and they were used for experiments after 1 week of acclimatization. All experiments using animals were performed with the supervision and approval of the Animal Care and Use Committee of Hokkaido University.

Isolation of Total RNA and Genomic DNA from Rat Liver. Total RNA was isolated from rat liver using Isogen (Nippon Gene, Toyama, Japan). Genomic DNA was isolated from rat liver using a DNeasy Tissue Kit (QIAGEN GmbH, Hilden, Germany). The concentration and purity of both DNA and RNA were determined by using a spectrophotometer. The integrity was examined by electrophoresis in a 1% agarose gel with ethidium bromide staining.

Quantitative Real-Time Polymerase Chain Reaction Analysis. The cDNA samples were obtained using 1 μg of total RNA as the template for reverse transcription with ReverTraAce and oligo(dt) 20 primer (Toyobo, Osaka, Japan). TaqMan MGB probes and primers for CYP2D3 and β-glucuronidase, as an endogenous control, were purchased from ABI (Applied Biosystems, Foster City, CA). According to the manufacturer’s instructions, a primer for CYP2D3 was designed around the boundary between exons 4 and 5. TaqMan polymerase chain reaction (PCR) reaction was performed using Real-time PCR Master Mix (Toyobo) and an automated DNA sequencer (ABI Prism 310 Genetic Analyzer) (Applied Biosystems) following the manufacturer’s instructions.

CYP2D3 Genotyping Using PCR-Restriction Fragment Length Polymorphism. The single PCR and restriction fragment length polymorphism (RFLP) methods were used to identify the wild-type and mutant CYP2D3 alleles. CYP2D3-specific PCR was accomplished with the primer pairs 5′-TCACATATCCCTGTATA-A3′ and 5′-TCATGTCAGTCTGGAGCT-3′, yielding a single 384-base pair (bp) product. The reaction mixture contained approximately 100 ng of genomic DNA, 0.5 μM of each primer, 0.2 μM of each dNTP, 2 mM MgCl2, and 1 U of ExTaq polymerase (Takara Bio, Shiga, Japan) in a total volume of 50 μl. Touchdown PCR was performed as follows: the first cycle of denaturation at 94°C for 60 s, annealing at 65°C for 30 s, elongation at 72°C for 30 s; the 2nd to 30th cycles of denaturation at 94°C for 30 s, annealing at 63 to 48.5°C for 30 s, elongation at 72°C for 30 s; the 31st cycle of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, elongation at 72°C for 30 s; and the final elongation at 72°C for 5 min. In touchdown PCR, the annealing temperature began at 63°C and was reduced by 0.5°C every cycle. Six microliters of each of the PCR products was then digested by the restriction enzyme XmnI (NEB, Ipswitch, MA) without further purification. The digested PCR products were electrophoresed in a 2% agarose gel with ethidium bromide staining. The fragment patterns determined the presence of the CYP2D3 wild-type and mutant alleles, as indicated in Fig. 2.

Results and Discussion

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one) is one of the benzodiazepines that are widely used in the treatment of anxiety disorders, depression, and insomnia. Inaba et al. (1988) reported interindividual variability of diazepam metab-
A frameshift mutation with a single thymine insertion at nucleotide 1269 in exon 8 (Fig. 1). This mutation consequently created a premature termination at codon 434 of the CYP2D3 gene. It is interesting to note that the region deleted by the premature termination codon contains the heme binding region and the highly conserved cysteine-containing portion of the P450 primary sequence in all species (Gonzalez, 1988). Deletion of the heme binding region, which is essential to maintain proper heme binding and active P450 enzymes, leads to the creation of a truncated protein that must function improperly or not at all. Therefore, the mutation in the sequence of CYP2D3 of DA and PM-W rats must plausibly be the cause of the significantly low diazepam p-hydroxylation. In contrast, SD and BN rats had no mutation in CYP2D3 cDNA, leading to the production of a functional protein. Nucleotide sequence analyses indicate that the genotypes of the extensive metabolizers (SD and BN rats) and that of poor metabolizers (DA and PM-W rats) are homozygous for wild and mutant types in the CYP2D3 gene, respectively. On the other hand, genomic DNA derived from the EM-W rat liver showed the heterozygosity of the CYP2D3 gene. This result is plausibly consistent with the fact that EM-W rats possess intermediate p-hydroxylation activity toward diazepam among the four rat strains.

We then developed a convenient and accurate genotyping assay based on PCR-RFLP to distinguish homozygote from heterozygote (Fig. 2). Using this assay, the genotype frequency among the four rat strains was determined (Table 1). The mutant homozygote (33/41, 80.5%) was more frequent than the heterozygote (8/41, 19.5%) in Wistar rats. In our previous report, we demonstrated that approximately 17% of outbred Wistar strain rats, referred to as EM-W in this article, showed high diazepam metabolism in their liver microsomes (Saito et al., 2004a). The genotype frequency reported in the current study is compatible with the phenotype frequency we previously reported.

Because the Wistar rats used in this study were from an outbred line, genetic heterogeneity was observed. Considering the fact that Wistar rats contribute to a large subset of strains such as Wistar-Kyoto rats, spontaneously hypertensive rats, and Wistar-Furth rats, it is reasonable to assume that the genetic variety is sustained in these populations. Although the identical mutation is detected in both the DA and Wistar rats, phylogenetic trees and a large-scale database suggest that DA rats are not closely related to Wistar rats (Thomas et al., 2003; Serikawa, 2004). We did not find any report in the literature on the relationship between DA and Wistar rats because the origin of DA rats remains uncertain. The lack of definitive knowledge about their origin has led to speculation that DA rats did not find any report in the literature on the relationship between DA and Wistar rats because the origin of DA rats remains uncertain.

Moreover, we reported that DA rats have a high level of expression of debrisoquine 4-hydroxylase polymorphism (Yamamoto et al., 1998). Intrastrain differences in the pharmacokinetics and pharmacodynamics of diazepam were documented among outbred Wistar rats (van der Laan et al., 1993; Bert et al., 2001). On the other hand, Mechan et al. (2002) reported strain differences between SD and DA rats in the pharmacodynamics of diazepam. The considerable differences in the plasma levels of diazepam and its metabolites indicated inter- and intrastrain variations in diazepam metabolism. We previously reported that the polymorphic expression of CYP2D3 in the liver caused the inter- and intrastrain differences in diazepam p-hydroxylation (Sakai et al., 2005). However, the mechanism underlying the polymorphic expression of CYP2D3 among the four rat strains remained to be described.

We initially analyzed the expression level of CYP2D3 mRNA by quantitative real-time PCR. However, the expressions of CYP2D3 mRNA in DA and PM-W rats were comparable with that of SD rats. To investigate whether CYP2D3 mRNA encodes the functional protein, we subsequently cloned and sequenced the full length of CYP2D3 cDNA. We found a mutation within a highly similar sequence region among the rat CYP2D subfamily, spanning exon 8, intron 8, and exon 9. In DA and PM-W rats, there was a common frameshift mutation with a single thymine insertion at nucleotide 1269.

![Fig. 2. Genotyping strategy using the PCR-RFLP method for CYP2D3 gene. A, horizontal arrows show specific primers used for the target amplification. The vertical arrow and vertical bar show the recognition site for the restriction enzyme Xmn1. Figures indicate the undigested and digested fragment lengths of the PCR product. B, lane 1 shows a 100-bp DNA ladder-size marker (Toyooho); lane 2 shows the undigested PCR product (384 bp); and lanes 3 to 5 show the RFLP of the PCR product amplified from BN, EM-W, and DA rat genomic DNA, respectively.](image)

### TABLE 1

<table>
<thead>
<tr>
<th>Genotype (Frequency)</th>
<th>Activity, Diazepam p-Hydroxylation pmol/min/mg protein</th>
</tr>
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<tbody>
<tr>
<td>Total</td>
<td>Wild Homozygote</td>
</tr>
<tr>
<td>SD</td>
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</tr>
<tr>
<td>BN</td>
<td>9</td>
</tr>
<tr>
<td>DA</td>
<td>8</td>
</tr>
<tr>
<td>PM-W</td>
<td>33</td>
</tr>
<tr>
<td>EM-W</td>
<td>8</td>
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* Significant difference from PM-W rats (P < 0.01).
CYP2D4 protein in liver microsomes (Sakai et al., 2005). These data suggest that DA rats have a unique expression pattern in the CYP2D subfamily.

In conclusion, we reported that the deficiency of CYP2D3 protein caused by the defect in the wild-type CYP2D3 gene results in the inter- and intrastrain differences in diazepam metabolism.

References


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