Characterization of the Novel Defective CYP2C9*24 Allele

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ABSTRACT:

CYP2C9 is one of the major drug-metabolizing enzymes, and it is involved in the oxidative metabolism of approximately 10% of clinically important drugs, among which some, such as the anticoagulant warfarin, have a narrow therapeutic index. The human CYP2C9 gene is highly polymorphic. We found a new sequence variation in exon 7 of the CYP2C9 gene (1060G>A) resulting in a substitution of acidic amino acid glutamate to basic lysine (E354K) when translated. The allele, designated CYP2C9*24, was present in heterozygous state in one warfarin-treated patient. To characterize the CYP2C9*24 allele, we expressed the wild-type and CYP2C9*24 protein in a recombinant yeast expression system and in mammalian cells (HEK-293). Carbon monoxide difference spectra were recorded on dithionite-reduced microsomes, and protein was determined by Western blotting. Transfection with CYP2C9.1 cDNA resulted in detectable CYP2C9 protein in yeast or HEK-293 cells, whereas only small amounts of the protein were detected in yeast transfected with CYP2C9*24 cDNA. A strong differential absorption peak at 450 nm was observed with microsomes of yeast transfected with CYP2C9*1 cDNA, whereas no peak was detected with microsomes of yeast transfected with CYP2C9.24 cDNA or empty pYeDP60 plasmid. These results suggest that CYP2C9*24 may be improperly folded, both in yeast and mammalian cells, resulting in improper heme incorporation and rapid intracellular degradation. The data obtained in the expression systems are consistent with our findings in vivo. In conclusion, we have identified a novel defective CYP2C9 variant allele of potential importance for drug metabolism in vivo.

CYP2C9 is involved in the metabolism of more than 100 currently used drugs, corresponding to about 10 to 20% of commonly prescribed drugs (Kirchheiner and Brockmoller, 2005). Some of them (e.g., anticoagulant warfarin) have a narrow therapeutic index (Miners and Birkett, 1998). Clinically available warfarin is a racemic mixture, whereas no peak was detected with microsomes of yeast transfected with CYP2C9.24 cDNA or empty pYeDP60 plasmid. These results suggest that CYP2C9*24 may be improperly folded, both in yeast and mammalian cells, resulting in improper heme incorporation and rapid intracellular degradation. The data obtained in the expression systems are consistent with our findings in vivo. In conclusion, we have identified a novel defective CYP2C9 variant allele of potential importance for drug metabolism in vivo.

Materials and Methods

Preparation of CYP2C9 Expression Constructs. The yeast expression vector pYeDP60, which contained the wild-type CYP2C9 cDNA (AstraZeneca, R&D, Umeå, Sweden), was used as a template to generate mutated CYP2C9*24 cDNA. QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce 1060G>A mutation with mutagenic oligonucleotides mut2C9_F (5'-CAGTGTCTGTGCTGCACAGGTCCAAGTTACATGTG-3') and mut2C9_R (5'-CAGATCTGGTCCTGACGACATCTG-3'). The CYP2C9*1 and CYP2C9*24 alleles were amplified from pYeDP60 vector using primers that introduced KpnI restriction site before the start codon (5'-ATAAGTGACCTGATCTTCTGTGGTC-3') and EcoRI site after the stop codon (5'-ATGATCTTCGACGCTGACGACAGCATG-3'). Fragments were purified by QiAquick Gel Extraction Kit (Qiagen, Valencia, CA) and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). All the constructs were verified by sequencing.

Expression in Yeast. pYeDP60 containing CYP2C9*1 or CYP2C9*24 cDNA were transfected by lithium acetate procedure in Saccharomyces cerevisiae strain W(R) that overexpresses yeast reductase (Pompon et al., 1996). Yeast cells transfected with empty pYeDP60 were used as a negative control. After 2 to 4 days of incubation at 28°C, the colonies were transferred to a new SGI (Mountain View, CA) plate (1 g/l casamino acids, 7 g/l yeast nitrogen base, 20 g/l glucose, and 20 mg/l tryptophan) making 2- to 3-cm lines and incubated for an additional 2 to 4 days at 28°C until the yeast had grown thick. Then the yeasts were transferred into 20 ml of SGI medium and grown overnight at 28°C until OD600 nm was around 10. The culture was poured into 230 ml of yeast peptone glycerol ethanol medium (10 g/l yeast extract, 10 g/l bacto-peptone, 5 g/l glucose, and 2% ethanol) and grown at 28°C to OD600 nm around 10 to 13. Induction was started by the addition of 28 ml of 20% galactose, and the growth was continued until OD600 nm reached 30 (Pompon et al., 1996).

The yeast cells were recovered by centrifugation, washed once with distilled water, and resuspended in Tris EDTA KCl buffer (50 mM Tris-HCL pH 7.4, 1 mM EDTA, and 0.1 M KCl). Cells were spun down again and resuspended in 2.5 ml of TES buffer (50 mM Tris-HCL, pH 7.4, 1 mM EDTA, and 0.6 M sorbitol), with added Complete protease inhibitor mixture (Roche Applied Science, Pentzberg, Germany). The yeast cells were mechanically disrupted using glass beads. Five milliliters of cold TES buffer was added to the crude extract three times, and the supernatant was withdrawn and centrifuged at 15,000g for 10 min at 4°C. The supernatant was then transferred to a new tube and centrifuged at 34,000g for 1 h at 4°C. The pellet, consisting of microsomes, was dissolved in 2.5 ml of cold Tris EDTA glycerol medium (50 mM Tris-HCL, pH 7.4, 1 mM EDTA, and 20% glycerol) (Pompon et al., 1996). The protein concentration was determined according to Lowry et al. (1951). The carbon monoxide-binding spectra analysis was recorded on dithionite-reduced microsomes (Omura and Sato, 1964). Yeast transfection and all the subsequent analysis were repeated three times.

Expression in HEK-293 Cells. Cells were grown at 37°C in atmosphere of 5% CO2 in minimum essential medium supplemented with fetal bovine serum, sodium pyruvate, nonessential amino acids, and antibiotics (Invitrogen). When HEK-293 cells reached 80 to 90% confluence, they were transiently transfected with pcDNA3.1 constructs using Lipofectamine 2000 transfection reagent (Invitrogen). Empty pcDNA3.1 was used as a negative control. After 60 h, the cells were harvested, homogenized, and fractionated by centrifugation (Karlgren et al., 2004). The protein concentration of microsomal fraction was determined according to Lowry et al. (1951), and the carbon monoxide-binding spectra analysis was recorded on dithionite-reduced microsomes (Omura and Sato, 1964). Transfection of HEK-293 cells and all the subsequent analysis were repeated three times.

Western Blot Analysis. Microsomes from transfected yeast or HEK-293 cells, corresponding to 30 μg of protein, were separated on 10% SDS-polyacrylamide gels and transferred to a Hybond-C extra membrane (Amer sham Biosciences, Uppsala, Sweden). The membrane was first incubated with a CYP2C9 antibody diluted 1:5000 (BD Bioscience, San Jose, CA) and then with the secondary antibody conjugated with horseradish peroxidase diluted 1:2000 (BD Bioscience). SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used for detection. Molecular weight marker was the BenchMark Prestained Protein Ladder (Invitrogen). Membranes were scanned using luminescence image analyzer LAS-3000 (Fujiﬁlm Life Science, Stamford, CT).

Results and Discussion

Most of the genes encoding enzymes involved in drug metabolism are genetically diverse. CYP genes, in particular, have higher density of polymorphic sites and higher genetic diversity than any other human genes examined. Among subfamilies involved in phase I drug metabolism, CYP2 displays the highest level of genetic diversity. It is particularly important to predict the function of nonsynonymous sequence variation in CYP genes and to correlate that function to phenotypic observation because of their role in drug metabolism and adverse drug reactions (Solus et al., 2004).

In the present study, we have characterized a rare variant CYP2C9*24 allele that was previously found in one warfarin-
western blot analysis of CYP2C9 using CYP2C9 antibody. Microsomes were prepared from heK-293 cells transfected with CYP2C9*1 and CYP2C9*24 cDNA or empty pcDNA3.1. Thirty micrograms of protein was loaded per lane.

radiation rate were recently reported in variant CYP1B1 allele with N453S substitution (Bandiera et al., 2005).

To further test this hypothesis, we heterologously expressed wild-type and mutated CYP2C9 proteins in mammalian HEK-293 cells. Carbon monoxide difference spectral analysis of the microsomal fraction obtained from transfected HEK-293 cells showed that only wild-type CYP2C9.1 protein was correctly folded (60 pmol/mg microsomal protein) (Fig. 2A), whereas no cytochrome P450 carbon monoxide spectrum was observed for the cells transfected with CYP2C9*24 cDNA or with empty pcDNA3.1 plasmid. Western blot analysis revealed that only cells transfected with wild-type construct produced detectable levels of CYP2C9.1 in all independent replications of transfection (Fig. 2B). These results were confirmative of those obtained from the yeast expression system. The only difference was that in the HEK-293 expression system not even a small amount of incorrectly folded protein could be detected. Similar discrepancies have been reported between yeast and mammalian systems (Oscarson et al., 1999), suggesting that yeast may be a more permissive expression system for poorly foldable proteins.

E354 is positioned in the carboxyl-terminal portion of the helix K and is a part of the absolutely conserved EXXR sequence, which may be involved in stabilizing the core structure. This sequence is buried in the interior of the protein and participates in a hydrogen bond network, together with the amino acid residues from the meander region (Fig. 3). Meander region is also highly conserved in structure (Graham and Peterson, 2002). The substitution of glutamate to lysine would disrupt the salt bridges between E354, R357, and M406 from the meander region. The bigger size of the lysine is also inconsistent with tight packing interactions with the amino acid residues that surround E354. Therefore, the substitution would probably result in disruption of the interaction of helix K with the meander region and would lead to protein misfolding or instability, which was also suggested by our experimental data.

In conclusion, our data suggest that the substitution of acidic amino acid glutamate to basic lysine may lead to incorrectly folded CYP2C9.24 protein that is rapidly degraded in both yeast and mammalian cells. Furthermore, our data support our observations of lower warfarin dose requirements and lower (S)-warfarin clearance in a patient with the CYP2C9*24 allele (Herman et al., 2006).

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