Drug Metabolism and Pharmacokinetics xxx (2017) 1–6

Contents lists available at ScienceDirect

Drug Metabolism and Pharmacokinetics

journal homepage: http://www.journals.elsevier.com/drug-metabolism-and-pharmacokinetics

Regular Article

Terfenadine metabolism of human cytochrome P450 2J2 containing genetic variations (G312R, P351L and P115L)

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ARTICLE INFO

Article history:
Received 1 August 2017
Received in revised form
22 September 2017
Accepted 30 October 2017
Available online xxx

Keywords:
Cytochrome P450
P450 2J2
Terfenadine
Genetic variation
Allele

ABSTRACT

The human cytochrome P450 2J2 is involved in several metabolic reactions, including the oxidation of important therapeutics and epoxidation of endogenous arachidonic acid. At least ten genetic variations of P450 2J2 have been identified, but their effects on enzymatic activity have not been clearly characterized. Here, we evaluated the functional effects of three genetic variations of P450 2J2 (G312R, P351L, and P115L). Recombinant enzymes of wild-type and three variant P450 2J2 were heterologously expressed in *Escherichia coli* and purified. P450 expression levels in the wild-type and two variants (P351L and P115L) were 142–231 nmol per liter culture, while the G312R variant showed no holoenzyme peak in the CO-binding spectra. Substrate binding titrations to terfenadine showed that the wild-type and two variants displayed K_d values of 0.90–2.2 μM, indicating tight substrate binding affinities. Steady-state kinetic analysis for *t*-butyl methyl hydroxylation of terfenadine indicated that two variant enzymes had similar k_{cat} and K_m values to wild-type P450 2J2. The locations of mutations in three-dimensional structural models indicated that the G312R is located in the I-helix region near the formal active site in P450 2J2 and its amino acid change affected the structural stability of the P450 heme environment.

1. Introduction

Human cytochrome P450 (P450, CYP) enzymes gained significant attention because they catalyze the oxidation reactions of major drugs as well as endogenous compounds, including eicosanoids, steroids, and vitamins [1]. Their catalytic activities are important in drug-drug interactions and endocrine function [2,3]. The P450 2 family includes approximately one-third of all human P450 enzymes, making it the largest family in humans [4]. The P450 2J subfamily was originally described by Kikuta and coworkers in 1991 [5] and includes a single gene, P450 2J2, in human. Its gene is located on the short arm of human chromosome 1, band p31.3-p31.2 [6]. Human P450 2J cDNA was first isolated from the liver, which showed high sequence identity to human P450 2A6, 2B6, and 2C10 [6,7]. Human P450 2J2 is generally considered an extrahepatic and is expressed at high levels in the heart, predominantly in cardiac myocytes and endothelial cells lining the small and large coronary arteries [4,7,8].

P450 2J2 has attracted much attention because it metabolizes arachidonic acid (AA), which can be converted into four different epoxyeicosatrienoic acids (EETs) with various biological activities that are particularly important in the cardiovascular system [9,10]. P450 2J2 was also shown to be rather proficient in the oxidation of a number of drugs [4]. It is involved in the metabolism of several antihistamine drugs including ebastine, terfenadine, and astemizole [11,12]. Its catalysis involves the hydroxylation of ebastine and terfenadine to produce hydroxyebastine and hydroxyterfenadine and the *O*-demethylation of astemizole to form desmethylastemizole [11,12].

At least nine allelic variants of P450 2J2 have been identified and named P450 2J2*2 to *10 (http://www.cypalleles.ki.se/). P450 2J2*2, *3, *4, *5, and *6 contain 427A>, 472C>T, 575T>A, 1024G>A, and 1210A>T mutations leading to T143A, R158C, I192N, D342N and N404Y, respectively [13]. P450 2J2*7 contains a G>T substation in the regulatory domain of transcription initiation site −76 [13,14]. In 2005, two novel P450 2J2 variations, P450 2J2*8 and *9 with frequencies of 0.8% and 0.18% in Koreans, respectively, were reported [15]. Additionally, P450 2J2*10 was recently identified in one fetal tissue and contains a 344C>T mutation in exon 2, leading to P115>L [16].

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https://doi.org/10.1016/j.dmpk.2017.10.004
1347-4367/

In this study, we analyzed the biochemical properties of three allelic variants of P450 2J2*8, *9, and *10 (G312R, P351L, and P115L) containing nonsynonymous single-nucleotide polymorphisms. The association of these genetic variations with clinical drug metabolism is important in numerous cardiovascular diseases.

2. Materials and methods

2.1. Chemicals and enzymes

Terfenadine, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and $NADP^+$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ni^{2+}-nitrilotriacetate (NTA) agarose was purchased from Qiagen (Hilden, Germany). Other chemicals were of the highest commercially available grade. *Escherichia coli* DH5α cells were purchased from Invitrogen (Carlsbad, CA, USA). Rat NADPH-P450 reductase was heterologously expressed in *E. coli* (HMS173) and purified as described previously [17,18].

2.2. Construction of expression plasmids for P450 2J2 genetic variations

cDNA of the human P450 2J2 gene was kindly provided by Professor Sang Seop Lee (Inje University, Korea). The construct of the pCW expression vector for P450 2J2 wild type was previously described [19]. Using site-directed mutagenesis with a QuikChange (Stratagene, La Jolla, CA, USA) site-directed mutagenesis kit, three variant clones of P450 2J2 were constructed. Primers used for the site-directed mutagenesis were 2J2*8 (5′-CCT CTT CTT TGC CAG AAC CGA GAC AAC-3′, 5′-GTT GTC TCG GTT CTG GCA AAG AAG AGG-3′), 2J2*9 (5′-CAG GGG CAG CAG CTG AGC ACA GCC GCC-3′, 5′-GGC GGC TGT GCT CAG CTG CTG CCC CTG-3′), and 2J2*10 (5′-CCG CCC CGT GAC CCT TAT GCG AGA ACA TA-3′, 5′-TAT GTT CTC GCA TAA GGG TCA CGG GGC GG-3′). The constructed variant clones were confirmed by nucleotide sequencing analysis and restriction enzyme digestion.

2.3. Expression of P450 2J2 genetic variants

Expression of P450 2J2 wild-type and variants was carried out as previously described with some modifications [19,20]. The plasmids containing P450 2J2 wild-type and variant clones were transformed into *E. coli* DH5α (possessing pGro EL/ES). One transformed colony was selected and then inoculated into 5 mL of LB liquid medium (containing 50 μg/mL ampicillin and 20 μg/mL kanamycin) and grown overnight at 37 °C with shaking at 200 rpm. The culture was transferred into 500 mL of Terrific broth medium containing 50 μg/mL ampicillin and 20 μg/mL kanamycin. The cultures were incubated at 37 °C with shaking at 230 rpm. When the OD_{600} reached 0.4–0.6, induction was carried out by adding 1.0 mM isopropyl β-D-thiogalactoside, 0.5 mM 5-aminolevulinic acid, 1.0 mM thiamine, 0.25 g/mL arabinose, and trace elements. The cultures were further incubated at 28 °C with shaking at 180 rpm for 48 h, and then harvested by centrifugation.

2.4. Purification of P450 2J2 genetic variants

P450 2J2 variant enzymes were purified as previously described with some modifications [20,21]. Briefly, the harvested cells were resuspended in lysozyme-containing TES buffer (50 mM Tris-acetate, 0.25 mM EDTA, 250 mM sucrose, pH 7.6) and incubated at 4 °C for 30 min. Cell pellets were centrifuged and sonicated in 100 mL of sonication buffer containing 10 mM potassium phosphate (pH 7.4), 20% glycerol (v/v), 6.0 mM Mg-acetate, 0.1 mM DTT, and 100 mM PMSF. Membrane fractions were prepared after ultracentrifugation and solubilized in 100 mM potassium phosphate (pH 7.4) buffer containing 20% glycerol (v/v), 1.0 mM EDTA, 10 mM β-mercaptoethanol, and 1% CHAPS (w/v). After ultracentrifugation, solubilized proteins in the supernatant were loaded into an Ni^{2+}-NTA agarose column that had been preequilibrated with 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 5 mM imidazole, and 0.5 M NaCl. After loading, the columns were washed with 20 mM imidazole and then eluted with 300 mM imidazole. The eluted fractions containing variant P450 2J2 proteins were dialyzed three times in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol at 4 °C.

2.5. Spectral binding titrations

Purified P450 2J2 wild-type and variant enzymes were diluted to 1 μM in 100 mM potassium phosphate buffer (pH 7.4) and divided into two glass cuvettes. The spectra (350–500 nm) were measured while adding the substrate terfenadine in a CARY 100 Varian spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The substrate concentration was plotted against the difference in absorbance between the maximum and minimum wavelengths.

2.6. Catalytic activity analysis

The catalytic activity of terfenadine hydroxylation was determined by high-performance liquid chromatography (HPLC) with some modifications as previously reported [19,22]. The reactions contained 150 pmol of purified P450 2J2 (wild-type or variants), 300 pmol of rat NADPH-P450 reductase, and 0.5 μg/μL of DLPC in 0.5 mL of 100 mM potassium phosphate buffer (pH 7.4), along with varying concentrations of substrate. After preincubation at 37 °C for 3 min, the reaction was started using an NADPH-generating system (100 mM glucose 6-phosphate, 10 mM $NADP^+$, 1 mg/mL glucose-6-phosphate dehydrogenase). Incubations were generally conducted for 10 min 37 °C and terminated by adding 0.05 mL 2 N HCl. The reactions were extracted using 1 mL of CH_2Cl_2 by vortex mixing and centrifugation at 1000× *g*, 15 min. The lower organic layer was transferred to a clean test tube and then the 50 μL of the samples were injected into the HPLC. HPLC analyses of the reaction metabolites were performed using an YL9101 vacuum degasser (Younglin, Korea), YL9110 quaternary pump (Younglin), and Alltima HPLC Reversed-Phase C18 (5 μm, 150 × 4.6 mm) column (Alltech, Albany, OR, USA). The reaction was monitored at 260 nm using an YL9120 UV/Vis detector (Younglin). Reaction products were analyzed over a gradient from 50% buffer A (0.1 M Tris acetate, pH 4.6) and 50% buffer B ($CH_3CN/CH_3OH/H_2O$ = 7:2:1, v/v) to 100% buffer B for 20 min at a flow rate of 0.9 mL/min. The metabolites were identified using phenacetin as an internal standard, and the peaks were integrated. Steady-state kinetic parameters were calculated using GraphPad Prism software (GraphPad, Inc., La Jolla, CA, USA).

2.7. NADPH oxidation assay

NADPH oxidation rates were measured using a phospholipid reconstituted system containing P450 2J2 and NADPH-P450 reductase [23]. Reconstituted enzymes were preincubated at 37 °C for 5 min in the presence or absence of substrates (100 μM of terfenadine). The reactions were initiated adding 10 μL of 10 mM NADPH, and reduction at A_{340} was monitored. Oxidation rates were calculated using an extinction coefficient of $\Delta\varepsilon_{340}$ = 6.22 $mM^{-1}cm^{-1}$.

Please cite this article in press as: Jeong D, et al., Terfenadine metabolism of human cytochrome P450 2J2 containing genetic variations (G312R, P351L and P115L), Drug Metabolism and Pharmacokinetics (2017), https://doi.org/10.1016/j.dmpk.2017.10.004