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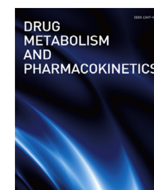

Regular Article

Biochemical analysis of recombinant CYP4A11 allelic variant enzymes: W126R, K276T and S353G

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ABSTRACT

Human CYP4A11 is the major ω−hydroxylase of fatty acids in the liver and kidneys. It produces 20-hydroxyeicosatetraenoic acid as well as hydroxylates fatty acids. In this study, we investigated the biochemical properties of three alleles of CYP4A11: W126R, K276T, and S353G. Site-directed mutagenesis of the wild type CYP4A11 was performed, to construct the W126R, K276T, and S353G variant clones. The CYP4A11 wild type and variant constructs were heterologously expressed in *Escherichia coli*. CO-binding spectra showed the expression of the wild type, K276T and S353G variants, indicating the functional P450 holoenzyme. The W126R variant was not expressed in *E. coli*. Binding affinities of lauric acid in K276T and S353G variants were stronger than that of wild type. Steady-state kinetics in the hydroxylation reaction of fatty acids were studied. The catalytic efficiencies (k_{cat}/K_m) of K276T and S353G variants in the reactions without cytochrome b_5 were approximately 2- and 4-fold higher, respectively, than that of wild type, and in the reactions with cytochrome b_5 they were approximately 2- and 3-fold higher, respectively. These results suggest that individuals carrying the alleles, K276T and S353G, might exhibit higher catalysis of CYP4A11, which may affect the endogenous metabolic products associated with regulation of blood pressure.

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1. Introduction

Human cytochrome P450 (P450, CYP) enzymes have garnered significant attention, because they catalyze the oxidative reactions of major drugs as well as important endogenous compounds, including eicosanoids, steroids, and vitamins [1]. There are 57 CYP enzymes in human genome and 12 of them (2J2, 2U1, 4A11, 4B1, 4F12, 4V2, 4F2, 4F3, 4F8, 5A1, and 8A1) catalyze fatty acids and eicosanoids [2]. CYP4A11 is a fatty acid hydroxylase that is expressed in human liver and kidneys. It produces 20-hydroxyeicosatetraenoic acid (20-HETE) from arachidonic acid as well as hydroxylates lauric acid to produce 12-hydroxy lauric acid as a major metabolite and 11-hydroxy lauric acid as a minor product [3].

There is no known clinical drug that is metabolized by CYP4A11, and collective interest has focused on the role of CYP4A11 in the regulation of cardiovascular system [2,4]. The most important genetic variation of CYP4A11 is T8590C (*rs1126742*). This substitution results in a Phe-to-Ser substitution at amino acid 434 which significantly reduces the catalytic 20-HETE synthase activity of CYP4A11, which is associated with hypertension and suggests that this CYP4A11 variant could be an important determinant in regulating blood pressure in humans [4]. However, Ward et al. reported that the urinary 20-HETE excretion has been shown to be lower in carriers of the C allele (TC and CC genotypes) than carriers of the T allele (TT genotype) but this substitution was not associated with blood pressure [5]. Human carriers of the C allele had higher diastolic blood pressure, aldosterone to renin ratio, and waist to hip ratio and the decreased 20-HETE was due to salt loading after adjustments for other covariates specially serum insulin concentration [6]. The frequency of T8590C is high in African Americans, followed by Asian, and low in Caucasians [7].

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The prosthetic heme group in CYP4A11 is covalently attached to a conserved glutamic acid residue in the I-helix [8,9]. This conserved glutamic acid is linked to a hydroxyl group on the heme 5-methyl group via an ester bond. The unique site of attachment involving the covalently linked heme may contribute to the distinct architecture of the substrate channel for the regioselective oxidation of fatty acids.

Genetic variations in metabolic enzymes can lead to altered responses to specific substrates. Specifically, allelic variations in the P450 enzymes have had the significant effect on the fate of xenobiotic or endogenous compounds [10,11]. At least nine allelic variants of CYP4A11 have been reported; however, the haplotype has not yet been determined (http://www.cypalleles.ki.se/). The variant, S353G, has been found in the Korean population with a frequency of 0.429; however, its functional characterization has not been studied [12]. In this study, we analyzed the biochemical properties of three allelic variants of CYP4A11: W126R, K276T, and S353G, containing nonsynonymous single nucleotide polymorphisms.

2. Materials and methods

2.1. Chemicals

Lauric acid, myristic acid, palmitic acid, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), *N*, *O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), 5-Aminolevulinic acid (ALA), $NADP^+$, and NADPH were purchased from SigmaAldrich (St. Louis, MO, USA). Ni^{2+}-nitrilotriacetate (NTA) agarose was purchased from Qiagen (Valencia, CA, USA). Other chemicals of the highest commercially available grade were used. *Escherichia coli* DH5α cells were purchased from Invitrogen (Life Technologies, Grand Island, NY, USA). Rat NADPH-P450 reductase (NPR) was heterologously expressed in *E. coli* (TOPP3 strain) and purified as described elsewhere [13].

2.2. Construction of CYP4A11 variants

We used a pCW bicistronic expression plasmid containing the coding sequence of CYP4A11 to construct expression vectors for the CYP4A11 variants. The general approach has been previously described [14,15]. Briefly, site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) with the following primers: 5′-CTGGCTCCAAGGATTGGGTA-3′, 5′-ATGGGTTAGGAACCTGGGTA-3′ (W126R); 5′- TCAACTACAGACGGAGGGGGAGC-3′, 5′-GCTCCCCCTCCGTCTGTAGTTGA-3′ (K276T); and 5′-GAGATCCACGGCCTCCTGGGTG-3′, 5′-CACCCAGGAGGCCGTGGATCTC-3′ (S353G). The open reading frames for CYP4A11 variants included 6 × His (polyhistidine-tag) at the C-terminals. The constructed variant clones were verified by nucleotide sequencing analysis.

2.3. Expression and purification of CYP4A11 variant enzymes

Expression and purification of CYP4A11 variant enzymes were carried out according to previously described methods with some modifications [16,17]. The *E. coli* DH5α strains transformed with pCW vectors were inoculated in LB medium containing 50 μg/mL ampicillin and incubated overnight at 37 °C. LB cultures were then inoculated in 500 mL of Terrific broth (TB) expression medium containing 50 μg/mL ampicillin. After incubation at 37 °C with shaking at 250 rpm for 4 h, the supplements (0.5 mM ALA, 1 mM Thiamin, and trace elements) were added, and the expression cultures were further grown at 28 °C with shaking at 200 rpm for 48 h. Bacterial inner membrane fractions containing CYP4A11 wild type and mutants were isolated from TB expression cultures, as described previously [14]. The isolated membrane fractions were solubilized in 100 mM potassium phosphate buffer (pH 7.4) including 20% glycerol, 0.1 mM EDTA, 10 mM β-mercaptoethanol, and 1.5% CHAPS (w/v) overnight at 4 °C. Purification of enzymes was performed using a Ni^{2+}-NTA affinity column eluted with 300 mM imidazole, as previously described [18,19].

2.4. Substrate binding assay

Purified CYP4A11 wild type and variant enzymes were diluted to a final concentration of 2 μM in 100 mM potassium phosphate buffer (pH 7.4) and divided between two glass cuvettes. Spectral changes (from 350 to 500 nm) were recorded with subsequent addition of fatty acids in methanol using a CARY 100 spectrophotometer (Varian). Binding affinities were estimated by fitting the data of absorbance difference to the quadratic equation using nonlinear regression analysis with Graph-Pad Prism software (Graph-Pad, San Diego, CA) [20].

$$\text{signal} = A_0 + \frac{\Delta A\sqrt{(K_d + [E] + [L])^2 + 4[E][L]}}{2[E]}$$

2.5. Catalytic activity assays

Catalytic activities of CYP4A11 variants in fatty acid hydroxylation were determined using the P450/NADPH-P450 reductase/phospholipid reconstituted systems [15,16]. The reaction mixture included 50 pmol purified CYP4A11 enzymes, 100 pmol rat cytochrome NADPH-P450 reductase (CPR), 100 pmol cytochrome b_5, and DLPC (50 μM) in 0.50 mL of 100 mM potassium phosphate buffer (pH 7.4), along with a specified amount of fatty acids. An NADPH-generating system was used to initiate reaction, after a 3-min preincubation at 37 °C. Incubations were generally performed for 20 min at 37 °C and terminated with 1 mL of CH_2Cl_2. The CH_2Cl_2 extract was dried under N_2 and then converted to trimethylsilyl derivatives by incubation with 50 μL of BSTFA at 70 °C for 10 min. The derivatized samples were analyzed on the Varian 240 ion-trap gas chromatograph-mass spectrometry system, and the hydroxylated products were determined as previously described [14]. Kinetic parameters were estimated from the fitted curves to the Michaelis–Menten equation using GraphPad Prism for nonlinear regression analysis [20].

$$\frac{v}{[E]_t} = \frac{k_{cat}[S]}{[S] + K_M}$$

3. Results

3.1. Expression and purification of recombinant CYP4A11 variant enzymes

Wild type CYP4A11 and all three variants were successfully constructed in the pCW bicistronic expression vectors, and recombinant CYP4A11 proteins were expressed in *E. coli*. The expression levels of wild type CYP4A11 and K276T and S353G variants in the whole cell culture were spectroscopically determined to be ~320, ~55, and ~200 nmol P450 L^{-1} culture medium, respectively (Fig. 1). However, no P450 holoenzyme spectrum was observed for the W126R variant (Fig. 1). Bacterial membrane fractions containing the CYP4A11 enzymes (wild

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