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The metabolism of lysophosphatidic acids by allelic variants of human soluble epoxide hydrolase

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ABSTRACT

Lysophosphatidic acids (LPAs) are phospholipids which have many physiological and pathophysiological functions. The human soluble epoxide hydrolase (sEH) plays a role in the metabolism of xenobiotics through its metabolism of aromatic hydrocarbon epoxides such as styrene oxide. sEH also has a phosphatase activity, and metabolizes LPAs. In this study, we investigated a purified wild-type (WT) and six allelic variants of sEH to evaluate differences in their activities toward LPAs. We found that the R103C and R287Q showed significantly lower activity than the WT sEH. We also found that the R103C and R287Q had significantly lower activity even when applied to only the N-terminal or C-terminal domain. The kinetic study determined that the R103C and R287Q had a lower *V*max/*K*m ratio toward stearoyl-LPA than the other variants. In a previous study, we found that WT sEH suppressed vascular endothelial growth factor (VEGF) mRNA in Hep3B cells; in the present experiments, all sEH variants except V442A suppressed VEGF mRNA levels in Hep3B cells. These results suggest that the R103C and R287Q have lower phosphatase activity, but that all the allelic variants have similar effects on VEGF suppression.

1. Introduction

Lysophosphatidic acids (LPAs) are phospholipids that act as important signaling molecules [1] and have been shown to function as substrates for the N-terminal domain of the human soluble epoxide hydrolase (sEH) [2,3]. LPA signaling contributes to a range of diseases, including neuropathic pain [4], cardiovascular diseases [5], cancer [6], fibrosis [7], infertility [8], and obesity [9]. The involvement of LPAs in signaling processes such as inflammatory response [10] and cell proliferation [11], suggests that these molecules would be good drug targets for the treatment of human diseases.

sEH is an enzyme with multiple biological functions and is involved in the metabolism of xenobiotics. The C-terminal domain of sEH catalyzes the hydrolysis of epoxides to their corresponding diols. Epoxides can be generated via oxidation of olefin or aromatic hydrocarbon by cytochrome P450s. sEH is present in the cytosolic and peroxisome fractions of the cell [12] and has a broad distribution in human tissues [13]. The endogenous substrates of sEH are epoxyeicosatrienoic acids (EETs), which play roles in the anti-inflammatory properties of endothelial cells [14], vasodilation [15], and cell proliferation [16], and sEH plays an important role in their proper functioning.

A single nucleotide polymorphism (SNP) variants of sEH have been identified, and several allelic variants of sEH have been shown to exhibit different epoxide hydrolase activities [17,18]. In human sEH, six allelic variants, K55R, R103C, C154Y, R287Q, V422A, and E470G, were identified [17]. With respect to phosphatase activity, Morisseau et al. showed that the sEH variant R287Q exhibited lower activity toward myristoyl-LPA than the wild-type (WT) [19]. However, the effect of allelic variants of human sEH on the phosphatase activities toward other LPAs remains unknown. The R103C amino acid substitution was associated with increased induction of cell death in cortical neurons in response to oxygen-glucose deprivation and re-oxygenation [20]. The R287Q was associated with increased plasma cholesterol levels in familial hypercholesterolemia [21] and with the onset on coronary artery calcification in Africa-American subjects [22]. R287Q has also been

Abbreviations: sEH, soluble epoxide hydrolase; sLPA, stearoyl L-α-lysophosphatidic acid (1-octadecanoyl-sn glycerol-3-phosphate) sodium salt; arachidonoyl LPA, arachidonoyl L-α-lysophosphatidic acid sodium salt; arachidoyl LPA, arachidoyl L-α-lysophosphatidic acid; GGPP, geranylgeranyl pyrophosphate ammonium salt; S1P, sphingosine-1-phosphate; 4-MUP, 4-methylumbelliferyl phosphate; RT-PCR, reverse-transcription polymerase chain reaction; VEGF, vascular endothelial growth factor; PHOME, 3-phenyl-cyano (6-methoxy-2-naphthalenyl) methyl ester-2-oxiraneacetic acid; *t*-SO, *trans* stilbene oxide.

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associated with insulin resistance in type 2 diabetic patients [23]. In addition, in human, the individuals which have a K55R variant have a higher risk of coronary heart diseases [24].

LPAs have been shown to function as substrates for the N-terminal phosphatase domain of sEH [2,3]. The functions of the phosphatase activities of sEH have been shown to include argumentation of the cholesterol synthesis in HepG2 cells [25]. In addition, we previously found that the phosphatase activity of sEH suppressed VEGF levels and cell growth in Hep3B cells [26]. In peritoneal mesothelial cells, LPA has been shown to induce VEGF expression in a concentration dependent manner [27]. Phosphatase activity of sEH has been linked to VEGF expression, but there is no evidence of physiologically relevant of allelic variants of sEH in VEGF expression. An examination of the actions of allelic variants of sEH on LPA hydrolysis could reveal other biological functions associated with the phosphatase activity.

In this study, we examined the mutated residues of sEH allelic variants to understand the effect of these variants on the metabolism of LPAs. These investigations may be useful for further clarifying the role of sEH allelic variants in regulating the hydrolysis of LPAs *in vivo*.

2. Materials and methods

2.1. Materials

Stearoyl L-α-lysophosphatidic acid (1-octadecanoyl-sn glycero-3-phosphate) sodium salt (s-LPA) was purchased from Avanti Polar Lipids (Birmingham, AL), arachidonoyl L-α-lysophosphatidic acid sodium salt (arachidonoyl-LPA) and arachidoyl L-α-lysophosphatidic acid (arachidoyl-LPA) were obtained from Echelon Biosciences Inc. (Salt Lake, UT), geranylgeranyl pyrophosphate ammonium salt (GGPP) was obtained from Sigma Chemical (St. Louis, MO), and sphingosine-1-phosphate (S1P) was kindly provided by Professor Katsumura of Kwansei Gakuin University. 3-phenyl-cyano (6-methoxy-2-napthalenyl) methyl ester-2-oxiraneacetic acid (PHOME) was purchased from Cayman Chemical (Ann Arbor, MI). Trans-stilbene oxide (t-SO) was obtained from Acros Organics (Fair Lawn, NJ). 4-methylumbelliferyl phosphate (4-MUP) was obtained from Wako Pure Chemicals (Osaka, Japan).

2.2. Preparation of the constructs of allelic variants of sEH for expression by Escherichia coli

The human WT and N-terminal domain (amino acids 1–221) of sEH in pET21a vector were constructed as described previously [2]. The primers used in this study are shown in Table 1. For the construction of sEH variants, the first half fragment was amplified with the primer sets 1 and 2 (K55R), 1 and 3 (R103C), 1 and 4 (C154Y), 1 and 5 (R287Q), 1 and 6 (V422A), and 1 and 7 (E470G), and the latter half fragment was amplified with the primer sets 8 and 9 (K55R), 8 and 10 (R103C), 8 and 11 (C154Y), 8 and 12 (R287Q), 8 and 13 (V422A), and 8 and 14 (E470G) using WT sEH cDNA as a template. The full-length of each variant was amplified with the primers 1 and 8 using fragments of the first half and the latter half as a template, and inserted into the pET21a vector with NdeI and XhoI sites. For construction of the C-terminal domain of sEH (amino acids 230–555), the fragment was amplified with the primers 15 and 16, and connected after the N-terminal 1st-5th amino acids of sEH with a BssHII site. The N-terminal allelic variants of sEH (N-K55R, N-R103C, N-C154Y) were amplified with the primers 1 and 16 using each allelic variant of full-length sEH as a template, and inserted into the pET21a vector. The C-terminal allelic variants of sEH in the pET21a vector (C-R287Q, C-V422A, and C-E470G) were prepared by insertion of the mutation into the C-terminal domain of sEH in the same way as for the full-length variants of sEH. The WT and all variants of the sEH protein were expressed in *E. coli* (BL21(DE3)codon+) for 24 h at 25 °C. The proteins were purified with an Ni-NTA agarose column (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified proteins were dialyzed against 50 mM Tris-HCL buffer, pH 7.5.

2.3. Cell culture and expression

The full-length WT or allelic variants of sEH (K55R, R103C, C154Y, R287Q, V422A, and E470G) were ligated into *Bam*HI/*Eco*RI sites of the pcDNA3.1(+) vector (Invitrogen). The pcDNA plasmids containing sEH allelic variants were transfected into Hep3B cells, and the transfected cells were selected by G418. Total RNA was extracted from cells with Isogen according to the manufacturer's instructions, and converted to cDNA by reverse-transcription. Real-time PCR was performed with SYBR premix Ex Taq II following the manufacturer's instructions. PCR was performed using a Thermal Cycler Dice Real Time System Single TP850 (Takara, Shiga, Japan). The primers for human *VEGF* (Acc No. NM_ AF022375) were 5′-TTCATGGATGTCTATCAGCG-3′ (forward) and 5′-CATCTCTCCTAT GTGCTGGC-3′ (reverse). The primers for human *histone* (Acc No. NM_003548) were 5′-TATCGGGCTCCAGCGGTCATGTC-3′ (forward) and 5′-GGATCGAAACGTGCAAAGCTGGAG-3′ (reverse).

2.4. Phosphatase activity assay and kinetics

Phosphate release was detected by the Biomol green assay. The Biomol green assay kit was purchased from Enzo Life Sciences (Plymouth Meeting, PA) and the assay was performed according to the manufacturer's instructions. Purified human sEH allelic variants (9.5 pmol) were pre-incubated for 5 min at 37 °C in 25 mM Bis Tris–HCl buffer, pH 7.0, containing 1 mM $MgCl_2$ and 0.01% BSA. Each substrate was added at the final concentration of 10 μM and incubated for 5 min at 37 °C. The reaction was stopped by the addition of Biomol green reagent and held at room temperature for 60 min. The resulting green color was measured by an EnVision 2104 Multilabel Reader (Perkin Elmer, Foster City, CA) at 630 nm. For comparison with the synthetic substrate, phosphatase activity was measured using 4-MUP in 25 mM Bis Tris–HCl buffer, pH 7.0, containing 1 mM $MgCl_2$ and 0.01% BSA. Purified full-length sEH (80 pmol) or N-terminal domain variants (80 pmol) were used. The reaction was started by addition of 4-MUP at a concentration of 0.5 mM. The reaction was performed at 37 °C for up to 60 min and the fluorescence intensity of the produced 4-methylumbelliferone was measured every 5 min by an EnVision 2104 Multilabel Reader at an excitation wavelength of 330 nm and emission wavelength of 465 nm. For the determination of kinetic parameters of sEH variants, phosphatase activities toward stearoyl-LPA (final concentration: 2, 4, 8, 15 and 20 μM) were measured, and the *K*m and *V*max were obtained using Prism Graphpad enzyme kinetic software as described previously [2].

2.5. Epoxide hydrolase activity assay

Epoxide hydrolase activities of the full-length and C-terminal allelic variants of sEH were measured with a fluorescent substrate, PHOME [28]. The full-length (1.6 pmol) and C-terminal domain (1.6 pmol) of sEH were reacted with 25 μM PHOME in 25 mM Bis Tris-HCL buffer, pH 7.0, containing 0.01% BSA at 30 °C. The reaction mixture was analyzed by an Envision 2014 Multilabel Reader every 1 min for 30 min at an excitation wavelength of 330 nm and emission wavelength of 465 nm. The fluorescence intensity of the reaction product, 6-metoxy-2-naphthaldehyde (6-MNA), was determined with a calibration curve prepared with the authentic