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Role of carboxylesterase 1 and impact of natural genetic variants on the hydrolysis of trandolapril

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ABSTRACT

Carboxylesterase 1 (CES1) and carboxylesterase 2 (CES2) are the major hydrolytic enzymes responsible for the metabolism of numerous therapeutic agents as well as endogenous substrates. CES1 and CES2 differ distinctly in their substrate specificity and tissue distribution. In this study, we investigated the role of CES1 and CES2 in converting the antihypertensive prodrug trandolapril to its more active form trandolaprilat, and determined the influence of two newly identified *CES1* mutations p.Gly143Glu and p.Asp260fs on trandolapril metabolism. Western blot analysis demonstrated that CES1 is expressed in human liver microsomes (HLM) but not in human intestinal microsomes (HIM). *In vitro* incubation studies were conducted to contrast the enzymatic activity of HLM as well as HIM upon trandolapril hydrolysis. Trandolapril was rapidly hydrolyzed to its principal active metabolite trandolaprilat after incubation with HLM. In contrast, in HIM, where CES2 is predominantly expressed, incubations did not produce any detectable trandolapril hydrolysis. Furthermore, hydrolysis of trandolapril catalyzed by wild type (WT) and mutant CES1 were assessed utilizing transfected Flp-In-293™ cells stably expressing WT CES1 and two variants. WT CES1 efficiently hydrolyzed trandolapril to trandolaprilat with V_{max} and K_m values of 103.6 ± 2.2 nmole/min/mg protein and 639.9 ± 32.9 μM, respectively. However, no appreciable trandolapril hydrolysis could be found after incubation with both p.Gly143Glu and p.Asp260fs variants. Thus, trandolapril appears to be a CES1 selective substrate while CES2 exerts little to no catalytic activity towards this compound. *CES1* mutations p.Gly143Glu and p.Asp260fs are essentially dysfunctional enzymes with regard to the conversion of trandolapril to its more active metabolite trandolaprilat.

1. Introduction

Carboxylesterases (CESs) are α/β hydrolase fold proteins and comprise a multigene superfamily [1]. CES isozymes are classified into four main subfamilies, CES1, CES2, CES3 and CES4 [2]. Among them, CES1 and CES2 are considered the two predominant CESs responsible for the hydrolysis of numerous xenobiotics and endogenous esters and amides. In humans,

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CES1 is primarily expressed in the liver but also found in other tissues including the heart, testis, macrophages, and lung epithelia. CES2 is primarily expressed in the small intestine (where CES1 expression is essentially absent), the colon, kidney, liver, heart, and brain [3]. CES1 and CES2 are further differentiated from one another with regard to substrate structural specificity. In general, CES1 preferentially hydrolyzes substrates with a small alcohol group and a large acyl group, while CES2 more readily catalyzes the hydrolysis of compounds with a relatively large alcohol group and small acyl constituent [4]. For instance, methylphenidate, a methyl ester psychostimulant widely used in the treatment of the neurobehavioral disorder attention-deficit hyperactivity disorder (ADHD), is exclusively metabolized by CES1 [5]. A further differentiation between these two enzymes is the ability of CES1 but not CES2 to catalyze transesterification reactions that have been observed for a number of substrates when combined with ethanol including methylphenidate, meperidine, and most notably cocaine and its conversion to its ethyl ester cocaethylene [6–8].

Recently, while conducting a single-dose pharmacokinetic study of racemic methylphenidate in normal volunteers ($n = 20$), grossly elevated methylphenidate plasma concentrations were observed in a single Caucasian subject [9]. This observation led to an investigation of the subject's *CES1* gene [10]. DNA sequencing identified two coding region single nucleotide mutations located in exons 4 and 6. The first was a substitution (p.Gly143Glu) in exon 4 at the second nucleotide (nt) of codon 143 (genomic: nt 9486; cDNA: nt 428), changing G to A (GGG → GAG). This mutation results in the nonconservative amino acid substitution of Glycine 143 to Glutamic acid. The second genetic variant identified (p.Asp260fs) was a deletion (T/–) occurring in exon 6 at the last nucleotide (genomic: nt 12,754; cDNA: nt 780) of codon 260 (GAT → GA-G). This deletion led to a frameshift mutation which changed Aspartic acid 260 to Glutamic acid and altered the next 39 residues from the WT sequence before truncating early at a premature stop codon. Expanded genotyping studies that captured a racially and ethnically diverse population ($n = 925$) established a minor allele frequency for p.Gly143Glu of 3.7%, 4.3%, 2.0%, and 0% in Caucasian, Black, Hispanic, and Asian populations, respectively. However, the p.Asp260fs has proven to be an extremely rare mutation since none of the 925 screened subjects was found to carry this variant. The functional consequences of both of these two mutants were examined by establishing cell lines expressing WT and mutant CES1. *In vitro* functional studies demonstrated the catalytic function of both p.Gly143Glu and p.Asp260fs are substantially impaired resulting in a complete loss of hydrolytic activity towards methylphenidate [10].

Trandolapril, an angiotensin-converting enzyme (ACE) inhibitor, is an antihypertensive agent in current clinical use that is formulated as an ethyl ester prodrug [11,12]. It is FDA-approved for the treatment of hypertension and for use in stable patients who have clinical evidence of left ventricular systolic dysfunction or symptoms of congestive heart failure within the first 2 days following an acute myocardial infarction. The compound is available as a single drug entity (Mavik®) and also marketed in a fixed-dose combination that includes the calcium channel blocker verapamil in an extended-release formulation (Tarka®). After oral administration, trandolapril is believed to be rapidly converted to its primary active form trandolaprilat in the liver (Fig. 1). Trandolaprilat is estimated to be eight-fold more potent than the parent compound with regard to ACE inhibition. It has been generally reported that non-specific hepatic esterases govern trandolapril activation (i.e. hydrolysis to trandolaprilat). However, the specific esterase(s) responsible for trandolapril metabolism has continued to remain a matter of speculation.

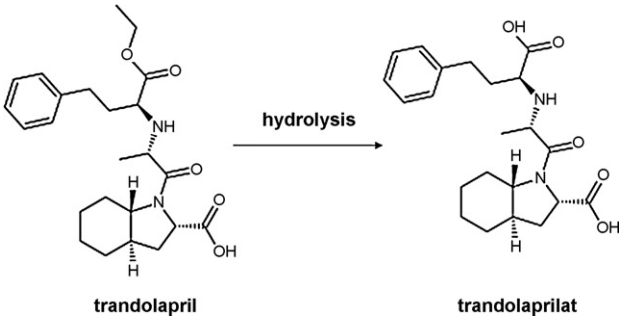

Fig. 1 – Activation of trandolapril through hydrolysis.

In the present study, the potential role of CES1 and CES2 upon the hydrolysis of trandolapril was examined by *in vitro* incubation with human liver microsomes (HLM) and human intestinal microsomes (HIM). Additionally, the influence of the previously identified CES1 mutations (i.e. p.Gly143Glu and p.Asp260fs) on the critical step of metabolic activation of trandolapril via hydrolysis to trandolaprilat was also investigated.

2. Materials and methods

2.1. Materials

Flp-In™-293 cells, a pcDNA5/FRT/V5-His TOPO®TA Expression Kit, phleomycin D1 (Zeocin™), hygromycin B, and Lipofectamine 2000™ were obtained from Invitrogen (Carlsbad, CA). *Taq* polymerase was purchased from Takara (*Takara EX Taq*™ HS, Shiga, Japan). Human *CES1A1* cDNA cloned into a pCMV-SPORT6 vector was from American Type Culture Collection (ATCC, Manassas, VA). Pooled HLM and HIM were obtained from BD Biosciences (Woburn, MA). *p*-Nitrophenyl acetate (PNPA) and *p*-nitrophenol (PNP) were purchased from Sigma (St. Louis, MO). Trandolapril and metabolite trandolaprilat were obtained from Toronto Research Chemicals Inc. (North York, ON. Canada). Acetonitrile, methanol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tris base, and Tween-20 were obtained from Fisher Scientific (Fair Lawn, NJ).

2.2. Construction of wild type and mutant CES1 plasmids

A TA cloning strategy was utilized to generate the *CES1* plasmid and has been described in detail elsewhere [10]. In brief, the human *CES1A1* gene was amplified from the *CES1A1* cDNA (ATCC, Manassas, VA) via *Taq* polymerase based PCR.

After gel-purification, and sequence confirmation, the PCR product was subcloned into a pcDNA5/FRT/V5-His-TOPO® vector with a stop codon after the *CES1* gene. The resulting plasmid was utilized as a template to generate the two mutations of interest, p.Gly143Glu and p.Asp260fs, utilizing the site-directed mutagenesis assay. All constructs were sequenced to confirm that the desired plasmids were obtained.

2.3. Establishment of cell lines stably expressing CES1 and selected mutations

Flp-In™-293 cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and 100 μg/ml phleomycin D1. The identified WT and mutant CES1 plasmids were co-transfected with the pOG44 plasmid at a ratio of 1:10 into Flp-In™-293 cells using the transfection reagent Lipofectamine 2000™ in serum free RPMI Medium 1640. Additionally, the self-ligated pcDNA5/FRT/V5-His-TOPO® vector was transfected into the cells as a vector control. The cell lines were established after hygromycin B selection.

2.4. Western blot analysis

Western blot analysis was conducted to determine CES1 and CES2 expression in HLM and HIM. HLM and HIM were diluted with PBS resulting in a protein concentration of 1 μg/μl. The samples were then boiled with 4 * XT sample buffer (Bio-Rad, Hercules, CA) for 5 min. An aliquot containing 10 or 20 μg total protein was separated by 12% Criterion XT Precast Gel (Bio-Rad, Hercules, CA), and the proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). After being blocked by 5% nonfat dry milk in Tris-Buffered Saline with Tween-20 (TBST) for 1 h at 25 °C, the membrane was incubated with CES1 primary antibody (Abcom, Cambridge, MA, 1:2000 diluted in 5% nonfat dry milk) or CES2 primary antibody (Abcom, Cambridge, MA, 1:1000 diluted in 5% nonfat dry milk) overnight at 4 °C. The membrane was then washed three times with TBST containing 5% nonfat dry milk, and subsequently incubated with secondary antibody (Abcom, Cambridge, MA) (goat anti-rabbit conjugated with horseradish peroxidase for CES1 and goat anti-mouse conjugated with horseradish peroxidase for CES2, 1:10,000 diluted in TBST containing 5% nonfat dry milk) for 1.5 h at 25 °C. Finally, after being washed three times in TBST, blots were developed using Pierce ECL Western Blotting Reagent (Pierce, Rockford, IL) and exposed to Amersham Hyperfilm™ ECL film (GE Healthcare, Buckinghamshire, UK). Additionally, anti-actin was included in this study as a loading control.

2.5. Enzymatic assays

Hydrolytic activity of HLM and HIM towards trandolapril was assessed by incubating trandolapril with the respective microsomes and measuring the formation of trandolaprilat formed via ester hydrolysis. The incubations of trandolapril were carried out in 1.5–ml Eppendorf tubes at a total volume of 100 μl. Prior to incubations, trandolapril solutions were prepared freshly in 50 μl reaction buffer (PBS containing 10 mM HEPES, pH 7.4) and then mixed with 50 μl of HLM or HIM with the final substrate and protein concentrations of 100 μM and 0.5 mg/ml, respectively. After incubation at 37 °C for 10 min, the reaction was terminated by adding 500 μl of methanol. Precipitated protein was then removed by centrifugation (20,000 × *g* for 5 min at 4 °C). Concentrations of the trandolapril metabolite trandolaprilat were determined utilizing an established HPLC method described below.

Additionally, the enzymatic activities of WT and mutant CES1 upon trandolapril hydrolysis were investigated utilizing the established cell lines stably expressing WT and each mutant CES1. After reaching approximately 95% confluence, the transfected cells were rinsed and harvested in PBS containing 10 mM HEPES (pH 7.4). The cell suspension was then sonicated and the supernatant 9000 (S9) fraction was collected by centrifugation at 9000 × *g* for 30 min at 4 °C. The protein concentration was determined using a Pierce BCA assay kit (Rockford, IL). The catalytic activity of collected S9 fractions on trandolapril hydrolysis was determined utilizing the methods described above with the exception of using the trandolapril concentrations ranging from 10 μM to 2000 μM.

PNPA, a standard non-specific hydrolase substrate, was included as a positive control. The hydrolysis of PNPA was conducted in 96-well culture plates held at 37 °C in a final volume of 200 μl. HLM, HIM, and the S9 fractions prepared from cells expressing WT and mutant CES1 were diluted in reaction buffer (PBS containing 10 mM HEPES, pH 7.4) with the protein concentration of 20 μg/ml, and preincubated at 37 °C for 10 min. The reaction was initiated by adding PNPA with a final concentration of 100 μM. The formation of PNP from PNPA through hydrolysis was recorded with the measurements of the absorbance at 405 nm by a microplate reader (ThermoMax, Molecular Devices, Sunnyvale, CA) following incubation at 37 °C in 30 min.

2.6. HPLC analysis

Trandolaprilat formated via enzyme-catalyzed hydrolysis of trandolapril was determined using high-performance liquid chromatography (HPLC). The HPLC system consisted of an Agilent 1100 module, a C18 reversed-phase column (250 mm × 4.6 mm, 5 μm) preceded by a 4 mm × 3 mm C18 guard column (Phenomenex, Torrance, CA), and a diode-array detector. The separation was performed using a mobile phase composed of acetonitrile and 20 mM KH_2PO_4 (pH 2.5), employing a gradient elution method in which the organic phase was initiated at 32% and increased to 41% over 14 min and returned to 32% at 15 min. The running time was set at 20 min. The retention time of trandolaprilat was 6.4 min with the flow rate at 1.0 ml/min, the column temperature at 40 °C, and the detection wavelength set at 210 nm. The inter-day and intra-day deviations of the assay were found to be less than 10%. The lower limit of quantification of trandolaprilat was determined to be 1 μM. A typical chromatogram is shown in Fig. 2.

2.7. Statistical analysis

All data are presented as the mean ± standard deviation (S.D.) of at least four independent experiments. Enzyme kinetic data were fit to the Michaelis–Menten equation, and kinetic parameters K_m and V_{max} were calculated using nonlinear

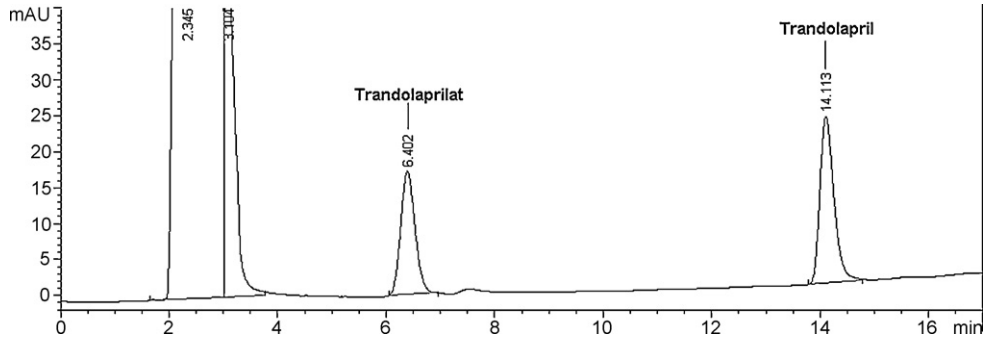

Fig. 2 – Representative chromatograph of trandolaprilat and trandolapril. Trandolaprilat was produced from the hydrolysis of trandolapril (200 μM) catalyzed by WT CES1 after incubation at 37 °C for 10 min.

regression analysis with Graphpad Prism software (Graphpad Software Inc., San Diego, CA).

3. Results

3.1. *CES1 and CES2 expression in HLM and HIM*

The western blot analysis showed that CES1 is strongly expressed in HLM but not in HIM (Fig. 3). The expression of CES2 was detected in both HLM and HIM. HIM exhibits significantly higher level of CES2 expression than HLM. These observations are consistent with previously published reports that CES1 is the major CES in the liver whereas CES2 predominates in the intestine in human [3,13].

3.2. *Trandolapril is hydrolyzed to trandolaprilat by liver but not intestinal microsomes*

Hydrolytic activity in the human liver is mainly attributed to CES1 which is abundantly expressed in the organ [4]. After incubation with HLM, trandolapril was rapidly hydrolyzed to its more active metabolite trandolaprilat. In contrast, in HIM, where CES2 is the predominant enzyme, incubations failed to produce any appreciable trandolapril hydrolysis (Fig. 4). As a comparator and positive control, PNPA, a sensitive non-specific hydrolase substrate, was efficiently hydrolyzed to its metabolite PNP by both HLM and HIM (Fig. 4). These data suggest that trandolapril serves as a readily hydrolyzed substrate of CES1 but not CES2.

3.3. *Hydrolytic activity of CES1 and selected natural mutants towards trandolapril and PNPA*

The cell lines stably expressing WT and each mutant CES1 were successfully established and applied to *in vitro* functional studies. As anticipated, PNPA was rapidly hydrolyzed by WT CES1 (Fig. 5). However, the hydrolytic activity of p.Gly143Glu was significantly decreased displaying a catalytic efficiency approximating 30% of that of WT enzyme (Figs. 5 and 6). Additionally, the frameshift mutation p.Asp260fs produced almost no hydrolytic activity, similar to that of vector transfected cells indicating a complete loss of CES1 activity (Figs. 5 and 6).

Trandolapril was efficiently metabolized by WT CES1 with the V_{max} and K_m values of 103.6 ± 2.2 nmole/min/mg protein and 639.9 ± 32.9 μM, respectively (Figs. 6 and 7). The efficiency of WT CES1 catalyzed hydrolysis of trandolapril was comparable to that of HLM (Figs. 4 and 6). Neither p.Gly143Glu nor p.Asp260fs produced any detectable trandolapril hydrolysis under the experimental conditions (Fig. 6).

4. Discussion

CESs expressed in the gastrointestinal tract and liver are estimated to contribute more than 90% of all hydrolytic

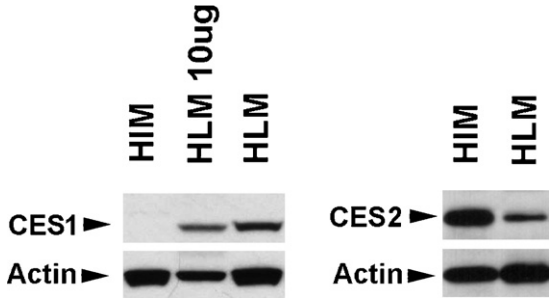

Fig. 3 – Western blot analysis of CES1 expression in HLM, and HIM. Each lane contains 20 μg total proteins unless otherwise specified. Anti-actin was included as a sample loading control.

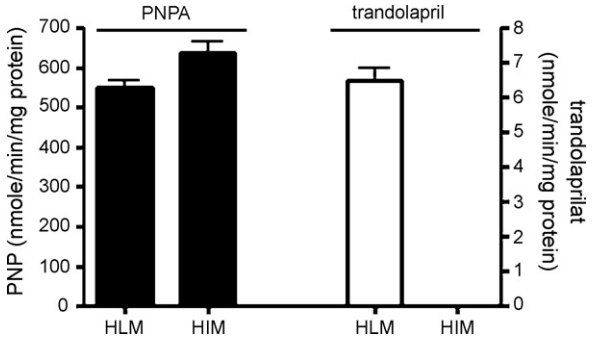

Fig. 4 – Hydrolysis of PNPA and trandolapril by HLM and HIM. The hydrolytic products of PNPA and trandolapril were determined after incubating the substrates with HLM and HIM at 37 °C for 10 min. Data were expressed as the mean ± S.D. (n = 4).

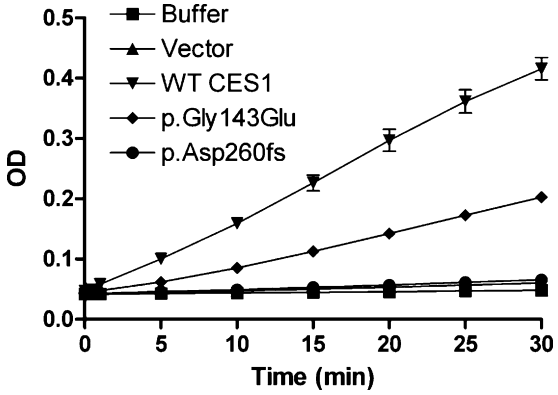

Fig. 5 – Time course of PNPA hydrolysis catalyzed by WT CES1 and its mutants p.Gly143Glu and p.Asp260fs. Optical density (OD) of PNPA metabolite PNP at 405 nm was monitored every 5 min in 30 min after incubating PNPA (100 μM) with the cell S9 fractions (10 μg/ml). Data are means ± S.D. for four independent experiments.

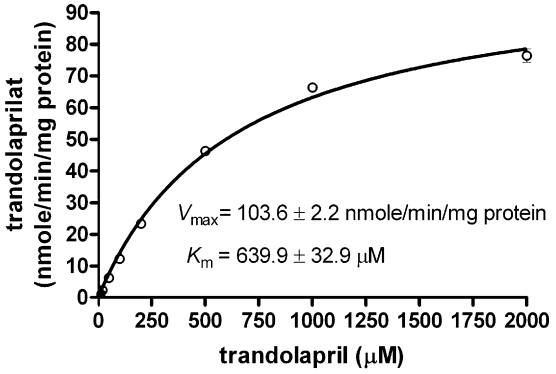

Fig. 7 – Enzyme kinetics study of trandolapril hydrolysis catalyzed by WT CES1. The hydrolysis of trandolapril was determined at the concentrations of 10 μM–2000 μM. The K_m and V_{max} values were calculated using nonlinear regression analysis with Graphpad Prism software. Data present means ± S.D. for four independent experiments.

activity in those tissues and play an important role in metabolizing (both deactivating and activating) many therapeutic agents, endogenous substrates, and potential toxins [4,14]. Many therapeutic agents with poor oral bioavailability characteristics are routinely formulated as ester prodrugs to be later activated by hydrolases including the CESs through the cleavage of ester bond in the intestine and/or liver and liberation of the active compound. Trandolapril, a member of the ACE inhibitor class of cardiovascular agents, is a relatively weak inhibitor of ACE in its parent form. However, its active metabolite, trandolaprilat exhibits high binding affinity for ACE and produces potent reversible inhibition of ACE thereby preventing the conversion of angiotensin I to the vasoactive peptide angiotensin II. Thus, as a prodrug, the therapeutic efficacy and potency of trandolapril is inherently dependent upon the presence of functional endogenous enzyme(s) to adequately hydrolyze it to trandolaprilat.

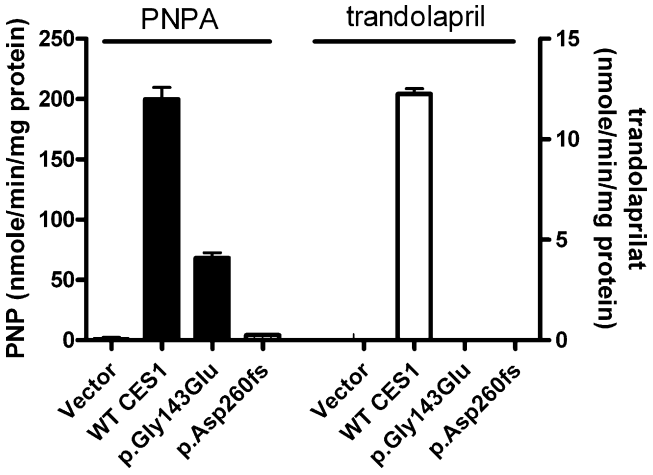

Fig. 6 – Enzymatic activity of WT CES1 and its mutations on hydrolyzing PNPA and trandolapril. The substrates PNPA (100 μM) and trandolapril (100 μM) were incubated with the cell S9 (0.5 mg/ml) at 37 °C for 10 min. The hydrolytic products PNP and trandolaprilat were determined, and normalized by time and protein contents. Values represent mean ± S.D. (n = 4).

CES1 is the predominant esterase expressed in the human liver and estimated to contribute approximately 80% of total hydrolytic activity in the organ while the remaining hydrolytic activity is attributed to CES2 and other esterases [4,15,16]. CES2 is abundantly expressed in the intestine where the expression CES1 is essentially undetectable [13]. The present study confirmed that CES1 is present in HLM but not in HIM while CES2 is the predominant CES in the intestine in human. In this study, trandolapril was determined to be hydrolyzed only by HLM but not by HIM, indicating CES1 but not CES2 is responsible for its metabolic activation. Structurally, trandolapril bears a relatively small alcohol group (Fig. 1). This observation is consistent with current theory regarding the substrate selectivity of CESs (i.e. CES1 prefers substrates with small alcohol groups and relatively large acyl group which is contrary to the general observations for CES2 substrates). Other reported selective CES1 substrates include methylphenidate (methyl group), meperidine (ethyl group) and temocapril (ethyl group) [5,17–19]. In comparison, PNPA, a non-specific CES substrate, was efficiently metabolized by both HLM and HIM.

The catalytic activity of CES1 towards trandolapril hydrolysis was further investigated utilizing the Flp-In™-293 cells transfected with the *CES1* gene. The present study demonstrated that the intrinsic expression and activity of CES1 in parent Flp-In™-293 cells are undetectable, which makes the cell suitable for establishing *CES1* transfected cell lines. The cells transfected with WT *CES1* strongly express the enzyme at a similar level to that found in HLM. Additionally, the hydrolytic activity observed in the cells transfected with WT CES1 was found to be comparable to that in HLM. This is consistent with the experimental results from the HLM and HIM incubation studies herein indicating that trandolapril is a selective substrate of CES1.

It was noted that the hydrolytic velocity of PNPA in WT CES1 transfected cells was lower than that in HLM. This substantial hydrolytic activity found in HLM might be attributed to the CES2 (albeit minimal) expressed in the liver since the catalytic efficiency of CES2 towards PNPA is about five-fold higher than that of CES1 [20].

Two CES1 variants p.Gly143Glu and p.Asp260fs were identified in our laboratory recently [10]. Both mutations display dramatically decreased enzymatic activity towards the selective CES1 substrate methylphenidate. In the present study, Flp-In^{TM}-293 cell lines transfected with each mutant *CES1* gene were established to determine the potential influence of these mutations on the activation of trandolapril. The frameshift mutation p.Asp260fs was found to be devoid of catalytic activity towards both PNPA and trandolapril. However, given that p.Asp260fs is an extremely rare mutation in all populations we have screened to date, the potential clinical significance of p.Asp260fs relative to trandolapril therapeutic use would appear almost nil. However, the substitution mutation p.Gly143Glu is a relatively common *CES1* variation in Caucasian, Black, Hispanic but perhaps not Asian populations. The enzymatic function of p.Gly143Glu in catalyzing trandolapril hydrolysis was undetectable under our experimental conditions. When a more sensitive but less specific substrate, PNPA (100 μM) was assessed, the catalytic activity of p.Gly143Glu was determined to be less than one-third of that of WT CES1.

In addition to the two *CES1* mutations we have previously identified, a number of variants have been reported even though their functional consequences remain unknown [21]. The *CES1A2* promoter region (~1 kb) was sequenced in 100 Japanese hypertensive subjects in a recent study [22]. Ten single nucleotide polymorphisms (SNPs) and one insertion/deletion were identified. The SNP haplotype was determined to be effective on Sp1 binding and transcriptional regulation [22]. An earlier report from the same group suggested a SNP in the promoter region of *CES1*, *A(−816)C*, is associated with increased transcription efficiency, and enhanced antihypertensive response to another ACE inhibitor prodrug, imidapril. This finding further suggests adequate and functional CES1 is essential to successful therapeutic outcomes when using a prodrug dependent upon the enzyme for activation [23].

In addition to its role in metabolizing many xenobiotic substances, CES1 also plays an important role in lipid metabolism [24,25]. Over-expression of CES1 in COS-7 cells resulted in significant increases of cholesteryl ester hydrolytic activity in the cells [26]. Thus, it is desirable to further explore the role of CES1 in lipid metabolism and the potential correlations between functional *CES1* mutations and abnormal lipid metabolism. Our laboratory has also recently initiated exploration of this area.

In conclusion, the present studies have demonstrated for the first time that trandolapril is a selective substrate of CES1. Hepatic CES1 is the major enzyme responsible for the activation of this prodrug. Furthermore, the identified *CES1* variations p.Gly143Glu and p.Asp260fs proved highly dysfunctional in terms of converting trandolapril to its active form trandolaprilat. Individuals carrying these mutations would likely experience difficulty in activating trandolapril to trandolaprilat, and thus display a decreased pharmacological response to trandolapril pharmacotherapy compared to individuals without these natural variants. Furthermore, there exists the potential for unanticipated problems with tolerability or toxicities due to elevated concentrations of the parent molecule. This study provides the first evidence that non-synonymous *CES1* mutations could lead to deficiency of the activation of this prodrug. Additional well designed clinical studies may be warranted to define the clinical significance of each mutation relative to the therapeutic response and potential adverse events related to trandolapril pharmacotherapy.

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