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ORIGINAL ARTICLE

Acylpeptide hydrolase (APEH) sequence variants with potential impact on the metabolism of the antiepileptic drug valproic acid

Despina Tsortouktzidis[1] · Kathleen Grundke[2] · Claudia Till[1,3] · Anne Korwitz-Reichelt[1] · Jörn Oliver Sass[1,2,3]

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Abstract

Acylpeptide hydrolase (APEH) is a serine protease involved in the recycling of amino acids from acylated peptides. Beyond that, APEH participates in the metabolism of the antiepileptic drug valproic acid (2-propylpentanoic acid; VPA) by catalyzing the hydrolysis of the VPA metabolite valproylglucuronide (VPA-G) to its aglycon. It has been shown that the inhibition of APEH by carbapenem antibiotics decreases therapeutic VPA levels by enhancing the urinary elimination of VPA in form of VPA-G. As various sequence variants of the *APEH* gene (which encodes the APEH protein) are listed in databases, but have not been functionally characterized yet, we assume, that some *APEH* sequence variants may have pharmacogenetic relevance due to their impaired cleavage of VPA-G. *APEH* sequence variants predicted to affect enzyme activity were selected from databases, and overexpressed in HEK293 cells (stable transfection), a cell line derived from human embryonic kidney cells. APEH activity in cell homogenates was determined spectrophotometrically by monitoring the hydrolysis of the synthetic substrate *N*-acetyl-L-alanine-nitroanilide. APEH enzyme activity and protein expression of the sequence variants were compared with those of APEH with the reference sequence. Three out of five tested missense sequence variants resulted in a considerable decrease of enzyme activity assessed with the standard substrate *N*-acetyl-L-alanine-nitroanilide, suggesting an effect on pharmacokinetics of VPA. Our work underlines the need to consider the *APEH* genotype in investigations of altered VPA metabolism.

Keywords Acylpeptide hydrolase · Aminoacylase · Valproic acid · Carbapenem · Polymorphism · Pharmacogenetics

Introduction

Acylpeptide hydrolase (APEH; EC 3.4.19.1), also known as *N*-acylaminoacylpeptide hydrolase, is a cytosolic protein that plays a role in protein metabolism by participating in amino acid recycling (Perrier et al. 2005). It is encoded by the *APEH* gene and catalyzes the hydrolysis of *N*-acylated peptides yielding an *N*-acyl-L-amino acid and a peptide with a free *N*-terminus. The subsequent removal of the acyl group by acylases such as aminoacylase 1 yields the free amino acid (Perrier et al. 2005; Sass et al. 2006). APEH is a tetrameric protein of 300 kDa and each subunit contains 732 amino acid residues (Scaloni et al. 1999). The enzyme is expressed in several cells and tissues including erythrocytes, kidney, liver and brain (Yamin et al. 2007).

APEH participates in different biological processes. It has been shown that the enzyme degrades oxidatively damaged proteins in erythrocyte membranes (Fujino et al. 2000). Furthermore, APEH works in coordination with the proteasome to clear cytotoxic, denatured proteins which would otherwise impair cellular function (Shimizu et al. 2004). It has also been demonstrated to be involved in single-stranded DNA repair and cell survival after exposure to H_2O_2, thus playing a role in DNA damage response (Zeng et al. 2017). Connections to cancer have been drawn not only because of deletions of a region comprising *APEH* in various small-cell lung carcinoma cells, but also because APEH inhibition was shown to affect

✉ Jörn Oliver Sass
joern.oliver.sass@h-brs.de

[1] Present address: Research Group Inborn Errors of Metabolism, Department of Natural Sciences,, Bonn-Rhein-Sieg University of Applied Sciences, von-Liebig-Str. 20, 53359 Rheinbach, Germany

[2] Division of Clinical Chemistry & Biochemistry, University Children's Hospital and Children's Research Center, Zürich, Switzerland

[3] Research Group Inborn Errors of Metabolism, Institute for Functional Gene Analytics (IFGA), Bonn-Rhein-Sieg University of Applied Sciences, Rheinbach, Germany

osteosarcoma cell viability via downregulation of the proteasome (Scaloni et al. 1992; Palumbo et al. 2016). Importantly, APEH endopeptidase activity participates in the degradation of β-amyloid peptides (Aβ) which accumulate in the brain of patients with Alzheimer disease (Yamin et al. 2007, 2009).

Notably, APEH is also involved in the metabolism of the widely-used antiepileptic drug valproic acid (2-propylpentanoic acid; VPA). Several studies (e.g., Nagai et al. 1997; De Turck et al. 1998; Miranda Herrero et al. 2015) have reported that concomitant treatment with valproic acid and carpabenem antibiotics leads to a decrease in VPA plasma levels that may cause loss of seizure control. Suzuki and co-workers have identified APEH as the key enzyme in human liver cytosol that hydrolyzes valproic acid glucuronide (VPA-G), thus releasing the active form of the drug valproic acid (VPA) (Suzuki et al. 2010; Suzuki et al. 2011). This finding was later confirmed in vivo in dogs and has been supported by a computational study of the competitive binding of valproic acid glucuronide and carbapenem antibiotics to APEH (Suzuki et al. 2016; Ishikawa et al. 2017).

Several *APEH* single nucleotide variants are listed in databases such as the ExAC Browser (http://exac.broadinstitute.org/). This raised the suspicion that humans exist who carry a genetic alteration in *APEH*. By affecting APEH enzyme activity this may have major effects on therapeutic application of valproic acid. Within this study, we have overexpressed and studied several *APEH* sequence variants in HEK293 cells, a human-derived cell line, which ensures authentic human posttranslational modifications of APEH.

Materials and methods

Selection of APEH sequence variants

Five *APEH* SNPs were selected from the ExAC Browser (http://exac.broadinstitute.org/) database (Table 1). They all represent missense mutations and were indicated to have damaging effects by at least one of the following programs: SIFT, PROVEAN, Polyphen2, Mutation Taster and Human Splicing Finder (http://provean.jcvi.org/genome_submit_2.php?species=human, http://genetics.bwh.harvard.edu/pph2/, http://www.mutationtaster.org/ and http://www.umd.be/HSF3/index.html, respectively). The selected sequence variants were: c.865G > C (predicted as p.(Gly289Arg); GenBank rs147272980), c.1352C > T (p.(Ser451Phe); rs202078335), c.1397G>A (p.(Arg466Gln); rs113937135), c.1409C > T (p.(Pro470Leu); rs147045459) and c.1622C > T (p.(Thr541Met); rs3816877) (Table 1). As controls, two more sequence variants were studied, one that introduces a stop codon in *APEH* and one that introduces a synonymous mutation: c.1008G > A (p.Trp336*; rs199629754) and c.1257C > G (p.Leu419=; rs201644578).

Plasmid constructs of APEH sequence variants

A pcDNA3.1+ vector carrying the open reading frame of human *APEH* (reference sequence GenBank NM_001640.3) was purchased from Genscript. This vector encoding the 'wild-type' enzyme was used as a template to produce APEH variants by site-directed mutagenesis. Site-directed mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit and the QuickChange XL-Mutagenesis Kit (Agilent Technologies). The newly generated constructs were transformed in X-Gold ultracompetent cells (Agilent Technologies). Generated constructs were verified by DNA sequencing analysis.

Stable transfection of HEK293 cells

Studies were performed in HEK293 cells (transformed human embryonic kidney cells), based on experience with previous studies on aminoacylase 1 (Sommer et al. 2011; Sass et al. 2016). Transfection of plasmid DNA into HEK293 cells was performed using Xfect™ transfection reagent (Clontech Laboratories) according to the manufacturer's instructions. Mock controls were transfected with the pcDNA3.1+ vector without insert ('empty vector'). 48 h after transfection the medium was changed to DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% heat-inactivated fetal bovine serum, 100 units penicillin G (sodium salt)/ml, 100 μg streptomycin sulphate/ml and 25 μg amphotericin B/ml in addition to the selection antibiotic G-418 (500 μg/ml culture medium).

APEH enzyme activity assay

APEH activity in cell homogenates was determined spectrophotometrically by monitoring the hydrolysis of the synthetic substrate *N*-acetyl-L-alanine-nitroanilide (Ac-Ala-pNA, or AANA) (Perrier et al. 2002). The assay was adapted to a microplate format with a final volume of 210 μL per well. After homogenization by ultrasound, protein supernatant was obtained by centrifugation at 13,000 *g* for 15 min at 4 °C. Different substrate concentrations were applied to determine kinetic parameters of APEH (final substrate concentrations in the reaction mix: 1.2, 2.4, 3.6, 4.8, 7.1, 9.5 and 14.3 mM). Absorbance change at 410 nm was monitored at 37 °C every minute for 25 min using an EON plate reader (BioTek). The absorbance coefficient ε_{410nm} = 8,800 M^{-1} cm^{-1} was used to calculate enzyme activity (Perrier et al. 2002). Total protein was determined using the Lowry method (Lowry et al. 1951). The kinetic parameters, maximum velocity of the enzyme (V_{max}) and Michaelis-Menten constant (K_m), were determined according to Lineweaver and Burk (Lineweaver and Burk 1934).

Table 1 Selected APEH sequence variants

APEH poly-morphisms	MAF	Protein prediction (score)				
		SIFT	PROVEAN	Poly-phen-2	MutationTaster	Human Splicing Finder
c.865G > C p.(Gly289Arg) (rs147272980)	C = 0.0001648	damaging (0.004)	deleterious (−2.91)	benign (0.187)	disease causing (125)	+2.92%
c.1352C > T p.(Ser451Phe) (rs202078335)	T = 0.0001763	damaging (0.010)	deleterious (−2.88)	benign (0.005)	disease causing (155)	−0.43%
c.1397G>A p.(Arg466Gln) (rs113937135)	A = 0.0006458	tolerated (0.388)	neutral (−0.43)	possibly damaging (0.530)	polymorphism (43) †	new site (+52.39%)
c.1409C > T p.(Pro470Leu) (rs147045459)	T = 0.0002488	damaging (0.005)	deleterious (−4.39)	possibly damaging (0.792)	disease causing (98)	+1.05%
c.1622C > T p.(Thr541Met) (rs3816877)	T = 0.003570	tolerated (0.128)	neutral (−0.63)	possibly damaging (0.947)	disease causing (81)	No difference between mutant and reference sequence
c.1257C > G p.(Leu419=) (rs201644578)	G = 0.00000827	tolerated (1.000)	neutral (0)	not applicable	polymorphism (32)	No difference between mutant and reference sequence
c.1008G > A p.(Trp336*) (rs199629754)	A = 0.000008 (TOPMed)§	not applicable	deleterious (−9.108)	not applicable	disease causing (6.0)	Site broken (−37.22%)

MAF = Minor Allele Frequency according to http://exac.broadinstitute.org/ or- if marked with §- TOPMed (the latter based on https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=199629754)

SIFT and PROVEAN (http://provean.jcvi.org/genome_submit_2.php?species=human)

Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/)

Mutation Taster (http://www.mutationtaster.org/)

Human Splicing Finder (http://www.umd.be/HSF3/index.html)

† Predicted to cause splice site change

Statistical comparisons were performed using paired Student's *t test* ($p \leq 0.001$ was labelled significant).

Immunoblot analyses

Briefly, cell homogenate supernatant (30 μg protein) was mixed with Laemmli buffer containing β-mercaptoethanol. The sample preparation was incubated for 5 min at 95 °C and subjected to SDS-PAGE analysis. Afterwards, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Trans-Blot® Turbo™ transfer system [Bio-Rad]). Membranes were blocked in skim milk (5% in TBS-T [10 mM Tris base, 150 mM NaCl]) for one hour and incubated with the corresponding primary antibody overnight at 4 °C (APEH: PAB22331, Abnova; GAPDH: GTX100118, Genetex). Subsequently, membranes were incubated for one hour with secondary antibodies (ECL anti rabbit IgG HRP). Immunoreactive proteins were detected using SuperSignal™ West Femto Maximum Sensitivity Substrate and visualized using MY ECL Imager (Invitrogen).

Results

Kinetic characteristics of overexpressed human 'wild-type' APEH

Employing the APEH enzyme activity assay, K_m and V_{max} of APEH 'wild-type' were determined in cell homogenate supernatant derived from three separate stable transfections ($N = 3$; $n \geq 4$ technical replicates). Mean ± standard deviation for K_m was 5.77 ± 1.29 mM. V_{max} was determined as 0.133 ± 0.023 mmol min^{-1} g^{-1} protein (Fig. 1). In mock controls (N = 3; n ≥ 4 technical replicates), expressing endogenous APEH only, K_m (6.96 ± 2.09 mM) resembled that of cells overexpressing APEH 'wild-type', while, as expected, V_{max} was much lower (0.009 ± 0.003 mmol min^{-1} g^{-1} protein).

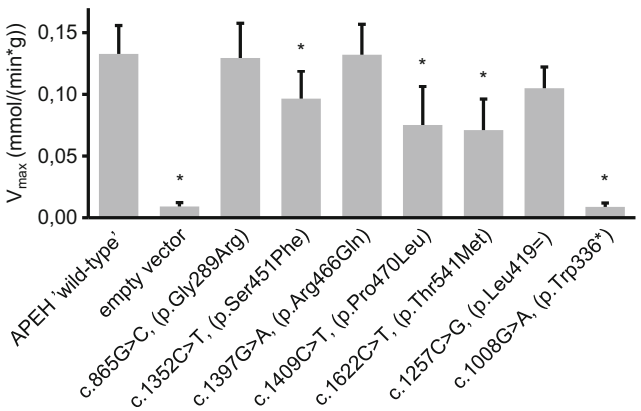

Fig. 1 APEH enzyme activity in HEK293 cells overexpressing *APEH* variants. V_{max} values for APEH obtained from HEK293 cells overexpressing *APEH* sequence variants and controls (*APEH* 'wild-type' and empty vector). The bars represent mean V_{max} values plus standard deviations derived from three (*APEH* 'wild-type' and empty vector) or two (*APEH* variants) independent transfections, each with $n \geq 4$ technical replicates. *: $p \leq 0.001$ in paired Student's *t test*

APEH sequence variants affect enzyme function

To study the effects of *APEH* sequence variants (Table 1) on enzyme activity, HEK293 cells overexpressing APEH variants were generated and APEH activity assays were performed. Cells expressing the APEH stop mutant p. (Trp336*) displayed endogenous APEH activity levels only (Fig. 1). As expected, overexpression of the synonymous sequence variant p. Leu419 = yielded an enzyme activity resembling that obtained for APEH 'wild-type'. However, overexpression of three of the five missense variants resulted in a pronounced decrease of V_{max}, on average down to 56%–68% of the wild-type value: c.1352C > T (p. (Ser451Phe)), c.1409C > T (p. (Pro470Leu)) and c.1622C > T (p. (Thr541Met)). The remaining two missense variants c.865G > C (p. (Gly289Arg)) and c.1397G > A (p. (Arg466Gln)) did not affect APEH function.

Notably, mean K_m values of all APEH variants were between 5.66 and 7.72 mM, resembling those of the recombinant human 'wild-type' enzyme and of the endogenous human enzyme of the mock control cells.

APEH variants are expressed to similar extent as APEH 'wild-type'

APEH expression in HEK293 cells overexpressing APEH variants was analyzed by immunoblotting (Fig. 2). Overexpression of the APEH stop mutant p. (Trp336*) resulted in APEH levels which were comparable to endogenous APEH expression only. However, no major change in protein expression was noted for any of the other mutations. Thus, their decreased enzyme activity levels can be attributed to APEH deficiency caused by *APEH* sequence variants rather than to decreased protein expression.

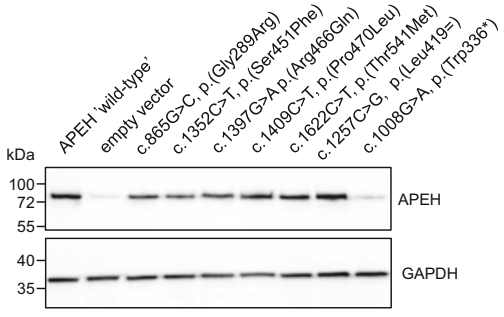

Fig. 2 APEH levels in HEK293 cells overexpressing *APEH* variants. A representative immunoblot of cell lysates derived from HEK293 cells transfected with *APEH* variants and controls is shown. GAPDH was used as loading control

Discussion

Our study searched for functional consequences of *APEH* sequence variants on APEH enzyme activity, as those may have pharmacogenetic implications for the treatment of patients with the antiepileptic drug valproic acid.

Overexpression of *APEH* with the reference sequence ('wild-type') resulted in a 15-fold increase of APEH activity compared with the control. This indicates that overexpression has been successful in all three performed transfections. Three of five missense mutations tested and predicted (by at least one established program) to be functionally relevant, displayed a considerable loss of APEH activity. In contrast, a synonymous mutation in *APEH* had no effect while a stop mutation essentially abolished enzyme activity.

Using the same commercially available substrate *N*-acetyl-L-alanine *p*-nintroanilide, APEH purified from rat liver and porcine intestinal mucosa has been analyzed previously (Kobayashi and Smith 1987; Raphel et al. 1999). Our mean K_m value for the human recombinant 'wild type' protein 5.77 ± 1.29 mM closely resembles the one reported for the rat liver enzyme (8.3 mM; Kobayashi and Smith 1987) and is similar to the one reported for APEH from porcine intestinal mucosa (1.80 mM; Raphel et al. 1999). However, both studies on purified enzyme yielded higher specific activities than we obtained for our rather crude samples.

While our experiments were being performed, a retrospective analysis including 149 southern Chinese epilepsy patients was published that demonstrated an effect on VPA pharmacokinetics in heterozygotes for the c.1622C > T (p. Thr541Met) sequence variant (Wen et al. 2016). This first in vivo report of its kind well supports our functional in vitro study which shows a decrease of APEH activity with this SNP to about half (56%) of the 'wild-type' activity. Furthermore, the findings by Wen et al. suggest that the loss of enzyme activity which we observed for mutants c.1352C > T (p. Ser451Phe) and c.1409C > T (p. Pro470Leu) may also be of relevance in vivo. Therefore, the fact that no obvious, easily accessible biomarker is available for APEH deficiency, should prompt *APEH* mutation analysis in patients who do not achieve usually reached therapeutic valproic acid levels or need particularly high drug doses to do so. This would help to personalize medicine and to avoid treatment that is based on a non-effective approach. Future, more complex, experiments should assess the activity of APEH sequence variants on VPA metabolite valproylglucuronide (VPA-G) instead the established synthetic substrate used here.

Due to its endopeptidase activity, APEH has also been associated with the degradation of β-amyloid peptides. Interestingly, decreased APEH activity levels have been observed in erythrocyte samples of Alzheimer disease patients when compared to controls (Yamin et al. 2007; Yamin et al. 2009; Palmieri et al. 2017). Beyond further studies of the APEH endopeptidase activity in the context of Alzheimer disease, coming research should address the question whether described *APEH* SNPs which result in functional impairment of APEH activity also play a role in Alzheimer disease, possibly as risk factors.

Conclusions

APEH sequence variants may have pharmacogenetic implications for the treatment of patients with the antiepileptic drug valproic acid.

Therefore, APEH mutation analysis is recommended in patients who do not achieve usually reached therapeutic valproic acid levels or need particularly high drug doses to do so. It remains to be studied whether *APEH* sequence variants which result in functional impairment of APEH activity also play a role in Alzheimer disease, possibly as risk factors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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