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Research paper

Four day inhibition of prolyl oligopeptidase causes significant changes in the peptidome of rat brain, liver and kidney

Jofre Tenorio-Laranga [a], Pekka T. Männistö [a], Markus Storvik [b], Pieter Van der Veken [c], J. Arturo García-Horsman [a,*]

[a] Division of Pharmacology and Toxicology, University of Helsinki, P.O. Box 56 (Viikinkaari 5E), 00014 Helsinki, Finland
[b] Department of Pharmacology and Toxicology, University of Eastern Finland, Kuopio, Finland
[c] Laboratory of Medicinal Chemistry, University of Antwerp, Wilrijk (Antwerp), Belgium

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ABSTRACT

Prolyl oligopeptidase (PREP) cleaves short peptides at the C-side of proline. Although several proline containing neuropeptides have been shown to be efficiently cleaved by PREP *in vitro*, the actual physiological substrates of this peptidase are still a matter of controversy. The aim of this study was to evaluate the changes in the peptidome of rat tissues caused by a repeated 4-day administration of the potent and specific PREP inhibitor KYP-2047, using our recently developed iTRAQ-based technique. We found tissue-dependent changes in the levels of specific subsets of peptides mainly derived from cytosolic proteins. Particularly in the kidney, where the levels of cytochrome *c* oxidase were found decreased, many of the altered peptides originated from mitochondrial proteins being involved in energy metabolism. However, in the hypothalamus, we found significant changes in peptides derived from hormone precursors. We could not confirm a role of PREP as the metabolising enzyme for β-endorphin, galanin, octadecaneuropeptide, neuropeptide–glutamic acid–isoleucine, substance P, somatostatin, enkephalin and neuropeptide Y. Furthermore, changes in the degradation patterns of some of these neuropeptides, and also most of those derived from other larger proteins, did not follow specificity to proline. After a 4-day treatment, we found a significant amount of peptides, all derived from secreted pro-proteins, being cleaved with pair of basic residue specificity. *In vitro* experiments indicated that PREP modifies the endogenous dibasic residue specific proteolysis, in a KYP-2047 sensitive way. These findings suggest that PREP may act indirectly within the routes leading to the specific peptide changes that we observed. The data reported here suggest a wider tissue specific physiological role of PREP rather than the mere metabolism of proline containing active peptides and hormones.

1. Introduction

Prolyl oligopeptidase (PREP) is a serine protease which cleaves peptides shorter than 30 amino acids at the carboxyl side of an internal proline. Although PREP is found in all tissues, it is localised only in specific cell types particularly in the brain, kidney and liver [1,2].

PREP has become an interesting drug target since administration of specific inhibitors has shown improvements in cognitive functions in different animal models [3]. Thus, PREP is probably involved in the cleavage of peptides involved in memory and learning like melanocyte-stimulating hormone, vasopressin, oxytocin, angiotensin II, or substance P [4]. Indeed, these peptides, and many other proline containing neuropeptides [4], are digested by PREP *in vitro*. However, with the current information, it has been difficult to relate the effects of PREP inhibitors *in vivo* with the proposed *in vitro* functions of the peptidase [5].

From previous peptidomic analysis of PREP inhibition [6–8], and circumstantial evidence on the effect of PREP inhibitors on peptide function [9], it can be suggested that PREP may have a role in the degradation of intracellular active peptides and proteins [10].

Abbreviations: ACBP, Acyl-CoA-binding protein; NEI, Neuropeptide–glutamic acid–isoleucine; PC, Pro-protein convertase; PREP, Prolyl oligopeptidase; VEGF, Vascular endothelial growth factor.

* Corresponding author. Tel.: +358 9 191 59459; fax: +358 9 191 59471.
E-mail address: Arturo.Garcia@helsinki.fi (J.A. García-Horsman).
URL: http://www.helsinki.fi/farmasia/farmakologia/english/mainpage.htm

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New efforts to identify novel peptides have relied on the development and application of peptidomic approaches based on mass spectrometry (MS) [37,38]. These methods are able to identify changes in the peptidome followed by experimental manipulations, for example after modulation of the activity of a particular enzyme [11–13]. MS-based techniques have the capacity to sensitively and rapidly detect multiple peptides without *a priori* knowledge of their identities, thus unbiased. This makes it a more suitable tool for the identification of unknown enzyme regulated peptides [14,15]. Previous peptidomic studies have demonstrated the value of this approach on PREP research, and have suggested the involvement of PREP in the degradation of hormones and mitochondrial protein precursors [6–8]. Pioneering studies analysed peptides generated from the *in vitro* PREP digestion of fractionated peptides form porcine brain homogenates [6]. Although, the treatment did not reflect physiological conditions, interesting peptides from intracellular proteins were identified, like those derived from myelin basic protein, α- and β-synucleins, haemoglobin, and from proteins connected with the inositol phospholipid signalling pathway and the Ca^{2+}-calmodulin system. Nolte et al. [7] analysed the peptidome of 1–4 h PREP inhibited mice and found many additional peptides, highlighting proline rich peptides, confirming PREP preference for shorter peptides and suggesting a previously unknown cleavage specificity. We have validated a peptidomic approach which considerably minimises post-mortem and tissue processing associated proteolysis, and increases peptide resolution by using a 2-dimensional RP-HPLC separation technique of the iTRAQ labelled peptides [8]. In that study, we reported changes due to an acute PREP inhibition, and found alterations in the levels of peptides from secreted proteins but also from other large proteins which included histones, proteasome components and respiratory complexes. All previous studies have been limited to the analysis of an acute PREP inhibition in the brain, and have drawn conclusions on few peptides found. These studies have not provided a comprehensive functional analysis of the data in search for clues on the biological or metabolic processes where PREP might be involved.

In an attempt to make the differences between control and PREP inhibited animals more obvious, and to take advantage of the bioinformatics tools, the present study reports the analysis of the peptidome of several brain areas and peripheral tissues from 4-day PREP inhibited rats. Accordingly, we have applied our iTRAQ labelling peptidomic approach [8] to analyse changes on peptide levels, using the most potent PREP inhibitor available, KYP-2047 [18]. We also performed a functional analysis of the data, and additional biochemical experiments to challenge some of the suggestions derived from this analysis. Finally we present a discussion in order to conclude on the physiological role of PREP.

2. Materials and methods

2.1. Chemicals

Chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified in the text. The PREP inhibitor KYP-2047 (4-phenylbutanoyl-L-prolyl-2(*S*)-cyanopyrrolidine) was synthesised at the Laboratory of Medicinal Chemistry (University of Antwerp) as previously described [17].

2.2. Animals

Male Wistar rats (Harlan, CPB, Zeist, The Netherlands) weighting 180–280 g were administrated intraperitoneally twice-a-day with 20 mg/kg of KYP-2047 in vehicle (0.5% DMSO in saline) or vehicle (control animals). The inhibitor is highly specific to PREP and has a good brain penetration and activity [16,18]. The measured compound levels in several brain areas are enough to inhibit PREP nearly 100% for several hours [19–21]. Effects of the 4-day twice-a-day treatment with KYP-2047 in general behaviour, food or water intake, relative to vehicle administrated animals, were not observed.

Altogether 15 animals were used, 9 of them were treated with KYP-2047 and 6 with vehicle. Four hours after the last injection, the animals were sacrificed and quickly dissected by three experienced surgeons taking several samples of the liver, kidney, hypothalamus, striatum, frontal cortex, cerebellum and the rest of the brain. Tissues were immediately flash frozen (liquid nitrogen). Samples were kept at −80 °C until processed for peptide extraction, or to assay peptidase activity or western immunoblotting.

2.3. Peptide extraction

Peptide extraction was performed as described before [8]. Briefly, each frozen rat tissue sample was powdered in a mortar at −80 °C and transferred to a pre-cooled tube. A volume of 300 μl/25 mg of tissue of boiling water was added, and boiled for 10 min. After centrifugation (16,000× *g*) the supernatant was separated and one volume of 0.25% acetic acid was added to the pellet, sonicated and spun at 20,000× *g* during 20 min at 4 °C. The resulting supernatant was decanted, combined with the boiling water fraction from above and filtered through a 10 kDa cut-off filter (Millipore, Billerica, MA, USA). After the extraction, the relative peptide concentration for each sample was measured at 280 nm (NanoDrop, Thermo Fisher, Waltham, MA, USA).

2.4. Sample pooling and iTRAQ labelling

After the peptide extraction, the different samples obtained were pooled in 4 groups as defined in Fig. 1. All the control samples (from 6 different animals) were pooled in one single group. Since, it is considered that pooling of sample is an appropriate biological average in proteomics studies, specially recommended when there is an interest of general characteristics of a population and when the sample obtained from one individual is insufficient [22]. The samples from treated animals were pooled in 3 groups. The 4 groups contained the same relative amount of peptides before the iTRAQ labelling.

We performed the iTRAQ labelling according to the protocol recommended by the manufacturer (Applied Biosystems, Framingham, MA, USA) as reported before [8]. Basically, vacuum dried samples (from 260 μl of a peptide solution of 1 UA) were reconstituted in 25 μl iTRAQ dissolution buffer. An aliquot (70 μl) of iTRAQ-reporter 114 was added to the control sample, and same aliquots of each iTRAQ-reporter, 115, 116 and 117, were added to the three different samples from treated animals. Mixtures were incubated at room temperature for 1 h and vacuum dried.

2.5. 2-D RP-HPLC–MS/MS analysis

The dual-pH reversed phase liquid chromatography coupled to tandem mass spectrometry (2-D RP-HPLC–MS/MS) was applied as described before [8]. Briefly, the peptides were separated by differential pH 2-dimensional-reverse phase-HPLC (2-D RP-HPLC), where the first dimension was at pH 11, and the second at pH 2, and the elution was directly applied to a nanospray source of a QSTAR XL instrument (Applied Biosystems, Framingham, MA, USA). Information-dependent acquisition analysis was carried out with acquisition cycles in mass spectrometry (MS) and tandem mass spectrometry (MS/MS) modes along all the chromatogram. The QSTAR XL was operated in information-dependent acquisition