Neurochemistry International 113 (2018) 112–119

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Concerted action of dipeptidyl peptidase IV and glutaminyl cyclase results in formation of pyroglutamate-modified amyloid peptides *in vitro*

Alvard Antonyan [a, *], Dagmar Schlenzig [b], Stephan Schilling [b], Marcel Naumann [b], Svetlana Sharoyan [a], Sona Mardanyan [a], Hans-Ulrich Demuth [b]

[a] H. Buniatian Institute of Biochemistry of Armenian NAS, Yerevan 0014, Armenia
[b] Fraunhofer Institute for Cell Therapy and Immunology, Department of Drug Design and Target Validation, Halle, Germany

ARTICLE INFO

Article history:
Received 1 September 2017
Received in revised form
29 November 2017
Accepted 4 December 2017
Available online 7 December 2017

Keywords:
Amyloid beta peptides
Dipeptidyl peptidase IV
Enzyme-substrate interactions
Glutaminyl cyclase
MALDI-TOF mass spectrometry

ABSTRACT

Compelling evidence suggests a crucial role of amyloid beta peptides (Aβ(1-40/42)) in the etiology of Alzheimer's disease (AD). The N-terminal truncation of Aβ(1-40/42) and their modification, e.g. by glutaminyl cyclase (QC), is expected to enhance the amyloid toxicity. In this work, the MALDI-TOF mass spectrometry application proved N-terminal cleavage of Aβ(1-40/42) by purified dipeptidyl peptidase IV (DPPIV) *in vitro* observed earlier. The subsequent transformation of resulted Aβ(3-40/42) to pE-Aβ(3-40/42) in QC catalyzed glutamate cyclization was manifested. Hence, consecutive conversion of Aβ(1-40/42) by DPPIV and QC can be assumed as a potential mechanism of formation of non-degrading pyroglutamated pE-Aβ(3-40/42), which might accumulate and contribute to AD progression. The *in vitro* acceleration of Aβ(1-40) aggregation in the simultaneous presence of DPPIV and QC was shown also.

1. Introduction

Amyloid beta peptides (Aβ(1-40/42) constitute the primary components of amyloid deposits found in the brain of Alzheimer's disease (AD) patients. The neurotoxicity of Aβ(1-40/42) correlates with their aggregation propensity (Varvel et al., 2008). An imbalance between the rate of synthesis and clearance of Aβ(1-40/42) is considered to be a possible contributor to the onset of AD (Bateman et al., 2006). Several pathways for Aβ(1-40/42) clearance include receptor-mediated cellular uptake, blood-brain barrier transport and direct proteolytic degradation by the action of peptidases. It is expected that proteolysis of Aβs could decrease their steady-state concentrations and reduce deposition in brain. A number of enzymes are capable of cleaving Aβs *in vitro* (Miners et al., 2008). Metabolism of Aβ(1-40/42) is considered as a physiologically relevant process. Neprilysin, metalloproteinases, insulin-degrading enzyme, angiotensin-converting enzyme, plasmin, etc. are suggested for the role of amyloid-degrading enzymes (Turner and Nalivaeva, 2007). Saido et al. (1995) have shown the predominance of the N- terminal truncated pE-Aβ(3-42) over the full-length form in the brain of AD patients. But Aβ fragments starting with residue 3 were never reported when APP processing was analyzed *in vitro* (Turner et al., 2003). Hence, some protease may produce cleavage at position 3 *in vivo*, encouraging the cyclization of N-terminal glutamate by glutaminyl cyclase and forming resistant to extracytoplasmic pyroglutamate aminopeptidases pE-Aβ(3-42). Glutaminyl cyclase (QC, EC 2.3.2.5) (Shirotani et al., 2002), an acyltransferase that catalyzes the conversion of N-terminal glutaminyl residues into pyroglutamic acid, is up-regulated in AD brain (Morawski et al., 2014). Hence, N-terminal truncation followed by QC catalyzed pE-modification might contribute to Aβ pathogenesis and toxicity (Saido et al., 1995; Piccini et al., 2005). The existence of a yet unknown protease, cleaving the peptide at position 3 and liberating the precursor for pGluformation by QC has been suggested (Piccini et al., 2005).

Abbreviations: Aβ, amyloid beta peptide; Aβ(1-40), amyloid beta peptide (1-40); Aβ(1-42), amyloid beta peptide (1-42); AD, Alzheimer's disease; DHB, 2,5-Dihydroxybenzoic acid; DPPIV, Dipeptidyl peptidase IV; Gln-AMC, L-Glutamine 7-amido-4-methylcoumarin; Gly-Pro-pNA, Gly-Pro p-nitroanilide toluene sulfonate salt; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; Ile-Thia, Ile-thiazolidide; pE-Aβ(3-40/42), pyroglutamated Aβ(3-40/42); QC, Glutaminyl cyclase; ThT, Thioflavin-T.

* Corresponding author. H. Buniatian Institute of Biochemistry of Armenian NAS, 5/1 P. Sevak Str., Yerevan 0014, Armenia.
E-mail address: biochem@ipia.sci.am (A. Antonyan).

https://doi.org/10.1016/j.neuint.2017.12.001
0197-0186/

Table 1
Sequences and masses of synthesized Aβ peptides.

Peptide	Sequence	$[M_{mono}+H]^+$
Aβ(1-40)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	4328.156
Aβ(1-42)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	4512.277
Aβ(3-40)	EFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	4142.092
Aβ(3-42)	EFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	4326.213

Dipeptidyl peptidase (DPP) family enzymes release N-terminal dipeptides from peptides and proteins with Pro or Ala in the penultimate position. The N-terminal sequence of Aβs contains an alanine residue at the second position, wherefrom it can be speculated that the action of any enzyme of this family might generate Aβ(3-40/42).

Indeed, Schilling et al. have shown the formation of Aβ(3-11) from the synthetic peptide Aβ(1-11) in the presence of porcine Dipeptidyl peptidase IV (DPPIV, EC3.4.14.5) (Schilling et al., 2004). Sharoyan with colleagues demonstrated the truncation of Aβ(1-16), Aβ(1-40) and Aβ(1-42) peptides by DPPIV from bovine kidney (Sharoyan et al., 2013; Movsisyan et al., 2013). Hence, DPPIV has the potential to represent the unknown protease, cleaving Aβs at position 3. As a result, these peptides become the substrates for Glu cyclization catalyzed by QC, as it was suggested by Saido and colleagues (Saido et al., 1995; Piccini et al., 2005).

The chemokines, neuropeptides, hormones and growth factors are among the known natural substrates of DPPIV, which modulates their biological activity (Boonacker and Van Noorden, 2003). BRI, amyloid peptide of familial British dementia, has been identified to be a substrate of DPPIV *in vivo* (Jost et al., 2009). The literature data indicate the presence of only traces of DPPIV in adult human brain (Bernstein et al., 1987; Wagner et al., 2008). However, the activity of the enzyme increases in the brain of animal models during stimulation of meningitis (Mitro and Lojda, 1988) and in neurons within the infarct area after transient focal cerebral ischemia (Röhnert et al., 2012). Hence, DPPIV can participate in the disease-related processes.

In the present work, the interplay of DPPIV and QC in the conversion of Aβ(1-40/42) into more aggregation-prone and toxic (resistant to peptidases) pyroglutaminated species pE-Aβ(3-40/42) was demonstrated *in vitro*.

2. Materials and methods

2.1. Chemicals and equipment

The electrophoretically homogenous DPPIV from bovine kidney cortex (Sharoyan et al., 2006) and recombinant human QC (Schilling et al., 2002) were purified in our laboratories. Gly-Pro-pNA and Gln-AMC, ThT and HFIP were purchased from Sigma LTD (USA). Ile-Thia was synthesized as described previously (Hildebrandt et al., 2001).

All aqueous solutions were prepared using ultrapure water (Milli-Q, Millipore, and Milford, U.S.A.). MALDI matrix 2,5-dihydroxybenzoic acid (DHB) and peptide calibration standard for MALDI-TOF mass spectrometry were obtained from Bruker Daltonics (Bremen, Germany). Acetonitrile (LC grade) and spectroscopic grade trifluoroacetic acid were purchased from Merck Millipore (Darmstadt, Germany).

Spectral measurements were performed using a Cary 60 spectrophotometer (USA) and microplate reader LabLine-022 (West Medica, Perchtoldsdorf, Austria). For fluorescence measurements, MPF-44A spectrofluorometer (Perkin Elmer, USA) and fluorescence plate reader (FLUOstar OPTIMA, BMG Labtech GmbH, Ortenberg, Germany) were applied using quartz cuvettes with light path 0.5–1 cm and microplates, respectively, in thermostatic holders. The standard temperature was 25 °C.

2.2. Peptide synthesis

The human type Aβ(1-40/42) and Aβ(3-40/42) peptides were synthesized as described previously (Schilling et al., 2006). The synthesized peptides, their amino acid sequence and the respective monoisotopic masses of the protonated molecules are summarized in Table 1:

2.3. DPPIV and QC reaction conditions

In the experiments, DPPIV and QC with specific activities 10 U/mg and 11 U/mg, respectively, were used. The degradation of Aβ(1-40/42) by DPPIV was investigated in 40 mM HEPES buffer, pH 8.0. The combined action of 50 μg/ml DPPIV and 597 μg/ml QC on Aβ(1-40/42) was studied in 40 mM Tris-HCl buffer, pH 7.4. In all experiments the concentration of Aβ(1-40/42) peptides was 20 μM. Reactions were carried out at 37 °C. For DPPIV inhibition, the Ile-Thia was used at a final concentration of 7 μM.

To identify the DPPIV- and QC-modified forms of Aβ(1-40/42) peptides, MALDI-TOF mass spectrometry analysis was used. Briefly: at the indicated time points, samples were removed from the assay tube, gently heated to 85 °C to stop the reaction and cooled. The samples taken at 0 min and those containing the heat-deactivated enzyme(s) served as controls. Before applying to MALDI-TOF analysis, the samples were purified using C18ZipTips (Millipore, Billerica, U.S.A.) according to the manufacturer's instructions. Elution solutions were mixed with DHB matrix (20 mg/ml in methanol) and applied to a MALDI ground steel target plate (Bruker Daltonics, Bremen, Germany) according to the dried-droplet preparation.

2.4. MALDI-TOF mass spectrometry

Mass spectra were acquired on an Autoflex Speed MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflector and positive ion mode with the acceleration voltage set to 19 kV. Ionization of Aβ peptides was achieved by irradiating the sample spot at different positions with a smart beam-II laser (Bruker Daltonics, Bremen, Germany). The smart beam-II laser had a pulse rate of 1 kHz and an emission wavelength of 355 nm. Each spectrum was an average of at least 5000 laser shots.

Spectra were processed with flex Analysis (Version 3.4, Bruker Daltonics, Bremen, Germany) by applying baseline subtraction with Top Hat algorithm and detection of the monoisotopic signals with SNAP algorithm (signal-to-noise threshold set to 6). Each spectrum was calibrated using a set of peptides (Peptide Calibration Standard II, Bruker Daltonics, Bremen, Germany) with known masses, applied with DHB matrix on the MALDI target plate in close vicinity to the sample spots and analyzed with the same laser energy.

2.5. Aggregation of amyloid peptides

Prior to use, the peptides were disaggregated in HFIP. After evaporation of HFIP, the peptides were dissolved in 0.1 M NaOH.