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http://dx.doi.org/10.1111/bcpt.13094

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Extracellular prolyl oligopeptidase derived from activated microglia is a potential neuroprotection target

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Running title: Prolyl oligopeptidase in neuroinflammation

Keywords: prolyl oligopeptidase; neuroinflammation; neuroprotection; microglia; astrocytes

Conflict of interest: none
Non-standard abbreviations:

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<th>Abbreviation</th>
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<tr>
<td>ac-PGP</td>
<td>N-acetylated proline glycine-proline</td>
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<td>AMC</td>
<td>7-amino-4-methylcoumarin</td>
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<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
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<td>IFNγ</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>Lipopolysaccharide</td>
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ABSTRACT

Prolyl oligopeptidase (PREP) is an abundant peptidase in the brain and periphery, but its physiological functions are still largely unknown. Recent findings point to a role for PREP in inflammatory processes. This study assesses the cellular and extracellular PREP activities in cultures of mouse primary cortical neurons, microglial cells and astrocytes, and immortalized microglial BV-2 cells under neuroinflammatory conditions induced by lipopolysaccharide (LPS) and interferon gamma (IFNγ). Furthermore, we evaluated the neuroprotective effect of a specific PREP inhibitor, KYP-2047, in a neuroinflammation model based on a co-culture of primary cortical neurons and activated BV-2 cells. The inflammatory insult reduced intracellular and increased extracellular PREP activity specifically in microglial cells, suggesting that activated microglia excretes active PREP. A targeted proteomics approach revealed upregulation in PREP protein levels in BV-2 cell growth medium, but downregulation in crude membrane bound PREP after LPS+IFNγ. In the co-culture of BV-2 cells and primary neurons, an increase in extracellular PREP activity was also detected after inflammation. KYP-2047 (10 µM) significantly protected neurons against microglial toxicity, and reduced the levels of the pro-inflammatory cytokine tumor necrosis factor alpha. In conclusion, these data point to an extracellular role for microglial PREP in the inflammatory process. Inhibition of PREP during neuroinflammation is a potential target for neuroprotection. Thus, PREP inhibitors may offer a novel therapeutic approach for the treatment of neurodegenerative disorders with an inflammatory component including Parkinson's and Alzheimer's diseases.
INTRODUCTION

Prolyl oligopeptidase (PREP; EC 3.4.21.26) is an 80 kDa serine protease that cleaves proline containing small peptides (1). PREP is an abundant peptidase in both the brain and periphery (2,3), but its physiological functions are still largely unknown. Earlier, PREP was associated with learning and memory processes because of its rather unique substrate specificity towards certain proline containing bioactive peptides involved in learning and memory, and numerous pharmacological PREP inhibitors were synthesized with the principal aim of developing anti-amnesic drugs (4). However, despite some promising results even in clinical studies (5), PREP inhibitors have not entered the market to date, probably due to a lack of efficacy as well as an incomplete understanding about the physiological functions of this enzyme. Nevertheless, several potential functions for PREP have been proposed over the last decade; it may be involved in α-synuclein aggregation in Parkinson’s disease (6,7), in angiogenesis (8), and in inflammatory responses (9,10). PREP has also been associated with neurodegenerative processes, since post mortem brain PREP activity has been observed to be altered in several neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and multiple sclerosis (11).

Microglia are resident immune cells in the CNS having wide variety of physiological functions e.g. tissue surveillance and synaptic remodeling. The acute inflammatory microglia activation has been suggested to promote clearance of pathogens and cellular debris and restore tissue homeostasis (12). However, chronic activation of microglia is harmful to the CNS and there is a convincing body of evidence pointing to a role for neuroinflammation in the pathogenesis of neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases (13–15). Microglia activation results in a flooding of surrounding tissue with inflammatory mediators, oxidizing free radicals, pro-apoptotic factors, matrix-degrading proteases and chemoattractant molecules (16). All of these activities contribute to the inflammatory process. Since neuroinflammation has been closely associated with neurodegenerative pathways (13–15), anti-inflammatory compounds may be potential disease-modifying treatment options for neurodegenerative diseases. Interestingly, there is now an extensive literature focused around the theme of PREP and neuroinflammation. The expression of PREP
protein in mouse brain has been demonstrated to be elevated in microglial cells and astrocytes after an inflammatory insult (10). Furthermore, PREP knockout mice were resistant to the microglia activating effect of lipopolysaccharide (LPS) treatment (17). Brain PREP levels were significantly elevated in an in vivo rat model of neuroinflammation, and notably, in rats experiencing neuroinflammation, a microdialysis approach revealed increased PREP activity in the brain extracellular space (18), although under normal conditions, the enzyme is known to be mainly cytosolic (19,20). The origin of this extracellular PREP activity remains unclear nor is it known why it is excreted into the extracellular space in inflammatory conditions. Elevated extracellular PREP activity has also been detected from different body fluids in diseases with an inflammatory component, including several forms of cancer (21–23), cystic fibrosis (9) and rheumatoid arthritis (24), hinting at a role for extracellular PREP in the remodeling of tissues at sites of inflammation. Moreover, PREP enzymatic activity has been recently associated with cleavage of extracellular matrix components (9,25). Although the contribution of PREP to the development of peripheral or neuronal inflammation is not well understood, one could postulate a specific extracellular role for PREP in the inflammatory process. Furthermore, it remains to be clarified whether specific PREP inhibitors have anti-inflammatory or neuroprotective properties in central nervous system (CNS) diseases with an inflammatory component.

To shed light on the behavior of PREP in neuroinflammatory conditions, we investigated the intra- and extra-cellular PREP enzymatic activities under LPS and interferon gamma (IFNγ) induced inflammation in cultures of mouse primary cortical neurons, microglial cells and astrocytes, and immortalized microglial BV-2 cells. We hypothesized that activated microglia might be the source of the extracellular PREP activity observed in previous studies. Furthermore, we assessed the neuroprotective effect of a specific PREP inhibitor, KYP-2047, in an in vitro model of neuroinflammation based on a co-culture of primary cortical neurons and microglial BV-2 cells activated with LPS and IFNγ.
MATERIALS AND METHODS

Ethical statement

The experimental procedures involving animals (primary cell harvest) were made in compliance with the European Commission Directive 2010/63/EU and approved by the Institutional Animal Care and Use Committee of University of Eastern Finland (Animal Usage Plan numbers: EKS-008-2016 and EKS-006-2017). All efforts were made to minimize the number of animals used and to minimize their suffering.

BV-2 cell cultures

Immortalized mouse microglial BV-2 cells were grown in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco), 2mM L-glutamine (Lonza) and 1% penicillin-streptomycin (P/S: 100 U/ml penicillin and 100 U/ml streptomycin, Lonza) in 15 cm cell culture plates (Nunc) as previously described (26). In the PREP activity measurement, ~4x10^5 cells were plated / well in 6-well plates in Neurobasal medium (Gibco) supplemented with 2 % B27, 2 mM L-glutamine and 1% P/S. After 2h, inflammation was induced by addition of 200 ng/ml LPS (Sigma) and 20 ng/ml of mouse IFNγ (Sigma). After 48 h, medium samples were collected and cultures were rinsed twice with ice cold phosphate buffered saline (PBS) (DPBS, Lonza) on ice. Cells were scraped in 100 µl of T-Per (ThermoScientific) and incubated on ice for 30 min. The lysates were centrifuged at 10,000 x g, 10 min, at +4°C and supernatants were collected to new tubes for PREP activity assays. In the targeted PREP proteomics assay, ~10^6 cells were plated in 10 cm dishes and treated similarly as the samples in the PREP activity measurement. After 48 h, cells were washed three times with PBS, scraped into ice cold PBS followed by centrifugation of the cells into a pellet and rapid freezing in liquid nitrogen.
**Mouse primary microglia and astrocyte cultures**

Neonatal (P0–P2, mixed gender) C57BL/6 WT mice were supplied by the Laboratory Animal Center of the University of Eastern Finland, Kuopio, Finland. The mice were housed in stainless steel cages and kept on a 12-h light/12-h dark cycle at an ambient temperature of 22°C. The pups were rapidly decapitated and the brains were dissected and meninges were removed. Brain tissue was dissociated using mechanical shearing and trypsin. Cells of two brains were plated on poly-L-lysine (Sigma) coated T75 culture flasks and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Lonza) supplemented with 10 % FBS (Gibco), 2 mM L-glutamine (Lonza) and 1 % P/S (Lonza). On the next day, the cells were washed three times with PBS (DPBS, Lonza) to remove cellular debris and cultured with DMEM supplemented with 10 % FBS, 2 mM L-glutamine (Lonza) and 1 % P/S (Lonza). After 7 days, mature microglia were shaken off from the astrocytic monolayer. Microglia were cultured in DMEM (with 10 % FBS, 2 mM L-glutamine and 1 % P/S). The remaining astrocytic monolayer was trypsinized using 0.05 % Trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco). Astrocytes were cultured in 15 cm dishes in F12/DMEM (Gibco) supplemented with 2 mM L-glutamine and 1 % P/S and subcultured until passage 4-5 in order to remove any microglial cells. In the PREP activity measurement, both primary microglia and astrocytes were treated with LPS and IFNγ for 48 h and the medium samples and cell lysates were collected similarly as in BV-2 cells. The purities of the primary microglial and primary astrocytic cultures were investigated by immunofluorescence staining. Cd11b monoclonal rat primary antibody (1:300; AbD Serotec) and Alexa Fluor 594 anti-rat secondary antibody (1:500; Abcam) were used for primary microglial cultures. Nuclei of the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1µg/ml; Sigma). A representative image (Supporting information figure S1A) shows that in primary microglial culture, practically all cells are Cd11b positive microglial cells confirming the high purity of the present microglial cultures. Primary astrocytic cultures were stained using glial fibrillary acidic protein (GFAP) monoclonal rat primary antibody (1:400; Invitrogen) and Alexa Fluor 594 anti-rat secondary antibody (1:1000; Invitrogen). In addition, to reveal possible microglial contamination, the same cultures were stained with ionized calcium-binding adapter molecule 1 (Iba1) polyclonal rabbit primary antibody.
A representative image (Supporting information figure S1B) shows that majority of the cells are indeed GFAP positive astrocyte cells. However, there are some Iba1 positive cells and cells with only DAPI staining, indicating that other cell types, such as microglia, oligodendrocytes and endothelial cells, are also present in the culture to some extent.

**Mouse primary cortical neuron cultures**

Primary cortical cultures were prepared as previously described in studies published by our group (27,28). Briefly, on embryonic day 18 (E18), pregnant C57BL/6 mice (age 4-6 months, Laboratory Animal Center of the University of Eastern Finland) were euthanized by cervical dislocation. Unborn pups were euthanized by rapid decapitation. Cortical brain tissues were dissected under the microscope and cells were harvested as described previously (27). Cells pooled together from different embryos were plated (18 x 104/cm²) on poly-D-lysine (PDL)-coated wells in 6-well or 48-well plates in serum-free Neurobasal medium (Gibco, Invitrogen) supplemented with 1 × B27 (Gibco), 2 mM L-glutamine (Lonza) and 1 % P/S (Lonza). Half of the medium was refreshed after 4 days in vitro (DIV4). In the measurement of PREP activity, cells were treated at DIV5 with LPS and IFNγ (200 ng/ml and 20 ng/ml, respectively) and samples were collected at DIV7 similarly as in BV-2 cells. We have characterized these primary neuronal cultures in detail in our previous studies (28) and identified that at DIV7, the cultures contain 80 % of microtubule-associated protein 2 (MAP2) positive cells, 12 % of GFAP positive astrocytes and 8 % of other cell types. We also confirmed by western blotting that LPS-IFNγ treatment does not induce astroglycosis in primary neurons nor in the co-culture of neurons and BV-2 cells as indicated by unchanged GFAP levels after treatment (data not shown).
Mouse primary cortical neuron and BV-2 cell co-culture

The neuroprotective effect of KYP-2047 was assessed in a co-culture of primary neurons and BV-2 microglial cells. This co-culture model was established based on the method described by Gresa-Arribas et al. (26) with minor modifications. Preparation and characterization of the co-cultures have been described previously (26–28). Briefly, mouse cortical neurons for the co-cultures were isolated and cultured in 48-well plates as described above. BV-2 microglial cells were seeded at a 1:5 ratio onto the primary cortical cultures on DIV5. The anti-inflammatory cytokine, interleukin 10 (IL-10, 50 ng/ml, Peprotech), and the inducible nitric oxide synthase (iNOS) inhibitor, 1400W (20 µM, Tocris), were used as positive controls. The PREP inhibitor KYP-2047 (0.1-10 µM) and positive controls were added 1 h after seeding of BV-2 cells to the co-cultures. Two hours after seeding BV-2 cells, inflammation was induced by addition of 200 ng/ml LPS and 20 ng/ml IFNγ and 48 h later, samples were collected (DIV7) similarly as described in BV-2 cells. KYP-2047 is a potent (Ki = 0.023 nM against pig PREP) (29) and specific (30) PREP inhibitor with favorable pharmacokinetic properties in vivo (31,32) and a long (4.8 h) enzyme-inhibitor complex dissociation half-life (29). PREP inhibitor concentrations in the viability assay were estimated based on our in vivo studies, where the C_{max} value of unbound KYP-2047 in brain extracellular fluid reached a level of approximately 1 µM after a single dose, a concentration that effectively inhibited rat brain PREP (32).

Viability assay

Neuronal viability in the mouse primary cortical neuron and BV-2 microglial co-cultures was assessed as described earlier (26). Briefly, wells were rinsed twice with PBS in order to remove any dead cells. The cells were fixed in 4 % paraformaldehyde in PBS for 20 min and then incubated in 0.3 % hydrogen peroxide in methanol to permeabilize the cells and block endogenous peroxidase activity. Incubation in blocking solution containing 1% bovine serum albumin and 10% horse serum for 20 min was used to prevent non-specific staining. Neurons were stained by incubation with mouse anti-MAP2 primary antibody (1:2000, Sigma) overnight at +4°C. Next, the cells were incubated with
biotinylated horse anti-mouse secondary antibody (1:500, Vector labs) for 1 h and ExtrAvidin-horseradish peroxidase (HRP) (1:500, Sigma) for another 1 h. The cells were washed three times for 10 min in PBS between the antibody incubations. All antibody dilutions were made in the blocking solution. Finally, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) Peroxidase Substrate solution (Vector Labs) was prepared following the manufacturer's instructions and added onto the cells. The absorbance was measured using a microtiter plate reader (ELx808, BioTek or Infinite M200, Tecan) at 405 nm and was directly proportional to the number of neurons in the wells. The measured background absorbance from co-cultures incubated without the anti-MAP2 primary antibody (negative controls) was averaged and subtracted from the absorbances in the other wells. The absorbance in the negative control wells was similar to the absorbance measured from wells without any cells. To assess the effect of KYP-2047 on neuronal viability, altogether 15-21 samples/treatment from three individual experiments were measured.

**LDH assay**

Cytotoxicity Detection kit (LDH) (Roche) was used in order to reveal any possible cytotoxicity of LPS and IFNγ or KYP-2047 treatment in BV-2 cells. Medium samples (50µl) from the cell cultures were used to assess the cytotoxicity according to the manufacturer’s protocol. Absorbance was measured at 450 nm with a microplate reader.

**TNF-α and NO measurements**

Mouse tumor necrosis factor alpha (TNF-α) ELISA Ready-SET-Go! kit (Affymetrix, San Diego, CA, USA) was used for the detection of TNF-α in the conditioned media. Nitric oxide (NO) levels were determined using the Griess Reagent Kit for Nitrite Determination (G-7921, Life Technologies). All kits were used as recommended by the manufacturers.
PREP activity measurement

PREP activity in cell culture and cell growth medium was measured fluorometrically using Suc-Gly-Pro-7-Amino-4-methylcoumarin (AMC) (Bachem AG, Switzerland) as the substrate for PREP. Briefly, cell medium was first concentrated from 3 mL down to 100 µl by using a centrifugal filter device (Amicon Ultra, molecular weight cut-off 30 kDa, Millipore Corporation, Billerica, MA, USA) and a Heraeus Minifuge (4000 × g, 20 min, +4°C). To accomplish buffer exchange, the remaining ultrafiltrate was reconstituted to the original sample volume with 0.1M Na-K-phosphate buffer (incubation buffer) and centrifuged again to 100 µl (4000 × g, 20 min, +4°C). Aliquots of 10 µl of the cell medium ultrafiltrates or cell lysates collected as described above were used in the PREP activity assays. The samples were pre-incubated for 30 min with 70 µl of incubation buffer at 32 °C in 96-well plates, and the enzyme reaction was initiated by adding 20 µl of 4 mM Suc-Gly-Pro-AMC. The plates were incubated for 180 min at 32°C, until the reaction was terminated by addition of 100 µl of 1 M sodium acetate buffer (pH 4.0). The formation of AMC was measured with a Victor2 fluorescence plate reader (PerkinElmer Inc., Waltham, MA, USA). The protein concentrations in the cell supernatants were measured using a BioRad Protein Assay kit (Biorad Laboratories, Hercules, CA, USA). The PREP activities in cell medium and cell lysate were normalized to the protein content of the corresponding cell lysate and calculated as mol AMC*min⁻¹*mg⁻¹ of protein.

Preparation of crude membrane and cytosolic cell fractions for targeted PREP proteomics assay

ProteoExtract® Subcellular Proteome Extraction Kit (Merck KGaA, Darmstadt, Germany) was used for the isolation of crude membrane and cytosolic fractions from the cell pellets following the manufacturer’s instructions. The protein concentrations in the fractions were determined as the mean of three samples by the BioRad Protein Assay and aliquots containing 50 µg of total protein were taken for further sample preparation.
The PREP protein expression in BV-2 cells was determined by means of targeted and quantitative liquid chromatography tandem-mass spectrometry (LC-MS/MS) based proteomics. The aliquots containing 50 µg of protein from crude membrane cell fractions were solubilized in 500 mM tris(hydroxymethyl)aminomethane (Tris)–hydrochloric acid (HCl) (pH 8.5), 7 M guanidine hydrochloride and 10 mM EDTA. The 50 µg protein aliquot from the cytosolic cell fractions were evaporated under nitrogen stream in cold water bath and solubilized in 9 µl of 100 mM Tris–HCl (pH 8.5) and 6 M urea. The cell growth medium samples were prepared by taking 10 µl aliquots and evaporating them under a nitrogen stream in a cold water bath, followed by solubilizing of the proteins in 9 µl 100 mM Tris–HCl (pH 8.5) and 6 M urea. After solubilizing, the proteins were S-carbamoylmethylated with iodoacetamide following dithiothreitol treatment. The alkylated proteins were precipitated using methanol and chloroform. The precipitates were dissolved in 6 M urea in 100 mM Tris–HCl (pH 8.5), diluted fivefold with 100 mM Tris–HCl (pH 8.5), spiked with isotopically labeled internal standard peptide VLYVQDSLEGAR (JPT Peptide Technologies GmbH, Berlin, Germany) resulting in final concentration of 3 fmol/µg. The samples were treated with Protease-Max surfactant (Promega, Madison, WI, USA) and lysyl endopeptidase (Lys-C: Wako Pure Chemical Industries, Osaka, Japan) at 30°C for 3 h. After which tosylphenylalanyl chloromethyl ketone-treated trypsin (Promega, Madison, WI, USA) at an enzyme/substrate ratio of 1:100 was added into the samples and incubated at 37°C for 16 h. The tryptic digests were mixed with 20 % (v/v) formic acid, and then centrifuged at 4°C and 14 000 × g for 5 min. The supernatant was mixed with water prior to LC-MS/MS analysis.

LC–MS/MS analysis was performed by coupling an Agilent 1290 Infinity LC (Agilent Technologies, Waldbronn, Germany) instrumentation to an Agilent 6495 Triple Quadrupole Mass Spectrometer with an electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA) using multiple reaction monitoring (MRM). The conditions used for the analysis were described in Gynther et al. (33). For the quantitation of the target protein, one unique peptide was chosen according to the in silico peptide selection criteria published by Uchida et al. (34). The PREP selective peptide
was monitored with four different selected reaction monitoring/MRM transitions (Supporting information Table S1) derived from a stable isotope-labeled peptide and the unlabeled natural peptide.

**Statistical analysis**

The number ‘n’ indicates the number of independent observations with different cell cultures and experimental conditions. Results in the text are expressed as mean ± standard deviation and in the figures as box and whiskers plots representing the median and the value of the highest and lowest observation. Statistical analyses were done with GraphPad Prism 5.03 software (GraphPad Software, San Diego, CA, USA) or SPSS Statistics (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY, IBM Corp.). Differences in PREP activities and protein levels between untreated and LPS+IFNγ treated groups were tested using unpaired two-sample two-tailed Student t-test (GraphPad Prism). The effects of different concentrations of KYP-2047 on neuronal viability and TNF-α levels were tested with one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc test (SPSS). For the variance analyses, the normal distribution of the data was tested with Shapiro-Wilk’s W test and the homogeneity of variances with Levene’s test (SPSS). Outliers were tested by Grubb’s test using online tools provided by GraphPad Software (https://www.graphpad.com/quickcalcs/grubbs2/). No data were excluded from the analyses. The criterion for statistical significance was set at p < 0.05.

**RESULTS**

*PREP enzymatic activity in different cell types and in cell culture mediums*

First, we investigated the cellular PREP enzymatic activities from untreated mouse primary neurons, primary microglial cells, primary astrocytes and microglial BV-2 cells (Figure 1A-D). The highest cellular PREP activity was detected in the primary neurons (4.8 ± 1.4 nmol/min/mg) followed by
BV-2 cells (4.7 ± 1.7 nmol*min⁻¹*mg⁻¹), primary astrocytes (3.2 ± 0.14 nmol*min⁻¹*mg⁻¹) and primary microglia (1.3 ± 0.06 nmol*min⁻¹*mg⁻¹). Next, we stimulated the cells with a combination of LPS and IFNγ. In primary neurons, there was a small non-significant decrease (to 4.2 ± 2.3 nmol*min⁻¹*mg⁻¹) in PREP activity after LPS+IFNγ treatment. Instead, in primary microglial cells and in BV-2 cells, LPS and IFNγ stimulation significantly reduced cellular PREP activity by 23 % (to 1.0 ± 0.1 nmol*min⁻¹*mg⁻¹; t-test: p = 0.0158) and 57 % (to 2.0 ± 0.7 nmol*min⁻¹*mg⁻¹; t-test: p = 0.0006), respectively. Notably, in primary astrocytes, LPS and IFNγ induced a significant 46 % increase (to 4.6 ± 0.2 nmol*min⁻¹*mg⁻¹; t-test: p = 0.0006) in PREP activity compared to untreated astrocytes. Next, we measured PREP activity in a co-culture of primary neurons and BV-2 cells with and without the presence of LPS and IFNγ (Figure 1E); the inflammatory stimulus reduced PREP activity by 17 %, but the difference from the untreated co-culture was statistically non-significant (4.7 ± 3.4 vs. 3.9 ± 2.5 nmol*min⁻¹*mg⁻¹ in untreated and stimulated co-cultures, respectively).

Based on the findings of reduced cellular PREP activities, we hypothesized that the microglial PREP enzyme activity had been excreted into the extracellular space after the neuroinflammatory stimulus. Thus, we measured PREP activities from the cell culture mediums with and without LPS and IFNγ (Figure 2A-E). Indeed, in primary microglial and BV-2 cultures, we were able to detect significant amounts of PREP enzymatic activity, and the activities were significantly upregulated after the inflammatory stimulus. Overall, the extracellular activities were low compared to the cellular activities (in the picomolar range vs. nanomolar in cells). In BV-2 cells, there was a 3.5-fold increase (from 199 ± 68 to 709 ± 181 pmol*min⁻¹*mg⁻¹; t-test p = 0.0103) in the extracellular activity after LPS and IFNγ stimulation. In primary microglia, the extracellular PREP activities were low, but LPS and IFNγ treatment induced a significant 7-fold increase (from 2.6 ± 1.2 to 18 ± 1.3 pmol*min⁻¹*mg⁻¹; t-test: p = 0.0001) in the extracellular activity. In primary neurons and astrocytes, we found no detectable PREP activities in the cell culture mediums. Instead, in a co-culture of primary neurons and BV-2 cells, LPS and IFNγ significantly upregulated extracellular PREP activity by 2.6 times (from 18.7 ± 4.1 to 49 ± 15 pmol*min⁻¹*mg⁻¹; t-test: p = 0.0305). Importantly, based on an LDH cytotoxicity assay, LPS and IFNγ treatment did not induce any significant cell death in BV-2 cell cultures as compared...
to control treatment (see Supporting information Figure S3A), indicating that PREP was not released to the extracellular space because of microglial cell damage.

Next, to clarify the origin of the extracellular PREP at cellular level, we investigated the PREP protein levels from the cytosolic and crude membrane fractions of the BV-2 cells. In addition, we wanted to confirm that the measured extracellular PREP activity was indeed attributable to PREP protein. For these purposes, we set up a targeted LC-MS/MS proteomics assay for detection of PREP protein from crude membrane and cytosol as well as from cell medium with and without LPS+IFNγ induced inflammation. PREP protein was present in both investigated cell fractions and in the cell medium (see Supporting information Figure S2 for representative chromatogram). Interestingly, the inflammatory stimulus induced a significant decrease in crude membrane bound PREP protein levels (from 0.49 ± 0.03 to 0.35 ± 0.02 fmol*µg\(^{-1}\) protein; t-test: p = 0.0033) (Figure 3A), whereas, there was a trend towards increased PREP levels in the cytosolic fraction (from 1.83 ± 1.19 to 2.90 ± 0.89 fmol*µg\(^{-1}\) protein) (Figure 3B) and a significant 2.5-fold increase in the extracellular PREP protein concentrations (from 0.04 ± 0.02 to 0.10 ± 0.02 fmol*µg\(^{-1}\) protein; t-test: p = 0.0132) in the LPS+IFNγ treated cells compared to control cells. Finally, a specific PREP inhibitor assay control (KYP-2047 at 1 µM concentration) was included to every PREP activity assay to confirm that the formed AMC resulted from PREP enzymatic activity. By this approach, we controlled that the substrate is not cleaved by some other peptidase in these experimental conditions. The results confirmed (data not shown) that the AMC production could be completely blocked by KYP-2047, confirming that the detected activities were indeed a result of functional PREP enzymatic activity.

The effect of PREP inhibition on neuronal viability and TNF-α levels

Next, we hypothesized that the extracellular PREP derived from activated microglia might contribute to microglial toxicity and thus could be a potential target for neuroprotection. Thus, we performed a pharmacological PREP inhibitor study in a co-culture model of mouse primary neurons and BV-2 cells. The detailed characteristics of the model have been published by Martiskainen et al. (28). BV-
2 cells were added to the mouse cortical neuron cultures after 5 DIV at the ratio 1:5 and neuroinflammation was induced using LPS and IFNγ. Activated BV-2 cells secrete proinflammatory cytokines and nitric oxide and induced a significant 50 % decrease in neuronal viability as compared to vehicle treated samples (LSD post hoc: p < 0.001 No LPS+IFNγ vs. control, Figure 4A). Positive controls IL-10 and 1400W worked as described previously (27,28). IL-10 decreased TNF-α levels ~60 % while 1400W reduced NO levels ~90 % and restored neuronal viability to the same level with vehicle treated samples (data not shown). There was a trend towards dose-dependent neuroprotection by the PREP inhibitor KYP-2047 (0.1-10 µM), and at 10 µM concentration, KYP-2047 significantly protected neurons against microglial toxicity (overall effect of treatment: one-way ANOVA: F_{4,80} = 27.308, p < 0.001; LSD post hoc vs. control: 1 µM p = 0.078; 10 µM p < 0.001; Figure 4A). Furthermore, KYP-2047 displayed anti-inflammatory properties by significantly reducing TNF-α levels in the co-culture at all examined concentrations (overall effect of treatment: one-way ANOVA: F_{4,15} = 111.044, p < 0.001; LSD post hoc vs. control: No LPS+IFNγ p < 0.001; 0.1 µM KYP-2047 p = 0.026; 1 µM KYP-2047 p = 0.01; 10 µM KYP-2047 p < 0.0001; Figure 4B). There was also a trend towards decreased NO levels with all KYP-2047 concentrations (data not shown). However, this decrease was not statistically significant. We also confirmed by an LDH cytotoxicity assay that KYP-2047 at 10 µM concentration was not cytotoxic to BV-2 cells (see Supporting information figure S3B).

**DISCUSSION**

By using the targeted proteomics approach and functional enzyme activity assay, we demonstrated that microglial BV-2 cells contain both PREP protein and enzyme activity. When primary or immortalized microglial cells were stimulated with LPS and IFNγ, the cellular PREP activities decreased and the extracellular activities increased, suggesting that the microglial cells excrete active PREP into the extracellular space upon inflammation. Instead, in primary neurons and astrocytes, PREP activity remained intracellular and was even upregulated in astrocytes in response to the inflammatory stimulus. However, the results obtained with the primary astrocytes should be interpreted with caution, because our immunofluorescence staining revealed that the astrocyte...
cultures, unlike the primary microglial cultures, contained some contaminating cell types. In a co-culture of primary neurons and BV-2 cells, a significant increase in extracellular PREP activity was observed upon inflammation, implying that activated microglia had been the source for the extracellular activity also in the co-culture. Notably, extracellular PREP activity in pmol*min$^{-1}$*mg$^{-1}$ appeared to be much lower in the co-culture than in the BV-2 cells simply because the activity was normalized to the total protein content of the corresponding cell lysate, and a large portion of the total protein in the co-culture is from the neurons that do not excrete PREP into the extracellular space, thus diluting the activity. The mechanism by which microglial cells extrude active PREP into the extracellular space is unclear since its protein sequence lacks the signal peptides associated with secretory pathways. Interestingly, crude membrane bound PREP protein levels in BV-2 cells decreased during inflammation, making it tempting to speculate that the increased extracellular activity is actually released from the membrane bound form of PREP. Indeed, it has been postulated that the membrane bound PREP is merely a transitory stage needed for the transportation of cytosolic PREP out of the cell (35).

Extracellular PREP activity has been detected in various human body fluids, although the activities have been low compared to the cellular levels (21) as also observed in the present study. Interestingly, elevations in human plasma PREP activities have been associated with several pathological conditions involving an inflammatory component, such as schizophrenia and depression (36). Elevated PREP activity has also been detected in the sputum of cystic fibrosis patients (9) and knee synovial fluid of patients with rheumatoid arthritis (24). Preclinically, extracellular brain PREP activity has been identified in a rat model of neuroinflammation (18). Thus, the present study raises the possibility that the increased extracellular PREP activity observed in these pathological conditions did originate from the immune cells at the site of inflammation. It remains to be clarified why extracellular PREP activity increases specifically in microglial cells during inflammation, but PREP appears to play a role in microglia activation, since PREP knockout mice show a lack of response to LPS at both behavioral and immunohistochemical levels (17). Furthermore, extracellular PREP has been associated with the cleavage of extracellular matrix components, such as collagen.
PREP overexpression in SH-SY5Y cells selectively activates matrix metalloprotease 9 (MMP-9), a key player in the extracellular matrix remodeling. The extracellular location of PREP upon neuroinflammation may also promote the extracellular cleavage of the classical PREP substrates, such as substance P, neurotensin, arginine-vasopressin and bradykinin (1), and PREP inhibitors might protect these peptides against cleavage and increase their brain levels in pathological conditions involving an inflammatory component.

Since PREP appears to have specific extracellular functions during inflammation that may be related to extracellular matrix remodeling, we hypothesized that specific PREP inhibitors might have neuroprotective effects upon neuroinflammatory stimulus. We tested this hypothesis in a co-culture of mouse primary neurons and microglial BV-2 cells treated with a specific PREP inhibitor, KYP-2047. Interestingly, we observed a trend towards dose-dependent increase in neuronal viability after KYP-2047 treatment, evidence of a potential neuroprotective effect for PREP inhibitors. Notably, KYP-2047 at 10 µM concentration was not cytotoxic to BV-2 cells, implicating that the reduced TNF-α secretion by KYP-2047 was not due to a reduction in BV-2 cells. Neuroprotection might be explained by an anti-inflammatory effect, as was preliminary demonstrated by decreased TNF-α levels after KYP-2047 treatment. However, to confirm the anti-inflammatory effect, future research should be undertaken to investigate the effect of PREP inhibition on multiple cytokine production in microglial cells. The neuroprotective effects are in agreement with the findings of Klegeris et al. (38), who reported an increase in the extracellular PREP activity in THP-1 monocytic cells after stimulation with LPS and IFNγ, and a partial neuroprotective effect with two PREP inhibitors. KYP-2047 readily penetrates the cell membranes and reaches the intracellular PREP (32), and thus, it remains to be clarified with a non-cell membrane-permeable PREP inhibitor whether the anti-inflammatory effect is dependent upon the inhibition of intra- or extracellular PREP. In summary, these results further support the idea that PREP inhibitors have potential as neuroprotective compounds in a heightened neuroinflammatory environment, possibly by regulating the activity of extracellular matrix-degrading proteases such as MMP-9 (25). However, further studies will be needed to investigate the effects of PREP inhibition on extracellular matrix degradation.
In conclusion, PREP enzymatic activity and protein levels in microglial cells are increased in the extracellular space after an inflammatory insult, suggesting an extracellular role for PREP in the inflammatory process. A PREP inhibitor protects primary neurons against the toxicity of activated microglia and exerts anti-inflammatory effects via reduced TNF-α levels. The exact mechanism of action of neuroprotection remains to be clarified and will require further testing of PREP inhibitors in in vivo models of neuroinflammation and microglial activation. These results indicate that PREP inhibitors may offer a novel therapeutic approach for the treatment of neurodegenerative disorders with an inflammatory component including Parkinson’s and Alzheimer’s diseases.

ACKNOWLEDGEMENTS

This study has been supported by grants from the Academy of Finland (T.N.: grant: 288659), the School of Pharmacy, University of Eastern Finland (A.J.J.: NFG grant) and Estonian Research Council (K.J.: Institutional research funding grant IUT2-3). The funding sources had no involvement in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. The authors would like to thank Mrs. Jaana Leskinen and Ms. Petra Mäkinen for their excellent technical assistance, Prof. Markus Forsberg for valuable discussions and Dr. Ewen MacDonald for the revision of the language.
REFERENCES


Figure 1: An inflammatory stimulus reduces cellular prolyl oligopeptidase (PREP) activity in microglial cells. Box and whisker plots of the cellular PREP activities (nmol*min⁻¹*mg⁻¹; median and the highest and lowest observation) in untreated and in lipopolysaccharide (LPS; 200 ng/ml) plus interferon gamma (IFNγ; 20 ng/ml) treated (for 48 h) mouse primary neurons (A), mouse primary microglial cells (B), mouse primary astrocytes (C), microglial BV-2 cells (D) and in a co-culture of primary neurons and BV-2 cells (E). (Unpaired t-test: *p < 0.05, ***p < 0.001 vs. untreated cells).
Figure 2: Prolyl oligopeptidase (PREP) activity in cell growth medium is increased in microglial cell cultures after an inflammatory stimulus. Box and whisker plots of the extracellular (cell growth medium) PREP activities (pmol*min⁻¹*mg⁻¹; median and the highest and lowest observation) in untreated and lipopolysaccharide (LPS; 200 ng/ml) plus interferon gamma (IFNγ; 20 ng/ml) treated (for 48 h) mouse primary neurons (A), mouse primary microglial cells (B), mouse primary astrocytes (C), microglial BV-2 cells (D) and in a co-culture of primary neurons and BV-2 cells (E). (Unpaired t-test: *p < 0.05, ***p < 0.001 vs. untreated cells).
Figure 3: An inflammatory stimulus induces a significant decrease in crude membrane bound prolyl oligopeptidase (PREP) protein levels and an increase in cell growth medium PREP levels in BV-2 microglial cells. Box and whisker plots of the PREP protein levels (fmol*µg⁻¹ total protein in the fraction; median and the highest and lowest observation) in crude membrane fraction (A), cytosol (B) and cell growth medium (C) in untreated and lipopolysaccharide (LPS; 200 ng/ml) plus interferon gamma (IFNγ; 20 ng/ml) treated (for 48 h) BV-2 cells. Note differences in the scales of the ordinate axes. (Unpaired t-test: *p < 0.05, **p < 0.01 vs. untreated cells).
Figure 4: Prolyl oligopeptidase (PREP) inhibitor treatment increases neuronal viability and decreases TNF-α levels in a dose-dependent manner in neuron-BV-2 neuroinflammation model. Box and whisker plots on the effects (median and the highest and lowest observation) of a PREP inhibitor KYP-2047 on neuronal viability (A) and TNF-α levels (B) in a co-culture of mouse primary neurons and BV-2 microglial cells activated with lipopolysaccharide (LPS; 200 ng/ml) and interferon gamma (IFNγ; 20 ng/ml) for 48 h. (One-way ANOVA + LSD post hoc test: **p < 0.01; #p < 0.05 vs. control, ###p < 0.001 vs. control. ***p < 0.001 vs. all other groups).
Extracellular prolyl oligopeptidase derived from activated microglia is a potential neuroprotection target

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SUPPORTING INFORMATION

Table S1. Probe peptide sequence and MRM transitions for the LC-MS/MS analysis of PREP

<table>
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<th>Protein</th>
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<th>MRM transitions (m/z)</th>
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<th>Q3.1</th>
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<td>1014.47</td>
<td>886.41</td>
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* denotes $^{13}$C and $^{15}$N labeled arginine
Figure S1: Assessment of the purity of mouse primary microglial and astrocyte cultures. A) Representative image of mouse primary microglial culture showing that practically all cells are Cd11b-positive (red) microglial cells. B) Representative image of mouse primary astrocyte culture indicating that majority of the cells are GFAP-positive (red) astrocyte cells. In addition, astrocyte culture contains other cell types like Iba1-positive (green) microglial cells and unidentified cells, such as oligodendrocytes and endothelial cells. Nuclei of the cells are stained with DAPI (blue)
Figure S2: Representative chromatogram of PREP selective peptide and isotopically labelled internal standard analysed from BV-2 cell cytosolic fraction. The chromatogram was created by Skyline 4.1 software.
Figure S3: Lipopolysaccharide (LPS; 200 ng/ml) and interferon gamma (IFNγ; 20 ng/ml) (A) or KYP-2047 (10 µM) (B) treatment for 48 h have no significant effect on BV-2 cell viability (Student's unpaired t-test: p > 0.05). Box and whisker plots (median ± the highest and lowest observation) on the effects of LPS+IFNγ (A) or 10 µM KYP-2047 (B) on BV-2 cell viability as assessed by an LDH assay.