Journal of Neuroscience Methods 204 (2012) 104–110

Contents lists available at SciVerse ScienceDirect

Journal of Neuroscience Methods

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

Basic Neuroscience

In situ prolyl oligopeptidase activity assay in neural cell cultures

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ARTICLE INFO

Article history:
Received 26 April 2011
Received in revised form 23 October 2011
Accepted 6 November 2011

Keywords:
Prolyl oligopeptidase inhibitors
Prolyl oligopeptidase activity

ABSTRACT

Prolyl oligopeptidase (PREP, E.C.3.4.21.26) is a cytosolic serine protease that hydrolyzes small (<3 kDa), proline-containing peptides on the carboxyl terminal side of proline residues, and is widely distributed in the brain. High PREP activity, due to aging or neurodegenerative disease, has been hypothesised to lead to an increased breakdown of neuropeptides, resulting in a decline of cognitive functions and an acceleration of neurodegeneration. Recent data have suggested that PREP involvement in neurodegeneration cannot be explained by its extracellular space proteolytic activity alone, but may involve intracellular PREP activities as well. In order to test this, appropriate methods for measuring PREP intracellular activity must first be developed. In the present study, we developed and validated an *in situ* PREP intracellular activity assay in primary rat cortical neurons, using nitroblue tetrazolium chloride salt (NBT) and a PREP specific substrate (S)-benzyl 2-(2-(4-hydroxynaphthalen-l-ylcarbanoyl)pyrrolidin-l-yl)-2-oxoethylcarbamate (UAMC-00682). This novel *in situ* PREP activity assay was further validated in neuroblastoma SH-SY5Y cells, under conditions of PREP overexpression and inhibited PREP expression. Using this assay, we demonstrated that PREP inhibitors, Z-Pro-Pro-aldehyde-dimethylacetal, Boc-Asn-Phe-Pro-aldehyde, and (S)-1-((S)-1-(4-phenylbutanoyl)-pyrrolidine-2-carbonyl)pyrrolidine-2-carbonitrile (KYP-2047), were able to inhibit intracellular PREP activity in primary rat cortical neurons. KYP-2047 was the most potent PREP inhibitor in all assay systems tested. The validated assay enables localization and quantification of *in situ* PREP activity in primary rat cortical neurons and neuroblastoma SH-SY5Y cells, as well allows testing cell permeability and efficiency of novel PREP inhibitors.

1. Introduction

Prolyl oligopeptidase (PREP, EC 3.4.21.26) is a serine protease found in a wide range of organisms (Venäläinen et al., 2004), and is highly expressed in the brain (Kato et al., 1980; Fuse et al., 1990; Myöhänen et al., 2007). PREP hydrolyses small (<3 kDa) peptides at the carboxyl-terminal (C-terminal) side of proline residues (Polgár, 2002). PREP is considered to be cytosolic, however PREP activity has been detected in biological fluids as well (García-Horsman et al., 2007). The crystal structure of PREP revealed a conserved β-propeller domain which blocks larger proteins, and only allows short (under 30 amino acid) peptides access to the catalytic core of the enzyme (Fülöp et al., 1998). PREP has been implicated in the metabolism of peptide hormones and neuropeptides, such as α-melanocyte-stimulating hormone, luteinizing hormone-releasing hormone, thyrotropin-releasing hormone, neurotensin, oxytocin, substance P and vasopressin (Momeni et al., 2005; Männisto et al., 2007). Increased PREP activity is associated with cell death in various neurodegenerative diseases, including Alzheimer's and Parkinson's disease (Mantle et al., 1996), suggesting that PREP inhibition could be a target for neuroprotection (Rossner et al., 2005; Puttonen et al., 2006). In fact, PREP inhibitors have been shown to protect against brain ischemia (Shishido et al., 1999), to reverse scopolamine-induced amnesia (Kamei et al., 1992), and to act as cognitive enhancers (Toide et al., 1995). PREP inhibitors reduced aggregation of α-synuclein (Brandt et al., 2008), as well the number of cells with α-synuclein inclusions in a cellular model of Parkinson's disease (Lambeir, 2011). These data indicate that PREP could be a target in the treatment of Parkinson's disease. In spite of the above described research efforts, the precise mechanism by which PREP affects the neurodegeneration processes remains unclear. Recently discovered intracellular roles associated with PREP seem to point to the existence of intracellular PREP activity, and suggest that PREP's intracellular activity may be

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doi:10.1016/j.jneumeth.2011.11.011

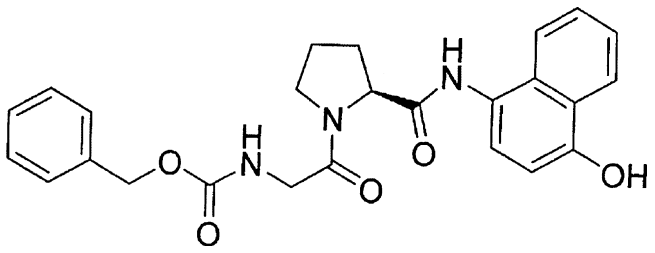

Fig. 1. Chemical structure of the PREP substrate UAMC-00682.

even more important than its extracellular activity. Intracellular localization of PREP in neuronal and glial cells was shown by Schulz et al. (2005). Previous studies demonstrated that PREP inhibition is associated with increased levels of intracellular inositol 1,4,5-triphosphate (Schulz et al., 2002) and intracellular PREP is involved in the regulation of peptide/protein secretion (Schulz et al., 2005). Recent studies also showed that PREP activities could involve functions connected to intracellular signalling and transport (Harwood, 2011; Morawski et al., 2011). To investigate these and other possible intracellular activities of PREP, an appropriate method for PREP intracellular activity measurements must be developed. The most widely used assay methods for measuring PREP activity use the fluorogenic substrate, Suc-Gly-Pro-AMC, or the chromogenic substrate, Z-Gly-Pro-β-naphtylamide (Kato et al., 1980; Irazusta et al., 2002), in either cell lysates or brain homogenates (cell free methods). However, these methods have significant limitations. Importantly, they do not allow the measurement of intracellular PREP activity in intact cells.

To address this problem, in the present study we developed and validated a novel method for measuring intracellular PREP activity *in situ*, using nitroblue tetrazolium chloride salt (NBT) and a specific, cell-permeable PREP substrate (S)-benzyl 2-(2-(4-hydroxynaphthalen-l-ylcarbanoyl)pyrrolidin-l-yl)-2-oxoethylcarbamate (UAMC-00682, Fig. 1) in primary rat cortical neurons and neuroblastoma SH-SY5Y cells. In addition, we tested the effects of a reported PREP inhibitor, KYP-2047 (Venäläinen et al., 2006), and two, commercially available PREP inhibitors, Z-Pro-Pro-aldehyde-dimethylacetal and Boc-Asn-Phe-Pro-aldehyde, on intracellular PREP activity in primary cortical neurons. To validate the method for measuring *in situ* PREP intracellular activity in cellular models, we measured *in situ* PREP activity in two neuroblastoma SH-SY5Y cell lines, one with overexpression of PREP and α-synuclein and another where PREP expression was knocked down by shRNA. Results were analysed using data obtained by classical cell free PREP activity assay using the chromogenic substrate, Z-Gly-Pro-pNA.

2. Materials and methods

2.1. Primary culture of rat cortical neurons

Primary cultures were prepared from 1-day-old Wistar rat pups, according to the method of Alho et al. (1988), with minor modifications. Briefly, cortices were dissected in ice-cold Krebs–Ringer solution (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM KH_2PO_4, 15 mM glucose, 20 mM HEPES, pH 7.4, containing 0.3% bovine serum albumin) and trypsinised in 0.08% trypsin-EDTA (Invitrogen, U.K.) for 10 min at 37 °C, followed by trituration in 0.008% DNAse I solution containing 0.05% soybean trypsin inhibitor (both obtained from Surgitech AS, Estonia). Cells were resuspended in Eagle's basal medium with Earle's salts (BME, Invitrogen, U.K.), containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, U.K.), 25 mM KCl, 2 mM GlutaMAX™-I (Invitrogen, U.K.) and 100 μg/mL gentamycin. Cells were plated onto poly-L-lysine (Sigma Chemical Co., MO, USA) coated plates and dishes at a density of 1.8×10^5 cells/cm^2. 2.5 h later, the medium was changed to Neurobasal™-A medium, containing 2 mM GlutaMAX™-I with B-27 supplement and 100 μg/mL gentamycin. Cultures were incubated for 6 days in a 5% CO_2/95% air atmosphere at 37 °C, and one-fifth of the culture medium was changed on DIV4 (days in vitro 4).

2.2. Overexpression of PREP in SH-SY5Y cells

2.2.1. Procedure for the double transfectants with a-syn and PREP overexpressing cells

Human PREP gene was cloned in a lentiviral vector and introduced into the α-synuclein-overexpressing cell line as described in Gerard et al. (2010). The lentiviral construct contained a cytomegalovirus promoter and allowed co expression of the hygromycin selection marker from the same transcript via an internal ribosomal entry site. In brief, 500,000 SH-SY5Y cells overexpressing α-synuclein were plated in a 6 well plate and grown in DMEM/GlutaMAX-I (Invitrogen, U.K.) completed with 1 μg/mL puromycin (Sigma Chemical Co, MO, USA). The next day, the vector was applied to the cells for 16 h, after which the vector-containing medium was replaced by DMEM/GlutaMAX-I (Invitrogen, U.K.) completed with 1 μg/mL puromycin and 200 μg/mL hygromycin B (Sigma Chemical Co, MO, USA). After 5 days of selection, the activity and protein levels of PREP were assayed using non-cellular activity measurements and western blot, respectively.

In the experiments on neuroblastoma SH-SY5Y cell lines, the following groups were used: wild type cells, α-synuclein overexpressing cells, and α-synuclein and PREP overexpressing cells (further in the text referred to as PREP overexpressing cells). The cells were grown in DMEM/GlutaMAX-I medium containing 15% FBS and 50 μg/mL gentamycin for wild type cells; 1 μg/mL puromycin for α-synuclein overexpressing cells and 1 μg/mL puromycin plus 200 μg/mL hygromycin B for PREP overexpressing cells.

2.3. Knocking down of PREP expression in SH-SY5Y cells

Human neuroblastoma SH-SY5Y cells were cultured in DMEM/F-12 medium (Invitrogen, U.K.) containing 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS, and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells were transfected with either empty vector (control) or validated PREP (shPREP) shRNA plasmid (SA Biosciences, MD, USA) using Lipofectamine™ 2000 (Invitrogen, U.K.) according to manufacturer's protocol. Briefly, 24 h before transfection, SH-SY5Y cells were plated in 24-well plates. The next day, at approximately 60–70% cell confluency, 0.8 μg of vector plasmid or shPREP plasmid and 1.2 μL Lipofectamine™ 2000 were diluted separately in 50 μL reduced serum medium (OptiMEM® I; Invitrogen, U.K.), incubated another 20 min and finally 100 μL transfection mix was added to each well containing 100 μL OptiMEM® I and incubated at 37 °C for 4 h, followed by replacement with fresh medium. For stable cell line generation, cells were cultured in DMEM/F-12 medium containing 500 μg/mL G418 48 h post-transfection. After three weeks, resistant colonies were pooled and maintained in 200 μg/mL G418 in DMEM/F-12 medium.

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2.4. In situ PREP activity assay and inhibition studies using PREP inhibitors

PREP activity was measured using a method modified from the method for the activity measurement of dipeptidyl peptidases in peripheral tissues and described by Schade et al. (2008). Primary cortical neurons were cultured for 6 days. On DIV6, cells were pre-incubated for 5 h 15 min with the following PREP inhibitors: (1) KYP-2047; (2) Z-Pro-Pro-aldehyde-dimethylacetal (Bachem AG, Switzerland); and (3) Boc-Asn-Phe-Pro-aldehyde (Bachem AG, Switzerland). All compounds were used in the concentrations between 0.01 and 100 μM. Nitroblue tetrazolium chloride salt (100 μM) (NBT, Sigma Chemical Co, MO, USA) and the PREP substrate UAMC-00682 (50 μM, designed and synthesized at the University of Antwerp, Belgium) (Fig. 1) were added to cultures for 105 min after the pre-incubation period with PREP inhibitors. UAMC-00628 and NBT were dissolved in DMSO. The final concentration of DMSO was not higher than 0.1%. Solvent control was used in every experiment. In experiments on neuroblastoma SH-SY5Y cells with overexpression of PREP, NBT (100 μM) and UAMC-00682 (20 μM) were added for 130 min. In experiments on neuroblastoma SH-SY5Y cells with knocked down PREP expression, NBT (100 μM) and UAMC-00682 (50 μM) were added for 260 min. To serve as a plate reader blank, 100 μM NBT without substrate was added to 4–6 wells for an appropriate incubation time. Cleavage of UAMC-00682 substrate by PREP releases a strong reducing agent, which reduces NBT to yield a diformazan. Diformazan crystals precipitate at sites of enzymatic activity and are visible as blue staining (Schade et al., 2008). After incubation with substrate and NBT, cells were washed with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) to remove extracellular diformazan crystals and dried for 10 min at 37 °C. Intracellular diformazan crystals were dissolved in 1:1 2 M KOH and DMSO (Choi et al., 2006) with gentle shaking and pipetting. Absorbance of the dissolved diformazan was measured at 620 nm using the plate reader (Tecan, Germany). Obtained absorbance data were compared to diformazan standard curve. Data were normalized to equal levels of protein. Sensitivity of the *in situ* PREP activity assay was evaluated by calculating the Michaelis–Menten constant (K_m, GraphPad Prism 5) using substrate concentration curve in primary cortical neurons.

2.5. Cell free PREP activity assay and inhibition studies using PREP inhibitors

In cultures of primary cortical neurons on DIV6 or in SH-SY5Y cells, PREP activity was measured according to a method described by Schulz et al. (2005), with minor modifications. Briefly, cells were washed twice with PBS and lysed in chilled hypotonic buffer (pH 7.5) containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 20 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT). Obtained lysates were centrifuged at 18,000 × *g* for 10 min at 4 °C and supernatants were transferred to new tubes. The protein concentration was determined by Bradford method (Bradford, 1976) and soluble protein was used immediately for PREP activity measurement. Equal amount of protein samples (10 μg) were mixed with assay buffer containing 25 μM chromogenic substrate Z-Gly-Pro-pNA (Bachem AG, Bubendorf). PREP inhibitors were added directly to the cell lysates together with substrate. The product absorbance was continuously measured 30 min at 405 nm using microplate reader (Sunrise™, Tecan, Germany).

2.6. Measurement of PREP protein levels by Western blotting

Neuroblastoma cells (SH-SY5Y) were lysed in 1 volume RIP-A lysis buffer (20 mM Tris–HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40 and 2 mM EDTA) containing protease and phosphatase inhibitors, before being manually homogenised. Lysed cells were incubated for 20 min on ice and centrifuged (16,000 × *g* for 20 min at 4 °C). The supernatants were resolved by electrophoresis on gradient SDS-polyacrylamide gel (lower part 12% gel for β-actin, upper part 8% gel for PREP). The proteins were transferred onto Hybond™-P PVDF Transfer Membranes (Amersham Biosciences, UK) in 0.1 M Tris-base, 0.192 M glycine and 20% (w/w) methanol using an electrophoretic transfer system. The membranes were blocked with 0.1% (w/w) Tween-20/TBS containing 0.5% (w/w) non-fat dried milk powder at room temperature in a SnapID Protein Detection System (Millipore, UK). After blocking, the membranes were incubated for 15 min at room temperature in the SnapID System with the chicken anti-PREP polyclonal antibody (1:1000; a generous gift from Dr. Arturo Garcia-Horsman, Division of Pharmacology and Toxicology, University of Helsinki, Finland, followed by incubation at room temperature for 15 min in the SnapID System with a secondary antibody: anti-chicken-HRP (1:400; Sigma Aldrich). The preparation, purification and specificity of polyclonal PREP antibody have been described previously (Venäläinen et al., 2002, 2006; Myöhänen et al., 2007, 2008). The membranes were incubated with the ECL detection reagent (ECL, Amersham, UK) for 5 min to visualize the proteins and then exposed to an autoradiography X-ray film (Amersham hyperfilm ECL, UK). To normalize the immunoreactivities of the proteins, the β-actin protein was measured on the same blot with a mouse monoclonal anti-β-actin antibody (dilution: 1:5000; Sigma Aldrich) followed by an anti-mouse HRP secondary antibody (1:400; Pierce, USA) for 15 min at room temperature in the SnapID System as an internal control for loading. The blots probed for the proteins of interest were densitometrically analysed using the Quantity One 710 System (BioRad) and the data are expressed as mean ± SEM of OD ratio.

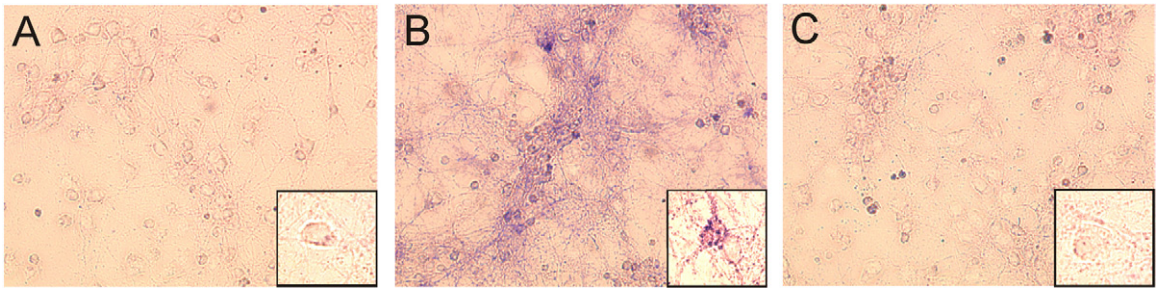

Fig. 2. Representative microphotographs of diformazan crystal formation in primary cortical neurons (DIV6) in: (A) negative control cell cultures in the presence of NBT (100 μM) without substrate; (B) positive control cell cultures in the presence of NBT (100 μM) and substrate UAMC-00682 (50 μM); (C) cell cultures treated with KYP-2047 (10 μM) by following addition of NBT and UAMC-00682. Inserts demonstrate the formation of diformazan crystals by magnification ×60.

2.7. Determination of protein concentrations

Total protein concentrations were measured in lysates using Bradford reagent (Bradford, 1976), purchased from Sigma Chemical Co (MO, USA), with bovine serum albumin as a standard. No NBT and substrate (UAMC-00682) were present in the samples used for protein assay.

2.8. Statistical analysis

IC50 values were calculated by nonlinear regression analysis (GraphPad Prism5 software). Data presented are the mean ± SEM, $n = 3$–10 data points from each experiment and the experiments were repeated 2–3 times. Data were analysed using Student's *t*-test.

3. Results

3.1. In situ PREP activity measurements in primary rat cortical neurons

Addition of the PREP substrate, UAMC-00682, and NBT to the cell culture medium, followed by incubation of the cells for 105 min in a 5% CO_2/95% air atmosphere at 37 °C, resulted in the appearance of blue diformazan crystals. Microscopic examination of formed crystals revealed intracellular as well as extracellular localisations. Extensive washing of the neuronal monolayer removed extracellular crystals, whereas intracellular crystals remained (Fig. 2B). Only weak crystal formation was observed in negative control cultures where UAMC-00682 was omitted (Fig. 2A) since only minimal nonspecific reduction of NBT in cells and in medium occurred. To quantify the intensity of the signal, crystals were dissolved in 50 μL 2 M KOH, followed by addition of 50 μL DMSO, and absorbance at 620 nm was measured in a micro-plate reader. The average OD_{620nm} in primary cortical neurons was measured to be 0.219 ± 0.07 ($n = 10$), approximately 2-fold higher than the negative control (0.113 ± 0.03, $n = 4$). Addition of PREP inhibitor KYP-2047 in high concentration (10 μM) reduced PREP activity to the negative control levels (0.117 ± 0.01, $n = 3$, Fig. 2C). For the evaluation of the sensitivity of *in situ* PREP activity assay K_m was calculated using substrate concentration curve and was shown to be 5.08 ± 0.71 μM.

3.2. Effect of PREP inhibitors on in situ and cell free PREP activity assays

IC50 values of PREP inhibitors measured using the *in situ* PREP activity assay are shown in Table 1. The most potent inhibitor was KYP-2047, with an IC50 value of 10 ± 1 nM. IC50 values for Z-Pro-Pro-aldehyde-dimethylacetal and Boc-Asn-Phe-Pro-aldehyde were 5111 and 2940, respectively.

Table 1
IC50 of PREP inhibitors in cell free and *in situ* assays.

PREP inhibitor	Cell free assay	*In situ* assay
	IC50 (nM) ± SEM	IC50 (nM) ± SEM
Z-Pro-Pro-aldehyde-dimethylacetal	733 ± 141	5111 ± 1
Boc-Asn-Phe-Pro-aldehyde	2040 ± 128	2940 ± 1
KYP-2047	0.29 ± 0.04	10 ± 1

Data are presented as average values ± SEM of three independent experiments. IC50 was calculated from nonlinear regression curve fit using GraphPad Prism5 software.

To compare the effects of inhibitors on PREP activity in lysates by cell free PREP activity assay, primary cortical neurons were lysed and PREP activity was measured in the absence and the presence of inhibitors, using Z-Gly-Pro-pNA, a chromogenic PREP substrate. Our experiments demonstrate high PREP activity in lysates prepared from primary rat cortical neurons. The average OD from positive control lysates was 0.122 ± 0.003 ($n = 3$), approximately 3-fold higher than the negative control (0.041 ± 0.001, $n = 3$). PREP inhibitors KYP-2047, Z-Pro-Pro-aldehyde-dimethylacetal and Boc-Asn-Phe-Pro-aldehyde (at concentrations between 0.01 nM and 10 μM), strongly inhibited PREP activity in cell lysates; with KYP-2047 displaying the strongest PREP inhibitory activity. It should be noted that IC50 values measured for the PREP inhibitors in this assay were 7.0, 1.4 and 34.5-fold (for Z-Pro-Pro-aldehyde-dimethylacetal, Boc-Asn-Phe-Pro-aldehyde and KYP-2047, respectively) lower than those measured from the *in situ* assay (Table 1).

3.3. Demonstration of in situ PREP activity in cells overexpressing PREP

All experiments were run on wild type, α-synuclein overexpressing and PREP overexpressing cells. In all cases no statistically significant differences between wild type and α-synuclein overexpressing cells were found regarding PREP activity and protein level and, therefore, wild type cells were used as a control for PREP overexpressing cells.

Cells transfected with the PREP containing plasmid demonstrated approximately 10 times more PREP protein than control cells transfected with empty vector (Fig. 3). PREP appeared on SDS-PAGE as a single band at around 80 kDa (Fig. 3A).

To measure PREP activity by cell free assay, lysates were prepared from PREP overexpressing cells. Obtained data showed a 12-fold increase in total PREP activity (Fig. 4A) compared to wild type cells. This corresponds to the levels of PREP protein levels seen on Western blots. Next we measured enzymatic activity of PREP using *in situ* assay. Experiments also demonstrated an increased activity of enzyme in PREP overexpressing cells, however this increase was much less pronounced using *in situ* method

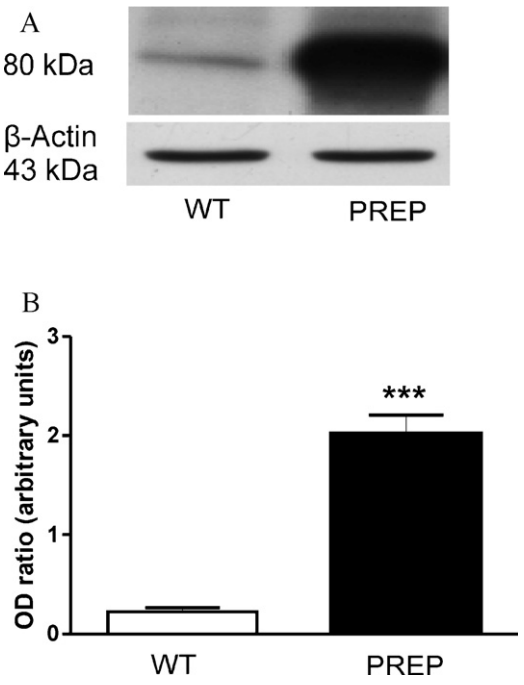

Fig. 3. (A) Representative Western blot of PREP in wild type (WT), and PREP overexpressing (PREP) SH-SY5Y cells. β-Actin was used as a loading control. (B) Immunoblotting analysis of the expression level of the PREP in wild type (WT), and PREP overexpressing (PREP) SH-SY5Y cells. Data are expressed as the mean OD ratio ± SEM ($^{***}p < 0.001$, *t*-test).

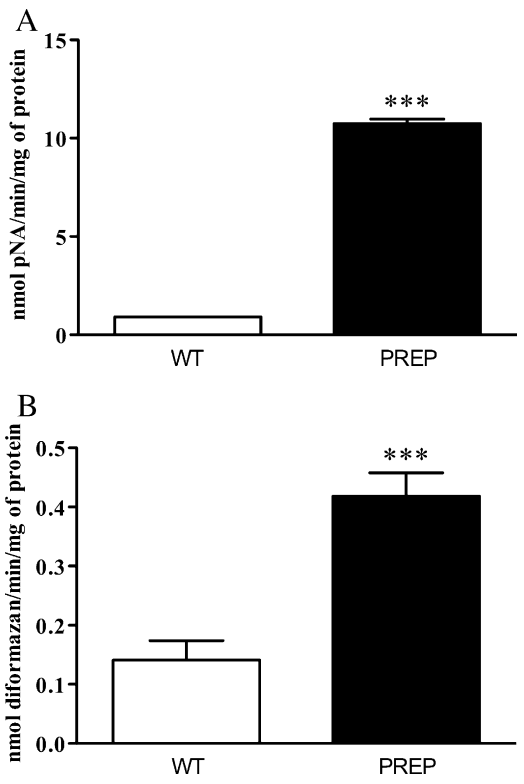

Fig. 4. (A) PREP activity measured using the non-cellular method and substrate Z-Gly-Pro-pNA in lysates of wild type (WT), and PREP overexpressing (PREP) SH-SY5Y cells; (B) PREP activity measured using the intracellular assay and substrate UAMC-00682. Data shown are the mean ± SEM ($^{***}p < 0.001$, *t*-test).

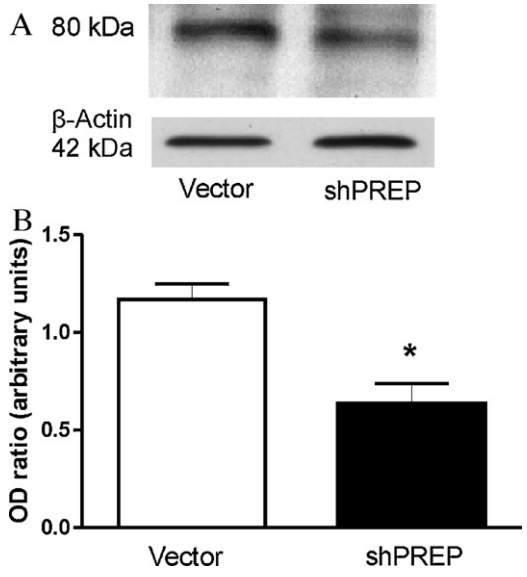

Fig. 5. (A) Representative Western blot of PREP protein levels in SH-SY5Y cells transfected with empty vector (control) and cells transfected with shPREP plasmid (shPREP). β-Actin was used as a loading control. (B) Immunoblotting analysis of the expression level of the PREP in the control cells and shPREP cells. Data are expressed as the mean OD ratio ± SEM ($^{*}p = 0.03$, *t*-test).

compared with the classical cell free assay. In PREP overexpressing cells, measured activity was 0.42 ± 0.04 nmol diformazan/min/mg of protein and in wild type cells – 0.14 ± 0.03 (Fig. 4B).

3.4. Demonstration of in situ PREP activity in cells with knocked down PREP expression

To reduce PREP expression, SH-SY5Y cells were transfected with a plasmid containing PREP specific shRNA. PREP shRNA transfection resulted in a decreased PREP protein levels by 40% as compared to the levels of PREP in control cells transfected with empty vector ($p = 0.03$, Fig. 5). Measurement of PREP activity by cell free method in lysates revealed a 2-fold decrease in total PREP activity in knocked down cells as compared to control (Fig. 6A). Next, we measured PREP activity *in situ*, using the PREP activity assay with UAMC-00682 as a substrate. *In situ* PREP activity in PREP knocked down cells was, approximately 3-fold lower than in cells transfected with empty vector (Fig. 6B).

4. Discussion

In the present study, we developed and validated a novel method for measuring intracellular activity of PREP *in situ* using NBT and a PREP specific substrate, UAMC-00682. The major difference of our new method from cell free activity assays in cell lysates is that the substrate UAMC-00682 added to living cells penetrates cell membranes and reacts with PREP under physiological conditions and enables activity assessment *in situ*. Moreover, *in situ* assay of PREP activity enables more precisely localize the sites of PREP activity on cellular levels and assess the ability of the inhibitors to penetrate cell membrane and affect enzymatic activity within the cell.

Analysis of the inhibitory activities of available PREP inhibitors in *in situ* assay and traditional cell free assay revealed that the IC50 of Boc-Asn-Phe-Pro-aldehyde obtained by both – *in situ* and cell free assays do not differ between assays, whereas the IC50 of KYP-2047 and Z-Pro-Pro-aldehyde-dimethylacetal obtained by cell free assay were much higher than the values obtained by *in situ* assay. This might be explained by the differences of compounds in their

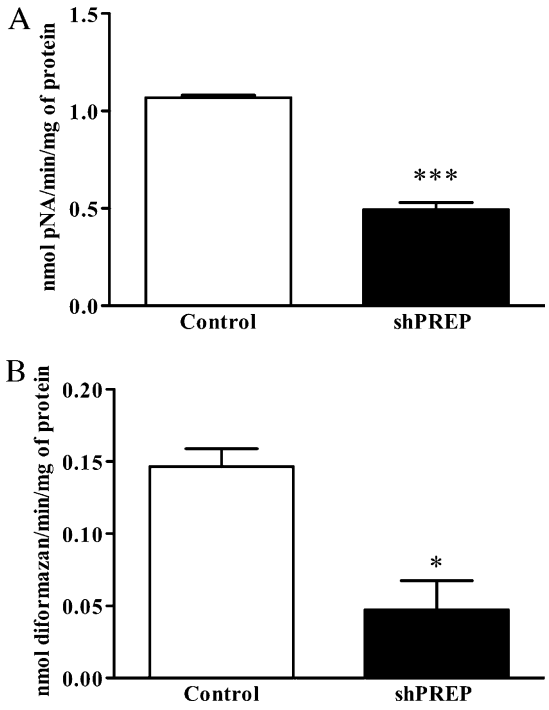

Fig. 6. (A) PREP activity measured using the non-cellular method and substrate Z-Gly-Pro-pNA in lysates of SH-SY5Y cells transfected with empty vector (control) and cells transfected with shPREP plasmid (shPREP); (B) PREP activity measured using the intracellular assay and substrate UAMC-00682 in Control and shPREP cells. Data represent the mean ± SEM. $^{*}p < 0.05$; $^{***}p < 0.001$ vs. Vector, *t*-test.

ability to penetrate cellular membranes. Thus, *in situ* method in combination with cell free method enables to assess the ability of compound to penetrate cell membranes and affect intracellular PREP activity. Results from both assays showed that a novel PREP inhibitor, KYP-2047, is the most potent PREP inhibitor tested, with an IC50 value approximately 400-fold smaller than the IC50 values measured for the other two tested PREP inhibitors. These data are in agreement with previously reported IC50 of KYP-2047 by Jarho et al. (2004), which was shown as 0.20 nM and is very similar to the value obtained in our study by cell free PREP activity assay (0.29 nM), but lower than the value obtained in our *in situ* assay (10 nM). IC50 of Z-Pro-Pro-aldehyde-dimethylacetal was reported as 120 nM by Augustyns et al. (1995) and is lower than the values obtained in our study by cell free and *in situ* assays. The difference could be due to the different experiment conditions in cell free assays and slow cellular permeability for this inhibitor in *in situ* assay.

Our *in situ* PREP activity assay was validated in neuroblastoma SH-SY5Y cellular models where PREP was overexpressed or knocked down using shRNA. In both systems *in situ* activity assay demonstrated corresponding changes in the activity of enzyme. There were some differences in the activity levels measured by *in situ* and cell free assays, which could be explained by the fact that the classical assay reaction is going on in solution. Described intracellular method needs NBT and substrate to enter the cell and, therefore, we suggest that the obtained difference is due to the differences in the concentrations of substrate penetrating the cell membrane. Furthermore, there might be differences in PREP activity in lysates and cells (some PREP is membrane bound). *In situ* assay measures the active form of the enzyme and provides the information about the localization of active enzyme in the cell or tissue. Cell free activity method measures the total potential activity within cells without any information about the enzyme activity in the cell before lysis. Our new *in situ* method could be helpful in clarifying the previously described differences between PREP expression and activity measured by cell free methods (Myöhänen et al., 2009). Taken together obtained results demonstrate that our *in situ* assay provides a tool for measuring *in situ* intracellular PREP activity, which can be compared to PREP activity measured by cell free (cell lysate) methods using substrate Z-Gly-Pro-pNA.

In conclusion, the present study demonstrates the validity of our method for PREP intracellular activity measurements *in situ*. The *in situ* method allows to measure PREP activity in physiological conditions and enables identification of the intracellular localization of PREP enzymatic activity. Moreover, our new method allows to assess efficiency of novel PREP inhibitors as well cellular permeability of PREP inhibitors and, therefore, using inhibitors with high and low permeability we can study the roles of intracellular vs. extracellular PREP. The validated *in situ* PREP activity assay on cell cultures might serve as a base for further PREP intracellular activity studies on brain sections.

Acknowledgements

This work was supported by funds from the European Community's Seventh Framework Programme (FP7) under Grant Agreement No. 223077i for the project "Oligopeptidase inhibitors in brain function and dysfunction: towards new therapeutic strategies for neuroprotection" and the Flanders Research Foundation (FWO, project G.0114.08) to A.M.L. We thank Dr. Zoltan Szeltner, Institute of Enzymology, Budapest, Hungary, for providing PREP protein as a kind gift, and for important advice.

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