In situ prolyl oligopeptidase activity assay in neural cell cultures

Prolyl oligopeptidase activity measurements in neural cells *in vitro*

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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 highly-enriched 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 a PREP intracellular activity assay in primary rat cortical neurons, using nitroblue tetrazolium chloride salt (NBT) and a PREP specific substrate UAMC-00682. This novel intracellular 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 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 intracellular PREP activity in primary rat cortical neurons and neuroblastoma SH-SY5Y cells.

1. Introduction

Prolyl oligopeptidase (PREP, EC 3.4.21.26) is a highly conserved cytosolic 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 (Polgar, 2002). The crystal structure of PREP revealed a conserved β-propeller domain which blocks larger proteins, and only allows small, approximately 30 amino acid long proteins or peptides access to the catalytic core of the enzyme (Fülop 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 processes 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). They also might be neuroprotective in Parkinson's disease by cleaving α-synuclein fragments (Brandt et al., 2005; Brandt et al., 2008). In spite of the above described research efforts, the precise mechanism by which PREP affects the neurodegeneration processes remains unclear. Furthermore, although

PREP has been shown to be primarily localised to the cytoplasm, suggesting additional intracellular functions (Schulz et al., 2002); little is known about the intracellular function(s) of this enzyme. Interestingly, PREP inhibition is associated with increased levels of intracellular inositol 1,4,5-triphosphate (Schulz et al., 2002). Recent findings indicate that both native and catalytically-dead PREP can form a complex with the synaptic growth-associated protein, GAP-43 (Di Daniel et al., 2009) and that PREP is involved in the regulation of peptide/protein secretion (Schulz et al., 2005). To investigate these and other possible intracellular activities of PREP, an appropriate method for PREP intracellular activity measurements must be developed.

PREP activity has been measured in cell lysates and brain homogenates using chromogenic or fluorogenic substrates (Kato et al., 1980; Irazusta et al., 2002). Although these assay methods are widely used, they have important limitations. Specifically, because these methods measure PREP activity only after cell lysis, they do not allow for discrimination between intracellular and extracellular PREP activity levels. To address this problem, in the present study we developed and validated a novel method for measuring intracellular PREP activity, using nitrobluetetrazolium chloride salt (NBT) and a specific, cell-permeable PREP substrate, UAMC-00682, in primary rat cortical neurons and neuroblastoma SH-SY5Y cells. In addition, we tested the effects of a reported PREP inhibitor, with the name KYP-2047, 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 this new method for measuring intracellular PREP activity in cellular models, we measured intracellular PREP activity in two neuroblastoma SH-SY5Y cell lines, one overexpressing PREP and the other with

PREP expression inhibited by shRNA. Results were compared to those obtained from a non-cellular PREP activity assay using the chromogenic substrate, Z-Gly-Pro-pNA.

2. Materials and methods

2.2. 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 K_2HPO_2, 15 mM glucose, 20 mM HEPES, pH 7.4, containing 0.3% bovine serum albumin) and trypsinised in 0.8% 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^{TM}-I (Invitrogen, U.K.) and 100 μg/mL gentamycin. Cells were plated onto poly-L-lysine (Sigma Chemical Co., MO, USA) coated 96-well plates at a density of $1.8x10^5$cells/cm^2. 2.5 hr later, the medium was changed to Neurobasal^{TM}-A medium, containing 2 mM GlutaMAX^{TM}-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).

2.3. Overexpression of PREP in SH-SY5Y cells

Three neuroblastoma SH-SY5Y cell lines: 1) wild type cells; 2) α-synuclein overexpressing cells; and 3) α-synuclein and PREP overexpressing cells, were grown in DMEM/GutaMAX-I (Invitrogen, U.K.) medium containing 15% FBS and 50 μg/mL gentamycin for wild type cells; 1 μg/mL puromycin (Sigma Chemical Co, MO, USA) for α-synuclein overexpressing cells; and 1 μg/mL puromycin plus 200 μg/mL hygromycin B (Sigma Chemical Co, MO, USA) for cells overexpressing both α-synuclein and PREP.

2.4. shRNA-silencing 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 control (vector) or PREP (shPREP) shRNA plasmids (SA Biosciences, MD, USA) using Lipofectamine™ 2000 (Invitrogen, U.K.) according to manufacturer's protocol. Briefly, 24 hours 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 were incubated with 50 μL reduced serum medium (OptiMEM® I; Invitrogen, U.K.) for 5 min. Subsequently, a mixture of 1.2 μL Lipofectamine™ 2000 and 50 μL OptiMEM® I was incubated for 5 min and then slowly added. After incubation for 20 min at room temperature, transfection mix was added to each well and incubated at 37°C for 4 hours, 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 hours post-transfection. After three weeks, resistant

colonies were pooled and maintained in 200 μg/mL G418 in DMEM/F-12 medium.

2.5. Intracellular PREP activity assay and inhibition studies using PREP inhibitors

PREP activity was measured using a method modified from Schade et al., 2008. Primary cortical neurons were cultured for 6 days. On DIV6, cells were incubated for 7 hours with the following PREP inhibitors: 1) KYP-2047; 2) Z-Pro-Pro-aldehyde-dimethylacetal (Bachem AG, Switzerland); and 3) Boc-Asn-Phe-Pro-aldehydeBachem AG, Switzerland). All compounds were used in the concentrations between 0.01-100 μM. Nitro blue tetrazolium chloride salt (100 μM) (NBT, Sigma Chemical Co, MO, USA) and the PREP substrate UAMC-00682 (50 μM) (Fig. 1) were added for the final 105 min. In experiments on neuroblastoma SH-SY5Y cells (wild type, overexpressing α-synuclein or overexpressing α-synuclein plus PREP), NBT (100 μM) and UAMC-00682 (20 μM) were added for the final 130 min. In experiments on neuroblastoma SH-SY5Y cells (shRNA silenced PREP or "vector only" control cells), NBT (100 μM) and UAMC-00682 (20 μM) were added for the final 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 the 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^{o}C. Intracellular diformazan crystals were dissolved in 1:1 2M KOH and DMSO (Hyung et al, 2006) with gentle

shaking and pipetting. Absorbance of the dissolved diformazan was measured at 620 nm using an ELISA plate reader (Tecan, Germany). For calculation of PREP intracellular activities, we prepared an NBT diformazan concentration curve (data not shown).

2.6. Non-cellular 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 14000 rpm for 10 min. All steps were performed on ice. The clarified supernatant, containing the soluble protein fraction, was transferred into a fresh tube and used immediately for enzyme activity measurements. 25 µL of protein samples (~5 µg protein) were mixed with 125 µL assay buffer containing 25 µM Z-Gly-Pro-pNA, a chromogenic PREP substrate (Bachem AG, Switzerland) and PREP inhibitors (at concentrations between 0.01-10000 nM). PREP substrate cleavage product absorbance was measured at 410 nm after 180 min using an ELISA plate reader (Tecan, Germany). For calculation of PREP activity in cell lysates, we prepared a p-Nitroanilide (p-NA) concentration curve (data not shown).

2.7. 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 2mM EDTA) containing

protease and phosphatase inhibitors, before being manually homogenised. Lysed cells were incubated for 20 min on ice and centrifuged (13,000 rpm for 20 min at 4 °C). Supernatants were resolved by 8% SDS-PAGE (total protein per lane was 20 g). Resolved proteins were transferred onto a Hybond™-P PVDF membrane (Amersham Biosciences, U.K.) in 0.1 M Tris-base, 0.192 M glycine and 20% (w/w) methanol, using an electrophoretic transfer system (100 V, 2 hours on ice).

Membranes were blocked with 0.1% (w/w) Tween 20/TBS (T-TBS) containing 0.5% (w/w) non-fat dried milk at room temperature for 1 h. After blocking, membranes were incubated overnight (+4 C) with chicken polyclonal anti-PREP antibody (a generous gift from Dr. Arturo Garcia-Horsman, Division of Pharmacology and Toxicology, University of Helsinki, Finland), 1:1000 dilution, followed by incubation with HRP-conjugated goat-anti-chicken secondary antibody for 2 hours at room temperature (1:10 000 dilution; Pierce, 185-8413, USA). Membranes were incubated with ECL detection reagent (ECL, Amersham, UK) for 5 min to visualise proteins and then exposed to autoradiography X-ray film (Amersham hyperfilm ECL, UK). Developed films were analysed by densitometry using a Quantity One 710 System (BioRad).

2.8. Determination of protein concentrations

Total protein concentrations were measured using Bradford reagent (Bradford, 1976), purchased from Sigma Chemical Co (MO, USA), with bovine serum albumin as a standard.

2.9. Statistical analysis

IC50 values were calculated by nonlinear regression analysis (GraphPad Prism5 software). Data presented are the mean ± SEM from 2-3 independent experiments. Data were analysed using Student's t-test.

3. Results

3.1. Intracellular 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 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). To quantify crystal formation, crystals were dissolved in 50 µL 2M 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).

3.2. Effect of PREP inhibitors on PREP activity in intracellular and non-cellular activity assays

IC50 values of PREP inhibitors measured using the intracellular 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 ± 1 nM and 2940± 1 nM, respectively.

To compare the effects of inhibitors on PREP activity in lysates, 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 6.9, 1.4 and 33.9-fold (for Z-Pro-Pro-aldehyde-dimethylacetal, Boc-Asn-Phe-Pro-aldehyde and KYP-2047, respectively) lower than those measured from the intracellular assay (Table 1).

Table 1. IC 50 of PREP inhibitors in non-cellular and intracellular assays

PREP inhibitor	***Non-cellular assay IC 50 (nM) ± SEM***	***Intracellular assay IC 50 (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. IC 50 were calculated from nonlinear regression curve fit using GraphPad Prism5 software.

3.3. Demonstration of intracellular PREP activity in cells overexpressing PREP

To further validate the intracellular assay, we measured intracellular PREP activity in neuroblastoma cells overexpressing PREP and α-synuclein, and compared obtained data with results from non-cellular assays of lysates prepared from these cells (Fig. 3A and 3B). PREP intracellular activity in cells overexpressing PREP + α-synuclein was measured to be 0.42 ± 0.04 nmol diformazan/min/mg of protein, at least 2-fold higher than intracellular PREP activity measured in wild type cells or cells expressing α-synuclein only (Fig. 3C).

3.4. Demonstration of intracellular PREP activity in cells with shRNA silenced PREP expression

To silence PREP expression, SH-SY5Y cells were transfected with a plasmid containing PREP specific shRNA. PREP shRNA transfection resulted in decreased PREP protein levels (Fig. 4A). Measurement of PREP activity in lysates prepared from PREP shRNA transfected cells revealed a 2-fold decrease in total PREP activity (Fig. 4B). Next, we measured PREP activity *in situ*, using the PREP intracellular assay with UAMC-00682 as a substrate. PREP intracellular activity in cells with silenced PREP expression was measured to be 0.05 ± 0.02 nmol diformazan/min/mg of protein, approximately 3-fold lower than in wild type cells (0.146± 0.01 nmol diformazan/min/mg) (Fig. 4C).

4. Discussion

The prevailing theory of the role of PREP in learning and memory states that PREP acts in the extracellular space by cleaving neuropeptides involved in regulation of cognitive

functions (Shishido et al., 1999). However, subsequent studies demonstrated that PREP also plays a role in regulating inositol turnover in human glial and neuronal cell lines (Schulz et al., 2002) and is able to bind to the C-terminus of α-tubulin (Schulz et al., 2005). Furthermore, more recent data suggests that PREP has an intracellular localisation, mainly in the perinuclear space of neuronal and glial cells (Schulz et al., 2005). Moreover, both native and catalytically-dead PREP co-immunoprecipitate with a synaptic growth-associated protein, GAP-43 (Di Daniel et al., 2008). All of these novel intracellular roles associated with PREP seem to point to the existence of intracellular PREP activity, and suggest that PREP's intracellular activity may be even more important than its extracellular activity. In fact, because of these findings, it was hypothesised that PREP may have important additional intracellular functions related to neurodegeneration. Therefore the development of an appropriate method to measure PREP intracellular activity is relevant.

The most widely used assay methods for measuring PREP activity use the chromogenic substrate, Suc-Gly-Pro-MCA, or the fluorogenic substrate, Z-Gly-Pro-β-naphtylamide (Kato et al., 1980; Irazusta et al., 2002), in either cell lysates or brain homogenates. However, these methods have significant limitations. Importantly, they do not allow for measurement of intracellular PREP activity in intact cells. In the present study, we developed and validated a novel method for measuring PREP intracellular activity, modified from a method described by Schade et al., (2008). Intracellular PREP activity was determined using NBT and a PREP specific substrate, UAMC-00682, which enables localisation of the site of PREP activity and discrimination of intracellular activity from extracelular activity. In this new assay, PREP inhibitors KYP-2047, Z-Pro-Pro-aldehyde-

dimethylacetal and Boc-Asn-Phe-Pro-aldehyde displayed inhibitory activity with IC50 values in the low micromolar range. It should be noted that the inhibitory activity of these PREP inhibitors in the intracellular assay is lower than in the traditional cell lysate assay, probably due to their lower cellular permeability. Results from the intracellular activity assay 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. KYP-2047 was also the most potent inhibitor of PREP activity in the traditional cell lysate based assay.

Our PREP intracellular activity assay was validated in neuroblastoma SH-SY5Y cellular models where: 1) PREP was overexpressed; or 2) the PREP gene was silenced using shRNA. Our results demonstrate that our intracellular assay provides a tool for measuring intracellular PREP activity, which can be compared to PREP activity measured by non-cellular (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. This method enables identification of the cellular localisation of PREP enzymatic activity, and was shown to be able to measure intracellular PREP activity in primary rat cortical neurons, and neuroblastoma cell lines.

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

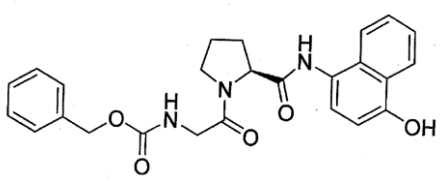


Fig. 2. Representative pictures of diformazan crystal formation in primary cortical neurons (DIV6) in: (B) positive control cell cultures in the presence of NBT (100 μM) and substrate UAMC-00682 (50 μM); and (A) in negative control cultures in the presence of NBT (100 μM) but without substrate.

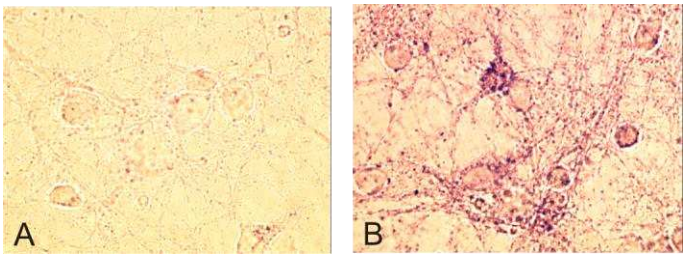


Fig. 3. (A) Representative Western blot of PREP protein levels in wild type (WT), α-synuclein (α-SYN) and PREP plus α-synuclein (PREP + α-SYN) overexpressing SH-SY5Y cells; (B) PREP activity measured using the non-cellular method and substrate Z-Gly-Pro-pNA in SH-SY5Y cell lysates; (C) PREP activity measured using the intracellular assay and substrate UAMC-00682 in SH-SY5Y cells. Data shown are the mean ± SEM. ***$p < 0.001$ vs. WT; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. α-SYN, t-test.

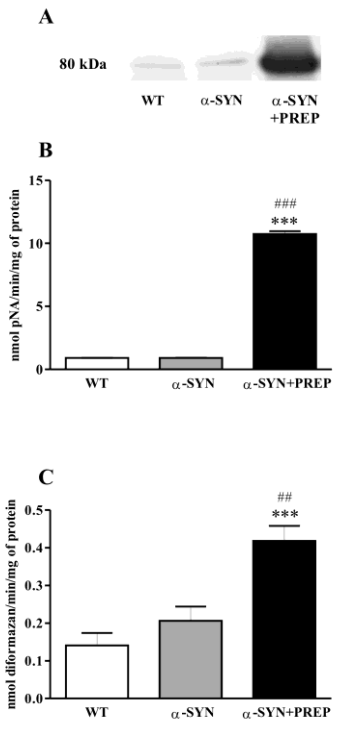
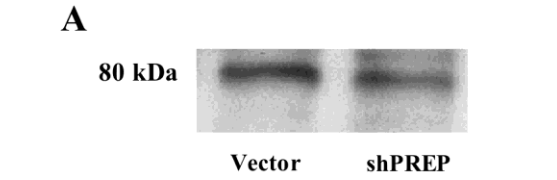
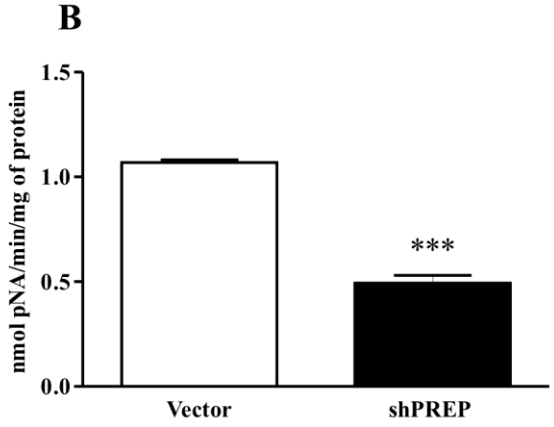
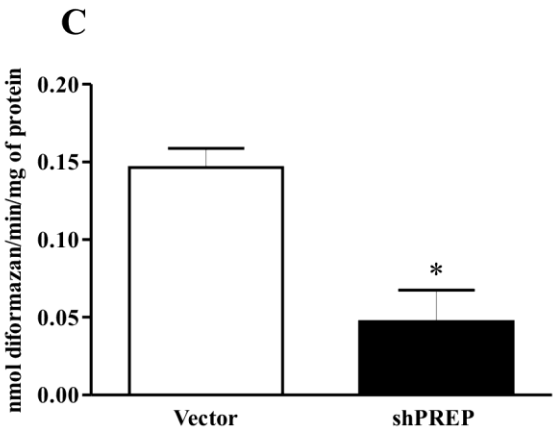


Fig. 4. (A) Representative Western blot of PREP protein levels in SH-SY5Y cells transfected with empty vector (Vector) and cells transfected with shPREP plasmid (shPREP); (B) PREP activity measured using the non-cellular method and substrate Z-Gly-Pro-pNA in SH-SY5Y cell lysates; (C) PREP activity measured using the intracellular assay and substrate UAMC-00682 in SH-SY5Y cells. Data represent the mean ± SEM. *$p < 0.05$; ***$p < 0.001$ vs. Vector, t-test.


A
80 kDa
Vector
shPREP


B
nmol pNA/min/mg of protein
1.5
1.0
0.5
0.0
***
Vector
shPREP


C
nmol diformazan/min/mg of protein
0.20
0.15
0.10
0.05
0.00
*
Vector
shPREP