



## Original article

# Prolyl oligopeptidase inhibition reduces oxidative stress via reducing NADPH oxidase activity by activating protein phosphatase 2A

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## ARTICLE INFO

## Keywords:

Oxidative stress  
Neurodegeneration  
Prolyl oligopeptidase  
Protein phosphatase 2A  
p47phox  
NADPH oxidase

## ABSTRACT

Oxidative stress (OS) is a common toxic feature in various neurodegenerative diseases. Therefore, reducing OS could provide a potential approach to achieve neuroprotection. Prolyl oligopeptidase (PREP) is a serine protease that is linked to neurodegeneration, as endogenous PREP inhibits autophagy and induces the accumulation of detrimental protein aggregates. As such, inhibition of PREP by a small-molecular inhibitor has provided neuroprotection in preclinical models of neurodegenerative diseases. In addition, PREP inhibition has been shown to reduce production of reactive oxygen species (ROS) and the absence of PREP blocks stress-induced ROS production. However, the mechanism behind PREP-related ROS regulation is not known. As we recently discovered PREP's physiological role as a protein phosphatase 2A (PP2A) regulator, we wanted to characterize PREP inhibition as an approach to reduce OS. We studied the impact of a PREP inhibitor, KYP-2047, on hydrogen peroxide and ferrous chloride induced ROS production and on cellular antioxidant response in HEK-293 and SH-SY5Y cells. In addition, we used HEK-293 and SH-SY5Y PREP knock-out cells to validate the role of PREP on stress-induced ROS production. We were able to show that absence of PREP almost entirely blocks the stress-induced ROS production in both cell lines. Reduced ROS production and smaller antioxidant response was also seen in both cell lines after PREP inhibition by 10 μM KYP-2047. Our results also revealed that the OS reducing mechanism of PREP inhibition is related to reduced activation of ROS producing NADPH oxidase through enhanced PP2A activation. In conclusion, our results suggest that PREP inhibition could also provide neuroprotection by reducing OS, thus broadening the scope of its beneficial effects on neurodegeneration.

## 1. Introduction

Oxidative stress (OS) is a state of imbalance between pro-oxidant and antioxidant homeostasis, which is represented by unregulated production of harmful reactive oxygen species (ROS) and/or insufficient cellular antioxidant defenses [1–3]. Although ROS are necessary for multiple physiological functions at lower concentrations, prolonged exposure to high ROS concentrations damages cellular macromolecules, which again can disrupt cellular membrane structures, DNA repair systems, and cause mitochondrial dysfunction, eventually leading to cell death [3–5]. The human brain, having its rich amounts of easily oxidizable unsaturated lipids, high oxygen consumption and metal ion

concentrations, and relatively low antioxidant capacity, is susceptible to OS [1,6]. Additionally, markers of oxidative damage can be seen in postmortem brains from patients who have suffered from neurodegenerative diseases [7–9].

Alzheimer's (AD) and Parkinson's diseases (PD) are examples of progressive neurodegenerative disorders that are characterized by accumulation of misfolded protein aggregates amyloid-β and hyperphosphorylated tau protein in AD and α-synuclein (α-Syn) in PD, respectively [10,11]. These pathological protein aggregates start accumulating as insufficiencies in defense mechanisms against atypical protein aggregation occur, i.e. in refolding chaperones and/or in ubiquitin-proteasome and autophagy systems responsible for protein

**Abbreviations:** ARE, antioxidant response element; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OS, oxidative stress; PREP, prolyl oligopeptidase; PREPko, PREP knock out; PP2A, protein phosphatase 2A; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; Nrf2, nuclear factor erythroid 2-related factor 2; 6-hydroxydopamine, 6-OHDA.

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<https://doi.org/10.1016/j.freeradbiomed.2021.04.001>

Received 9 February 2021; Received in revised form 19 March 2021; Accepted 1 April 2021

Available online 7 April 2021

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degradation [12–15]. It is thought that formation of the larger pathological protein inclusions, such as Lewy bodies, is actually a protective mechanism and that the aggregation intermediates are responsible for the cellular damage and consequent neuronal loss [16–18]. Additionally, OS is also having an essential role in neurodegeneration, and it has been shown that there is a link between abnormal protein aggregation intermediates and oxidative damage in different neurodegenerative diseases [9,17,18]. The exact mechanism remaining unclear, it has been suggested that the toxic protein aggregation intermediates can generate ROS via interactions with redox-active metal ions [9]. On the contrary, it is also known that OS can enhance protein aggregation, indicating that the interaction between OS and protein aggregation is a bidirectional phenomenon [19]. Taken together, OS is an essential toxic feature in neurodegeneration.

Prolyl oligopeptidase (PREP) is a serine protease that is widely expressed throughout the body, including the brain, where its highest expression and activity has been observed in nigrostriatal and cortical neurons [20–23]. Additionally, its expression and activity have been shown to increase with aging [23,24]. Increased PREP activity is also linked to neurodegenerative diseases accompanied by formation of pathological protein inclusions, as it is able to increase protein aggregation and to disturb autophagy-mediated protein degradation [23,25,26]. PREP is also shown to colocalize with  $\alpha$ -synuclein,  $\beta$ -amyloid, and tau protein in post-mortem brain samples from AD and PD patients [27]. Based on these findings, our group has shown in several studies that the small molecular PREP inhibitor KYP-2047 has a neuroprotective effect [25,26,28,29], which is a consequence from diminished pathological protein aggregation and enhanced protein clearance. The effects of PREP inhibition are unrelated to its proteolytic functions but are more likely related to the conformational stabilization that follows the binding of the inhibitor [30]. In addition, the endogenous PREP acts as a negative regulator for protein phosphatase 2A (PP2A), whereas PREP inhibition increases PP2A activity [31]. This is thought to be the mechanism by which PREP inhibition conveys its effects.

Puttonen et al. [32] have showed that pre-treatment with two different PREP inhibitors (JTP-4819 and Z-Pro-Prolinal) reduced 6-hydroxydopamine (6-OHDA)-induced ROS production in CV1-P cells by altering nuclear translocation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). However, GAPDH translocation to the nucleus is in the downstream of the ROS production pathway [33], and therefore the mechanisms for how PREP regulated ROS production remained unclear. In addition, our group made an earlier observation that lack of PREP completely abolished ROS production in  $\alpha$ -Syn transfected HEK-293 PREPko cells after induction of OS, whereas the wild type cells had a significant increase in their production [34]. The absence of PREP resulted also in reduced induction of enzymatic antioxidants (antioxidant response) in these cells. As we have now discovered a more accurate mechanism for the physiological function of PREP as a PP2A regulator [31], these findings have evoked our interest in studying PREP's role in cellular redox regulation. Hence, aim of this study was to verify the role of PREP, and more importantly, its enzymatic inhibition by small molecular inhibitor on OS. Stress induced ROS production and antioxidant response to OS were studied using two different cell lines, HEK-293 and SH-SY5Y cells. The potential mechanism behind PREP inhibition-mediated decrease on OS was also probed. Here, we studied if the mechanism is a PP2A related phenomenon and if the effect could result from direct inhibition of the membrane bound ROS generating NADPH oxidase (NOX) [35], as PP2A has been shown to reduce its activity via dephosphorylation of p47phox subunit [36]. In addition, the stress-related nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway activation was also checked after oxidative stress and PREP inhibitor treatment, as activation of this pathway can be seen as a general cellular switch against oxidative stress [37].

## 2. Materials and methods

### 2.1. Cell culture

Human embryonic kidney (HEK-293) and human neuroblastoma (SH-SY5Y) cell lines were used in this study. The cells were obtained from ATCC (Manassas, VA, USA). In addition to wild type cells, HEK-293 PREPko [34] and SH-SY5Y PREPko [31] cells were used. In brief, the stable PREPko cells were created by using a CRISPR/Cas9 construct targeting 3rd exon of PREP gene. HEK-293 cells were cultured in full Eagle's medium (DMEM; #D6429, Sigma) with additional 10% (v/v) fetal bovine serum (FBS; #16000–044, ThermoFisher Scientific) and 1% L-glutamine-penicillin-streptomycin solution (15140122; ThermoFisher Scientific). HEK-293 PREPko cells were cultured in the same medium, except with 20% (v/v) FBS. SH-SY5Y cells were cultured in Dulbecco's modified Eagle medium (DMEM-Glutamax; #31966021; ThermoFisher Scientific) with 15% (v/v) FBS for wild types and 30% (v/v) for PREPko cells, 1% non-essential amino acids (NEAA; #11140050; ThermoFisher Scientific) and 50  $\mu$ g/ml Gentamycin (15750–045; ThermoFisher Scientific). During the culturing, the cells were kept in a humidified incubator at +37 °C with 5% CO<sub>2</sub> and used in passages 3–15.

### 2.2. Inducing the oxidative stress

Oxidative stress (OS) was induced to the cells by treating them with culturing medium including 100  $\mu$ M hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and 10 mM ferrous chloride (FeCl<sub>2</sub>) in order to achieve the formation on highly reactive hydroxyl radicals (HO<sup>•</sup>) via Fenton reaction [34,38,39]. Oxidative stress (OS) medium was prepared by first diluting 30% (w/w) H<sub>2</sub>O<sub>2</sub> (H1009; Merck) in sterile PBS to make 1 mM intermediate dilution and weighing FeCl<sub>2</sub> (Iron (II) chloride tetrahydrate; 44939-50G; Sigma-Aldrich) and dissolving it into a separate amount of sterile PBS to prepare 1 M intermediate dilution. These intermediate dilutions were then added to the right amount of culturing medium at 1:100 ratio. After vigorous vortexing, the OS medium was filtered (17598-K; Minisart NML Syringe Filter Steril, 0.45  $\mu$ m Pore Size; Sartorius) and added to the cells with or without concurrent treatment compounds. The cells in the control wells received only fresh cell growth medium during OS-induction. The PREP inhibitor, KYP-2047 (4-phenylbutanoyl-1-prolyl-2(S)-cyanopyrrolidine), was synthesized in the School of Pharmacy (synthesized 2016; stability checked by NMR 2018 and 2020), University of Eastern Finland, as previously described in [40]. Stock solution of KYP-2047 was 100 mM in dimethyl sulfoxide (DMSO) and it was diluted with PBS to 1 mM concentration prior to dilution to the final concentration in the cell medium.

### 2.3. ROS detection assay

Stress induced ROS production was studied using the DCFDA cellular ROS detection assay kit (Abcam, ab113851) according to the protocol provided with it. Briefly, HEK-293 or SH-SY5Y cells (wild type or PREPko) were plated on poly-L-lysine (Poly-L-lysine solution 0.01%; P4832; Sigma-Aldrich) pre-coated clear bottom black-walled 96-well plate (30,000 cells per well) and incubated overnight. The following day the wells were washed with buffer solution provided by the kit and a diluted DCFDA solution was added. The plate was incubated with the DCFDA for 45min. Meanwhile, the OS solution with or without other treatment compounds were prepared. In this assay, OS solution was prepared in phenol red free DMEM (PRF-MEM; 21063029; Gibco) without any additional FBS to minimize the error producing interactions with the DCFDA [41]. After the incubation, the DCFDA solution was removed from the wells, and replaced with the treatment solutions or fresh PRF-MEM in control wells. The plate was incubated with treatment solutions for 3h, after which the ROS proportional fluorescence signal was measured with Victor<sup>2</sup> multilabel counter (PerkinElmer; excitation/emission 485nm/535 nm).

## 2.4. Antioxidant response

**Treatments and cell lysates.** HEK-293 (wild type) or SH-SY5Y (wild type or PREPko) cells were plated on a 6-well plate at a seeding density of 500,000 cells per well and incubated overnight. The following day OS solution with treatment compounds were prepared in normal growth medium and added to the cells after removal of the old growth medium. Cells in the control wells received fresh normal growth medium. The cells were treated either for 3 h or for 24 h with OS + 1  $\mu$ M or 10  $\mu$ M KYP-2047. After the treatments, the cells were lysed with mRIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl) containing 1:100 HALT protease inhibitor cocktail (Product#78429, ThermoFisher Scientific) and HALT phosphatase inhibitor cocktail (Product# 87786, ThermoFisher Scientific). The lysates were then sonicated and centrifuged at 13,300 rpm at +4 °C for 15 min. The supernatants were collected and the protein amounts were measured with the BCA method (ThermoFisher Scientific).

**Western blot.** OS induced increase in the protein levels of three enzymatic antioxidants (catalase, superoxide dismutase 1 (SOD1), thioredoxing) that were measured by Western blot. For standard SDS-PAGE, 25  $\mu$ g of protein per sample was loaded on 12% Mini-PROTEAN TGX precast gels (#4561044; Bio-Rad). The gels were transferred to PVDF membranes (*Trans-bolt Turbo Midi* 0.2  $\mu$ m, Product# 1704157, Bio-Rad) using Trans-blot Turbo Transfer System (Bio-Rad). Oxidative stress defense (catalase, SOD1, TRX, smooth muscle actin) Western blot cocktail (1:250 in 5% skim milk; ab179843, Abcam) was used to detect the enzymatic antioxidants.  $\beta$ -actin was used as a loading control (1:2000 in 5% skim milk; ab8227, Abcam). Goat anti-rabbit HRP-conjugated secondary antibody (1:2000; #31460, ThermoFisher Scientific) was used as a secondary antibody for both primary antibodies. The membranes were stripped (WesternSure ECL stripping buffer 5x; part No 926–92000, Li-Cor) before  $\beta$ -actin determination. To verify that bands were in the linear range of detection, increasing exposure time and automatic detection of saturated pixels in ImageLab software (version 6.01, Bio-Rad) was used. Thereafter, images were converted to 8-bit grayscale format, and the optical densities (OD) of the bands were measured by ImageJ (histogram area analysis; version 1.52i; National Institute of Health, Bethesda, MD, USA). The OD obtained from each band was normalized against the corresponding  $\beta$ -actin band. The control group (non-treated) of each studied protein was set 100% (green dashed line in the figures), and treatments were compared to control group of the current protein. All Western blot analysis were done with at least three technical replicates and with two biological replicates (at least three different Western blot assays). In addition, the researcher performed the Western blot analysis blinded for the order of the treatments and bands in the membrane, and analysis was performed by a researcher who did not perform the actual SDS-PAGE.

## 2.5. PP2A activation during OS

PP2A activation during OS was studied by determining the protein levels of total PP2A and Tyr307 phosphorylated PP2A (pPP2A; inactive form) in OS and PREP inhibitor treated SH-SY5Y wild type or PREPko cells. Treatments were done and cell lysates prepared as above. Total PP2A and pPP2A protein levels were determined with Western blot on separate membranes. Primary antibodies used for total PP2A and pPP2A: Anti-PP2A Antibody, C subunit, clone 1D6 (1:5000 in 5% skim milk; 05–421, Merck) and Phospho-PP2A alpha, Tyr307 (1:500 in 5% skim milk; PA5-36874, ThermoFisher Scientific). HRP-conjugated goat anti-mouse secondary antibody (1:2000 in 5% skim milk; #31430, ThermoFisher Scientific) and HRP-conjugated goat anti-rabbit secondary antibody (1:2000 in 5% skim milk; #31460, ThermoFisher Scientific), was used with total pPP2A. The pPP2A antibody used was shown to recognize inactive PP2A after okadaic acid treatment [31].

## 2.6. NADPH oxidase activation

**Western blot.** The activity of ROS generating NADPH oxidase 2 (NOX2) was studied by determining the protein levels of NOX2 regulating subunit p47phox and its Ser345 phosphorylated form (required for NOX2 activation [35]) in OS and PREP inhibitor treated SH-SY5Y wild type cells. The cells were treated and lysed as above, and Western blot was performed. Primary antibodies used here were anti-NCF1/p47phox (1:1000 in 5% skim milk; ab795, AB\_306163, Abcam) and anti-phospho-p47phox (pSer 345) (1:500 in 5% skim milk; SAB4504721, Sigma-Aldrich). Secondary antibody used with total p47phox was HRP-conjugated donkey anti-goat secondary antibody (1:2000 in 5% skim milk; PA-28805, Invitrogen) and for phospho-p47phox HRP-conjugated goat anti-rabbit secondary antibody (1:2000 in 5% skim milk; #31460, ThermoFisher Scientific).

**NADPH/NADP<sup>+</sup> assay.** The bioluminescent NADP/NADPH-Glo Assay (G9081, Promega) was used to detect total and reduced nicotinamide adenine dinucleotide phosphates (NADP<sup>+</sup> and NADPH) and their ratio in OS and PREP inhibitor treated samples. HEK-293 wild type cells were plated on 6-well plates and treated as described above for 3 h or 24 h. After the treatments, the cells were washed with PBS, collected in falcon tubes, and centrifuged at 9000 rpm for 2 min. The supernatants were discarded, and the pellets were resuspended in PBS. Resuspended samples were transferred on a white-walled 96-well plate for the actual NADPH/NADP<sup>+</sup> assay. Relative NADPH and NADP<sup>+</sup> amounts from individual samples were determined in separate wells according to the protocol provided with the kit. The luminescence signal, relative to the amount of NADPH or NADP<sup>+</sup>, was measured with Varioskan Lux multimode microplate reader. The signals were normalized to non-stressed control groups.

## 2.7. Nrf2 pathway activation

**Treatments and nuclear extracts.** SH-SY5Y wild type cells were plated in T25 flasks (2  $\times$  10<sup>6</sup> cells per bottle) and incubated overnight. The following day cells were treated with OS and, with or without, concurrent 10  $\mu$ M KYP-2047 for 24h. The cells in control groups received only fresh normal growth medium. After the treatments, the cells were collected in microcentrifuge tubes for nuclear extraction. Nuclear extracts were prepared with a nuclear extraction kit (ab113474). The protein amounts from the nuclear extracts were measured with the Bradford method.

**Nrf2 transcription factor assay.** The colorimetric Nrf2 Transcription Factor Assay Kit (Abcam, ab207223) was used to study the activation of Nrf2 pathway from nuclear extracts of stress and PREP inhibitor treated SH-SY5Y wild type cells. The Nrf2 assay itself combined a quick ELISA format with a sensitive and specific assay for transcription factor activation. The assay provided a colorimetric readout at OD 450 nm that was proportional to the amount of activated Nrf2 transcription factor in the sample. The values were normalized to non-stressed control groups.

## 2.8. Statistical analysis

IBM® SPSS® Statistics (version 24, 64-bit edition) was used for statistical analysis and GraphPad Prism (version 7.05) for preparing the figures. Two-way ANOVA was used to analyze data from the experiment where differences in stress induced ROS production were studied between wild type and PREPko cells, as well as the data from NADPH/NADP<sup>+</sup> assay where NADPH and NADP<sup>+</sup> were shown separately. One-way ANOVA and Bonferroni multiple comparisons were used for analysis of data from the ROS production after PREP inhibition and Nrf2 pathway activation experiments. In case the homogeneity of variances was violated (Levene's test for equality of variances), Welch's ANOVA with Games-Howell *post hoc* test was used instead of the regular one-way ANOVA. Western blot and NADPH/NADP<sup>+</sup> assay ratio data were normalized to control treatment, i.e. non-stressed groups, and analyzed

with independent samples *t*-test.

### 3. Results

#### 3.1. OS induced ROS production

OS induced ROS production was studied with the DCFDA fluorescent probe -based method that provided fluorescent signal proportional to the produced ROS. ROS production was studied in two different cell lines. In SH-SY5Y cells, lack of PREP resulted in significantly lower stress induced ROS production compared to wild type cells ( $F_{1,36} = 76.76$ ,  $p < 0.001$ , two-way ANOVA) (Fig. 1A). The same observation was made with PREPko and wild type HEK-293 cells ( $F_{1,20} = 45.89$ ,  $p < 0.001$ , two-way ANOVA) (Fig. 1B). In addition, the impact of PREP inhibition on ROS production was studied in wild type cells by treating the cells with KYP-2047 concurrently with OS. In SH-SY5Y cells, the 10  $\mu\text{M}$  concentration KYP-2047 treatment decreased ROS production significantly compared to the vehicle treated group ( $p < 0.001$ ), whereas the 1  $\mu\text{M}$  concentration failed to do this ( $p = 0.884$ ) ( $F_{3, 45.557} = 24.48$ ,  $p < 0.001$ , Welch's ANOVA, Games-Howell *post hoc* test) (Fig. 1C). In HEK-293 cells, KYP-2047 decreased ROS production significantly with 1  $\mu\text{M}$  concentration compared to the vehicle treated group ( $p = 0.002$ ) but not with the 10  $\mu\text{M}$  concentration ( $p = 0.129$ ) even though there was a clear apparent decrease in this group as well ( $F_{3, 8.51} = 64.74$ ,  $p < 0.001$ , Welch's ANOVA, Games-Howell *post hoc* test) (Fig. 1D).

#### 3.2. OS induced antioxidant response

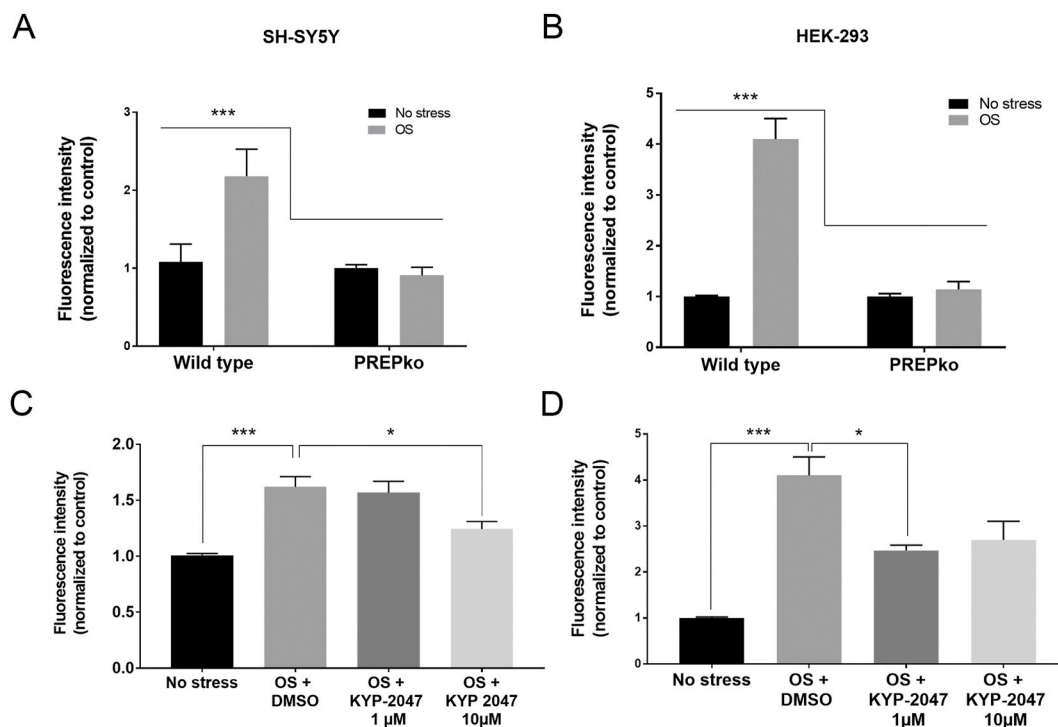
OS induced antioxidant response, and the effect of PREP inhibition on it, was studied in HEK-293 (wild type) and SH-SY5Y (wild type and PREPko) cells by determining protein levels of three enzymatic antioxidants (catalase, SOD1, thioredoxin) after 3 h or 24 h stress. In the 3 h stress group with HEK-293 cells, the increase in the protein levels of antioxidant enzymes remained modest, but still PREP inhibition resulted

in an apparent decrease in all three enzymes (Fig. 2A–C). After 24 h stress, the increase was clear in vehicle treated groups, and PREP inhibition resulted again in a decrease in all three enzymes (Fig. 2D–F) Moreover, the decrease in thioredoxin was statistically significant ( $t_9 = 2.889$ ,  $p = 0.0018$ ) (Fig. 2F).

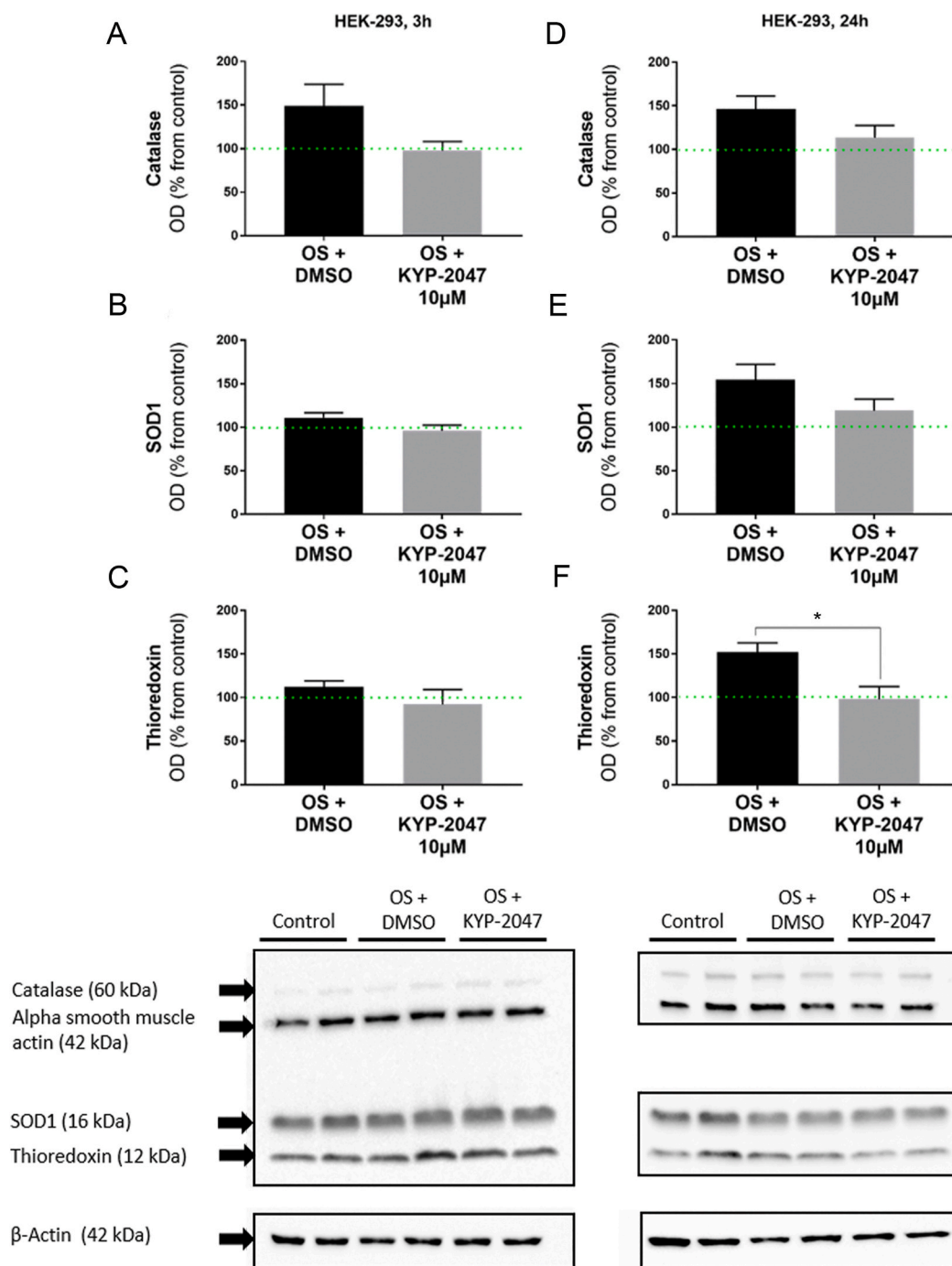
In SH-SY5Y cells, PREP inhibition was not able to decrease the antioxidant protein levels at the 1  $\mu\text{M}$  concentration, similar to ROS production (data not shown), but treatment with the 10  $\mu\text{M}$  concentration did result in significant decreases in catalase ( $t_{29} = 2.064$ ,  $p = 0.048$ ) and SOD1 ( $t_{27} = 2.129$ ,  $p = 0.043$ ) compared to vehicle treatment in the 3 h treatment group (Fig. 3A–B). There was only an apparent decrease in thioredoxin levels after 3 h treatment. In cells treated with 24 h stress, PREP inhibition decreased SOD1 ( $t_{14} = 2.792$ ,  $p = 0.014$ ) and thioredoxin ( $t_{16} = 2.296$ ,  $p = 0.036$ ) protein levels significantly. Further, catalase levels seemed to be close to the control in both vehicle and PREP inhibitor groups after 24 h treatment. In addition, we observed that 3 h treatment either only with OS or OS concurrently with PREP inhibitor did not result in induced antioxidant response in SH-SY5Y PREPko cells (Supplementary Fig. 1).

#### 3.3. PP2A activation during OS

Phosphorylation of Tyr307 in the C-tail of the catalytic subunit of PP2A (PP2Ac) prevents assembly of the active holoenzyme, thus inhibiting PP2A [42]. In our previous study, we validated the pPP2A antibody, and correlation with elevated pPP2A signal was seen after inhibition of PP2A with okadaic acid [31]. PP2A activation was measured by determining the protein levels of total PP2A and the inactive pPP2A from cell lysates prepared from SH-SY5Y wild type cells treated with OS and PREP inhibitor for 3 h or 24 h. PREP inhibitor decreased the ratio of phosphorylated PP2A to total PP2A significantly, i.e. enhanced PP2A activity, compared to vehicle after 3 h treatment ( $t_{17} = 3.782$ ,  $p = 0.001$ ) (Fig. 4B). The ratio was also decreased after 24 h treatment ( $t_{19} = 2.344$ ,  $p = 0.03$ ) (Fig. 4D). Fig. 4A and C presents the



**Fig. 1.** Lack of PREP and PREP inhibition reduce ROS production under oxidative stress. ROS production in SH-SY5Y (A) and HEK-293 (B) wild type and PREPko cells after treatment with 3 h OS. In both cell lines, lack of PREP blocked the stress induced ROS production. ROS production in 3 h stress and PREP inhibitor (KYP-2047) treated SH-SY5Y (C) and HEK-293 (D) wild type cells. Bars represent group means  $\pm$  SEM. \*\*\* $p < 0.001$ , two-way ANOVA (A–B). \* $p < 0.05$ , \*\*\* $p < 0.001$ , Welch's ANOVA with Games-Howell *post hoc* test (C–D).



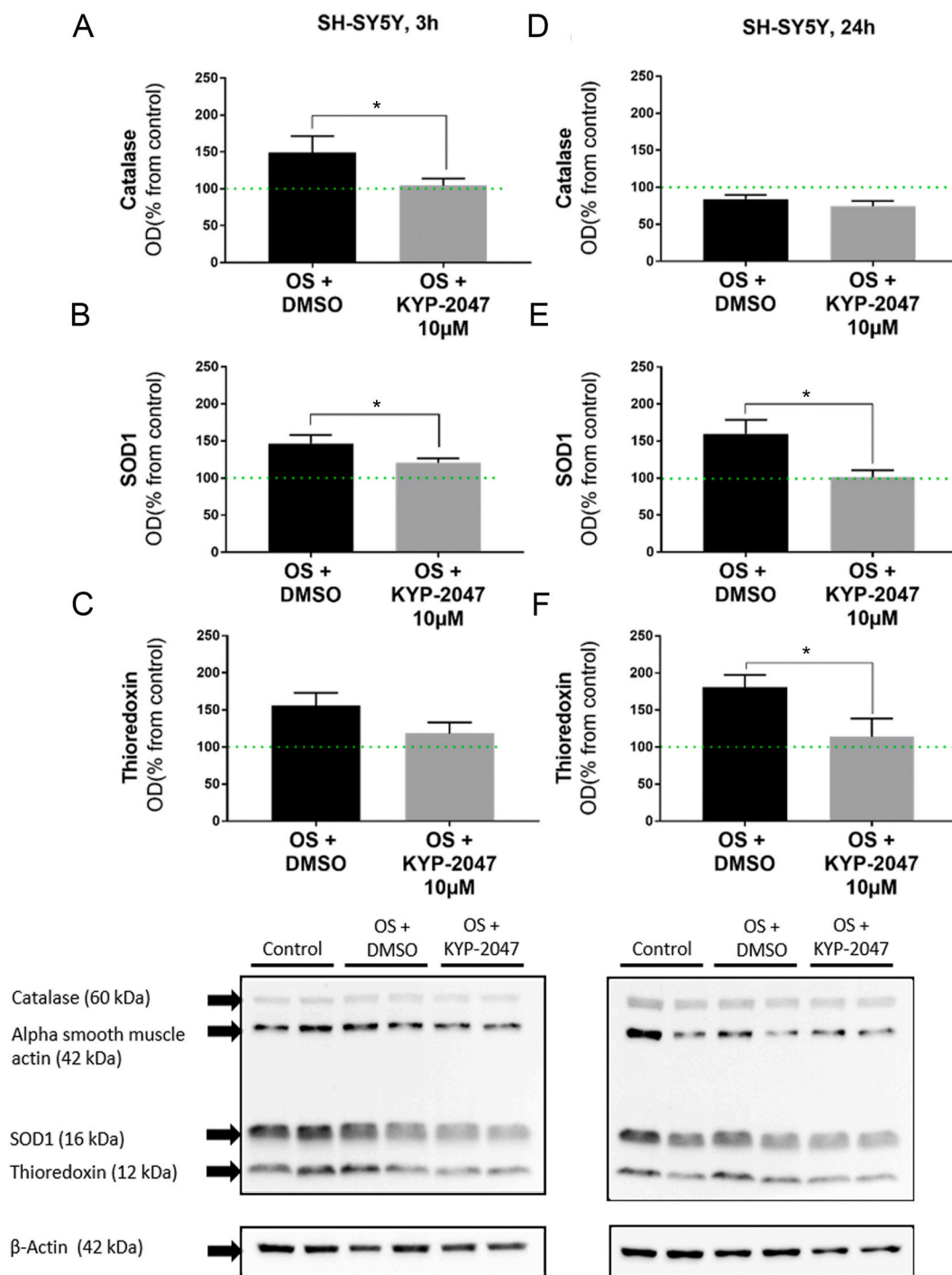
**Fig. 2.** Antioxidant response in HEK-293 wild type cells. Protein levels of A) catalase B) SOD1 and C) thioredoxin after 3h treatment with OS with or without concurrent PREP inhibitor (10 µM KYP-2047). D), E) and F) levels of the enzymatic antioxidants after 24h treatment. Bars represent group means normalized to non-stressed control set at 100% ± SEM, \* $p < 0.05$ , independent samples  $t$ -test.

results as separate protein levels of total PP2A and phospho-PP2A. Furthermore, we were not able to observe changes in PP2A activity after 3 h treatment with OS alone or concurrently with PREP inhibitor in SH-SY5Y PREPko cells (Supplementary Fig. 2).

### 3.4. NADPH oxidase activation

ROS producing NADPH oxidase activation was studied by measuring the total p47phox subunit and the activated Ser345 phosphorylated p47phox protein levels from cell lysates of OS and PREP inhibitor treated SH-SY5Y cells. Assembly of active NOX2 requires extensive

p47phox subunit phosphorylation, thus phosphorylation of p47phox indicates NOX2 activation [35]. Here we observed a significant decrease in the ratio of phosphorylated p47phox to total p47phox between PREP inhibition and vehicle treatment after 3 h treatment, indicating reduced NOX activation ( $t_{20} = 2.25$ ,  $p = 0.0359$ ) (Fig. 5B). Moreover, the indirect method we used for measuring NADPH oxidase activity, i.e. NADPH turnover to the oxidized form  $\text{NADP}^+$ , showed significant differences in luminescence proportional NADPH conversion to  $\text{NADP}^+$  after 3 h treatment with PREP inhibitor compared to vehicle treated group ( $F_{1,20} = 9.86$ ,  $p < 0.005$ , two-way ANOVA) (Fig. 6A). This was also seen as an increase in NADPH/ $\text{NADP}^+$  ratio ( $t_{10} = -2.545$ ,  $p = 0.029$ ) (Fig. 6B).



**Fig. 3.** Antioxidant response in SH-SY5Y wild type cells. Protein levels of A) catalase B) SOD1 and C) thioredoxin after 3h treatment with OS with or without concurrent PREP inhibitor (10 µM KYP-2047). D), E) and F) levels of the enzymatic antioxidants after 24h treatment. Bars represent group means normalized to non-stressed control set at 100% ± SEM, \*p < 0.05, independent samples *t*-test.

The effect was not seen after 24 h treatment (data not shown). These assays provided information about first line ROS production by NADPH oxidases after stress induction [35].

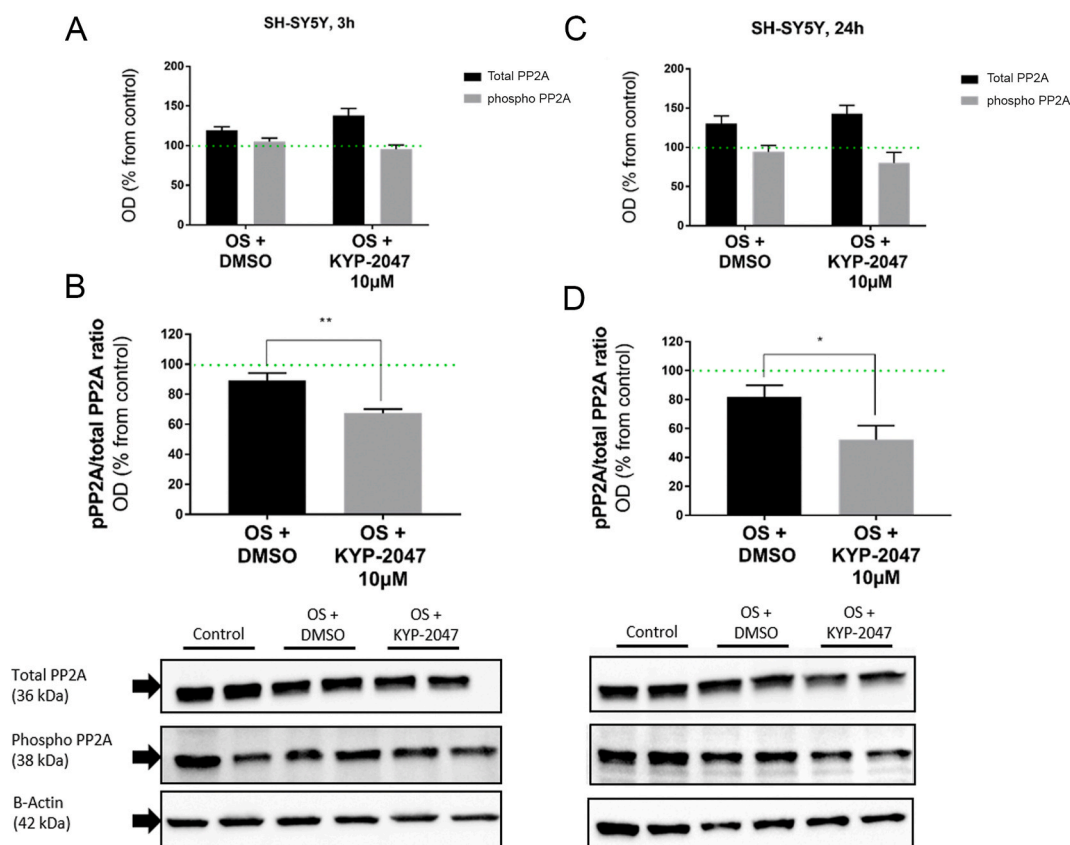
### 3.5. Nrf2 pathway activation

During Nrf2 pathway activation, e.g. under OS conditions, the Nrf2 transcription factor translocates to the nucleus and activates the antioxidant response element (ARE) -mediated gene expression [37]. Nrf2 transcription factor's nuclear translocation (Nrf2 activation) was

studied in nuclear extracts from SH-SY5Y wild type cells treated with or without OS and PREP inhibitor for 24 h. As expected, OS caused an increase in Nrf2 activation. However, the effect of PREP inhibition lacked statistical significance (Supplementary Fig. 3).

## 4. Discussion

Despite their differences, many neurodegenerative diseases with proteinopathies involve OS in their pathologies [43]. Excessive amounts of ROS during OS can enhance pathological protein aggregation, and on



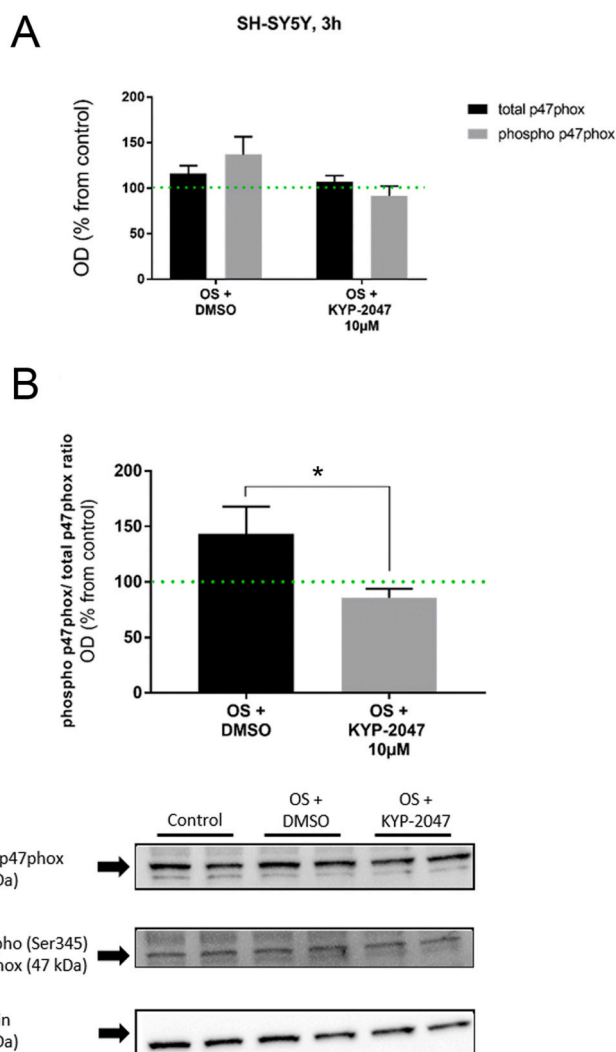
**Fig. 4.** PP2A activation during OS. Protein amounts of total PP2A and phospho-PP2A after A) 3 h treatment with OS and PREP inhibitor and C) 24 h treatment. B) and D) present the results as phospho-PP2A to total PP2A ratios, (independent samples *t*-test). Bars represent group means normalized to non-stressed control set at 100% ± SEM, \**p* < 0.05, \*\**p* < 0.01.

the other hand, early protein aggregation intermediates can induce OS, indicating that there is a clear link between these two phenomena [9,17,18]. ROS can directly cause damage to macromolecular cellular structures but, in addition, they are involved in modulation of various cellular functions, such as mitogen-activated protein (MAP) kinase cascade activation, calcium mobilization, and apoptosis program activation [43,44]. During OS when the amount of ROS is significantly increased, these cellular functions get disturbed and, together with the direct ROS-mediated damage participate in neurotoxicity. Therefore, blocking OS would be a promising and rational approach to achieve neuroprotection.

PREP has already proven to have an impact on ROS production and cellular antioxidant response proteins in  $\alpha$ -Syn transfected HEK-293 PREPko cells after induction of OS [34]. Here we managed to recreate the effect on ROS production in two different cell lines (SH-SY5Y and HEK-293) after induction of OS without  $\alpha$ -Syn transfection. Lack of PREP resulted in significantly diminished ROS production in both cell lines compared to wild type cells, and additionally, we observed a similar kind lack of cellular antioxidant response in SH-SY5Y PREPko cells we have previously seen in HEK-293 PREPko cells [34]. More interestingly, PREP inhibition resulted in a similar effects in both wild type cell lines. In SH-SY5Y cells, a significant decrease in ROS production was achieved with the 10  $\mu$ M PREP inhibitor (KYP-2047) concentration, whereas in HEK-293 cells, a significant decrease was already achieved with the 1  $\mu$ M concentration. Furthermore, we were able to observe reduced OS as a diminished antioxidant response after PREP inhibitor treatment (Figs. 2 and 3). Here we studied three different enzymatic antioxidants. Briefly, SOD1 participates in antioxidation by catalyzing the dismutation of superoxide ( $O_2^{\cdot-}$ ) radicals generating hydrogen peroxide that is further converted to water, for instance, by catalase [45]. Thioredoxin, in turn, reverses the disulfide formation of

proteins caused by OS, and its expression is induced by oxidative stimuli [46]. The effect on antioxidant response was not that clear in HEK-293 cells, even though a general decrease was seen (Fig. 2). With HEK-293's the differences appear to be more evident after 24 h treatment and here a significant decrease in thioredoxin protein levels can be observed. In SH-SY5Y cells, a reduced antioxidant response can be seen after both 3 h and 24 h treatments. After the 3 h treatment there was significant decreases in catalase and SOD1 protein levels, whereas decrease in thioredoxin revealed only an apparent decrease. In turn, after the 24 h treatment a significant reduced antioxidant response can be seen as decreased SOD1 and thioredoxin levels. Interestingly, at this time point catalase protein levels remain low in both vehicle and PREP inhibitor treatment groups. However, cellular responses to OS vary between different cell lines and they are dependent on the duration of the treatment [47], which might explain the lack of catalase induction in SH-SY5Y cells after the 24 h treatment. Based on these results about reduced ROS production and diminished antioxidant response after PREP inhibition, we can say that PREP has a role in cellular redox regulation and PREP inhibition can reduce OS.

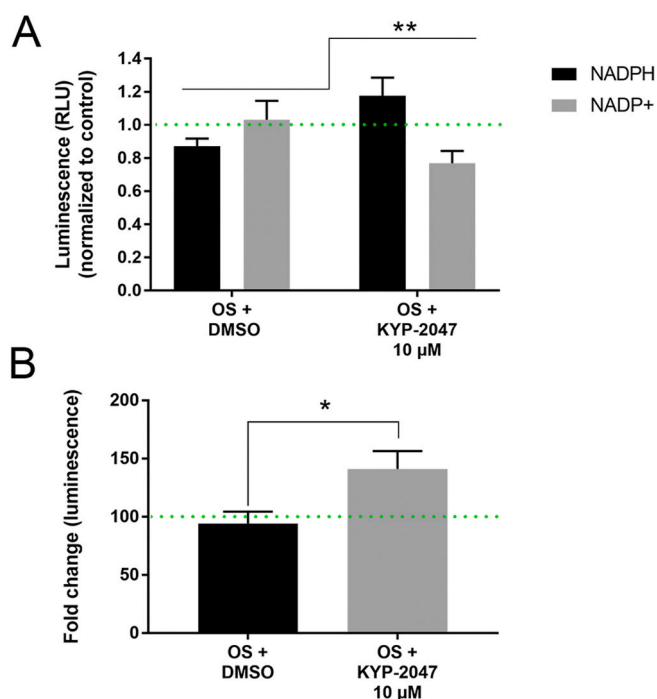
In addition to the ability of PREP inhibition to reduce OS, we were also able to point the mechanism by which PREP inhibition conveys its OS reducing effect, i.e. reduction of ROS producing NOX activity. NADPH oxidases (NOX) are superoxide anion producing enzymes that need an electron from NADPH to be able to reduce oxygen, and especially NOX2 is an enzyme isoform that is expressed in various brain regions and is associated with AD and PD -related neurodegeneration [35,48,49]. As mentioned above, NOX2 assembly and activation requires subunit p47phox phosphorylation [50]. Here we showed that PREP inhibition during OS caused a significant decrease in phospho-p47phox to total p47phox ratio after 3 h treatment compared to vehicle treated group, indicating reduced NOX activation (Fig. 5B).



**Fig. 5.** PREP inhibition reduces OS induced p47phox phosphorylation. Protein amounts of total p47phox and phospho-p47phox after 3 h treatment with OS and PREP inhibitor (A) and results represented as phospho-p47phox to total p47phox ratio (B). Bars represent group means normalized to non-stressed control set at 100%  $\pm$  SEM, \* $p < 0.05$  independent samples *t*-test.

Moreover, the data about NADPH turnover to NADP<sup>+</sup> revealed also a significant difference between PREP inhibition and vehicle treatment. PREP inhibition was able to reduce NADPH turnover to its oxidized form after the 3 h treatment (Fig. 6), indirectly indicating to a reduced NOX activity. These results suggest that PREP inhibition reduces ROS production and consequent OS via diminishing NOX activity.

Here we showed that treating the SH-SY5Y wild type cells with OS, increased PP2A activity in both chosen time points (Fig. 4) measured by the protein level ratios of inactive Tyr307 phosphorylated PP2A to total PP2A, indicating that PP2A activation is related to the cellular response to OS. ROS-mediated PP2A activation has also been shown in the study by Ding et al. [51], which supports this finding. Concurrent PREP inhibition resulted in even more prominent PP2A activity after both 3 h and 24 h treatments (Fig. 4). PREP inhibition has already proven to increase autophagy via a PP2A-mediated mechanism [52], and these results suggest that reduction of OS might also be mediated by this property. Moreover, increase in PP2A activation could not be seen in PREPko cells during stress, further indicating the role of PREP in OS and as a physiological regulator of PP2A. The role of PP2A in defense against OS is not clear, but PP2A activation has been shown to lead to dephosphorylation of the p47phox subunit of the ROS producing NOX



**Fig. 6.** PREP inhibition reduces NADPH turnover during oxidative stress. Indirect indicator of NADPH oxidase activation represented by turnover of NADPH to NADP<sup>+</sup> during OS conditions. Proportional amounts of NADPH and NADP<sup>+</sup> represented on separate columns (A) and as NADPH to NADP<sup>+</sup> ratios (B). Bars represent group means normalized to non-stressed control set at 100%  $\pm$  SEM, \*\* $p < 0.01$ . two-way ANOVA (A), \* $p < 0.05$  independent samples *t*-test (B).

[36], which also supports our results of the decrease in NOX activation after PREP inhibition, i.e. according to these results PREP inhibition during OS increases PP2A activity which leads to reduced p47phox phosphorylation and NOX activity. Furthermore, PP2A has been suggested to protect cells against OS via other mechanisms as well [51,53–55], but further studies need to be conducted to figure out the other possible mechanism how PP2A activation could be related to OS reduction.

The Nrf2 signaling pathway is typically activated by OS [56]. When the pathway is activated, Nrf2 transcription factor dissociates from Keap 1, that keeps it in the cytosol, and translocates into the nucleus where it binds to antioxidant response elements (AREs) and initiates transcription of various proteins capable of reducing OS, e.g. thioredoxin, catalase, SOD1, and proteins involved in NADPH synthesis [56,57]. As this pathway can be seen as a general switch against OS, we decided to study if PREP inhibition had an effect on its activation. The results show that OS itself increases the amount of translocated Nrf2 after 24 h treatment, which could be expected. Further, PREP inhibition failed to produce significant differences in nuclear translocation of Nrf2 measured with the colorimetric ELISA assay we used. Nrf2 pathway activation is widely considered a promising approach to tackle OS in various neurodegenerative disorders [56,58], and thus, it was interesting to assess if PREP inhibition would reduce OS via Nrf2 activation.

## 5. Conclusions

To conclude, PREP is involved in cellular redox regulation as its absence prevents stress stimulated ROS production and cellular antioxidant response, effects we could see here in two different cell lines (SH-SY5Y and HEK-293) and in one of our earlier studies [34]. Here we showed that inhibition of PREP produces a similar kind of effect in ROS production and cellular antioxidant response in both cell lines. According to our results, the mechanism by which PREP inhibition reduces



OS is related to its ability to reduce ROS producing NOX activity, which we observed as reduced p47phox phosphorylation and diminished NADPH turnover to NADP<sup>+</sup>. Moreover, our results revealed enhanced PP2A activation after treatment with PREP inhibitor during OS, which might be the cause behind decreased p47phox phosphorylation and consequent reduced NOX activation. PREP inhibition has already proven to be neuroprotective in cellular and animal models of neurodegeneration accompanied by abnormal protein aggregation, as it can enhance protein clearance by inducing autophagy and prevent formation of detrimental protein aggregates [25,26,28,29]. Now, these recent results indicate that PREP inhibition can also provide neuronal protection by reducing OS that is a common toxic factor for all neurodegenerative diseases. That being said, PREP inhibition could be an approach to tackle various common detrimental features among neurodegenerative diseases with abnormal protein aggregation.

## Funding

This study was supported by grants from Academy of Finland (grant 318327), Jane and Aatos Erkko Foundation and Sigrid Juselius Foundation to Timo Myöhänen. Funding bodies did not participate in the study design nor in the collection, analysis, and interpretation of data, nor in writing of the report.

## Language revision

Manuscript of this article was sent to Dr. Katrina Rätty for proofreading.

## Data statement

All raw data used to generate the results of this study are available from corresponding author by request.

## Declaration of competing interest

None.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.freeradbiomed.2021.04.001>.

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