Neurotoxicology 73 (2019) 112–119

Contents lists available at ScienceDirect

Neurotoxicology

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

Full Length Article

Activation of the immunoproteasome protects SH-SY5Y cells from the toxicity of rotenone

Congcong Sun[a], Mingshu Mo[b], Yun Wang[c], Wenfei Yu[a], Chengyuan Song[a], Xingbang Wang[a], Si Chen[a], Yiming Liu[a,d,*]

[a] Department of Neurology, Qilu Hospital of Shandong University, Jinan, 250012, China
[b] Department of Neurology, First Affiliated Hospital of Guangzhou Medical University, Guangzhou, 510120, China
[c] Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou Medical University, Xuzhou, 221000, China
[d] Brain Science Research Institute, Shandong University, Jinan, 250012, China

ARTICLE INFO

Keywords:
Immunoproteasome
PSMB9
Parkinson's disease
Rotenone

ABSTRACT

This study investigated the expression and role of immunoproteasome (i-proteasome) in a cell model of Parkinson's disease (PD). The cytotoxicity of rotenone was measured by CCK-8 assay. The i-proteasome β1i subunit PSMB9 was suppressed by a specific shRNA or transfected with an overexpression plasmid in the SH-SY5Y cells. Under the exposure to rotenone or not, the expression of constitutive proteasome β subunits, i-proteasome βi subunits, antigen presentation related proteins, α-syn and TH were detected by Western blot in PSMB9-silenced or -overexpressed cells, and the proteasomal activities were detected by fluorogenic peptide substrates. The location of i-proteasome βi subunits and α-syn were detected by immunofluorescence staining. The levels of ROS, GSH and MDA were measured by commercial kits. Cell apoptosis was detected by flow cytometry. Besides impairing the constitutive proteasomes, rotenone induced the expression of βi subunits of i-proteasome and antigen presentation related proteins such as TAP1, TAP2 and MHC-I. Silencing or over-expressing PSMB9 had no obvious effect on the levels of other subunits, but could regulate the chymotrypsin-like activity of 20S proteasome and the expression of TAP1, TAP2 and MHC-I. Three βi subunits (PSMB9, PSMB10, PSMB8) of i-proteasome were all co-localized with α-syn. PSMB9 knockdown aggravated accumulation of α-syn, degradation of TH, release of ROS, increased level of MDA, decreased level of GSH and eventually promoted apoptosis in SH-SY5Y cells after rotenone treatment, while over-expression of PSMB9 could attenuate these toxic effects of rotenone. I-proteasome is activated in SH-SY5Y cells treated with rotenone and may play a neuroprotective role.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease in elderly people (Goedert and Compston, 2018). The pathologic changes, which are characterized by loss of dopaminergic neurons and deposition of abnormal α-synuclein (α-syn) in the surviving neurons, appear long before the onset of the classic movement disorders (Surmeier et al., 2017). Ubiquitin-proteasome pathway, which plays a key role in maintaining protein homeostasis, is impaired under these stresses, and subsequently induces the formation of harmful protein aggregates (Mckinnon et al., 2015).

Proteasomes consist of two main components: the catalytic 20S core particle and the 19S regulator particle. The catalytic 20S core particle contains three active subunits of β1, β2 and β5, which have caspase-like, trypsin-like and chymotrypsin-like peptidase activities, respectively (Eskandari et al., 2017). These subunits could be replaced by homologous subunits β1i (PSMB9), β2i (PSMB10) and β5i (PSMB8), and the constitutive proteasome transforms into an immunoproteasome (i-proteasome) under oxidative stress or inflammation conditions (Kaur and Batra, 2016). I-proteasome is a subtype of proteasome which could degrade proteins and present optimal peptides to antigen presenting cells. Genetic polymorphisms in i-proteasome have been linked to neurodegenerative disease. For example, single nucleotide polymorphisms located in PSMB9 could influence the proteasome activity in

Abbreviations: i-proteasome, immunoproteasome; PD, Parkinson's disease; α-syn, α-synuclein; AD, Alzheimer's disease; SD, standard deviation

* Corresponding author at: Department of Neurology, Qilu Hospital of Shandong University and Brain Science Research Institute, Shandong University, No.107, Wenhua West Road., Jinan, 250012, China.
E-mail address: yimingliuqilu@163.com (Y. Liu).

https://doi.org/10.1016/j.neuro.2019.03.004
Received 11 September 2018; Received in revised form 13 March 2019; Accepted 18 March 2019
Available online 20 March 2019
0161-813X/

Alzheimer's disease (AD) (Mishto et al., 2006). A previous study has reported that rs17587 variants in PSMB9 gene might contribute to the susceptibility of sporadic PD in Chinese females (Mo et al., 2016).

Growing evidence shows that i-proteasome could be activated and might be involved in the pathogenesis of neurodegenerative disease with abnormal protein aggregation. For example, increased levels of PSMB9 and PSMB8 have been detected in cortex and striatum of patients with Huntington's disease (Díaz-Hernández et al., 2003). Increased i-proteasome activity is found in human AD samples (Orre et al., 2013). A trend of increased PSMB8 expression in remaining TH positive cells in Multiple system atrophy and Progressive supranuclear palsy has been reported (Bukhatwa et al., 2010). However, the expression of i-proteasomes in PD is less studied and the results are controversial as yet. Bukhatwa et al detected no obvious change of PSMB8 expression in remaining TH positive cells in PD samples compared to control (Bukhatwa et al., 2010), while Ugras et al found increased levels and activity of PSMB8 in postmortem human brains with PD (Ugras et al., 2018).

Besides modulating inflammation, i-proteasome possess antioxidant capacity and proteolytic activity (Johnston-Carey et al., 2015; Kimura et al., 2015). Augmented immunoproteasome function may contribute to lifespan diff ;erences in mice and among primate species (Pickering et al., 2015). To investigate the role of i-proteasome in the pathophysiology of PD, we detected the exprssion and founction of βi subunits of i-proteasome in SH-SY5Y cells with rotenone toxicty, which is widely used to imitate the pathogenesis of PD (Johnson and Bobrovskaya, 2015).

2. Materials and methods

2.1. Cell culture and rotenone treatment

The SH-SY5Y cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and was cultured in Dulbecco's modified Eagle's high glucose medium (DMEM, Life Technologies, Rockville, MD, USA), supplemented with 10% fetal bovine serum (FBS, Invitrogen, CA, USA) in a humidified incubator with 5% CO_2 at 37 °C.

Rotenone (Sigma-Aldrich, St Louis, MO, USA) was prepared in DMSO (Sigma-Aldrich, St Louis, MO, USA) and stocked at a concentration of 50 mM. Different concentrations of rotenone (0 nM, 10 nM, 100 nM, 500 nM, 1 u M, 10 u M) were diluted with DMEM respectively. Different incubation time (0 h, 3 h, 6 h, 12 h, 24 h, 48 h) was carried out according to corresponding experiments and with vehicle as control.

2.2. CCK-8 assay

Cells (5×10^3 cells/well) were seeded in a 96-well plate and incubated overnight. Then cells were treated with vehicles or different doses of rotenone (0–10 u M). Cell viability was detected with Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Briefly, at different time points (0–48 h), 10 μL of CCK-8 solution was added to each well and the incubation continued for another 2 h at 37 °C. Absorbance was measured by a multimode microplate reader (Thermo Fisher Scientific Inc., MA, USA) at 450 nm. Cell viability was expressed as a percentage in relative to the absorbance of control cells.

2.3. ShRNA and overexpression plasmid transfection

The PSMB9 was suppressed by a specific shRNA in PLKO.1-EGFP-puro vector (Wuhan Miaoling Bioscience & Technology Co., Ltd., China) with target sequences of 5′-CGCTTCACCACAGACGCTATT-3′, 5′-CTGCAAATGTGGTGAGAAAT-3′ and 5′-CATCTACCTGGTCACTAT TAC-3′. The coding sequence of human PSMB9 (Accession No: NM_002800) was amplified by PCR and the obtained fragment was subsequently cloned into a pECMV-3xFLAG-N vector (Wuhan Miaoling Bioscience & Technology Co., Ltd, China), which contains the neomycin selection marker. The vectors without the target gene and target of βeta-actin were used as the negative control and positive control, respectively. Empty plasmids were used as the mock control. All plasmids were verified by DNA sequencing. The plasmids were transfected with Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) following the manufacturer's instructions.

2.4. Western blot

After treating with vehicles or 500 nM rotenone for 24 h, the whole-cell lysate was prepared by a Mammalian Cell Extraction Kit (Biovision, CA, USA). The protein concentration was measured with a Bicinchoninic Acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., MA, USA). Soluble proteins (50 μg) were separated by 8–12% SDS-polyacrylamide gels, and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). Primary antibodies were as follows: rabbit anti-20 S proteasome β1(1: 1000, Abcam, Cambridge, MA, USA), mouse anti-20 S proteasome β2 (1: 1,000, Abcam, Cambridge, MA, USA), rabbit anti-20 S proteasome β5 (1: 1000, Abcam, Cambridge, MA, USA), rabbit anti-PSMB9 (1: 1,000, Abcam, Cambridge, MA, USA), rabbit anti-PSMB10 (1: 1,000, Abcam, Cambridge, MA, USA), mouse anti-PSMB8 (1: 200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse anti-TAP1 (1: 2,00, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit anti-TAP2 (1: 1,000, Abcam, Cambridge, MA, USA), rabbit anti-rat MHC-I (1:1000, Abcam, Cambridge, MA, USA), rabbit anti-TH (1: 1,000, Abcam, Cambridge, MA, USA), mouse anti-Alpha-synuclein (1: 1,000, Abcam, Cambridge, MA, USA) and mouse anti- β-actin(1:10,000, Proteintech Group Inc., Chicago, IL, USA). Subsequently, membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG (KPL, MD, USA) secondary antibodies. Proteins were detected by SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., MA, USA). The densities were calculated as target protein expression/ β-actin expression ratios with Image J 1.42q software (U.S. National Institutes of Health).

2.5. Proteasome activity measurement

Cells were treated with vehicles or 500 nM rotenone for 24 h. Then, the proteasomal activity was measured with 20S proteasome activity assay kit (Chemicon-Millipore, Billerica, MA, USA). Briefly, the cells were sonicated in cell lysis buffer (Thermo Scientific Pierce, Rockford, IL, USA). A BCA Protein Assay Kit (Thermo Fisher Scientifc, Rockford, USA) was used to determine protein concentration. The isolated protein (50 μg) was incubated with buffer and 50 μM fluorogenic substrates (Z-LLE-AMC for caspase-like activity, Suc-LLVY-AMC for chymotrypsin-like activity, and Z-ARR-AMC for trypsin-like activity) at 37 °C for 1 h. Production of hydrolyzed AMC was measured at a multimode microplate reader (Thermo Fisher Scientific Inc., MA, USA, Ex/Em: 380/460 nm). Cells treated with vehicles were used as control. The proteasome activity was expressed as a percentage in relative to the absorbance of control cells.

2.6. Immunofluorescence staining

Cells treated with vehicles or 500 nM rotenone for 24 h were fixed with 4% paraformaldehyde and permeabilized with 0.01 M PBS containing 0.1% Triton X-100. After blocking with 10% goat serum, cells were incubated overnight at 4 °C with rabbit anti-PSMB9 (1:500, Abcam, Cambridge, MA, USA), rabbit anti-PSMB10 (1:500, Abcam, Cambridge, MA, USA), rabbit anti-PSMB8 (1:500, Abcam, Cambridge, MA, USA) and mouse anti-Alpha-synuclein (1:300, Abcam) primary antibodies. Alexa Fluor 594-conjugated goat anti-mouse antibody and FITC-conjugated goat anti-rabbit antibody (1:1,000, Jackson

Immunoresearch Inc., West Grove, PA, USA) were used as secondary antibodies. Cells were examined under a LEICA TCS SP5 MP confocal laser scanning microscope (Leica, Heidelberg, Germany).

2.7. Measurement of reactive oxygen species (ROS), GSH and MDA

Cells were treated with vehicles or rotenone (500 nM) for 24 h and then incubated with DCFH-DA (Reactive oxygen species assay kit, Nanjing Jiancheng BioEngineering Institute, China) at a final concentration of 10 μmol/l for 30 min at 37 °C. Fluorescence was recorded at 488 nm excitation and 525 nm emission wavelengths by the multimode microplate reader (Thermo Fisher Scientific Inc., MA, USA).

Following the protocols of two commercial kits (Reduced glutathione (GSH) assay kit and Cell Malondialdehyde (MDA) assay kit, Nanjing Jiancheng BioEngineering Institute, China), GSH and MDA levels in the treated cells were quantified spectrophotometrically at the wavelengths of 405 and 532 nm, respectively, by the multimode microplate reader (Thermo Fisher Scientific Inc., MA, USA).

The intracellular ROS, GSH and MDA levels were expressed as a percentage in relative to the absorbance of control cells.

2.8. Flow cytometry

Cells (5×10^5 cells/well) were seeded in six-well plates and treated with vehicles or rotenone (500 nM) for 24 h, respectively. Then, the cells were harvested, washed with cold PBS, and double-stained using an Annexin V–FITC/PI apoptosis detection kit (KeyGen BioTECH, Nanjing, China). The number of apoptotic cells was evaluated by flow cytometry (BD Biosciences, CA, USA).

2.9. Statistical analysis

All analysis was performed with SPSS software (version 12.0; SPSS Inc., Chicago, IL, USA). The results were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by Tukey's test or Games–Howell test was used to compare the data among multiple groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxic effect of rotenone on SH-SY5Y cells

To determine the cytotoxicity of rotenone on SH-SY5Y cells, a cell viability test at deferent drug concentrations (0–10 μM) and deferent time (0–48 h) was performed using CCK-8 assay. Compared with controls, cell viability was obviously decreased at 100 nM rotenone for 24 h, and a concentration-dependent reduction of cell viability was observed after rotenone treatment (Fig. 1A). Rotenone at 500 nM was chosen to conduct a time course study, and a time-dependent reduction of cell viability was also observed (Fig. 1B). After exposing to 500 nM rotenone for 24 h, cell viability significantly decreased to 55.35 ± 7.02% compared to controls ($P < 0.01$). These results indicate that rotenone has toxic effect on SH-SY5Y cells in a time- and dose-depended manner. Cells were treated with 500 nM rotenone (the volume ratio of DMSO was 0.001%) for 24h before they were used for the following experiments.

3.2. Expression of β subunits in the constitutive proteasome and i-proteasome in SH-SY5Y cells treated with rotenone

To examine whether variation of proteasome is involved in SH-SY5Y cells with rotenone toxicity, the expression of β subunits in the constitutive proteasome and i-proteasome were determined by Western blot. After exposing to 500 nM rotenone for 24 h, the expression of β1, β2 and β5 subunits in the constitutive proteasome was decreased obviously compared to that of control ($P < 0.05$, respectively, Fig. 2A and 2B), while the expression of β1i (PSMB9), β2i (PSMB10) and β5i (PAMB8) subunits in the i-proteasome was increased dramatically compared with that of control ($P < 0.05$, respectively, Fig. 3A and B). These results indicate that rotenone induces the expression of i-proteasome with constitutive proteasome lesion.

3.3. PSMB9 inhibition or overexpression does not affect the expression of other subunits

To confirm the impact of PSMB9 expression on the other subunits, cells were transfected with PSMB9 shRNA or PSMB9 overexpression plasmid (Fig. S1) and then treated with vehicles or 500 nM rotenone for 24 h. There was no obvious difference in protein level of other subunits (β1, β2, β5, PSMB10 and PSMB8) among cells with PSMB9 inhibition or overexpression under normal condition (Fig. 2A and C, Fig. 3A and C) or rotenone toxicity (Fig. 2A and D, Fig. 3A and D). This suggests that PSMB9 has no obvious effect on the expression of other subunits in constitutive proteasome or i-proteasome.

3.4. Rotenone impairs the 20S proteasomal activities and the chymotrypsin-like activity could be regulated by PSMB9

The 20S proteasome activities of SH-SY5Y cells were analyzed through three fluorogenic substrates. After the treatment with 500 nM rotenone for 24 h, the caspase-like activity, the trypsin-like activity and the chymotrypsin-like activity were impaired significantly compared to that of controls ($P < 0.05$, respectively, Fig. 4A).

PSMB9 knockdown resulted in a decline of chymotrypsin-like activity ($P < 0.05$, compared with the controls) and subsequent rotenone exposure further dropped it significantly (Fig. 4B). On the contrary, compared with controls, PSMB9 over expression elevated the chymotrypsin-like activity significantly but subsequent rotenone exposure dropped it (Fig. 4C). No obvious effect was observed for the caspase-like activity and trypsin-like activity with PSMB9 knockdown or

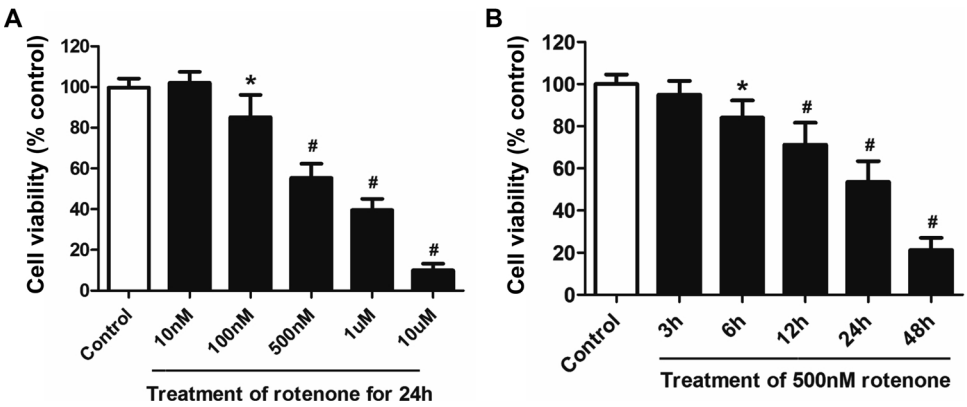

Fig. 1. Cytotoxic effect of rotenone on SH-SY5Y cells. SH-SY5Y cells were treated with (A) 0- 10 μM rotenone for 24 h or (B) 500 nM rotenone for 0–48 h. Cell viability was measured by CCK8 assay and expressed as percentage relative to control group. Data was presented as mean ± SD from three independent experiments. $^{*}P < 0.05$ compared to the control group, $^{\#}P < 0.01$ compared to the control group.

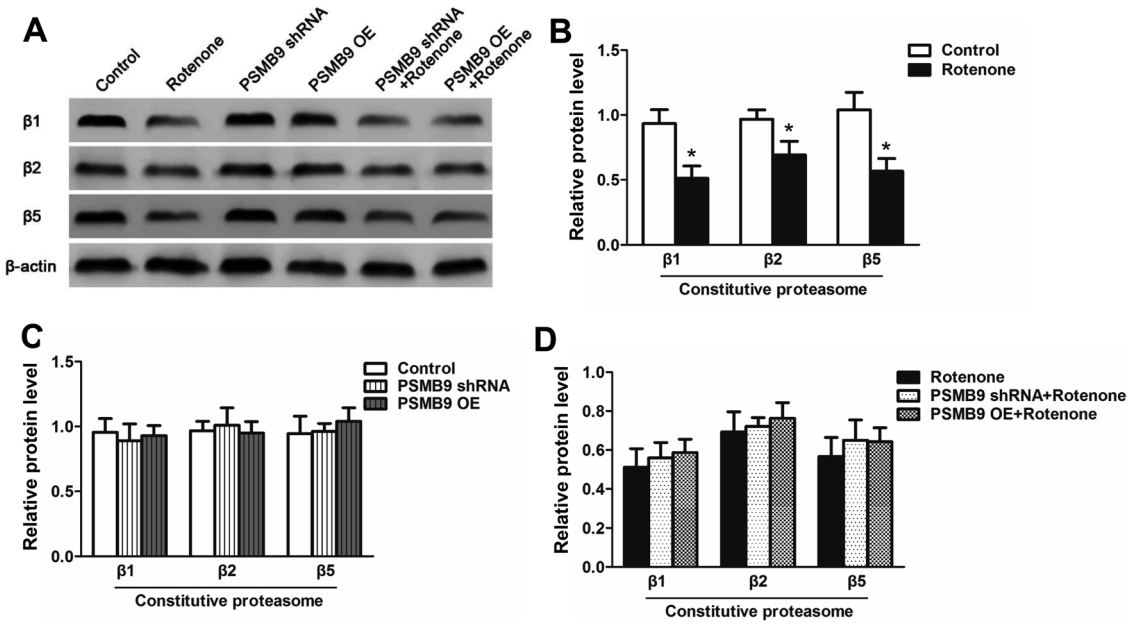

Fig. 2. The expression of β subunits of constitutive proteasome was decreased in SH-SY5Y cells with rotenone toxicity. The expression of β subunits of constitutive proteasome were detected by Western blot (A) in cells treated with vehicle or 500 nM rotenone for 24 h (B) after PSMB9 knockdown or overexpression (OE) (C and D). Cells with neither transfection nor rotenone treatment served as control. The relative band intensities of β subunits were normalized to the expression of β-actin. Data was presented as mean ± SD. $^{*}P < 0.05$ compared to the control group.

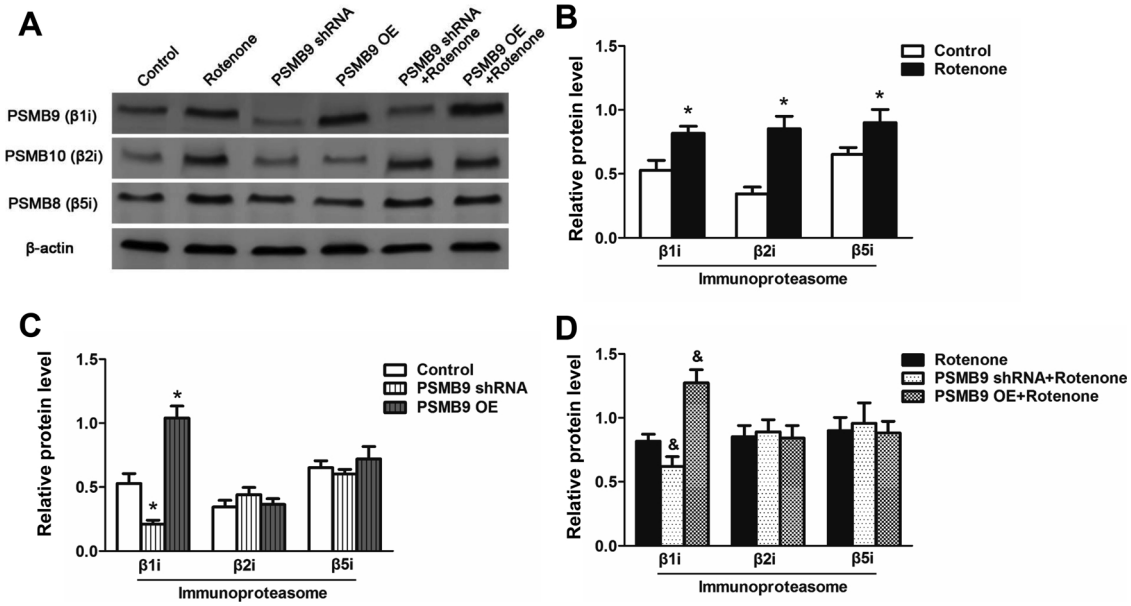

Fig. 3. Rotenone induced the expression of βi subunits of immunoproteasome (i-proteasome) in SH-SY5Y cells. The expression of βi subunits of i-proteasome were detected by Western blot (A) in cells treated with vehicle or 500 nM rotenone for 24 h (B) after PSMB9 knockdown or overexpression (OE) (C and D). Cells with neither transfection nor rotenone treatment served as control. The relative band intensities of βi subunits were normalized to the expression of β-actin. Data was presented as mean ± SD. $^{*}P < 0.05$ compared to the control group. $^{\&}P < 0.05$ compared to the rotenone-alone treatment group.

overexpression. All these results point to that rotenone impaired the 20S proteasome activities significantly, and the chymotrypsin-like activity could be regulated positively by the expression of PSMB9.

3.5. Rotenone induces the expression of antigen presentation related proteins (TAP1, TAP2 and MHC-I), which could be regulated by the expression of PSMB9

To explore the downstream reactions after the activation of i-proteasome, several proteins involved in antigen presentation were detected by Western blot. The results showed obvious up-regulation on the expressions of TAP-1, TAP2 and MHC-I ($P < 0.05$, compared with controls, respectively), accompanying with the activation of i-proteasome, after exposing to rotenone (Fig. 5A, B).

PSMB9 knockdown or overexpression had no obvious effect on the expression of TAP-1, TAP-2 and MHC-I under normal conditions (Fig. 5C). When the cells exposing to 500 nM rotenone for 24 h, PSMB9 knockdown prevented the activation of TAP-1, TAP-2 and MHC-I induced by rotenone, but only the changes in TAP-1 and TAP-2 had statistical significance ($P < 0.05$, compared with rotenone alone group, respectively) (Fig. 5D). Over-expression of PSMB9 effectively promoted the activation of TAP-1, TAP-2 and MHC-I induced by rotenone

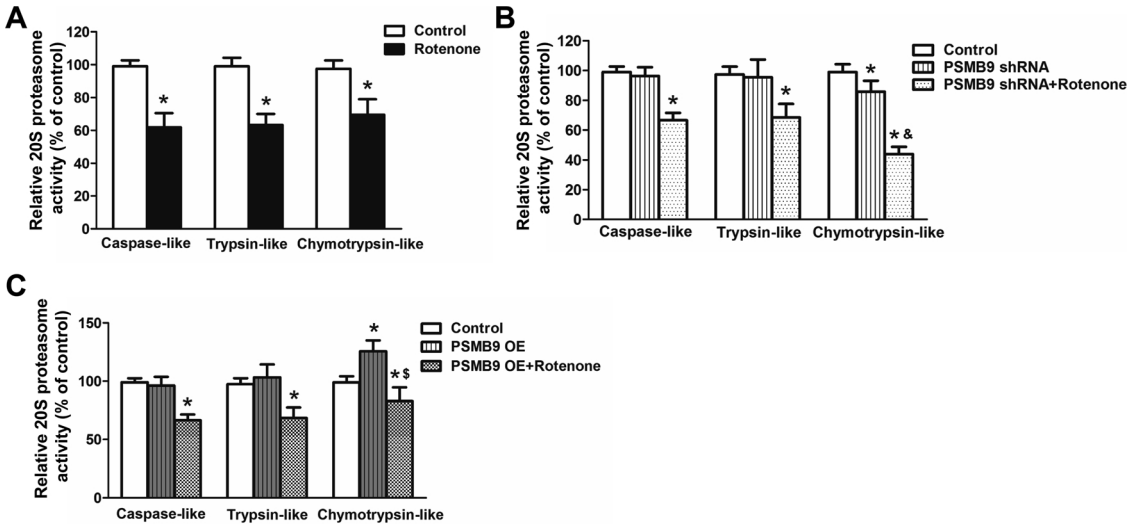

Fig. 4. Rotenone impaired the 20S proteasome activities in SH-SY5Y cells, and the chymotrypsin-like activity was regulated by PSMB9. The caspase-like activity, trypsin-like activity and chymotrypsin-like activity were detected through fluorogenic substrate in cells treated with vehicle or 500 nM rotenone for 24 h (A) after PSMB9 knockdown or overexpression (OE) (B–C). Cells with neither transfection nor rotenone treatment served as control. The data was expressed as percentage relative to control group and presented as mean ± SD from three independent experiments. $^{*}P < 0.05$ compared to the control group. $^{\&}P < 0.05$ compared to the PSMB9 knockdown alone group. $^{\$}P < 0.05$ compared to the PSMB9 overexpression alone group.

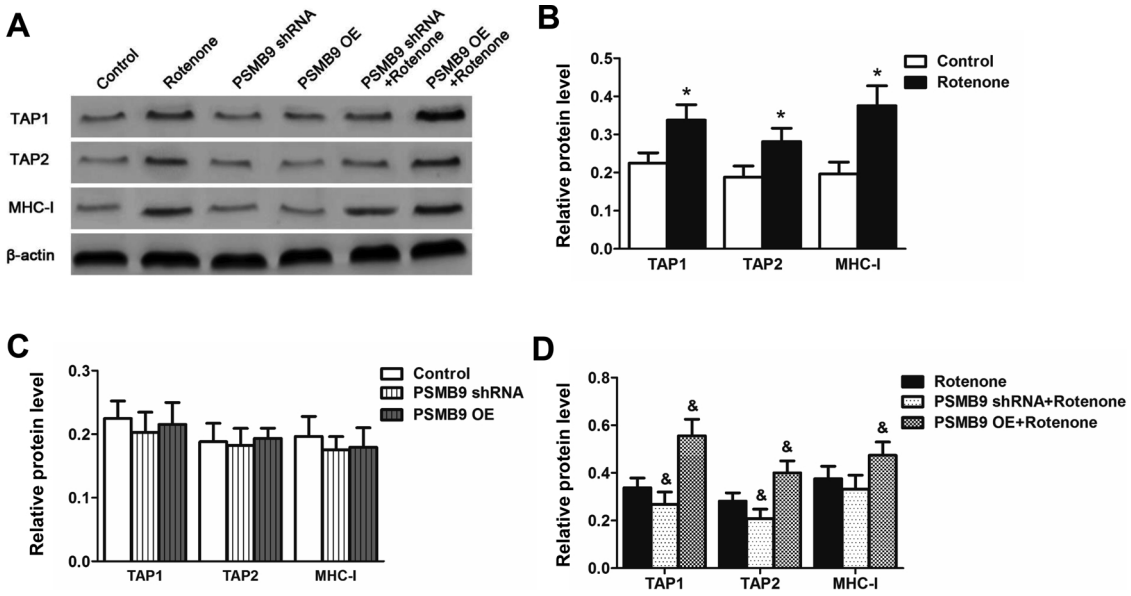

Fig. 5. Rotenone induced antigen presentation related proteins expression in SH-SY5Y cells, which could be modulated by PSMB9. TAP1, TAP2 and MHC-I protein expressions were determined by Western blot (A) in cells treated with vehicle or 500 nM rotenone for 24 h (B) after PSMB9 knockdown or overexpression (OE) (C–D). Cells with neither transfection nor rotenone treatment served as control. The relative band intensities of TAP1, TAP2 and MHC-I were normalized to the expression of β-actin. Data was presented as mean ± SD. $^{*}P < 0.05$ compared to the control group. $^{\&}P < 0.05$ compared to the rotenone alone treatment group.

($P < 0.05$, compared with rotenone alone group, respectively) (Fig. 5D). These results indicate that PSMB9 regulate the antigen presentation related proteins induced by rotenone in SH-SY5Y cells

3.6. PSMB9 attenuates rotenone-induced accumulation of α-syn and loss of TH

The locations of βi subunits of i-proteasome and α-syn were observed through immunofluorescence. Three active subunits (β1i PSMB9, β2i PSMB10 and β5i PSMB8) of i-proteasome were seldom expressed in cells under normal conditions (Fig. S2). However, after treated with rotenone, they were expressed and all co-localized with α-syn (Fig. 6A). The expression of α-syn and TH were further assessed by Western blot (Fig. 6B). Exposing to rotenone led to obvious upregulation of α-syn along with dereased expression of TH ($P < 0.05$, compared with controls, Fig. 6B, C). PSMB9 knockdown or over-expression had no obvious effect on the expression of α-syn or TH under normal condition (Fig. 6D). But when cells were exposed to rotenone, PSMB9 knockdown resulted in further increased expression of α-syn and degradation of TH ($P < 0.05$, compared with rotenone alone group, respectively), while over-expression of PSMB9 could alleviate these changes efficently ($P < 0.05$, compared with rotenone alone group, respectively) (Fig. 6E). This suggests that PSMB9 could protect cells from rotenone toxicity, preventing the accumulation of α-syn and loss of TH.

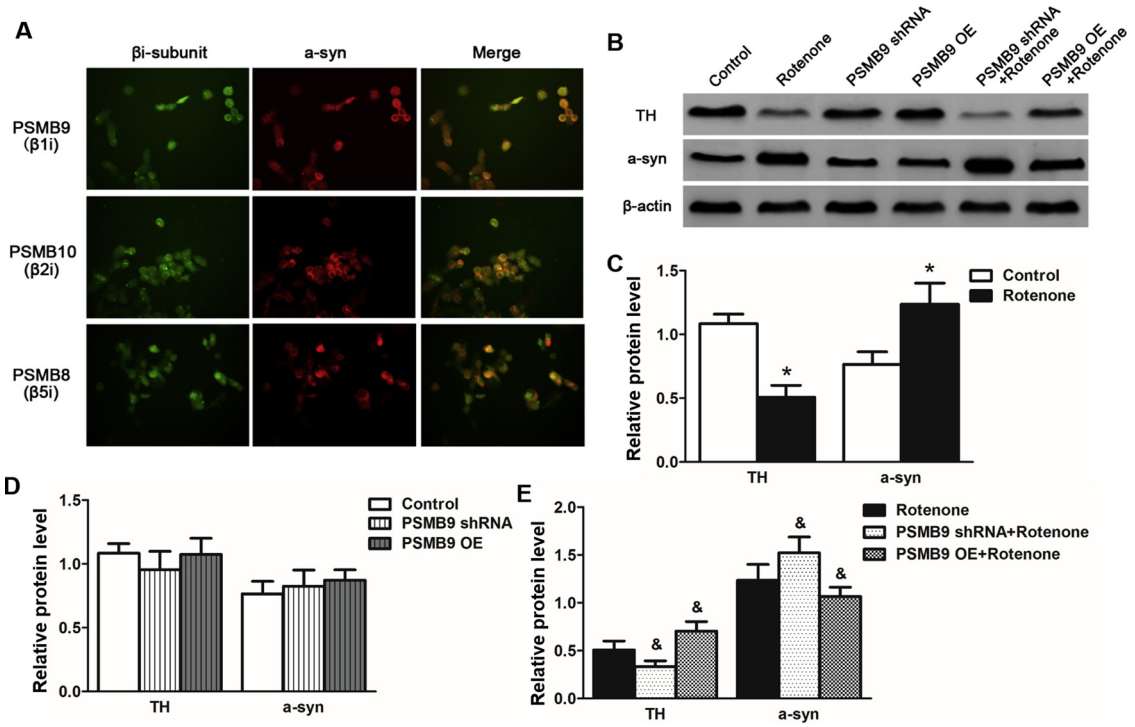

Fig. 6. PMSB9 alleviated the accumulation of α-syn and loss of TH in SH-SY5Y cells induced by rotenone. (A) The distribution of i-proteasome was examined by immunofluorescence, showing that PSMB9 (β1i), PSMB10 (β2i) and PSMB8 (β5i) (green) were all co-localized with α-syn (red). All images were captured by a fluorescence microscope (× 400). (B) The expression of α-syn and TH were detected by Western blot in cells treated withvehicle or 500 nM rotenone for 24 h (C) after PSMB9 knockdown or overexpression (OE) (D–E). Cells with neither transfection nor rotenone treatment served as control. The relative band intensities of α-syn and TH were normalized to the expression of β-actin. Data was presented as mean ± SD. $^{*}P < 0.05$ compared to the control group. $^{\&}P < 0.05$ compared to the rotenone alone treatment group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.7. PSMB9 antagonizes rotenone-induced oxidative stress

To explore the antioxidation of i-proteasome, the levels of ROS, MDA and GSH were detected in cells treated with vehicle or rotenone after PSMB9 konckdown or overexpression. Rotenone induced the release of ROS, increased the level of MDA and decreased the level of GSH significantly ($P < 0.05$, compared with controls, respectively) (Fig. 7). PSMB9 konckdown exacerbated the oxidative stress damage induced by rotenone in SH-SY5Y cells. Compared with the rotenone treatment on non-interfered cells, PSMB9 knockdown caused a more release of ROS, a higher level of MDA and a lower level of GSH in cells ($P < 0.05$, respectively) (Fig. 7). On the contrary, overexpression of PSMB9 could effectively reduce the oxidative stress induced by rotenone. Compared with the rotenone treatment on non-interfered cells, PSMB9 overexpression caused a decreased release of ROS, a lower level of MDA and a higher level of GSH in cells ($P < 0.05$, respectively) (Fig. 7). This indicates that PSMB9 could antagonize the oxidative stress induced by rotenone.

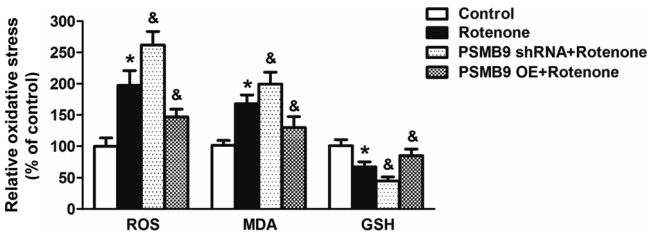

Fig. 7. PSMB9 alleviated oxidative stress in SH-SY5Y cells induced by rotenone. The levels of ROS, MDA and GSH were measured by commercially available kits in cells treated with vehicle or 500 nM rotenone for 24 h after PSMB9 knockdown or overexpression (OE). Cells with neither transfection nor rotenone treatment served as control. Data was presented as mean ± SD from three independent experiments. $^{*}P < 0.05$ compared to the control group. $^{\&}P < 0.05$ compared to the rotenone alone treatment group.

3.8. PSMB9 protects cells fromapoptosis induced by rotenone

To assess the effect of activated i-proteasome on cells with rotenone exposure, flow cytometry was performed to detect cell apoptosis. As shown in Fig. 8, the percentage of apoptosis cells in the control group was 3.97 ± 1.50%, while that in rotenone treated cells was 22.61 ± 3.94%. PSMB9 knockdown aggravated the rotenone induced apoptosis to 33.78 ± 4.86%, while PSMB9 overexpression could reduce the rotenone induced apoptosis to 12.50 ± 3.07%. PSMB9 knockdown or overexpression had no obvious effect on cell apoptosis in normal conditions. These results showed that PSMB9 could protect cells from apoptosis induced by rotenone efficiently.

4. Discussion

Consistent with previous reports (Jang et al., 2016; Pal et al., 2014), we found that rotenone could induce the production of ROS, decrease of GSH and increase of MDA. Oxidative stress promotes protein aggregate formation and aggravates the damage of ubiquitin-proteasome pathway (Bragoszewski et al., 2017). The ubiquitin–proteasome system is vital for regulating endogenous protein degradation in neurodegenerative diseases (Ross et al., 2015). In this study, obviously inhibited proteasome activitiy and increased degradation of proteasome subunits were detected in the high-dose rotenone-induced acute cell injury. The accumulation of α-syn and the the apoptosis of SH-SH5Y cells were observed at the same time. Proteasomes are the key protease complexes responsible for the ubiquitin-dependent degradation of proteins (Gu and Enenkel, 2014). Proteasome activity declines during aging, and proteasomal dysfunction is associated with late-onset disorders (Saez

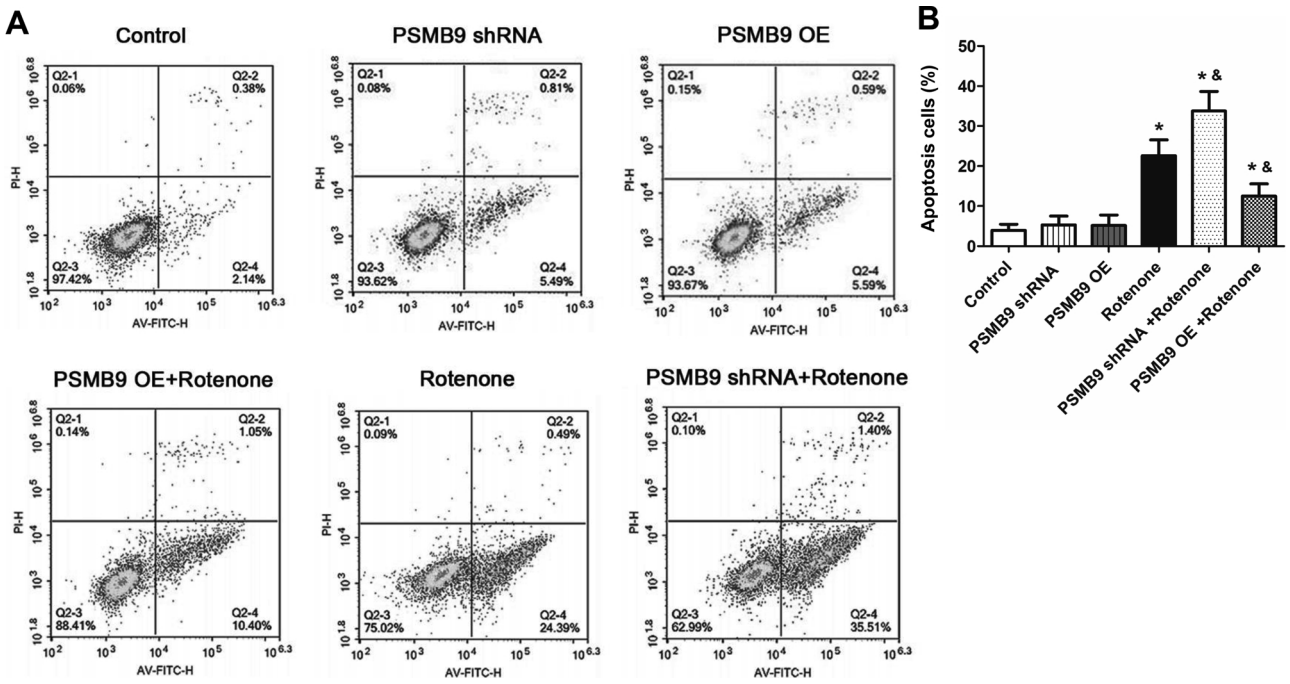

Fig. 8. PSMB9 prevented cell apoptosis in SH-SY5Y cells induced by rotenone. (A) Apoptosis in cells treated with vehicle or 500 nM rotenone for 24 h after PSMB9 knockdown or overexpression (OE) were determined by flow cytometry. Cells with neither transfection nor rotenone treatment served as control. (B) Data was presented as mean ± SD from three independent experiments. $^{*}P < 0.05$ compared to the control group. $^{\&}P < 0.05$ compared to the rotenone alone treatment group.

and Vilchez, 2014). The baseline of i-proteasome is low in non-immune tissues, but it can be significantly up-regulated when cells suffer inflammation or oxidative stress (Ferrington and Gregerson, 2012). The incresed expression of βi subunits of i-proteasome had been detected in cells with rotenone exposure in our trials. Assembly of the i-proteasome is more effective than that of the constitutive proteasome (Marques et al., 2009). The two subtypes of proteasome were coexisted in the SH-SY5Y cells with rotenone toxicity, which contributes to adapt the pathological conditions. However, such changes did not avert the impaired proteasomal activities. In this study, PSMB9 knockdown or overexpression did not affect other subunits expression, but it regulated the chymotrypsin-like activity of 20S proteasome positively. Deficiency of PSMB9 was not able to suppress all chymotrypsin-like activity because both PSMB9 and PSMB8 have chymotrypsin-like activity. It is reported that the i-proteasome actually degrades oxidized proteins with an activity and selectivity equal to that of the constitutive proteasome (Nathan et al., 2013). Proteasome isoforms exhibit only quantitative differences in cleavage and epitope generation (Ferrington and Gregerson, 2012). There is a general agreement that the i-proteasome has increased tryptic and chymotryptic activities, but reduced post glutamyl peptide hydrolyzing activities for peptides compared with the constitutive proteasome (Schröter and Adjaye, 2014). Integrated beta subunits is essential to maximize the functions of i-proteasome (Huber et al., 2012). PSMB9 knock-out mice had reduced proteasome activities and increased levels of oxidatively damaged proteins in brain (Ding et al., 2006). We preliminarily estimated that cleavage sites existed in the sequence of α-syn based on the models specific to predict the cleavage sites by the i-proteasome (Diez-Rivero et al., 2010). In this study, the immunofluorescence staining showed that three beta subunits of i-proteasome all co-localized with α-syn. PSMB9 knockdown before exposing to rotenone led to aggravated oxidative stress, upregulation of α-syn, loss of TH and eventualy promoted cell apoptosis. By contrary, PSMB9 overexpression before exposing to rotenone could protect cells through againsting oxidative damage and attenuating α-syn accumulation.

A recent study has reported that the i-proteasome degrades α-syn aggregates and generates potentially antigenic peptides, which is a potential effective pathway for elimination of α-syn aggregates (Ugras et al., 2018). The aggregated α-syn with prion-like ability produces spreading pathology and activates both the innate and adaptive immune systems in PD (Allen Reish and Standaert, 2015; Peelaerts et al., 2018). The primary role of the i-proteasome is to process antigens for the presentation on MHC I to CD8 + T lymphocytes (McCarthy and Weinberg, 2015). Short peptides processed by the i-proteasome translocate to the endoplasmic reticulum through TAP-1 and TAP-2, where it is loaded onto the MHC-I complexes and then transported to the cell surface for recognition by CD8 + T cells as part of immune surveillance (Thomas and Tampe, 2017). The peptides presented on MHC-II molecules are primarily derived from the degradation of exogenous antigens (Forsyth and Eisenlohr, 2016). PSMB9, PSMB8, TAP1 and TAP2 genes are all mapped to the MHC class II region, and genetic polymorphisms in MHC-I and MHC-II region has a relevant role in the risk of PD among different racial types (Nalls et al., 2014; Witoelar et al., 2017). MHC-II immunolabeling microglia has long been shown in the substantia nigra of patients with PD (Kannarkat et al., 2013). However, only in recent years, MHC-I expression and antigen display in catecholaminergic neurons triggered by microglial activation or high cytosolic dopamine has been determined (Cebrian et al., 2014a, b). It is a remarkble discovery that explained why catecholaminergic neurons, a kind of non-immune cells, are susceptible to T-cell-mediated degeneration. In this study, a significantly increased expression of antigen related proteins TAP-1, TAP-2 and MHC-I in SH-SY5Y cells was observed under the rotenone treatment, implying that the antigen presentation pathways could be activated when i-proteasome is expressed. However, apart from TAP1 and TAP2, MHC-I were not exactly in line with the down- or up-regulation of PSMB9, which possibly because other pathways are also involved the activation of MHC molecules such as mitochondrial antigen presentation and endoplasmic reticulum stress (Matheoud et al., 2016; Osorio et al., 2018). As a study reported that T cells of PD patients could recognize α-synuclein peptides (Sulzer et al., 2017), we have reasons to believe that α-syn aggregates could be degraded by i-proteasome and then the peptides are presented to T-cells through MHC

molecules. The process would be proceeded individually on account of polymorphisms lie in the MHC and T cell receptor gene regions. Since i-proteasome degrades protein aggregates efficiently and provides optimal peptides to MHC complexes (McCarthy and Weinberg, 2015; Ugras et al., 2018), that can prevent the accumulation of protein as well as trigger the cell-killing effects of specific T lymphocytes (Cebrian et al., 2014b). It is worth noting that i-proteasome also expressed in microglias, and activated i-proteasome are related to inflammatory reactions (Chen et al., 2015; Wagner et al., 2017). Further researches are needed to determine whether the protective roles of the activated i-proteasome would be reserved *in vivo*.

In conclusion, the present study shows that the immunoproteasome is activated in SH-SY5Y cells following rotenone treatment. Our resluts also show that PSMB9 protects against rotenone induced toxicity by suppressing oxidative damage and α-syn accumulation.

Disclosure

None.

Acknowledgments

This work was supported by the National Natural Science Foundation of China [grant numbers 81501100, 81471292 and 81771373]; Shandong Provincial Natural Science Foundation, China [grant number BS2015YY04]; and the Projects of Medical and Health Technology Development Program in Shandong Province [grant number 2014WSB20030].

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2019.03.004.

References

Allen Reish, H.E., Standaert, D.G., 2015. Role of α-synuclein in inducing innate and adaptive immunity in Parkinson disease. J. Parkinsons Dis. 5, 1–19.
Bragoszewski, P., Turek, M., Chacinska, A., 2017. Control of mitochondrial biogenesis and function by the ubiquitin–proteasome system. Open Biol. 7, 170007.
Bukhatwa, S., Zeng, B.Y., Rose, S., Jenner, P., 2010. A comparison of changes in proteasomal subunit expression in the substantia nigra in Parkinson's disease, multiple system atrophy and progressive supranuclear palsy. Brain Res. 1326, 174.
Cebrian, C., Loike, J.D., Sulzer, D., 2014a. Neuronal MHC-I expression and its implications in synaptic function, axonal regeneration and Parkinson's and other brain diseases. Front. Neuroanat. 8, 114.
Cebrian, C., Zucca, F.A., Mauri, P., Steinbeck, J.A., Studer, L., Scherzer, C.R., et al., 2014b. MHC-I expression renders catecholaminergic neurons susceptible to T-cell-mediated degeneration. Nat. Commun. 5, 3633.
Chen, X., Zhang, X., Wang, Y., Lei, H., Su, H., Zeng, J., et al., 2015. Inhibition of immunoproteasome reduces infarction volume and attenuates inflammatory reaction in a rat model of ischemic stroke. Cell Death Dis. 6, e1626.
Díaz-Hernández, M., Hernández, F., Martín-Aparicio, E., Gómez-Ramos, P., Morán, M.A., Castaño, J.G., et al., 2003. Neuronal induction of the immunoproteasome in Huntington's disease. J. Neurosci. 23 (37), 11653–11661. https://doi.org/10.1523/JNEUROSCI.23-37-11653.2003.
Diez-Rivero, C.M., Lafuente, E.M., Reche, P.A., 2010. Computational analysis and modeling of cleavage by the immunoproteasome and the constitutive proteasome. BMC Bioinf. 11, 479.
Ding, Q., Martin, S., Dimayuga, E., Brucekeller, A.J., Keller, J.N., 2006. LMP2 knock-out mice have reduced proteasome activities and increased levels of oxidatively damaged proteins. Antioxid. Redox Signal. 8, 130.
Eskandari, S.K., Seelen, M.A.J., Lin, G., Azzi, J.R., 2017. The immunoproteasome: an old player with a novel and emerging role in alloimmunity. Am. J. Transplant. 17, 3033–3039.
Ferrington, D.A., Gregerson, D.S., 2012. Immunoproteasomes: structure, function, and antigen presentation. Prog. Mol. Biol. Transl. Sci. 109, 75–112.
Forsyth, K.S., Eisenlohr, L.C., 2016. Giving CD4+ T cells the slip: viral interference with MHC class II-restricted antigen processing and presentation. Curr. Opin. Immunol. 40, 123–129.
Goedert, M., Compston, A., 2018. Parkinson's disease - the story of an eponym. Nat. Rev. Neurol. 14, 57–62.
Gu, Z.C., Enenkel, C., 2014. Proteasome assembly. Cell. Mol. Life Sci. 71, 4729–4745.
Huber, E.M., Basler, M., Schwab, R., Heinemeyer, W., Kirk, C.J., Groettrup, M., et al., 2012. Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity. Cell 148, 727–738.
Jang, W., Kim, H.J., Li, H., Jo, K.D., Lee, M.K., Yang, H.O., 2016. The neuroprotective effect of erythropoietin on rotenone-induced neurotoxicity in SH-SY5Y cells through the induction of autophagy. Mol. Neurobiol. 53, 3812–3821.
Johnson, M.E., Bobrovskaya, L., 2015. An update on the rotenone models of Parkinson's disease: their ability to reproduce the features of clinical disease and model gene–environment interactions. Neurotoxicology 46, 101–116.
Johnston-Carey, H.K., Pomatto, L.C., Davies, K.J., 2015. The Immunoproteasome in oxidative stress, aging, and disease. Crit. Rev. Biochem. Mol. Biol. 51, 268–281.
Kannarkat, G.T., Boss, J.M., Tansey, M.G., 2013. The role of innate and adaptive immunity in Parkinson's disease. J. Parkinsons Dis. 3, 493–514.
Kaur, G., Batra, S., 2016. Emerging role of immunoproteasomes in pathophysiology. Immunol. Cell Biol. 94, 812–820.
Kimura, H., Caturegli, P., Takahashi, M., Suzuki, K., 2015. New insights into the function of the immunoproteasome in immune and nonimmune cells. J. Immunol. Res. 2015, 541984.
Marques, A.J., Palanimurugan, R., Matias, A.C., Ramos, P.C., Dohmen, R.J., 2009. Catalytic mechanism and assembly of the proteasome. Chem. Rev. 109, 1509–1536.
Matheoud, D., Sugiura, A., Bellemare-Pelletier, A., Laplante, A., Rondeau, C., Chemali, M., et al., 2016. Parkinson's disease-related proteins PINK1 and parkin repress mitochondrial antigen presentation. Cell 166, 314–327.
McCarthy, M.K., Weinberg, J.B., 2015. The immunoproteasome and viral infection: a complex regulator of inflammation. Front. Microbiol. 6, 21.
Mckinnon, C., Goold, R., Andre, R., Devoy, A., Ortega, Z., Moonga, J., et al., 2015. Prion-mediated neurodegeneration is associated with early impairment of the ubiquitin–-proteasome system. Acta Neuropathol. 131, 411–425.
Mishto, M., Bellavista, E., Santoro, A., Stolzing, A., Ligorio, C., Nacmias, B., et al., 2006. Immunoproteasome and LMP2 polymorphism in aged and Alzheimer's disease brains. Neurobiol. Aging 27, 54–66.
Mo, M.S., Huang, W., Sun, C.C., Zhang, L.M., Cen, L., Xiao, Y.S., et al., 2016. Association analysis of proteasome subunits and transporter associated with antigen processing on chinese patients with parkinson's disease. Chin. Med. J. (Engl). 129, 1053–1058.
Nalls, M.A., Nathan, P., Lill, C.M., Do CB, Hernandez D.G., Mohamad, S., et al., 2014. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. Nat. Genet. 46, 989–993.
Nathan, J.A., Spinnenhirn, V., Schmidtke, G., Basler, M., Groettrup, M., Goldberg, A.L., 2013. Immuno- and constitutive proteasomes do not differ in their abilities to degrade ubiquitinated proteins. Cell 152, 1184–1194.
Orre, M., Kamphuis, W., Dooves, S., Kooijman, L., Chan, E.T., Kirk, C.J., et al., 2013. Reactive glia show increased immunoproteasome activity in Alzheimer's disease. Brain 136, 1415–1431.
Osorio, F., Lambrecht, B.N., Janssens, S., 2018. Antigen presentation unfolded: identifying convergence points between the UPR and antigen presentation pathways. Curr. Opin. Immunol. 52, 100–107.
Pal, R., Monroe, T.O., Palmieri, M., Sardiello, M., Rodney, G.G., 2014. Rotenone induces neurotoxicity through Rac1-dependent activation of NADPH oxidase in SHSY-5Y cells. FEBS Lett. 588, 472–481.
Peelaerts, W., Bousset, L., Baekelandt, V., Melki, R., 2018. α-Synuclein strains and seeding in Parkinson's disease, incidental Lewy body disease, dementia with Lewy bodies and multiple system atrophy: similarities and differences. Cell Tissue Res. 373, 195–212.
Pickering, A.M., Lehr, M., Miller, R.A., 2015. Lifespan of mice and primates correlates with immunoproteasome expression. J. Clin. Invest. 125, 2059–2068.
Ross, J.M., Olson, L., Coppotelli, G., 2015. Mitochondrial and ubiquitin proteasome system dysfunction in ageing and disease: two sides of the same coin? Int. J. Mol. Sci. 16, 19458–19476.
Saez, I., Vilchez, D., 2014. The mechanistic links between proteasome activity, aging and age-related diseases. Curr. Genomics 15.
Schröter, F., Adjaye, J., 2014. The proteasome complex and the maintenance of pluripotency: sustain the fate by mopping up? Stem Cell Res. Ther. 5, 24.
Sulzer, D., Alcalay, R.N., Garretti, F., Cote, L., Kanter, E., Agin-Liebes, J., et al., 2017. T cells from patients with Parkinson's disease recognize α-synuclein peptides. Nature 546, 656–661.
Surmeier, D.J., Obeso, J.A., Halliday, G.M., 2017. Selective neuronal vulnerability in Parkinson disease. Nat. Rev. Neurosci. 18, 101–113.
Thomas, C., Tampe, R., 2017. Structure of the TAPBPR-MHC I complex defines the mechanism of peptide loading and editing. Science 358, 1060–1064.
Ugras, S., Daniels, M.J., Fazelinia, H., Gould, N.S., Yocum, A.K., Luk, K.C., et al., 2018. Induction of the immunoproteasome subunit Lmp7 links proteostasis and immunity in α-Synuclein aggregation disorders. Ebiomedicine.
Wagner, L.K., Gilling, K.E., Schormann, E., Kloetzel, P.M., Heppner, F.L., Krüger, E., et al., 2017. Immunoproteasome deficiency alters microglial cytokine response and improves cognitive deficits in Alzheimer's disease-like APPPS1 mice. Acta Neuropathol. Commun. 5, 52.
Witoelar, A., Jansen, I.E., Wang, Y., Desikan, R.S., Gibbs, J.R., Blauwendraat, C., et al., 2017. Genome-wide pleiotropy between parkinson disease and autoimmune diseases. JAMA Neurol. 74, 780–792.