Neurochemistry International 125 (2019) 127–135

Contents lists available at ScienceDirect

Neurochemistry International

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

Iron-induced oxidative stress contributes to α-synuclein phosphorylation and up-regulation via polo-like kinase 2 and casein kinase 2

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ARTICLE INFO

Keywords:
Parkinson's disease
Iron
α-synuclein
Phosphorylation
Oxidative stress

ABSTRACT

α-Synuclein plays a central role in synucleinopathies pathogenesis such as Parkinson's disease (PD). Phosphorylation is the most common and important protein modification linked to α-synuclein pathologies. There is mounting evidence suggested iron and α-synuclein are closely related in PD. We previously reported iron up-regulated α-synuclein mRNA levels and induced α-synuclein aggregation. In the present study, we aimed to investigate whether and how phosphorylation was involved in iron-induced α-synuclein regulations. The results showed that iron could induce pS129 α-synuclein (phosphorylation at Ser129) and α-synuclein upregulation in the substantia nigra of iron-overloaded rats and iron-treated SH-SY5Y cells, accompanied by the elevated levels of polo-like kinase 2 (PLK2) and casein kinase 2 (CK2). Over-expression of CK2 or PLK2 induced pS129 α-synuclein up-regulation and inhibitors of CK2 or PLK2 could suppress iron-induced α-synuclein phosphorylation. Antioxidant NAC could fully block iron-induced upregulation of CK2, PLK2 and pS129 α-synuclein levels, indicating oxidative stress plays a critical role in iron-induced α-synuclein phosphorylation. However, iron-induced α-synuclein up-regulation could only be partially blocked by CK2/PLK2 inhibitor or NAC. These findings demonstrate that iron-induced oxidative stress is largely responsible for α-synuclein phosphorylation and upregulation via CK2 and PLK2, and α-synuclein upregulation is not fully phosphorylation-dependent.

1. Introduction

Parkinson's disease (PD) is a common and adult-onset progressive neurodegenerative disorder, mainly affecting the population over the age of 65. PD is clinically characterized by rest tremor, bradykinesia, rigidity and postural instability. Neuropathological hallmarks of PD include the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the formation of Lewy bodies (LBs) (Jellinger, 2012; Spillantini et al., 1997). α-Synuclein is the main component of LBs, which has been found extensively in both familial and sporadic PD cases. The aberrant aggregation of α-synuclein is regarded as the crucial player in PD pathogenesis. Several post-translational modifications of α-synuclein have already been identified, including phosphorylation (Hasegawa et al., 2002), acetylation (Fauvet et al., 2012), truncation (Muntane et al., 2012), ubiquitination (Shimura et al., 2001), nitration (Giasson et al., 2000), glycosylation (Guerrero et al., 2013) and SUMO (Dorval and Fraser, 2006). Among these, phosphorylation is the most common and important protein modification linked to α-synuclein pathologies. Only 4% of the soluble, monomeric α-synuclein appears to be phosphorylated under physiological conditions *in vivo*, however, approximately 90% is phosphorylated at serine 129 (pS129 α-synuclein) in LB lesions (Anderson et al., 2006; Hasegawa et al., 2002). Mounting evidence suggests that the phosphorylated status of α-synuclein either promotes its aggregation or can be secondary to aggregates formation, while still not fully elucidated (Azeredo da Silveira et al., 2009; Chen and Feany, 2005; Oueslati et al., 2013; Smith et al., 2005).

In addition to α-synuclein aggregation in the affected neurons in PD, elevated iron in the region and the individual dopaminergic neurons were observed, although it is still under debate whether iron is a

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https://doi.org/10.1016/j.neuint.2019.02.016
Received 1 September 2018; Received in revised form 13 February 2019; Accepted 21 February 2019
Available online 22 February 2019
0197-0186/

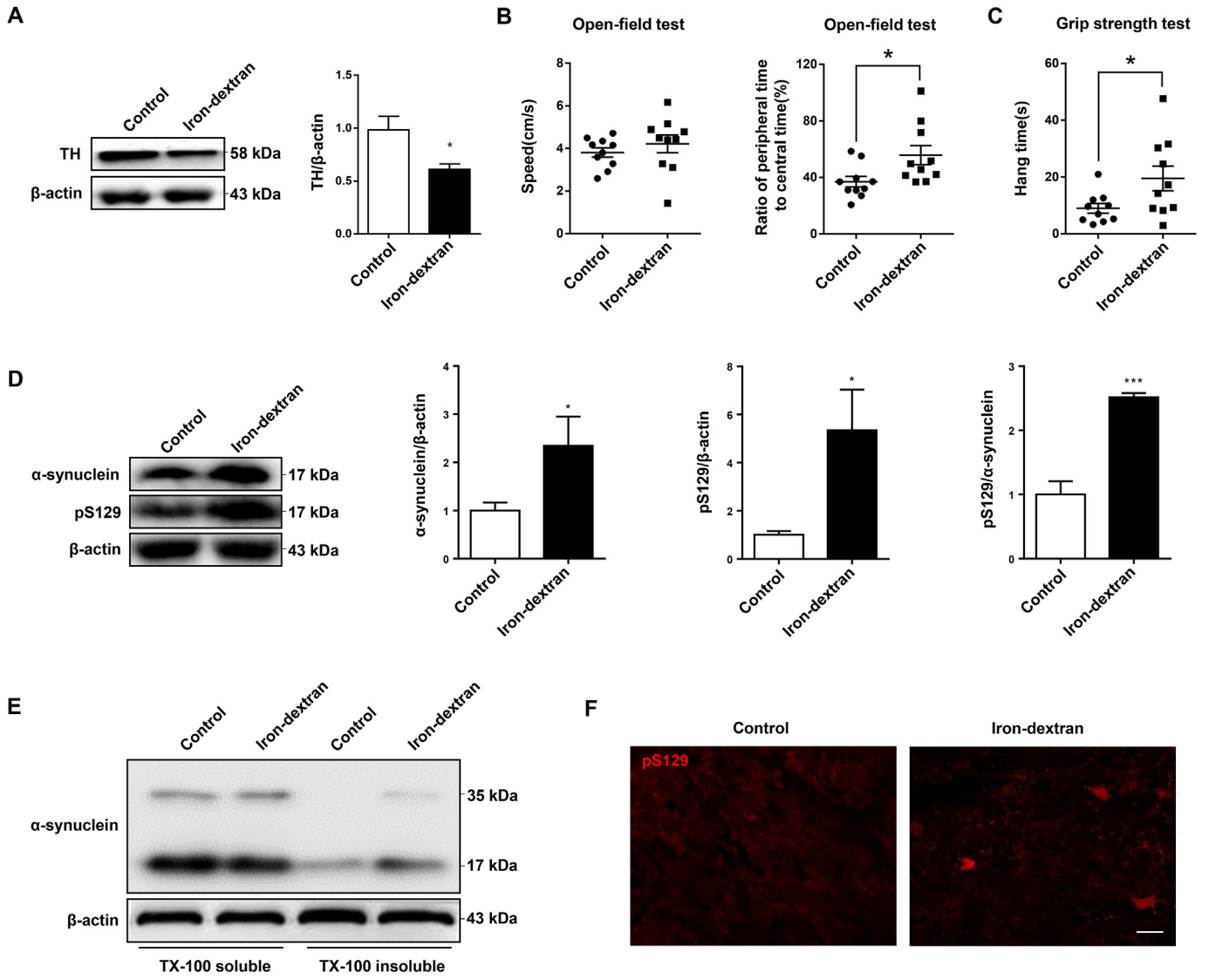

Fig. 1. Iron induced α-synuclein phosphorylation and upregulation *in vivo*. (A) Western blot analysis of TH expression in the SNpc of rats with iron-dextran administration for 4 weeks and controls. (B) Analysis of the average walking speed and the ratio of time spending in the peripheral zone to central zone in open-field test. (C) Grip strength test in each group. (D) Western blot analysis of α-synuclein and pS129 α-synuclein levels, as well as the ratio of pS129 α-synuclein to α-synuclein in each group. (E) Representative immunoblots of high-molecular-weight α-synuclein species in the TritonX-100 insoluble fraction in each group. (F) Immunofluorescence staining of pS129 α-synuclein in each group. Bar, 20 μm. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$, compared with the control group.

primary cause or just released from the degenerated neurons (Belaidi and Bush, 2016; Healy et al., 2017; Jiang et al., 2017; Zucca et al., 2017). As early as the beginning of this century, it was reported that LBs in the SNpc of PD cases showed strong iron (II or III) labeling; however, those in cases of LB variants of Alzheimer's disease were unstained (Castellani et al., 2000). This provided the powerful histological link of iron deposit and α-synuclein pathology in PD. The relationship between these two factors was then investigated by *in vitro* studies that iron caused α-synuclein aggregation and aggravated its toxicity (Ostrerova-Golts et al., 2000; Uversky et al., 2001). A direct interaction of iron metabolism and α-synuclein expression is achieved through (1) the conformational changes and fibril formation of α-synuclein combined with iron directly and ligand bridge formation (Kostka et al., 2008; Uversky, 2007); (2) a putative iron regulatory elements (IREs) in the 5′-untranslated regions (UTRs) of mRNA (Friedlich et al., 2007). We previously reported that iron up-regulated α-synuclein mRNA levels in SK-N-SH dopaminergic cells (Qu et al., 2018). Iron also was able to induce α-synuclein aggregation and up-regulation dependent on oxidative stress (Li et al., 2011). However, whether and how phosphorylation participates in this process is not clear.

In the present study, we set out to explore the phosphorylation (pS129 α-synuclein) and expression of α-synuclein in both iron-overloaded rats and SH-SY5Y cells. Two phosphorylation related kinases, polo-like kinase 2 (PLK2) and casein kinase 2 (CK2), which could mediate α-synuclein phosphorylation at Ser129 specifically (Inglis et al., 2009; Mbefo et al., 2010; Smith et al., 2005; Waxman and Giasson, 2008, 2011), were also investigated. We found that iron induced α-synuclein phosphorylation and up-regulation both *in vivo* and *in vitro*. Antioxidant N-acetylcysteine (NAC), as well as CK2 and/or PLK2 inhibitors could fully block α-synuclein phosphorylation, however, only partially block its up-regulation.

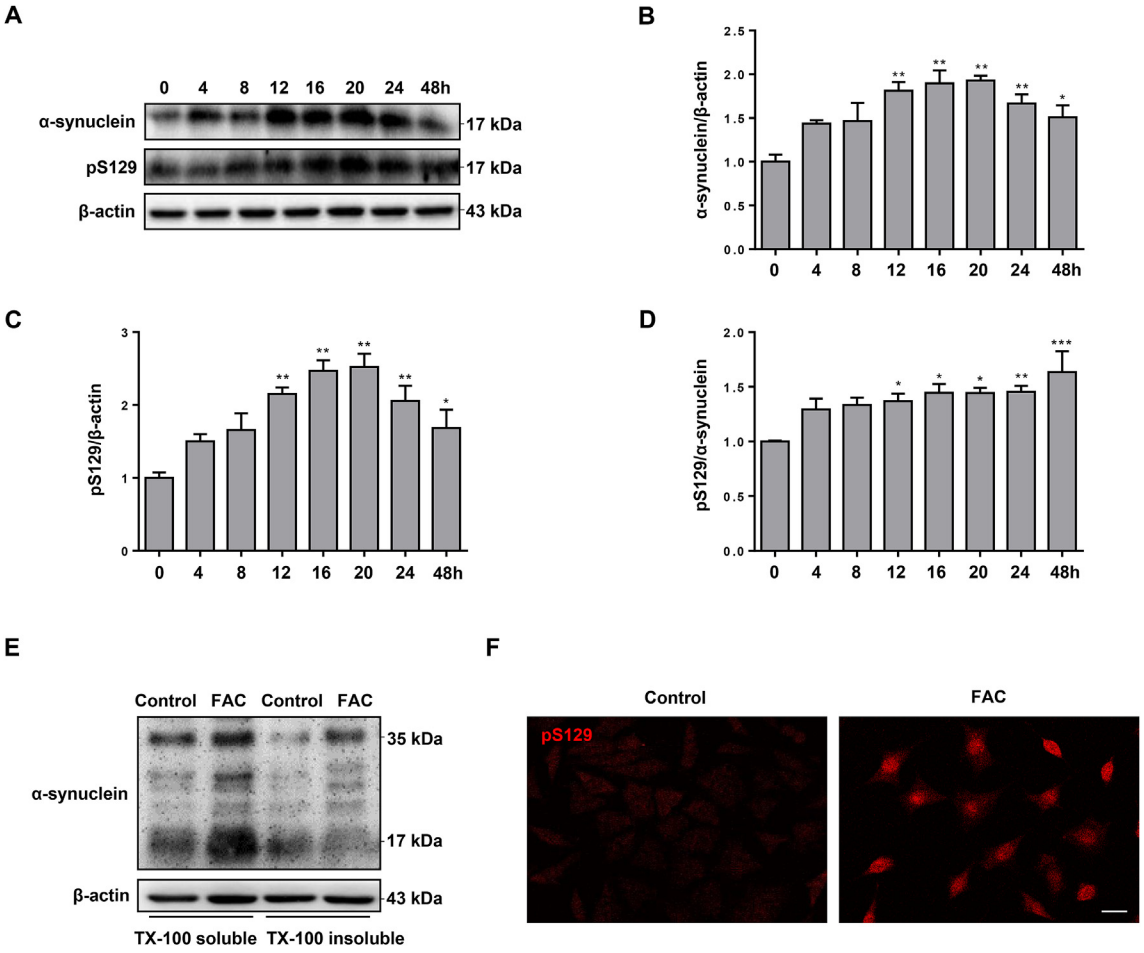

Fig. 2. Iron induced α-synuclein phosphorylation and upregulation *in vitro*. (A–D) Western blot analysis of α-synuclein and pS129 α-synuclein levels, as well as the ratio of pS129 α-synuclein to α-synuclein with treatment with FAC for different time. (E) Representative immunoblots of high-molecular-weight α-synuclein species in the TritonX-100 insoluble fraction in FAC and control groups. (F) Immunofluorescence staining of pS129 α-synuclein in FAC and control groups. Bar, 50 μm. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with 0 h group.

2. Materials and methods

2.1. Pharmacological agents

Iron-dextran, ferric ammonium citrate (FAC), N-Acetyl-L-cysteine (NAC), tetrabromocinnamic acid (TBCA), 3-Methyladenine (3-MA), MG-132, phosphatase Inhibitor Cocktail 2 and 3, primary antibodies rabbit anti-pS129 α-synuclein and tyrosine hydroxylase (TH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BI2536 was from Axon Medchem BV (Groningen, The Netherlands). Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (Ham, D-MEM/F-12), Opti-MEM® Reduced-Serum Medium and fetal bovine serum (FBS) were from Gibco (Grand Island, NY, USA). Lipofectamine™ 3000 was from Invitrogen (Carlsbad, CA, USA). The primary antibodies against α-synuclein, CK2 and p62 were obtained from Cell Signaling Technology (Danvers, MA, USA); rabbit anti-PLK2 was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA); rabbit anti-LC3 was from Novus Biologicals (Littleton, CO, USA) and rabbit anti-β-actin was from Bioss (Beijing, China). All other chemicals and regents were of the highest grade available from local commercial sources.

2.2. Animals and treatment

Animals were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Ethical Committee of the Medical College of Qingdao University. Healthy male Wistar rats were housed with a 12-h light-darkness cycle at room temperature (22 ± 2 °C) and with free access to water and standard food. Chronic iron-overloaded rats models were performed as previously described (Jiang et al., 2006, 2007; Legssyer et al., 2003; Marques et al., 2015; Sun et al., 2012). Briefly, young male Wistar rats weighing 70–80 g were given 10 mg/day of elemental iron dextran 3 days a week for 4 weeks to develop chronic iron overload. Rats in the control group were given intraperitoneal injections of equivalent volumes of saline accordingly.

2.3. Behavioral tests

2.3.1. Open-field test

The open-field test can be adapted to assess general behavior and locomotor activity in a novel environment. Rats were gently placed in the center of a dedicated black box (55 × 55 × 50 cm), and locomotor behavior was video-recorded for 10 min (Liu et al., 2018; Zhang et al., 2015). Average walking speed and percentages of time spent in each zone were calculated for each animal.

2.3.2. Grip strength test

Grip strength, used to assess limb rigidity, was measured by recording the time for the rats hanging on a steel wire. The wire was 2 mm in diameter and 35 mm in length and was suspended 50 cm above the horizontal surface of the ground (Liu et al., 2018). This test was performed three times and the average measurement was used.

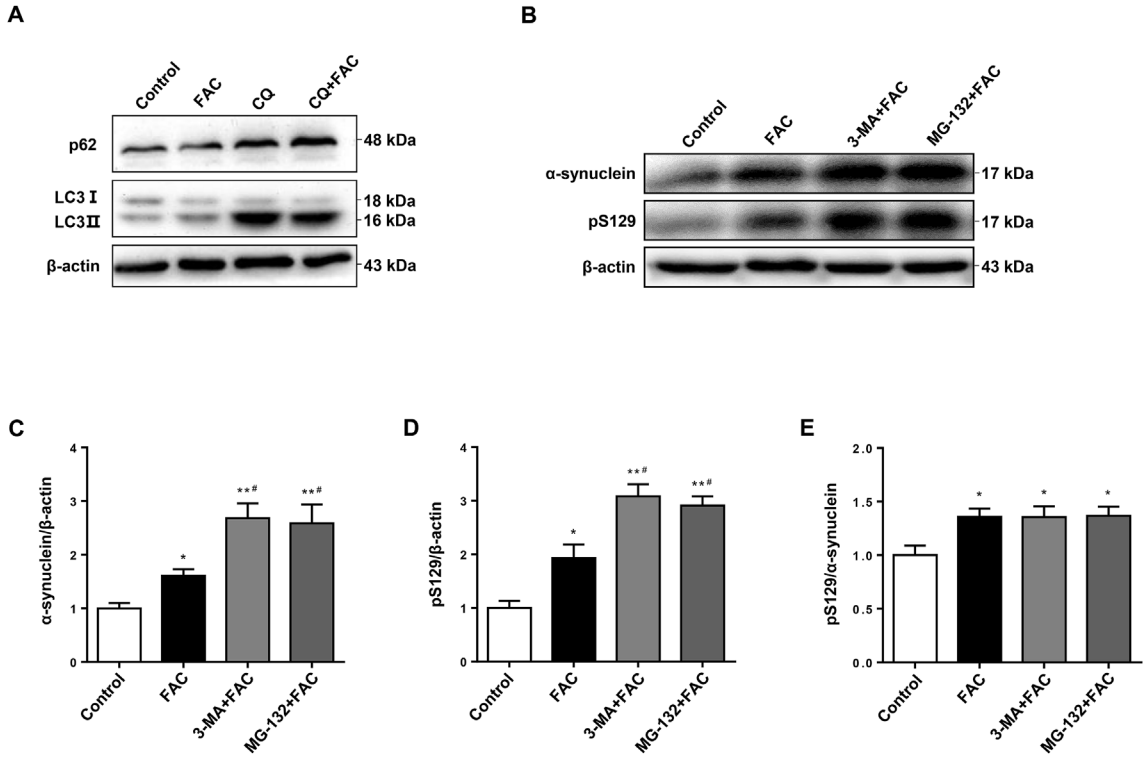

Fig. 3. Impaired degradation induced elevated α-synuclein and pS129 α-synuclein levels. (A) LC3 and p62 levels in FAC treated cells with or without CQ treatment. (B–E) Western blot analysis of α-synuclein and pS129 α-synuclein levels with autophagy or proteasome inhibitor, as well as the ratio of pS129 α-synuclein to α-synuclein in each group. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group. $^{\#}P < 0.05$, compared with the FAC group.

Observers were blinded to animal conditions.

2.4. Cell culture and treatment

SH-SY5Y dopaminergic cells were cultured at 37 °C and 5% atmospheric CO_2 in DMEM growth medium supplemented with 10% FBS and 1% penicillin/streptomycin 100 × solution. When grow to 80% confluence, cells were detached with 0.25% trypsin plus 0.02% EDTA, resuspended in DMEM with 10% FBS supplemented, and subcultivated into 6-well plastic plates for experiments. SH-SY5Y cells were co-incubated with 1 mmol/L FAC for 24 h with or without 0.5 mmol/L NAC, 20 μmol/L TBCA, 1 μmol/L BI2536, 5 mmol/L 3-MA, 10 μmol/L MG-132 or 30 μmol/L chloroquine (CQ) pretreatment for 30 min. To explore the role of CK2 and PLK2 overexpression on α-synuclein and pS129 α-synuclein levels, SH-SY5Y cells were transfected with CMV-MCS-3FLAG-SV40-Neomycin CSNK2A1(CK2) or CMV-MCS-3FLAG-SV40-Neomycin PLK2 (GENECHEM, Shanghai, China) at 70–80% confluence using Lipofectamine™ 3000 reagent according to the manufacturer's instructions, with CMV-MCS-3FLAG-SV40-Neomycin vector as a control group, and the samples were collected 24 h after transfection.

2.5. Western blotting

SNpc tissues dissected from the brain of rats and SH-SY5Y cells at the end of treatment were lysed with lysis buffer containing 50 mmol/L Tris (PH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and sodium orthovanadate, sodium fluoride, EDTA, leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and phosphatase inhibitors (25 mmol/L beta-glycerophosphate, phosphatase inhibitor cocktail 2 and 3). The harvested lysate was centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatant was used for analysis. Protein concentration was determined with a BCA protein assay kit (CWBIO, China). Samples were then prepared with loading buffer and denatured by incubating samples at 95 °C for 5 min. A total of 25 μg of protein was separated on SDS polyacrylamide gels and transferred onto PVDF membranes (Millipore, MA, USA). After being blocked with 10% (w/v) non-fat milk or 5% (w/v) BSA for 2 h at room temperature, membranes were incubated overnight at 4 °C with the primary antibodies against α-synuclein (1:800), pS129 α-synuclein (1:800), TH (1:1000), CK2 (1:800), PLK2 (1:800), LC3 (1:1000) or p62 (1:1000) and then probed with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Texas, USA) at a dilution of 1:10,000 for 2 h at room temperature. β-actin was detected by an anti-β-actin monoclonal antibody (1:10,000) using a similar procedure to confirm equal protein loading across samples. Cross-reactivity was visualized using ECL western blotting detection reagents (Millipore, USA) and then was analyzed through scanning densitometry by a UVP BioDoc-It Imaging System (UVP, Upland, USA).

2.6. α-Synuclein aggregation assay

Tissues dissected from the brain of rats and SH-SY5Y cells at the end of treatment were resuspended in ice-cold TritonX-100 lysis buffer (50 mmol/L Tris, pH 7.4, 175 mmol/L NaCl, 5 mmol/L EDTA, 1% TritonX-100 [Sigma, V900502], 1 mmol/L PMSF), and incubated on ice for 30 min. Samples were centrifuged at 100,000 x g for 30 min at 4 °C and the resulting supernatant fraction was deemed the TritonX-100-soluble fraction. The pellet was resuspended in lysis buffer containing 2% SDS and following sonication on ice was designated as the TritonX-100-insoluble fraction.

2.7. pS129 α-synuclein immunofluorescence staining

SN sections were stained with pS129 α-synuclein antibody. After three washes in 0.01% phosphate-buffered saline (PBS; pH 7.4) and blocked in 10% goat serum for 30 min, sections were incubated overnight with the primary antibody of pS129 α-synuclein (1:1000). Then washed three times with PBS and incubated in the second antibody of

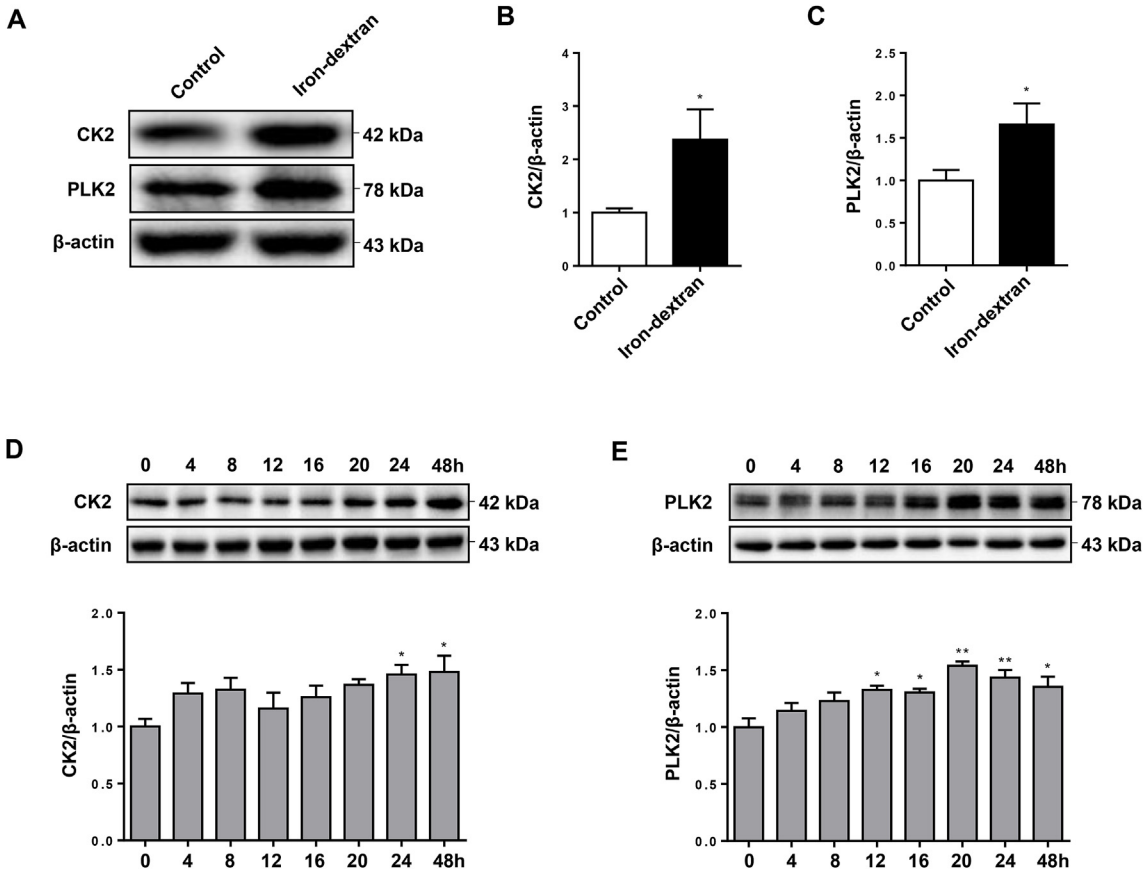

Fig. 4. Iron induced CK2 and PLK2 upregulation in iron-overloaded rats and SH-SY5Y cells. (A) Representative immunoblots of CK2 and PLK2 expression in the SNpc of rats with iron-dextran administration for 4 weeks and controls. (B, C) Quantitative determination of CK2 and PLK2 levels in each group. Data are presented as mean ± SEM. $^{*}P < 0.05$, compared with control. (D, E) Western blot analysis of CK2 and PLK2 expression at different time points in SH-SY5Y cells with FAC treatment. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with 0 h group.

Alexa Fluor ® 594 donkey anti-rabbit IgG for 3 h at room temperature. Then sections were mounted with 70% glycerin and examined using a fluorescence microscope (ZEISS, Germany).

Cells were treated with FAC for 24 h, followed by washing once with 0.01% PBS. All the steps were done at room temperature unless stated otherwise. Briefly, cells grown on glass coverslips were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Coverslips were blocked in 10% goat serum for 30 min. Cells were incubated in pS129 α-synuclein antibody (1:1000) in PBS overnight. After three washes in PBS, each for 10 min, secondary antibody in PBS was added for 2 h. The fluorescence signals were visualized with a confocal laser scanning microscope (Olympus, Japan).

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 6.0). All results were presented as mean ± SEM of at least three independent experiments. Unpaired *t*-test was used to compare differences between two groups. One-way analysis of variance (ANOVA) was used to compare differences between means in more than two groups supported by multiple comparisons, followed by Dunnet *t*-test. Results were considered significant when $P < 0.05$.

3. Results

3.1. Iron induced α-synuclein phosphorylation and upregulation in vivo

Chronic peripheral iron dextran overload was proved to induce iron deposit and dopaminergic neurons degeneration in the SNpc (Jiang et al., 2006, 2007; Sun et al., 2012). TH protein levels were decreased in chronic iron-dextran-treated rats, compared with control group (Fig. 1 A). We then examined effects of iron-dextran on motor functions. In the open-field test, although the speed recorded was unchanged in the iron dextran intoxicated rats, the animals spent more time in the periphery zone of the open field (Fig. 1 B). In the grip strength test, mean suspension time in the iron-overloaded group was significantly longer than that in the sham group, indicating the abnormality of limb rigidity (Fig. 1 C). These results indicated that iron-overload could induce motor dysfunction.

We analyzed the expression and phosphorylation levels of α-synuclein in the SNpc of rats. Results showed that iron induced significant increase of α-synuclein levels, pS129 α-synuclein levels, as well as the ratio of pS129 α-synuclein to α-synuclein in the SNpc of rats with iron-dextran administration for 4 weeks (Fig. 1 D). To confirm whether iron-dextran-treatment could induce α-synuclein aggregation, the presence of high molecular weight α-synuclein species was examined by western blotting. We used solubility in 1% TritonX-100 to separate soluble from insoluble forms. The accumulation of high molecular weight α-synuclein species was increased in the TritonX-100 insoluble fraction in iron-dextran-treated rats (Fig. 1 E). Immunofluorescence staining were applied to examine pS129 α-synuclein in the SNpc. Results showed that obvious pS129 α-synuclein aggregates were observed in chronic iron-dextran-treated rats and almost none pS129 α-synuclein aggregates could be observed in control group (Fig. 1 F).

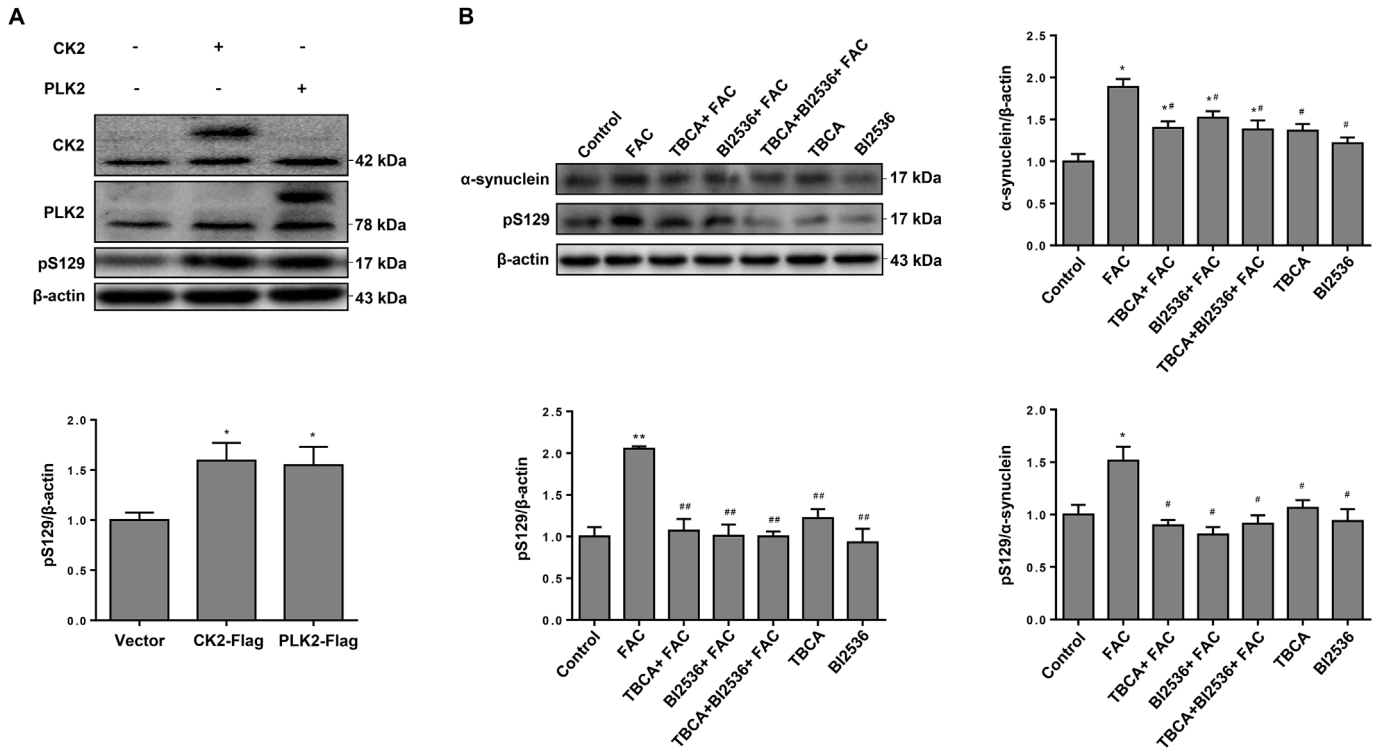

Fig. 5. Effects of CK2 and PLK2 on iron-induced α-synuclein phosphorylation in SH-SY5Y cells. (A) Western blot analysis of pS129 α-synuclein expression in SH-SY5Y cells with CK2 or PLK2 plasmid transfection. Data are presented as mean ± SEM. $^{*}P < 0.05$, compared with vector group. (B) Western blot analysis of α-synuclein and pS129 α-synuclein levels, as well as the ratio of pS129 α-synuclein to α-synuclein in each group. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the FAC group.

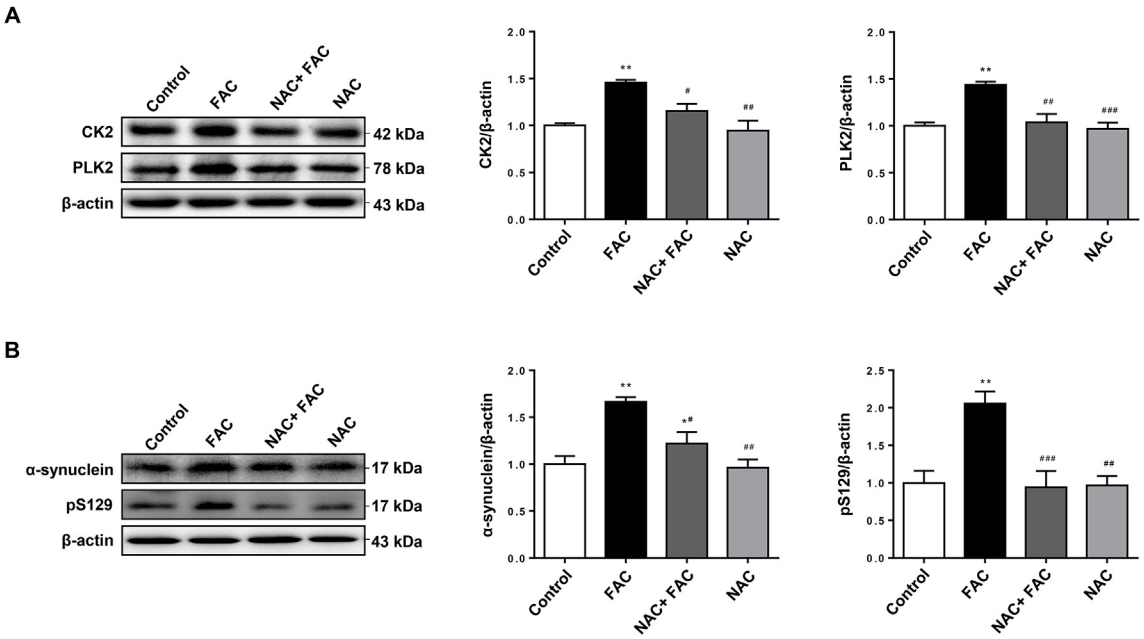

Fig. 6. NAC suppressed iron-induced CK2, PLK2, α-synuclein and pS129 α-synuclein upregulation in SH-SY5Y cells. (A) Western blot analysis of CK2 and PLK2 expression in FAC-treated SH-SY5Y cells with NAC pretreatment for 30 min. (B) Western blot analysis of α-synuclein and pS129 α-synuclein levels in each group. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the FAC group.

3.2. Iron induced α-synuclein phosphorylation, aggregation and upregulation in vitro

We then applied FAC, an iron overload reagent in SH-SY5Y cells. We previously reported 1 mmol/L FAC treatment for 24 h had minor effects on cell viability (> 90%), as tested by MTT analysis and could induce α-synuclein up-regulation and aggregation (Li et al., 2011). Therefore, it was chosen for the present study. α-Synuclein and pS129 α-synuclein levels in FAC-treated cells were detected at 4 h, 8 h, 12 h, 16 h, 20 h, 24 h or 48 h. α-Synuclein protein levels began to increase at 12 h and peaked at 20 h, although there was a tendency to increase at 4 h. A less extent up-regulation was observed at 24 h and 48 h (Fig. 2 A, B). pS129

α-synuclein levels showed a similar pattern at 12 h, 16 h, 20 h, 24 h and 48 h after FAC treatment (Fig. 2 A, C). However, the ratio of pS129 α-synuclein to α-synuclein in FAC-treated cells was consistently going up within 48 h (Fig. 2 D). These results indicated that iron could induce α-synuclein phosphorylation and up-regulation. We used western blotting to detect the presence of high molecular weight α-synuclein species in SH-SY5Y cells. The accumulation of high molecular weight α-synuclein species was increased in the TritonX-100 insoluble fraction in FAC-treated SH-SY5Y cells (Fig. 2 E). pS129 immunofluorescence staining was applied in SH-SY5Y cells. Results showed that obvious pS129 α-synuclein aggregates were observed in FAC-treated SH-SY5Y cells for 24 h, especially around the cellular nucleus. And almost none pS129 α-synuclein aggregates could be observed in control group (Fig. 2 F).

3.3. Impaired degradation contributed to elevated α-synuclein and pS129 α-synuclein levels

We detected LC3 and p62 levels in SH-SY5Y cells with FAC treatment for 24 h. CQ was used to inhibit autophagy and the results showed that FAC induced elevated ratio of LC3II/LC3I compared with control group, although co-administration with iron and CQ did not differ too much from the CQ treated group (Fig. 3 A). This is consistent with other reports that iron overload impaired autophagic flux and induced autophagy dysfunction (Fernandez et al., 2016; Wan et al., 2017). Then 5 mmol/L autophagy inhibitor 3-MA, as well as 10 μmol/L proteasome inhibitor MG-132 were applied and the results showed inhibition of degradation led to further increased α-synuclein and phosphorylated α-synuclein protein levels (Fig. 3 B–E).

3.4. CK2 and PLK2 were involved in iron-induced α-synuclein phosphorylation

We then investigated whether CK2 and PLK2 participated in iron-induced α-synuclein phosphorylation. Both CK2 and PLK2 levels were up-regulated in the SNpc of rats with iron-dextran administration for 4 weeks (Fig. 4 A–C). In FAC-treated SH-SY5Y cells, PLK2 showed a time-dependent up-regulation beginning at 12 h (Fig. 4 E). CK2 showed a tendency of up-regulation, however, only significant at 24 h and 48 h (Fig. 4 D). It seems there was an earlier response of PLK2 compared with CK2 in the iron-treated cells.

As we expected, transfection with CMV-MCS-3FLAG-SV40-Neomycin CSNK2A1(CK2) or CMV-MCS-3FLAG-SV40-Neomycin PLK2 in SH-SY5Y cells for 24 h induced pS129 α-synuclein up-regulation, indicating CK2/PLK2 up-regulation could trigger α-synuclein phosphorylation (Fig. 5 A).

To further elucidate the role of CK2 and PLK2 in α-synuclein phosphorylation induced by iron, SH-SY5Y cells were pretreated with 20 μmol/L TBCA (CK2 inhibitor) or 1 μmol/L BI2536 (PLK2 inhibitor) for 30 min and then co-incubated with 1 mmol/L FAC for 24 h. Although α-synuclein up-regulation in FAC-treated cells were partially inhibited with TBCA or BI2536 pretreatment, pS129 α-synuclein and the ratio of pS129 α-synuclein to α-synuclein up-regulation were fully inhibited with TBCA or BI2536 pretreatment (Fig. 5 B). The results indicated that CK2 or PLK2 inhibition could suppress iron-induced α-synuclein upregulation and phosphorylation.

3.5. Oxidative stress participated in iron-induced CK2/PLK2 upregulation and α-synuclein phosphorylation

Iron overload was supposed to elicit oxidative stress inside the cells. We then investigated whether PLK2/CK2 up-regulation was due to iron-induced pro-oxidant environment. Free radical scavenger NAC was applied 30 min before 1 mmol/L FAC incubation for 24 h. The results showed that NAC pretreatment fully blocked iron-induced upregulation of CK2 and PLK2 levels (Fig. 6 A). The upregulation of α-synuclein and pS129 α-synuclein induced by FAC was also suppressed with NAC pretreatment (Fig. 6 B). The results indicated that iron-induced oxidative stress was responsible for the kinases upregulation and thus α-synuclein phosphorylation.

4. Discussion

In the present study, we reported iron could induce α-synuclein and pS129 α-synuclein up-regulation *in vivo* and *in vitro*, accompanied by the elevated levels of CK2 and PLK2. Impaired degradation may contribute to the elevated α-synuclein and pS129 α-synuclein levels. Antioxidant NAC could fully block iron-induced kinases up-regulation and α-synuclein phosphorylation, which hinted that oxidative stress played a critical role in these processes. However, iron-induced α-synuclein up-regulation was not fully phosphorylation-dependent.

Aberrant expression and aggregation of α-synuclein is regarded as the crucial player in PD pathogenesis (Lawand et al., 2015). Several points mutations of α-synuclein, e.g. A53T, A30P, E46K, A53E, G51D and H50Q, were identified to be responsible for familial PD (Fredenburg et al., 2007; Ghosh et al., 2014; Khalaf et al., 2014; Kiely et al., 2013; Kruger et al., 1998; Zarranz et al., 2004). In sporadic PD without identified α-synuclein gene mutation, aggregated and phosphorylated α-synuclein were the main components in LBs and Lewy neuritis (LNs) in affected neurons. Meanwhile, elevated iron was consistently found in the degenerated SNpc of PD patients as well as animal models reviewed recently (Belaidi and Bush, 2016; Jiang et al., 2017; Zucca et al., 2017). We previously reported in the chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -treated PD mice, increased iron levels associated with α-synuclein up-regulation were observed only in the SNpc rather than ventral tegmental area (VTA), indicating iron and α-synuclein were involved in the selective degeneration of dopaminergic neurons in the SNpc. In the present study, we first adopted a peripheral iron dextran overload model, which was proved to induce iron deposit and dopaminergic neurons degeneration in the SNpc (Jiang et al., 2006, 2007; Sun et al., 2012). Iron overload induced significant α-synuclein up-regulation, together with pS129 α-synuclein up-regulation in the SNpc, suggesting iron promotes α-synuclein expression and phosphorylation *in vivo*. Aggregated pS129 α-synuclein was also observed in the SNpc of iron-dextran rats.

In vivo results were further supported by the *in vitro* results that FAC, an iron overload reagent, up-regulated α-synuclein expression and phosphorylation in SH-SY5Y cells. It is worth noting that the ratio of pS129 α-synuclein/α-synuclein showed a consistently time-dependent increase, although α-synuclein levels were actually at the peak at a later time, reinforcing phosphorylation is important to understand the process in iron-induced α-synuclein pathologies. Except for the possible effects on α-synuclein synthesis (Qu et al., 2018), we observed elevated ratio of LC3II/LC3I induced by iron; which might be due to the impaired autophagic flux. Therefore, the impaired degradation could not be excluded considering iron overload impaired autophagic flux and induced autophagy dysfunction (Fernandez et al., 2016; Wan et al., 2017).

Several kinases are involved in phosphorylation of α-synuclein. G protein-coupled receptor kinases (GRKs) (Hara et al., 2013; Pronin et al., 2000), CKs (Okochi et al., 2000; Waxman and Giasson, 2008) or PLKs (Inglis et al., 2009; Mbefo et al., 2010) were linked to phosphorylating α-synuclein at Ser129. In a rat model of familial PD, degeneration of dopaminergic neurons could be constrained by inhibiting the kinases that phosphorylate α-synuclein at Ser129, suggesting the roles of these kinases in α-synuclein pathologies (Sato et al., 2011). CK2 and PLK2 were most frequently investigated in the modulation of α-synuclein phosphorylation. Cellular toxin etoposide or proteasomal inhibition increased CK2 activity in SH-SY5Y cells, which results in elevated pS129 α-synuclein levels (Waxman and Giasson, 2008). On the contrary, CK2 inhibition decreased pS129 α-synuclein immunoreactivity in QBI293 cells (Waxman and Giasson, 2011). Heparin, a potent and relatively specific inhibitor of CK2, was able to inhibit

phosphorylation of α-synuclein in brain extracts (Ishii et al., 2007). PLK2 was regarded as an important kinase in α-synuclein phosphorylation at Ser129 both *in vivo* and *in vitro*. Except for this activity under physiological conditions (Bergeron et al., 2014), in brains of Alzheimer's disease and Lewy body disease patients increased PLK2 protein levels is accompanied by increased pS129 α-synuclein protein levels, which could further verify the effects of PLK2 on diseases (Mbefo et al., 2010). Recently, PLK2 is reported to mediate α-synuclein phosphorylation and degradation, thus capable of reducing α-synuclein protein expression and suppressing its toxicity (Dahmene et al., 2017; Oueslati et al., 2013). Highly selective inhibitors of PLK2 had suitable physicochemical and pharmacokinetic properties to reduce α-synuclein phosphorylation in rat brain (Aubele et al., 2013). In the present study, iron overload induced CK2 and PLK2 up-regulation both *in vivo* and *in vitro*. Inhibitors of these two kinases could fully block pS129 α-synuclein up-regulation in iron overloaded SH-SY5Y cells, whereas overexpression could trigger the phosphorylation, suggesting that iron-induced CK2 and PLK2 were responsible for the process of α-synuclein phosphorylation.

Excess iron induces oxidative stress via formation of hydroxyl radicals and this reinforces the pro-oxidant circumstances in at-risk dopaminergic neurons (Hare and Double, 2016). Oxidative damage, e.g. treatment with H_2O_2, increased both α-synuclein upregulation and α-synuclein phosphorylation (Guan et al., 2017; Smith et al., 2005). As phosphokinases important for α-synuclein phosphorylation, both CK2 and PLK2 could be induced by oxidative stress (Li et al., 2014; Takahashi et al., 2007). Transcription of the PLK2 gene is responsive to increased oxidative stress; accordingly, PLK2 protein levels were induced by oxidative stress. We applied NAC, which could fully scavenge iron generated reactive oxygen species (ROS), to explore whether oxidative stress participates in kinases upregulation and α-synuclein phosphorylation induced by iron. The fully blockages suggested iron-induced oxidative stress was crucial in these processes. Consistent with our previous results (Li et al., 2011), clearance of oxidative stress could not completely eliminated iron-induced α-synuclein up-regulation. The results further indicated that phosphorylation might be proceedings of increased α-synuclein expression rather than a compensatory response; considering inhibitions of the kinases could not fully block α-synuclein up-regulation.

In summary, our data demonstrate that iron overload could induce α-synuclein phosphorylation and expression via up-regulating CK2 and PLK2 levels. Oxidative stress is largely responsible for regulating these processes; and α-synuclein upregulation by iron is not fully phosphorylation-dependent. These results provide evidence for further elucidating the interaction between iron and α-synuclein and intervention strategies in PD.

Conflict of interests

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (81430024, 31871049, 31800893, 31771124), Excellent Innovative Team of Shandong Province and Taishan Scholars Construction Project.

References

Anderson, J.P., Walker, D.E., Goldstein, J.M., de Laat, R., Banducci, K., Caccavello, R.J., Barbour, R., Huang, J., Kling, K., Lee, M., Diep, L., Keim, P.S., Shen, X., Chataway, T., Schlossmacher, M.G., Seubert, P., Schenk, D., Sinha, S., Gai, W.P., Chilcote, T.J., 2006. Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. J. Biol. Chem. 281, 29739–29752.

Aubele, D.L., Hom, R.K., Adler, M., Galemmo Jr., R.A., Bowers, S., Truong, A.P., Pan, H., Beroza, P., Neitz, R.J., Yao, N., Lin, M., Tonn, G., Zhang, H., Bova, M.P., Ren, Z., Tam, D., Ruslim, L., Baker, J., Diep, L., Fitzgerald, K., Hoffman, J., Motter, R., Fauss, D., Tanaka, P., Dappen, M., Jagodzinski, J., Chan, W., Konradi, A.W., Latimer, L., Zhu, Y.L., Sham, H.L., Anderson, J.P., Bergeron, M., Artis, D.R., 2013. Selective and brain-permeable polo-like kinase-2 (Plk-2) inhibitors that reduce alpha-synuclein phosphorylation in rat brain. ChemMedChem 8, 1295–1313.

Azeredo da Silveira, S., Schneider, B.L., Cifuentes-Diaz, C., Sage, D., Abbas-Terki, T., Iwatsubo, T., Unser, M., Aebischer, P., 2009. Phosphorylation does not prompt, nor prevent, the formation of alpha-synuclein toxic species in a rat model of Parkinson's disease. Hum. Mol. Genet. 18, 872–887.

Belaidi, A.A., Bush, A.I., 2016. Iron neurochemistry in Alzheimer's disease and Parkinson's disease: targets for therapeutics. J. Neurochem. 139 (Suppl. 1), 179–197.

Bergeron, M., Motter, R., Tanaka, P., Fauss, D., Babcock, M., Chiou, S.S., Nelson, S., San Pablo, F., Anderson, J.P., 2014. *In vivo* modulation of polo-like kinases supports a key role for PLK2 in Ser129 alpha-synuclein phosphorylation in mouse brain. Neuroscience 256, 72–82.

Castellani, R.J., Siedlak, S.L., Perry, G., Smith, M.A., 2000. Sequestration of iron by Lewy bodies in Parkinson's disease. Acta Neuropathol. 100, 111–114.

Chen, L., Feany, M.B., 2005. Alpha-synuclein phosphorylation controls neurotoxicity and inclusion formation in a Drosophila model of Parkinson disease. Nat. Neurosci. 8, 657–663.

Dahmene, M., Berard, M., Oueslati, A., 2017. Dissecting the molecular pathway involved in PLK2 kinase-mediated alpha-Synuclein-selective autophagic degradation. J. Biol. Chem. 292, 3919–3928.

Dorval, V., Fraser, P.E., 2006. Small ubiquitin-like modifier (SUMO) modification of natively unfolded proteins tau and alpha-synuclein. J. Biol. Chem. 281, 9919–9924.

Fauvet, B., Fares, M.B., Samuel, F., Dikiy, I., Tandon, A., Eliezer, D., Lashuel, H.A., 2012. Characterization of semisynthetic and naturally Nalpha-acetylated alpha-synuclein *in vitro* and in intact cells: implications for aggregation and cellular properties of alpha-synuclein. J. Biol. Chem. 287, 28243–28262.

Fernandez, B., Fdez, E., Gomez-Suaga, P., Gil, F., Molina-Villalba, I., Ferrer, I., Patel, S., Churchill, G.C., Hilfiker, S., 2016. Iron overload causes endolysosomal deficits modulated by NAADP-regulated 2-pore channels and RAB7A. Autophagy 12, 1487–1506.

Fredenburg, R.A., Rospigliosi, C., Meray, R.K., Kessler, J.C., Lashuel, H.A., Eliezer, D., Lansbury Jr., P.T., 2007. The impact of the E46K mutation on the properties of alpha-synuclein in its monomeric and oligomeric states. Biochemistry 46, 7107–7118.

Friedlich, A.L., Tanzi, R.E., Rogers, J.T., 2007. The 5'-untranslated region of Parkinson's disease alpha-synuclein messengerRNA contains a predicted iron responsive element. Mol. Psychiatr. 12, 222–223.

Ghosh, D., Sahay, S., Ranjan, P., Salot, S., Mohite, G.M., Singh, P.K., Dwivedi, S., Carvalho, E., Banerjee, R., Kumar, A., Maji, S.K., 2014. The newly discovered Parkinson's disease associated Finnish mutation (A53E) attenuates alpha-synuclein aggregation and membrane binding. Biochemistry 53, 6419–6421.

Giasson, B.I., Duda, J.E., Murray, I.V., Chen, Q., Souza, J.M., Hurtig, H.I., Ischiropoulos, H., Trojanowski, J.Q., Lee, V.M., 2000. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. Science 290, 985–989.

Guan, H., Yang, H., Yang, M., Yanagisawa, D., Bellier, J.P., Mori, M., Takahata, S., Nonaka, T., Zhao, S., Tooyama, I., 2017. Mitochondrial ferritin protects SH-SY5Y cells against H2O2-induced oxidative stress and modulates alpha-synuclein expression. Exp. Neurol. 291, 51–61.

Guerrero, E., Vasudevaraju, P., Hegde, M.L., Britton, G.B., Rao, K.S., 2013. Recent advances in alpha-synuclein functions, advanced glycation, and toxicity: implications for Parkinson's disease. Mol. Neurobiol. 47, 525–536.

Hara, S., Arawaka, S., Sato, H., Machiya, Y., Cui, C., Sasaki, A., Koyama, S., Kato, T., 2013. Serine 129 phosphorylation of membrane-associated alpha-synuclein modulates dopamine transporter function in a G protein-coupled receptor kinase-dependent manner. Mol. Biol. Cell 24, 1649–1660 S1641-1643.

Hare, D.J., Double, K.L., 2016. Iron and dopamine: a toxic couple. Brain 139, 1026–1035.

Hasegawa, M., Fujiwara, H., Nonaka, T., Wakabayashi, K., Takahashi, H., Lee, V.M., Trojanowski, J.Q., Mann, D., Iwatsubo, T., 2002. Phosphorylated alpha-synuclein is ubiquitinated in alpha-synucleinopathy lesions. J. Biol. Chem. 277, 49071–49076.

Healy, S., McMahon, J.M., FitzGerald, U., 2017. Modelling iron mismanagement in neurodegenerative disease *in vitro*: paradigms, pitfalls, possibilities & practical considerations. Prog Neurobiol 158, 1–14.

Inglis, K.J., Chereau, D., Brigham, E.F., Chiou, S.S., Schobel, S., Frigon, N.L., Yu, M., Caccavello, R.J., Nelson, S., Motter, R., Wright, S., Chian, D., Santiago, P., Soriano, F., Ramos, C., Powell, K., Goldstein, J.M., Babcock, M., Yednock, T., Bard, F., Basi, G.S., Sham, H., Chilcote, T.J., McConlogue, L., Griswold-Prenner, I., Anderson, J.P., 2009. Polo-like kinase 2 (PLK2) phosphorylates alpha-synuclein at serine 129 in central nervous system. J. Biol. Chem. 284, 2598–2602.

Ishii, A., Nonaka, T., Taniguchi, S., Saito, T., Arai, T., Mann, D., Iwatsubo, T., Hisanaga, S., Goedert, M., Hasegawa, M., 2007. Casein kinase 2 is the major enzyme in brain that phosphorylates Ser129 of human alpha-synuclein: implication for alpha-synucleinopathies. FEBS Lett. 581, 4711–4717.

Jellinger, K.A., 2012. Neuropathology of sporadic Parkinson's disease: evaluation and changes of concepts. Mov. Disord. 27, 8–30.

Jiang, H., Luan, Z., Wang, J., Xie, J., 2006. Neuroprotective effects of iron chelator Desferal on dopaminergic neurons in the substantia nigra of rats with iron-overload. Neurochem. Int. 49, 605–609.

Jiang, H., Song, N., Wang, J., Ren, L.Y., Xie, J.X., 2007. Peripheral iron dextran induced degeneration of dopaminergic neurons in rat substantia nigra. Neurochem. Int. 51, 32–36.

Jiang, H., Wang, J., Rogers, J., Xie, J., 2017. Brain iron metabolism dysfunction in Parkinson's disease. Mol. Neurobiol. 54, 3078–3101.

Khalaf, O., Fauvet, B., Oueslati, A., Dikiy, I., Mahul-Mellier, A.L., Ruggeri, F.S., Mbefo, M.K., Vercruysse, F., Dietler, G., Lee, S.J., Eliezer, D., Lashuel, H.A., 2014. The H50Q mutation enhances alpha-synuclein aggregation, secretion, and toxicity. J. Biol. Chem. 289, 21856–21876.

Kiely, A.P., Asi, Y.T., Kara, E., Limousin, P., Ling, H., Lewis, P., Proukakis, C., Quinn, N., Lees, A.J., Hardy, J., Revesz, T., Houlden, H., Holton, J.L., 2013. alpha-Synucleinopathy associated with G51D SNCA mutation: a link between Parkinson's disease and multiple system atrophy? Acta Neuropathol. 125, 753–769.

Kostka, M., Hogen, T., Danzer, K.M., Levin, J., Habeck, M., Wirth, A., Wagner, R., Glabe, C.G., Finger, S., Heinzelmann, U., Garidel, P., Duan, W., Ross, C.A., Kretzschmar, H., Giese, A., 2008. Single particle characterization of iron-induced pore-forming alpha-synuclein oligomers. J. Biol. Chem. 283, 10992–11003.

Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L., Riess, O., 1998. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat. Genet. 18, 106–108.

Lawand, N.B., Saade, N.E., El-Agnaf, O.M., Safieh-Garabedian, B., 2015. Targeting alpha-synuclein as a therapeutic strategy for Parkinson's disease. Expert Opin. Ther. Targets 19, 1351–1360.

Legssyer, R., Geisser, P., McArdle, H., Crichton, R.R., Ward, R.J., 2003. Comparison of injectable iron complexes in their ability to iron load tissues and to induce oxidative stress. Biometals 16, 425–433.

Li, J., Ma, W., Wang, P.Y., Hurley, P.J., Bunz, F., Hwang, P.M., 2014. Polo-like kinase 2 activates an antioxidant pathway to promote the survival of cells with mitochondrial dysfunction. Free Radic. Biol. Med. 73, 270–277.

Li, W., Jiang, H., Song, N., Xie, J., 2011. Oxidative stress partially contributes to iron-induced alpha-synuclein aggregation in SK-N-SH cells. Neurotox. Res. 19, 435–442.

Liu, H., Jia, L., Chen, X., Shi, L., Xie, J., 2018. The Kv7/KCNQ channel blocker XE991 protects nigral dopaminergic neurons in the 6-hydroxydopamine rat model of Parkinson's disease. Brain Res. Bull. 137, 132–139.

Marques, V.B., Nascimento, T.B., Ribeiro Jr., R.F., Broseghini-Filho, G.B., Rossi, E.M., Graceli, J.B., dos Santos, L., 2015. Chronic iron overload in rats increases vascular reactivity by increasing oxidative stress and reducing nitric oxide bioavailability. Life Sci. 143, 89–97.

Mbefo, M.K., Paleologou, K.E., Boucharaba, A., Oueslati, A., Schell, H., Fournier, M., Olschewski, D., Yin, G., Zweckstetter, M., Masliah, E., Kahle, P.J., Hirling, H., Lashuel, H.A., 2010. Phosphorylation of synucleins by members of the Polo-like kinase family. J. Biol. Chem. 285, 2807–2822.

Muntane, G., Ferrer, I., Martinez-Vicente, M., 2012. alpha-synuclein phosphorylation and truncation are normal events in the adult human brain. Neuroscience 200, 106–119.

Okochi, M., Walter, J., Koyama, A., Nakajo, S., Baba, M., Iwatsubo, T., Meijer, L., Kahle, P.J., Haass, C., 2000. Constitutive phosphorylation of the Parkinson's disease associated alpha-synuclein. J. Biol. Chem. 275, 390–397.

Ostrerova-Golts, N., Petrucelli, L., Hardy, J., Lee, J.M., Farer, M., Wolozin, B., 2000. The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity. J. Neurosci. 20, 6048–6054.

Oueslati, A., Schneider, B.L., Aebischer, P., Lashuel, H.A., 2013. Polo-like kinase 2 regulates selective autophagic alpha-synuclein clearance and suppresses its toxicity *in vivo*. Proc. Natl. Acad. Sci. U. S. A. 110, E3945–E3954.

Pronin, A.N., Morris, A.J., Surguchov, A., Benovic, J.L., 2000. Synucleins are a novel class of substrates for G protein-coupled receptor kinases. J. Biol. Chem. 275, 26515–26522.

Qu, L., Xu, H., Jia, W., Jiang, H., Xie, J., 2018. Rosmarinic acid protects against MPTP-induced toxicity and inhibits iron-induced alpha-synuclein aggregation. Neuropharmacology 144, 291–300.

Sato, H., Arawaka, S., Hara, S., Fukushima, S., Koga, K., Koyama, S., Kato, T., 2011. Authentically phosphorylated alpha-synuclein at Ser129 accelerates neurodegeneration in a rat model of familial Parkinson's disease. J. Neurosci. 31, 16884–16894.

Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S., Selkoe, D.J., 2001. Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. Science 293, 263–269.

Smith, W.W., Margolis, R.L., Li, X., Troncoso, J.C., Lee, M.K., Dawson, V.L., Dawson, T.M., Iwatsubo, T., Ross, C.A., 2005. Alpha-synuclein phosphorylation enhances eosinophilic cytoplasmic inclusion formation in SH-SY5Y cells. J. Neurosci. 25, 5544–5552.

Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R., Goedert, M., 1997. Alpha-synuclein in Lewy bodies. Nature 388, 839–840.

Sun, C., Song, N., Xie, A., Xie, J., Jiang, H., 2012. High hepcidin level accounts for the nigral iron accumulation in acute peripheral iron intoxication rats. Toxicol. Lett. 212, 276–281.

Takahashi, M., Ko, L.W., Kulathingal, J., Jiang, P., Sevlever, D., Yen, S.H., 2007. Oxidative stress-induced phosphorylation, degradation and aggregation of alpha-synuclein are linked to upregulated CK2 and cathepsin D. Eur. J. Neurosci. 26, 863–874.

Uversky, V.N., 2007. Neuropathology, biochemistry, and biophysics of alpha-synuclein aggregation. J. Neurochem. 103, 17–37.

Uversky, V.N., Li, J., Fink, A.L., 2001. Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular NK between Parkinson's disease and heavy metal exposure. J. Biol. Chem. 276, 44284–44296.

Wan, W., Jin, L., Wang, Z., Wang, L., Fei, G., Ye, F., Pan, X., Wang, C., Zhong, C., 2017. Iron deposition leads to neuronal alpha-synuclein pathology by inducing autophagy dysfunction. Front. Neurol. 8, 1.

Waxman, E.A., Giasson, B.I., 2008. Specificity and regulation of casein kinase-mediated phosphorylation of alpha-synuclein. J. Neuropathol. Exp. Neurol. 67, 402–416.

Waxman, E.A., Giasson, B.I., 2011. Characterization of kinases involved in the phosphorylation of aggregated alpha-synuclein. J. Neurosci. Res. 89, 231–247.

Zarranz, J.J., Alegre, J., Gomez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B., Llorens, V., Gomez Tortosa, E., del Ser, T., Munoz, D.G., de Yebenes, J.G., 2004. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann. Neurol. 55, 164–173.

Zhang, Z., Du, X., Xu, H., Xie, J., Jiang, H., 2015. Lesion of medullary catecholaminergic neurons is associated with cardiovascular dysfunction in rotenone-induced Parkinson's disease rats. Eur. J. Neurosci. 42, 2346–2355.

Zucca, F.A., Segura-Aguilar, J., Ferrari, E., Munoz, P., Paris, I., Sulzer, D., Sarna, T., Dasella, L., Zecca, L., 2017. Interactions of iron, dopamine and neuromelanin pathways in brain aging and Parkinson's disease. Prog Neurobiol 155, 96–119.