Neuroprotective effect of treadmill exercise possibly via regulation of lysosomal degradation molecules in mice with pharmacologically induced Parkinson’s disease

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Abstract
Dysfunction of mitophagy, which is a selective degradation of defective mitochondria for quality control, is known to be implicated in the pathogenesis of Parkinson’s disease (PD). However, how treadmill exercise (TE) regulates mitophagy-related molecules in PD remains to be elucidated. Therefore, we aimed to investigate how TE regulates α-synuclein (α-syn)-induced neurotoxicity and mitophagy-related molecules in the nigro-striatal region of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mice. Our data showed that TE exhibited a significant restoration of tyrosine hydroxylase and motor coordination with suppression of α-syn expression, hallmarks of PD, possibly via up-regulation of lysosomal degradation molecules, LAMP-2 and cathepsin L, with down-regulation of p62, LC3-II/LC3-I ratio, PINK1 and parkin in the substantia nigra of MPTP mice. Therefore, these results suggest that treadmill exercise can be used as a non-invasive intervention to improve the pathological features and maintain a healthier mitochondrial network through appropriate elimination of defective mitochondria in PD.

Keywords Parkinson’s disease · α-Synuclein · Tyrosine hydroxylase · Treadmill exercise · PINK1/Parkin · Mitophagy · Lysosomal degradation

Introduction
Parkinson’s disease is a geriatric illness that shows neurobehavioral function defects resulting from specific neuronal degeneration of substantia nigra pars compacta (SNpc). The role of α-syn in cellular toxicity, which is a hallmark of PD, is unclear, but aberrant expression of α-syn is generally recognized to be toxic to dopaminergic neurons [1].

Many studies have suggested that a diverse range of molecular mechanisms, including mitochondrial dysfunction, the ubiquitin–proteasome system, aberrant autophagy, and oxidative stress contribute to the loss of dopaminergic neurons with aggregation of α-syn [2–4]. More detailed analyses reported that abnormal accumulation of α-syn could induce oxidative stress by disrupting mitochondria, resulting in neuronal cell death with accumulation of defective mitochondria [5, 6].

Mitophagy, defined as the selective degradation of dysfunctional mitochondria, exists to regulate cellular homeostasis. Interestingly, it has been discovered that α-syn-induced cellular toxicity might be associated with mitophagy [7], and overexpression of α-syn induces defective mitophagic clearance (mitophagic flux), preceding dopaminergic neuronal cell death [8]. In addition, the activation of mitophagy through diverse stimuli promotes neuroprotective effects [9] and attenuates mitochondrial dysfunction [10]. These results imply that the regulation of the
mitophagic process might contribute to a decrease in aberrant expression of α-syn, alleviating the progressive loss of dopaminergic neurons. However, the underlying mechanism of this phenomenon needs to be fully elucidated in PD.

Since most PD patients are only aware of the disease after clinical symptoms have appeared, the period of treatment is delayed. Physical exercise (PE), which is one of the strategies used against neurodegenerative diseases, including Alzheimer’s disease (AD), Huntington’s disease (HD) and PD, has been suggested as an effective intervention for prevention as well as treatment [11]. Moreover, it has been discovered that PE ameliorates pathological features such as α-syn, tyrosine hydroxylase (TH) and motor behavioral deficits in an animal model of PD [12].

More detailed research has shown that, as a protective mechanism for reversing the diverse pathological features, PE regulates the mitophagy process [13]. However, it still remains to be fully elucidated as to whether TE mitigates motor function and pathological features and how mitophagy-related molecules are regulated in PD. Therefore, this study aimed to investigate whether 8 weeks of treadmill exercise ameliorates motor behavioral deficits and pathological features through the regulation of mitophagy signaling molecules in the SN of MPTP-induced mouse model PD.

**Experimental methods**

**Animals**

All animal experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee at Korea National Sport University (KNSU-IACUC-2014-02). Thirty young adult (8-week-old) male C57BL/6 mice were purchased from Samtako (Osan, Korea). The mice were maintained at a 12:12 h dark–light cycle environment, housed at 22 ± 2 °C with 50% relative humidity, and had free access to standard chow diet (Purina Mills, Seoul, Korea) ad libitum.

**MPTP-induced mouse model of Parkinson’s disease**

To induce the mouse model of Parkinson’s disease, young adult (8-week-old) male C57BL/6 J mice were injected with a total of 10 doses of 25 mg/kg MPTP (Sigma-Aldrich, St. Louis, MO) and 250 mg/kg Probenecid (Sigma-Aldrich, St. Louis, MO) for 5 weeks by intraperitoneal injection. Then, we anesthetized the MPTP mice (n = 10) at the end of the 5 weeks of MPTP administration to provide clues to the pathogenesis of PD. The other ten control mice received the same dose of normal saline.

**Treadmill exercise**

At 13 weeks of age, MPTP-induced PD mice and the control mice were divided into one of the following groups until the age of 21 weeks: C57BL/6 mice with saline injection (CON, n = 10), MPTP mice in sedentary condition (MPTP, n = 10) and MPTP mice on treadmill exercise (MPTP-TE, n = 10). Standard laboratory cages (330 × 180 × 140 mm) were used for all mice. In addition, treadmill exercise was performed using a Rodent Treadmill (8 lanes, Dae-myung Scientific Co, Ltd, Korea). Pre-exercise was performed at 8 m/min, 30 min/day for 5 days to familiarize the mice with the treadmill-exercise environment. After this period, exercise protocol (6 m/min for the first 5 min, 9 m/min for the next 5 min, 12 m/min for the next 20 min, 15 m/min for the next 5 min, and 12 m/min for the last 5 min) was conducted 5 days a week at a gradient of 0° for 8 weeks [14, 15]. However, a sedentary group remained in their home cage throughout the course of the experiment.

**Rota-rod test for behavioral testing**

In this behavioral test, mice were made to walk on top of a rotating cylinder to evaluate their motor coordination function and sense of balance before and after the experiment. A Rota-rod (JD-A-07RA5, Jeung Do Bio & Plant Co, Ltd) was used to gradually increase the speed every 30 s, and the time when the test animal fell off was recorded. First, the mice were allowed to get acclimated to the rotating cylinder moving at 10 rpm for 120 s. Rota-rod tests were performed before and after 8 weeks of treadmill exercise. The speed was increased by 5–40 rpm and two sessions were measured to calculate the average. The maximum performance time was limited to 300 s.

**Brain tissue extraction and fixation**

All animal models were anesthetized by inhaling CO₂ gas, and then we extracted the brain tissue sample and separated the tissue (substantia nigra). All samples were stored at – 80 °C in a deep freezer (SANYO, Japan) until needed for biochemical assay. Extraction of brain tissues for immunohistochemical analysis proceeded as follows. After opening the thoracic cavity, 0.1 M phosphate buffer saline (PBS) and 4% paraformaldehyde (PFA) solution mixed in 0.1 M phosphate buffer solution were perfused through the left ventricle. Fixed brains were extracted and placed in 4% PFA solution for 12 h, and then it was precipitated for 5 straight days in 30% sucrose solution. Serial coronal
sections sliced into 40-μm-thick slices were made by using a freezing microtome (Leica, Nussloch, Germany).

**Isolation of brain mitochondria fraction**

Mitochondria were extracted by using a commercial Mitochondrial Extraction Kit (NOVUS, IMGENEX Corporation, San Diego, CA). Following the homogenization of brain tissue by using 1 ml of homogenizing buffer per 70 mg, it was centrifuged at 3000 rpm for 10 min at 4 °C. Extracted supernatant was again centrifuged at 12,000 rpm for 30 min at 4 °C. After that, the supernatant (cytosolic fraction) was extracted. The pellet left was centrifuged with 1 ml of 1× suspension buffer at 12,000 rpm for 10 min at 4 °C. After that, the pellet left after discarding the supernatant was mixed with 1 ml of suspension buffer, and again centrifuged at 12,000 rpm for 10 min at 4 °C. The pellet left following the elimination of supernatant was mixed with 1 ml of mitochondrial lysis buffer for 30 min at 4 °C. The supernatant (mitochondrial fraction) obtained by centrifuging the extracted supernatant (mitochondrial extract) at 12,000 rpm for 5 min at 4 °C was extracted.

**Western blotting**

After the protein sample was loaded into the stacking gel well in the Mini-Protein II dual-slab apparatus (Bio-Rad, CA, USA) with a standard marker (Page Ruler Pre-stained Protein Ladder #SM0671-Fermertas) at a concentration of 30 μg total protein, the protein sample was electrophoresed at 80 V. A polyvinylidene difluoride (PVDF) membrane (Immuno-Blot, PVDF, Bio-Rad, CA, USA) was reacted to induce membrane activation for 2 min. Proteins were transferred for 1 h at a constant voltage of 200 mA. Each membrane was blocked in 3% BSA, followed by incubation with the primary antibody: α-synuclein (BD bioscience, 1:1000); tyrosine hydroxylase (Millipore, 1:1000); PINK1 (Abcam, 1:1000); parkin (Abcam, 1:1000); p62 (Cell Signaling, 1:1000); LC3A/B (Abcam, 1:1000); LAMP2 (Santa Cruz, 1:1000); cathepsin L (Abcam, 1:1000); Tom 20 (Santa Cruz, 1:1000); β-actin (Santa Cruz, 1:1000) diluted in 3% BSA for 12 h at 4 °C. Following washing each membrane with 0.05% TBS-T solution, it was incubated with the secondary antibody: anti-α-synuclein (BD bioscience, USA) at 4 °C. The next day, tissue samples were incubated with the secondary antibody: biotinylated anti-rabbit IgG (H + L) (Vector laboratories, Inc, BA-1000, USA). Avidin–biotin–peroxidase complex (Vectastain-Elite ABC kit, Vector Laboratories) was used to react to the tissues at 37 °C. Brain tissues were visualized using DAB peroxidase substrate solution (SK-4100, Vector Laboratories, USA) for 3–5 min. Visualized tissue samples were mounted on gelatin-coated slide glass and immersed in 80, 90, 100% Et-OH for dehydration, and then cleared with 80, 90, 100% xylene. Finally, the slide glasses were covered using Permount medium and cover glasses. Images were observed using an optical microscope (Leica Microsystems, DM 2500, Germany).

**Immunohistochemistry**

In this study, brain tissue sections (40 μm) of an animal model were analyzed by using a free-floating method. Antigen retrieval was performed by boiling the sections in 0.01 M sodium citrate buffer at 67 °C for 1 h. Five percent normal donkey serum (2309032, Millipore, USA) was used to block non-specific bindings for 1 h at room temperature. The sections were then incubated overnight with the primary antibody: anti-TH (Millipore, AB152, Germany) at 4 °C. The next day, tissue samples were incubated with the secondary antibody: biotinylated anti-rabbit IgG (H + L) (Vector laboratories, Inc, BA-1000, USA). Avidin–biotin–peroxidase complex (Vectastain-Elite ABC kit, Vector Laboratories) was used to react to the tissues at 37 °C. Brain tissues were visualized using DAB peroxidase substrate solution (SK-4100, Vector Laboratories, USA) for 3–5 min. Visualized tissue samples were mounted on gelatin-coated slide glass and immersed in 80, 90, 100% Et-OH for dehydration, and then cleared with 80, 90, 100% xylene. Finally, the slide glasses were covered using Permount medium and cover glasses. Images were observed using an optical microscope (Leica Microsystems, TCS SP8, Germany).

**Immunofluorescence staining**

Brain samples (40 μm) of each group were washed out with 10 mM phosphate buffer saline (PBS). Antigen retrieval was performed by boiling the sections in 0.01 M sodium citrate buffer at 90 °C for 1 h. Ten percent normal donkey serum (2309032, Millipore, USA) was used to block non-specific bindings for 1 h at room temperature. The sections were then incubated overnight with the primary antibody: anti-α-synuclein (BD bioscience, USA) at 4 °C. The following day, tissue samples were incubated with the secondary antibody: Cy3-conjugated donkey anti-mouse (Jackson Immunocohemicals, West Grove, PA, USA). Analysis of results was conducted by using an immunofluorescence microscope (Leica Microsystems, TCS SP8, Germany).

**Detection and quantification of immunolabeled cells**

The counting of immunolabeled positive cells in response to the antibodies was carried out manually, and the results for all animals in the group were averaged. The definition of the area was based on the bregma point, referring to the...
Mouse Brain Atlas: C57BL/6 J (website; http://www.mbl.org/atlas170/atlas170_frame.html).

Statistical analysis

Data were analyzed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). All values are expressed as mean ± SEM. Statistical significance was determined using an independent t-test or a one-way ANOVA when comparing the groups. A least significant difference (LSD) post hoc test was followed for all pair-wise multiple comparisons if a statistically significant group main effect was found. Differences were considered to be statistically significant at α = 0.05. In addition, the results ( % of expression levels derived from each experiment) obtained by western blotting analysis (six mice per group) were used for correlation analysis.

Results

Treadmill exercise ameliorates motor behavioral deficits in MPTP mice

Figure 1 shows the results of the rota-rod test as behavioral assessment. MPTP mice showed significant reduction in the retention time compared with saline-treated control mice on the rota-rod test (independent t-test, t = 18.56, df = 28, p < 0.001, Fig. 1a). On the other hand, MPTP mice observed after treadmill exercise showed significant improvement in the retention time as compared to MPTP mice [LSD post hoc test, F(2,29) = 35.37, p < 0.001, one-way ANOVA, Fig. 1b], suggesting that treadmill exercise restores motor behavioral deficits while MPTP administration promotes motor coordination dysfunction.

Treadmill exercise suppresses α-syn expression in substantia nigra and striatum of MPTP mice

To determine whether treadmill exercise down-regulates the expression of α-syn protein in MPTP mice (since aggregation of α-syn is one of the neuropathological hallmarks of PD, being directly toxic to neurons [16]), we performed a western blot followed by immunofluorescence analysis. One-way ANOVA of α-syn data indicated significant effects for the groups (SN, F(2,17) = 21.18, p < 0.001). As shown in Fig. 2a, b, MPTP mice with treadmill exercise had a significantly lower level of α-syn than MPTP mice in the substantia nigra (SN). In addition, we analyzed substantia nigral and striatal α-syn immunoreactivity with higher magnification. As shown in Fig. 2c, immunolabeling with α-syn specific antibody revealed that the density of nigro-striatal α-syn in MPTP mice was notably increased whereas the density of nigro-striatal α-syn was decreased after treadmill exercise. We further analyzed the numbers of α-syn-positive cells (Fig. 2d, e). One-way ANOVA of α-syn-positive cells data indicated significant effects for the groups (SN, F(2,11) = 8.62, p < 0.01; STR, F(2,11) = 18.33, p < 0.001). MPTP mice with treadmill exercise had a significantly lower number of α-syn-positive cells than MPTP mice in the SN and STR, respectively.

Western blot and immunohistochemical analysis of tyrosine hydroxylase in the substantia nigra of MPTP mice after treadmill exercise

To determine whether treadmill exercise increases the expression of tyrosine hydroxylase (TH) protein as an enzyme converting into L-dopa in the dopaminergic system, we performed western blot followed by immunohistochemical analysis. One-way ANOVA of TH data indicated significant effects for the groups (F(2,17) = 138.51, p < 0.001). As shown in Fig. 3a, MPTP mice that were subjected to treadmill exercise had a higher expression level of TH than MPTP mice in the SN. Moreover, we analyzed TH immunoreactivity at a higher magnification. As shown in Fig. 3c, immunostaining with a TH specific antibody revealed that...
the density of TH in the SN region of MPTP mice was notably decreased whereas the density of TH was increased after treadmill exercise. We further analyzed the numbers of TH-positive neurons (Fig. 3d). One-way ANOVA of TH-positive neurons data indicated significant effects for the groups (SN, \( F_{(2,11)} = 23.38, p < 0.001 \)). MPTP mice with treadmill exercise had a significantly higher number of TH-positive neurons than MPTP mice in the SN.

The effect of treadmill exercise on the mitophagy-related molecules in substantia nigra of MPTP mice

We investigated whether or not the mitophagy-related factors are altered in MPTP mice and then additionally whether treadmill exercise induced a neuroprotective effect.
by regulating the mitophagic flux. One-way ANOVA of mitochondrial PINK1 and parkin data indicated significant effects for the groups (PINK1, $F_{(2,17)} = 7.87, p < 0.01$; parkin, $F_{(2,17)} = 5.63, p < 0.05$). As shown in Fig. 4a, c, d, MPTP mice which were subjected to treadmill exercise had a lower level of SN mitochondrial PINK1 and parkin than MPTP mice. In addition, one-way ANOVA of p62 expression level indicated significant effects for the groups (p62, $F_{(2,17)} = 10.38, p < 0.001$). As shown in Fig. 4b, e, f, MPTP mice with treadmill exercise had a significantly lower level of mitochondrial p62 than MPTP mice in the SN.

The effect of treadmill exercise on the lysosomal degradation process in the substantia nigra of MPTP mice

We investigated whether or not lysosomal degradation-related factors are altered in MPTP mice and then whether treadmill exercise would modulate the lysosomal degradation process. One-way ANOVA of LAMP2 and cathepsin L data indicated significant effects for the groups (LAMP2, $F_{(2,17)} = 9.68, p < 0.01$; cathepsin L, $F_{(2,17)} = 9.69, p < 0.01$). As shown in Fig. 5a–c, MPTP mice with treadmill exercise had a significantly higher level of SN mitochondrial LAMP2 and cathepsin L than MPTP mice. As shown in
Fig. 5d–e, the LAMP2 \( (r = -0.79, p < 0.001) \) and cathepsin L \( (r = -0.87, p < 0.001) \) levels were both negatively correlated with α-synuclein protein expression.

**Discussion**

The dysfunction of mitophagy, which is a selective degradation of defective mitochondria for quality control, has been reported to be one of the pathological mechanisms contributing to the pathogenesis of PD \[8, 9\]. PE as a protective methodology to reverse the various pathological situations is known to exhibit neuroprotective effects, possibly via regulation of mitophagy signaling \[13, 17\]. However, the underlying mechanisms of the exercise-induced neuroprotective effect involved in the regulation of mitophagy-related molecules remain largely unknown in PD. The present study demonstrated that TE restored motor function and loss of dopaminergic neurons in the SN of MPTP-induced mouse model of PD, accompanying the down-regulation of aberrantly expressed α-syn. In addition, we found that TE may prevent the accumulation of dysfunctional mitochondria by ameliorating the lysosomal degradation (i.e., mitophagic clearance).

To clarify the effect of TE on the neurological and behavioral deficits involved in PD, MPTP and probenecid was administered to mice, which is known to replicate the Parkinsonian syndrome in rodents and humans \[18, 19\]. In this study, the effects of TE on diverse pathological features of PD was evaluated by rotarod test (retention time) and immunoblot analysis, and our data revealed that TE restored the behavioral deficit and expression of TH with suppression of α-syn expression in the SN. These results are in agreement with previous reports \[20, 21\] and suggest that, as a result of exercise-induced decreased expression of α-syn, TE prevents neurotoxicity, mitigating the loss of dopaminergic neurons.

A number of studies has reported that mitophagy is mediated by complex mechanisms through interaction with mitochondrial fusion, fission \[22\] and that the proteins...
PTEN-induced kinase 1 (PINK1) and E3 ubiquitin ligase (parkin) play a fundamental role [23, 24]. In the present study, our novel findings revealed that, as shown by increased PINK1, parkin, p62, LC3-II/I ratio and decreased lysosomal degradation-related molecules such as LAMP2 and cathepsin L in the SN of MPTP mice were increased after treadmill exercise, β-actin was probed as an internal control. By contrast, TE resulted in down-regulation of PINK1, parkin and p62 protein expression with the improvement of molecules associated with the lysosomal degradation process. Consequently, our data suggest that the increase of mitophagy-related molecules in the MPTP group is not interpreted as an activation of excessive mitophagy signaling but could be interpreted as the accumulation of impaired mitochondria by interrupting the appropriate elimination of dysfunctional mitochondria or mitophagosomes [25–28].

There are many different interpretations of LC3 immunoblots that may exaggerate the effect of the autophagy-lysosomal process; however, the conversion of LC3-I to LC3-II is widely used in many studies as an indicator of phagophore formation in autophagy and/or mitophagy [30, 31]. Although we could not exclude the differences in our experimental approach, our results regarding the LC3-II/LC3-I expression ratio are in conflict with a previous study that reported an increase in LC3-II expression induced by exercise [13, 28]. Therefore, further investigations of these molecules in PD are needed.

Also, a limitation of our study is that we did not consider the effect of the skeletomuscular system affected by PE that could exhibit a neuroprotective effect in PD. It is well known that functional abnormalities of skeletal muscle as well as the nervous system exist in patients with PD, and mitochondrial dysfunction in muscle cells is one of the representative pathological features [31–34]. However, studies on skeletal...
muscle in PD are very limited. Therefore, subsequent studies on the aforementioned points should be conducted, and it would be very meaningful to study the connection and effect between the response of skeletal muscle and functional changes of the brain due to exercise adaptation in patients with PD.

In conclusion, our results, which require further confirmation, demonstrated that TE in a neurotoxin-induced mouse model effectively prevented motor function deficit, loss of dopaminergic neurons and increased α-syn expression, and that the TE may additionally produce neuroprotective effects by improving the lysosomal degradation process, forestalling the accumulation of impaired mitochondria in the SN. Taken together, regulating mitophagic clearance by exercise intervention may be one of the multi-faceted strategies of mitigating the progression of PD, with an improvement in pathological features.

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Author contributions DJH, JHK, and JYC conceived and designed the research; DJH, KCK, and CDH performed experiments; DJH, JHK, and JYC analyzed data and interpreted results of experiments; DJH, SDS, HSU, and JHJ prepared figures; DJH, JHK, HSU, and JYC edited and revised the manuscript; JYC approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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