Original Paper

Allicin Protects PC12 Cells Against 6-OHDA-Induced Oxidative Stress and Mitochondrial Dysfunction via Regulating Mitochondrial Dynamics

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Abstract

Background: Parkinson disease (PD) is a common adult-onset neurodegenerative disorder, and PD related neuronal injury is associated with oxidative stress and mitochondrial dysfunction. Allicin, the main biologically active compound derived from garlic, has been shown to exert various anti-oxidative and anti-apoptotic activities in *in vitro* and *in vivo* studies. **Methods:** The present study aimed to investigate the potential protective role of allicin in an *in vitro* PD model induced by 6-hydroxydopamine (6-OHDA) in PC12 cells. The protective effects were measured by cell viability, decreased lactate dehydrogenase (LDH) release and flow cytometry, and the anti-oxidative activity was determined by reactive oxygen species (ROS) generation, lipid peroxidation and the endogenous antioxidant enzyme activities. Mitochondrial function in PC12 cells was detected by mitochondrial membrane potential (MMP) collapse, cytochrome c release, mitochondrial ATP synthesis, and the mitochondrial Ca^{2+} buffering capacity. To investigate the potential mechanism, we also measured the expression of mitochondrial biogenesis factors, mitochondrial morphological dynamic changes, as well as detected mitochondrial dynamic proteins by western blot. **Results:** We found that allicin treatment significant increased cell viability, and decreased LDH release and apoptotic cell death after 6-OHDA exposure. Allicin also inhibited ROS generation, reduced lipid peroxidation and preserved the endogenous antioxidant enzyme activities. These protective effects were associated with suppressed mitochondrial dysfunction, as evidenced by decreased MMP collapse and cytochrome c release, preserved mitochondrial ATP synthesis, and the promotion of mitochondrial Ca^{2+} buffering capacity. In addition, allicin significantly enhanced mitochondrial biogenesis and prevented fragmentation of mitochondrial network after 6-OHDA treatment. The results of western blot analysis showed that the 6-OHDA induced decrease in the expression of optic atrophy type 1 (Opa-1), increase in mitochondrial fission 1

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(Fis-1) and dynamin-related protein 1 (Drp-1) were all partially revised by allicin. ***Conclusion:*** In summary, our data strongly suggested that allicin treatment can exert protective effects against PD related neuronal injury through inhibiting oxidative stress and mitochondrial dysfunction with dynamic changes.

Introduction

Parkinson's disease (PD) is a common adult-onset neurodegenerative disorder that affects more than 3% people over the age of 65 [1]. There are an estimated 1 million PD patients in the United States and tens of millions PD patients worldwide [2]. It is clinically characterized by the tetrad of motor manifestations of tremor, rigidity, slowness of voluntary movements, and poor balance. Despite the findings of many promising neuroprotective agents for PD based on laboratory studies in the past decades, nearly all of them failed when tested in clinical trials.

The characteristic pathogenesis of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the loss of dopamine input to the striatum [3, 4]. Although the precise mechanisms underlying neuronal injury in PD are still not fully elucidated, convincing evidence from *in vitro* and *in vivo* PD models and the analysis of human genetics showed that genetic factors, infections, immunological abnormalities, aging, oxidative stress, and toxins (endogenous and exogenous) might all be involved in the development of PD [5-7]. Among these factors, oxidative stress and impaired mitochondrial function have been considered to play important roles in PD development [8]. Many pharmacological compounds targeting neuronal oxidative stress and mitochondrial dysfunction have been shown to be candidate agents for PD treatment.

Garlic, also known as Allium sativum, has been used as a general food and a valuable folk medicine in oriental for a long time [9]. As a safe and economic option, garlic has been proposed as an effective herbal therapeutic, as it is a strong anti-oxidative and anti-inflammatory agent having beneficial effects on immune system [10, 11]. Allicin (diallyl thiosulfinate) is a major component of garlic and a precursor of many secondary products formed in aged garlic and crushed garlic preparations. It is produced during the crushing of garlic cloves resulting in a chemical interaction between the non-protein amino acid alliin and the enzyme allicinase [12]. Allicin became an object of interest due to its potential to confer several health beneficial effects, including anti-inflammatory, anti-microbial, anti-fungal, anti-parasitic, anti-hypertensive and anti-cancer activities. Previous published data indicated that allicin protects cells against oxidative stress by inducing the generation of antioxidant products, thereby reducing cytotoxic substances and scavenging free radicals [13]. Recently, researchers demonstrated that allicin exerts neuroprotective activities against traumatic or ischemic neuronal injury through regulating oxidative stress and apoptosis related cascades [14-16]. In this study, we aimed to examine the potential protective effects of allicin on 6-hydroxydopamine (6-OHDA) induced cytotoxicity in PC12 cells, a well characterized in vitro PD model, as well as the related underlying mechanisms with focus on oxidative stress and mitochondrial dysfunction.

Materials and Methods

Materials

Allicin (purity > 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco (Gaithersburg, MD, USA). The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kits were obtained from Promega (Madison, WI, USA). The LDH release kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The CXP cell quest software was obtained from Beckman-Coulter (Madison, WI, USA). The MDA assay kits were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The bicinchoninic acid (BCA) protein assay kits were obtained

from Pierce (Rockford, IL, USA). The primary Opa-1, Mfn-1, Drp-1 and Fis-1 antibodies were obtained from Cell Signaling Technology (Rockford, IL, USA). The fluorescent dye dichlorofluorescein diacetate (DCF-DA), rhodamine 123 (Rh123), mitochondrial tracker (Mitotracker), 6-OHDA and diamidino-phenyl-indole (DAPI) were purchased from Sigma (St. Louis, MO, USA).

Cell culture

PC12 cells were purchased from the Institute of Biochemistry and Cell Biology, SIBS, CAS. The cells were grown in DMEM plus 10% foetal bovine serum and 1% antibiotics (penicillin/streptomycin, Sigma) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The medium was changed every 2-3 days.

Cell viability assay

Cell viability assay was performed by using a 3-(4, 5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) that can be reduced to purple-coloured formazans by intact cells [17]. After various treatments, cell viability was assessed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, in accordance with the manufacturer's instructions. The absorbance was measured with an automatic microplate reader (Safire) at a wavelength of 492 nm. Results are presented as a percentage of the control.

Lactate dehydrogenase (LDH) assay

Cytotoxicity was determined by LDH release using a diagnostic kit according following the manufacturer's instructions. Briefly, 50 µl of supernatant from each well was collected to assay LDH release. The samples were incubated with reduced form of nicotinamide-adenine dinucleotid (NADH) and pyruvate for 15 min at 37°C and the reaction was stopped by adding 0.4 M NaOH. The activity of LDH was calculated from the absorbance at 440 nm and background absorbance from culture medium that was not used for any cell cultures was subtracted from all absorbance measurements. LDH release was defined as ratio of LDH in the media to total LDH and normalized to the fold of control.

Flow cytometry

Cultured PC12 cells were harvested 24 h after exposure to 6-OHDA, washed with ice-cold Ca^{2+} free phosphate buffered saline (PBS), and re-suspended in binding buffer. Cell suspension was transferred into a tube and double-stained for 15 min with Alexa Fluor 488-conjugated Annexin V (AV) and propidium iodide (PI) at room temperature in the dark. After addition of 400 µl binding buffer, the stained cells were analyzed by an FC500 flow cytometer with the fluorescence emission at 530nm and >575 nm. The CXP cell quest software was used to count the number of cells in B2 (AV^+/PI^+, the late phase apoptotic cells) and B4 (AV^+/PI^-, the early phase apoptotic cells), and analyzed the results.

Measurement of intracellular reactive oxygen species (ROS) production

Intracellular ROS levels were quantified using the H_2DCF-DA probe (Sigma). Briefly, PC12 cells were incubated with H_2DCF-DA (10 µM) for 1 h at 37°C in the dark, and then re-suspended in PBS. Intracellular ROS production was detected using the fluorescence intensity of H_2DCF-DA in an Olympus BX60 microscope and fluorescence was read using an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

Measurement of oxidative products

The MDA content was measured by a commercial kit (Ann Arbor, MI, USA). Protein carbonyl content was assayed as we previously described [18] using a commercial ELISA kit (Cell Biolabs, USA). All standards and samples were run in duplicate.

Measurement of anti-oxidant enzyme activity

The enzyme activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione S-transferase (GST) were measured according to the technical manual of the detection kits (Cayman Chemical, USA). Protein concentration was determined by using BCA protein kits. The enzyme activities were then normalized to the corresponding protein concentration for each group, and expressed as the percentage of control.

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Measurement of mitochondrial membrane potential (MMP)

MMP was measured using the fluorescent dye Rh123 as reported previously [19]. Rh123 was added to PC12 cells to achieve a final concentration of 10 μM for 30 min at 37°C after the cells had been treated and washed with PBS. The fluorescence was observed by using an Olympus BX60 microscope with the appropriate fluorescence filters (excitation wavelength of 480 nm and emission wavelength of 530 nm).

Quantification of cytochrome c release

Cytochrome c release into the cytoplasm was assessed after subcellular fraction preparation. PC12 cells were washed with ice-cold PBS for three times and lysed with a lysis buffer containing protease inhibitors (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 0.2 % deoxycholic acid and 1:100 protease inhibitor cocktail). The cell lysate was centrifuged for 10 min at 750 g at 4°C, and the pellets containing the nuclei and unbroken cells were discarded. The supernatant was then centrifuged at 15 000 g for 15 min. The resulting supernatant was removed and used as the cytosolic fraction. The pellet fraction containing mitochondria was further incubated with PBS containing 0.5% Trition X-100 for 10 min at 4°C. After centrifugation at 16 000 g for 10 min, the supernatant was collected as mitochondrial fraction. The levels of cytochrome c in cytosolic and mitochondrial fractions were measured using the Quantikine M Cytochrome C Immunoassay kit obtained from R&D Systems (Minneapolis, MN, USA). Data were expressed as ng/mg protein.

Measurement of ATP synthesis

Isolated mitochondria were utilized to measure ATP synthesis with a luciferase/ luciferin-based system as described elsewhere [20]. Thirty μg of mitochondria- enriched pellets were re-suspended in 100 μl of buffer A (150 mM KCl, 25 mM Tris-HCl, 2 mM potassium phosphate, 0.1 mM $MgCl_2$, pH 7.4) with 0.1 % bovine serum albumin (BSA), 1 mM malate, 1 mM glutamate and buffer B (containing 0.8 mM luciferin and 20 mg/ml luciferase in 0.5 M Tris-acetate pH 7.75). The reaction was initiated by addition of 0.1 mM ADP and monitored for 5 min using a microplate reader.

Measurement of mitochondrial calcium buffering capacity

Mitochondrial calcium buffering capacity was estimated with the Ca^{2+} sensitive Calcium Green 5N fluorescent dye. Incubation medium was composed of 125 mM KCl, 20 mM HEPES (pH 7.2), 2 mM KH_2PO_4, 2 mM $MgCl_2$, 5 mM succinate, 1 μM rotenone and 0.2 mM ADP, with 1 μg/mL oligomycin and 1 μM Calcium Green 5N. Bolus additions of $CaCl_2$ were made to the 60 μg of mitochondria in suspension in 30 nM increments and changes in Calcium Green 5N fluorescence were recorded at an emission of 532 nm.

Determination of mitochondrial DNA (mtDNA) content

Long fragment polymerase chain reaction (PCR) was used to quantify the relative abundance of intact mtDNA as previously described [21]. As an internal standard, rat DNA derived from normal rat spinal cord tissues was added to the PCR reaction mixture for each sample. The primers used for the amplification of 14.3 kbp mitochondrial genomes for rat were: forward, 5'-ATATTTTCACTGCTGAGTCCCGTGG-3'; reverse, 5'-AATTTCGGTTGGGGTGACCTCGGAG-3'. Band densitometry was semi- quantitatively analyzed using Image J software.

Real-time RT-PCR

Total RNA was isolated from the spinal cord tissues using Trizol according to the manufacturer's instructions. A 2-3 μg template RNA was used to synthesize the first strand of cDNA using a reverse transcription kit purchased from Takara. Real-time PCR of cDNA was performed using the forward and reverse primer sequences: PGC-1: forward, 5'-GTGCAGCCAAGACTCTGTATGG-3'; reverse, 5'-GTCCAGGTCATTCACATCAA-GTTC-3'; NRF-1: forward, 5'-TTACTCTGCTGTGGCTGATGG-3'; reverse, 5'-CCTCTGATG- CTTGCGTCGTCT-3'; TFAM: forward, 5'-GAAAGCACAAATCAAGAGGAG-3'; reverse, 5'- CTGCTTTTCATCATGAGACAG-3'; GAPDH: forward, 5'-GGGTCAGAAGGATTCCTATG-3'; reverse, 5'-GGTCTCAAACATGATCTGGG-3'. Data were analyzed using a comparative critical threshold (Ct) method where the amount of target normalized to the amount of endogenous control and relative to the control samples.

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Immunocytochemistry (ICC)

After being fixed with 4% paraformaldehyde for 15 min at room temperature, PC12 cells were washed with NaCl/Pi permeabilized with 0.2% Triton X-100, and incubated with Mitotracker and DAPI for 30 min at 4°C. After mounting in fluoromount media (Sigma-Aldrich Co. LLC), the slides were visualized under a confocal microscope and the images were recorded. Images were captured with an Olympus FV10i Confocal Microscope (Tokyo, Japan). At least six images of each group were taken by an evaluator blinded to the experimental conditions.

Western blot analysis

Equivalent amounts of protein was loaded and separated by 10% SDS-PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat milk solution in tris-buffered saline with 0.1% Triton X-100 (TBST) for 1 h, and then incubated overnight at 4°C with the primary Opa-1 antibody (1:1000), mitofusin 1 (Mfn-1, 1:1000), Drp-1 (1:800), Fis-1 (1:700) or β-actin (1:600) antibody dilutions in TBST. After that the membranes were washed and incubated with secondary antibody for 1 h at room temperature. Immunoreactivity was detected with Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). The Image J analysis software (Scion Corporation) was used to quantify the optical density of each band.

Statistical analysis

Statistical analysis was performed using SPSS 16.0, a statistical software package. Statistical evaluation of the data was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons or unpaired t test (two groups). A value of $p < 0.05$ was considered statistically significant.

Results

Allicin attenuates 6-OHDA-induced cytotoxicity

To test the safety of allicin in our *in vitro* model, PC12 cells were treated with different concentrations of allicin, and the results showed that treatment with allicin less than 0.5 mM did not change the cell viability at 24 h in PC12 cells (Fig .1A). Allicin at 1 mM significantly decreased the cell viability of PC12 cells from 12 h to 72 h in a time-dependent manner, whereas 50 μM allicin had no toxic effects up to 72 h after treatment (Fig. 1B). Thus, allicin at 50 μM was used in the following experiments.

To examine the potential protective effects of allicin, PC12 cells were pretreated with 50 μM allicin for 2 h, and exposed to 100 μM 6-OHDA for additional 24 h. The results showed that allicin obviously mitigated the 6-OHDA-induced decrease in cell viability (Fig. 1C) and increase in LDH release (Fig. 1D). In addition, we also detected the apoptotic cell death after 6-OHDA exposure using flow cytometry (Fig. 1E), and the results showed that the number of AV^+/PI^- and AV^+/PI^+ cells in allicin pretreated group was lower than that in 6-OHDA treated group (Fig. 1F), indicating the anti-apoptotic activity of allicin in 6-OHDA induced cytotoxicity.

Allicin reduces 6-OHDA-induced oxidative stress

We then sought to further examine the effect of allicin on oxidative stress, which was known to play important roles in 6-OHDA-induced cytotoxicity. As compared with 6-OHDA treated group, allicin pretreatment significantly decreased the intracellular ROS production at 24 h after injury (Fig. 2A), and an approximate 50% reduction in intracellular ROS generation was observed (Fig. 2B). Lipid peroxidation was estimated by determining the MDA formation, and the results showed that administration of allicin significantly alleviated MDA generation in PC12 cells (Fig. 2C). In addition, we also identified oxidative protein damage by the assessment of protein carbonyl. As shown in Fig .2D, protein carbonyl levels in PC12 cells subjected to 6-OHDA showed a 3-fold increase when compared with control cells, which was significantly alleviated by allicin treatment. To test the effects of allicin on the endogenous antioxidant system, the enzymatic activities of SOD, CAT, GST and GPx
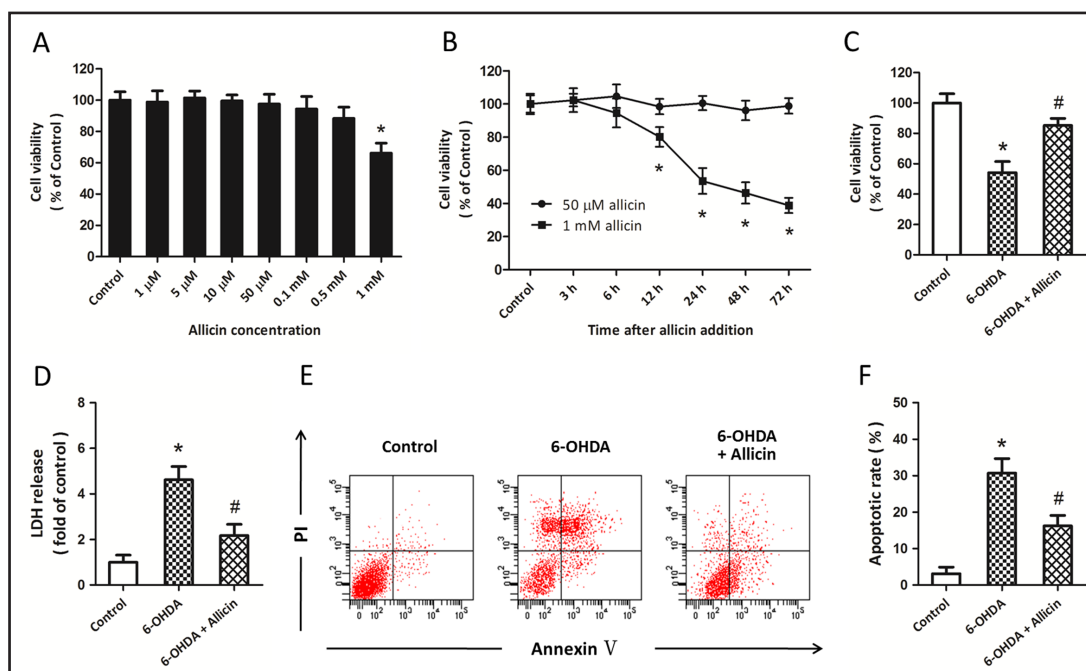
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Fig. 1. Allicin attenuates 6-OHDA-induced cytotoxicity. PC12 cells were treated with allicin at different concentrations, and the cell viability was detected at 24 h (A). PC12 cells were treated with 50 μM or 1mM allicin, and the cell viability was detected at different time points (B). PC12 cells were treated with 50 μM allicin at 30 min before exposure to 100 μM 6-OHDA, and the cell viability (C) and LDH release (D) were measured at 24 h. The apoptotic cell death were detected by flow cytometry (E), and the apoptotic rate was calculated (F). The results of cell viability were presented as % of control (the O.D. value), and the results of LDH were presented as fold of control (the O.D. value). Data are shown as mean ± SEM of five experiments. $^*p < 0.05$ vs. Control group. $^\#p < 0.05$ vs. 6-OHDA group.

were measured after 6-OHDA exposure in PC12 cells, and the results showed that 6-OHDA-induced damage in antioxidant system were partially prevented by allicin (Fig. 2E).

Allicin inhibits 6-OHDA-induced mitochondrial dysfunction

To determine if allicin has protective effect against mitochondrial dysfunction, the changes of MMP levels were measured using the fluorescent dye Rh123, and the results showed that 6-OHDA-induced loss of MMP was partially prevented by allicin treatment (Fig. 3A). The release of cytochrome c into cytoplasm was detected by an immunoassay kit after subcellular fraction preparation, and a significant increase in mitochondrial cytochrome c levels (Fig. 3B) and decrease in cytoplasmic cytochrome c level (Fig. 3C) were observed in allicin-treated PC12 cells. In addition, allicin partly rescued the decrease in mitochondrial ATP production in isolated mitochondria from 6-OHDA treated PC12 cells (Fig. 3D). We also examined the calcium buffering capacity in isolated mitochondria following allicin and 6-OHDA treatment. As shown in Fig. 3E, the peaks corresponded to sequential bolus additions of 30 nM of Ca^{2+}, and the downward deflections reflected mitochondrial Ca^{2+} uptake. 6-OHDA exposure resulted in a ~60% reduction in Ca^{2+} buffering capacity in isolated mitochondria, whereas allicin pretreatment significantly preserved the Ca^{2+} buffering capacity compared to that in 6-OHDA treated PC12 cells (Fig. 3F).

Allicin preserves mitochondrial biogenesis after 6-OHDA treatment

Inhibition of mitochondrial dysfunction and preservation of mitochondrial ATP generation can be results of increased mitochondrial biogenesis, which is defined as the growth and division of mitochondria. Therefore, the long fragment PCR was used to quantify the total amount of intact mtDNA, a correlate of mitochondrial biogenesis (Fig. 4A). 6-OHDA
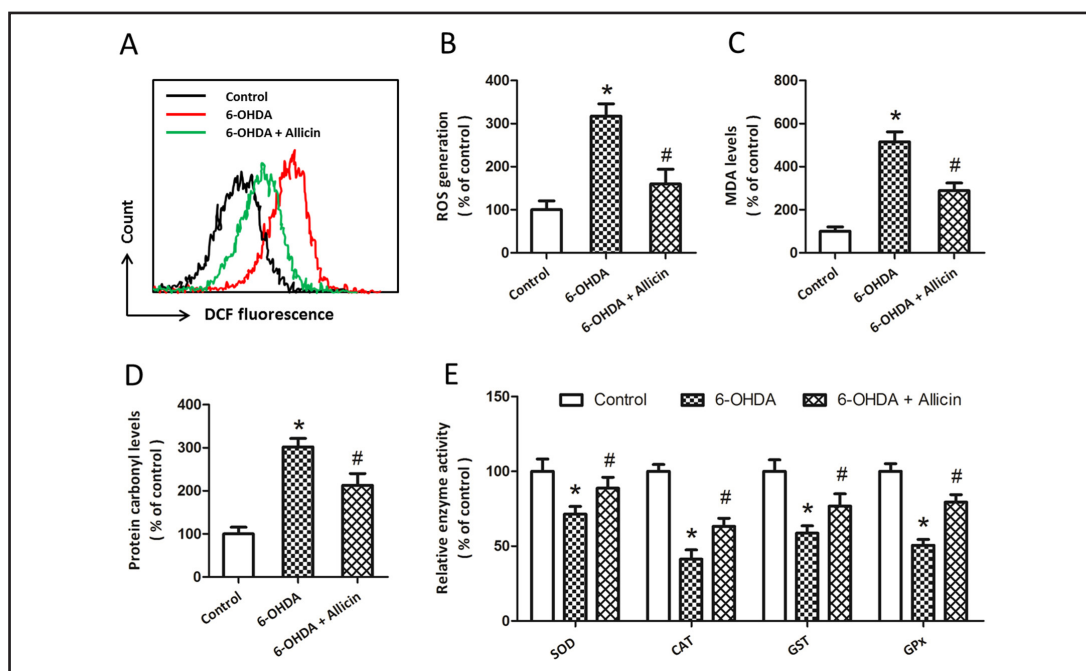
Fig. 2. Allicin reduces 6-OHDA-induced oxidative stress. PC12 cells were treated with 50 μM allicin at 30 min before exposure to 100 μM 6-OHDA. The intracellular ROS production was detected by DCF fluorescence staining (A), and calculated (B). The expression levels of MDA (C), protein carbonyl (D), and the activities of endogenous antioxidant enzymes (E) were measured. The results of ROS generation were presented as % of control (the DCF intensities), and the results of 2B to 2D were presented as % of control (the O.D. value). Data are shown as mean ± SEM of five experiments. $^*p < 0.05$ vs. Control group. $^#p < 0.05$ vs. 6-OHDA group.

exposure resulted in a ~30% reduction in mtDNA content, which was obviously attenuated by allicin treatment. We also examined mRNA levels of three transcription factors considered essential for mitochondrial gene expression in mammals. We found that 6-OHDA exposure caused significant decreases in the expression levels of PGC-1, NRF-1 and TFAM. As shown in Fig. 4B, the 6-OHDA-induced inhibition of all these transcription factors were partially prevented by allicin treatment, indicating the preservation of mitochondrial biogenesis in PC12 cells after allicin treatment.

Effects of allicin on mitochondrial morphological dynamic changes

Mitochondria are depicted as sausage-shaped organelles floating freely in the cytoplasm. We further investigated dynamic changes of mitochondrial morphology in PC12 cells after 6-OHDA and allicin treatment. To confirm the morphological dynamic changes in mitochondria, PC12 cells were strained with a fluorescent dye, MitoTracker red. The mitochondrial morphology of PC12 cells in the normal state without any treatment primarily exhibited tubular networks. After incubation with 6-OHDA, the mitochondria were prevalent in shorter and smaller size, indicating mitochondrial fragmentation. As shown in Fig. 5, these mitochondrial morphological changes were attenuated by allicin pretreatment.

Effects of allicin on the expression of mitochondrial dynamic proteins

Mitochondrial morphology is likely to be the result of the interplay between mitochondrial division and fusion. Mitochondrial fusion is controlled by Mfn-1 and Opa-1, whereas mitochondrial fission is controlled by Drp-1 and Fis-1. To investigate whether mitochondrial fission and fusion were affected by 6-OHDA and allicin treatment, the expression of these factors were analyzed by western blot (Fig. 6A). We found a significant decrease in Opa-1 and Mfn-1 after 6-OHDA exposure, and allicin partially prevented the decrease of Opa-1, with
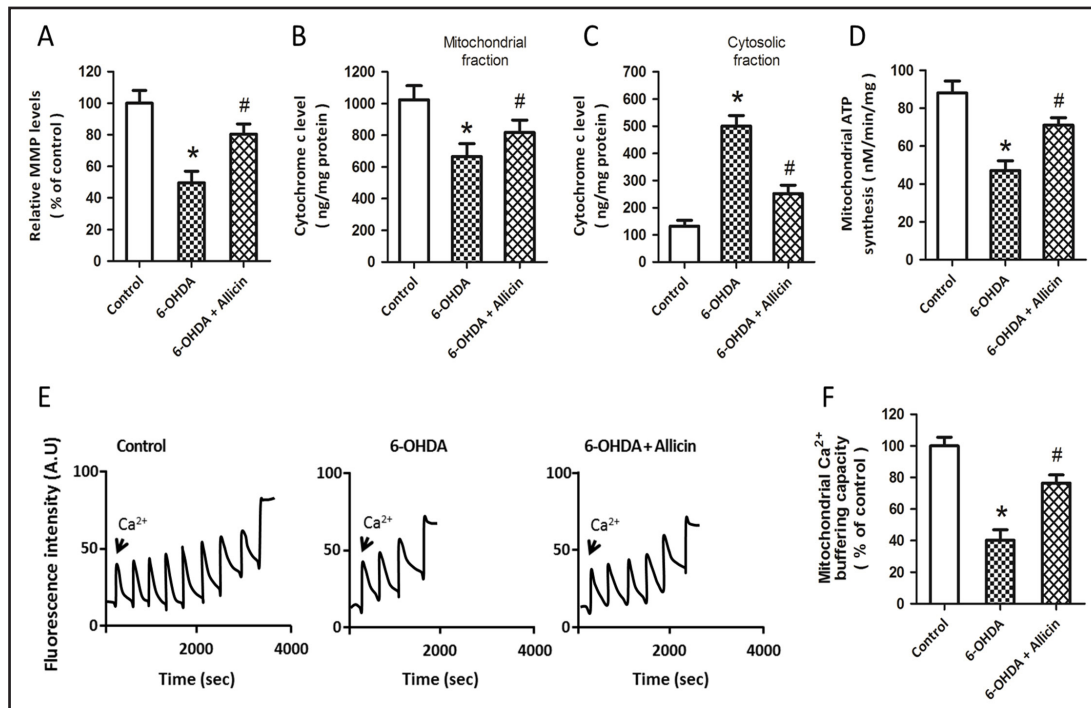
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Fig. 3. Allicin inhibits 6-OHDA-induced mitochondrial dysfunction. PC12 cells were treated with 50 µM allicin at 30 min before exposure to 100 µM 6-OHDA. The MMP levels (A), mitochondrial cytochrome c (B) and cytosolic cytochrome c (C) were measured. After allicin and 6-OHDA treatment, mitochondria in each group were isolated and purified. Levels of ATP synthesis were determined (D). Relative Ca^{2+} uptake capacity of isolated mitochondria were determined (E) and calculated (F). The results of 3A and 3F were presented as % of control (the fluorescence intensities). Data are shown as mean ± SEM of five experiments. $^*p < 0.05$ vs. Control group. $^#p < 0.05$ vs. 6-OHDA group.
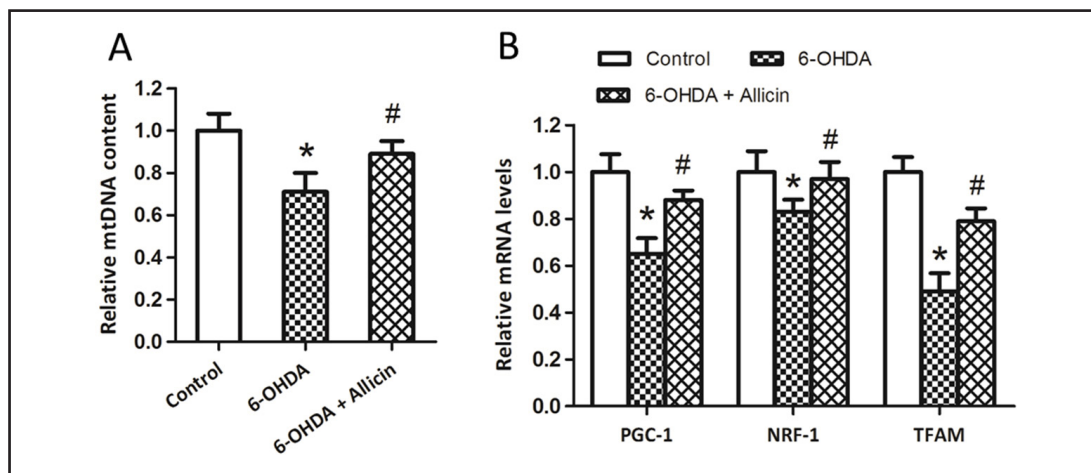

Fig. 4. Allicin preserves mitochondrial biogenesis after 6-OHDA treatment. PC12 cells were treated with 50 µM allicin at 30 min before exposure to 100 µM 6-OHDA. The relative mitochondrial DNA content was quantified by long fragment PCR (A), and the mRNA expression of mitochondrial biogenesis factors was measured by real-time RT-PCR (B). Data are shown as mean ± SEM of five experiments. $^*p < 0.05$ vs. Control group. $^#p < 0.05$ vs. 6-OHDA group.

no effect on Mfn-1. As for fission proteins, both Drp-1 and Fis-1 were increased by 6-OHDA exposure, whereas the expression of these two factors in allicin pretreated cells were lower than that in 6-OHDA treated cells.
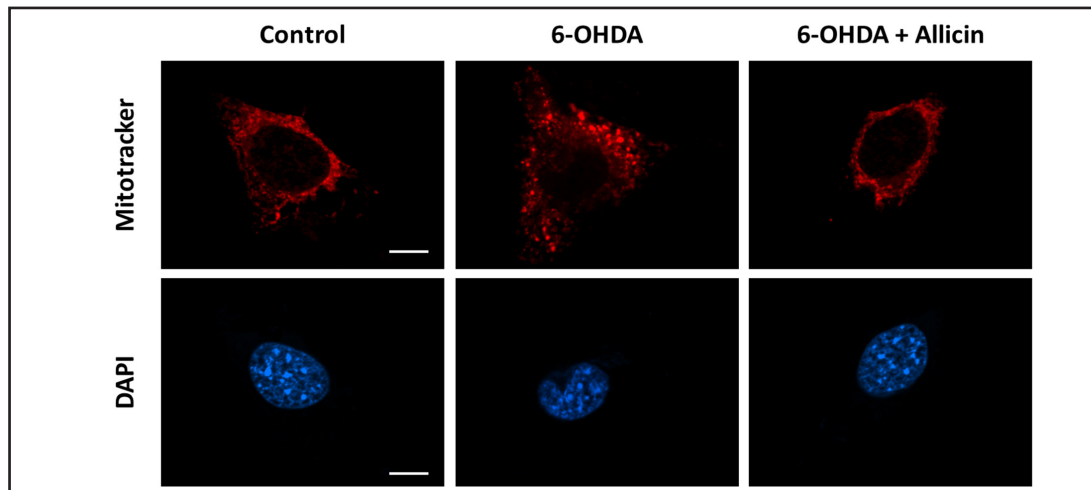
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Fig. 5. Effects of allicin on mitochondrial morphological dynamic changes. PC12 cells were treated with 50 μM allicin at 30 min before exposure to 100 μM 6-OHDA. The mitochondrial morphological dynamic changes were detected by Mitotracker staining (red), and DAPI staining (blue) was used to detect the nuclei. Scale bars: 10 μm. Data are representative of three similar experiments.
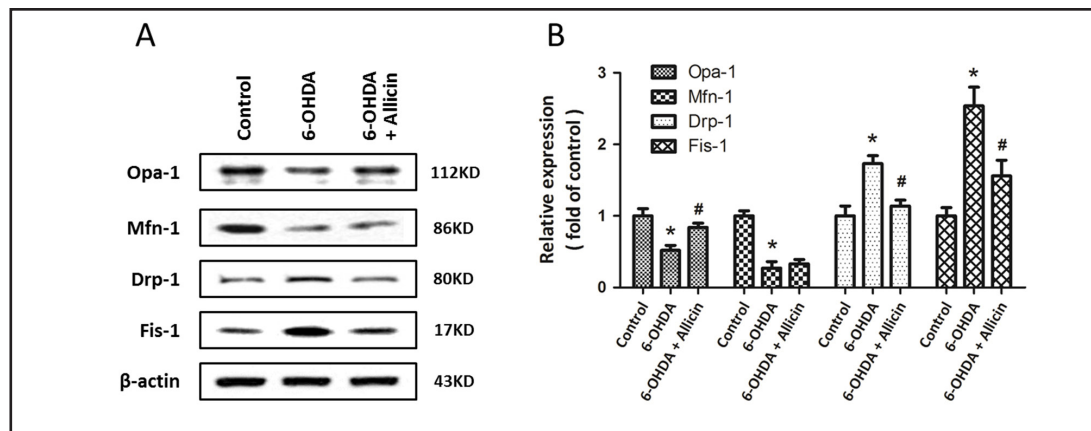

Fig. 6. Effects of allicin on the expression of mitochondrial dynamic proteins. PC12 cells were treated with 50 μM allicin at 30 min before exposure to 100 μM 6-OHDA. The expression of mitochondrial dynamic proteins, including Opa-1, Mfn-1, Drp-1 and Fis-1, were detected by western blot (A), and calculated (B). The results of western blot analysis were presented as fold of control (the optical densities). Data are shown as mean ± SEM of five experiments. $^*p < 0.05$ vs. Control group. $^\#p < 0.05$ vs. 6-OHDA group.

Discussion

Four decades ago, 6-OHDA, also known as oxidopamine, was found to cause degeneration of dopaminergic neurons in the SNpc, accompanied by marked postural bias and turning asymmetry following systemic administration [22]. The neuronal damage induced by 6-OHDA is mainly due to its accumulation in the mitochondria, and it has remained the most commonly used in vitro PD model to determine the possible neuroprotective compounds and investigate the underlying mechanism [23]. The present study evaluated the neuroprotective effect of allicin against 6-OHDA induced cytotoxicity in PC12 cells, an *in vitro* PD related neuronal injury model. This beneficial effect is thought to be brought about by the inhibition of oxidative stress, as the 6-OHDA induced increase of oxidative products (MDA and protein carbonyl) expression and decrease of antioxidant enzymes activity were both attenuated after allicin administration. In addition, allicin significantly prevented mitochondrial

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dysfunction after 6-OHDA treatment through regulating mitochondrial biogenesis and mitochondrial dynamics.

Once inside cells, 6-OHDA undergoes auto-oxidation or metabolic degradation and produces hydrogen peroxide, superoxide, and hydroxyl radicals. This process causes lipid peroxidation, protein oxidation, and DNA oxidation and finally results in oxidative stress, mitochondrial dysfunction, and apoptosis [24]. In the present study, increased oxidative stress, as evidenced by ROS overproduction, significant increases in MDA and protein carbonyl levels, was found in PC12 cells after 6-OHDA treatment, which was consistent with the results of previous reports [25]. Oxidative stress related apoptotic cell death occurs when ROS production overwhelms the anti-oxidative ability or the endogenous anti-oxidative system was destroyed [26]. Our results showed that 6-OHDA induced apoptosis in PC12 cells were accompanied by a striking decrease in the activities of SOD, CAT, GST and GPx. These endogenous antioxidants constitute the initial anti-oxidative defense systems, which will scavenge ROS induced by oxidative stress. Preservation of the endogenous antioxidants activity would play a protective role against apoptosis under oxidative stress conditions. Allicin is a physiologically active molecule with many potential health benefits. Previous studies showed that allicin could effectively reduce intracellular ROS of heart by 50% in aortic-banded mice with cardiac hypertrophy, and inhibit ROS generation in cardiac myocytes by 60% at 10 μM in an in vitro manner [27]. Allicin helps to fight against oxidative stress by directly raising reduced glutathione (GSH) content in the cell and indirectly increasing GSH by allicin derivatives, such as S-allylmercaptoglutathione and S-allylmercaptocysteine [13, 28]. In consistent with these previous findings, our present data strongly support that allicin can protect against PD related neuronal injury by enhancing the antioxidant status via lowering the level of ROS and stimulating the production of anti-oxidative enzymes.

Mitochondria have long attracted the attention of biomedical researchers because of their role in human diseases, and pathology, toxicology and genetics suggest that mitochondrial dysfunction is an etiological factor in PD [29, 30]. Mitochondria are one of the major sources of ROS, and are highly susceptible to oxidative damage because ROS damage mitochondrial enzymes directly, cause mtDNA mutation and alter mitochondrial membrane permeability. The proteins that are reported to be related to PD, including PTEN-induced putative kinase 1 (PINK1), DJ-1, α-synuclein, leucine-rich repeat kinase 2 (LRRK2) and parkin, are either mitochondrial proteins or are associated with mitochondria [31]. Furthermore, several particular mtDNA polymorphisms and haplotypes have been reported to be associated with the risk of PD, and mutations in mtDNA or in the nuclear-encoded mtDNA polymerase-G (POLG) cause PD-like symptoms [32]. The involvement of mitochondrial dysfunction in the process of PD related neuronal death is also supported by epidemiological studies which showed the role of several neurotoxins, some of which are proved to be mitochondria targeted agents, in the occurrence of PD [33, 34]. In the present study, collapse of MMP, release of cytochrome c, decrease of mitochondrial ATP generation, as well as damaged mitochondrial Ca^{2+} buffering capacity were observed after 6-OHDA exposure in PC12 cells, which was consistent with previous studies [35].

The mitochondrion is a key regulator of the metabolic activity of the cell, and it is reckoned that higher mitochondrial copy number (or higher mitochondrial mass) is protective for cells [36]. Increased mitochondrial ATP production, the most important marker to assess preservation of mitochondrial function, can be the result of increased mitochondrial biogenesis. Mitochondrial biogenesis is defined as the growth and division of mitochondria, and can be activated by numerous different signals during times of cellular stress or in response to environmental stimuli [37]. When the stimuli exceed the tolerable threshold, the mitochondrial mass will be damaged and elicit apoptotic cell death by inducing a variety of mitochondrial cascades, including changes in electron transport, collapse of MMP, altered cellular oxidation-reduction potential, release of caspase family proteins, and participation of pro- and anti-apoptotic B-cell non-Hodgkin lymphoma-2 (Bcl-2) family proteins [38, 39]. The activation of specific transcription factors encoded by nuclear genes, such as proliferator-activated receptor gamma coactivator 1 (PGC-1),

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nuclear respiratory factors 1 (NRF-1) and mitochondrial transcription factor A (TFAM), are demonstrated to control biogenesis and division of mitochondria under pathological conditions. In the present study, 24 h exposure of 6-OHDA induced a significant decrease in mtDNA and these three transcriptional factors, whereas all these changes were attenuated by allicin, indicating that preservation of mitochondrial biogenesis might be account for the allicin-induced protection against oxidative stress and mitochondrial dysfunction in our in vitro model.

One of the most important means that might adapt mitochondrial function to various conditions of living cells is dynamic structural changes of the mitochondrial network, including continuous remodeling by fusion and fission events [40]. These dynamic processes are of particular importance in neuronal cells because of their post-mitotic state and long processes with higher energy requirements, and dis-regulation in both fission and fusion proteins have been associated with central nervous system diseases [41, 42]. In vertebrates, high-molecular weight GTPases, such as Mfn-1, Opa-1, Drp-1 and Fis-1, are key components involved in regulating the mitochondrial morphologic dynamics, and these factors have been linked to the cellular death program of apoptosis through the caspase associated pathway [43]. Inhibition of mitochondrial fragmentation by activation of Mfn-1 or Opa-1 was shown to antagonize apoptosis progression, whereas the pharmacologic inhibitor of Drp-1, mdivi-1, inhibited tBid-dependent cytochrome c release from isolated mitochondria [44]. In the present study, individual mitochondria disclosed a fragmented structure and an abnormal distribution accumulating around the peri-nuclear area were more prominent in 6-OHDA treated PC12 cells compared with those cells with allicin treatment. In addition, the 6-OHDA induced increase in Drp-1 and Fis-1, as well as decrease in Opa-1 were all partially prevented by allicin, indicating that allicin induced protection against 6-OHDA induced toxicity is mediated by preservation of the balance between mitochondrial fission and fusion.

There are some limitations to our study. First, undifferentiated PC12 cells were used in the present study and 6-OHDA-induced cytotoxicity was performed to mimic PD related neuronal injury in vitro. The PC12 cell line, which was derived from a rat pheochromocytoma, maintains a differentiated neuroendocrine phenotype and has been widely used as a convenient model system for a wide variety of cell biological studies [45]. However, without differentiation upon exposure to nerve growth factor (NGF), it is less relevant to the study of neuronal communication and neuronal injury mechanisms [46, 47]. Thus, it needs to be further investigated that whether the conclusion in the present study can also apply to differentiated PC12 cells or primary cultured neurons. In addition, preservation of mitochondrial biogenesis and differently regulated mitochondrial dynamics were observed in our in vitro model after allicin treatment, which was accompanied by enhanced mitochondrial function, but whether allicin-induced regulation of mitochondrial dynamic proteins accounts for its protective effects, and the relationship between allicin-induced modulation of mitochondrial dynamic proteins and endogenous antioxidant systems are not fully determined. Previous studies showed that allicin protects cells against oxidative stress by inducing the generation of antioxidant products, thereby reducing cytotoxic substances and scavenging various free radicals [13], and the potential underlying mechanisms might be associated with several downstream signaling cascades, such as NF-E2-related factor-2 (Nrf-2) pathway [48-50]. Thus, the modulation of mitochondrial dynamics and its interaction with endogenous antioxidant systems, as well as the potential involved signaling cascades, need more extensive study in allicin-treated cells.

Conclusions

In conclusion, the findings from the present study indicate that allicin, an active substance of garlic, might be an anti-oxidative agent to protect against PD-related neuronal injury by affecting endogenous antioxidant system and mitochondrial function. These novel protective effects elicited by allicin were accompanied by the balance of mitochondrial fission/fusion and preservation of mitochondrial biogenesis. These data strongly support

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the hypothesis that allicin might represent an ideal therapeutic candidate for PD as well as other neurological diseases where oxidative stress and endogenous antioxidant system play a major role.

Disclosure Statement

There is no conflict of interest.

Acknowledgements

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