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SIRT3 deacetylated and increased citrate synthase activity in PD model

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ABSTRACT

SIRT3 have been found to be neuroprotective in many neurological diseases, but its detail mechanism is only partially understood. In this study, MPP^+ was used to treat SH-SY5Y cells as the cellular model of PD to test the role of SIRT3 and the mechanism may be involved in. We focused on the changes and relationship between SIRT3 and the key mitochondrial enzymes citrate synthase (CS) and isocitrate dehydrogenase 2 (IDH2). We found MPP^+ decreased SIRT3 expression. And our results showed that the enzymatic activities of CS and IDH2 were significantly reduced in MPP^+ treatment cells, while protein acetylation of CS and IDH2 increased. However overexpressed-SIRT3 partially reversed at least, the decline of CS activity and the increase of CS protein acetylation. IDH2 did not showed the same changes. The study suggested that SIRT3 deacetylated and activated CS activity. Hence, we conclude that SIRT3 exhibits neuroprotection via deacetylating and increasing mitochondrial enzyme activities.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, as well as the most common movement disorder associated with the elderly [1]. PD, affects more than 1% of the population over the age of 65 years and more than 4% of the population by the age of 85 years [2]. So far there are no effective treatments or preventive therapies [3]. Therefore, it's of great importance to study the underlying mechanisms of dopaminergic (DA) neuronal degeneration and develop the relevant neuroprotective/therapeutic strategies. There are various factors involved in PD pathogenesis, of which mitochondrial dysfunction is the most remarkable one. It is generally accepted that DA neurons turn sensitive to mitochondrial dysfunction particularly in energy status. Neuronal damage has been closely connected with the reduction of intracellular adenosine triphosphate (ATP) level in the brain of PD patients, and in models of PD [4,5]. Given that mitochondrial-based oxidative damage is a major postulated mechanism for PD pathogenesis [6], many investigators are focusing on mitochondrial enzymes.

Sirtuins, NAD^+-dependent protein deacetylases, participates in systemic metabolic regulation, of which SIRT3, SIRT4, and SIRT5 are mitochondrial localized [3]. As the most principal one, SIRT3 is expected to participate in PD progression [3], but how it works still has been unknown. Recent findings suggest that SIRT3 deacetylates and activates mitochondrial enzymes, of which involved in fatty acid β-oxidation, amino acid metabolism, the electron transport chain, and antioxidant defenses [7].

Accumulating evidences have suggested that protein acetylation, as a major post-translational modification, is recognized as a major regulatory mechanism in mitochondrial function and neuronal health [8]. Our previous study found that MPP^+ treatment decreased the expression of SIRT1 of DA neuron, which weakened the deacetylation to H3K14 and strengthened the combination of H3K14 with HIF1α promoter, and then increased the transcription activity of HIF1α [9].

Citrate synthase (CS), isocitrate dehydrogenase 2 (IDH2) and pyruvatede hydrogenase (PDH) are the gatekeeper enzymes of the citric acid cycle (TCA), which occur in the mitochondrial matrix and generate high-energy product for oxidative phosphorylation. Impaired CS activity could thus impact mitochondrial function and exacerbate age-related hearing loss [10]. Johnson et al. found that a

Abbreviations: PD, Parkinson's disease; DA, dopaminergic; ATP, adenosine triphosphate; CS, citrate synthase; IDH2, isocitrate dehydrogenase 2; TCA, citric acid cycle; PDH, pyruvatede hydrogenase; DMEM, Dulbecco's Modified Eagle's medium; CCK8, cell counting kit-8; FBS, fetal bovine serum; PBS, phosphate buffered saline; MPP^+, methyl 4 phenylpyridinium; SIRT3, silent information regulator 3.

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missense mutation of CS is responsible for the *ahl4*-related hearing loss of A/J strain mice [10]. Other researchers found that PDH α1 subunit can be deacetylated by SIRT3 in vitro and in vivo, altering the activity of PDH as well as tumor cell metabolism [11]. Whether CS, IDH2 and PDH are participated in PD or associate with SIRT3 are unclear. The aim of this study is to examine the effects of SIRT3 on PD pathogenesis and uncover the molecular mechanisms involved.

2. Materials and methods

2.1. Cell culture and treatments

SH-SY5Y cells were maintained in Dulbecco's modified eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (GIBCO, Gaithersburg, MD, USA) and grown in a CO_2 incubator maintained at atmospheric oxygen levels and 5% CO_2. MPP^+ (Sigma–Aldrich, St. Louis, MO, USA) stocks were dissolved in phosphate buffered saline (PBS) at a stock concentration at 125 mM which was stored at −20 °C. MPP^+ was further diluted in serum free DMEM to achieve the final concentrations.

2.2. CCK8 cell viability assay

Cell viability was measured with CCK8 assay (Cell Counting Kit-8; Beyotime, Shanghai, China). SH-SY5Y cells were seeded into 96-well plates with 5000 cells in each well. After all groups of cells were subjected to various conditions, 10 μL CCK8 was added to each well for another 1 h at 37 °C. The well containing only the culture medium was regarded as blank. Absorption was measured at 450 nm using a microplate reader (Bio Tek, VT, USA), and the results were expressed as percentages of control values. The cell inhibition rate was calculated as 1 − [(mean OD of one group - blank)/(mean OD of the control - blank)]. All the experiments were independently repeated at least three times.

2.3. Mitochondrial membrane potential (MMP)

MMP was determined by incubating the cells in a medium containing 1 μg/ml hochest (Thermo scientific) at 37 °C for 10 min without light. Then the cells were incubated in a 1 μg/ml TMRM (Thermo scientific) 37 °C for 30 min. Wash cells gently three times with warm buffer, followed by cellomics arrayscan VTI (Thermo scientific).

2.4. Measurement of ATP level

After the treatments, cells were harvested. And the ATP Kit (Thermo scientific) was utilized to determine the ATP level according to the manufacturer's instructions. Briefly lysed with the assay buffer, followed by centrifugation at 12000*g* for 10 min at 4 °C. Lastly, the level of ATP was measured by mixing appropriate supernatant with reaction mix to each of the wells. Mix well using a horizontal shaker. Incubate the plate at room temperature for 30 min. Protect the plate from light during the incubation. Measure the absorbance at 570 nm (A570). Using the corrected measurement, determine the amount of ATP present in the sample from the standard curve.

2.5. Immuoblotting assay

After specific treatment, total proteins were lysed in IP buffer (Beyotime, Shanghai, China), including protease inhibitors. The protein homogenates were separated nextly via 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (PVDF). After blocking the PVDF membranes were incubated overnight with special primary antibodies: anti-SIRT3, anti-IDH2 (Cell Signaling Technology, Inc., Danvers, MA, USA), anti-CS (Abcam, San Francisco, USA), and anti-acetyl-Lysine (Millipore), respectively. βactin (Protein Tech Group, Chicago, USA) was used to performed to confirm equal protein loading. Then PVDF membranes were washed with TBST (1 × Tris-buffered saline, 0.1% Tween-20) for 3 times and 10min for each. Followed by incubating with secondary antibodies for 1 h at room temperature and washing with TBST. Immunoreactive bands were then visualized using the ECL reagent (Protein Tech Group, Chicago, USA). The densities of the immunoblotting bands were semiquantified using Image J software (National Institutes of Health).

2.6. In vitro deacetylation assay

For immunoprecipitation (IP) experiments, a kit from Proteintech was utilized according to the manufacturer's instructions. Extracted proteins were incubated with special primary antibodies (CS, IDH2) at 4 °C overnight with rotation, followed by precipitation with protein A sepharose beads. Immunoprecipitates were then washed and subjected to SDS-PAGE separation and incubated with antibodies raised against anti-CS, anti-IDH2 and anti-acetyl-Lysine. Subsequent immunoblotting procedures were the same as described above.

2.7. RNA extraction and analysis by quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA samples were conducted with DNase I (Sigma-Aldrich), quantified, then reverse-transcribed into cDNA using a ReverTra Ace-α first strand cDNA synthesis kit (Toyobo Co., Ltd., Osaka, Japan). qRT-PCR was performed utilizing a RealPlex4 real-time PCR detection system (Eppendorf Co., Ltd. Hamburg, Germany), with SYBR-Green real-time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) as the detection dye. 18s rRNA was used as a comparative threshold cycle (Ct) normalizer to determine relative gene expression. For all samples, the Ct values of the genes were normalized using the formula ΔCt = Ct_genes − Ct_18s rRNA. To calculate relative expression levels, the following formula was used ΔΔCt = ΔCt_all_groups −ΔCt_blank control group. The values, which were used to plot relative expression of markers, were calculated using the formula $2^{-\Delta\Delta Ct}$. The following primers were used: SIRT3-F:CACAGTCTGCCAAAGACCCT; SIRT3-R: AGAACACAATGTCGGGCTTC; CS-F: AATTCGTGGAGGAAGCACTG; CS-R:ATGGCTTTACTTACTGCGGC; IDH2-F: ACCTCGCAAGAGCAGCC; IDH2-R: TGAACTGCCAGATAATACGGG; 18s rRNA-F: CAGCCACCCGAGATTGAGCA; 18s RNA-R:TAGTAGCGACGGGCGGTGTG.

2.8. Annexin V-FITC/PI assay

An Annexin V-FITC/PI kit (Beyotime, Shanghai, China) was using to evaluate apoptosis according to the manufacturer's instructions. After treatment under various conditions for each group, cells (approximately 10^5) were harvested and washed twice with PBS. Annexin V-FITC binding buffer (195 μL) was added to the resuspended cells, then 5 μL Annexin V-FITC was followed. After vortexing gently, the cells were incubated for 30 min at 37 °C without light. Centrifuged the cells at 1000 × g for 5 min, then removed the supernatant liquid. Followed by 190 μL binding buffer and 10 μL propidium iodide (PI) was added to the cells. Flow cytometry was chose to count the number of cells which underwent apoptosis.

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