

RESEARCH PAPER



NaSH increases SIRT1 activity and autophagy flux through sulfhydrylation to protect SH-SY5Y cells induced by MPP~+

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ABSTRACT

Parkinson's disease (PD) is one of the most prevailing aging diseases around the world. The present study was to investigate the potential effect of hydrogen sulfide (H₂S) and silent mating type information regulation 2 homolog 1 (SIRT1) in MPP~+ induced SH-SY5Y cells and its underlying mechanisms in PD. SH-SY5Y cells were induced by MPP~+ and treated with the H₂S donor NaHS to detect the effect of H₂S on the molecular behaviors of MPP~+ induced SH-SY5Y cells. NaHS reduced the apoptosis rate and expressions of MDA, 4-HNE and p62, while increased cell viability, autophagy flux and expressions of LC3 II/I and Beclin1 in MPP~+ induced SH-SY5Y cells. Then, levels of autophagy-related proteins and inflammation-related proteins (TNF- α , IL-1 β) were detected, indicating that Chloroquine and Sirtinol reversed the protective effect of H₂S on SH-SY5Y cells induced by MPP~+. We further explored the particular function of H₂S, SH-SY5Y cells treated with MPP~+, NaHS chloroquine, and SIRT1 inhibitor (Sirtinol). The results showed that H₂S increased SIRT1 expression and sulfhydrylation. Finally, a PD mouse model verified the above results. In a word, H₂S ameliorated SIRT1 activity through acceleration of SIRT1 sulfhydrylation to increase the autophagy flux and attenuate damage of SH-SY5Y cells induced by MPP~+. H₂S and SIRT1 activator might be a target in the treatment of PD patients.

ARTICLE HISTORY

Received 6 December 2019
Revised 9 June 2020
Accepted 3 July 2020

KEYWORDS

Hydrogen sulfide; MPP~+; Parkinson's disease; SH-SY5Y; SIRT1; autophagy flux

1. Introduction

Parkinson's disease (PD) is one of the most prevailing neuro-degenerative motion disorders, which is tightly correlated with remarkable motor deficits [1]. Its main motor symptoms include bradykinesia/muscular atrophy, rigidity, postural instability and tremor, but the clinical manifestations are other motor/non-motor disorders [2]. It is estimated that 1% of the population over 60 y old worldwide is presently living with serious and even fatal PD [3]. There are drugs that can increase or stabilize the brain dopamine, but they cannot block or slow down the PD progress [4]. Autophagy is a highly conserved intracellular degradation process, which underlies various neurodegenerative disorders in the central nervous system [5]. Promotion of autophagy maintains neuron survival *in vivo* and *in vitro*, and accelerates the elimination of protein aggregates in the models of PD and Alzheimer's disease (AD) [6]. In addition, it is suggested by different studies that

mediation of autophagy might be a prospective therapeutic scheme for PD patients [7,8]. Besides, human SH-SY5Y cell line shows numerous dopaminergic neuron properties, which is widely employed to study toxicity induced by 1-methyl-4-phenylpyridinium (MPP~+), and a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is extensively used as PD inducer *in vitro* [4]. Therefore, we used SH-SY5Y cells treated with MPP~+ as the target of autophagy therapies in PD.

Hydrogen sulfide (H₂S), a metabolic poison normally emitting malodor, participates in significant physiological functions in cancers, cardiovascular systems, inflammatory procedures and neuroprotection [9]. Inhaled H₂S or NaHS has been presented to exert neuroprotection in the rodent models of PD induced by neurotoxins [10]. Besides, Sirtuins (SIRTs) are known as the nicotinic adenine dinucleotide-dependent deacetylases targeting forkhead transcription factor O3 α , one of the downstream factors of SIRT1 [11]. SIRT1 belongs to the Sirtuin family, which

partakes in different bioprocesses including oxidative stress, cell proliferation, cancer development, and pyroptosis [12]. SIRT1 also extensively exists in adult brain and has been acknowledged to exert protective functions in neurodegenerative disorders, including PD [13]. Interestingly, H₂S upregulates SIRT1 and protects HT22 cells against neuronal senescence caused by high glucose through improving autophagy flux [14]. By upregulating SIRT1, H₂S can protect kidney cells from further damage of diabetes and reduce reactive oxygen species (ROS), fasting blood glucose and apoptosis, thus protecting renal cells against further damage led by diabetes mellitus [15]. However, the relationship between H₂S and SIRT1 in PD is rarely elucidated. Therefore, we established an SH-SY5Y cell model treated with MPP^{~+} to investigate the interaction of H₂S and SIRT1 and their influence on autophagy.

2. Materials and methods

2.1. Ethics statement

All animal measurements were under the guidance on used animals and supervised by the ethic committee of The Second Hospital of Harbin Medical University. This study minimized the number and pain of experimental animals as much as possible.

2.2. Cell culture and grouping

Human neuroblastoma cell line SH-SY5Y was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin (ThermoFisher Scientific Inc., Rockford, IL, USA).

MPP^{~+} was used to simulate the microenvironment of PD [16]. SH-SY5Y cells were treated with MPP^{~+} at different concentrations of 100, 200, 300, 400, 500, 600, and 700 μmol/L (D048, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 24 h, and the optimal concentration was determined as 500 μmol/L. Then, cells were assigned into blank group (SH-SY5Y cells were cultured normally without any reagent treatment), MPP^{~+} group (SH-SY5Y cells were cultured with 500 μmol/L MPP^{~+}), MPP^{~+} + NaHS

groups (SH-SY5Y cells were respectively pre-treated with 50, 100, 200 or 400 μmol/L NaHS for 30 min and then treated with 500 μmol/L MPP^{~+} for 24 h), MPP^{~+} + NaHS + Chloroquine (CQ) group (SH-SY5Y cells were pre-treated with 200 μmol/L NaHS for 30 min and then treated with 500 μmol/L MPP^{~+} and CQ for 24 h), and MPP^{~+} + NaHS + Sirtinol group (SH-SY5Y cells were pre-treated with 200 μmol/L NaHS for 30 min and then treated with 500 μmol/L MPP^{~+} and 17 μg/mL Sirtinol) for 24 h. NaHS was purchased from Sigma-Aldrich, and CQ and Sirtinol were purchased from MedChemExpress (NJ, USA).

2.3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cells in logarithmic growth phase after different treatments were used for preparing the single cell suspension at the density of 1×10^5 cells/mL, and then the cells were seeded into the 96-well plate at the density of 200 μL/well. After 48 h of incubation, 20 μL of 5 mg/mL MTT solution (Sigma-Aldrich) was added to detect cell viability. Next, the cells were supplemented with 150 μL dimethyl sulfoxide followed by dissolving the crystals at room temperature for 15 min. The optical density (OD) of each well was measured at a wavelength of 490 nm using a microplate reader.

2.4. Flow cytometry

The cells in each group were seeded into the 12-well plate (8×10^4 cells/well). After proper treatment, the cells were treated following the instructions of annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). The cell apoptosis was detected using the flow cytometer.

2.5. Hoechst 33342 staining

The cells in each group were washed with phosphate buffered saline (PBS), fixed with 4% formaldehyde and stained with 10 μg/mL Hoechst 33342 for 15 min at room temperature. After washing with PBS, the cells were observed and recorded

under the fluorescence microscope (Olympus, Tokyo, Japan).

2.6. Enzyme linked immunosorbent assay (ELISA)

The expressions of malondialdehyde (MDA) and 4-Hydroxynonenal (4-HNE) in SH-SY5Y cells were detected in strict accordance with the instructions of the human MDA ELISA kit (kt98244, MSK Biotechnology Co., Ltd., Wuhan, Hubei, China) and human 4-HNE ELISA kit (kt99767, MSK Biotechnology).

2.7. Monomeric red fluorescent protein (mRFP)-green fluorescent protein (GFP)-microtubule-associated protein1 light chain 3 (LC3) fusion protein tracing

mRFP-GFP-LC3 was purchased from HanBio Co., Ltd. (Shanghai, China). SH-SY5Y cells were cultured in the 24-well plate (1×10^5 cell/well). When cell confluence reached 70%-80%, the cells were incubated with adenovirus mRFP-GFP-LC3 (adjusting the virus multiplicity of infection to 100) for 2 h. After PBS washing, the cells were cultured in a complete culture medium overnight. The transfection efficiency of adenovirus mRFP-GFP-LC3 was observed and verified under the fluorescence microscope. Next, the cells were treated for subsequent experiments, and then observed and photographed under the fluorescence microscope (BX51, Olympus).

2.8. Western blot analysis

SH-SY5Y cells were lysed in cold radioimmunoprecipitation assay (RIPA) containing a mixture of protease inhibitors (Sigma-Aldrich) for 30 min. The lysate was centrifuged at 16000 g for 20 min at 4°C to collect the supernatant. Pierce bicinchoninic acid (BCA) protein assay kit (Beyotime) was used to quantify protein concentration. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were sealed in 5% skimmed milk at room temperature for 2 h, incubated overnight

with the primary antibodies at 4°C, then incubated with the secondary antibody for 1 h, and developed with chemiluminescence reagent using Bio-Rad Gel Dol EZ (Bio-Rad, Inc., Hercules, CA, USA). Image J software was used to analyze the gray value of the target band (National Institutes of Health, Bethesda, Maryland, USA). The antibodies used were as follows: p62 (1:1000, ab56416, Abcam, Cambridge, MA, USA), LC3B (1:1000, ab48394, Abcam), Beclin1 (1:1000, ab62557, Abcam), SIRT1 (1:1000, ab110304, Abcam), immunoglobulin G (IgG) (1:1000, ab205719, Abcam), β -actin (1:1500, ab8227, Abcam), nuclear factor kappa B (NF- κ B) p65 (1:1000, ab16502, Abcam), and p53 (1:1000, ab26, Abcam).

2.9. Biotin switch assay

Cells were homogenized in HEN buffer [250 mM Hepes-NaOH (pH 7.7), 1 mM EDTA and 0.1 mM neocuproine] supplemented with 1% proteinase inhibitor cocktail and 100 μ M deferoxamine. Next, the cells were centrifuged at $14000 \times g$ for 15 min at 4°C. The samples were added with blocking buffer (HEN buffer adjusted to 2.5% SDS and 20 mM methyl methanethiosulfonate), and then incubated at 50°C for 20 min along with gentle and frequent vortexing devoid of foaming. Proteins were precipitated at -20°C for 1 h after the addition of acetone. After centrifugation at $2000 \times g$ for 10 min, the acetone was removed. The protein pellets were re-suspended with the HEN buffer and incubated with 1% SDS and 30 mM biotin-disodium 1-hydroxypentylidiphosphonate for 3 h at 25°C. Afterward, the biotinylated proteins were precipitated by streptavidin-agarose beads, followed by the washes with HEN buffer and PBS solution. Subsequently, the biotinylated proteins were eluted by SDS-PAGE gel and analyzed by Western blot analysis with anti SIRT1 antibody.

2.10. Establishment of a PD model in mice

The establishment of a PD model in mice was based on the method in the literature [17]. C57/BL6 mice (8 wk-old) were purchased from Shanghai Hengrui Pharmaceutical Co., Ltd.

[SYXK (Hu) 2017-0015, Shanghai, China]. Twenty-four mice were randomly allocated into four groups ($n = 6$), namely control group (mice were injected with saline), PD group [mice were intraperitoneally injected with MPTP (14 mg/kg, m0896, Sigma-Aldrich) every 2 h within 8 h], NaHS group (mice were intraperitoneally injected with 5.6 mg/kg NaHS) and PD + NaHS group (mice were intraperitoneally injected with 5.6 mg/kg NaHS 30 min before MPTP injection). All mice were raised in specific pathogen-free (SPF) grade environment with food and water. Two weeks after MPTP injection, three mice in each group were randomly selected for behavioral tests. The remaining three mice were sacrificed by cervical dislocation. Brain tissues were taken out and ground into homogenate for ELISA or western blot analysis.

2.11. Behavioral tests

The rotarod test, beam walk test, and grid walking test were conducted to evaluate the mouse behaviors according to the methods as reported by Yuan *et al.* [17].

2.12. Statistical analysis

The Statistical Package for the Social Sciences (SPSS) 21.0 (IBM Corp. Chicago, IL, USA) was applied for data analysis. According to Kolmogorov–Smirnov detection, the data are in normal distribution and expressed as mean \pm standard deviation. Differences between two groups were evaluated using the *t*-test while the differences among multiple groups were compared using one-way or two-way analysis of variance (ANOVA). Tukey's multiple comparisons test was used for the pairwise comparison after ANOVA analysis. The *p* value was obtained by a two-tailed test and $p < 0.05$ indicated a significant difference.

3. Results

3.1. H₂S protected SH-SY5Y cells induced by MPP~+

After the induction of SH-SY5Y cells by MPP~+ with different concentrations (100, 200, 300, 400,

500, 600, and 700 $\mu\text{mol/L}$), the optimal concentration of MPP~+ was selected as 500 $\mu\text{mol/L}$ (Figure 1a). Then, it was found that H₂S had protective effects on SH-SY5Y cells induced by MPP~+ after the pretreatment of different concentrations of H₂S donor NaHS (50, 100, 200, and 400 $\mu\text{mol/L}$). The optimal concentration of NaHS was 200 $\mu\text{mol/L}$ (Figure 1b). After 24 h of MPP~+ treatment, SH-SY5Y cells had neural retraction and membrane blistering under microscope; NaHS could reduce the cell morphological changes, while NaHS treatment alone did not change the cell morphology (Figure 1c). In addition, NaHS treatment reduced the apoptosis rate of SH-SY5Y cells induced by MPP~+ (Figure 1d/e) and the accumulation of cytotoxic substances MDA and 4-HNE (figure 1f), while NaHS treatment alone had no effect on the apoptosis rate and the accumulation of MDA and 4-HNE in SH-SY5Y cells. These results showed that H₂S could protect SH-SY5Y cells induced by MPP~+.

3.2. H₂S increased autophagy flux of SH-SY5Y cells damaged by MPP~+

Autophagy is essential for obtaining cell homeostasis and related to a variety of neurodegenerative diseases [5]. By tracing with MRFP-GFP-LC3, we found that autophagosome in SH-SY5Y cells induced by MPP~+ was significantly reduced and NaHS treatment reversed the trends (Figure 2a). Western blot analysis was used to detect the expressions of autophagy-related proteins (p62, LC3 and Beclin 1). The results showed that the expressions of LC3 II/I and Beclin1 decreased significantly (all $p < 0.05$) and the expression of p62 increased notably ($p < 0.05$) in SH-SY5Y cells induced by MPP~+, and these trends were reversed after NaHS treatment (Figure 2b).

3.3. Blocking autophagy flux reversed the protective effect of H₂S on SH-SY5Y cells induced by MPP~+

CQ is a lysosomal inhibitor. Under the action of CQ, the autophagosome of MPP~+ induced SH-SY5Y cells treated with NaHS reduced (Figure 3a), with decreased expressions of LC3 II/I and Beclin1 and

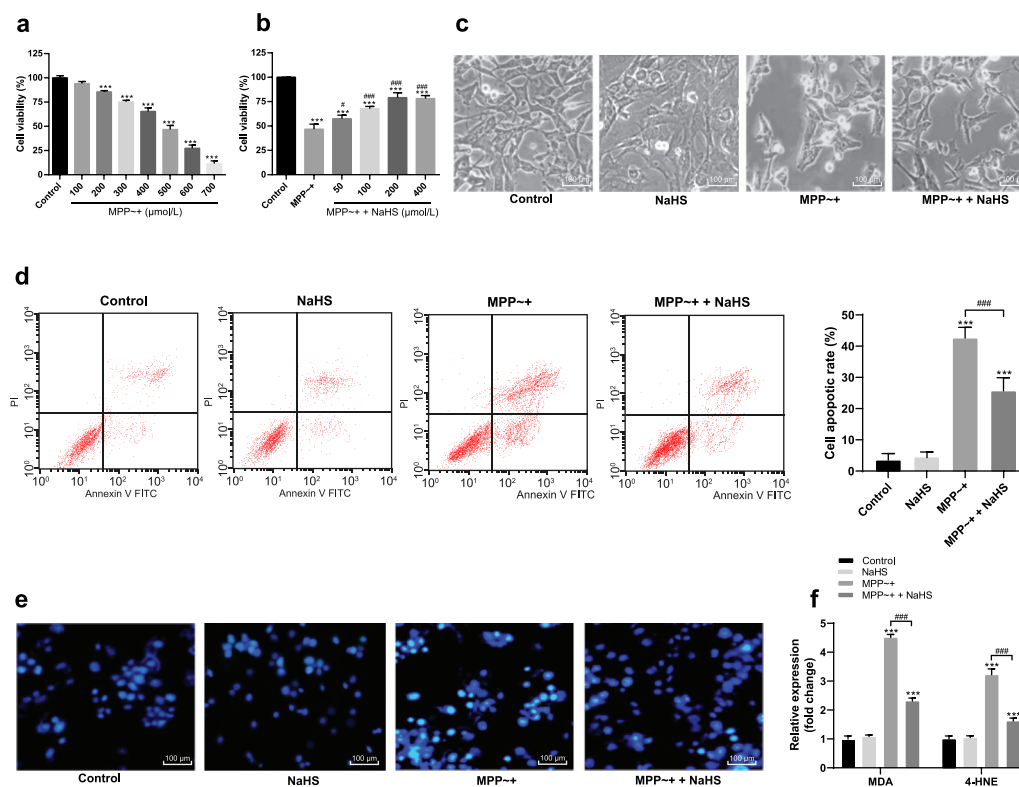


Figure 1. H₂S protected SH-SY5Y cells from MPP~+ damages. (a). SH-SY5Y cell viability treated with different concentrations of MPP~+ detected by MTT assay; (b). SH-SY5Y cell viability treated with different concentrations of MPP~+ + NaHS detected by MTT assay; (c). SH-SY5Y morphology observed under an optical microscope: SH-SY5Y cells had neural retraction and membrane blistering; NaHS could reduce the cell morphological changes, while NaHS treatment alone did not change the cell morphology; (d). cell apoptosis detected by flow cytometry; (e). cell apoptosis detected by Hoechst 33342 staining; (f). MDA and 4-HNE levels detected by ELISA. *** $p < 0.001$ vs. the control group, # $p < 0.05$, ### $p < 0.001$ vs. the MPP~+ group. Data in panels (a), (b), and (d) were analyzed by one-way ANOVA and data in panel (f) were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test for post hoc test. Repetitions = 3.

increased p62 expression (all $p < 0.05$) (Figure 3b). Compared with MPP~+ induced SH-SY5Y cells treated with NaHS, CQ significantly increased the SH-SY5Y cell apoptosis rate ($p < 0.05$) (Figure 3c/D), as well as the contents of MDA and 4-HNE ($p < 0.05$) (Figure 3e). It was suggested that blocking autophagy could reverse the protective effect of H₂S on MPP~+ induced SH-SY5Y cells.

3.4. H₂S increased the SIRT1 activity in SH-SY5Y cells induced by MPP~+ through mediation of SIRT1 sulfhydrylation

SIRT1 can improve autophagy flux, and the loss or mutation of SIRT1 can promote the pathological development of PD [18]. The expression of SIRT1 in MPP~+ induced SH-SY5Y cells decreased markedly compared with that of untreated SH-

SY5Y cells ($p < 0.05$), and increased after NaHS pretreatment ($p < 0.05$) (Figure 4a). The results of biotin switch assay revealed that H₂S increased the sulfhydrylation of SIRT1 in SH-SY5Y cells (Figure 4b). NaHS inhibited the expression of target genes of SIRT1 (p53 and NF- κ B p65) (Figure 4c), and reduced the levels of inflammatory factors in SH-SY5Y cells induced by MPP~+ (Figure 4d).

3.5. Sirtinol inhibited SIRT1 activity and reversed the effect of H₂S on autophagy flux of SH-SY5Y cells induced by MPP~+

After NaHS pretreatment, Sirtinol (an inhibitor of SIRT1) was added to SH-SY5Y cells to inhibit the activity of SIRT1 (Figure 5a), which reversed the effect of NaHS treatment on the autophagy flux of SH-SY5Y cells induced by MPP~+ (Figure 5b),

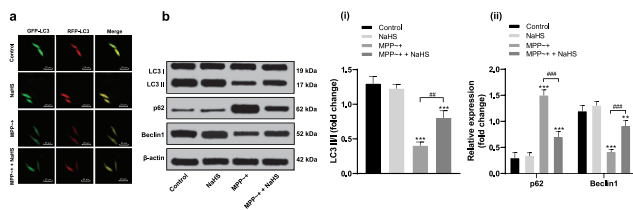


Figure 2. H₂S restored the damaged autophagy flux in SH-SY5Y cells induced by MPP~+. (a). mRFP-GFP-LC3 fluorescence tracing (LC3 fluorescence indicated the aggregation of autophagosome in cells); B. the expressions of autophagy-related proteins (p62, LC3 and Beclin 1) detected by Western blot analysis. ***p* < 0.01, ****p* < 0.001 vs. the control group; ###*p* < 0.001 vs. the MPP~+ group. Data in panel B(i) were analyzed by one-way ANOVA and data in panel B(ii) were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test for post hoc test. Repetitions = 3.

reduced the levels of LC3 II/I and Beclin1, and increased the expression of p62 (all *p* < 0.05) (Figure 5c/d).

3.6. Sirtinol reversed the protective effect of H₂S on SH-SY5Y cells induced by MPP~+

After SIRT1 inhibition, the apoptosis rate of SH-SY5Y cells induced by MPP~+ increased significantly (*p* < 0.05) (Figure 6a); the contents of MDA and 4-HNE in cells increased significantly (*p* < 0.05) (Figure 6b), and the levels of inflammatory-related factors increased (*p* < 0.05) (Figure

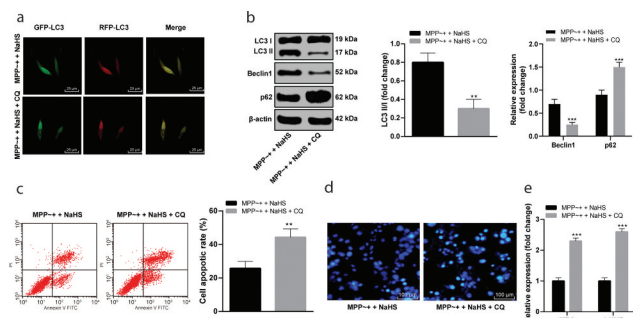


Figure 3. CQ blocked autophagy in SH-SY5Y cells. (a). mRFP-GFP-LC3 fluorescence tracing (fluorescence point of LC3 in SH-SY5Y cells decreased under the effect of CQ); (b). the expressions of autophagy-related proteins in the SH-SY5Y cells after CQ treatment detected by Western blot analysis; (c). the SH-SY5Y cell apoptosis rate detected by flow cytometry; (d). the apoptosis detected by Hoechst 33342 staining (the stronger the blue fluorescence was, the higher the apoptosis rate); (e). the levels of the MDA and 4-HNE measured by ELISA. ***p* < 0.01, ****p* < 0.001 vs. the MPP~+ + NaHS group. Data in panels B (i) and C were analyzed by *t*-test; data in panels B(ii) and E were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test for post hoc test. Repetitions = 3.

6c). It was suggested that Sirtinol reversed the protective effect of H₂S on SH-SY5Y cells induced by MPP~+.

3.7. H₂S increased the SIRT1 activity in SH-SY5Y cells induced by MPP~+ through sulfhydrylation to ameliorate PD in vivo

Later, a PD mouse model was established. After intraperitoneal injection of NaHS, the SIRT1 sulfhydrylation in the brain tissues of mice and SIRT1 activity increased (Figure 7a/b). Various behavior tests of mice showed that NaHS injection alleviated clinical symptoms of PD (Figure 7c-E), and reduced the levels of inflammatory factors in the brain tissues of PD mice (figure 7f).

4. Discussion

PD is the most ubiquitous neurodegenerative disease that influences the motor system with the incidence ranging from 5 to over 35 per 100,000 people worldwide [19]. H₂S has been widely considered as a toxic gas in the scientific community for over 300 y, but understanding of this small molecule has changed since H₂S is found to involve in the physiological and pathological mechanism of the brain [20]. SIRT1 was suggested to suppress the synuclein aggregate formation in

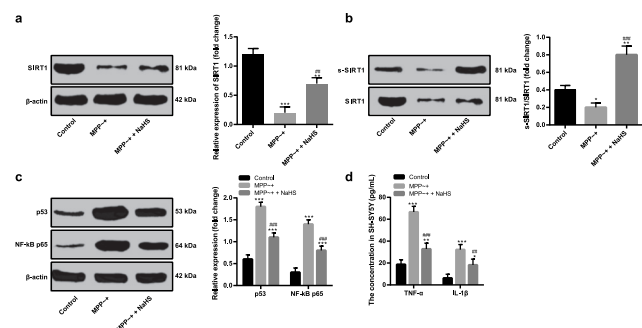


Figure 4. H₂S increased SIRT1 sulfhydrylation in SH-SY5Y cells. (a/c). the expressions of SIRT1, p53, NF-κB p65 in the SH-SY5Y cells detected by Western blot analysis; (b). the degree of SIRT1 sulfhydrylation in SH-SY5Y cells detected by biotin switch assay; (d). the levels of TNF-α and IL-1β detected by ELISA. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. the control group; ##*p* < 0.01, ###*p* < 0.001 vs. the MPP~+ group. Data in panels (a) and (b) were analyzed by one-way ANOVA; data in panels (c) and (d) were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test for post hoc test. Repetitions = 3.

PD and it was also correlated with H₂S in neuronal senescence and diabetes [14,15,21]. Herein, this study aims to investigate the roles of H₂S and SIRT1 in PD, and drew a conclusion that H₂S ameliorated SIRT1 activity through acceleration of SIRT1 sulphydration to increase the autophagy flux and attenuate damage of SH-SY5Y cells induced by MPP⁺.

Generally speaking, there are two releasing models of H₂S donors: instant-releasing and slow-releasing [22]. Our study uses NaHS as the donor of H₂S, belonging to the follower of the first model which has quite low relevance to a physiological situation. NaHS, an H₂S donor, was found to protect PC12 cells against MPP⁺-induced apoptosis and cytotoxicity [23]. First of all, we found that H₂S protected SH-SY5Y cells induced by MPP⁺ through increasing autophagy flux. NaHS reduced the apoptosis rate and the accumulation of cytotoxic substances MDA and 4-HNE, while increased the autophagy flux and expression of LC3 II/I and Beclin1 in SH-SY5Y cells induced by MPP⁺. Exogenous application of H₂S protected the SH-SY5Y cells against 6-hydroxydopamine-induced or rotenone-induced cell

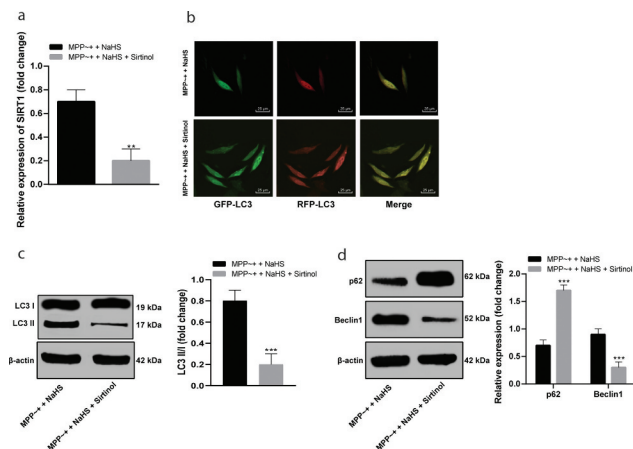


Figure 5. Sirtinol suppressed SIRT1 activity and affected autophagy flux in SH-SY5Y cells. (a/c/d). the expressions of SIRT1 and autophagy-related proteins in SH-SY5Y cells induced by MPP⁺ after Sirtinol treatment detected by Western blot analysis; (b). mRFP-GFP-LC3 fluorescence tracing (LC3 fluorescence point in SH-SY5Y cells decreased under the action of Sirtinol). ***p* < 0.01, ****p* < 0.001 vs. the MPP⁺ + NaHS group. Data in in panels (a) and (c) were analyzed by *t*-test; data in panel (d) were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test for post hoc test. Repetitions = 3.

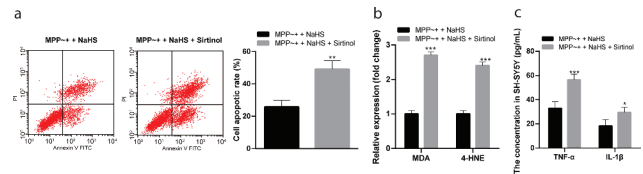


Figure 6. Sirtinol reversed the protective effect of H₂S on SH-SY5Y cells induced by MPP⁺. (a). cell apoptosis detected by flow cytometry; (b/c). the expressions of MDA, 4-HNE, TNF-α and IL-1β detected by ELISA. **p* < 0.05, ***p* < 0.01 vs. the MPP⁺ + NaHS group. Data in panel (a) were analyzed by *t*-test; data in panels (b) and (c) were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test for post hoc test. Repetitions = 3.

apoptosis and death [24]. In addition, inhaled NaHS or H₂S was presented to show neuroprotection in rodent models of PD induced by neurotoxins [10]. Exogenous H₂S increased the protein expression of Beclin1 and the ratio of LC3 II/I so as to promote autophagy blocked by oleic acid [25]. Shefa U *et al.* suggested that H₂S protects neurons against oxidative stresses through down-regulation of ROS and the aggregation of lipid peroxidation products [26]. There was a study also indicating that H₂S neuroprotection is, partly because of its anti-inflammation, antioxidation, and anti-apoptosis properties [27]. Now, more and more sulphydration targets are proved which provides new direction to known effects of H₂S by

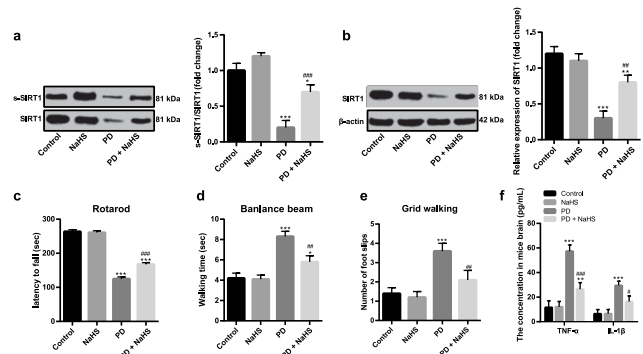


Figure 7. NaHS injection alleviated clinical symptoms of PD. (a). the degree of sulphydration in brain tissues of PD mice detected by biotin switch assay; (b). SIRT1 expression in brain tissues of PD mice detected by Western blot analysis; (c–e). results of rotarod test, beam walk test, and grid walking test; (f). TNF-α and IL-1β expression in brain tissues of PD mice detected by ELISA. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. the control group; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs. the PD group. Data in panels (a–e) were analyzed by one-way ANOVA and data in panel F were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test for post hoc test. *n* = 3.

shedding deeper sights on its biological functions [28]. Chloroquine, a potent inhibitor of autophagy, has been employed in human patients for a variety of purposes [29]. CQ blocks autophagy by preventing lysosomal acidification and autophagosome such as Beclin1 and LC3II/LC3I degradation and fusion [30,31]. The results in this study also revealed that CQ reversed the protective effect of H₂S on SH-SY5Y cells induced by MPP~+.

H₂S is a special SIRT1 activator through direct sulfhydrylation, and SIRT1 sulfhydrylation contributes to H₂S inhibition of endothelial inflammation [32]. Then, this study discovered that H₂S increased the SIRT1 activity in SH-SY5Y cells induced by MPP~+ through mediation of SIRT1 sulfhydrylation. Upregulation of hippocampal SIRT1 improved the role of H₂S in the cognitive function of rats treated by homocysteine, which also associated with inhibition of the endoplasmic reticulum stress [33–35]. Upregulation of SIRT1 also upregulated of antioxidant enzyme and improve the resistance of NaHS on oxidative stress injury induced by H₂O₂ [36]. Through promotion of signal transducers and activators of transcription 3 (STAT3) deacetylation regulated by SIRT1, H₂S could inhibit inflammatory hepatic hepcidin [37]. H₂S exerts a direct anti-apoptosis function in H9c2 cardiomyocytes through the SIRT1 signaling pathway [38]. H₂S is related to aging by suppressing free-radical reactions and activating SIRT1, and H₂S was revealed to own therapeutic potential in age-correlated diseases [39].

Besides, SIRT1 expression was reduced in SH-SY5Y cells induced by MPP~+ and PD mice. Inhibition of SIRT1 reversed the protective effect of H₂S on SH-SY5Y cells induced by MPP~+. SIRT1 is an effective mediator of autophagy degradation and mitotic signaling pathways, thereby blocking the α -synuclein toxicity in PD [40]. A study suggested that microRNA-200a induced down-regulation of SIRT1 to accelerate apoptosis of DA neurons, and contributed to pathogenesis of PD [41]. SIRT1 expression markedly reduced not only in primary culture neurons treated by neurotoxin MPP~+, environmental factor PD models, and MPTP-treated mice but also in the genetic factor PD models [42]. Reliable research

indicates that SIRT1 expresses in a lower level in dopaminergic neurons in PD model and protects neurons against neurotoxicity [13]. Autophagy can be triggered by SIRT1 overexpression, which is vital for nerve regeneration for its promotion of functional nerve reinnervation [43]. What's more, *in vivo* experiments were conducted. The results indicated that intraperitoneal injection of NaHS increased the activity of SIRT1 in the brain of PD mice. Various behavioral tests of mice showed that NaHS injection could alleviate clinical symptoms of PD.

In summary, it was uncovered that H₂S promoted SIRT1 activity through acceleration of SIRT1 sulfhydrylation to increase the autophagy flux and ameliorate damage of SH-SY5Y cells induced by MPP~+. Collectively, this article provides an overview of the physiological functions and effects of H₂S and SIRT1 on PD, and proposes the potential health and therapeutic benefits of H₂S and SIRT1. The main function of SIRT1 is acetylation, but the theme of our research is hydro-sulfidation. We will conduct a more in-depth and comprehensive research on SIRT1 acetylation in the future. On the other hand, there are certain limitations in using NaHS as the donor of H₂S. For example, the instant-releasing of H₂S fails to simulate the slow and continuous generation process of H₂S *in vivo* [44]. The instant-releasing of H₂S may result in inaccurate results and harmful effects on human body [45]. We can't use slow-releasing H₂S donors to perform our research in a short period of time due to the limitation of research conditions. Hence, the in-depth research on slow-releasing H₂S donors is also deemed essential in the future.

Disclosure statement

All authors declare that there is no conflict of interests in this study.

Funding

This work was supported by the Young and Middle-aged Innovation Fund of Second Hospital of Harbin Medical University [KYCX2018-13]; the Heilongjiang Postdoctoral Scientific Research Developmental Fund [LBH-Q18094].

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