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Full-length Article

Impaired CBS-H_2S signaling axis contributes to MPTP-induced neurodegeneration in a mouse model of Parkinson's disease

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ABSTRACT

Hydrogen sulfide (H_2S), a novel neuromodulator, is linked to the pathogenesis of several neurodegenerative disorders. Exogenous application of H_2S exerts neuroprotection via anti-inflammation and anti-oxidative stress in animal and cellular models of Parkinson's disease (PD). However, the role of endogenous H_2S and the contribution of its various synthases in PD remain unclear. In the present study, we found a decline of plasma and striatal sulfide level in 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced PD mouse model. Interestingly, among the three H_2S generating enzymes, only cystathionine β-synthase (CBS) expression was largely reduced in the striatum of MPTP-treated mice. The *in vitro* study confirmed a significant decrease of CBS expression in 1-methyl-4-phenylpyridinium (MPP^+)-stimulated astrocytes and microglia, but not in neurons or SH-SY5Y dopaminergic cells. Striatal CBS overexpression, elicited by stereotaxic delivery with *Cbs* gene using recombinant adeno-associated-virus (*rAAV-Cbs*), successfully enhanced the sulfide level in the striatum and partially rescued the MPTP-induced dopaminergic neurotoxicity in the midbrain. Specifically, striatal CBS overexpression alleviated the motor deficits and dopaminergic neuron losses in the nigro-striatal pathway, with a concomitant inhibition of glial activation in MPTP-treated mice. Furthermore, compared to *rAAV-Vector*, *rAAV-Cbs* injection reduced the aberrant accumulation of nitric oxide and 3-nitrotyrosine (an indicator of protein nitration) in the striatum of MPTP-treated mice. Notably, it also attenuated the increase of nitrated α-synuclein level in MPTP mice. The *in vitro* study demonstrated that lentivirus-mediated CBS overexpression elevated the sulfide generation in glial cells. Moreover, glial CBS overexpression offered protection to midbrain dopaminergic neurons through repressing nitric oxide overproduction in both glial and neuronal cells induced by MPP^+. Taken together, our data suggest that impaired CBS-H_2S axis may contribute to the pathogenesis of PD, and that modulation of this axis may become a novel therapeutic approach for PD.

1. Introduction

Parkinson's disease (PD) is a common degenerative movement disorder, characterized by the loss of nigrostriatal dopaminergic neurons and formation of cytoplasmic inclusion body that is largely comprised of α-synuclein (α-syn). Microglia activation and inflammatory mediators have been detected in the brains of PD patients and animal models (Gerhard et al., 2006; McGeer et al., 1988). However, its etiology remains elusive. Several pathogenic factors such as mitochondria dysfunction, neuroinflammation, circadian dysfunction, and autophagy impairment are considered to participate in PD pathogenesis (Li et al., 2017; Winslow et al., 2010). To date, no disease-modifying treatment is available. Therefore, extensive and intensive studies on the pathogenesis of PD are needed.

Hydrogen sulfide (H_2S) is the third member of the gasotransmitter family. It is endogenously synthesized by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST). Other non-

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enzymatic sources such as the gut microbes also contribute to H_2S generation. It regulates a variety of physiologic and pathologic processes such as blood pressure, inflammation and redox signaling (Hu et al., 2011). It also serves as a neuromodulator in the nervous system (Abe and Kimura, 1996), and dysregulation of its metabolism may participate in neurodegeneration. Exogenous H_2S was demonstrated to exert neuroprotection in different rodent models of PD, acting by resolving inflammation and oxidative stress (Hu et al., 2010; Kida et al., 2011). Moreover, a PD-related protein PARKIN is regulated by sulfhydration, a post-translational modification mediated by H_2S (Vandiver et al., 2013). Sulfhydration occurs in the same residue but functions in an opposite way of nitrosylation. Sulfhydration increases while nitrosylation decreases the catalytic activity of PARKIN. PD is associated with mutations in *PARK2* (human PARKIN encoding gene) that abolish PARKIN sulfhydration, which is diminished in the postmortem brain of PD patients. All these studies indicate a possible role of H_2S in PD pathogenesis.

However, measurement of H_2S generation in PD patient is lacking. And few studies examine the role of endogenous H_2S in PD using genetic approaches although the beneficial effect of exogenous H_2S has been reported. Moreover, the commonly used H_2S donor NaHS causes a rapid release of H_2S in aqueous solution, which may not mimic the biologically synthesized H_2S and its regulation *in vivo*. These may limit our understanding of endogenous H_2S function in PD. Therefore in this study we examined the possible changes of H_2S and its synthases, and explored the role of endogenous H_2S, based on genetic modulation, in MPTP-induced PD mouse model.

Here we report a decline of striatal and plasma H_2S level in MPTP-induced PD model, along with CBS downregulation in the striatum and glial cells. Overexpression of CBS via recombinant adeno-associated-virus (rAAV) delivery of *Cbs* gene into the striatum was found to protect against dopaminergic neuron degeneration and motor deficits induced by MPTP. Moreover, CBS overexpression that resulted in an elevation of H_2S production, inhibited the neuroinflammatory response and nitrated modification of α-syn in the striatum of MPTP-treated mice. Thus, genetic or pharmacologic modulation of CBS-H_2S signaling may have therapeutic implications for PD.

2. Materials and methods

2.1. Reagents and antibodies

MPTP (M0896) and MPP^+ (D048) were obtained from Sigma, USA. rAAV and lentiviral vector used in this study were provided by Obio Technology (Shanghai, China).

The primary antibodies were listed as follows: anti-CBS (Santa Cruz, H300-67154, USA), anti-3-MST (Sigma, HPA001240, USA), anti-CSE (Abnova, H00001491-M01, Taiwan), anti-tyrosine hydroxylase (anti-TH, Sigma T1299, USA), anti-Flag (Immunoway, YG0004, USA), anti-neuronal nitric oxide (NO) synthase nNOS (anti-nNOS, BD 610308, USA), anti-nitro-α-syn (Thermo scientific, MA5-16142, USA), anti-nitrotyrosine (Milipore, MAB5404, USA), anti-Iba-1 (Abcam 5076 for western blot; Wako 019-19741 for immunostaining), anti-β-actin (Sigma, A3584, USA), anti-β-tubulin (Sigma, T0198, USA), and anti-GFAP (DAKO, z033429-2, Japan). All the secondary antibodies for western blot were from Jackson lab (USA).

2.2. Animals and treatment

C57/BL6 mice (8-week-old male) were purchased from Shanghai Laboratory Animal Center (China) and housed in a SPF-grade animal room with food and water ad libitum. *rAAV2/9* was used for transgene expression of mouse *Cbs* under the control of the CAG promoter. To explore the possible role of CBS in PD pathogenesis, *rAAV-Cbs-3FLAG* (1.28E+10 v.g.) or the corresponding vector *rAAV-3FLAG* were bilaterally injected into the striatum (coordinates relative to bregma: AP + 0.4 mm; ML ± 2 mm; DV −3.5 mm) via stereotaxic apparatus. On the 4th day after surgery, MPTP (14 mg/kg) or saline was intraperitoneally (i.p.) injected at 2-h intervals over an 8-h period. All experimental procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee of Soochow University.

2.3. Cell cultures

Glia-enriched primary cultures were prepared from newborn mice as previously described (Hu et al., 2007). In brief, the midbrains were dissected, minced and trypsinized in Hank's solution after removing meninges. Tissues were filtered through a 200-μm mesh and plated in flasks. The culture media were replaced every three days. Once reaching confluence, microglia were shaked off by mechanical agitation, collected and seeded for further study. The lower layer that remains in the culture flask was deemed as astrocytes-enriched culture and used for further study.

Midbrain neuron culture was prepared from embryonic day 12–13 C57/BL6 mice. In brief, ventral mesencephalon was dissected and minced in phosphate buffered saline (PBS). Tissues were then trypsinized at 37 °C for 10 min. Next, trypsin was neutralized by DMEM/F12 medium with 20% fetal bovine serum (FBS). After passing through 200 μm mesh filters, cells were seeded at a density of 6.5×10^5 per cm^2 on poly-L-ornithine/laminin-coated coverslips with 2% B27 and 1% glutmax neurobasal medium. Every three days half of the culture media were changed. The cultures were maintained at 37 °C with 5% CO_2 and 95% oxygen in an incubator and used for study at about ten days later.

Human neuroblastoma SH-SY5Y cells and PC12 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin in an incubator. MES23.5 cells were grown in DMEM/F12 supplemented with 5% FBS, 1% penicillin/streptomycin and Sato's chemically defined medium to a final concentration of 5 μg/ml insulin, 5 μg/ml transferring, 48.6 μg/ml pyruvic acid, 6.3 ng/ml progesterone, 5 ng/ml sodium selenite, and 4 μg/ml putrescine at 37 °C in an incubator.

2.4. Behavioral tests

Behavioral tests were performed two weeks after MPTP injection by the experimenters blindly to the treatments.

2.4.1. Rotarod test

Rotarod test was conducted to evaluate the motor coordination ability of the mice. The apparatus consists of a four-lane rotating rod (1.25 inch in diameter), which allows us to test four mice at a time. The mice were trained twice daily for two successive days (12 rpm on the first day and 14 rpm on the second day) before the test. The rotational speed was increased to 20 rpm on the third day in a test session. The time to stay on the rotating bar was recorded. Each mouse was subjected to three trials, at 10 min intervals with a maximum of 5 min per trial. Once the mouse fell off the rod, the recorder was stopped. The motor coordination ability was estimated according to the average time that each mouse held on the rotating rod during three trials.

2.4.2. Beam walk

Motor coordination and balance was also evaluated by beam walking test. Briefly, each mouse was placed on a batten and tempted with fodder to cross a wooded balance beam (64 cm long, 1.5 cm wide, 15 cm high). If a mouse failed, the test was stopped

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