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Full paper

Polysulfide protects midbrain dopaminergic neurons from MPP^+-induced degeneration via enhancement of glutathione biosynthesis

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ABSTRACT

Polysulfides are endogenous sulfur-containing molecular species that may regulate various cellular functions. Here we examined the effect of polysulfides exogenously applied to rat midbrain slice cultures, to address their potential neuroprotective actions. Na_2S_3 at concentrations of 10 μM or higher prevented 1-methyl-4-phenylpyridinium (MPP^+)-induced loss of dopaminergic neurons. Na_2S_4 at 10 μM also protected dopaminergic neurons from MPP^+ cytotoxicity, whereas Na_2S and Na_2S_2 at the same concentration had no significant effect. We also found that Na_2S_3 (10 μM) prevented MPP^+-induced increase in intracellular reactive oxygen species as detected by 2′,7′-dichlorofluorescein fluorescence. In addition, the protective effect of Na_2S_3 was abolished by L-buthionine sulfoximine, an inhibitor of glutathione synthesis. In cellular models of neurons (SH-SY5Y cells) and glial cells (C6 cells), Na_2S_3 (30 and 100 μM) increased expression of mRNAs encoding the subunits of glutamate cysteine ligase, the rate-limiting enzyme for glutathione biosynthesis. Consistently, the cellular content of total glutathione was increased by Na_2S_3, and the effect was more prominent in SH-SY5Y cells than in C6 cells. These results suggest that polysulfides are efficient neuroprotectants superior to monosulfur species such as H_2S and HS^-, and that the neuroprotective effect of polysulfides is mediated by upregulation of glutathione biosynthesis.

1. Introduction

Parkinson disease (PD) is characterized by progressive and selective loss of dopaminergic neurons in the midbrain substantia nigra.[1] Among various factors considered to participate in the pathogenic events, mitochondrial dysfunction has been known as an important cause of degeneration of dopaminergic neurons.[2] 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is commonly used for reproducing PD pathology in experimental animals. MPTP is converted to 1-methyl-4-phenylpyridinium (MPP^+), which interferes with mitochondrial respiration via inhibition of complex I.[1] Mitochondrial dysfunction is accompanied by generation of reactive oxygen species (ROS), and increased oxidative stress plays a major role in the pathogenesis of PD.[3,4]

On the other hand, glutathione (GSH) constitutes the endogenous antioxidative system.[3] Notably, the substantia nigra of PD patients contains smaller amount of GSH than that of healthy controls.[5] Both neurons and glial cells can synthesize GSH in the brain.[6] Formation of γ-glutamylcysteine is catalyzed by glutamate cysteine ligase (GCL), and then, formation of GSH is catalyzed by glutathione synthase.[7] GCL is considered the rate-limiting enzyme, which is composed of catalytic subunit (GCLC) and modifier subunit (GCLM).[7] Expression of GCLC and GCLM is regulated by antioxidant-response element (ARE),[8] and nuclear factor erythroid 2-related factor 2 (Nrf2) binds to ARE to activate transcription.[9] Either depletion of GSH by an irreversible GCL inhibitor L-buthionine sulphoximine (BSO) or deficiency of Nrf2 accelerates degeneration of dopaminergic neurons in mice.[10,11]

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Hydrogen sulfide (H_2S) has been receiving increasing attention[12–14] and is now recognized as a signaling molecule in mammalian tissues.[15,16] Several enzymes such as cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfur transferase along with cysteine aminotransferase have been identified as components of biosynthetic pathways for H_2S.[17] Interestingly, recent reports indicate that H_2S exists in mammalian tissues as forms of persulfide (RSSH) or polysulfides (RSS_nH, RSS_nSR).[12,17] Indeed, persulfide and polysulfides can be generated from H_2S in the presence of oxygen.[18,19] CBS and CSE can produce persulfide and polysulfides from L-cysteine.[20] These facts imply that some of the biological actions of H_2S may actually be mediated by other molecular species. For example, H_2S modulates transient receptor potential ankyrin 1 (TRPA1) channels,[21] but polysulfides modulate TRP channel activity in rat astrocytes more potently than H_2S.[22]

H_2S affords neuroprotection in neurodegeneration models induced by oxidative stress,[23–25] but whether polysulfides produce superior neuroprotective actions as compared to H_2S is unknown. We set out for the present study to address the neuroprotective effect of polysulfides on midbrain dopaminergic neurons.

2. Materials and methods

2.1. Organotypic midbrain slice cultures

Procedures involving experimental animals were approved by Kumamoto University ethics committee on animal experiments and were conformed to the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society. Organotypic midbrain slice cultures were prepared according to the methods described previously.[26] Briefly, brain hemispheres obtained from 2–3-day-old neonatal Wistar rats (Nihon SLC, Shizuoka, Japan) were cut into coronal slices with 350 μm thickness. Midbrain slices were transferred onto microporous membranes (Millicell-CM, Merck Millipore, Darmstadt, Germany) in six-well plates. Culture medium, consisting of 50% minimum essential medium/HEPES, 25% Hanks' balanced salt solution and 25% heat-inactivated horse serum (Life Technologies, Tokyo, Japan) supplemented with 6.5 mg/ml glucose, 2 mM L-glutamine and 100 U/ml penicillin-G/100 μg/ml streptomycin, was supplied at a volume of 700 μl per well. The culture medium was exchanged with fresh medium on the next day of culture preparation, and thereafter, every 2–3 d. Slices were maintained in a 34 °C, 5% CO_2 humidified atmosphere.

At 19–20 d in vitro, slices were treated with drug-containing serum-free medium. Serum-free medium consisted of 75% minimum essential medium and 25% Hanks' balanced salt solution supplemented with 6.5 mg/ml glucose, 2 mM L-glutamine and 10 U/ml penicillin-G/10 μg/ml streptomycin.

MPP^+ and ML385 were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium sulfide (Na_2S), sodium disulfide (Na_2S_2), sodium trisulfide (Na_2S_3) and sodium tetrasulfide (Na_2S_4) were obtained from Dojindo (Kumamoto, Japan). BSO was obtained from Wako Pure Chemicals (Osaka, Japan).

2.2. Immunohistochemistry

Slices were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer containing 4% sucrose at 4 °C for 2 h, and processed for tyrosine hydroxylase (TH) immunohistochemistry.[26] Rabbit anti-TH polyclonal antibody (1:500, Merck Millipore) was used as a primary antibody, and biotinylated anti-rabbit IgG from goat (1:200, Vector Laboratories, Burlingame, CA, USA) was used as a secondary antibody. After secondary antibody incubation, cultures were treated with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories), and then peroxidase was visualized with diaminobenzidine and H_2O_2. TH-positive cells possessing round or oval cell bodies and dendrite of twice as long as cell diameter were considered as viable dopaminergic neurons. The number of dopaminergic neurons in an area of 570 μm × 750 μm in individual slices was counted.

2.3. Measurement of intracellular ROS

After drug treatment, slices were washed with serum-free medium and loaded with 30 μM 2′,7′-dichlorodihydrofluorescein diacetate for 30 min at 34 °C. Images of fluorescence of 2′,7′-dichlorofluorescein (DCF) were taken by BIOREVO (KEYENCE Japan, Osaka, Japan). Fluorescence intensity of individual slices was determined by using NIH Image J software.

2.4. Cell culture

Human neuroblastoma SH-SY5Y cell line and rat astrocytoma C6 cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). SH-SY5Y cells were maintained in DMEM/F-12 medium (1:1) supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 U/ml penicillin-G/100 μg/ml streptomycin, at 37 °C in an atmosphere of 5% CO_2. C6 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 U/ml penicillin-G/100 μg/ml streptomycin, at 37 °C in an atmosphere of 5% CO_2. Before drug treatment, SH-SY5Y cells and C6 cells at 70–80% confluency were exposed for 30 min to serum-free DMEM/F-12 medium and DMEM medium, respectively.

2.5. RNA isolation and quantitative real-time reverse transcription polymerase chain reaction

Total mRNA was isolated from SH-SY5Y cells and C6 cells by RNAiso Plus® (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. Reverse transcription was performed with PrimeScript® RT Master Mix (TaKaRa). Real-time polymerase chain reaction (PCR) was performed with SYBR® Premix Ex Taq II™ (TaKaRa) on a CFX Connect™ real-time PCR analysis system (BioRad, Tokyo, Japan). The thermal cycling program consisted of one cycle at 95 °C for 30 s, and then 40 cycles at 95 °C for 15 s, at 60 °C for 45 s, at 72 °C for 30 s. The primer sequences were as listed in Table 1. Data were analyzed by the comparative threshold cycle method.

2.6. Western blotting

Western blotting of proteins in whole cell lysates of SH-SY5Y cells was performed as described previously,[27] with mouse anti-GCLC antibody (1:1000, Abnova, Taipei City, Taiwan), rabbit anti-GCLM antibody (1:1000, GeneTex Inc., Irvine, CA, USA), and mouse anti-β-actin (1:5000, Sigma). After incubation with

Table 1
Primers used for quantitative RT-PCR.

Gene name	Primer sequences
GAPDH	forward 5′-ACCATCTTCCAGGAGCGAGA-3′ reverse 5′-CAGTCTTCTGGGTGGCAGTG-3′
human GCLC	forward 5′-TGAGATTTAAGCCCCCTCCT-3′ reverse 5′-TTGGGATCAGTCCAGGAAAC-3′
human GCLM	forward 5′-TTTGGTCAGGGAGTTTCCAG-3′ reverse 5′-ACACAGCAGGAGGCAAGATT-3′
rat GCLC	forward 5′-TTACCGAGGCTACGTGTCAGAC-3′ reverse 5′-TGTCGATGGTCAGGTCGATGTC-3′
rat GCLM	forward 5′-AATCAGCCCTGATTTGGTCAGG-3′ reverse 5′-CCAGCTGTGCAACTCCAAGGAC-3′

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