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Polysulfide promotes neuroblastoma cell differentiation by accelerating calcium influx

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ABSTRACT

Polysulfides are a typical type of bound sulfur, which is physiologically stable form of sulfur species, derived from the hydrogen sulfide (H_2S) that is generated endogenously in cells. We previously reported that bound sulfur protects neuronal cells from oxidative injury. In the present study, we demonstrated that polysulfides inhibited cell growth and promoted neurite outgrowth in mouse neuroblastoma Neuro2A (N2A) cells. However, Na_2S showed no effect on neurite outgrowth in N2A cells. Furthermore, 2-APB and SKF96365, which are typical transient receptor potential (TRP) channel inhibitors, suppressed the neurite outgrowth induced by Na_2S_4. These new findings suggest that bound sulfur could induce neurite outgrowth and cell differentiation of N2A cells by accelerating calcium influx.
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1. Introduction

Bound sulfur species (polysulfides, persulfide, elemental sulfur, etc.) are known storage forms of hydrogen sulfide (H_2S) in mammalian tissues [1–3] (Fig. 1A). It has been suggested that H_2S is produced from L-cysteine by three enzymatic pathways in mammalian tissues, cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and cysteine aminotransferase (CAT)/3-mercaptopyruvate sulfurtransferase (3MST) [4–6]. Additionally, a previous study revealed a novel pathway of H_2S production from D-cysteine consisting of D-amino acid oxidase (DAO) and 3MST [7]. Recently, many studies that examine the physiological roles of H_2S have been reported [8].

In particular, studies of H_2S in the central nerve system are very active. H_2S was first found to induce hippocampal long-term potentiation (LTP) by enhancing the activity of NMDA receptors [8]. Furthermore, Kimura and coworkers found that H_2S protects neuronal cells from oxidative damage [9] and induces calcium influx in astrocytes, which is mediated by activating transient receptor potential (TRP) channels [10]. In addition, a recent study indicated that endogenous H_2S is stored as "bound sulfur" in mammalian tissues [1]. Stipanuk et al. examined the possible existence of active reduced sulfur as a stored form of sulfide generated from the desulfurization pathway, the use of which depends on the physiological reaction in mammalian tissues [11]. Although, Westley et al. proposed "sulfane sulfur" as a labile and highly reactive sulfur atom covalently bound to another sulfur atom with an oxidation state of 0 or -1 [12], Ogasawara et al. redefined the unclear term "sulfane sulfur" as bound sulfur that is rapidly liberated as sulfide by reduction with dithiothreitol (DTT) and established a novel method to determine bound sulfur [1]. Although studies of endogenous H_2S have recently increased, the physiological function of bound sulfur still remains poorly understood. However, more recent studies have shown that the reactivity of polysulfides with the transient receptor potential ankyrin 1 (TRPA1) channel is 320 times more potent than that of H_2S in astrocytes in the rat brain [13]. Additionally, we revealed that polysulfides protect neural cells from oxidative damage through the activation of the Nuclear factor erythroid-2-related factor-2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) system [3]. Furthermore, recent studies have indicated that functions which have been attributed to H_2S in many previous reports may be mediated by

Abbreviation: N2A, neuro2a; TRP, transient receptor potential; Nrf2, nuclear factor erythroid-2-related factor-2; Keap1, kelch-like ECH-associated protein 1; RA, retinoic acid; CSE, cystathionine γ-lyase; CBS, cystathionine β-synthase; CAT, cysteine aminotransferase; 3MST, 3-mercaptopyruvate sulfurtransferase; DAO, D-amino acid oxidase; 2-APB, 2-aminoethoxydiphenyl borate.

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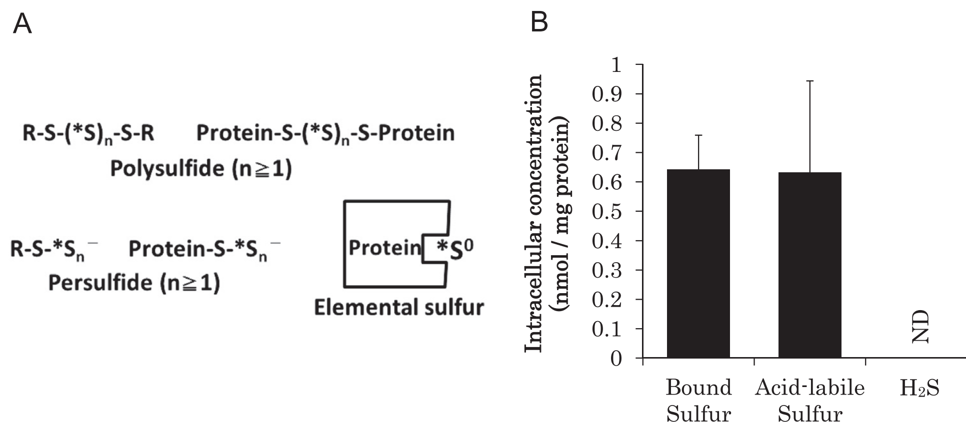

Fig. 1. Bound sulfur concentration in N2A cells. (A) Various types of physiological bound sulfur species. (B) Bound sulfur, acid-labile sulfur and free hydrogen sulfide were measured in N2A cells as described in Section 2. Values indicate means ± S.D. (n = 3).

bound sulfur species, such as polysulfides [14]. Thus, the physiologically relevant functions of bound sulfur have been recognized.

In this study, to show a novel role of bound sulfur in neuronal cells, we examined the effect of polysulfides on the induction of neurite outgrowth and neuronal differentiation in N2A cells.

2. Materials and methods

2.1. Chemicals

Sodium tetrasulfide (Na_2S_4 (99%)) was obtained from Kojundo Chemical lab. Co. (Saitama, Japan), sodium sulfide nonahydrate (Na_2S $9H_2O$ (>98%)) was purchased from Wako Pure Chemical Co. (Osaka, Japan), and 2-aminoethoxydiphenyl borate (2-APB) and all-trans retinoic acid (RA) were purchased from Sigma (St. Louis, MO, USA). Na_2S_4 solution was freshly prepared and used in a day. 2-APB was dissolved in MilliQ grade water. Na_2S was dissolved in phosphate-buffered saline. SKF96365 and RA were dissolved in dimethyl sulfoxide (DMSO).

2.2. Cell culture

Mouse neuroblastoma Neuro2A (N2A) cells were purchased from the ATCC. N2A cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 25 U/ml penicillin, 25 μg/ml streptomycin and 10 mM HEPES (pH 7.4) and were maintained at 37 °C and 5% CO_2.

2.3. Cell treatment

N2A cells were treated with Na_2S_4 as described previously [3]. Na_2S_4 was dissolved in MilliQ water. Briefly, N2A cells were seeded on 6-well tissue culture plates at a density of 0.5×10^4 cells/cm^2 in DMEM containing 10% FBS. The next day, the medium was changed to DMEM containing 2% FBS, and the cells were treated with the indicated concentrations of Na_2S_4 for the indicated times. The control group was treated with vehicle only.

2.4. Determination of bound sulfur

The intracellular level of bound sulfur was measured by a method described previously [3]. Briefly, N2A cells treated with Na_2S_4 were washed with ice-cold PBS and resuspended in lysis buffer [10 mM potassium phosphate buffer (pH7.4), 0.5% Triton X-100, protease inhibitor cocktail complete (EDTA free, Roche Diagnostics), 10 mM hydroxylamine, and 10 mM benzoic acid]. The cell lysate was centrifuged at 4 °C at 12,000 g for 10 min, and then the supernatant was recovered. For the measurement of H_2S released from bound sulfur species, the supernatant was added to an equivalent amount of 15 mM DTT in 0.2 M Tris–HCl (pH 9.0) in a 15-ml centrifugation tube, sealed with Parafilm and then incubated at 37 °C for 50 min. After adding 0.2 ml of 1 M sodium citrate buffer (pH 6.0), the mixtures were incubated with shaking at 125 rpm on a rotary shaker (NR-3, Taitec, Tokyo, Japan) at 37 °C for 10 min. The bound sulfur level was calculated by a calibration curve obtained using Na_2S as a standard.

2.5. Cell growth assay

N2A cells treated with Na_2S_4 or vehicle were harvested, and viable cells were counted with a hemocytometer after staining with trypan blue.

2.6. Measurement of neurite outgrowth

Neurite outgrowth was observed under a phase-contrast light microscope (Olympus IX71, Olympus Optical Co. Ltd, Tokyo, Japan or Leica DMI4000B microscope, attached to a Leica DFC340 FX digital camera, GmbH, Wetzlar, Germany) with a 10 × or 20 × objective. Neurites were identified as cell processes greater than two cell body diameters in length. The percentage of cells bearing neurites was calculated by counting 180 cells in six randomly chosen fields per well. Neurite length was defined as the distance from the cell body to the tips of neurites. The length of the longest neurite was measured in at least 50 cells in five randomly chosen fields using ImageJ software. Each experiment was repeated three times.

2.7. Measurement of intracellular calcium

Intracellular calcium responses were measured using Calcium Kit Fluo 4 (Dojindo, Kumamoto, Japan) according to the manufacture's protocol. Briefly, N2A cells were treated with Na_2S_4 for 72 h in the absence or presence of 2-APB. After incubation, the medium was washed out and then cells were loaded with the florescent calcium indicator Fluo 4-AM in loading buffer containing 0.04 % Pluronic F-127, 1.25 mmol/l Probenecid at 37 °C for 1 h in the dark. After second incubation, loading buffer was removed and replaced by recording medium containing 1.25 mmol/l Probenecid, Hoechst 33342 for total cell demarcation and the cells incubated at 37 °C for

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