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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.

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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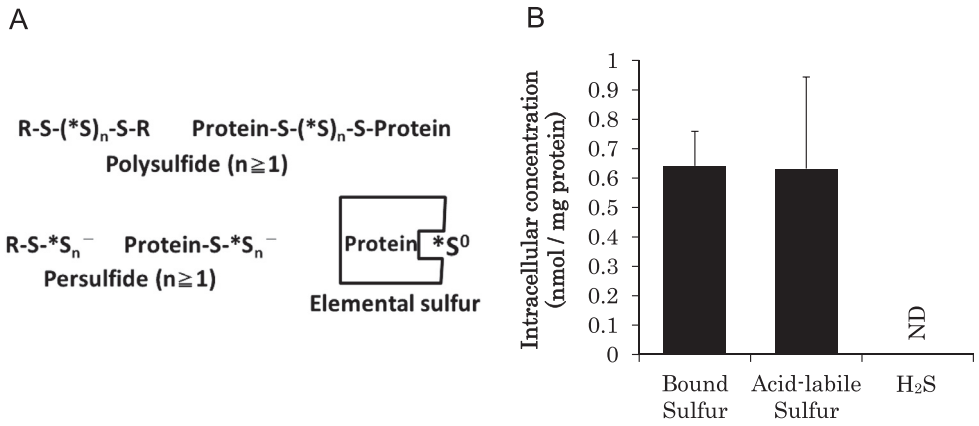

Fig. 1. Bound sulfur concentration in N2A cells. (A) Various types of physiological bound sulfur species. (B) Bound sulfur, acid-labile sulfur and free hydrogen sulfide were measured in N2A cells as described in Section 2. Values indicate means ± S.D. (n = 3).

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

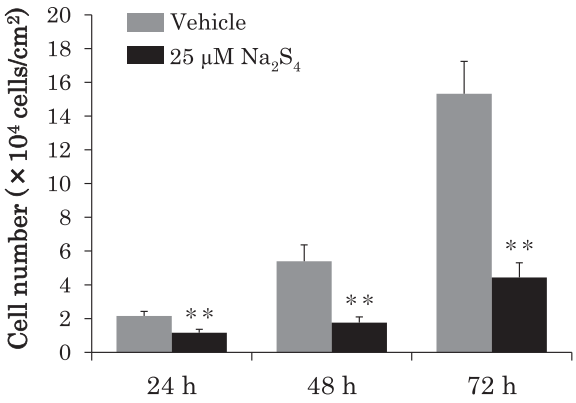

Fig. 2. Na_2S_4 suppresses cell growth in N2A cells. Cells were treated with or without 25 μM Na_2S_4 for the indicated time periods in DMEM supplemented 2% FBS. Cell numbers were counted as described in Section 2. Values indicate means ± S.D. $^{**}P < 0.01$ (n = 6).

30 min in the dark. Plates were scanned for fluorescent determination using IN Cell Analyzer 2200 (GE Healthcare). Images were acquired in nine fields per well at 20 × magnification in combination with two detection channels. Hoechst 33342 was visualized in the blue channel (4′,6-diamidino-2-phenylindole filter) while Fluo-4 was visualized in the green channel (FITC filter). The percentage of Fluo-4 positive cells for each image was calculated as follows: the percentage of Fluo-4 positive cells = (number of Fluo-4 positive cells/number of Hoechst 33342 stained cells) × 100. The percentage of Fluo-4 positive cells were normalized to vehicle control.

2.8. Statistical analysis

Values are presented as the mean ± S.D or ± S.E.M. The data were evaluated using Student's *t*-test ($p < 0.05$ was considered a statistically significant difference).

3. Results and discussion

3.1. An additional low level of polysulfide promotes neurite outgrowth of N2A cells

The mouse neuroblastoma cell line N2A is widely used to study the mechanism of neuronal differentiation. N2A cells cease to proliferate and can be induced to differentiate, as shown by neurite outgrowth [15]. For example, serum withdrawal or all-trans retinoic acid (RA) addition are well known induction events that cause neurite outgrowth resulting in the differentiation of N2A cells into neuronal cells [16,17]. However, it is unclear whether bound sulfur effects neurite outgrowth and cell differentiation.

In the present study, we first determined the level of bound sulfur, acid-labile sulfur derived from iron–sulfur clusters, and free H_2S in N2A cells. Free H_2S was not detected by our method in N2A cells, while bound sulfur existed in approximately 0.65 nM/mg of protein (=approximately 10 μM) (Fig. 1B).

We treated N2A cells with 25 μM Na_2S_4 in culture medium containing 2% FBS. As shown in Fig. 2, when N2A cells were cultured

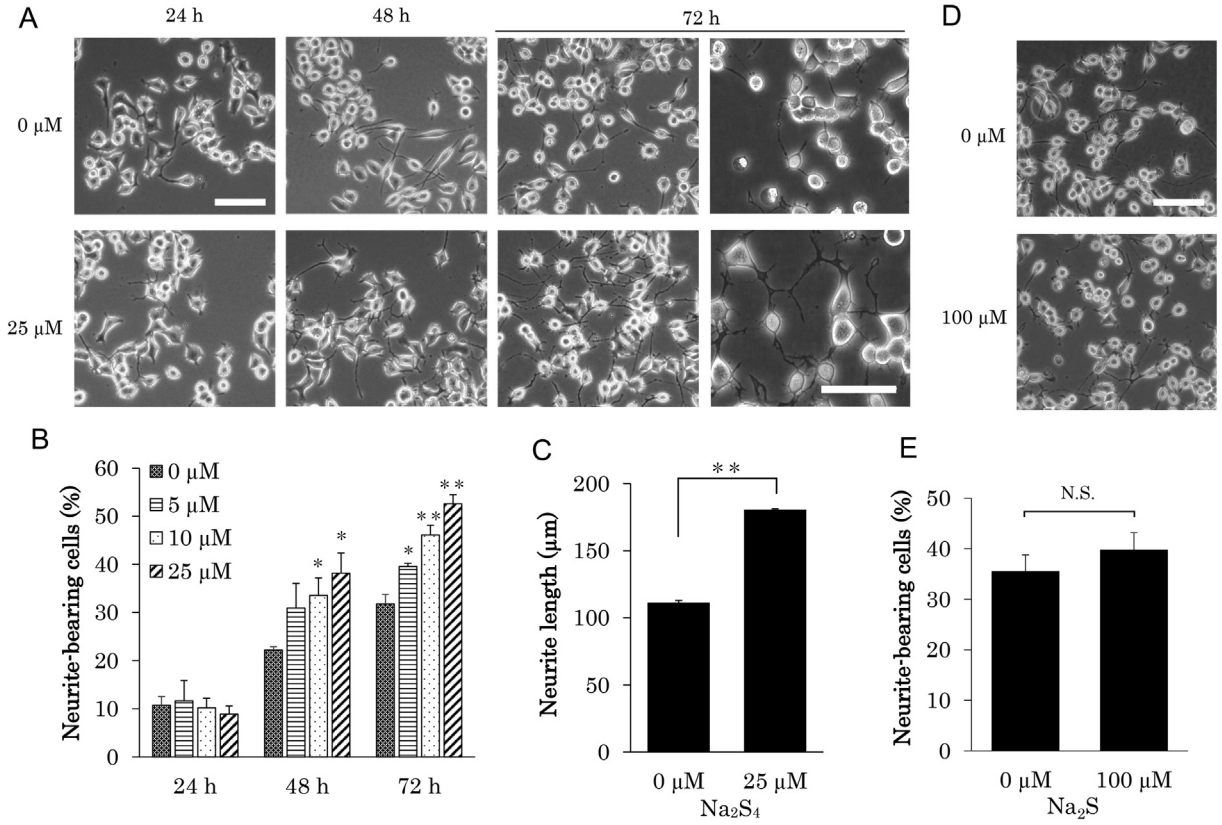

Fig. 3. Na_2S_4 promotes neurite outgrowth in N2A cells. (A, B, C) Cells were treated with the indicated concentration of Na_2S_4 for the indicated time periods in DMEM supplemented with 2% FBS. (A) Images show typical fields of cells observed using light microscopy. (B) The number of neurite-bearing cells and (C) neurite length were analyzed as described in Section 2. (D, E) Cells were treated with or without Na_2S for 72 h in DMEM supplemented with 2% FBS. (D) Images show typical fields of cells observed using light microscopy. (E) Neurite-bearing cells were analyzed as described in Section 2. Scale bar: 50 μm. Values indicate means ± S.E.M. N.S.: not significant $^{*}P < 0.05$ and $^{**}P < 0.01$.

in the presence of 25 μM Na_2S_4 for 24 h, 48 h, and 72 h, cell growth was significantly suppressed compared to untreated cells. To investigate the dose and time responses of polysulfide treatment on neurite outgrowth in N2A cells, we added Na_2S_4 (5, 10, and 25 μM) to N2A cells for 24, 48, and 72 h in 2% FBS-containing culture medium. Although neurite outgrowth in untreated cells cultured in 2% FBS-containing medium was little facilitated by the withdrawal of FBS, treatment with 25 μM Na_2S_4 for 72 h significantly increased the number of neurite-bearing cells compared to untreated cells (Fig. 3A and B). Additionally, treatment with Na_2S_4 for 72 h remarkably extended neurite length compared to untreated cells (Fig. 3A and C). Because increases in neurite outgrowth are considered evidence of differentiation in neuroblastoma cells, these results indicate that polysulfides significantly induce cell differentiation of N2A cells.

A previous report demonstrated that high concentrations of H_2S treatment (NaHS, 1.5–13.5 mM) promote neurite outgrowth in NG108-15 cells [18]. However, endogenous concentrations of H_2S in the low nanomolar range have been reported [19]. We examined the influence of H_2S on neurite outgrowth in N2A cells with Na_2S, a compound with a much higher purity than NaHS and an H_2S precursor. When N2A cells were treated with 100 μM Na_2S for 72 h, no morphological changes were observed (Fig. 3D and E). However, the present study clearly demonstrates that polysulfides induce neurite outgrowth at concentrations ranging from 5 μM to 25 μM in culture medium. Moreover, recent reports have suggested that H_2S is oxidized to polysulfides (H_2S_n) (n = 2–7) [20] and that NaHS solutions contain polysulfides [14]. Thus, the apparent effects of millimolar concentrations of NaHS may be exerted by the additional polysulfides that are oxidation products of H_2S. Because bound sulfur exists in N2A cells at a concentration of approximately 10 μM (Fig. 1B) and Kimura et al. have indicated that polysulfides exist in the brain at a concentration of approximately 20 μM [20], our results suggest that endogenous bound sulfur could be related to neurite outgrowth in cells and to the differentiation of neuroblastomas.

3.2. Na_2S_4-induced neurite outgrowth is affected by the intracellular calcium concentration

We found that Na_2S_4 increased neurite outgrowth in N2A cells. It is well known that calcium plays a critical role in neuronal differentiation and neurite outgrowth [21]. Thus, to investigate the involvement of intracellular calcium signaling on polysulfide-induced neurite outgrowth increases, we examined the influence of 2-APB and SKF96365, which are known to block calcium entry mediated by several types of TRP channels [22,23], on Na_2S_4-induced neurite outgrowth.

Firstly, we confirmed that intracellular calcium level in N2A cells was increased by the treatment of Na_2S_4 and this increase was significantly inhibited by co-treatment of 2-APB (Fig. 4A and B). Subsequently, we found co-treatment with 2-APB or SKF96365 radically inhibited Na_2S_4-induced neurite outgrowth, while RA-induced neurite outgrowth was not affected by the presence of either calcium blocker (Fig. 4C–F). These results suggest that treatment with Na_2S_4 leads to an increase in the intracellular

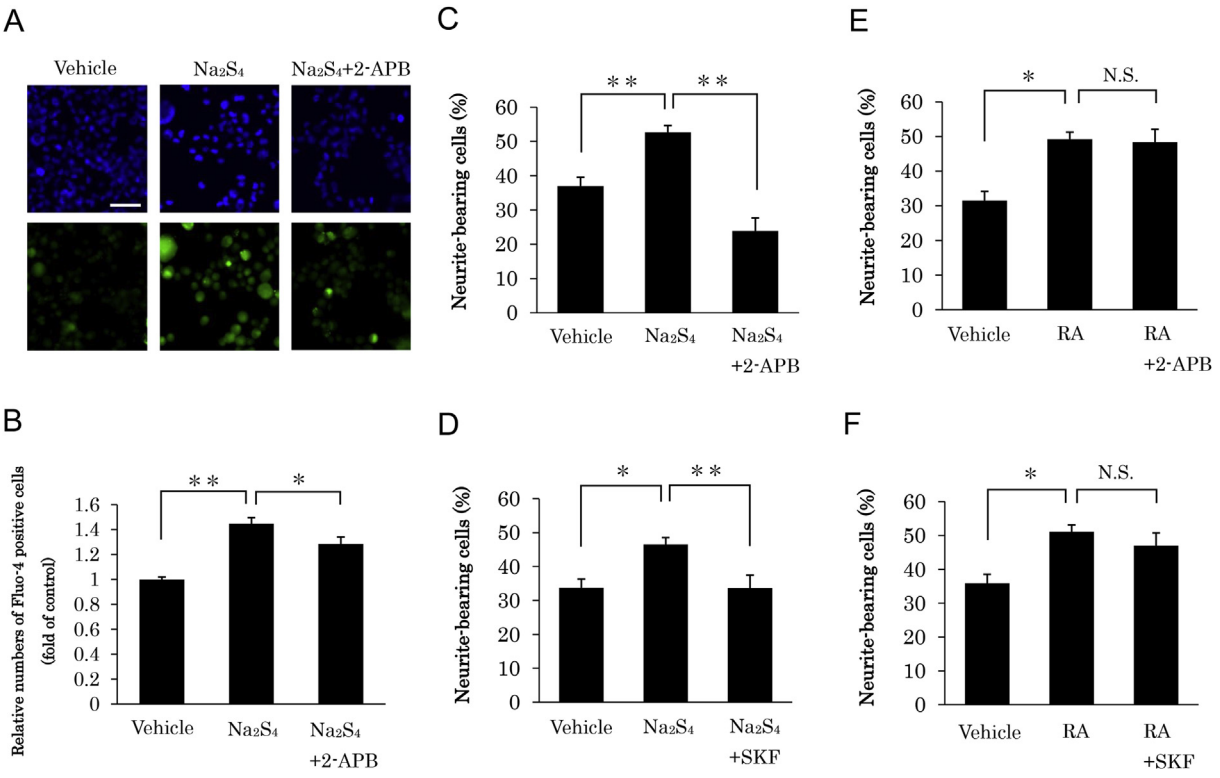

Fig. 4. Effects of various inhibitors of calcium influx on Na_2S_4-induced neurite outgrowth. (A, B) Cells were treated with 25 μM Na_2S_4 for 72 h in the presence of 5 μM 2-APB or vehicle alone (vehicle: water). (A) Images show typical fields of Hoechst 33342 stained cells (Blue) and Fluo-4 positive cells (Green) observed using IN Cell Analyzer 2200. Scale bar: 50 μm. (B) Quantification of Fluo-4 positive cells normalized to vehicle control (n = 6). The percentage of Fluo-4 positive cells were calculated as described in Section 2. Values indicate means±S.E.M. *$P < 0.05$ and **$P < 0.01$. (C, D) Cells were treated with 25 μM Na_2S_4 for 72 h in the presence of (C) 5 μM 2-APB or vehicle alone (vehicle: water) or (D) 200 μM SKF or vehicle alone (vehicle: DMSO) in DMEM supplemented with 2% FBS. (E, F) Cells were treated with 20 μM RA for 72 h with (E) 5 μM 2-APB or vehicle alone (vehicle: water) or (F) 200 μM SKF or vehicle alone (vehicle: DMSO) in DMEM supplemented with 2% FBS. Neurite-bearing cells were analyzed as described in Section 2. RA; Retinoic acid, SKF; SKF96365. Values indicate means±S.E.M. N.S.: not significant *$P < 0.05$ and **$P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

calcium level, and the calcium entry seems to be required for the induction of neurite outgrowth caused by Na_2S_4. Thus, the mechanism of neurite outgrowth with Na_2S_4 may be different from that of RA-induced neurite outgrowth in N2A cells. A previous report suggested that polysulfides induce calcium influx in astrocytes by modulating TRPA1 activity [13]. Furthermore, it has been suggested that TRP channels are closely related to neurite outgrowth in neuronal cells [24]. Because 2-APB and SKF96365 also inhibit calcium influx by blocking other channels and receptors in addition to TRP channels [25–27], our results indicate the possibility that the neurite outgrowth and differentiation caused by polysulfides depend on other calcium influx pathways. Additionally, we recently reported that polysulfide activates the Nrf2/Keap1 system and the PI3K/AKT signaling pathway. Many reports have also shown that the activation of Nrf2 and the PI3K/AKT signaling pathway induce neurite outgrowth and differentiation in neuroblastoma cells [28–30]. Thus, we speculate that differentiation in response to polysulfides may be partially mediated by activating Nrf2 and the PI3K/AKT signaling pathway.

In conclusion, the present study demonstrated that polysulfides might induce neurite outgrowth and differentiation in N2A cells by accelerating calcium influx. These observations are important for examining the novel physiological role of bound sulfur species, such as polysulfides, and the mechanism of neurite outgrowth in neuronal cells. Moreover, neuroblastoma is the second most common form of tumor in early childhood, and the prognosis of these patients is currently poor [31]. Our results indicate that bound sulfur species may be a novel differentiation inducer in neuroblastomas and may be important for determining a novel mechanism of differentiation in neuroblastoma cells.

Conflict of interest

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.133.

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