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Polysulfide exerts a protective effect against cytotoxicity caused by *t*-buthylhydroperoxide through Nrf2 signaling in neuroblastoma cells

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ABSTRACT

Polysulfide is a bound sulfur species derived from endogenous H_2S. When mouse neuroblastoma, Neuro2A cells were exposed to *tert*-butyl hydroperoxide after treatment with polysulfide, a significant decline in cell toxicity was observed. Rapid uptake of polysulfides induced translocation of Nrf2 into the nucleus, resulting in acceleration of GSH synthesis and HO-1 expression. We demonstrated that polysulfide reversibly modified Keap1 to form oxidized dimers and induced the translocation of Nrf2. Moreover, polysulfide treatment accelerated Akt phosphorylation, which is a known pathway of Nrf2 phosphorylation. Thus, polysulfide may mediate the activation of Nrf2 signaling, thereby exerting protective effects against oxidative damage in Neuro2A cells.
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1. Introduction

Hydrogen sulfide (H_2S) is the third endogenously produced neuromodulator to be identified as a cell signaling molecule in nerve cells [1]. As shown in Fig. 1, cysteine is the major source of H_2S in mammals, and the conversion of cysteine to H_2S is catalyzed by the enzymes cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), cysteine aminotransferase (CAT), and 3-mercaptopyruvate sulfurtransferase (3MST).

H_2S is produced from cysteine or homocysteine and was initially found to be a neuromodulator [2]. It facilitates hippocampal long-term potentiation by enhancing *N*-methyl-D-aspartic acid receptor activity in neurons and increasing the influx of Ca^{2+} into astrocytes [3]. H_2S may function in a wide range of physiological effects in mammalian tissue that contribute to cellular homeostasis and protect the cell against oxidative stress, apoptosis, and necrosis [4]. The protective effects of H_2S against oxidative stress in neurons were first reported by Kimura and Kimura [5]. In their study, H_2S was reported to protect primary neuronal cultures from death in a well-studied model of oxidative stress caused by glutamate, a process called oxidative glutamate toxicity or oxytosis [5].

Over 20 years ago, we developed a sensitive method for the determination of H_2S liberated by reduction and applied this method to mammalian tissue [6–8]. As a result of these experiments, we termed "bound sulfur" to describe the preferred state of endogenous H_2S [7]. Bound sulfur is defined as sulfur that is rapidly liberated as sulfide by reduction with dithiothreitol (DTT), as shown in Fig. 1. Moreover, we found that rat liver cytosolic CSE catalyzed the generation of bound sulfur in the presence of cystine or cysteine [9]. In addition, in vitro modification of sulfhydryl-dependent enzymes was found to occur as a result of their interaction with bound sulfur, which had high reactivity and relative specificity for thiol residues [9]. Although physiologically active sulfur species (bound sulfur) have long been recognized in living organisms [8,10,11], their biological function remains poorly understood so far.

Despite the fact that H_2S is recognized to be neuroprotective [12,13] and serves as a protective mediator for various tissues [14–16], the similar function of bound sulfur is not well known. Therefore, further study is required to resolve the individual mechanisms by which H_2S and bound sulfur may perform physiological roles.

Abbreviations: Nrf2, nuclear factor-erythroid 2 p45-related factor 2; Keap1, Kelch-like ECH-associated protein-1; GSH, glutathione; HO-1, heme oxygenase 1; *t*-BHP, *tert*-buthylhydroperoxide; DTT, dithiothreitol

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Recent evidence suggests that NF-E2 p45-related factor 2 (Nrf2) is targeted for rapid degradation by the ubiquitin–proteasome pathway, and electrophoretic compounds, heavy metals, and ROS inhibit Kelch-like ECH-associated protein-1 (Keap1)-dependent regulation and activate the translocation of Nrf2 to the nucleus, thereby inducing the expression of several regulated genes including heme oxygenase 1 (HO-1) [17–19]. Although the signaling molecule H_2S is a poorly characterized messenger that may regulate the Keap1-Nrf2 pathway, Calvert et al. reported that the cardioprotective effects of H_2S were mediated, in large part, by a combination of antioxidant and anti-apoptotic signaling, and this study highlights a novel signaling cascade involving Nrf2 [20].

In the present study, we investigated the cytoprotective effects of polysulfides, which are typical bound sulfur species, against reactive oxygen species (ROS) in cultured mouse neuroblastoma cells. Furthermore, we attempted to establish whether polysulfide requires the Nrf2/Keap1 regulation redox system to protect cells from ROS and whether activation of Nrf2 by polysulfide involves the antagonism of Keap1, a redox-sensitive ubiquitin ligase substrate adaptor that represses Nrf2 under normal homeostatic conditions.

2. Materials and methods

2.1. Materials

Sodium tetrasulfide (Na_2S_4) was purchased from Kojundo Chemical Lab. Co. (Saitama, Japan), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and DTT were purchased from Wako Pure Chemical Co. (Osaka, Japan). Buthionine sulfoximine (BSO), *t*-butyl hydroquinone (*t*-BHQ), and *t*-butyl hydroperoxide (*t*-BHP) were purchased from Sigma (St. Louis, MO, USA). The protease inhibitor and phosphatase inhibitor cocktails were obtained from Roche Applied Science (Darmstat, Germany). The cytosolic and nuclear fractions were prepared using the NE-PER Nuclear and Cytosolic Extraction Reagents (Thermo Scientific, Rockford, IL, USA).

2.2. Cell culture

Mouse neuroblastoma Neuro2A (N2A) cells were purchased from 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). The cells were cultured and maintained at 37 °C under 5% CO_2.

2.3. Cell treatment

N2A cells were treated with Na_2S_4 or *t*-BHQ. Briefly, N2A cells were seeded on culture dishes at a density of $0.5–1 \times 10^4$ cells/cm^2 in DMEM containing 10% FBS. The medium was changed to DMEM containing 2% FBS after 24 h, and the cells were treated with various concentrations of Na_2S_4 or 50 μM *t*-BHQ for various times. After eliminating the excess Na_2S_4 or *t*-BHQ in the medium, N2A cells were washed twice and collected for preparation of cell lysates.

2.4. Determination of bound sulfur

Intracellular levels of bound sulfur were measured using a previously described method [21]. Briefly, N2A cells treated with Na_2S_4 were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in lysis buffer [10 mM potassium phosphate buffer (pH 7.4), 0.5% Triton X-100, protease inhibitor cocktail complete (EDTA free, Roche Applied Science, Darmstadt, Germany), 10 mM hydroxylamine, and 10 mM benzoic acid]. The lysate was then centrifuged at 12 000g for 10 min at 4 °C, and the resultant supernatant was recovered. For the measurement of H_2S released from bound sulfur, 15 mM DTT in 0.2 M Tris–HCl (pH 9.0) was added to an equivalent amount of the sample in a 15 ml centrifugation tube; the tube was then sealed with Parafilm and incubated at 37 °C for 50 min. After adding 0.2 ml 1 M sodium citrate buffer (pH 6.0), each mixture was incubated at 37 °C for 10 min with shaking at 125 rpm on a rotary shaker to facilitate the release of

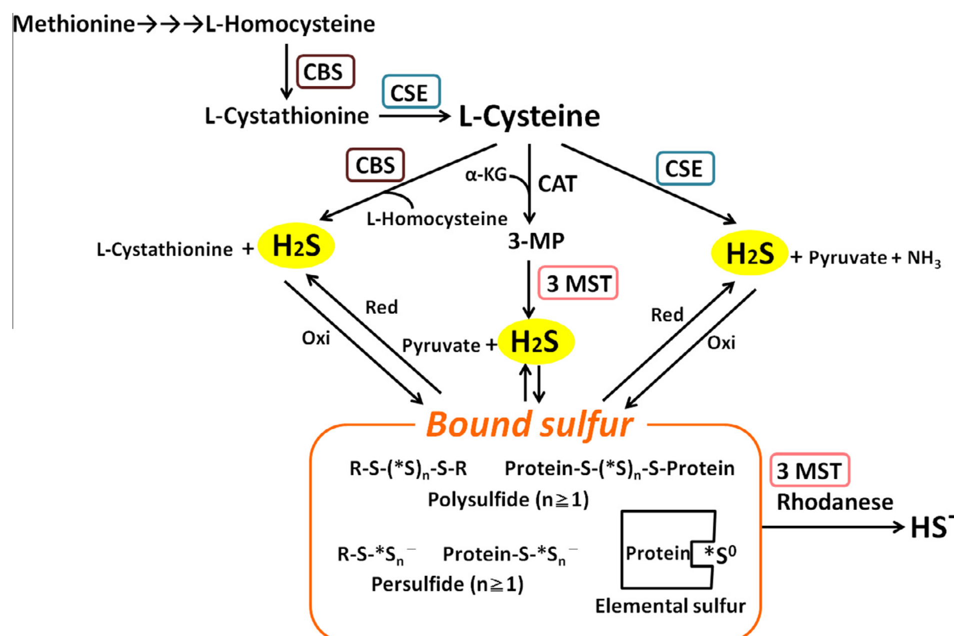

Fig. 1. Bound sulfur generation pathway of cysteine catabolism in mammals. CBS, cystathionine β synthase; CSE, cystathionine γ lyase; 3MST, 3-mercaptopyruvate sulfurtransferase; CAT, cysteine aminotransferase; α-KG, α-ketoglutarate; 3-MP: 3-mercaptopyruvate; *, bound sulfur; R, alkyl or H.

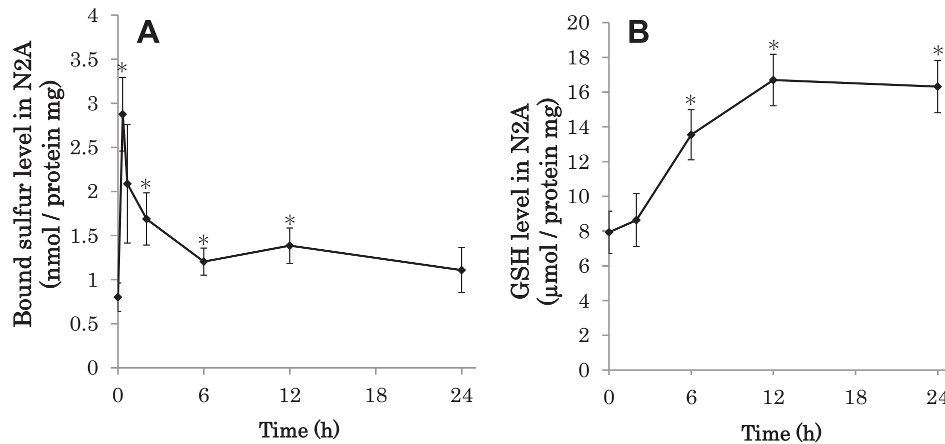

Fig. 2. Variations in bound sulfur levels in N2A cells treated with Na_2S_4 for different lengths of time. (A) Cells were treated with 25 μM Na_2S_4 for the indicated time periods. Cell lysates were prepared, as described in the Section 2, to measure the amount of bound sulfur at any given point in time. $^{*}P < 0.05$ compared to the control level. Data are shown as the mean values ± S.D. ($n = 4$). (B) Time course of intracellular GSH levels in N2A cells treated with 25 μM Na_2S_4 for the indicated time periods. GSH was measured using DTNB, as described in the Section 2. $^{*}P < 0.05$ compared to the value obtained at 0 h. Data are shown as the mean values ± S.D. ($n = 3$). All data are representative of at least three independent experiments.

H_2S gas from the aqueous phase. H_2S concentrations were then determined using gas chromatography.

2.5. GSH determination

Cellular GSH levels were determined using the DTNB method [22]. Briefly, cytosolic fractions were prepared using the NE-PER™ nuclear and cytoplasmic extraction reagents (Thermo Scientific), according to the manufacturer's instructions. After the addition of 5-sulfosalicyclic acid to each cytoplasmic fraction, the lysate samples were centrifuged at 4 °C at 15000 rpm. A portion (50 μl) of the supernatant was added to 0.2 ml 0.25 M Tris–HCl (pH 8.2) buffer containing 20 mM EDTA; then, 10 mM DTNB was added to the solution and incubated at room temperature for 15 min. The absorbance of each sample was measured at 405 nm using a microplate reader (BIO-RAD model 680).

2.6. Western blot analysis

N2A cells were treated with or without Na_2S_4 for various timeframes. After incubation, the cells were washed with ice-cold PBS and were resuspended in lysis buffer [20 mM HEPES (pH 7.4), 0.5% Triton X-100, 0.5 mM EDTA, and a protease inhibitor cocktail (Roche Applied Science, Darmstadt, Germany)]. Each lysate sample was centrifuged at 4 °C at 12000 rpm, after which the supernatant was collected as the whole cell extract. Nuclear and cytoplasmic extracts were then prepared using the NE-PER™ nuclear and cytoplasmic extraction reagents (Thermo Science), according to the manufacturer's instructions. A total of 10–20 μg of protein extract from N2A cells was boiled for 5 min in SDS sample buffer [2% sodium dodecyl sulfate, 62.5 mM Tris–HCl (pH 6.8), 10% glycerol, and 0.001% bromophenol blue]. The proteins were then separated using 10% or 12.5% polyacrylamide gels and then transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked using EZ Block (ATTO, Tokyo, Japan), after which the membrane was incubated with the primary antibodies Nrf2 (1:500) or Keap1 (1:1000) (Santa Cruz Biotechnology, Inc. Dallas, TX, USA), HO-1 (1:1000) (Enzo Life Sciences, Inc. Farmingdale, NY, USA), β-actin (1:2000) or lamin B1 (1:5000) (Abcam, Cambridge, MA, USA), Akt (1:1000) or p-Akt (Ser473) (1:1000) (Cell Signaling Technology, Inc. Danvers, MA, USA). After washing with PBS containing 0.1% Triton-X 100, each membrane was incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody, including HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, and HRP-conjugated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, USA). Chemiluminescence from the protein bands was detected using Crescendo Western Reagents (Millipore Co.) in a Light Capture II device (ATTO, Tokyo, Japan). Quantification of results was performed by densitometry using CS Analyzer version 3.0 software (ATTO, Tokyo, Japan).

2.7. Dephosphorylation with λ protein phosphatase

The nuclear extracts were incubated with λ protein phosphatase (Upstate, Charlottesville, VA, USA) at 9.86 units of enzyme/μg of protein extract diluted in the λ protein phosphatase buffer [50 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, and 2 mM $MnCl_2$] at 30 °C for 45 min. After incubation, Nrf2 was detected by Western blotting as described above in the Westernblot analysis section.

2.8. Non-reducing SDS–PAGE

Westernblot analysis for Keap1 was performed under non-reducing conditions. according to the method reported by Forrquet et al. [23]. N2A cells were lysed in redox lysis buffer [0.1 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.2% deoxycholic acid, 5% Nonidet P-40, 0.2 mM NaF, 0.2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail, and 40 mM NEM]. The lysate samples were then centrifuged at 12000 rpm at 4 °C, after which the supernatant was collected as the redox whole cell extract. An aliquot of the extract was diluted in sample buffer without DTT, while another aliquot was diluted in sample buffer containing DTT. After heat denaturation, protein separation was performed using a 10% gel, and Keap1 was detected using Westernblot analysis.

2.9. LDH assay

N2A cells were seeded in a 48-well plate at a density of 1×10^4 cells/cm^2 in DMEM medium containing 10% FBS. After 24 h, the medium was changed to DMEM containing 2% FBS, and the cells were treated with various concentration of Na_2S_4. After incubation for 2 h, the medium was changed to the DMEM containing 10% FBS, and the cells were treated with 100 μM *t*-BHP. LDH leakage was measured using a Cytotoxicity Detection Kit (TAKARA BIO Inc.), according to the manufacturer's instructions. The absorbance of each sample was measured at 490 nm with a microplate reader.

2.10. Statistical analysis

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

3. Results

3.1. Measurement of bound sulfur and GSH levels in N2A cells

Bound sulfur levels were monitored during treatment of N2A cells with Na_2S_4 for 24 h. As shown in Fig. 2, a significantly

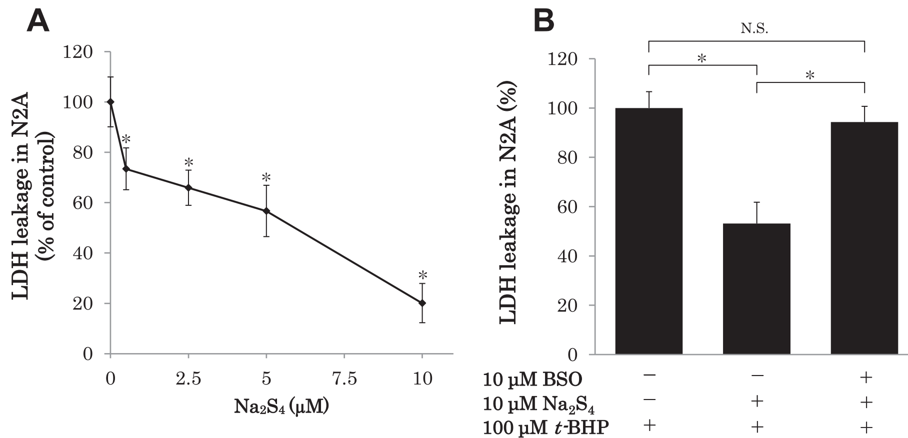

Fig. 3. Cellular damage evaluated by LDH assay. (A) N2A cells were treated with the indicated concentrations of Na_2S_4 for 2 h in DMEM containing 2% FBS and were then exposed to 100 μM *t*-BHP for 6 h in DMEM containing 10% FBS. (B) N2A cells pre-treated with or without 10 μM BSO for 1 h were treated with 25 μM Na_2S_4 for 2 h and then exposed to 100 μM *t*-BHP for 6 h. LDH leakage was measured, as described in the Section 2. $^*P < 0.05$, N.S.; not significant. Data are shown as the mean values ± S.D. ($n = 7$).

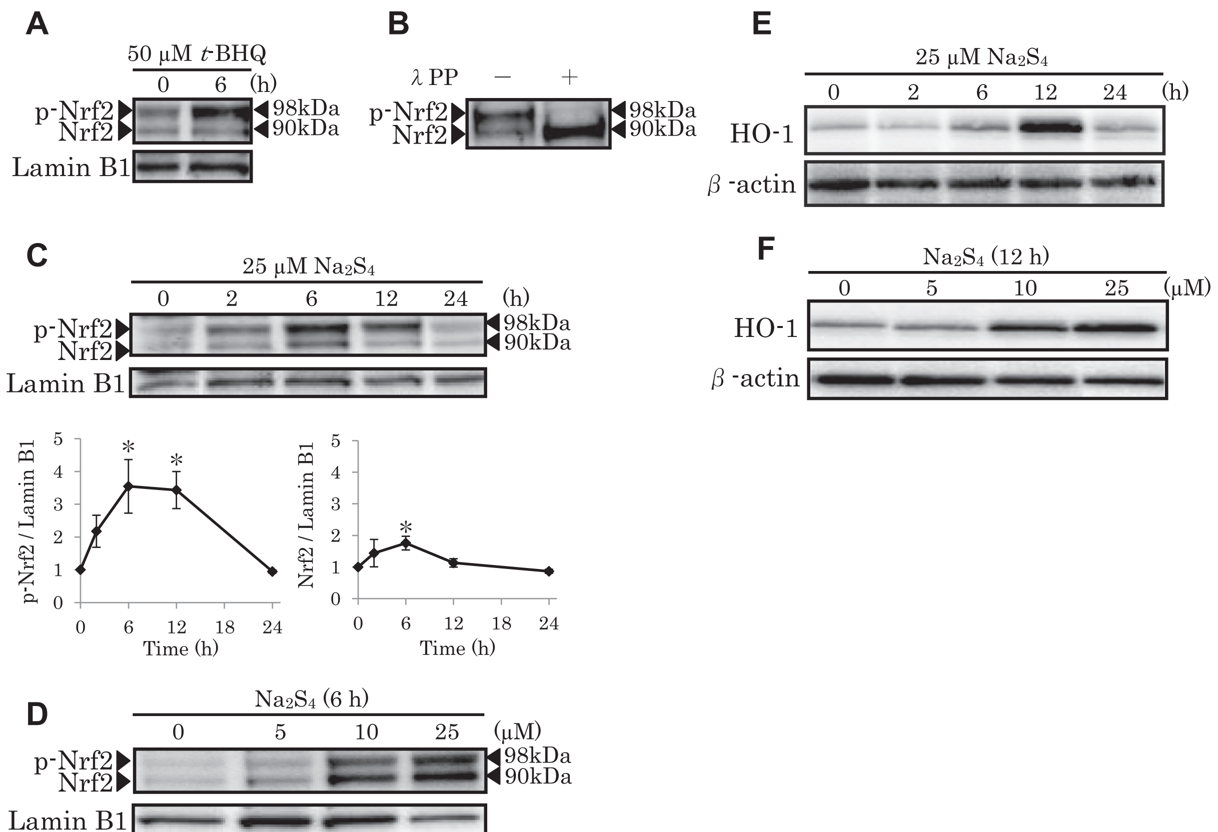

Fig. 4. Accumulation of Nrf2 in the nuclear fraction and the expression of HO-1 in N2A cells. (A) Cells were treated with 50 μM *t*-BHP. (B) N2A cells were treated with 25 μM Na_2S_4 for 12 h, and nuclear fractions were incubated with or without λ protein phosphatase (λ PP) , as indicated, at 30 °C for 45 min before Western blot analysis to detect phosphorylated Nrf2 protein (top panel). Lamin B1 was used as the loading control (bottom panel). (C and D) Cells were exposed to 25 μM Na_2S_4 for (C) the indicated time periods or (D) treated with the indicated concentrations of Na_2S_4 for 6 h. (E and F) N2A cells were treated with 25 μM Na_2S_4 for (E) the indicated time periods or (F) treated with the indicated concentrations of Na_2S_4 for 12 h, and HO-1 protein expression was analyzed. Nuclear and cytosolic fractions were prepared, as described in the Section 2. Westernblot analysis was performed on nuclear protein and cytosolic protein with Nrf2 (A–D) and HO-1 (E and F) antibodies. Lamin B1 was used as the nuclear loading control (bottom panel) (C and D). β-actin was used as the cytoplasmic loading control (bottom panel) (E and F). All data are representative of at least three independent experiments. $^*P < 0.05$ compared to the value obtained at 24 h. Data are shown as the mean values ± S.D. ($n = 8$).

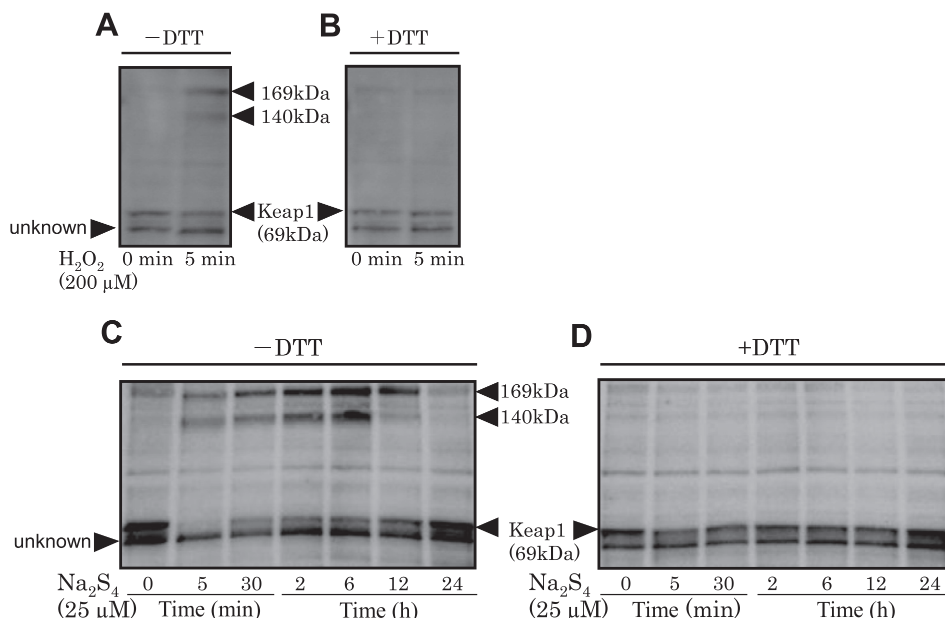

Fig. 5. Modification of Keap1 protein within N2A cells. (A) Cells were treated with 200 μM H_2O_2 for the indicated time periods. Keap1 protein was detected in the N2A cells treated with 25 μM Na_2S_4 for the indicated time periods. (B and C) Keap1 protein was detected by Western blot analysis under (B) non-reducing conditions and (C) reducing conditions. (D and E) N2A cells were treated with the indicated concentrations of Na_2S_4 for 5 min and then detected by Western blot analysis under (D) non-reducing conditions and (E) reducing conditions. Whole cell lysate for the non-reducing conditions was prepared, as described in the Section 2. All data are representative of at least three independent experiments.

increased level of bound sulfur contents was observed at 5 min when compared with the untreated levels in the N2A cells. In addition, we observed no significant increase of bound sulfur at 5 min in Na_2S_4 treated cell membrane compared to that from non-treated cells. The initially elevated level (over three times) of intracellular bound sulfur gradually declined after 2 h during the treatment with Na_2S_4 (Fig. 2A). After 24 h of treatment, the level of bound sulfur returned to basal levels in the N2A cells.

Next, we measured a dose-dependent increase in intracellular GSH levels in N2A cells treated with Na_2S_4 (Fig. 2B). After 25 μM Na_2S_4 was added to the cells and cultured for 12 h, a peak in intracellular GSH was observed; the level gradually decreased and returned to basal levels by 24 h after treatment with Na_2S_4 (Fig. 2B).

3.2. Effect of polysulfide on t-BHP-induced oxidative damage

We investigated whether Na_2S_4 might have a protective effect against *t*-BHP-induced oxidative damage. Exposure to 100 μM *t*-BHP caused significant LDH leakage in N2A cells (Fig. 3). Pre-treating N2A cells with Na_2S_4 produced a dose-dependent increase in LDH leakage. This protective effect was shown following treatment with 0.5–10 μM Na_2S_4. The cytotoxicity induced by *t*-BHP was suppressed by approximately 60% following treatment with 10 μM of Na_2S_4 (Fig. 3A).

To estimate a relationship between the increase of GSH induced by Na_2S_4 and expressing the protective effect for cytotoxicity induced by *t*-BHP exposure, we pre-treated N2A cells by BSO, which is an inhibitor of GSH synthesis, together with Na_2S_4 followed by exposure to *t*-BHP. As shown in Fig. 3B, the protective effect of Na_2S_4 was significantly suppressed by pretreatment with BSO.

3.3. Translocation of Nrf2 to the nucleus after treatment with polysulfide

Treatment with Na_2S_4 increases in the levels of GSH in the cell (Fig. 2) and also protects against *t*-BHP-induced oxidative damage (Fig. 3). As it is well-known that the Keap1/Nrf2 pathway is a key system that protects cells from oxidative stress, we focused on the effect of Na_2S_4 treatment on Nrf2 activation, which caused GSH synthesis. Protein levels of Nrf2 were analyzed in the nuclear fraction of N2A cells using Westernblot analysis. The intensity of two bands (90 and 98 kDa) increased in response to *t*-BHQ treatment, which is a potent inducer of Nrf2-dependent gene expression (Fig. 4A). As shown in Fig. 4B, when samples of the nuclear fraction were dephosphorylated using λ protein phosphatase, the 98 kDa band disappeared and the 90 kDa band was rather

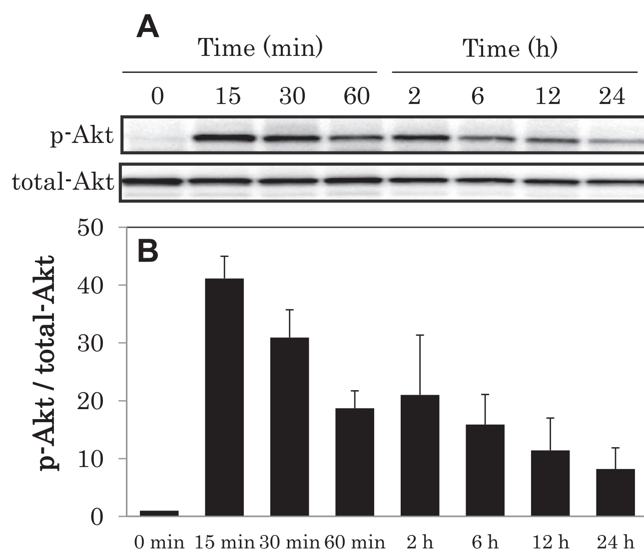

Fig. 6. Analysis of the PI3K/Akt pathway in N2A cells treated with Na_2S_4. (A) N2A cells were treated with 25 μM Na_2S_4 for the indicated time periods, and the levels of Akt and phospho-Akt were examined in whole cell extract. Samples of whole cell lysate were prepared, as described in the Section 2. Western blot analyses were performed using Akt (top panel) or phospho-Akt (bottom panel) antibodies. All data are representative of at least three independent experiments. (B) Quantitative analysis of phospho-Akt (Ser473) levels was performed, as described in the Section 2. Data are shown as the mean values ± S.D. (n = 3).

increased When N2A cells were treated with Na_2S_4, a significant increase in nuclear phosphorylated Nrf2 (98 kDa) was observed after 2–12 h of treatment, after which the levels returned to basal levels by 24 h (Fig. 4C). The amount of Nrf2 that accumulated after 6 h of treatment occurred in a Na_2S_4 concentration-dependent manner (Fig. 4D). Furthermore, treatment with Na_2S_4 increased HO-1 protein levels within the cytosolic fractions, as shown in Fig. 4E. The level of HO-1 protein peaked at 12 h of treatment with 25 μM Na_2S_4 (Fig. 4F). This increase in HO-1 expression correlated with the translocation of Nrf2 to the nucleus in a time- and concentration-dependent manner.

3.4. Polysulfide induces disulfide formation of Keap1

We treated N2A cells with either 25 μM Na_2S_4 for 5 min–12 h or 200 μM H_2O_2 for 5 min. Following treatment with H_2O_2 (Fig. 5A, B) or Na_2S_4 (Fig. 5C, D), three variable bands of 69 kDa, 140 kDa, and 169 kDa were detected for Keap1 using Westernblot analysis under non-reducing conditions. Although an unknown band (68 kDa) was detected, the band did not change after reduction with DTT. The intensity of the 69 kDa band corresponding to Keap1 was transiently reduced when cells were treated with 25 μM Na_2S_4 for 5 min-12 h under non-reducing conditions (Fig. 5C). Only the 69 kDa band was detected at the same expression level regardless of the Na_2S_4 treatment duration under reducing conditions (Fig. 5D).

3.5. Polysulfide activates the PI3K/Akt pathway

A number of reports have indicated that phosphorylation of Nrf2 could be a key regulator of its translocation to the nucleus from the cytoplasm. We therefore investigated phosphorylation pathways relating to Nrf2. As shown in Fig. 6, the p-Akt/total-Akt ratio was significantly increased during 15 min-6 h of treatment with Na_2S_4, after which the ratio returned to baseline levels at 24 h. To assess any correlation between the accumulation of phosphorylated Nrf2 within the nucleus and the activation of the PI3K/Akt pathway by Na_2S_4 treatment, 15 μM LY294002, a PI3K inhibitor, suppressed the nuclear accumulation of phosphorylated Nrf2 induced by treatment with 25 μM Na_2S_4 for 6 h (Fig. 7A). In addition, pretreatment with 15 μM LY294002 suppressed the increase in HO-1 expression that was otherwise observed after Na_2S_4 treatment for 12 h (Fig. 7B).

4. Discussion

We recently reported that polysulfides induced Ca^{2+} influx by activating transient receptor potential (TRP) A1 channels, which have sensitive thiol residues, in rat astrocytes, and a maximal response was induced at 0.5 μM, which is 1/320 of the concentration of H_2S required to achieve a response of similar magnitude [24]. These findings support the notion that bound sulfur species, such as polysulfides, have direct reactivity independent of H_2S. Furthermore, a recent report determined that evidence that has been attributed to H_2S in many previous reports may in fact have been mediated by polysulfides [25]. This study suggests that polysulfide formation, rather than the sulfide itself, is the in vivo agent of "H_2S signaling". Moreover, Toohey also noted that the previously implicated reaction for protein sulfhydration (protein – SH + H_2S → protein – SSH) is not a balanced chemical equation [26].

Wang and coworkers recently suggested that H_2S causes the activation of Nrf2 as an intracellular response to cell senescence through the S-sulfhydration of Keap1 in mouse embryonic fibroblast cells [27]. They proposed the conformational changes of

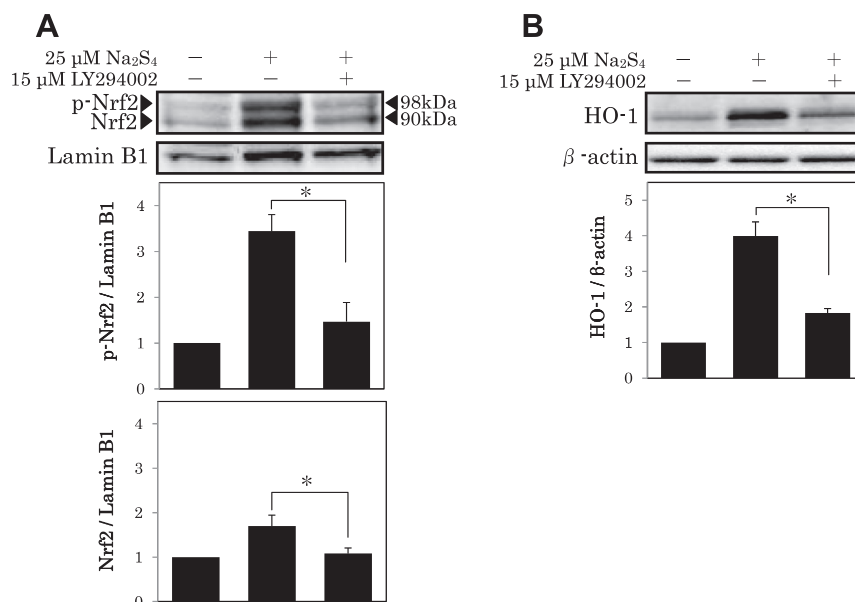

Fig. 7. Effects of PI3K/Akt inhibition on the polysulfide-induced Nrf2 accumulation and HO-1 expression in N2A cells. (A) N2A cells were pre-treated for 30 min with or without 15 μM LY294002 and were then treated with 25 μM Na_2S_4 for 6 h. The nuclear fractions were harvested, the Nrf2 protein levels were analyzed. The nuclear fractions were prepared, as described in the Section 2. Western blot analyses were performed using the Nrf2 antibody (top panel), and Lamin B1 was used as the nuclear loading control (bottom panel). All data are representative of at least three independent experiments. Quantitative analyses of phospho-Nrf2 (98 kDa) and Nrf2 (90 kDa) protein levels were performed, as described in the Section 2. $^{*}P < 0.05$ compared to the value obtained following treatment with 25 μM Na_2S_4 alone. Data are shown as the mean values ± S.D. ($n = 3$). (B) N2A cells were pre-treated for 30 min with or without 15 μM LY294002 and were then treated with 25 μM Na_2S_4 for an additional 12 h. HO-1 protein levels were analyzed within the cytosolic fractions of the cells. Cytosolic fractions were prepared, as described in the Section 2. Western blot analyses were performed with HO-1 antibody (top panel), and β-actin was used as the cytosolic loading control (bottom panel). All data are representative of at least three independent experiments. Quantitative analysis of HO-1 protein levels was performed, as described in the Section 2. $^{*}P < 0.05$ compared to the level obtained following treatment with 25 μM Na_2S_4 alone. Data shown are the mean values ± S.D. ($n = 3$).

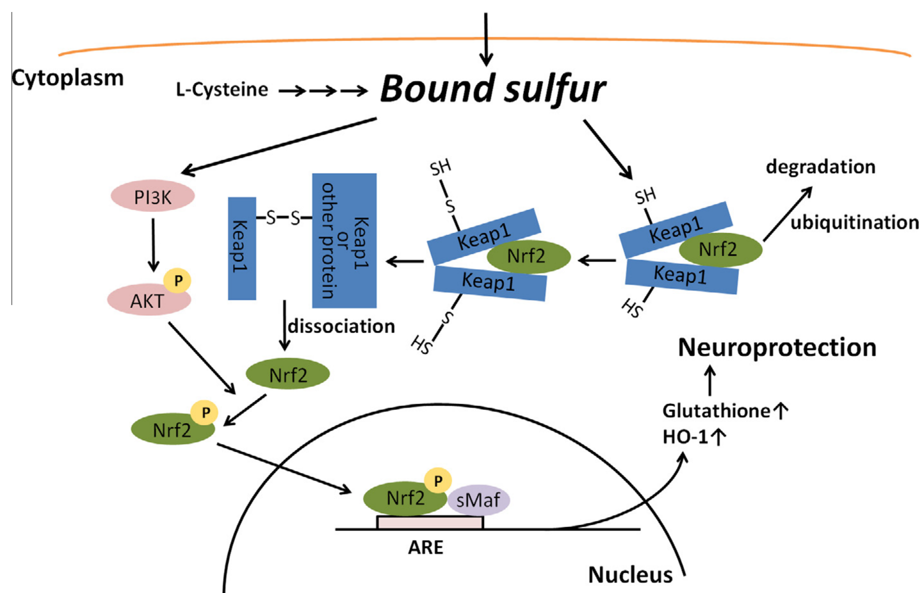

Fig. 8. Proposed mechanism of Nrf2 accumulation in N2A cells induced by bound sulfur species.

Keap1 that are caused by the formation of S-sulfhydrated residues on the Keap1 sequences. However, direct evidence of S-sulfhydration by reaction with H_2S was not shown, and therefore, the mechanism connecting conformational changes in Keap1 to Nrf2 activation is unclear.

Thus, we investigated whether polysulfides, a typical bound sulfur species, function as signaling molecules and protect cells against oxidative stress through the activation of the Nrf2/Keap1 pathway. In the present study, we first investigated the possibility that polysulfides are taken up by nerve cells. Interestingly, we found that polysulfides were quickly incorporated into the nerve cells within a few minutes. This indicated that bound sulfur species may produce HS^- or persulfides by reacting with the SH groups of proteins or thiol compounds in the cells. Furthermore, intracellular levels of bound sulfur significantly have begun to decrease at 2 h compared to the levels observed after a few minutes of exposure. Based on these findings, we theorized that polysulfides are either oxidized or secreted from cells or are changed into a form that is not recovered as H_2S following reduction with DTT.

In this study, we observed a marked increase in GSH levels in N2A cells after Na_2S_4 treatment. GSH levels within cells are typically in the millimolar range and act as reducing agents to protect cells from oxidative stress [28]. Therefore, we expected that the observed increase in intracellular GSH resulted in protective effects against various oxidative damages.

Based on our finding that Na_2S_4 increased GSH levels within N2A cells, we then focused on the protective effect of Na_2S_4 against ROS-induced oxidative cell injury in nerve cells. Pre-treatment with Na_2S_4 markedly suppressed oxidative damage and membrane damage caused by ROS exposure. Even the low concentration of 0.5 μM Na_2S_4 had a significant protective effect (Fig. 3). Therefore, we suggest that exogenous or endogenous polysulfides may exert physiological effects within the cells.

Because increases in intracellular GSH as a result of Na_2S_4 treatment with might confer cytoprotective effects against ROS, we examined the effects of polysulfides on the Keap1-Nrf2 system. When N2A cells were treated with Na_2S_4, significant accumulation of Nrf2 was observed within the nuclei; additionally, the expression of HO-1, a typical Nrf2-regulatory gene, increased [29]. HO-1 is prominently induced by various oxidative stress conditions in many different cells, and HO-1-deficient embryonic fibroblasts are hypersensitive to the cytotoxicity of both hemin and hydrogen peroxide [30]. It is well understood that the observed accumulation of Nrf2 within the nuclei precedes an increase in intracellular GSH levels; this is because GCL, the rate-limiting enzyme of GSH synthesis, is regulated by Nrf2 [31].

It has recently been reported that when the SH groups of Keap1 are oxidized by reacting with H_2O_2, disulfide bonds are formed either between or within Keap1 molecules, thereby altering their tertiary structure [23]. Therefore, Keap1 might be regulated by transient persulfideration (S-sulfhydration) and sequential disulfide formation as Keap1 is known to have active thiol residues.

In the present study, we analyzed the structural changes of Keap1 in N2A cells following treatment with Na_2S_4 using SDS–PAGE analysis under non-reducing conditions. Similar to the results obtained after treatment with H_2O_2, Western blot analysis showed a decrease in the 69 kDa band, as well as the formation of two new bands at 140 kDa and 169 kDa after Na_2S_4 treatment (Fig. 5). Because the molecular mass of Keap1 is 69 kDa, we presume that the 140 kDa band represents a Keap1 homodimer. Because the 169 kDa band also disappeared following DTT reduction, this band may have resulted from the formation of an intermolecular disulfide bond between Keap1 and another protein with an SH group. Thus, our results strongly suggest that Na_2S_4 is rapidly taken into cells where it reacts with the SH groups of Keap1, resulting in transient persulfide formation prior to the formation of intermolecular disulfides involving the Keap1 molecule. These disulfide bond formations are linked to changes in the tertiary structure of Keap1 and promote the release of Nrf2. It has been reported that phosphorylated Nrf2 escapes degradation in the cytoplasm and is stably translocated into the nucleus [32].

Multiple pathways are thought to be involved in Nrf2 phosphorylation. Although we did not observe any changes in Erk1/2 phosphorylation following Na_2S_4 treatment, Akt phosphorylation was clearly observed after Na_2S_4 treatment in N2A cells (Fig. 6). When we specifically inhibited Akt phosphorylation, the accumulation of phosphorylated Nrf2 in the nucleus due to Na_2S_4 treatment was suppressed, and the Nrf2-regulated HO-1 expression was also suppressed (Fig. 7). These results suggest that the translocation of Nrf2 into the nucleus as a result of polysulfide exposure is connected with the PI3K/Akt pathway in N2A cells (Fig. 8).

In conclusion, bound sulfur species, such as those in polysulfides, induce the translocation of Nrf2 into the nucleus, thereby increasing the antioxidant capacity of neuroblastoma cell to protect against oxidative damage due to excessive ROS (Fig. 8). Thus, bound sulfur may be a signal to regulate Keap1–Nrf2 pathway when its concentration is changed to oxidative stress in the cell. Our findings also support the results of a recent report [25] that showed that polysulfides efficiently promote persulfide formation, after which disulfides are immediately formed between the SH groups of many proteins. Moreover, the present study suggests that bound sulfur is a reactive species and is not just a storage form of H_2S, and therefore, its physiological relevance should be discriminated from that of H_2S. Further study in vivo is required to clear the significance of bound sulfur to protect neural cell.

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