RESEARCH PAPER

Sulfite protects neurons from oxidative stress

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BACKGROUND AND PURPOSE

Hydrogen sulfide (H_2S) and polysulfides (H_2S_n) are signalling molecules that mediate various physiological responses including cytoprotection. Their oxidized metabolite sulfite (SO_3^{2-}) is found in blood and tissues. However, its physiological role remains unclear. In this study, we investigated the cytoprotective effect of sulfite on neurons exposed to oxidative stress caused by high concentrations of the neurotransmitter glutamate, known as oxytosis.

EXPERIMENTAL APPROACH

Concentrations of sulfite as well as those of cysteine and GSH in rats were measured by HPLC. Cytoprotective effects of sulfite on primary cultures of rat neurons against oxytosis was examined by WST-8 cytoprotective and LDH cytotoxicity assays and compared with that of H_2S , H_2S_n and thiosulfate.

KEY RESULTS

Free sulfite, present at approximately 2 μM in the rat brain, converts cystine to cysteine more efficiently than H_2S and H_2S_n and facilitates transport of cysteine into cells. Physiological concentrations of sulfite protected neurons from oxytosis and were accompanied by increased intracellular concentrations of cysteine and GSH probably due to converting extracellular cystine to cysteine, more efficiently than H_2S and H_2S_n . In contrast, thiosulfate only slightly protected neurons from oxytosis.

CONCLUSIONS AND IMPLICATIONS

Our present data have shown sulfite to be a novel cytoprotective molecule against oxytosis, through maintaining cysteine levels in the extracellular milieu, leading to increased intracellular cysteine and GSH. Although there may be adverse clinical effects in sensitive individuals, our results provide a new insight into the therapeutic application of sulfite to neuronal diseases caused by oxidative stress.

LINKED ARTICLES

This article is part of a themed section on Chemical Biology of Reactive Sulfur Species. To view the other articles in this section visit <http://onlinelibrary.wiley.com/doi/10.1111/bph.v176.4/issuetoc>

Abbreviations

3MST, 3-mercaptopyruvate sulfurtransferase; CAT, cysteine aminotransferase; CBS, cystathionine β -synthase; CSE, cystathionine γ -lyase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; HPLC, high-performance liquid chromatography; Keap1, Kelch ECH-associating protein 1; LDH, lactate dehydrogenase; mBBR, monobromobimane; MEM, modified Eagle's medium; Nrf2, nuclear factor erythroid 2-related factor 2; PTEN, tumour suppressor phosphatase and tensin homologue; SQR, Sulfur quinone oxidoreductase; WST-8, 4-3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate sodium salt

Introduction

Hydrogen sulfide (H_2S), which is produced **cystathionine β -synthase (CBS)**, **cystathionine γ -lyase (CSE)**, and **3-mercaptopyruvate sulfurtransferase (MPST)** together with **cysteine aminotransferase (CAT)**, regulates neuronal transmission, vascular tone and inflammation; confers cytoprotection against oxidative stress; and acts as an oxygen sensors (Abe and Kimura, 1996; Hosoki *et al.*, 1997; Zhao *et al.*, 2001; Kimura and Kimura, 2004; Zanardo *et al.*, 2006; Olson *et al.*, 2006; Elrod *et al.*, 2007; Shibuya *et al.*, 2009; Singh *et al.*, 2009; Peng *et al.*, 2010).

Polysulfides (H_2S_n), which have a higher number of (sulfane) sulfur than H_2S , are produced by 3MST and by the chemical interaction between H_2S and oxidants including **NO** (Kimura *et al.*, 2013; Eberhardt *et al.*, 2014; Cortese-Krott *et al.*, 2015; Kimura *et al.*, 2015; Miyamoto *et al.*, 2017; Kimura *et al.*, 2017). They activate **TRPA1** cation channels and **protein kinase $G1\alpha$** to regulate the vascular tone (Kimura *et al.*, 2013; Stubbert *et al.*, 2014; Kimura *et al.*, 2015; Kimura *et al.*, 2017). Moreover, H_2S_n regulate the activity of a **tumour suppressor phosphatase and tensin homologue** and GAPDH (Greiner *et al.*, 2013; Jarosz *et al.*, 2015).

There are two forms of glutamate toxicity: receptor-initiated excitotoxicity (Choi, 1988) and non-receptor-mediated oxidative glutamate toxicity called oxytosis (Murphy *et al.*, 1989; Tan *et al.*, 2001), which is a well-studied programmed cell death pathway that is independent of **ionotropic glutamate receptors** (Murphy *et al.*, 1989; Tan *et al.*, 2001; Maher and Davis, 1996; Kimura and Kimura, 2004). Oxytosis is induced by high concentrations of extracellular glutamate in newly plated primary cultures of neurons that have not yet expressed ionotropic glutamate receptors (Choi, 1987; Schubert and Piasecki, 2001; Kimura and Kimura, 2004). High concentrations of glutamate suppress the **cystine/glutamate transporter xCT** (SLC7A11) to decrease the incorporation of cystine, which is reduced to cysteine, a substrate to produce an intracellular major antioxidant **GSH** (Bannai and Kitamura, 1980), resulting in the vulnerability of cells to oxidative stress.

H_2S protects neurons by increasing the intracellular levels of GSH through enhancing the activity of xCT as well as glutamate cysteine ligase, a rate-limiting enzyme for GSH production (Bannai and Kitamura, 1980; Kimura and Kimura, 2004; Kimura *et al.*, 2010). H_2S also facilitates the cysteine transport possibly through the **excitatory amino acid transporter EAAT3 (SLC1A1)**, which was initially identified as transporting aspartate and glutamate, but not through the **alanine/serine/cysteine (ASC) transporter** (Chen and Swanson, 2003; Kimura and Kimura, 2004; Kimura *et al.*, 2010). On the other hand, H_2S_n protects cells by activating a Kelch ECH-associated protein 1 (**Keap1**)/nuclear factor erythroid 2-related factor 2 (**Nrf2**) pathway through the sulfuration of Keap1, leading to the activation of antioxidant genes including those for GSH production (Koike *et al.*, 2013).

Endogenous sulfite is present in the serum under physiological conditions, at concentrations typically between 0.2 and 4.87 μM (Togawa *et al.*, 1992; Ji *et al.*, 1995; Kajiyama *et al.*, 2000; Meng *et al.*, 2005). The extracellular milieu where cystine should be a dominant form of cysteine, is more oxidized than the intracellular milieu and the serum contains

approximately 11–19 μM of cysteine and 40–77 μM of cystine (Brigham *et al.*, 1960; Jones *et al.*, 2011). Sulfite reacts with cystine to convert it into cysteine which can be more efficiently transported into neurons than cystine and used for GSH production (Clarke, 1932; Kimura *et al.*, 2010). Sulfite can also be involved in maintaining cysteine levels in the extracellular milieu such as serum.

Sulfite protects hepatocytes against the toxicity of menadione, a precursor of vitamin K (Sun *et al.*, 1990), and suppresses the formation of amyloid and aggregation of transthyretin, which transports thyroid hormones and retinal (Gales *et al.*, 2007). Sulfite additives are used widely in food as antioxidant and preservatives. However, exposure to sulfite has been reported to cause a range of adverse reactions including dermatitis, flushing, asthma and anaphylactic reactions in sensitive individuals (Vally and Misso, 2012).

Thiosulfate, another oxidized product of H_2S and H_2S_n , was recently reported to protect neuroblastoma cells and primary neuronal cultures from oxygen glucose deprivation followed by reoxygenation and was proposed as a chemical entity to mediate H_2S cytoprotection (Marutani *et al.*, 2015). Sulfite was a more effective protector of neurons against oxytosis than thiosulfate.

Our present results showed that sulfite increased cysteine levels in the extracellular milieu and the intracellular cysteine and GSH to protect neurons from oxidative stress induced by oxytosis. It also provided a new insight into the therapeutic approach to neuronal diseases caused by oxidative stress.

Methods

Measurement of endogenous sulfite

Brains of postnatal day10 Sprague Dawley rats were homogenized in 9 volume of 50 mM Tris HCl buffer (pH 8.5). Homogenates were purged with nitrogen gas before sealing with a cap then centrifuged at 9,600 $\times g$ for 10 min at 4°C. The supernatant was transferred to new tubes and incubated for 30 min at room temperature in the presence or absence of 100 mM NaBH₄. Samples (100 μL) of reduced (total sulfite = free + bound forms) or non-reduced (free form) materials were derivatized with monobromobimane (mBBR). The derivatized samples were then analysed by HPLC as described below for intracellular cysteine.

Cell culture and toxicity assay

All animal care and experimental procedures were approved by the National Institute of Neuroscience Animal Care and Use Committee of National Center of Neurology and Psychiatry (Approval number: 2017020). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). Primary cortical neurons were prepared from embryonic day 17 Sprague–Dawley rats as described (Kimura and Kimura, 2004). In brief, cells were dissociated from the cortex and maintained in modified Eagle's medium (MEM) supplemented with 30 mM glucose, 2 mM glutamine, 1 mM pyruvate and 10% fetal calf serum in atmosphere with 10% CO₂. The next day, the medium was changed to MEM supplemented with N1 (Sigma-Aldrich, St. Louis, MO, USA). For toxicity studies, cells were seeded on

poly-D-lysine-coated 96-well microtiter dishes at 5×10^4 cells per 100 μL in each well and exposed to 1 mM glutamate in the presence or absence of Na_2S , Na_2SO_3 , Na_2SO_4 , Na_2S_2 , Na_2S_3 and $\text{Na}_2\text{S}_2\text{O}_3$ for 24 h. The WST-8 viability assay was performed with the cell-counting kit-8 (Dojindo, Kumamoto, Japan) 24 h after the application of glutamate together with Na_2S , Na_2S_2 , Na_2S_3 , Na_2SO_3 , $\text{Na}_2\text{S}_2\text{O}_3$ and Na_2SO_4 . WST-8 (10 μL of 10 mM) was added to each well, the cells were incubated for 2 h at 37°C , and absorption values at 450 nm were measured.

The results obtained by WST-8 assay were confirmed by LDH assays. Twenty four hours after the addition of glutamate together with Na_2S , Na_2S_2 , Na_2S_3 , Na_2SO_3 , $\text{Na}_2\text{S}_2\text{O}_3$ and Na_2SO_4 , the microtiter plate was centrifuged at $250 \times g$ for 10 min, and the cell supernatant was used for the LDH assay [Cytotoxicity Detection kit (LDH), Roche Diagnostics, Basel, Switzerland]. Background control is medium only, and high control is cells lysed with Triton X-100 (final 2%) to determine the maximum releasable LDH enzyme activity. Low control is untreated cells used to determine the spontaneous LDH release. Per cent toxicity was calculated by the equation,

$$\text{Cytotoxicity}\% = \frac{(\text{Sample OD490} - \text{low control})}{(\text{High control} - \text{low control})} \times 100$$

Measurement of intracellular cysteine and GSH levels

The intracellular amount of cysteine and GSH was measured by the method described previously (Kimura and Kimura, 2004). Briefly, 4 h after the treatment of glutamate in the presence or absence of Na_2S , Na_2S_2 , Na_2S_3 , Na_2SO_3 , $\text{Na}_2\text{S}_2\text{O}_3$ or Na_2SO_4 , cells were washed twice with ice-cold PBS and harvested in phosphate buffer (0.1 M NaH_2PO_4 , pH 5.8, 2 mM EDTA). After sonication, cell lysates were centrifuged at $16\,000 \times g$ for 10 min, and supernatants were derivatized for HPLC. Samples of the supernatant (75 μL) were mixed with 0.5 M CHES [2-(cyclohexylamino)-ethanesulfonic acid], pH 8.4, then derivatized with 4 μL of 50 mM mBBR for 15 min in the dark. The reaction was terminated by adding 10 μL of 30% (v/v) acetic acid. Samples were analysed with a Waters Symmetry C18 (250 $\times$ 4.6 mm ID) column. The mBBR adduct was monitored by scanning fluorescence detector (Waters 474) with an excitation wavelength at 370 nm and an emission wavelength at 485 nm.

Measurement of cysteine levels in the culture medium

The amount of cysteine in culture medium was measured by the method described previously (Kimura *et al.*, 2010). In brief times (2, 10, 20, 60, 120 and 240 min) after the addition of Na_2S , Na_2S_2 , Na_2S_3 , Na_2SO_3 , $\text{Na}_2\text{S}_2\text{O}_3$ or Na_2SO_4 to the medium, 75 μL of culture medium was collected in a 1.5 mL Eppendorf tube containing 26 μL of 0.5 M CHES (pH 8.4) then derivatized with mBB. The derivatized samples were then analysed by HPLC as described above for intracellular cysteine.

Calcium imaging

The cells were washed once with basal salt solution (BSS) consisting of 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgCl_2 , 5.5 mM glucose and 10 mM HEPES-NaOH (pH 7.3) and were loaded with 1 μM calcium green-1/acetoxymethyl ester (Thermo Fischer Scientific, Waltham, MA, USA) and 0.01% (v/v) cremophorEL (Nacalai Tesque, Kyoto, Japan) in BSS solution for 50 min at 37°C . The dishes were mounted on an upright fixed stage microscope (Leica DMLFS; Leica Microsystems, Wetzlar, Germany) and perfused at 2 $\text{mL} \cdot \text{min}^{-1}$. The images were acquired using a water-immersion objective (20 $\times$; 0.5 NA; Leica Microsystems) and a CCD camera (C4742-95-12ER; Hamamatsu Photonics, Shizuoka, Japan). The frame duration ranged from 147 to 207 ms, and each image was acquired at 4 s intervals and 4 $\times$ 4 binning. Images were acquired and analysed using the Aquacosmos software ver. 2.0 (Hamamatsu Photonics). Changes in the intracellular calcium concentrations were monitored as a change in the fluorescence intensity (F) relative to the control image (F_0) that was acquired before stimulation.

Data and statistical analysis

All results are expressed as mean $\pm$ SEM. The data from multiple groups were compared by using Statcel2 software (OMS Japan) for one-way ANOVA with *post hoc* testing by using Turkey-Kramer test. Statistical analysis between two groups was performed by Student's *t*-test. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Materials

Na_2S , Na_2SO_3 , Na_2SO_4 were from Wako Pure Chemical Industries Ltd (Osaka, Japan); Na_2S_2 , Na_2S_3 from Dojindo, and $\text{Na}_2\text{S}_2\text{O}_3$ from Sigma-Aldrich, who also supplied glutamate, as L-glutamic acid monosodium salt and CHES (2-[cyclohexylamino]-ethanesulfonic acid). Monobromobimane (mBBR) was supplied by Millipore Corp. (Billerica, USA).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c,d).

Results

Endogenous concentrations of sulfite in rat brain

The endogenous concentrations of sulfite in the brain homogenates of neonatal (10 day old) rats were determined using HPLC. The concentration of free sulfite is approximately 2 μM and comprised approximately 6% of the total sulfite, including a bound form (Figure 1).

Figure 1

Concentrations of endogenous sulfite in the rat brain. Free and total sulfite in the brain were measured by HPLC ($n = 8$). All data shown are means $\pm$ SEM and are expressed as nmol g^{-1} of tissue (wet weight).

Sulfite protects neurons from oxytosis

Because sulfite has been reported to protect hepatocytes from menadione-induced cytotoxicity (Sun *et al.*, 1990), we examined its protective effects against oxytosis in primary neuronal cultures compared with that of H_2S and H_2S_n using a cell survival assay based on WST-8, a tetrazolium salt that is reduced by the dehydrogenase activity of cells to form intensely coloured formazan proportional to the number of living cells (Ishiyama *et al.*, 1997; Umemura and Kimura, 2007). After 24 h of exposure of neurons to 1 mM glutamate, WST-8 values decreased to approximately 55% of controls (Figure 2A). The addition of 1 μM of sulfite (as Na_2SO_3) completely protected the neurons against oxytosis (Figure 2A). In contrast, much higher concentrations (10 μM) of Na_2S and Na_2S_3 were required to exert complete protection, while 3 μM of Na_2S_2 was required for complete protection (Figure 2B–D). These observations suggested that sulfite efficiently protects neurons from oxytosis.

In the absence of glutamate, Na_2SO_3 barely altered the values obtained in the WST-8 assay, whereas Na_2S , Na_2S_2

Figure 2

Comparison of the cytoprotective effect of sulfite, H_2S , H_2S_2 and H_2S_3 . WST-8 values were shown for primary neuronal cultures 24 h after the addition of 1 mM glutamate (Glu) or without glutamate (–Glu) together with Na_2SO_3 (A), Na_2S (B), Na_2S_2 (C) and Na_2S_3 (D) ($n = 8$). $*P < 0.05$, as indicated; Student's *t*-test. LDH values were shown for primary neuronal cultures 24 h after the addition of 1 mM glutamate (Glu) or no glutamate (–Glu) together with Na_2SO_3 (E), Na_2S (F), Na_2S_2 (G), and Na_2S_3 (H) ($n = 8$). Cytotoxicity [% = (Sample OD490 – low control) / (high control – low control) $\times$ 100] showed negative values at higher concentrations of Na_2SO_3 , Na_2S , Na_2S_2 , and Na_2S_3 . Low control corresponds to untreated cells used to determine spontaneous LDH release. The negative cytotoxicity % values are caused by the values of samples being lower than the low control value. Even the spontaneous release of LDH was suppressed by Na_2SO_3 , Na_2S , Na_2S_2 , and Na_2S_3 possibly due to their cytoprotective effects on cells. All data shown are means $\pm$ SEM. In A–D, $*P < 0.05$, significantly different as indicated; Tukey–Kramer test. In E–H, $\#P < 0.05$, significantly different as indicated, for values with glutamate. OD490, optical density at 490 nm.

and Na_2S_3 at concentrations greater than 10 μM , increased these values above the control levels (Figure 2). Because primary neuronal cultures do not proliferate, WST-8 values above the control level may not reflect an increase in the number of cells, but the reducing activity of WST-8 in neurons, as we previously reported on H_2S (Umemura and Kimura, 2007).

The cytoprotective effect of sulfite was further examined by using the LDH assay. As a stable cytoplasmic enzyme present in all types of cells, LDH is released into the cell culture medium through damage to plasma membranes. Thus levels of released LDH are proportional to the number of damaged cells. Both WST-8 and LDH assays have been performed to complementarily assess cell viability (Kimura *et al.*, 2006). The results indicated that 1 μM of Na_2SO_3 significantly suppressed oxytosis, which was completely suppressed at a concentration of 3 μM (Figure 2E). Similar changes were observed with Na_2S and Na_2S_2 , whereas a higher concentration of Na_2S_3 (10 μM) was required to suppress oxytosis (Figure 2F–H). At 100 μM , both Na_2SO_3 and Na_2S_3 exerted toxic effects, similar to those observed in the WST-8 assay (Figure 2A, D, E, H). These observations confirmed the cytoprotective effect of sulfite at low concentrations.

Because cytotoxicity (%) is calculated as (sample OD490 – low control)/(high control – low control), the negative values observed in Figure 2E–H are ascribed to sample values being lower than the low control values at concentrations greater than 3 μM . This observation suggests that sulfite, H_2S and H_2S_n can even suppress the spontaneous LDH release.

Increase in the levels of intracellular cysteine

Na_2S increases the transport of cystine/cysteine and production of GSH, which peaks at 4 h after Na_2S addition (Kimura and Kimura, 2004). Because Na_2SO_3 protected neurons from oxytosis more efficiently than did Na_2S , Na_2S_2 and Na_2S_3 , we examined the possibility that sulfite increases the intracellular levels of cysteine and GSH more effectively than do Na_2S , Na_2S_2 and Na_2S_3 .

The results revealed that incubation with extracellular sulfite markedly increased the intracellular concentrations of cysteine in neurons. At 10 μM , Na_2SO_3 and Na_2S_3 , but not Na_2S and Na_2S_2 , significantly increased intracellular cysteine concentrations (Figure 3A–C). At 30 μM , both Na_2SO_3 and Na_2S_3 increased cysteine concentrations to more than

Figure 3

Increase in the intracellular concentrations of cysteine by sulfite compared with that by H_2S , H_2S_2 and H_2S_3 , and a time course of the conversion of cystine to cysteine in the culture medium induced by sulfur containing molecules. Intracellular concentrations of cysteine in primary neuronal cultures were measured by HPLC 4 h after the addition of Na_2SO_3 (A), Na_2S (B), Na_2S_2 (C), and Na_2S_3 (D), with or without glutamate (Glu). All data shown are means $\pm$ SEM ($n = 5$). * $P < 0.05$, significantly different as indicated, for values without glutamate; Tukey–Kramer test. # $P < 0.05$, significantly different as indicated, for values with glutamate. Furthermore, 10 μM (E) and 100 μM (F) sulfur-containing molecules indicated in (E) were applied to MEM and incubated in an atmosphere with 10% CO_2 , and the concentrations of cysteine in the medium were measured. All data shown are means $\pm$ SEM ($n = 9$).

3-fold, even in the presence of glutamate, whereas Na_2S and Na_2S_2 elicited much weaker effects (Figure 3A–D).

Sulfite reacts with cystine to produce cysteine and S-cystinesulfonate (Clarke, 1932). Cysteine can be transported into cells by the cysteine transporters more efficiently than cystine transported by cystine/glutamate antiporter (Bussolati *et al.*, 1992; Kimura *et al.*, 2010). It is possible that the conversion of cystine to cysteine in the culture medium, corresponding to the extracellular milieu such as serum, is involved in the sulfite-induced increase in the intracellular concentrations of cysteine.

The ability of sulfite to generate cysteine from cystine was examined by applying Na_2SO_3 to the culture medium in the absence of cells and was compared with the effects of Na_2S , Na_2S_2 , Na_2S_3 , $\text{Na}_2\text{S}_2\text{O}_3$ and Na_2SO_4 . Our results revealed that Na_2SO_3 efficiently converted cystine to cysteine in the medium, whereas Na_2S_2 and Na_2S_3 resulted in approximately one-third of the production induced by Na_2SO_3 (Figure 3E, F); 10 and 100 μM Na_2SO_3 generated approximately 7 and 54 μM cysteine, respectively, suggesting that approximately 50% of the applied concentrations of Na_2SO_3 were converted to generate cysteine in the medium. These observations suggested that the increase in the extracellular concentrations of cysteine may facilitate its transport into cells.

Increase in the levels of intracellular GSH

Because Na_2SO_3 increases the intracellular concentrations of cysteine, a component of GSH, it is possible that Na_2SO_3 also increases the production of GSH. At 1, 3 and 10 μM , Na_2SO_3 increased GSH concentrations significantly to higher levels than incubation with 10 μM Na_2S_2 or Na_2S_3 , whereas 10 μM Na_2S did not significantly change intracellular GSH. These results showed that low concentrations of Na_2SO_3 efficiently increased the neuronal concentrations of GSH (Figure 4). Under oxytosis conditions (in the presence of 1 mM glutamate), GSH concentrations were decreased to approximately 70% of controls. This depressed levels of GSH was significantly raised by 3 μM Na_2S_3 , 10 μM Na_2SO_3 or Na_2S_2 , and 30 μM Na_2S (Figure 4). These observations suggested that sulfite increased the intracellular concentrations of GSH efficiently even under conditions of oxytosis.

Sulfuration is not involved in the cytoprotective effect of sulfite

The polysulfides, H_2S_n , can sulfurate cysteine residues of target proteins, followed by the formation of cysteine disulfide bonds, leading to conformational changes that modify the activity of the protein (Kimura, 2015). For example, H_2S_n activate TRPA1 channels by sulfurating two cysteine residues at the amino terminus of the channels (Kimura *et al.*, 2013; Hatakeyama *et al.*, 2015). Sulfuration of the Keap1/Nrf2 complex leads to the induction of GSH production (Koike *et al.*, 2013). Sulfuration is a chemical reaction, that takes places rapidly and is maintained, while the induced molecules remain in the medium. We examined whether sulfite can sulfurate cysteine residues by assessing the activation of TRPA1 channels in astrocytes as a sensitive assay (Kimura *et al.*, 2013; Hatakeyama *et al.*, 2015). In our present experiments, even at 500 μM , Na_2SO_3 did not activate TRPA1 channels (Figure 5). In contrast, 10 μM Na_2S_3 , which sulfurates cysteine residues, effectively activated the channels (Kimura *et al.*, 2013; Hatakeyama *et al.*, 2015). These observations indicated that sulfite does not sulfurate cysteine residues and suggested that sulfuration of cysteine residues of proteins such as Keap1 may not be involved in the cytoprotective effect of sulfite.

Thiosulfate exerts a weak cytoprotective effect against oxytosis

The cytoprotective effect of H_2S against oxygen glucose deprivation followed by reoxygenation was reported to be mediated by thiosulfate sequentially produced by the oxidation of H_2S in culture medium (Marutani *et al.*, 2015). We examined whether or not thiosulfate protects neurons from oxytosis. The sequential generation of sulfite and thiosulfate from Na_2S in a culture medium was examined in the absence of cells. The concentrations of Na_2S were markedly decreased immediately after its addition to the medium, as previously reported (Kimura *et al.*, 2006). The concentrations of sulfite gradually increased, peaked 30 min after Na_2S addition and declined thereafter (Figure 6A). In contrast, the concentrations of thiosulfate started increasing 1 h after the addition of Na_2S and peaked after 2 h (Figure 6A).

Figure 4

Increase in the intracellular concentrations of GSH after incubation with sulfite, compared with those after H_2S , H_2S_2 , or H_2S_3 . Intracellular concentrations of GSH in primary neuronal cultures were measured by HPLC 4 h after the application of Na_2SO_3 (A), Na_2S (B), Na_2S_2 (C), and Na_2S_3 (D) with or without glutamate. All data shown are means $\pm$ SEM ($n = 5$). * $P < 0.05$, significantly different as indicated, for values without glutamate; Tukey–Kramer test. # $P < 0.05$, significantly different as indicated, for values with glutamate.

Figure 5

The inability of sulfite to sulfurate the cysteine residues. Ca^{2+} influx induced in astrocytes was used as an index of sulfuration on cysteine residues of TRPA1 channels (Kimura *et al.*, 2013; Hatakeyama *et al.*, 2015). Na_2S_3 induced Ca^{2+} influx (A), but even 500 μM of Na_2SO_3 did not (B). Responses from seven individual cells were superimposed. (C) The comparison between Na_2S_3 (six to seven cells picked up for Ca^{2+} imaging from each plate, and a total number of cells was 62) and Na_2SO_3 (number of cells: 84) with respect to the induction of Ca^{2+} influx in astrocytes. ATP was applied as a positive control. All data are expressed as mean $\pm$ standard error of the mean.

The cytoprotective effect of culture medium at 30 min (containing approximately 42 μM sulfite, 20 μM H_2S , and 12 μM thiosulfate) and at 2 h (18 μM sulfite, 2 μM H_2S , and 18 μM thiosulfate) after the addition of Na_2S , was examined by adding them to neuronal cultures. The medium 30 min after adding Na_2S markedly protected neurons (approximately 150%) from oxytosis, whereas only a weak effect was observed after the 2 h medium (approximately 50%; Figure 6B).

Changes in the intracellular concentrations of cysteine and GSH were also examined, on adding 30 min or 2 h medium to neuronal cultures. Both cysteine and GSH showed a tendency to be more increased by 30 min medium than by 2 h medium (Figure 6C, D). These observations suggest that sulfite rather than thiosulfate exerts cytoprotective effect against oxytosis.

Because the medium contains a mixture of H_2S , sulfite and thiosulfate at 30 min and 2 h after the addition of Na_2S , the effect of thiosulfate alone was examined. At 30 μM , thiosulfate showed a weak cytoprotective effect against oxytosis in primary neuronal cultures, as shown by the WST-8 and LDH assays (Figure 7A, C). The intracellular concentrations of cysteine and GSH were not significantly increased in the presence of thiosulfate compared with the controls (Figure 7D, E). We also determined the effect of sulfate, which neither showed cytoprotective effects nor increased GSH production (Figure 7). These observations confirmed that sulfite, rather than thiosulfate, exerts the cytoprotective effects on neurons against oxytosis.

Discussion

Sulfite increases the intracellular concentrations of cysteine and GSH

Sulfite generates cysteine three times more effectively than do H_2S , H_2S_2 , and H_2S_3 (Figures 3E and F). The serum

concentrations of sulfite (0.2–4.87 μM) previously reported (Togawa *et al.*, 1992; Ji *et al.* 1995; Kajiyama *et al.*, 2000) may therefore facilitate the conversion of cystine to cysteine. While the modified Eagle's medium (MEM) contains cystine but not cysteine, serum contains both cysteine and cystine at 11–19 and 40–77 μM , respectively (Brigham *et al.*, 1960). The intracellular cysteine can be provided not only by cystine transported through the cystine/glutamate antiporter but also by the cysteine transporter (Bannai and Kitamura, 1980; Kimura and Kimura, 2004; Kimura, *et al.*, 2006 and 2010). These observations suggested that sulfite may play a physiological role in maintaining cysteine in the extracellular milieu.

An experiment to show suppression of sulfite neuroprotection by pharmacological or siRNA inhibition of the cysteine transporters such as xCT and EAAT3 (Murphy *et al.*, 1989; Tan *et al.*, 2001; Kimura and Kimura, 2004; Kimura *et al.*, 2010) would support the cytoprotective effect of sulfite against oxytosis. However, both these transporters are suppressed by glutamate, and their inhibitors are glutamate analogues (Chan and Swanson, 2003). Suppression of transporter activity by inhibitors or siRNA must induce oxytosis-like cell death. For these reasons, it is difficult to show the suppression of the reinstating effect of sulfite on oxytosis by using inhibitors or siRNAs against these transporters.

Sulfite increased the levels of GSH more effectively than did H_2S , H_2S_2 , and H_2S_3 (Figure 4). Polysulfides, such as H_2S_2 and H_2S_3 , can facilitate the production of GSH through the activation of Nrf2. In contrast, because sulfite cannot sulfurate cysteine residues, proteins such as Nrf2 whose activity is regulated by nucleophiles, including compounds that induce sulfuration, may not be involved in the effect of sulfite (Figure 5) (Kimura *et al.*, 2013; Hatakeyama *et al.*, 2015). The increase in GSH levels by sulfite may partly be induced by increased cysteine in the extracellular milieu. It is possible that sulfite enhances the

Figure 6

H₂S is oxidized to sulfite and thiosulfate in culture medium. (A) The levels of H₂S, sulfite (SO₃²⁻), and thiosulfate (S₂O₃²⁻) after the application of 100 μM Na₂S to the culture medium are shown (*n* = 6). o/n, overnight incubation. (B) The cytoprotective effect of culture medium 30 min and 2 h after the addition of 100 μM Na₂S on primary cultures of cortical neurons was measured by the WST-8 assay (*n* = 6). (C, D) Increase in intracellular cysteine (*n* = 7) (C) and GSH (*n* = 6) (D) was measured by HPLC 4 h after the application of medium 30 min and 2 h. *P < 0.05, significantly different as indicated; Tukey–Kramer test. All data shown are means ± SEM.

activity of GSH-producing enzymes in addition to increasing cysteine concentrations.

Cytoprotective effect of sulfite on neurons

Sulfite protected neurons without reducing WST-8 as observed in cells treated with H₂S, H₂S₂ and H₂S₃ (Figure 2) (Umemura and Kimura, 2007). The mechanism has not been elucidated yet. Because H₂S, H₂S₂ and H₂S₃ were not in the culture medium 24 h after their addition, a possibility of the reduction of WST-8 formazan by the remaining H₂S, H₂S₂ and H₂S₃ could be excluded (Umemura and Kimura, 2007). The intracellular reducing substances, such as cysteine and GSH, were increased by sulfite 4 h after its addition, while the levels of both cysteine and GSH returned to the control level after 24 h (Kimura and Kimura, 2004). Therefore, cysteine and GSH may not be involved in the reduction of WST-8. Because H₂S₂ and H₂S₃ readily react with cysteine and GSH to

produce cysteine–persulfide, GSH–persulfide and persulfurated cysteine residues of proteins that have a strong reducing activity and more stable than H₂S₂ and H₂S₃, they may be involved in the reduction of WST-8 (Kimura *et al.*, 2017). Because sulfite does not sulfurate cysteine, GSH or cysteine residues, we assumed that it did not increase WST-8 values through persulfide species (Figure 5).

At 1 μM, sulfite effectively protected neurons from oxytosis (Figure 2). The endogenous concentration of free sulfite in rat brain is approximately 2 μM, which is in the range of the effective concentrations (Figure 1). Sulfite may be involved in protecting neurons from oxidative stress. Although bound sulfite is more abundant than the free form, it may not contribute to the cytoprotective effect (Figure 1) (Meng *et al.*, 2005).

It is also possible that sulfite may be taken up into cells and react with cysteine disulfide bonds in proteins to modify

Figure 7

A weak protective effect of thiosulfate against oxytosis. The cytoprotective effect of $\text{Na}_2\text{S}_2\text{O}_3$ ($n = 5$) (A) and Na_2SO_4 ($n = 5$) (B) measured by the WST-8 cell survival assay. $*P < 0.05$, significantly different as indicated; Student's *t*-test. (C) Suppression of glutamate cytotoxicity by $\text{Na}_2\text{S}_2\text{O}_3$ and Na_2SO_4 was measured by the LDH assay ($n = 8$). Increase in the concentrations of cysteine ($n = 6$) (D) and GSH ($n = 6$) (E) induced by $\text{Na}_2\text{S}_2\text{O}_3$ and Na_2SO_4 was measured by HPLC. All data shown are means $\pm$ SEM. $*P < 0.05$, significantly different as indicated; Tukey–Kramer test.

their activity. The enzyme 3MST produces H_2S_n , cysteine-persulfide, glutathione-persulfide and persulfurated cysteine residues of proteins that can subsequently produce cysteine disulfide bonds (Kimura *et al.*, 2015; Kimura *et al.*, 2017; Koike *et al.*, 2017; Nagahara *et al.*, 2018). The expression of 3MST increases the levels of bound sulfane sulfur, which consists of H_2S_n , cysteine-persulfide, GSH-persulfide and per-sulfurated cysteine residues of proteins, while the brains of 3MST knockout mice show markedly decreased levels of bound sulfane sulfur (Shibuya *et al.*, 2009; Kimura *et al.*, 2017). 3MST supplies persulfide to thiolation of tRNA, and cysteinyl t-RNA synthetase also produces these persulfurated molecules (Lipsett *et al.*, 1967; Wong *et al.*, 1974; Frasdorf *et al.*, 2014; Akaike *et al.*, 2017). For example, H_2S_n activate TRPA1 channels by sulfurating two cysteine residues at the amino terminus of the channels (Kimura *et al.*, 2013). H_2S_n also regulate antioxidant genes by sulfurating two cysteine residues of Keap1 to release Nrf2 from Keap1/Nrf2 complex (Koike *et al.*, 2013). The activity of these proteins can be modified by sulfite transported into cells.

Sulfite protects neurons more efficiently than does thiosulfate

Thiosulfate, which was reported to protect neurons from oxygen glucose deprivation and reoxygenation, showed relatively weak protection against oxytosis (Figure 7A, C). GSH levels were slightly reinstated by thiosulfate, leaving cysteine levels unchanged (Figure 7D, E). Marutani *et al.* (2015) reported that H_2S is oxidized to thiosulfate, which was proposed as the chemical entity mediating the protective effect on neurons. However, the present study revealed that H_2S was converted to sulfite before thiosulfate (Figure 6A), and sulfite exerted protective effects against oxytosis in neurons much more effectively than did thiosulfate (Figures 2A, E, 3A and 7). Sulfite markedly increased intracellular GSH, while thiosulfate did not (Figures 4A and 7E). Thiosulfate was reported to significantly increase the levels of GSH in SH-SY5Y cells (Marutani *et al.*, 2015), but not in primary neuronal cultures in the present study (Figure 7E). The discrepancy may be caused by the difference between a cell line and primary neuronal cultures.

Therapeutic potential and possible adverse effects of sulfite

S-cysteinesulfonate produced from sulfite and cystine activates **NMDA receptors** as does glutamate (Kumar *et al.*, 2018). Substantial neuronal excitotoxic injury is caused with 2 to 5 μM glutamate (Mark *et al.*, 2001), and the pathophysiological extracellular concentrations of glutamate in human traumatic brain injury patients are approximately 20 μM (Bullock *et al.*, 1998). Sulfite applied to culture medium generated approximately a half of its concentration as cysteine and the counterpart S-cysteinesulfonate (Figure 3E). A low concentration of sulfite (1 μM) is enough to protect neurons from oxytosis (Figure 2A, B), and only approximately 0.5 μM S-cysteinesulfonate can be produced at this concentration of sulfite. These observations suggest that any excitotoxic effects due to the presence of S-cysteinesulfonate could be minimal. However, the administration of sulfite would need special attention in individuals sensitive to sulfite and those with molybdenum cofactor deficiency whose sulfite oxidase is deficient (Vally and Misso, 2012; Kumar *et al.*, 2018). The adverse effect of S-cysteinesulfonate on neurons may be alleviated by the application of antagonists to NMDA receptors.

Cytoprotective effects of sulfite have been reported for cells other than neurons. Sulfite protected hepatocytes from oxidative stress caused by menadione (Sun *et al.*, 1990). Sulfite competes with GSH for conjugation with menadione and thus preserves intracellular GSH to suppress the reactive oxygen species formed by menadione.

In summary, our present results show clearly that sulfite protected neurons in culture from oxytosis and that this effect was accompanied by an increase in the intracellular levels of cysteine and GSH. The present study has provided a new insight into the protection of cells from oxidative stress and indicated a therapeutic potential of sulfite for treating diseases caused by oxidative stress.

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Author contributions

Y.K. and H.K. conceived and designed the study. Y.K. conducted the experiments and analysed the data. Y.K., N.S. and H.K. discussed the study. Y.K. and H.K. authored the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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