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ORIGINAL PAPER

Cellular toxicity of zinc can be attenuated by sodium hydrogen sulfide in neuronal SH-SY5Y cell

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Abstract

Backgrounds: Although zinc acts as a major regulator of neuronal physiology, its dyshomeostasis may cause neuronal cell death. Hydrogen sulfide (H_2S) has been reported to attenuate ischemic brain injury and to suppress Zn^{2+}-induced neuronal cell death, but the underlying mechanisms have not been elucidated.
Methods: We determined the direct Zn^{2+}-chelating capacity of sodium hydrogen sulfide (NaHS), an H_2S donor, using specific Zn^{2+} fluorescent dyes (Zinpyr-1 and Zinquin) in SH-SY5Y cells.
Results: NaHS significantly suppressed the Zn^{2+}-mediated increase in the fluorescence intensities of Zinpyr-1 and Zinquin in a dose-dependent manner. NaHS significantly inhibited cell death induced by extracellular or intracellular Zn^{2+} overload. Furthermore, Zn^{2+}-mediated increases in the phosphorylation of glycogen synthase kinase-3β and protein kinase C were highly suppressed by NaHS treatment.
Conclusion: These results demonstrate that NaHS has the capacity to chelate extracellular and intracellular Zn^{2+}, and could therefore be used in the protection against Zn^{2+} neurotoxicity.

Keywords: Hydrogen sulfide, SH-SY5Y, Zinc, Cell death, Zinquin, Zinpyr-1, Chelation, Glycogen synthase kinase-3β, Neutral red uptake

Introduction

Hydrogen sulfide (H_2S) is a water-soluble gas, which in water consists of inorganic sulfides, such as undissociated H_2S, the hydrosulfide anion (HS^-), and the sulfide anion ($S2^-$)[1]. In the brain, the level of free H_2S is assumed to be less than 10 μM, depending on the analytical methods used for determination[2]. H_2S is synthesized mainly by cystathionine β-synthase and mercaptopyruvate sulfurtransferase (MPST)[3]. H_2S can be stored in the brain, either as acid-labile sulphur in the mitochondria or as bound sulphur (including polysulfides and persulfides) in the cytoplasm, the latter being the main location[2]. In the brain, H_2S is either slowly released from, or stored as bound sulphur, unlike its release and binding in the heart and the liver[2]. Previously, H_2S was identified as a gaseous toxin, because of its high binding affinity for oxygen transporting proteins (e.g., hemoglobin and myoglobin) and mitochondrial cytochrome C oxidase, resulting in a blockade of cellular respiration[4]. However, similar to nitric oxide and carbon monoxide[1], H_2S is involved in regulating various biological functions, including ion channels and mitochondrial functions[5-7]. Moreover, numerous protective cellular roles for H_2S have been highlighted recently, and there have been several attempts to use H_2S as a therapeutic target for intervention in brain diseases[5].

Zinc is an essential trace element that participates in transcription, protein synthesis, and signal transduction, including synaptic transmission during neuronal activity[8-11]. The brain has a high zinc content with a non-uniform distribution[12,13]. Compared to the total zinc concentration in the brain, which is estimated to be approximately 150 μmol/L (about ten-fold greater than the serum zinc level), the concentration of the free zinc ion (Zn^{2+}) is estimated at a subnanomolar level, based on the cytosol of cultured neurons and extracellular brain fluids. If the cellular level of Zn^{2+} is depleted, numerous cellular functions become impaired.

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Conversely, a high burden of Zn^{2+}, due to toxins (e.g., zinc oxide nanoparticles), or overload from mitochondrial dysfunction and oxidative stress, causes neuronal cell death[14-16]. Additionally, post-synaptic accumulation of intracellular Zn^{2+} contributes to the neuronal injury observed in some forms of cerebral ischemia[17] and in a Parkinson's disease (PD) model[18], as well as in post-mortem brains of PD patients[19].

Recently, H_2S has been reported to ameliorate ischemic brain injury and to suppress Zn^{2+}-induced neuronal cell death[20]. Sodium hydrogen sulfide (NaHS) is widely used in cellular and whole animal experimental systems[21]. Although the exact protective role of H_2S against Zn^{2+}-induced toxicity is not clear, it has been suggested to be associated with its inhibitory effect on Zn^{2+} entry into the cells[20]. In line with that report, we determined the direct Zn^{2+}-chelating potential of NaHS both in an *in vitro* experimental setting and in human neuronal SH-SY5Y cells. First, the *in vitro* Zn^{2+} scavenging activity of NaHS was determined using two Zn^{2+}-specific fluorescent dyes, Zinquin[22] and Zinpyr-1[23]. Intracellular Zn^{2+} scavenging activity was also assessed in SH-SY5Y cells after exogenous loading of Zn^{2+}. The protective cellular capacity of NaHS against extracellular (via 750 μM $ZnCl_2$) and intracellular Zn^{2+}(via 10 μM $ZnCl_2$ with a zinc ionophore) overload was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction, neutral red (NR) uptake and propium iodide (PI) uptake assay in SH-SY5Y cells. The Zn^{2+}-chelating activity of NaHS was further determined by the change in the Zn^{2+}-mediated increase of protein kinase C (PKC) and glycogen synthase kinase-3β (GSK-3β) phosphorylation by immunoblotting.

Materials & Methods

Chemicals and reagents

NaHS, $ZnCl_2$ propium iodide (PI), neutral red (NR), Zinquin, Zinpyr-1, 2-mercaptopyridine N-oxide (Pyrithione), and N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; a Zn^{2+}-specific chelator) were purchased from Sigma (St Louis, MO). Tetramethylrhodamine ethyl ester (TMRE) was obtained from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's Medium (DMEM)/Ham F-12 (1:1), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY). Phospho-GSK-3β (Ser9), phospho-PKCα/β II (Thr638/641), phospho-(Ser/Thr) Phe, and β-tubulin antibodies were obtained from Cell Signalling Technology (Beverley, MA).

Cell culture

The human neuroblastoma cell line, SH-SY5Y, obtained from the American Type Culture Collection (ATCC CRL-2266, Manassas, VA), was maintained in DMEM/Ham's F-12 (1:1) supplemented with 10% FBS and penicillin (0.05 U/mL)/streptomycin (0.05 mg/mL) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Cell viability assay

SH-SY5Y cells were treated with different concentrations of $ZnCl_2$ (0 to 1000 μM) in the presence and absence of NaHS (0-10 mM) for the times indicated in the results. Then, cell viability was measured using the MTT reduction assay, as described previously[24], and the NR uptake assay, which quantifies the amount of the supravital dye NR taken-up by the lysosome[25]. To induce intracellular Zn^{2+} overload, cells were co-treated with a specific Zn^{2+} ionophore, pyrithione (Pyr) and $ZnCl_2$. Cells were exposed to 10 μM $ZnCl_2$ with 4 μM Pyr in the presence or absence of NaHS for 4 hours. Then, cell viability was determined by the MTT assay. At the end of each treatment, MTT (100 μg/mL) or NR (40 μg/mL) were added to each well, and the cells were further incubated at 37°C for 1 hour. Then, the water-insoluble formazan was dissolved in DMSO. For the NR uptake assay, cells were washed with phosphate buffered saline (PBS), and then, the NR taken-up by the cells was dissolved in a destaining solution containing 50:49:1 of ethanol: distilled water: acetic acid. Colorimetric determination of MTT reduction and NR uptake was measured at 550 nm using a Spectramax M^{2e} microplate Reader (Molecular Device, Menlo Park, CA). Cell viability is expressed as the percentage of the control value.

Propium iodide uptake assay

PI (10 μg/mL) was loaded onto SH-SY5Y cells, and then cells were treated with 750 μM $ZnCl_2$ or 10 μM $ZnCl_2$ plus 4 μM Pyr in the presence of NaHS (0, 3 and 5 mM) for 8 hours. Cells were visualized under an Olympus fluorescent microscope IX71 (Tokyo, Japan) and representative images were captured using MetaVue Software (Molecular Device).

Determination of Zinquin and Zinpyr-1 fluorescence *in vitro*

A solution of 50 μM Zinquin (previously dissolved in DMSO) in PBS was prepared. After the addition of 10 μM, cells were further treated with various concentrations of NaHS for 20 minutes at 30°C. Then, the intensity of Zinquin fluorescence was measured with

an excitation of 380 nm and an emission of 495 nm using a Spectramax M^{2e}. A 5 μM solution of Zinpyr-1 in PBS was prepared. After the addition of 10 μM $ZnCl_2$, different concentrations of NaHS were treated for 20 minutes at 30°C. Then, the intensity of Zinpyr-1 fluorescence was measured with an excitation of 485 nm and an emission of 595 nm using a Spectramax M^{2e}. The fluorescence intensity of either Zinquin staining alone or Zinpyr-1 staining alone was set at 100%. Results were expressed as the percentage of the respective control value.

Determination of cellular Zinpyr-1 fluorescence

SH-SY5Y cells, grown in a 100-mm dish, were stained with 25 μM Zinpyr-1 for 1 hour. After trypsinization, cells were collected by centrifugation at 350 × g for 5 min, and then suspended in Hanks' Balanced Salt solution containing 2% FBS. Cells (5×10^4 cells per well) were transferred into a fluorescence microplate, and then, treated with 10 μM $ZnCl_2$ and 4 μM Pyrithione for 20 minutes at 30°C. The change in the fluorescence intensity of Zinpyr-1 was determined 20 minutes after the addition of NaHS. The fluorescence intensity of Zinpyr-1 staining alone was set at 100%. Results were then expressed as the percentage of the fluorescence intensity of Zinpyr-1 alone. To determine the intracellular Zn^{2+} chelating activity of NaHS, SH-SY5Y cells were stained with 25 μM Zinpyr-1 dissolved in FBS-free culture medium for 1 hour at 37°C. After treating cells with 750 μM $ZnCl_2$ or 10 μM $ZnCl_2$ plus 4 μM Pyr for 30 minutes, culture media were replaced with fresh media containing NaHS (0, 3 and 5 mM). After 30 minutes, changes in the green fluorescence were determined under a fluorescent microscope

Determination of TMRE fluorescence

TMRE is a specific fluorescent indicator for the mitochondrial transmembrane potential (MMP). Cells were stained with 200 nM TMRE dissolved in FBS-free culture medium for 1 hour at 37°C. Cells were then treated with 750 μM $ZnCl_2$ or 10 μM $ZnCl_2$ plus 4 μM Pyr in the presence of NaHS (3 and 5 mM), or in the absence of it, for 4 hours. Changes in red fluorescence reflecting the MMP were determined using a fluorescent microscope.

Immunoblot analysis

SH-SY5Y cells were treated with 750 μM $ZnCl_2$ for 4 hours or 10 μM $ZnCl_2$ with 4 μM pyrithione for 4 hours, in the presence of NaHS at different doses, and in the absence of it. After washing twice with cold PBS, cells were lysed in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS), containing protease/phosphatase inhibitor cocktails (Sigma). The protein concentration was measured by the Pierce BCA protein assay kit (Thermo, Rockford, IL), and fifty micrograms of protein were electrophoresed through a 10% SDS-PAGE gel, and transferred to an Immobilon1-P polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany). Binding of each primary antibody was detected with a secondary antibody and visualized using enhanced chemiluminescence. Equal loading of the samples was confirmed by reprobing the membranes with an anti β-tubulin antibody.

Statistical analysis

Data were expressed as the mean ± S.E.M. Significance was determined by one-way analysis of variance (ANOVA) for comparisons between individual groups using SigmaPlot (Systat Software, Inc. San Jose, CA). A statistical probability of $P < 0.05$ was considered significant.

Results

NaHS protects SH-SY5Y cells against Zn^{2+}-induced cell death

We first examined the toxic effect of NaHS on SH-SY5Y cells using the MTT reduction assay. As shown in Figure 1, up to 25 mM NaHS did not result in any significant cell death within the first 24 hours of treatment. When the same concentration of NaCl was added to the culture medium to exclude the possibility of a NaHS-mediated osmotic change, there was no significant cell death (data not shown). Exogenous Zn^{2+}, supplied in the form of $ZnCl_2$ for 24 hours, caused significant cell death at concentrations above 100 μM, which was determined using the MTT assay (Figure 2A). In subsequent experiments, we selected 750 μM Zn^{2+} as the treatment dose to mimic the acute phase of Zn^{2+} overload and to further determine changes in cell viability with exposure time. SH-SY5Y cells, exposed to 750 μM Zn^{2+} for 3 hours, exhibited significant cell death and failed to recover from the Zn^{2+} insult (Figure 2B). However, cell death induced by Zn^{2+} overload (750 μM) was significantly inhibited in the presence of NaHS (1-10 mM), as depicted in Figure 3A. The MTT-based cell viability measurement is largely dependent on mitochondrial activity[26], but its reduction can be interfered with by highly reductive compounds[27]. NaHS was able to weakly reduce MTT *in vitro* (data not shown). There is the possibility that Zn^{2+}, as well as NaHS, may directly affect mitochondrial function[28]

and thereby, cause interference in the MTT reduction assay. Thus, a neutral red (NR) uptake assay was also conducted to determine the protective effect of NaHS against Zn^{2+}-mediated cell toxicity. Similar to the results of the MTT reduction assay, the protective effect of NaHS was further confirmed by the NR uptake assay (Figure 3B). Zn^{2+} overload can be induced by extracellular or intracellular sources. To mimic intracellular Zn^{2+} overload, SH-SY5Y cells were treated in the presence or absence of NaHS with a combination of $ZnCl_2$ and pyrithione (Pyr), which is a Zn^{2+}-specific ionophore, thus causing an increase in intracellular Zn^{2+}. As shown in Figure 3C, NaHS was found to suppress intracellular Zn^{2+} overload-mediated induced cell death. These results suggest that NaHS can protect cells against both extracellular and intracellular Zn^{2+} insult. In addition, the PI uptake assay and TMRE staining were conducted to further determine the protective effect of NaHS against Zn^{2+}-induced cell death. It is due to compromised membrane integrity that dead cells uptake PI, and thus emit a red fluorescence. PI-stained cells exposed to 750 μM $ZnCl_2$ (Figure 4B) and 10 μM $ZnCl_2$ plus 4 μM Pyr (Figure 4C) were greatly increased at 8 hours. However, NaHS treatment was found to suppress the Zn^{2+}-induced cell death, resulting in decreased occurrence of PI-stained cells. A decrease in the MMP is another hallmark of cell death caused by toxic insults. Assessment of mMP by TMRE staining (Figure 5), revealed that both 750 μM $ZnCl_2$ and 10 μM $ZnCl_2$ plus 4 μM Pyr caused a decrease in red fluorescence, compared with the untreated control cells. NaHS treatment was also found to block the Zn^{2+}-induced loss of mMP. Interestingly, the morphologies of SH-SY5Y cells exposed to both 750 μM $ZnCl_2$ and 10 μM $ZnCl_2$ plus 4 μM Pyr at 4 hours were almost round (Figure 5), but PI-stained cells were less detectable (data not shown). These results imply that the collapse of mMP by Zn^{2+} overload is rather an earlier step than that of PI-uptake in the cell. Collectively, these results indicate that the decrease in cell viability shown by the MTT and NR uptake assays (Fig-

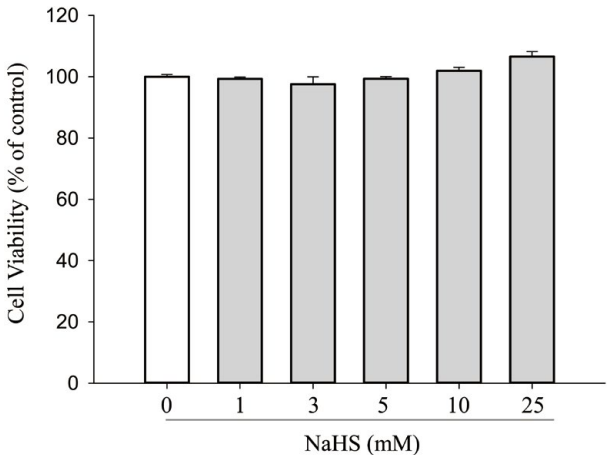

Figure 1. Cytotoxicity of NaHS. SH-SY5Y cells were treated with various concentrations of NaHS for 24 hours. Cell viability was determined by the MTT assay. Results are expressed as a percentage of the value reported for the untreated control cells. Data are the mean ± SEM (for each group, n = 5). There was no significant difference among the groups. NaHS; sodium hydrogen sulfide.

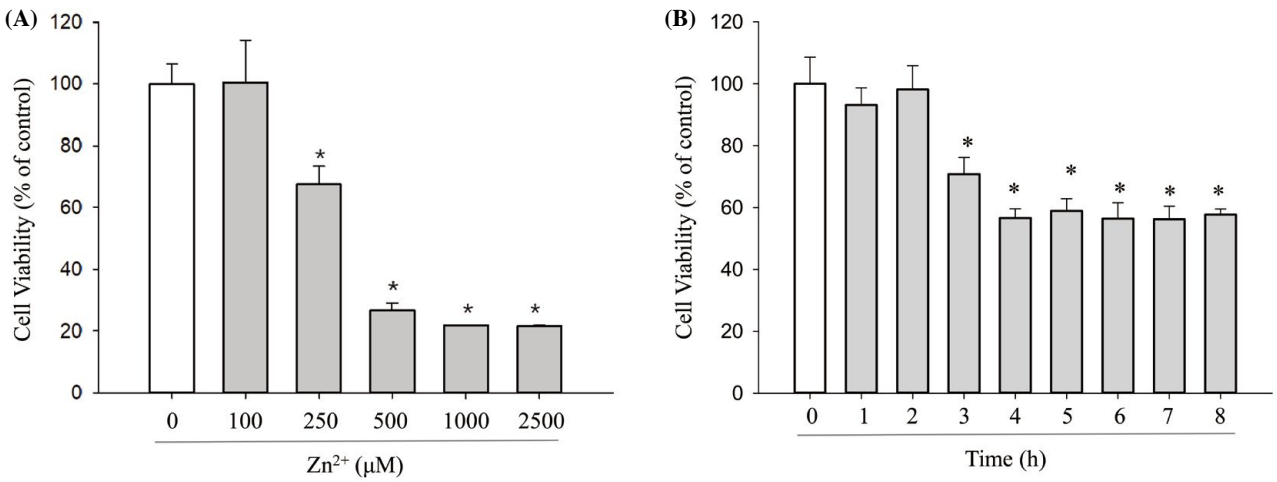

Figure 2. Cytotoxicity of exogenous Zn^{2+} insult. SH-SY5Y cells were treated with various concentrations of $ZnCl_2$ for 24 hours, and then, cell viability was determined by the MTT assay (A). To assess the cytotoxicity of Zn^{2+}, cells were treated with 750 μM $ZnCl_2$ for the indicated time and replaced with fresh medium to remove any remaining $ZnCl_2$ from the medium. Cell viability was determined by the MTT assay at the 24th hour (B). Results are expressed as a percentage of the value reported for the untreated control cells. Data are the mean ± SEM (for each group, n = 5). *$P < 0.05$ versus the untreated control.

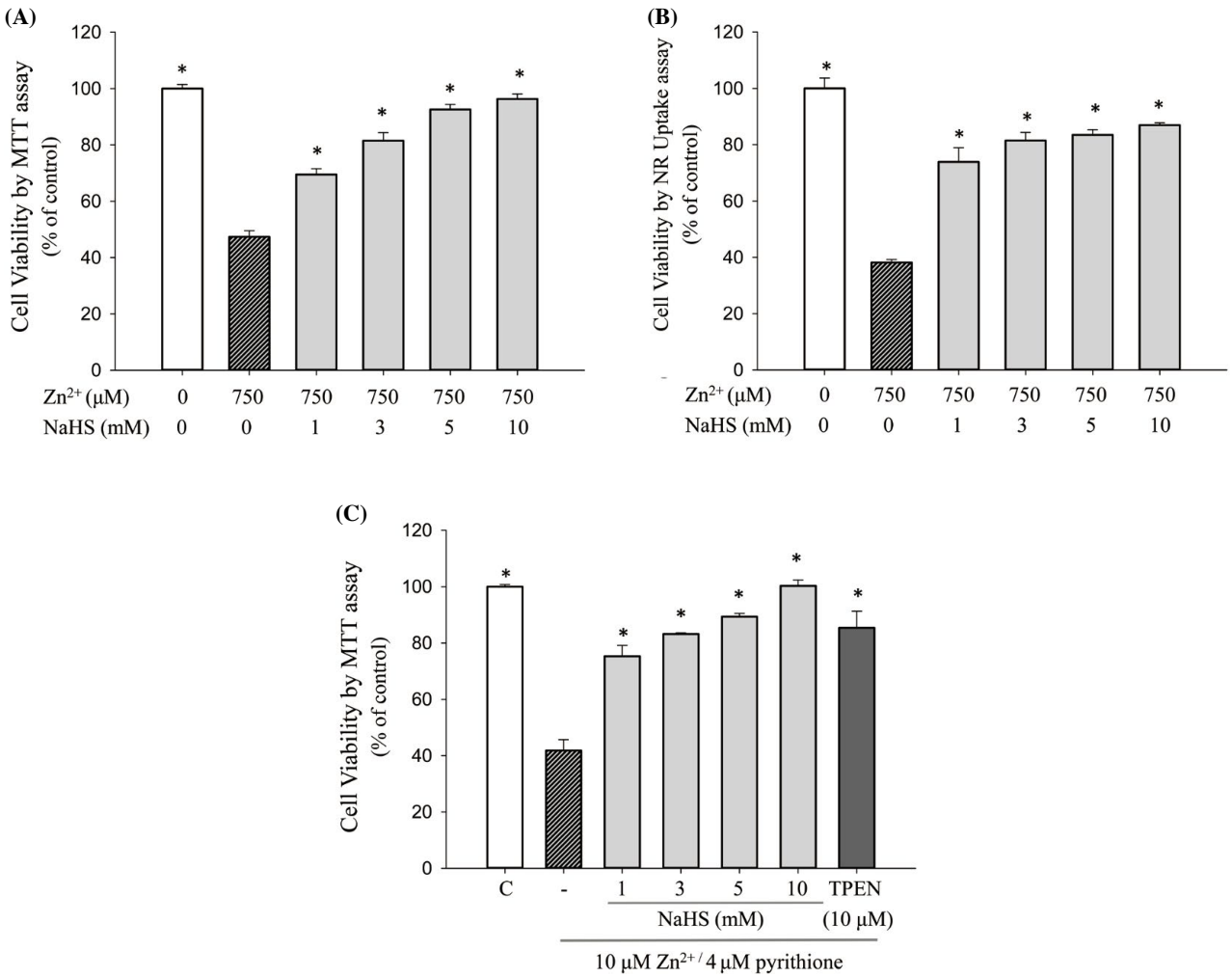

Figure 3. Protective effect of NaHS against Zn^{2+}-induced cell death. SH-SY5Y cells were treated with 750 μM $ZnCl_2$ for 4 hours in the presence of NaHS at different doses, or in the absence of it. Cell viability was determined by the MTT assay (A) and the neutral red (NR) uptake assay (B). To induce intracellular Zn^{2+} overload, cells were co-treated with pyrithione (Pyr), a specific Zn^{2+} ionophore, and $ZnCl_2$. Here, TPEN was used as a Zn^{2+}-specific chelator. SH-SY5Y cells were exposed to 10 μM $ZnCl_2$ with 4 μM Pyr in the presence or absence of NaHS for 4 hours. Then, cell viability was determined by the MTT assay (C). Results are expressed as the percentage of the value of the untreated control group. Data are the mean ± SEM (each group, n = 5). *$P < 0.05$ versus the 750 μM $ZnCl_2$-treated group or the 10 μM $ZnCl_2$ with 4 μM Pyr-treated group.

ure 3) was associated not with the arrest in proliferation but with cell death. However, it is not clear whether the mode of Zn^{2+}-induced cell death observed in the present study corresponds to apoptosis or necrosis.

NaHS has the capacity to chelate Zn^{2+}

NaHS prevented SH-SY5Y cell death caused by both extracellular and intracellular Zn^{2+} overload (Figures 3-5). One of the underlying protective mechanisms of NaHS against Zn^{2+} overload may be associated with its direct Zn^{2+}-chelating capacity. The effect of NaHS on Zn^{2+} levels was determined *in vitro* by a fluorometric method after staining with Zinpyr-1 and Zinquin, which are highly specific zinc probes. The Zn^{2+}-mediated increase in the fluorescence intensities of Zinpyr-1 (Figure 6A) and Zinquin (Figure 6B) was significantly decreased in the presence of NaHS in a dose-dependent manner. The Zn^{2+}-chelating capacity of NaHS at 1 mM was similar to that of 25 μM TPEN. This result implies that NaHS may chelate Zn^{2+} with a lower affinity than TPEN. Next, the intracellular Zn^{2+} scavenging capacity of NaHS was tested in SH-SY5Y cells, after treating cells with 10 μM $ZnCl_2$ plus 4 μM Pyr, using a fluorometric method. TPEN completely suppressed intracellular Zn^{2+} levels, as determined by the changes in the fluorescence intensity of Zinpyr-1.

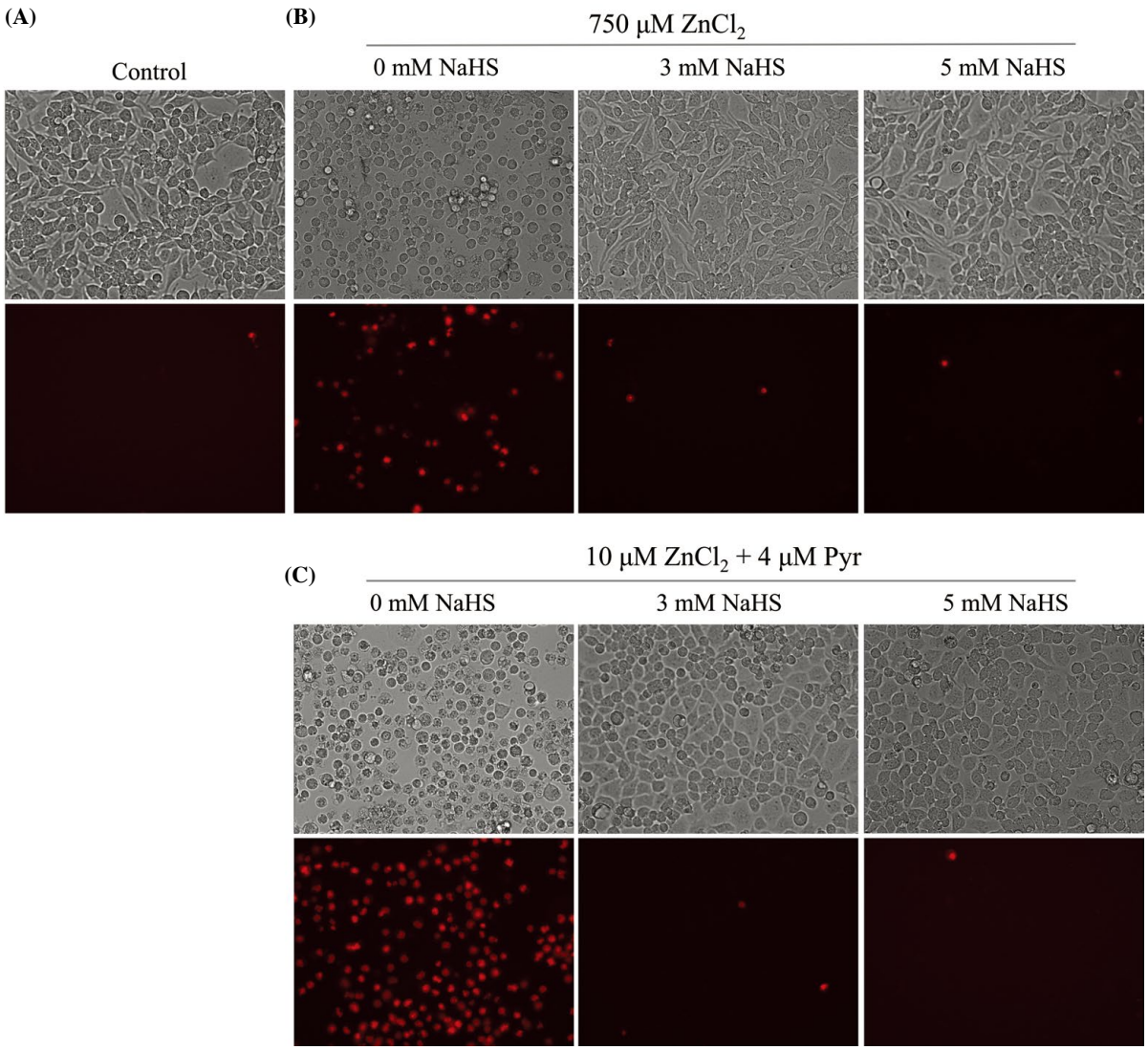

Figure 4. Propium iodide Staining. SH-SY5Y cells were preloaded with PI (10 μg/mL) and treated with 750 μM $ZnCl_2$ or 10 μM $ZnCl_2$ plus 4 μM Pyr for 8 hours in the presence of NaHS (0, 3, 5 mM). Dead cells uptake PI and emit red fluorescence. Cells were visualized under an Olympus fluorescent microscope IX71. Representative images were captured using MetaVue Software. (A) untreated control. (B) 750 μM $ZnCl_2$ in the presence of NaHS. (C) 10 μM $ZnCl_2$ plus 4 μM Pyr in the presence of NaHS. The upper and the lower panels show an image from bright field and from fluorescent microscopy (results obtained from duplicate experiments), respectively. Original magnification, ×200. NaHS; sodium hydrogen sulfide, PI; propium iodide, Pyr; pyrithione (zinc ionophore).

NaHS was also found to suppress the intracellular levels of Zn^{2+} in a dose-dependent manner (Figure 7), but a high dose of NaHS was required, compared with the *in vitro* Zn^{2+}-chelating activity shown in Figure 6. Next, we tested the direct intracellular Zn^{2+}-scavenging activity of NaHS using fluorescent microscopy. The green fluorescence of Zinpyr-1, which indicates the level of free Zn^{2+}, was highly increased after exposure to 750 μM $ZnCl_2$ (Figure 8B) and 10 μM $ZnCl_2$ plus 4 μM Pyr (Figure 8C). However, this increase in Zinpyr-1 fluorescence was markedly decreased by subsequent treatment with NaHS (3 and 5 mM). This result suggests that NaHS can scavenge intracellular Zn^{2+} directly, but its scavenging capacity may be rather weak, because the intensity of Zinpyr-1 fluorescence is not completely prevented even in the presence of NaHS. Nevertheless, it still showed higher fluorescent intensity than that of the untreated control (Figure 8A). This result indicates that NaHS possesses an intracellular Zn^{2+}-chelating capacity, and therefore can be

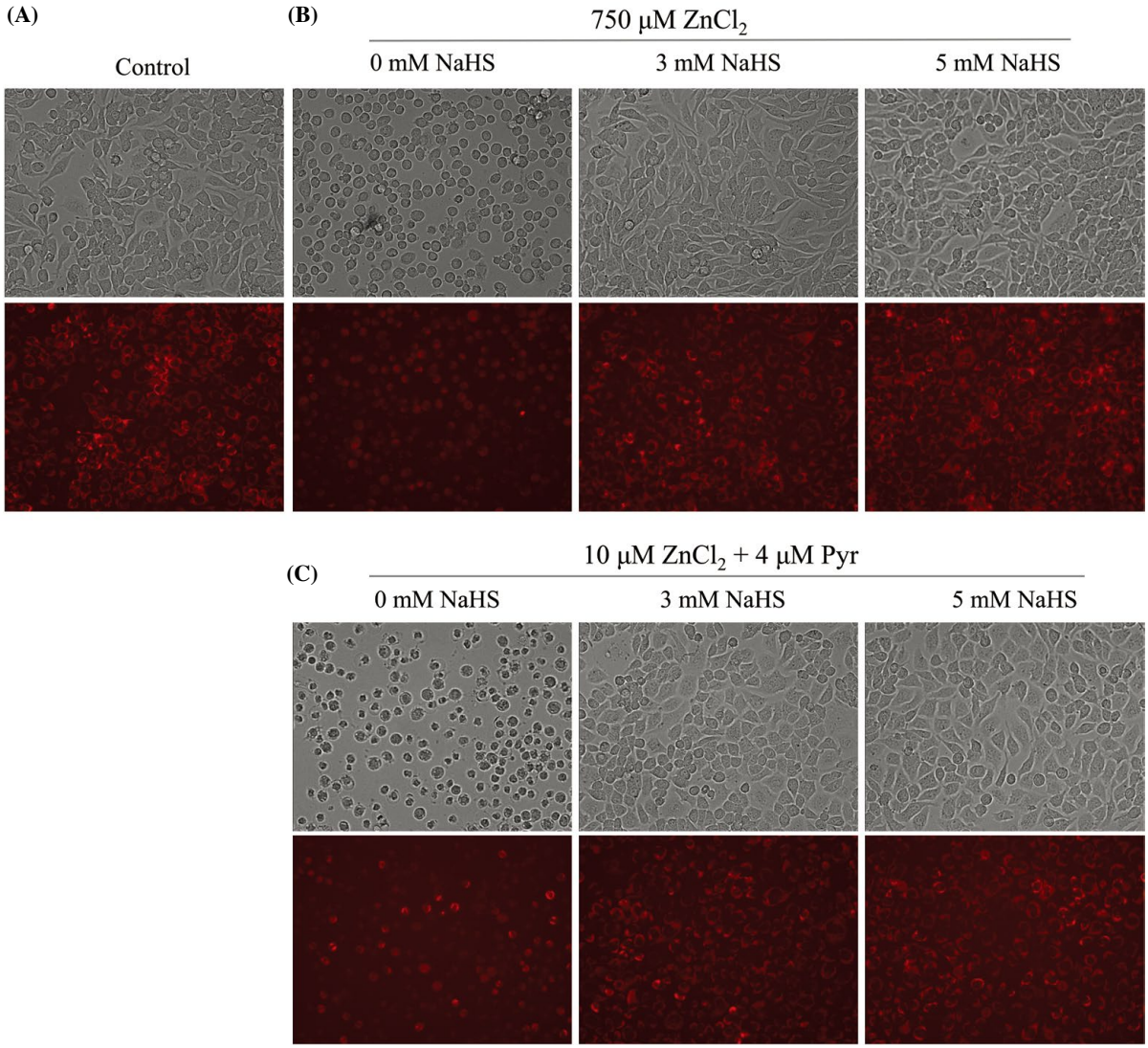

Figure 5. TMRE staining. After staining with TMRE (200 nM), a specific fluorescent indicator for mitochondrial membrane potential, SH-SY5Y cells were treated with 750 μM $ZnCl_2$ or 10 μM $ZnCl_2$ plus 4 μM Pyr for 4 hours in the presence of NaHS (0, 3, 5 mM). Cells were visualized under an Olympus fluorescent microscope IX71. Representative images were captured using MetaVue Software. (A) untreated control. (B) 750 μM $ZnCl_2$ in the presence of NaHS. (C) 10 μM $ZnCl_2$ plus 4 μM Pyr in the presence of NaHS. The upper and the lower panels show an image from bright field and from fluorescent microscopy (results obtained from duplicate experiments), respectively. Original magnification, ×200. NaHS; sodium hydrogen sulfide, Pyr; pyrithione (zinc ionophore), TMRE; tetramethylrhodamine ethyl ester.

used in the cellular protection against Zn^{2+} overload in SH-SY5Y cells.

NaHS suppresses Zn^{2+}-mediated increases in the phosphorylation of Ser/Thr, GSK-3β and PKC

Zn^{2+} can evoke numerous signalling pathways in complex manners[29]. For example, it is well known that Zn^{2+} can strongly induce the phosphorylation of GSK-3β[30] and PKC[31]. If NaHS can chelate Zn^{2+}, then the Zn^{2+}-induced increase in the phosphorylation of GSK-3β and PKC should be suppressed in the presence of NaHS. To test this, SH-SY5Y cells were treated either with 750 μM $ZnCl_2$ or with 10 μM $ZnCl_2$ combined with 4 μM Pyr in the presence and absence of NaHS (1-10 mM). Next, changes in the phosphorylation of serine/threonine residue at proteins (phospho-Ser/Thr), GSK-3β or PKC were determined by immunoblotting (Figure 9). Upon treatment with 750 μM $ZnCl_2$, the level of phospho-Ser/Thr was greatly increased com-

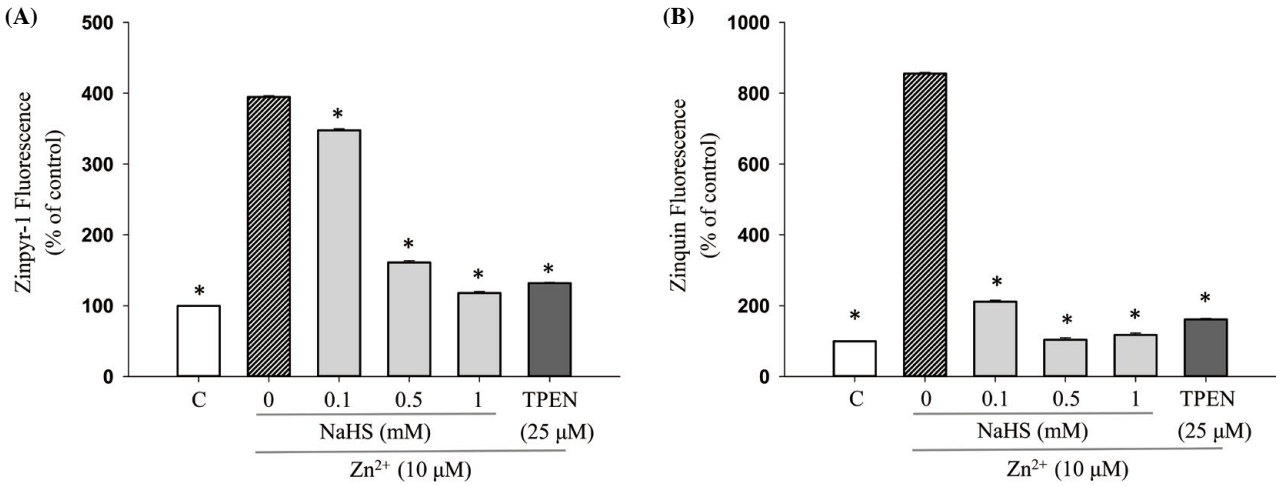

Figure 6. *In vitro* Zn^{2+}-chelating activity of NaHS. To determine the changes in Zn^{2+} fluorescence in the presence or absence of NaHS, Zn^{2+}-specific fluorescent probes, Zinpyr-1 and Zinquin, were used. Both 5 μM Zinpyr-1 (A) and 50 μM Zinquin (B) dissolved in PBS, were incubated with 10 μM $ZnCl_2$ at 30°C, and then, various concentrations of NaHS were added for 20 minutes. Changes in the fluorescence intensities of Zinpyr-1 (excitation: 485 nm; emission: 595 nm) and Zinquin (excitation: 380 nm; emission: 495 nm) were measured by a Spectramax M^{2e}. Results are expressed as the percentage of Zinpyr-1 or Zinquin staining alone. Data are the mean ± SEM (for each group, n = 5). *$P < 0.05$ versus the 10 μM $ZnCl_2$-treated group.

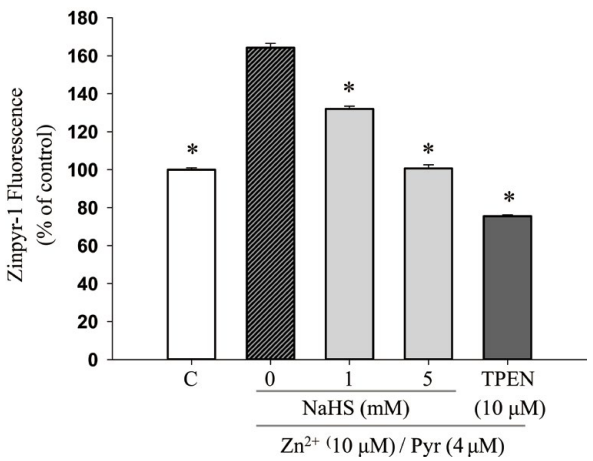

Figure 7. Intracellular Zn^{2+}-chelating activity of NaHS. SH-SY5Y cells were stained with 25 μM Zinpyr-1 for 1 hour at 37°C. Cells were harvested and then suspended in Hanks' Balanced Salt solution containing 2% FBS. After treatment with 10 μM $ZnCl_2$ and 4 μM zinc pyrithione (Pyr) for 20 min at 30°C, cells were further incubated with various concentrations of NaHS for 10 minutes. Then, the change in the fluorescence of Zinpyr-1 (excitation: 485 nm; emission: 595 nm) was measured using a Spectramax M^{2e}. Results are expressed as the percentage of the value reported for the untreated control. Data are the mean ± SEM (for each group, n = 5). *$P < 0.05$ versus the 10 μM $ZnCl_2$-treated group.

pared with the untreated control cells. However, NaHS treatment suppressed that increase in the level of phospho-Ser/Thr (Figure 9A). In addition, NaHS blocked the Zn^{2+}-mediated increase in the phosphorylation of GSK-3β (Figure 9C) and PKC (Figure 9B and C). These results also suggest that NaHS can chelate Zn^{2+}, and therefore, in this experimental setting, Zn^{2+}-mediated signalling pathways cannot be initiated due to lowered level of Zn^{2+}.

Discussion

In this study, we found that NaHS can offer neuroprotection against extracellular and intracellular Zn^{2+} overload-induced cell death in SH-SY5Y cells. This protective feature of NaHS appears to be associated with its Zn^{2+} scavenging activity, because the extracellular and/or intracellular Zn^{2+} level was, at least in part, suppressed in the presence of NaHS.

Besides an overdose of zinc, excessive Zn^{2+} is released from nerve terminals, following cerebral ischemia that causes brain injury[17,32]. Aberrant control of Zn^{2+} content may cause dopaminergic neuron death, which contributes to the progression of Parkinson's disease[18]. These pathological conditions, which may be associated with an excessive presence of Zn^{2+} in the brain, could be ameliorated by a supply of H_2S[21,33,34]. H_2S constitutes an endogenously-produced gas in the central nervous system, and is considered to be an important mediator in a myriad of neural functions, including neuroprotection, which has been attributed to its antioxidant, anti-apoptotic, vasculoprotective,

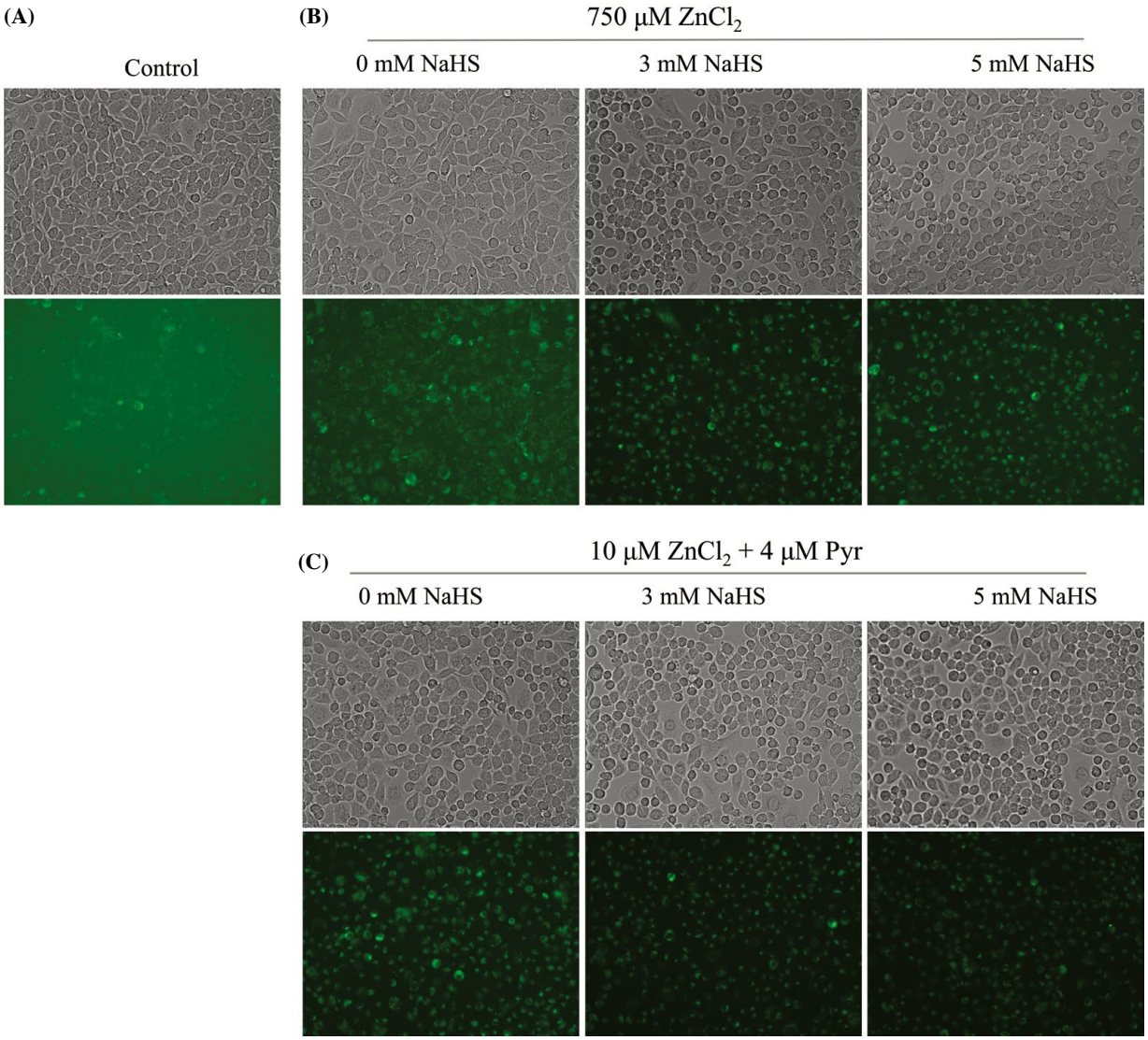

Figure 8. Intracellular Zinpyr-1 Staining. After staining with 25 μM Zinpyr-1 for 1 hour at 37°C, SH-SY5Y cells were treated with 750 μM $ZnCl_2$ or 10 μM $ZnCl_2$ plus 4 μM Pyr for 30 minutes to increase intracellular Zn^{2+} content. After washing, cells were treated with NaHS (0, 3, 5 mM) for 30 minutes and then cells were visualized under an Olympus fluorescent microscope IX71. Representative images were captured using MetaVue Software. (A) untreated control. (B) 750 μM $ZnCl_2$ in the presence of NaHS. (C) 10 μM $ZnCl_2$ plus 4 μM Pyr in the presence of NaHS. The upper and the lower panels show an image from bright field and fluorescent microscopy (results obtained from duplicate experiments), respectively. Original magnification, ×200. NaHS; sodium hydrogen sulfide, Pyr; pyrithione (zinc ionophore).

and anti-inflammatory properties, as reviewed in detail elsewhere[1,35-38]. Moreover, deficiency of H_2S is strongly associated with many neurological conditions, such as stroke, Alzheimer's and Parkinson's diseases[37,38]. Although its underlying mechanisms need to be studied further, a number of clinical trials have been conducted to explore novel interventions in these pathophysiological conditions[34].

In this experiment, NaHS was found to inhibit the loss of mMP (Figure 5) and cell death (Figures 3 and 4) caused by excessive extracellular and intracellular Zn^{2+}. It has been suggested that Zn^{2+} cytotoxicity in SH-SY5Y cells can be suppressed by the application of NaHS, an H_2S donor[20]. In a previous report, the authors suggested that the NaHS protection against Zn^{2+} cytotoxicity may be exerted by inhibiting the entry of Zn^{2+} into the cells. In this study, however, we identified that NaHS can directly chelate Zn^{2+}. This was determined by Zn^{2+}-specific fluorescent probes, namely Zinpyr-1 and Zinquin (Figures 6-8). Compared

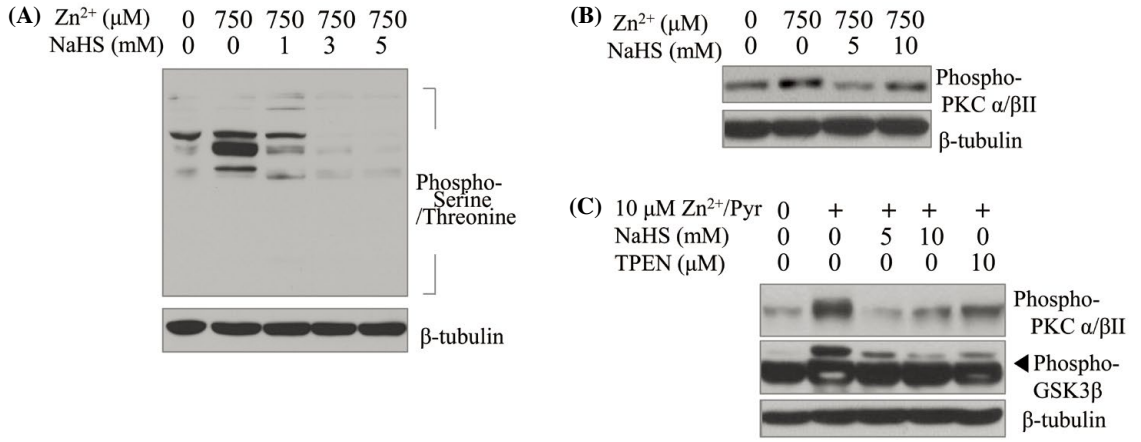

Figure 9. Immunoblot analysis. SH-SY5Y cells were treated with either 750 μM $ZnCl_2$ for 4 hours (A and B) or with 10 μM $ZnCl_2$ plus 4 μM pyrithione (Pyr) for 4 hours (C) in the presence or absence of NaHS. Whole cell lysates were prepared and subjected to immunoblot analysis for phosphor-serine/threonine (A), phosphor-PKC α/βII (B) and phospho-GSK3β (C). β-tubulin was used as a protein loading control. The image shows a representative immunoblot from three independent experiments. GSK-3β: glycogen synthase kinase-3β; PKC: protein kinase C.

with a previous report[20], the novel finding of this study is that the cytoprotective effect of NaHS against Zn^{2+} insult was shown to be mediated by its Zn^{2+} scavenging activity, along with suppression of the entry of Zn^{2+} into the cell. The Zn^{2+}-chelating capacity of NaHS was found to be involved in the attenuation of toxicity caused by extracellular and intracellular Zn^{2+} overload. Moreover, the intracellular Zn^{2+} scavenging activity of NaHS, as shown in Figures 6-8, provides an insight into the regulatory role of endogenous H_2S in the handling of Zn^{2+}. Zinc acts as either an antioxidant or a pro-oxidant in cellular systems. Moreover, zinc deficiency and zinc excess cause cellular oxidative stress[39]. Unfortunately, the possible role of NaHS in the oxidative stress caused by Zn^{2+} has not been investigated. It is speculated that Zn^{2+} insult may cause oxidative stress and lead to cell death. But this type of Zn^{2+} toxicity might not be initiated in the presence of NaHS, due to its Zn^{2+} chelating activity. However, experimental demonstration of this will be needed in future studies.

When Zn^{2+} is depleted by TPEN, a specific zinc-chelator, cells undergo cellular death[40,41]. Unlike cell death caused by TPEN treatment, NaHS was found to chelate Zn^{2+}, similar to TPEN, but it did not cause any significant cell death up to a concentration of 25 mM (Figure 1). It is unclear why NaHS is not cytotoxic, despite its Zn^{2+}-chelating capacity. This raises the possibility that NaHS may control the sequestration of Zn^{2+} in the cell in an unidentified manner, or serve as a temporal Zn^{2+} storage pool. However, in order to clearly elucidate the role of NaHS (or H_2S) in the regulation of Zn^{2+} levels, more extensive work is needed. It should be mentioned that previous reports have addressed the cytotoxicity of NaHS above a concentration of 1 mM[42]. However, in this study, NaHS up to 25 mM did not significantly induce cell death in SH-SY5Y cells at 24 hour. The protective effect of NaHS against Zn^{2+}-induced cell death was assessed by the MTT and NR uptake assays, which are based on mitochondrial activity and on the retention of lysosomal integrity, respectively. If NaHS at a given dose displays cytotoxic effects on SH-SY5Y cells, then cell viability should be lower in the presence than in the absence of it. It was assumed that the doses of NaHS used in this experiment were not cytotoxic, but a clear explanation for this could not be provided.

NaHS dissolved in water produces H_2S, HS^-, and other such compounds. It is unclear which form of NaHS, dissolved in biological systems, is truly involved in the direct scavenging of Zn^{2+}. Indeed, the protective role of NaHS in brain ischemia-reperfusion injury, or other conditions, is not limited to its Zn^{2+}-chelating activity, because short-lasting metabolic inhibition, followed by restoration of mitochondrial electron transport[21], in addition to the previously mentioned protective roles of H_2S, are likely to be involved also. Alternatively, it has been suggested that H_2S can be used to detoxify the cell from cadmium excess, as shown in plants[43]. It would be interesting to determine whether the chelating potential of NaHS is limited to Zn^{2+} or extends to other heavy metals, such as cadmium or copper. Future work is needed to elucidate the exact Zn^{2+}-chelating mode of NaHS and its involvement in the regulation of intracellular Zn^{2+} content.

Conclusion

Collectively, our results indicate that NaHS can directly scavenge extracellular and intracellular Zn^{2+}, and thus, this capacity of NaHS (or H_2S) underlies the protective potential against Zn^{2+} overload-mediated cell death. In addition, the Zn^{2+} chelating activity of NaHS does not seem to affect cell viability.

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