Neuroprotective effects of hydrogen sulfide on sodium azide-induced autophagic cell death in PC12 cells

HAIYAN SHAN[1,2*], YANG CHU[3*], PAN CHANG[4*], LIJUN YANG[3], YI WANG[3], SHAOHUA ZHU[3], MINGYANG ZHANG[1,3] and LUYANG TAO[3]

[1]Shanghai Key Laboratory of Forensic Medicine, Institute of Forensic Science, Ministry of Justice, Shanghai 200063; [2]Department of Obstetrics and Gynecology, North of Suzhou Municipal Hospital, Suzhou, Jiangsu 215008; [3]Institute of Forensic Sciences, Soochow University, Suzhou, Jiangsu 215123; [4]Central Laboratory, Second Affiliated Hospital of Xi'an Medical College, Xi'an, Shaanxi 710038, P.R. China

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Abstract. Sodium azide (NaN_3) is a chemical of rapidly growing commercial importance. It is very acutely toxic and inhibits cytochrome oxidase (COX) by binding irreversibly to the heme cofactor. A previous study from our group demonstrated that hydrogen sulfide (H_2S), the third endogenous gaseous mediator identified, had protective effects against neuronal damage induced by traumatic brain injury (TBI). It is well-known that TBI can reduce the activity of COX and have detrimental effects on the central nervous system metabolism. Therefore, in the present study, it was hypothesized that H_2S may provide neuroprotection against NaN_3 toxicity. The current results revealed that NaN_3 treatment induced non-apoptotic cell death, namely autophagic cell death, in PC12 cells. Expression of the endogenous H_2S-producing enzymes, cystathionine-β-synthase and 3-mercaptopyruvate sulfurtransferase, decreased in a dose-dependent manner following NaN_3 treatment. Pretreatment with H_2S markedly attenuated the NaN_3-induced cell viability loss and autophagic cell death in a dose-dependent manner. The present study suggests that H_2S-based strategies may have future potential in the prevention and/or therapy of neuronal damage following NaN_3 exposure.

Introduction

Traumatic brain injury (TBI), a form of acquired brain injury, is one of the leading causes of death in children and adults worldwide. It causes long-term disability with a broad spectrum of symptoms, including headache, confusion, dizziness, blurred vision or tired eyes, ringing in the ears, bad taste in the mouth, fatigue or lethargy, a change in sleep patterns, behavioral or mood changes, and trouble with memory, concentration, attention, or thinking (1). TBI is documented to have detrimental effects on the central nervous system (CNS) metabolism, including the depression of mitochondrial oxidative phosphorylation. Studies have also provided evidence that TBI can suppress the activity of the cytochrome oxidase (COX) and inhibition of COX is an important aspect of trauma pathology (2-4).

Sodium azide (NaN_3) is a colorless, explosive, and highly toxic salt that is soluble in water. Its principal toxic action is in inhibiting the function of COX in the mitochondrial electron transport chain (5). The tissue-specific inhibition of COX by NaN_3 could serve as a useful research tool for the evaluation of cell death *in vivo* and *in vitro*. A growing body of evidence suggests that neuronal cell death serves a pivotal role in the TBI process (6-8). Autophagy is known as one of the critical cellular homeostatic mechanisms. Dysregulation of autophagy contributes to neuronal cell death following TBI (9). Previous studies from our group have demonstrated that autophagic cell death could be induced by TBI and therefore, this pathway may serve as a target for future treatments (10,11). Another study has reported that NaN_3 could lead to apoptosis in primary cortical neuronal cells (12). Evidence is accumulating that non-apoptotic cell death is associated with neuronal damage induced by NaN_3 in primary cortical neuron cultures (13). However, whether NaN_3 may induce autophagic cell death remains poorly understood.

Research into the biology of hydrogen sulfide (H_2S) over the last decade has exponentially increased our understanding of the way in which this gasotransmitter influences physiological and pathophysiological processes in a wide range of biological systems (14). H_2S has long been hypothesized to be an environmental pollutant which is a colorless, flammable, water-soluble gas characterized by a peculiar smell of rotten eggs (15). H_2S is also produced endogenously in mammals, including humans. In particular, cystathionine-β-synthase (CBS) in the central nervous system and cystathionine-γ-lyase (CSE) in the

Correspondence to: Dr Mingyang Zhang or Dr Luyang Tao, Institute of Forensic Sciences, Soochow University, 178 Ganjiang East Road, Suzhou, Jiangsu 215123, P.R. China
E-mail: mingyangzhang@suda.edu.cn
E-mail: taoluyang@suda.edu.cn

*Contributed equally

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cardiovascular system are the key enzymes mostly responsible for the endogenous generation of H_2S (16). 3-mercaptopyruvate sulfurtransferase (3-MST) is also known to be a significant producer of endogenous H_2S in the brain (17). H_2S has recently been regarded as a novel gasotransmitter, possessing very important physiological and pharmacological functions in the brain: enhancing N-methyl-D-aspartate receptor-mediated responses, facilitating the induction of hippocampal long-term potentiation, and inhibiting synaptic transmission in the hippocampus (16,18). However, it is not known whether H_2S participates in cell physiology and autophagic pathways in the neuronal cells treated with NaN_3.

In the present study, the features of the neuronal damage induced by NaN_3 treatment were investigated. Then, the potential neuroprotective activity of H_2S and its effect on autophagic cell death were investigated following NaN_3 treatment in neuron-like rat pheochromocytoma (PC12) cells. This is the first study demonstrating that NaN_3 can induce autophagic cell death in PC12 cells and that H_2S can suppress this effect. The present findings may help to gain a better insight into the physiological functions of H_2S in the normal and injured conditions and its association with the cellular and molecular mechanisms underlying nervous system lesion and repair.

Materials and methods

Cell culture. Rat pheochromocytoma PC12 cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). PC12 cells were grown on polystyrene tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 5% fetal bovine serum (FBS; Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), supplemented with 2 mmol/l glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 95% air-5% CO_2. Prior to differentiation, the medium was changed twice a week and the cells were subcultured at a ratio of 1:4 once a week. For differentiation, the cells were washed and incubated in fresh medium containing nerve growth factor (NGF; final concentration of 50 ng/ml) for 48 h at 37˚C in a cell incubator. The addition and concentration of NGF in the media was maintained throughout all experiments and cell were used between passages 3-8.

Cell injury model. For simulating injury, the DMEM medium was removed, PC12 cells were washed twice with glucose-free Earle's balanced salt solution (pH 7.5), and changed to glucose-free DMEM medium without FBS prior to treatment. Then neurotoxic damage was induced by adding the indicated concentrations of NaN_3 for different periods of time in the cultured cells. Cells were preincubated with the indicated concentrations of sodium hydrosulfide (NaHS), as a donor of H_2S, for 30 min prior to NaN_3 treatment and maintained throughout the entire experiment. NaHS was dissolved in saline and was freshly prepared just before use. The stock solutions were directly added into the bath solution to achieve the final concentration. Control cultures were maintained in DMEM medium for the same duration and were left untreated. The concentrations of all reagents were maintained throughout the injury period.

Determination of cell viability. The viability of PC12 cells was determined by Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's instructions. PC12 cells were cultured in 96-well plates at 37˚C under an atmosphere of 5% CO_2 and 95% air. At the end of treatment, CCK-8 reagent (10 μl) was added to each well of the plates and then the plates were incubated at 37˚C for 3-4 h in the incubator. Absorbance at a wavelength of 450 nm was measured with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The mean optical density (OD) of 6 replicate wells in each of the indicated groups were calculated, and the cell viability was expressed as a % of the control. All experiments were performed in triplicate and repeated three independent times.

Nuclear staining for assessment of cell death. Chromosomal condensation and morphological changes in the nucleus of PC12 cells were observed by DAPI and propidium iodide (PI) staining. The PC12 cells were fixed with 4% paraformaldehyde for 10 min. Following three rinses with PBS, the cells were stained with 10 μg/ml DAPI for 10 min. PI (10 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was diluted in 0.9% NaCl to a working concentration of 40 μg/ml. For detection of PI-labeled cells, cells were fixed in 100% ethanol for 10 min at room temperature, then were stained with PI for 30 min at room temperature, washed 3 times in PBS and coverslipped with Permount (Biomeda Corporation, Burlingame, CA, USA), and photographed on a Nikon Eclipse Ti-S fluorescence microscope (Nikon Corporation, Tokyo, Japan) using excitation/emission filters at 568/585 nm for PI. Viable cells exhibited a normal nucleus size and uniform fluorescence in the DAPI channel, whereas dead cells exhibited PI/DAPI double positive staining and condensed nuclei. Colocalization and morphometric measurements were performed using ImageJ software, version 1.6 (National Institutes of Health, Bethesda, MD, USA). To quantify the immunoreacted cells, the fluorescence intensity was measured in 10 randomly selected images. Data were obtained from at least three independent experiment.

Immunofluorescence analysis. PC12 cells in 24-well plates were fixed with 4% paraformaldehyde for 15 min at room temperature and washed thrice with PBS for 10 min. The cells were then blocked with 5% donkey serum (Gibco; Thermo Fisher Scientific, Inc.) with 0.3% Triton X-100 and 5% bovine serum albumin (BSA) for 2 h at room temperature. Cells were incubated with rabbit polyclonal primary antibodies targeting microtubule-associated protein 1A/1B-light chain 3 (LC3; cat. no. ab48394; 1:100; Abcam, Cambridge, UK) overnight at 4˚C, followed by a mixture of fluorescein isothiocyanate-conjugated secondary antibodies (1:200; cat. no. 131699; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h at room temperature. Following three washes with PBS for 10 min each, 40 μg/ml PI was added for 30 min at room temperature. Following three further washes with PBS for 10 min each, the coverslips were mounted using Antifade Mounting Medium (Beyotime Institute of Biotechnology, Haimen, China) and observed with an Eclipse Ti-S fluorescence microscope (Nikon Corporation). Colocalization and morphometric measurements were performed using ImageJ software, version 1.6. To

quantify the immunoreacted cells, the fluorescence intensity was measured in 10 randomly selected images. Data were obtained from at least three independent experiment.

Western blot analysis. The cells were homogenized in lysis buffer (1% NP-40, 50 mmol/l Tris PH 7.5, 5 mmol/l EDTA, 1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mmol/l phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, and 1 μg/ml leupeptin) and the lysates were centrifuged at 15,000 x g for 20 min at 4˚C. Following determination of protein concentration with a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 50 μg of total protein was subjected to 12% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) by a transfer apparatus at 90 V for 1 h. The membrane was then blocked with 5% non-fat milk for 2 h at room temperature and incubated overnight at 4˚C with primary antibody against CBS (1:200; cat. no. sc-67154; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), 3-MST (1:400; cat. no. sc-374326; Santa Cruz Biotechnology, Inc.), LC3 (1:3,000; cat. no. ab48394; Abcam, Cambridge, UK), Beclin-1 (1:500; cat. no. mb0030; Bioworld Technology, Inc., St. Louis Park, MN, USA), sequestosome 1 (also known as P62; 1:1,000; cat. no. ab56416; Abcam), or β-actin (1:10,000; cat. no. jla20; Merck KGaA). Following incubation with horseradish peroxidase-conjugated secondary antibodies (A0208, A0216; Beyotime Institute of Biotechnology, Haimen, China), protein signals were visualized using an enhanced chemiluminescence (ECL) system (cat. no. 32106; Pierce; Thermo Fisher Scientific, Inc.). For semiquantitative analysis, protein bands detected by ECL were scanned into Adobe Photoshop CS6 (Adobe Systems, Inc., San Jose, CA, USA) and analyzed using ImageJ software, version 1.6.

Statistical analysis. All statistical analyses were conducted with SPSS statistical software version 16.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as means ± standard error of the mean. The statistical significance of differences between groups was determined by one-way analysis of variance followed by Tukey's post hoc multiple comparison tests, or Student t-test for two means comparisons. $P<0.05$ was considered to indicate a statistically significant difference. Each experiment consisted of at least three replicates per condition.

Results

NaN_3 causes cytotoxicity to PC12 cells. The effect of NaN_3 on the viability of PC12 cells was measured by CCK-8 assay following treatment with a range of concentrations from 5 to 100 mmol/l and for different durations (1, 3, 6, 12, 18 and 24 h). As presented in Fig. 1, treatment of PC12 cells with NaN_3 for 12 h at concentrations from 20 to 100 mmol/l resulted in a concentration-dependent reduction of cell viability. Further analysis demonstrated that the cell survival decrease caused by treatment with 30 mmol/l NaN_3 was also time-dependent (Fig. 2).

NaN_3 induces autophagic cell death in PC12 cells. The cell morphology of the treated PC12 cells was analyzed by PI/DAPI staining in order to evaluate cell death. By phase-contrast

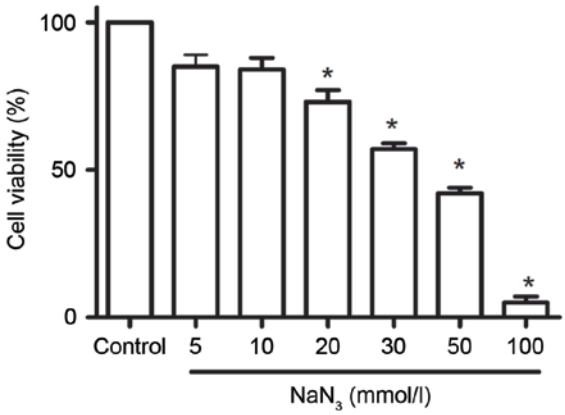

Figure 1. Concentration effect of NaN_3 on cell viability in PC12 cells. PC12 cells were treated with 5, 10, 20, 30, 50 or 100 mmol/l NaN_3 for 12 h and cell viability was determined by Cell Counting Kit-8 assay. Data are presented as means ± standard error of the mean (n=3). *$P<0.05$ vs. control. NaN_3, sodium azide.

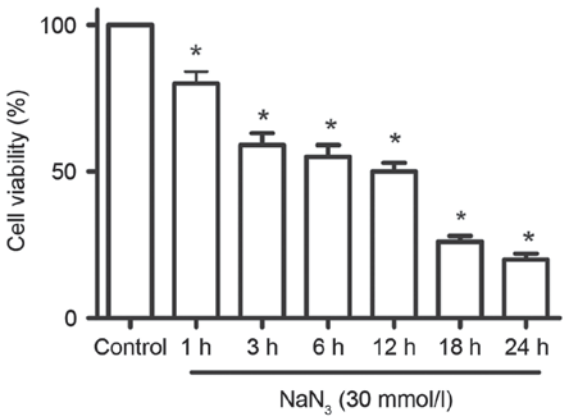

Figure 2. Time effect of NaN_3 on cell viability in PC12 cells. PC12 cells were treated with 30 mmol/l NaN_3 for 1, 3, 6, 12, 18 or 24 h and cell viability was determined by Cell Counting Kit-8 assay. Data are presented as means ± standard error of the mean (n=3). *$P<0.05$ vs. control. NaN_3, sodium azide.

microscopy, PC12 cells treated with 30 mmol/l NaN_3 for 12 h appeared more round to oval in shape, compared with the long spindle-like shape of untreated control PC12 cells (Fig. 3A). Following DAPI/PI double staining, the nuclei of control cells appeared round to oval, with a separate pattern of blue fluorescence (DAPI) and red fluorescence (PI). Upon NaN_3 treatment, nuclei became increasingly bright, decreased in size, and condensed into round bodies (Fig. 3A). To distinguish which type of cell death was induced by NaN_3 treatment, neuronal cultures were examined following staining for PI and for the autophagy marker LC3. The results indicated that LC3/PI double-positive cells existed in both the control and NaN_3-injured group (Fig. 3A). Quantification of the microscopy images confirmed that treatment of PC12 cells with NaN_3 resulted in a significant increase in both the number of PI/DAPI-positive cells (Fig. 3B) and the number of PI/LC3 double-positive cells (Fig. 3C), compared with control, suggesting that NaN_3 induced autophagic cell death in PC12 cells.

Effect of NaN_3 on CBS and 3-MST protein expression in PC12 cells. To examine the expression levels of the endogenous

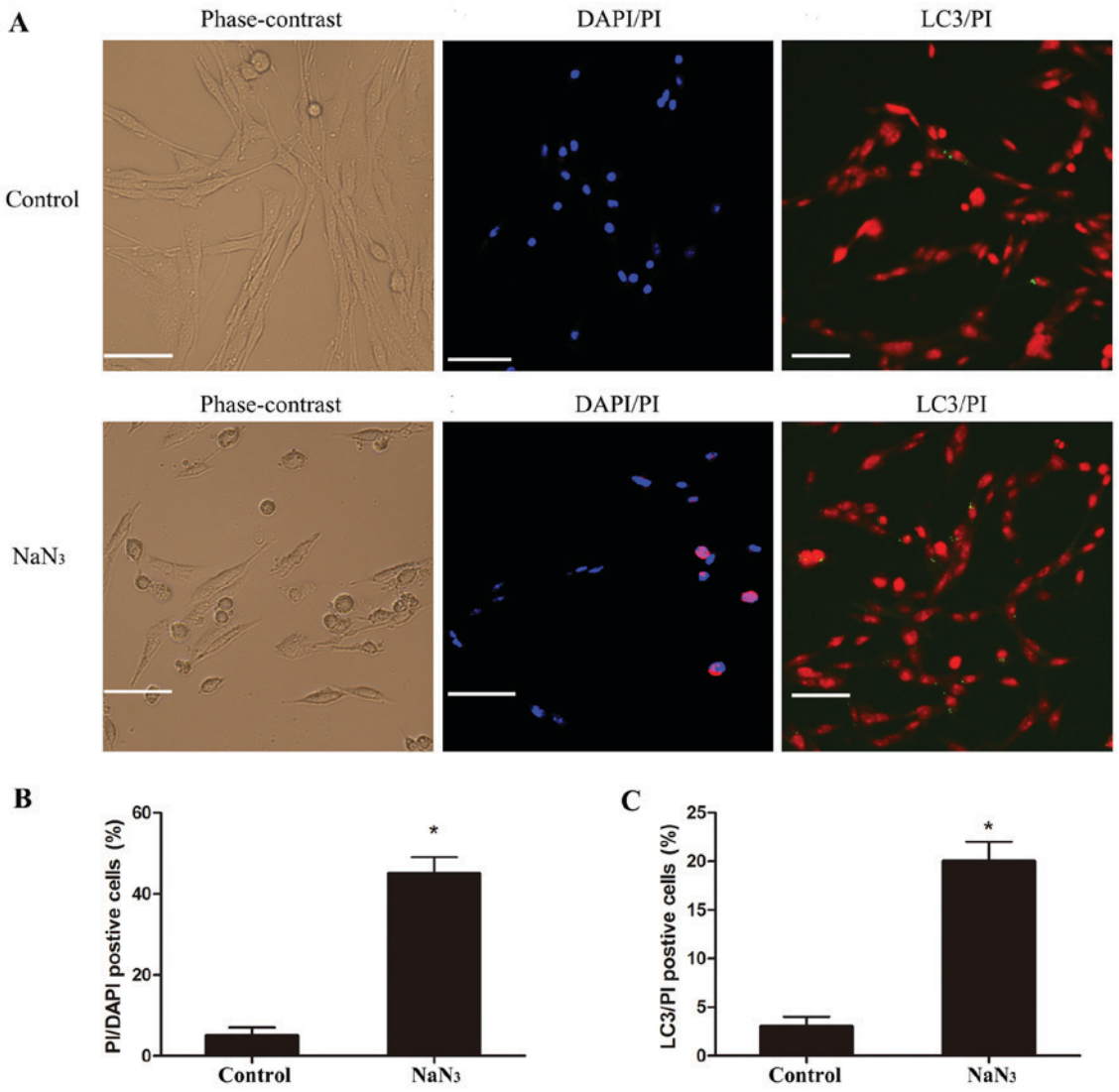

Figure 3. Effect of NaN_3 on cell morphology and autophagic cell death in PC12 cells. PC12 cells were treated with 30 mmol/l NaN_3 and then examined by immunostaining and microscopy. Cell death was evaluated by double PI/DAPI staining. Authophagic cell death was evaluated by double LC3/PI staining. (A) Representative images from phase-contrast microscopy and from double-stained fluorescent cells (DAPI, blue; PI, red; LC3, green). Scale bar, 30 μm. (B) Quantification of the PI/DAPI double-positive cells as % of total. (C) Quantification of the LC3/PI double-positive cells as % of total. Data are presented as means ± standard error of the mean (n=3). $^*P<0.05$ vs. control. NaN_3, sodium azide; PI, propidium iodide; LC3, microtubule-associated protein 1A/1B-light chain 3.

H_2S-producing enzymes, expression of CBS and 3-MST proteins was analyzed by western blotting in control untreated cells and cells treated with different concentrations of NaN_3. The expression levels of both CBS and 3-MST proteins decreased in a dose-dependent manner following NaN_3 treatment compared with control (Fig. 4).

H_2S protects PC12 cells against NaN_3-induced cytotoxicity. To investigate the effect of H_2S on NaN_3-induced cytotoxicity, cell viability was analyzed by CCK-8 assay. As illustrated in Fig. 5, treatment with NaN_3 at concentrations of 30 mmol/l for 12 h significantly attenuated cell viability. The cytotoxic effects of NaN_3 on PC12 cells were significantly blocked by pretreatment with 100 and 200 μmol/l NaHS for 30 min (Fig. 5). At 200 μmol/l, NaHS alone did not affect the viability of PC12 cells (Fig. 5).

H_2S suppresses NaN_3-induced autophagic cell death in PC12 cells. Next, the effects of H_2S on NaN_3-induced autophagic cell death were assessed, by examining the protein expression levels of LC3, Beclin-1 and P62 with western blot analysis (Fig. 6A). Treatment of PC12 cells with 30 mmol/l NaN_3 for 12 h significantly increased the protein expression levels of LC3 (Fig. 6B) and Beclin-1 (Fig. 6C), but decreased P62 expression (Fig. 6D) compared with control untreated cells. However, Pretreatment with 200 μmol/l NaHS significantly abolished the sodium azide-induced decrease of P62 expression and the increase of LC-3 and Beclin-1 expression (Fig. 6). These results indicated that H_2S was able to block the NaN_3-elicited downregulation of P62 expression and upregulation of LC3 and Beclin-1 expression.

The effect of NaHS pretreatment on autophagic cell death in PC12 cells was further examined by LC3/PI double staining and microscopy analysis. As illustrated in Fig. 7, following treatment with NaN_3 (30 mmol/l, 12 h), the number of LC3/PI double-positive cells significantly increased compared with the control untreated group. Pretreatment with NaHS (200 μmol/l) dramatically ameliorated this NaN_3-induced increase of PI/LC3 double-positive cells (Fig. 7), suggesting that H_2S

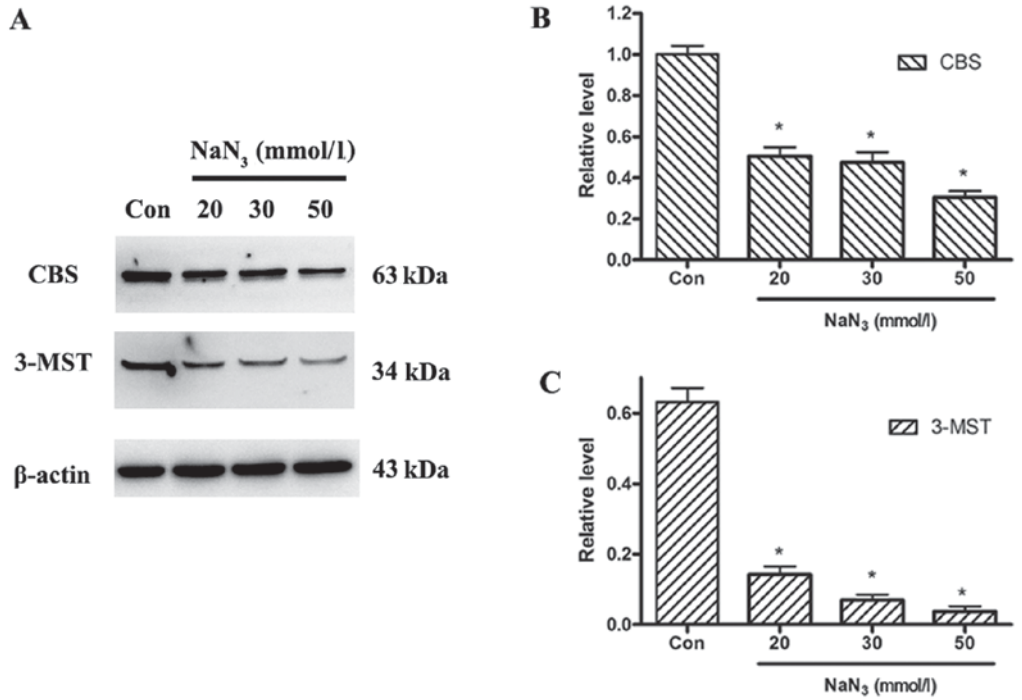

Figure 4. Effect of NaN_3 on protein expression of CBS and 3-MST in PC12 cells. Cells were treated with different concentrations of NaN_3 for 12 h, and then analyzed by (A) western blotting for CBS and 3-MST. The relative protein expression of (B) CBS and (C) 3-MST was calculated, with β-actin as the loading control. Representative images and quantification as mean protein levels relative to β-actin ± standard error of the mean (n=3). *P<0.05 vs. control. NaN_3, sodium azide; CBS, cystathionine-β-synthase; 3-MST, 3-mercaptopyruvate sulfurtransferase; Con, control.

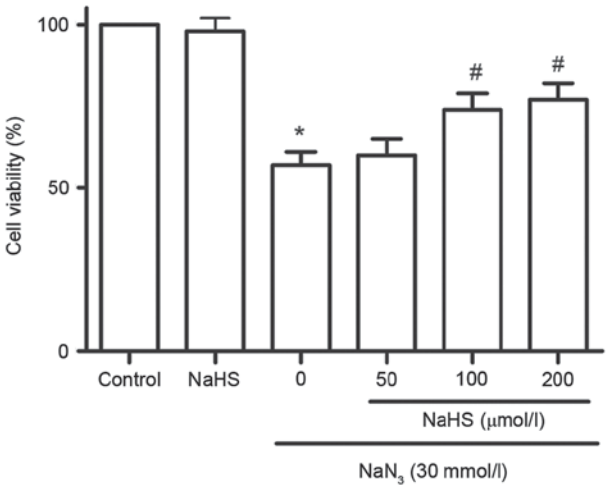

Figure 5. Effect of H_2S on NaN_3-induced cytotoxicity in PC12 cells. PC12 cells were treated with NaN_3 (30 mmol/l) for 12 h in the absence or presence of NaHS (50, 100 or 200 mmol/l) as a donor for H_2S, and then cell viability was determined by CCK-8 assay. Data are presented as means ± standard error of the mean (n=6). *P<0.05 vs. control; #P<0.05 vs. NaN_3 alone. H_2S, hydrogen sulfide; NaN_3, sodium azide; NaHS, sodium hydrosulfide.

protected the cells from autophagic cell death. The control and NaHS-treated groups displayed few LC3/PI double-positive cells (Fig. 7).

Discussion

The present study examined the cell damage occurring in cultured PC12 cells exposed to NaN_3. Due to the ability of NaN_3 to induce cell death through inhibition of the electron transfer between COX and oxygen, this *in vitro* model represents an interesting tool in neurotoxicity studies. In the present study, the possible molecular mechanisms underlying the neuroprotective effects of H_2S to protect against NaN_3-induced neuron cell injury were investigated. The results demonstrated a concentration-dependent loss of cell viability induced by NaN_3. To explore whether autophagic cell death was induced by NaN_3, double immunofluorescence staining was performed for the autophagy marker LC3 and for PI. Microscopy analysis indicated that LC3 positive staining was partly colocalized with PI (a cell death marker), implying that a proportion of dying cells were undergoing autophagy, which is one of the mechanisms of NaN_3-induced neurotoxicity. This finding is the first report of NaN_3 inducing autophagic cell death in PC12 cells. In addition, exposure of PC12 cells to NaN_3 downregulated the expression of the endogenous H_2S synthases (CBS and 3-MST) in a concentration-dependent manner, suggesting that H_2S was involved in the pathophysiology of NaN_3-induced cell injury. Furthermore, NaHS, a H_2S donor was demonstrated to prevent the NaN_3-exerted upregulation of LC3 and Beclin-1 expression and downregulation of P62 expression. A significant reduction in the number of LC3/PI double-positive cells was also observed following pretreatment with NaHS, suggesting that H_2S may have yielded a protective effect against NaN_3-mediated autophagic cell death. Taken together, the present findings suggest that H_2S may be an important protective factor against NaN_3-induced neurotoxicity by modulating the autophagic cell death pathway.

NaN_3 is a highly reactive white crystalline powder used in industry, which is also used as a preservative in aqueous laboratory reagents and biologic fluids, and as a fuel in automobile airbag gas generates (19). It is also a broad-spectrum biocide

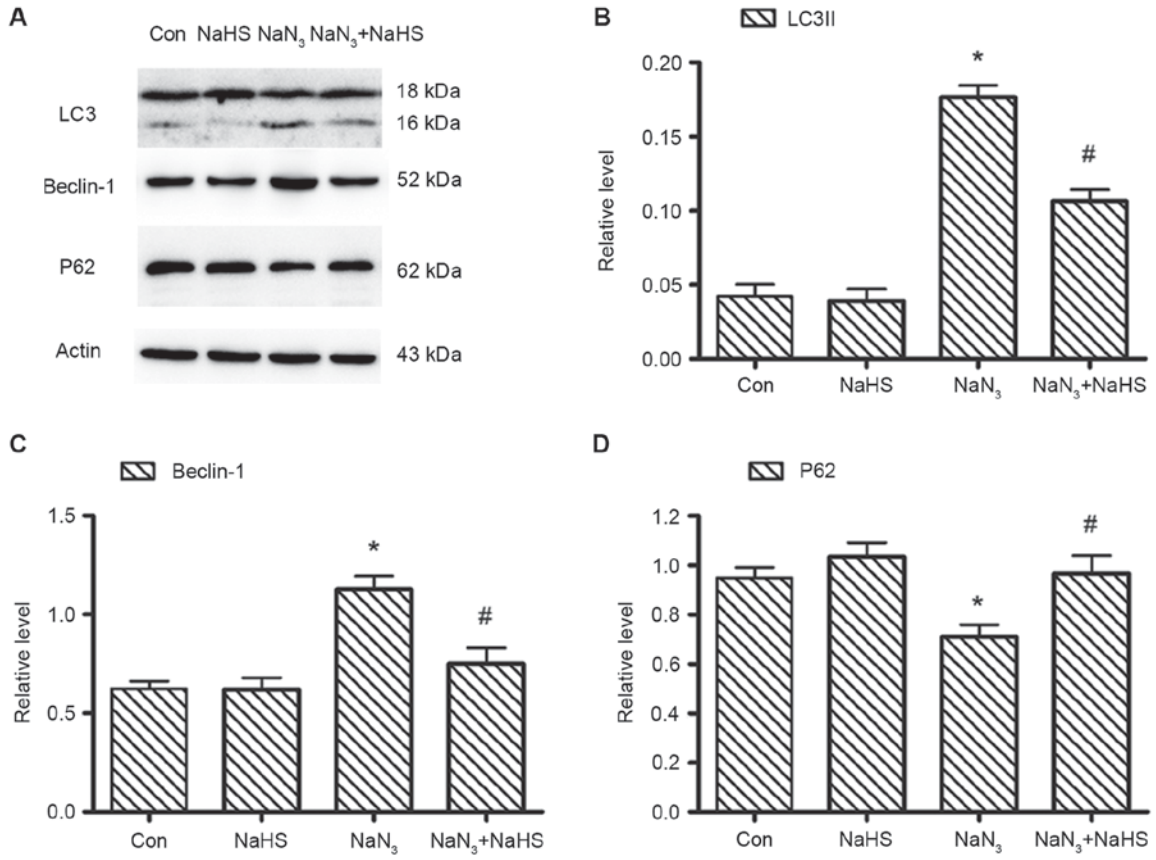

Figure 6. Effect of H_2S on NaN_3-induced autophagy-related protein expression in PC12 cells. Cells were treated with NaN_3 (30 mmol/l) for 12 h in the absence or presence of NaHS (200 mmol/l) as a donor for H_2S. Cells were then analyzed for protein expression of LC3, Beclin-1 and P62 by western blotting. (A) Representative images. (B-D) Quantification of protein levels relative to β-actin. Data are presented as means ± standard error of the mean (n=3). *P<0.05 vs. control; $^\#$P<0.05 vs. NaN_3 alone. H_2S, hydrogen sulfide; NaN_3, sodium azide; NaHS, sodium hydrosulfide; LC3, microtubule-associated protein 1A/1B-light chain 3; P62, sequestosome 1; Con, control.

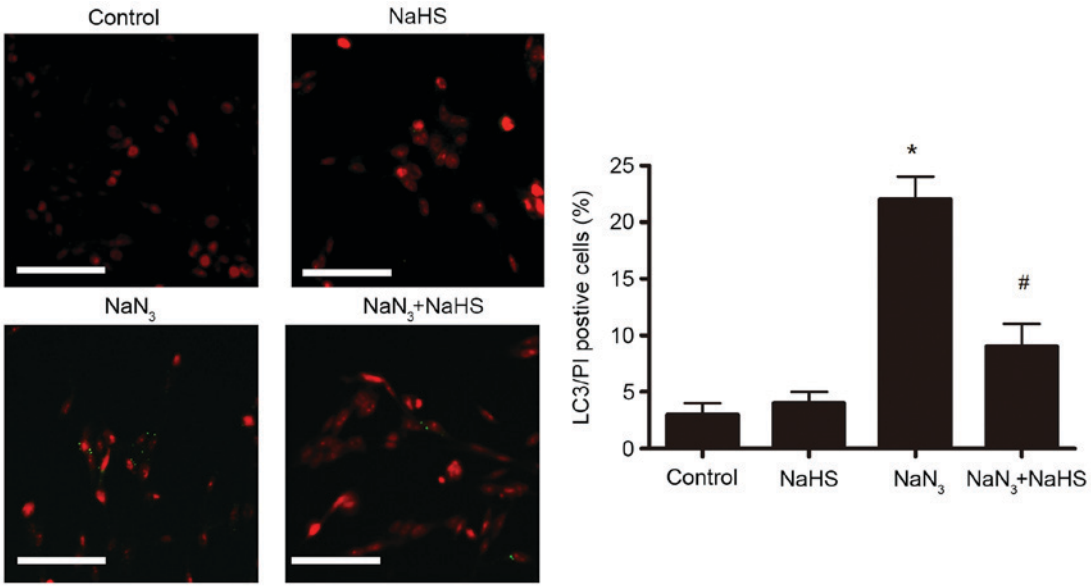

Figure 7. Effect of H_2S on NaN_3-induced autophagic cell death in PC12 cells. Autophagic cell death in PC12 cells was examined by staining with LC3 (green) and PI (red) and microscopy analysis. Representative images and quantification as mean % LC3/PI double-positive cells per total ± standard error of the mean (n=3). Scale bars, 50 μm. *P<0.05 vs. control; $^\#$P<0.05 vs. NaN_3 alone. H_2S, hydrogen sulfide; NaN_3, sodium azide; LC3, microtubule-associated protein 1A/1B-light chain 3; PI, propidium iodide; NaHS, sodium hydrosulfide.

used in research and agriculture. NaN_3, as a COX inhibitor, has been extensively considered as a useful tool to study different pathological conditions. Mitochondrial energy metabolism has been hypothesized to be a determining element to interpret

impaired neuron function, reduced molecular turnover, and enhanced cell death (20,21). Inhibition of mitochondrial COX has been reported to induce cell death in a variety of cells. Programmed cell death can be classified into apoptosis, necrosis, and autophagic cell death, and emerging evidence suggests that all three may be important modes of cell death in neural stem/progenitor cells (22). Previous studies suggested that NaN_3 could induce neuronal apoptosis and necrosis, which was associated with the mitochondrial pathway (11,13). The role of NaN_3 in apoptosis and necrosis has been the subject of extensive investigation, however its role in autophagic cell death remains poorly understood (23-25).

PC12 cells, which are generally considered to have neuronal-like characteristics, appear to be more sensitive to NaN_3 compared with other neural tumor cell lines. To induce hypoxia/hypoglycemia or oxidative stress, NaN_3 concentrations used in PC12 cells range from 1 mM to 10 mM (26,27). In order to induce autophagic cell death, high concentrations of NaN_3 were employed in the present study. Increased autophagy is observed in several experimental injury models (28,29). However, it is not known whether the role of autophagy is protective or detrimental in neural cell injury. It is possible that the role of autophagy following cell injury is dependent upon the cell's capacity to respond in relation to the cumulative burden of damaged or dysfunctional macromolecules and organelles. If the increase in autophagic capacity is insufficient, augmenting autophagy would likely be beneficial. The increase in autophagic capacity is in excess, and inhibiting autophagy may be beneficial. Thus, the role of autophagy may be dictated by whether or not it can meet intracellular demands. Examining cell viability is important in order to evaluate if the cells are still physiologically responsive, or if they are likely to be entering cell death. Therefore, in the present study the overall toxic effects of NaN_3 were evaluated by monitoring cell viability in PC12 cells following treatment. Under more severe stress conditions (30 mM NaN3), when PC12 cell viability was severely affected, an accumulation of autophagic cell death was observed.

The concept of autophagic cell death was first established based on observations of increased autophagic markers in dying cells (30). LC3, an autophagosomal ortholog of yeast *Atg8*, is one of the most reliable markers in the study of autophagy induction (7). Beclin-1, the mammalian orthologue of yeast *Atg6*, has a central role in autophagy (31). More recently, autophagy flux has been assessed in several injury models based on the levels of the autophagic substrate protein P62 (32). P62 is an adaptor protein that directs ubiquitinated cargo to autophagosomes for degradation. As P62 is degraded along with its cargo, when autophagy flux is increased, its protein levels decrease; conversely, when autophagy flux is inhibited, P62 levels increase (33). The present results demonstrated that treatment with NaN_3 significantly increased the amount of LC3 and Beclin-1 expression but decreased P62 expression, suggesting that autophagy flux was increased in the NaN_3-induced injury *in vitro* model. To investigate whether an increase in autophagy was beneficial or detrimental, fluorescence microscopy analysis of LC3/PI double staining was performed in PC12 cells. The results indicated that LC3-positive cells were partly colocalized with PI, implying that a proportion of dying cells were undergoing some degree of autophagy. The nuclei of LC3/PI-positive cells appeared round, which is consistent with autophagic cell death, and they were neither shrunken nor fragmented as is observed in apoptotic nuclei, demonstrating that NaN_3 could induce autophagic cell death. The present results demonstrated that NaN_3 induced autophagic cell death, which is consistent with prior reports that NaN_3 induces non-apoptotic cell death (13).

Selvatici *et al* (13) reported that mitochondrial dysfunction induced by NaN_3 provides a common platform for investigating the mechanisms of neuronal injury, useful for screening potential protective agents against neuronal death. H_2S has increasingly been recognized as an important signaling molecule of comparable importance to nitric oxide and carbon monoxide in mammalian systems (34). In addition to its function as a signal molecule, H_2S also functions as a cytoprotectant in neurons and cardiac muscle (17). The neuroprotective properties of H_2S have been the focus of extensive research for decades (35). Previous reports from our group demonstrated that endogenous H_2S is involved in neuronal autophagy in TBI mice (10,36,37). Endogenous H_2S is generated by three distinct enzymatic pathways mediated by CSE, CBS and 3-MST (18). CBS and 3-MST are expressed in the brain, while CSE is widely located in other organs (38). Miyamoto *et al* (39) reported that CSE is not expressed in PC12 cells and that the 3-MST pathway is primarily responsible for H_2S production in PC12 cells. In order to explore whether H_2S was involved in NaN_3-induced autophagy, the expression of endogenous H_2S synthases, CBS and 3-MST, was analyzed by western blotting. Exposure of PC12 cells to NaN_3 downregulated the expression of CBS and 3-MST in a concentration-dependent manner, suggesting that H_2S may be involved in NaN_3-induced cell injury. In addition, pretreatment with NaHS, a donor for H_2S, reduced the number of LC3/PI double-positive cells, suggesting that H_2S may have protective effects against NaN_3-mediated autophagic cell death.

The potential neuroprotective mechanism of H_2S may be that it reduces oxidative stress. Biochemical studies have demonstrated that NaN_3 inhibits COX and interferes with cellular respiration (40). The mitochondrial respiratory chain is one of the most important sites of reactive oxygen species (ROS) production under physiological and pathological conditions (41). ROS generation is considered as a cause of cell death mediated by mitochondrial electron transport chain inhibitors in dopaminergic neuronal cells (42). Oxidative stress caused by overproduction of ROS is detrimental and one of the etiological factors of many neurologic diseases (43). H_2S protects various tissues and organs from oxidative stress and also scavenges ROS (18,44,45). H_2S also upregulates the transcription of antioxidant genes to exert its cytoprotective effect. The kelch-like ECH associated protein 1 (Keap1)-nuclear factor E2-related factor 2 (Nrf2) pathway is the major regulator of cytoprotective responses to oxidative and electrophilic stress (46). The translocation of Nrf2 to the nucleus has been suggested as a molecular mechanism for the H_2S-mediated protection (47,48). Further study is necessary to clarify the mechanisms underlying the protective effects of H_2S on the Keap1-Nrf2 pathway in a NaN_3-induced cell injury model.

Although H_2S showed a promising neuroprotective effect in NaN_3-mediated cell injury in the present study, further research is underway to identify selective autophagy regulators

that may serve as potential targets for treatment. In summary, the present results suggested an active role for H_2S in the process of autophagy induced by NaN_3. This is the first report of autophagic cell death in PC12 cells induced by NaN_3. The present data may provide a potential novel pathway to elucidate the underlying molecular and cellular mechanisms of CNS following inhibition of COX and potential novel strategies for the treatment of CNS diseases. Further studies, such as examining other H_2S biosynthesis enzymes of the trans-sulfuration pathway, other signal transduction pathways involved in the H_2S protective effect, and the functional consequences of H_2S on cellular autophagy and its downstream signaling targets, will strengthen these conclusions.

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