Hydrogen sulfide prevents homocysteine-induced endoplasmic reticulum stress in PC12 cells by upregulating SIRT-1

CHUN-YAN WANG[1*], WEI ZOU[2*], XIAO-YU LIANG[2,3], ZHI-SHENG JIANG[1], XIANG LI[3], HAI-JUN WEI[3], YI-YUN TANG[3], PING ZHANG[2] and XIAO-QING TANG[2-4]

[1]The Institute of Cardiovascular Disease, Medical College, [2]Department of Neurology, Nanhua Affiliated Hospital, [3]Institute of Neuroscience, Medical College, University of South China; [4]Hunan Province Cooperative Innovation Center for Molecular Target New Drug Study, Hengyang, Hunan 421001, P.R. China

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Abstract. It was previously confirmed that hydrogen sulfide (H_2S) has a neuroprotective effect, preventing homocysteine-induced neurotoxicity. However, the exact molecular mechanisms underlying this protective effect remain to be fully elucidated. Endoplasmic reticulum (ER) stress contributes to homocysteine-induced neurotoxicity. Silent mating type information regulator 2 homolog 1 (SIRT-1) can attenuate ER stress, exerting its neuroprotective effect. Therefore, the present study aimed to investigate whether H_2S protects PC12 cells against homocysteine-induced ER stress and whether SIRT-1 mediates this protective effect of H_2S. Western blotting was used to detect the expression of SIRT-1, glucose-regulated protein 78 (GRP78), and cleaved caspase-12 in PC12 cells. It was observed that sodium hydrosulfide (NaHS), an exogenous H_2S donor, significantly attenuated the homocysteine-induced ER stress responses, including increases in the protein expression levels of GRP78 and cleaved caspase-12. Simultaneously, NaHS upregulated the expression of SIRT-1 and reversed the homocysteine-induced downregulation of SIRT-1 in PC12 cells. Sirtinol, a specific inhibitor of SIRT-1, eliminated the protective effects of H_2S in homocysteine-induced ER stress. These data indicated that H_2S prevented homocysteine-induced ER stress via enhancing the expression of SIRT-1. These findings offer novel insight into the protective mechanisms of H_2S against homocysteine-induced neurotoxicity.

Correspondence to: Professor Xiao-Qing Tang, Institute of Neuroscience, Medical College, University of South China, 28 West Changsheng Road, Hengyang, Hunan 421001, P.R. China
E-mail: tangxq-usc@qq.com

Professor Zhi-Sheng Jiang, The Institute of Cardiovascular Disease, Medical College, University of South China, 28 West Changsheng Road, Hengyang, Hunan 421001, P.R. China
E-mail: zsjiang2005@163.com

*Contributed equally

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Introduction

Homocysteine, a thiol-containing amino acid, is generated by the demethylation of methionine (1,2). It has been established that an elevated level of circulating homocysteine is an independent risk factor for Alzheimer's disease (AD) (3-8), and there is increasing evidence that homocysteine directly causes neurotoxicity in multiple neuronal types (9-11). In addition, it is known that endoplasmic reticulum (ER) stress is closely associated with the development and pathology of AD, which has the typical characteristics of inclusion bodies, abnormal formation and misfolded protein aggregation (12-14). It has also been reported that homocysteine leads to ER stress in neuronal cells (15-18), which suggests that ER stress-mediated homocysteine-induced neurotoxicity may be vital in the pathogenesis of AD. Therefore, the suppression of ER stress may provide a promising approach for the treatment of homocysteine-dependent neurodegenerative diseases.

Hydrogen sulfide (H_2S) is considered to be a novel endogenous neuroprotectant (19-23). Of note, data from our previous study demonstrated that the disturbance of endogenous H_2S generation was involved in the neurotoxicity of homocysteine (24), and that H_2S ameliorated homocysteine induced-neurotoxicity (25), indicating the potential of H_2S-based prevention and treatment for neuronal injury induced by homocysteine exposure. Based on the importance of ER stress in the neurotoxicity of homocysteine, the present study aimed to expand on current understanding of the protective effects of H_2S in homocysteine-elicited neurotoxicity by examining the effects of H_2S on homocysteine-induced ER stress and the underlying mechanisms.

Sirtuins are nicotinamide adenine dinucleotide-dependent histone deacetylases, which counter aging, having a broad spectrum of metabolic and stress-tolerance functions. Emerging evidence has confirmed that silent mating type information regulator 2 homolog 1 (SIRT-1), one of the seven mammalian sirtuins, is directly involved in the neuronal protective effect against cellular damage and stressful perturbations in neurological diseases, including AD (26-29), amyotrophic lateral sclerosis (28), Huntington's disease (30,31) and Parkinson's disease (32). Furthermore, it has been reported that SIRT-1 mediates the neuroprotective effect of paliperidone against

MK-801-induced neuronal damage (33) and hyperbaric oxygen preconditioning-induced ischemic tolerance in the rat brain (34). Of note, previous studies have suggested that SIRT-1 exhibits its beneficial effects in neuroprotection via alleviation of the ER stress response (35,36). Therefore, the present study investigated whether SIRT-1 contributes to the protective effects of H_2S against homocysteine-induced ER stress.

The results of the present study revealed that H_2S prevented homocysteine-induced ER stress and increased the protein expression of SIRT-1 in PC12 cells. Sirtinol, a specific inhibitor of SIRT-1, eliminated the inhibitory effects of H_2S against homocysteine-induced ER stress in the PC12 cells. These findings indicated that H_2S protects PC12 cells against homocysteine-induced ER stress via upregulating the expression of SIRT-1.

Materials and methods

Materials. Sodium hydrosulfide (NaHS), an exogenous donor of H_2S, homocysteine and sirtinol, a specific inhibitor of SIRT-1, were supplied by Sigma-Aldrich (cat. no. S7942; Merck KGaA, Darmstadt, Germany). Specific antibody against SIRT-1 (cat. no. ab110304) was purchased from Abcam (Cambridge, UK). Specific antibody against glucose-regulated protein 78 (GRP78; cat. no. S1931) was obtained from Epitomics (Burlingame, CA, USA). Specific antibody against cleaved caspase-12 (cat. no. C7611) was supplied by Sigma-Aldrich (Merck KGaA); β-actin polyclonal antibody (cat. no. 20536-1-AP) and goat anti-rat immunoglobulin (Ig)G (cat. no. SA00001-2) or goat anti-mouse IgG (cat. no. SA00001-1) antibody were obtained from ProteinTech Group, Inc. (Chicago, IL, USA). RPMI-1640 medium, fetal bovine serum (FBS) and horse serum were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture. The PC12 cells (American Type Culture Collection; CRL-1721), provided by Sun Yat-sen University Experimental Animal Center (Guangzhou, China), were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated horse serum and 5% FBS (v/v) at 37˚C, in an atmosphere containing 5% CO_2 and 95% air. The culture medium was replaced every 2-3 days.

Western blot analysis. The PC12 cells, treated as described above, were homogenized in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing phenylmethylsulphonyl fluoride (1 mM) for 30 min 4˚C and the supernatants was obtained by centrifugation at 5,000 x g for 10 min at 4˚C. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equivalent quantities of protein (50 μg) were separated by SDS-PAGE on a 12% gel. The proteins were then transferred onto polyvinylidene fluoride membranes, and the membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature. The membranes were then incubated with primary antibodies specific for blocking solution, containing primary antibodies against SIRT-1 (1:2,000), GRP78 (1:2,000), cleaved caspase-12 (1:2,000) and β-actin (1:5,000) overnight at 4˚C. Following washing with TBST three times, the membranes with SIRT-1 were incubated in peroxidase-conjugated affinipure goat anti-mouse IgG (1:5,000) and others were incubated in anti-rabbit secondary antibodies (1:5,000) in blocking solution for 2 h at 25˚C and then washed in TBST buffer. The bands of protein were visualized using an enhanced chemiluminescence reaction solution (solution 1:0.1 M Tris-HCl, luminol and p-coumaric acid; solution 2:0.1 M Tris-HCl and hydrogen peroxide) for 2 min, and quantified using an image analysis system equipped withBIO-1D software (v4.62; VilberLourmat, Marne-la-Vallée, France).

Statistical analysis. Data are expressed as the mean ± standard error of the mean. The significance of differences in different groups was assessed using one-way analyses of variance followed by the least significant difference test using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Homocysteine induces ER stress in PC12 cells. To investigate whether homocysteine induces ER stress in PC12 cells, the present study measured the expression levels of ER stress-related proteins, including GRP78 and cleaved caspase-12, in homocysteine-treated PC12 cells using western blot analysis. It was found that treatment with homocysteine (1.25, 2.5 or 5 mM for 24 h) significantly increased the expression levels of GRP78 (Fig. 1A) and cleaved caspase-12 (Fig. 1B) in the PC12 cells, which indicated that homocysteine-induced ER stress in the PC12 cells.

H_2S protects PC12 cells from homocysteine-induced ER stress. To determine whether H_2S protects PC12 cells against homocysteine-induced ER stress, the present study examined the effects of H_2S on the protein levels of GRP78 and cleaved caspase-12 in homocysteine-exposed PC12 cells. As shown in Fig. 2, cotreatment of the PC12 cells with NaHS (200 or 400 μM) significantly downregulated the expression levels of GRP78 (Fig. 2A) and cleaved caspase-12 (Fig. 2B), compared with the cells treated with 5 mM of homocysteine for 24 h. This indicated that H_2S had a protective effect in homocysteine-induced ER stress.

H_2S upregulates the expression of SIRT-1 in PC12 cells. As shown in Fig. 3A, treatment with homocysteine (1.25, 2.5 or 5 mM, for 24 h) significantly decreased the expression levels of SIRT-1 in the PC12 cells, which indicated that homocysteine downregulated the protein expression of SIRT-1 in PC12 cells. Treatment with NaHS at concentrations of 200 and 400 μM for 24 h dose-dependently increased the expression of SIRT-1 in the PC12 cells (Fig. 3B), and also significantly increased the expression of SIRT-1 in the homocysteine-exposed (5 mM for 24 h) PC12 cells (Fig. 3C), which indicated the promoting effect of H_2S on the expression of SIRT-1 in PC12 cells.

Inhibition of SIRT-1 eliminates the beneficent effect of H_2S against homocysteine-induced ER stress in PC12 cells. To further investigate whether the protective effect of H_2S on homocysteine-induced ER stress in PC12 cells was through the upregulation of SIRT-1, the present study used sirtinol,

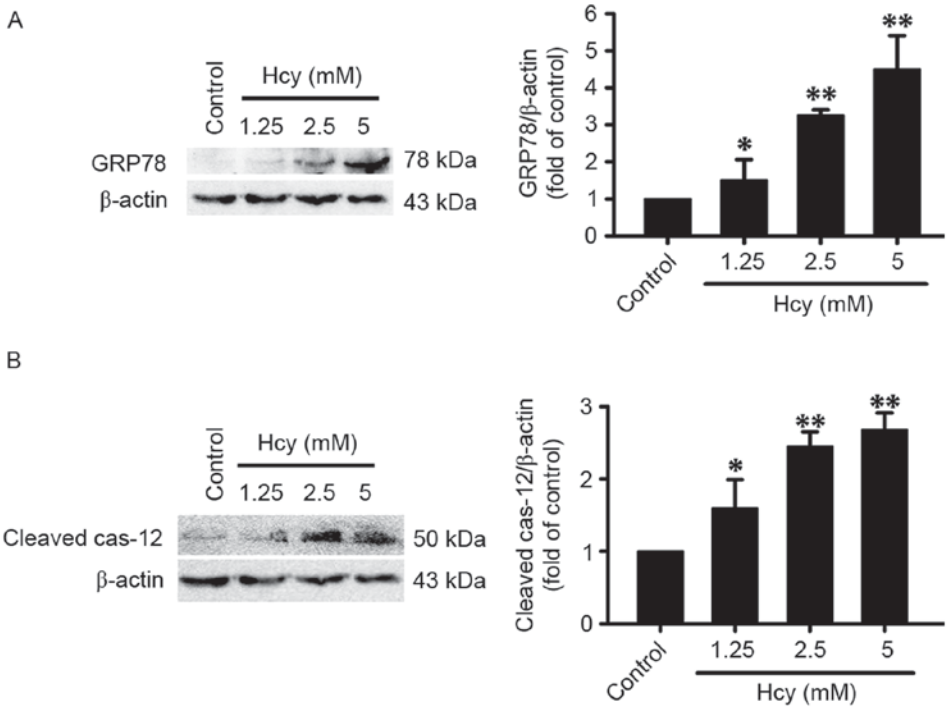

Figure 1. Hcy increases the protein expression levels of GRP78 and cleaved caspase-12 in PC12 cells. PC12 cells were treated with Hcy (1.25, 2.5 and 5 mM) for 24 h. The protein expression levels of (A) GRP78 and (B) cleaved caspase-12 in PC12 cells were measured using western blot analysis. β-actin was used as a loading control. The results were normalized to the percentage of β-actin and expressed as the fold of the control group. Values are expressed as the mean ± standard error of the mean of three independent experiments. *P<0.05 and **P<0.01, compared with the control group. Hcy, homocysteine; GRP78, glucose-regulated protein 78; cas-12, caspase-12.

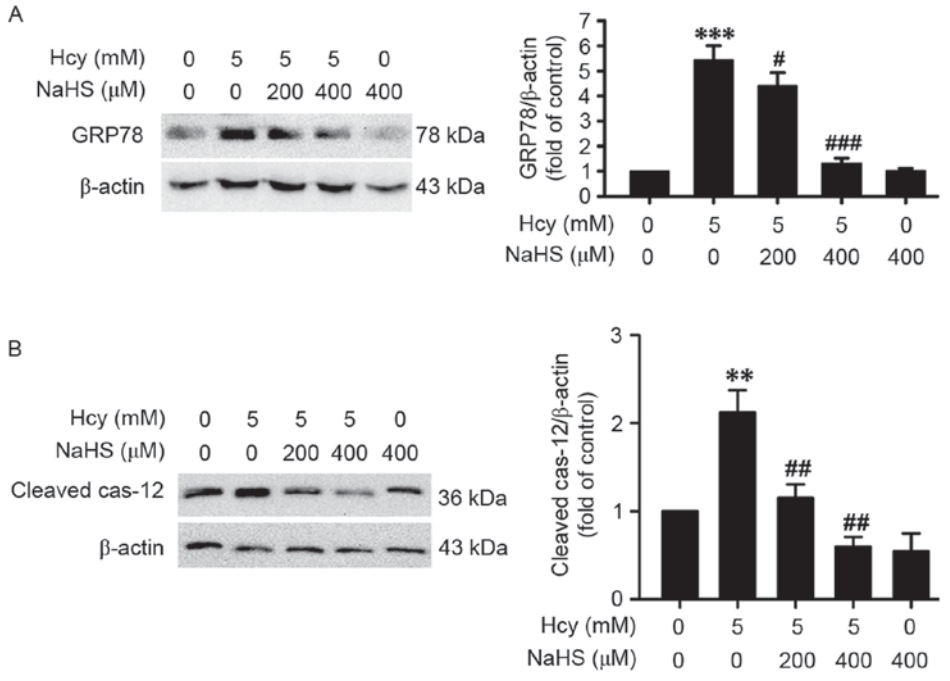

Figure 2. NaHS decreases endoplasmic reticulum stress in homocysteine-treated PC12 cells. Following cotreatment with NaHS (200 or 400 μM) and homocysteine (5 mM) for 24 h, the protein expression levels of (A) GRP78 and (B) cleaved caspase-12 in PC12 cells were detected using western blot analysis. β-actin was used as a loading control. The results were normalized to the percentage of β-actin and expressed as the fold of the control group. Values are expressed as the mean ± standard error of the mean of three independent experiments. **P<0.01 and ***P<0.001, compared with the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001, compared with the Hcy only group. Hcy, homocysteine; NaHS, sodium hydrosulfide; GRP78, glucose-regulated protein 78; cas-12, caspase-12.

a specific inhibitor of SIRT-1, to examine the effect of H2S on ER stress under homocysteine treatment. The PC12 cells were pretreated with sirtinol (15 μM) for 30 min prior to the administration of NaHS (200 μM). The results demonstrated that sirtinol (15 μM) treatment inhibited the NaHS (200 μM)-induced suppression of GRP78 (Fig. 4A) and

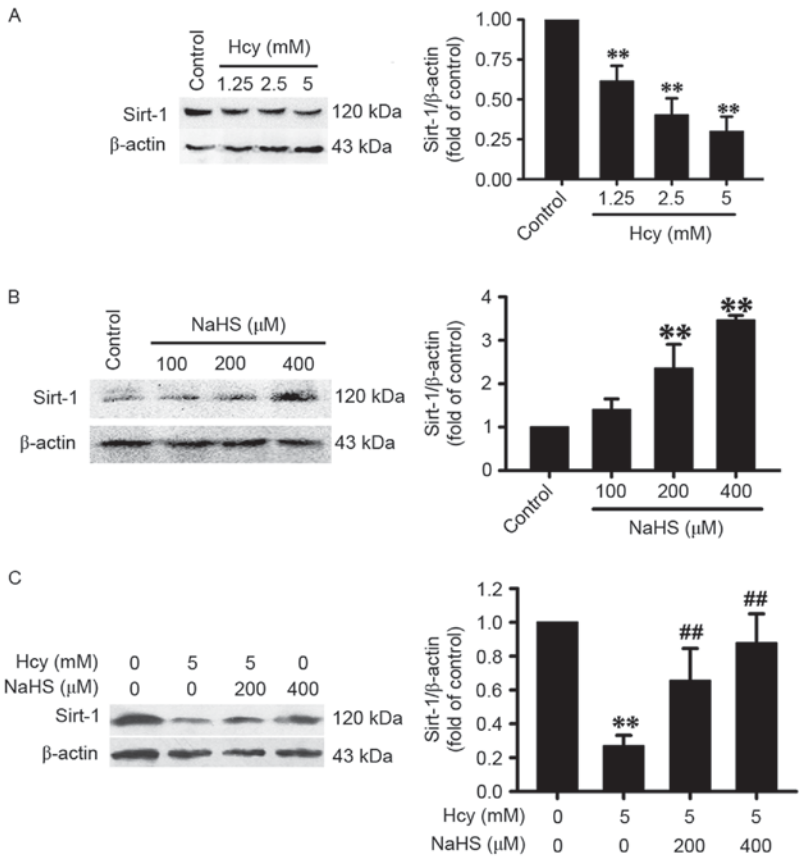

Figure 3. NaHS increases the protein expression of SIRT-1 in PC12 cells. (A) PC12 cells were incubated with Hcy (1.25, 2.5 or 5 mM) for 24 h. (B) PC12 cells were pretreated with NaHS (100, 200 or 400 μM) for 24 h. (C) PC12 cells were co-treated with NaHS (200 or 400 μM) and Hcy (5 mM) for 24 h. The protein expression levels of SIRT-1 in PC12 cells were assessed using western blot analysis. β-actin was used as a loading control. The results were normalized to the percentage of β-actin and expressed as the fold of the control group. Values are expressed as the mean ± standard error of the mean of three independent experiments. **P<0.01, compared with the control group; $^{\#\#}$P<0.01, compared with the Hcy only group. Hcy, homocysteine; NaHS, sodium hydrosulfide; SIRT-1 silent mating type information regulator 2 homolog 1.

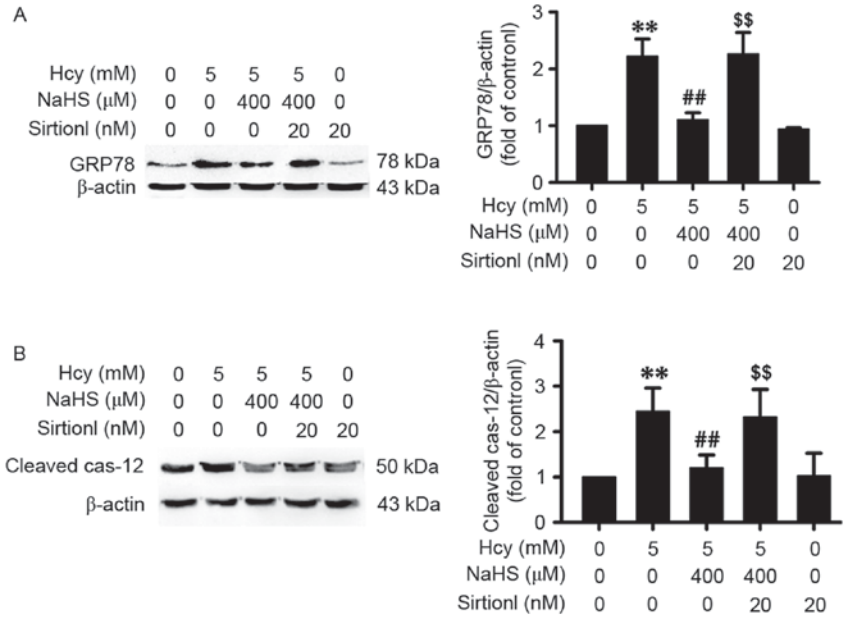

Figure 4. Sirtinol, a specific SIRT-1 inhibitor, reverses hydrogen sulfide-induced endoplasmic reticulum stress in Hcy-treated PC12 cells. PC12 cells were pre-incubated with sirtinol (15 μM) for 30 min prior to cotreatment with NaHS (400 μM) and Hcy (5 mM) for 24 h. The protein expression levels of (A) GRP78 and (B) cleaved caspase-12 in PC12 cells were measured using western blot analysis, respectively. β-actin was used as a loading control. The results were normalized to the percentage of β-actin and expressed as the fold of the control group. Values are expressed as the mean ± standard error of the mean of three independent experiments. **P<0.01, compared with the control group; $^{\#\#}$P<0.01, compared with the Hcy only group; $^{\$\$}$P<0.01, compared with the NaHS and Hcy cotreatment group. Hcy, homocysteine; NaHS, sodium hydrosulfide; SIRT-1 silent mating type information regulator 2 homolog 1; GRP78, glucose-regulated protein 78; cas-12, caspase-12.

cleaved caspase-12 (Fig. 4B) in the homocysteine-exposed PC12 cells. Treatment with sirtinol alone did not affect the expression of these two proteins. These results indicated that the inhibition of SIRT-1 eliminated the protective effect of H_2S against homocysteine-induced ER stress.

Discussion

In our previous study, it was demonstrated that H_2S had a protective effect against homocysteine-induced neurotoxicity (25). In addition, the involvement of abnormal ER stress has been shown to be prominent in the neurotoxicity of homocysteine (15,16). Therefore, the present study was designed to investigate whether the protective role of H_2S in the neurotoxicity of homocysteine was associated with regulating neuronal ER stress, and the underlying mechanisms were investigated. The main findings of the present study were as follows: i) H_2S markedly inhibited homocysteine-induced ER stress in the PC12 cells; ii) H_2S enhanced the protein level of SIRT-1 in the presence or absence of homocysteine treatment; and iii) sirtinol, an inhibitor of SIRT-1, eliminated the inhibitory effect of H_2S on homocysteine-induced ER stress. These findings suggested the protective role of H_2S against homocysteine-induced ER stress by enhancing the expression of SIRT-1.

Increasing evidence has confirmed the neurotoxic effects of homocysteine (37-40), which are associated with ER stress (15,16). It is known that GRP78 is an ER-chaperone protein involved in the modulation of ER dynamic homeostasis (41,42). Pro-caspase-12 is located on the cytoplasmic region of ER and is proteolytically activated during excess ER stress (43-45). GRP78 and caspase-12 are two important markers of ER stress. In the present study, the effects of homocysteine on the protein expression levels of GRP78 and cleaved caspase-12 in PC12 cells were examined. It was demonstrated that homocysteine upregulated the protein levels of GRP78 and cleaved caspase-12 in the PC12 cells. These results indicated that homocysteine was able to elevate ER stress in the PC12 cells. It is known that ER stress is involved in the pathogenic effects of homocysteine in several diseases, including cardiovascular disease (46), apoptosis of osteoblastic cells (47), insulin resistance of adipose tissue (48), and type 2 diabetes mellitus (49). Therefore, ER stress may be a common intermediate pathway in the homocysteine-induced pathogenic effects in tissues and cells.

H_2S is a protective gaseous signaling molecule. In our previous study, it was demonstrated that H_2S prevented homocysteine-induced neurotoxicity in PC12 cells (25). To improve current understanding of the protective role of H_2S in the neurotoxicity of homocysteine, the present study investigated whether H_2S suppresses the homocysteine-induced upregulatory effect on the expression levels of GRP78 and cleaved caspase-12. The results showed that NaHS (the donor of H_2S) downregulated the expression levels of GRP78 and cleaved caspase-12 in the homocysteine-exposed PC12 cells, which indicated that H_2S was able to suppress homocysteine-induced ER stress. Previous studies have demonstrated that H_2S prevents ER stress in doxorubicin-induced cardiotoxicity (50) and 6-hydroxydopamine-induced neurotoxicity (51). These previous findings offer a reasonable explanation for the results obtained in the present study. Furthermore, Wei *et al* (46) revealed the protective effect of H_2S against homocysteine-induced cardiomyocytic ER stress. In the present study, the inhibitory role of H_2S in homocysteine-induced ER stress was further confirmed in the PC12 cells. Therefore, the regulation of ER stress offers insights into the protective effect of H_2S against homocysteine neurotoxicity.

The present study also examined the possible underlying signaling mechanisms for the protective effect of H_2S against homocysteine-induced ER stress. SIRT-1is involved in lifespan modulation (52-54) and orchestrates diverse biological processes, including cell survival, differentiation and metabolism (55,56). SIRT-1 is considered to be a vital modulator of cellular defenses and survival in response to stress (57,58). SIRT-1 is also expressed in the brain. Accumulating evidence has indicated that the upregulation of SIRT-1 rescues neurons in acute and chronic neurological diseases (28,31,59). It has also been found that impaired SIRT1-deacetylation induces ER stress (60) and that the overexpression of SIRT-1 attenuates ER stress (35,36). This suggests that SIRT-1 is important in counteracting ER stress, therefore, the present study focused on the effect of homocysteine on the expression of SIRT-1, and the role of H_2S in the expression of SIRT-1 in PC12 cells treated with or without homocysteine. The results showed that the expression of SIRT-1 was downregulated in the homocysteine-exposed PC12 cells, which indicated the involvement of downregulated SIRT-1 in the increase of ER stress induced by homocysteine. In addition, NaHS was found to increase the protein expression of SIRT-1 in PC12 cells. It was also found that NaHS inhibited the homocysteine-induced decrease in the protein expression of SIRT-1 in PC12 cells. These results suggested that the upregulation of SIRT-1 contributed to the beneficent effects of H_2S on homocysteine-induced ER stress. To confirm whether SIRT-1 mediates the protective effect of H_2S against homocysteine-induced ER stress, the present study examined whether the inhibition of SIRT-1 eliminates the protective effect of H_2S against ER stress induced by homocysteine. The results confirmed that the inhibition of SIRT-1 inhibited the reversal effect of H_2S on the homocysteine-increased protein expression of GRP78 and cleaved caspase-12 in PC12 cells. Taken together, these results suggested that the upregulation of SIRT-1 mediated the H_2S-induced protective effects against homocysteine-induced ER stress.

In conclusion, the present study demonstrated that H_2S was able to overcome homocysteine-induced ER stress and increases in the protein expression of SIRT-1 in PC12 cells. Inhibiting SIRT-1 reversed the protective effect of H_2S against ER stress elicited by homocysteine in PC12 cells. These results suggested that H_2S had the ability to inhibit homocysteine-induced ER stress and that the upregulation of SIRT-1 mediated this protective effect of H_2S. These findings shed light on the molecular mechanism underlying the protective role of H_2S in the neurotoxicity of homocysteine. Homocysteine is an independent risk factor for AD (3-7) and ER stress is a crucial process in the pathogenesis of AD (12-14). Therefore, the results of the present study suggested that positive intervention of SIRT-1 may have profound therapeutic benefits against homocysteine-dependent neurodegenerative diseases.

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