Neuroprotective effects of sodium hydrosulfide against β-amyloid-induced neurotoxicity

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Abstract. Alzheimer's disease (AD) is known to be caused by the accumulation of amyloid-β peptide (Aβ). The accumulation of Aβ has been shown to cause learning and memory impairment in rats, and it has been shown that hydrogen sulfide donors, such as sodium hydrosulfide (NaHS) can attenuate these effects. However, the underlying mechanisms have not yet been fully eludicated. This study was designed to investigate whether NaHS attenuates the inflammation and apoptosis induced by Aβ. We demonstrated that NaHS attenuated $A\beta_{25-35}$-induced neuronal reduction and apoptosis, and inhibited the activation of pro-caspase-3. It also decreased the protein expresion of phosphodiesterase 5 (PDE5) in the hippocampus of the rats. In addition, NaHS upregulated the expression of peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ, but it did not affect the expression of PPAR-β. Moreover, the $A\beta_{25-35}$-exposed rats exhibited a decrease in IκB-α degradation and an increase in nuclear factor-κB (NF-κB) p65 phosphorylation levels, whereas these effects were attenuated by NaHS. Our data suggest that NaHS prevents Aβ-induced neurotoxicity via the upregulation of PPAR-α and PPAR-γ and the inhibition of PDE5. Hence NaHS may prove to be beneficial in the treatment of AD.

Introduction

Alzheimer's disease (AD) has become the fourth leading lethal disease among the elderly following cancer, heart disease and stroke. AD is an age-related neurodegenerative disorder, which is typically characterized by the deposition of β-amyloid plaques, neurofibrillary tangles (NFTs) and neuronal loss (1). These pathological characteristics of the disease lead to the progressive loss of memory, which causes cognitive dysfunction. The neurotoxicity of amyloid-β (Aβ) peptides has been widely accepted to be responsible for the pathogenesis of AD (2). In fact, both *in vitro* and *in vivo* findings have demonstrated that Aβ fragments promote a marked neuroinflammatory response, accounting for the synthesis of various cytokines and pro-inflammatory mediators (3,4). It is believed that the inflammatory process, once initiated, may contribute independently to neuronal dysfunction and cell death (5). The nuclear receptors known as peroxisome proliferator-activated receptors (PPARs), which antagonize the effects of the pro-inflammatory transcription factor, nuclear factor-κB (NF-κB), regulate the expression of many genes which encode proteins that play a decisive role in the process of inflammation (6). The three PPAR isotypes, PPAR-α, PPAR-β/δ and PPAR-γ, are expressed in all cell types in the brain (7). Numerous studies have described the neuroprotective properties of PPAR-α and PPAR-γ agonists in different models of neurological diseases, and propose PPAR-dependent mechanisms for their mode of action. The efficiency of PPAR-β/δ agonists has previously been reviewed in animal models of neurodegenerative diseases (8). However, the biology of PPAR-β/δ in the brain is less understood compared to PPAR-α and PPAR-γ. Some scholars have proposed that PPAR-γ is an opportunistic therapeutic target in patients with mild cognitive impairment (MCI)/AD and concomitant insulin dysregulation; the co-morbidity of insulin resistance is shared by both AD and diabetes (9-11). Indeed, PPAR-γ agonists, such as rosiglitazone (RSG) have been shown to improve cognitive function in some patients with early-stage AD, as well as in several animal models of AD (12-14).

A number of researchers have demonstrated the involvement of the cyclic guanosine monophosphate (cGMP) pathway in learning and memory (15-17). Of note, sildenafil (Viagra), a specific phosphodiesterase 5 (PDE5) inhibitor, has been shown to increase cGMP levels by inhibiting its degradation and is widely used as the selective drug for the treatment of erectile dysfunction and pulmonary hypertension. It has recently been proposed as a molecule for use in the treatment a variety of disorders, including AD and aging (18). In addition, the age-related decline of cognitive functions is thought to be associated with an increase in neuronal apoptosis (19), a process of programmed cell death that may result in pathological processes, such as degeneration (20,21). Many proteins are involved in the process of apoptosis, such as Bcl-2 family members, caspases and many more (22,23). Caspase-3 stimulates the formation of Aβ by affecting amyloid precursor

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protein (APP), a single transmembrane protein, via the cleavage of protease to generate Aβ (24). To date, specific treatment for AD is unavailable. Thus, it is urgent to further explore novel treatment strategies for AD.

Hydrogen sulfide (H_2S) is a well known gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO) (25). H_2S is primarily produced in the brain from the cysteine precursor by the cystathionine β-synthase (CBS) and cystathione γ-lyase (CGL) enzymes (26). CBS, is highly distributed in the hippocampus (27). 3-Mercaptopyruvate sulfurtransferase (3MST) is a third enzyme also responsible for the generation of endogenous H_2S (28,29). H_2S has been gradually confirmed to be a new type of neuromodulator involved in multiple physiological nerve functions. It has been previously demonstrated that H_2S exerts a variety of effects (including antioxidant, anti-inflammatory and anti-apoptotic effects) in animal models or neuronal and glial cells in AD, Parkinson's disease and other diseases (30-33). The levels of H_2S are markedly decreased in patients with AD. Moreover, there is an association between the levels of H_2S and the severity of AD (34). Recent data have demonstrated that exogenous H_2S significantly improves spatial learning and memory impairment induced by $A\beta_{25-35}$, and exerts anti-inflammatory and anti-apoptotic effects (35). These findings suggest the possible involvement of H_2S in attenuating the pathogenesis of AD. However, the possible and corresponding molecular mechanisms of action of H_2S as an anti-inflammatory and anti-apoptotic agent in a rat model of $A\beta_{25-35}$-induced neurotoxicity have not yet been fully elucidated.

Therefore, the present study was designed to investigate the effects of NaHS on $A\beta_{25-35}$-induced neurotoxicity and further explore its underlying mechanisms of action.

Materials and methods

Animals. Healthy male SPF Sprague-Dawley (SD) rats (weighing 220 to 250 g) were obtained from the Animal Center of the Third Military Medical University (Chongqing, China) (certificate no. SCXK20020003). The animals were maintained under a 12 h light/dark cycle in temperature (23 ±1˚C) and humidity (relative, 60%)-controlled rooms and allowed free access to food and water. All experiments were performed according to the National Institutes of Health Guidelines for Humane Use and Care (Eighth Edition), and the Current Guide for the Care and Use of Laboratory Animals under a protocol approved by Zunyi Medical University Animal Studies Committee.

Experimental design and treatment. Forty-two rats were randomly assigned to 3 groups as follows: the sham-operated group, the $A\beta_{25-35}$ group and the $A\beta_{25-35}$ + NaHS group (n=14 rats per group). $A\beta_{25-35}$ was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in sterilized saline at a concentration of 2 μg/μl, and then incubated at 37˚C for 7 days prior to injection in order to allow aggregation. The animals were intraperitoneal injected with chloral hydrate (40 mg/kg) anesthesia and placed in a stereotaxic device (SR-6N; Narishige, Tokyo, Japan). Aggregated $A\beta_{25-35}$ was injected into the rats in accordance with a previously pubished protocol (36). A midline incision was made on the head skin of the rats following routine sterilization, exposing the periosteum, and then, using a 5 μl microsyringe injector, $A\beta_{25-35}$ was injected into the bilateral CA1 subregion at the following coordinates: 3.3 mm posterior to thye bregma, 2 mm lateral to the sagittal suture, 3 mm beneath the surface of brain. Rats in the sham-operated group were injected with normal sterilized saline. The rats were injected with 5 μl $A\beta_{25-35}$ or 5 μl sterilized normal saline in each bilateral CA1 subregion at a rate of 1 μl/min. The needle was left for 5 min after injection. NaHS (Sigma-Aldrich) was continuously intraperitoneally injected at a dose of 5 mg/kg for 15 days. Rats in the sham-operated and $A\beta_{25-35}$ group were administrated the same volume of normal saline.

Nissl staining. Four rats from each group were randomly selected and were anesthetized and sacrificed by intracardiac perfusion with 0.1 M phosphate buffer containing 0.4% heparin. The brains were carefully removed following decapitation and transferred into ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.38), and fixed in 4% paraformaldehyde for 48 h, and then embedded in paraffin. The conventional paraffin-embedded tissue sections were stained with toluidine blue (Solarbio, Beijing, China). The Nissl bodies were stained blue-purple under a light microscope (KS300; Zeiss-Kontron, Göttingen, Germany). Neurons in the hippocampus from each group were counted as previously described (37). Neurons in the area of the CA1 region of the hippocampus were counted using 5 equally spaced coronal sections passing through the hippocampus for each brain.

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining. Cells undergoing apoptosis induced by $A\beta_{25-35}$ were detected by TUNEL staining using an *In Situ* Cell Death Detection kit, Fluorescein (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer's instructions. In order to block endogenous peroxidase activity, the sections were immersed in 3% H_2O_2 for 15 min in the dark. After being washed 3 times in phosphate-buffered saline (PBS) for 5 min each, the sections were treated with proteinase K solution (20 μg/ml in 10 mM Tris/HCl, pH 7.6) at 37˚C for 15 min. They were then incubated for 60 min at 37˚C with TUNEL reaction mixture. The sections were then washed again and incubated for 30 min at 37˚C with converter-POD. The sections were rinsed in PBS, treated with DAB substrate solution and washed again with PBS. The sections were viewed and counted under a light microscope (BX43; Olympus Corporation, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA) for the detection of PDE5. The content of PDE5 in the hippocampus was measured by ELISA. Six rats from each group were randomly selected and sacrificed, and the right hippocampus was collected for ELISA. Hippocampal tissues were homogenized (1:5, w:v) in 0.01 M PBS (pH 7.4) and centrifuged (3,000 rpm at 4˚C for 20 min), as previously described (38). The supernatant was stored at -80˚C for subsequent determination. The protein levels of homogenate samples were analyzed using the BCA protein assay kit (Biocolor Biotechnology, Shanghai, China). PDE5 (Shanghai Jiang Lai Biotechnology Co., Ltd., Shanghai, China) was quantified in these samples using the PDE5 ELISA kit according to the manufacturer's instructions.

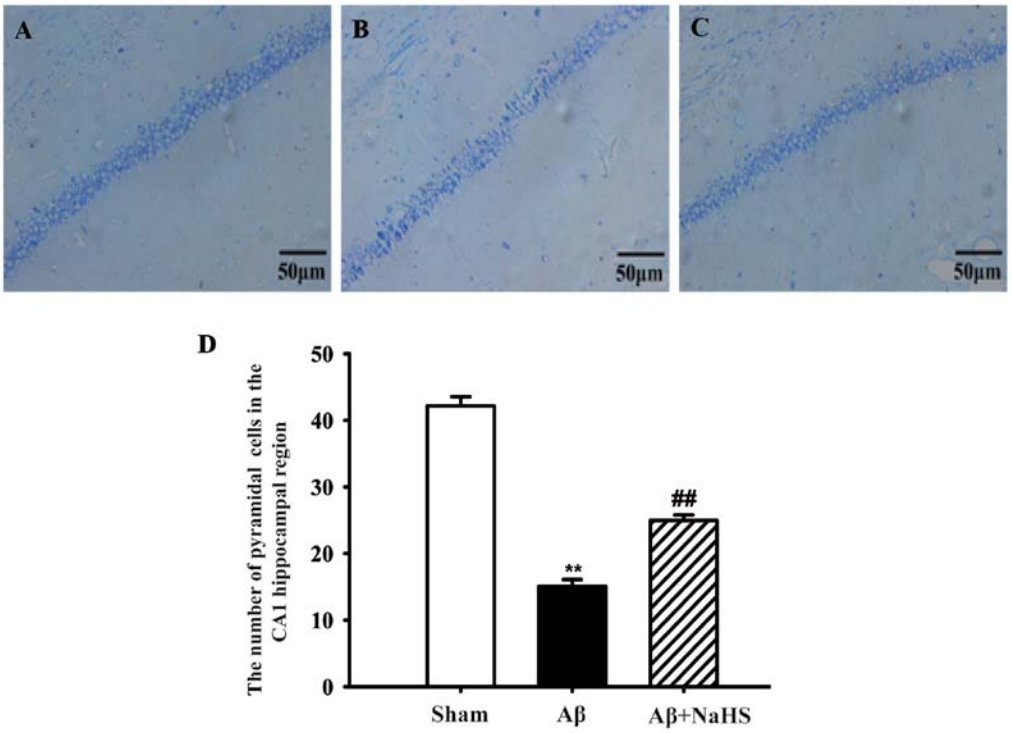

Figure 1. Effect of sodium hydrosulfide (NaHS) on neuronal damage in the hippocampal CA1 region in rats subjected to $A\beta_{25-35}$-induced neurotoxicity by Nissl staining in each group. (A) No neuronal impairment was discovered in the hippocampus of rats from the sham-operated group. (B) In the $A\beta_{25-35}$ group, a large quantity of pyknotic cells was detected. On a more serious note, some neuronal cells even disappeared in the hippocampus compared with the sham-operated group. (C) Damaged neurons significantly ameliorated following treatment with NaHS. Scale bar, 50 μm. (D) Quantification of Nissl bodies in the hippocampal CA1 region. Data are presented as the means ± SEM (n=4 rats in each group, for triplicate experiments). **P<0.01 vs. sham-operated (sham) group; $^{\#\#}$P<0.01 vs. $A\beta_{25-35}$.

Western blot analysis. The protein expression of PPAR-α (ab8934), PPAR-β (ab137724), PPAR-γ (ab19481) and active + pro-caspase-3 (ab47131) (all from Abcam, Cambridge, UK), p-NF-κB p65 (#3033), NF-κB p65 (#8242) and IκB-α (#9242) (all from Cell Signaling Technology, Danvers, MA, USA) and β-actin (AF0003; Beyotime Biotechnology, Nanjing, China) was analyzed by western blot analysis. Three rats from each group were sacrificed and the right hippocampal tissues were dissected and immediately frozen at -80˚C. The frozen tissues were sliced into small sections and homogenized on ice in cold radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 0.5% deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, 2 mM phenylmethylsulfonyl fluoride and 50 mM Tris-hydrochloric acid, pH 7.4) containing protease and phosphatase inhibitor cocktail. Following homogenization, the dissolved proteins were gathered by centrifugation for 30 min at 10,000 x g. The supernatant was collected and the protein concentration was then determined using the BCA protein assay kit (Biocolor Biotechnology). The protein (30 μg) was then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore Trading Co., Ltd., Bedford, MA, USA). Blotting membranes were incubated with 3% bovine serum albumin (BSA) in Tris-buffered saline with Tween-20 (TBST) (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5) and then probed with a primary antibody against PPAR-α (1:2,000), PPAR-β (1:3,000), PPAR-γ (1:2,000), p-NF-κB p65 (1;1,000), NF-κB p65 (1:1,000), IκB-α (1;1,000), active and pro-caspase-3 (1:2,000) and β-actin (1:5,000; Beyotime Institute of Biotechnology) at 4˚C overnight. After washing, the membranes were incubated with appropriate horseradish peroxidase-coupled secondary antibodies for 2 h at room temperature. The blots were then revealed using the ECL select kit (Beyotime Institute of Biotechnology) and exposed to Gel Imaging (Bio-Rad, Hercules, CA, USA).

Statistical analysis. All data are presented as the means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to examine statistical comparisons between groups. Post hoc comparisons were performed by LSD with equal variances, and by Dunnett's T3 with unequal variances. All analyses were performed using SPSS 16.0 software. In all cases, a value of P<0.05 was considered to indicate a statistically significant difference.

Results

NaHS attenuates $A\beta_{25-35}$-induced neuronal cell death in the hippocampus of rats. Nissl staining was utilized to evaluate the effects of NaHS on $A\beta_{25-35}$-induced neuronal cell death in the hippocampus. Healthy neurons in the CA1 region in the hippocampus were observed in the sham-operated group. The pyramidal layer of cells was neatly and closely arranged and the structure was clear. However, following the injection of $A\beta_{25-35}$, typical neuropathological changes were observed, including the pyknosis of the pyramidal layer of cells and appreciable neuronal cell loss or disappearance. However, treatment with NaHS reduced neuronal morphological impairment compared to exposure to $A\beta_{25-35}$ alone (Fig. 1). On the whole, our results suggest that the administration of NaHS

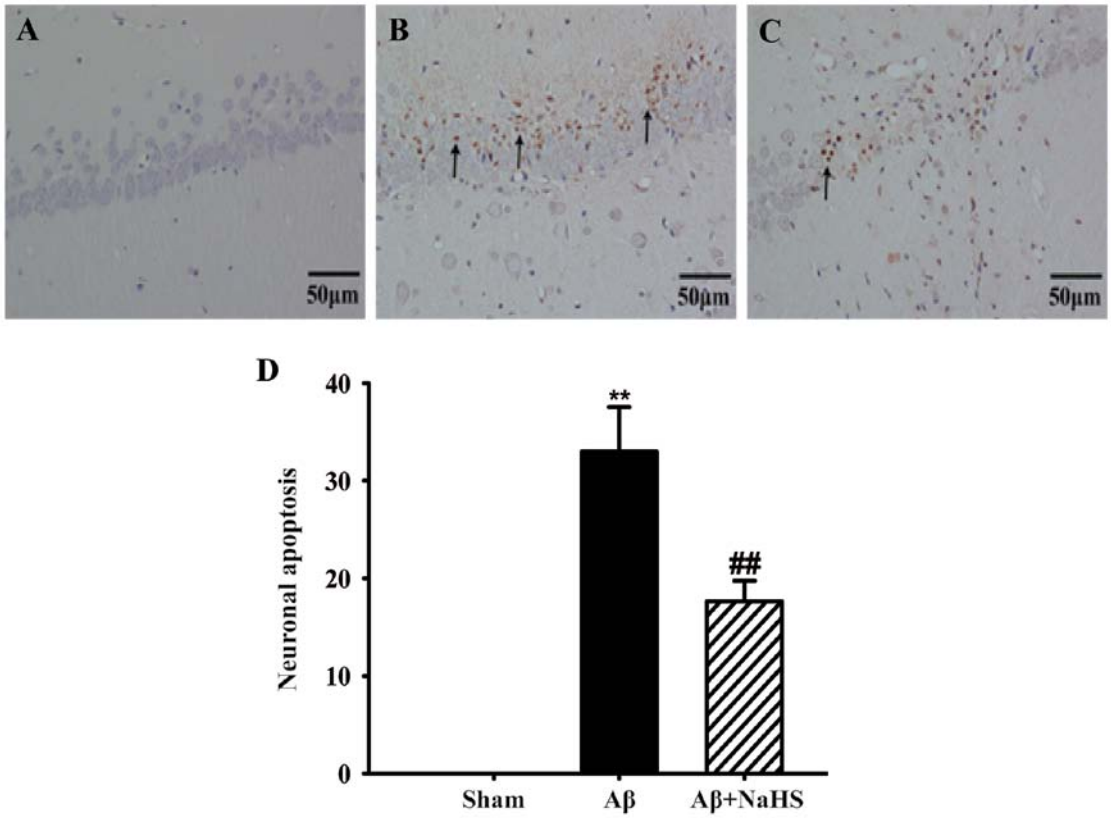

Figure 2. Effect of sodium hydrosulfide (NaHS) on cell apoptosis in the hippocampal CA1 region of rats subjected to $A\beta_{25-35}$-induced neurotoxicity by TUNEL staining in each group. (A) No apoptotic cells were observed in the hippocampus of rats from the sham-operated group. (B) Apoptotic cell death was increased in the $A\beta_{25-35}$-exposed rat brains. (C) NaHS treatment significantly decreased the number of apoptotic cells. Arrows indicate apoptotic cells which were stained in brown. Scale bar, 50 μm. (D) Quantification of apoptotic cell in the hippocampal CA1 region. Data are presented as the means ± SEM (n=4 rats in each group, for triplicate experiments). **P<0.01 vs. sham-operated (sham) group ; $^{\#\#}$P<0.01 vs. $A\beta_{25-35}$ group.

attenuates $A\beta_{25-35}$-induced neuronal loss in the hippocampus of rats.

NaHS suppresses $A\beta_{25-35}$-induced cell apoptosis in the hippocampus of rats. $A\beta_{25-35}$-induced cell apoptosis in the hippocampus of rats was detected by TUNEL staining. There was no TUNEL reaction in the hippocampus of the rats from the sham-operated group and examination revealed morphologically normal neurons. There was an increase in the number of TUNEL-positive pyramidal neurons after the $A\beta_{25-35}$ injection. However, treatment with NaHS markedly reduced the number of TUNEL-positive neurons (Fig. 2). These results indicate that NaHS suppresses $A\beta_{25-35}$-induced cell apoptosis in the hippocampus of rats.

NaHS inhibits the activation of caspase-3 in the hippocampus of rats. To further examine the protective effects of NaHS against $A\beta_{25-35}$-induced apoptosis, the protein levels of pro-caspase-3 and active-caspase-3 were examined by western blot analysis. The protein level of pro-caspase-3 was decreased after the $A\beta_{25-35}$ injection compared with the sham-operated group (P<0.01; Fig. 3). However, treatment with NaHS increased the expression of pro-caspase-3 in contrast to the $A\beta_{25-35}$ group (P<0.05). On the contrary, the $A\beta_{25-35}$ injection markedly increased the level of active-caspase-3 compared with the sham-operated group (P<0.01). The administration of NaHS significantly inhibited the protein level of active-caspase-3 compared with the $A\beta_{25-35}$ group (P<0.01). Taken together, our results indicate that NaHS prevents the $A\beta_{25-35}$-induced the activation of pro-caspase-3, and thereafter decreases the level of active-caspase-3 in the hippocampus.

NaHS decreases the protein content of PDE5 in the hippocampus of rats. The protein content of PDE5 in the hippocampus of the rats was detected by ELISA. The rats in the $A\beta_{25-35}$ group had a higher PDE5 protein level compared with the rats in the sham-operated group (P<0.01; Fig. 4). By contrast, treatment with NaHS significantly reduced the PDE5 protein level in the hippocampus of the rats compared to exposure to $A\beta_{25-35}$ alone (P<0.01; Fig. 4). Our data thus indicate that NaHS inhibits PDE5 protein expression in the hippocampus induced by $A\beta_{25-35}$.

NaHS upregulates the expression of PPAR-α and PPAR-γ, but not that of PPAR-β in the hippocampus. To determine whether the expression of PPARs is associated with the protective effects of NaHS against $A\beta_{25-35}$-induced neurotoxicity, the protein levels of PPAR-α, PPAR-β and PPAR-γ were determined by western blot analysis. The PPAR-α level in the $A\beta_{25-35}$ group was higher than that in the sham-operated group (P<0.05), but PPAR-α expression significantly increased further when the rats were treated with NaHS (P<0.05; Fig. 5A and B). As regards the PPAR-β protein level, there was no significant difference between the $A\beta_{25-35}$, sham and $A\beta_{25-35}$ + NaHS groups (Fig. 5A

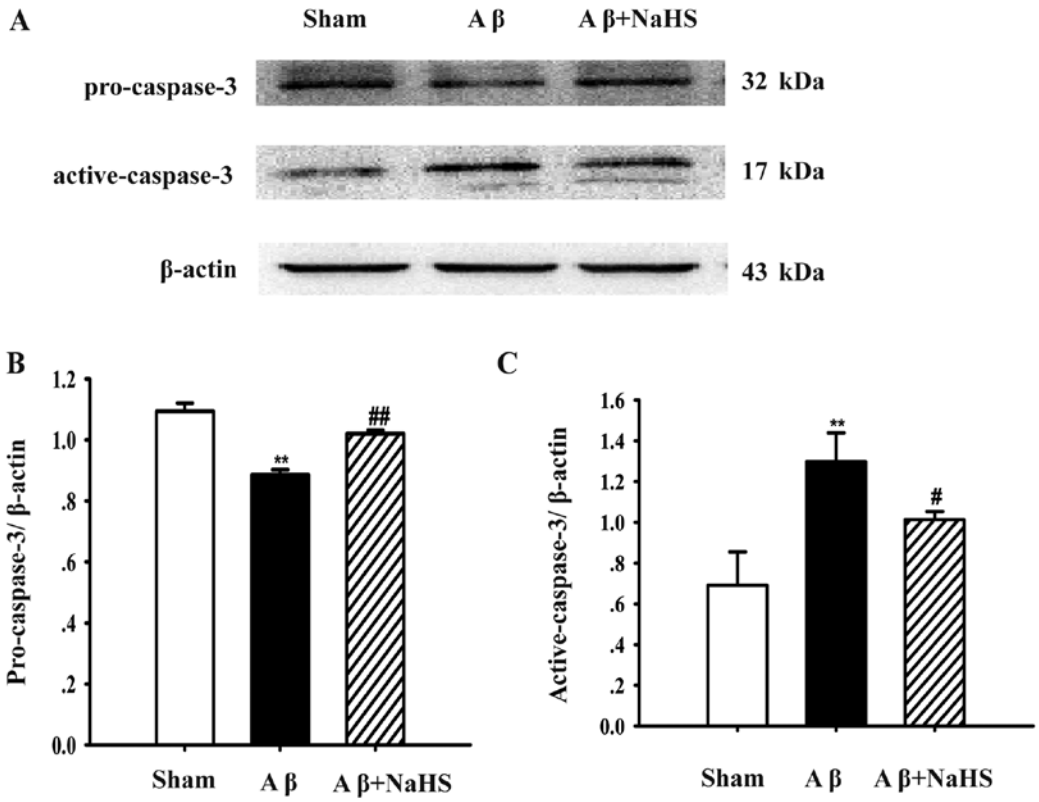

Figure 3. Effects of sodium hydrosulfide (NaHS) on the protein levels of pro-caspase-3 and cleaved-caspase-3 in the hippocampus after the injection of $A\beta_{25-35}$ for 15 days. (A) Protein contents were analyzed by western blot analysis for the sham-operated, $A\beta_{25-35}$ and $A\beta_{25-35}$ + NaHS groups, respectively. (B) Quantification of pro-caspase-3 protein. (C) Quantification of active-caspase-3 protein. The relative optical density was normalized to β-actin. Data are presented as the means ± SEM (n=3 rats in each group, for triplicate experiments). **P<0.01 vs. sham-operated (sham) group; $^{\#}$P<0.01 or $^{\#\#}$P<0.05 vs. $A\beta_{25-35}$ group.

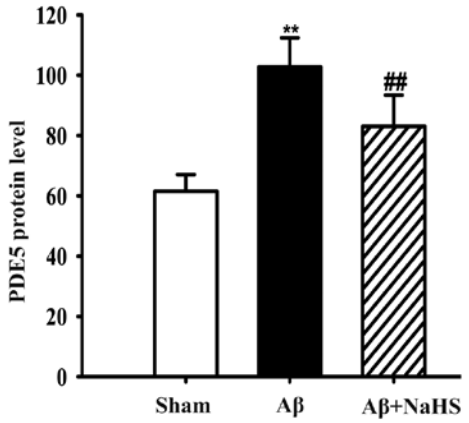

Figure 4. Effect of sodium hydrosulfide (NaHS) on the phosphodiesterase 5 (PDE5) protein content in the hippocampus. PDE5 protein level in the hippocampus was distinctly improved compared with the sham-operated group after the injection $A\beta_{25-35}$ for 15 days, whereas NaHS treatment decreased the PDE5 protein level compared with the $A\beta_{25-35}$ group. Data are presented as the means ± SEM (n=6 rats per group). **P<0.01 vs. sham-operated group; $^{\#\#}$P<0.01 vs. $A\beta_{25-35}$ group.

and D). As regards PPAR-γ protein expression, $A\beta_{25-35}$ injection significantly increased the protein level compared with the sham-operated group (P<0.01). Treatment with NaHS further enhanced the expression of PPAR-γ compared to exposure to $A\beta_{25-35}$ alone (P<0.01; Fig. 5A and C). These results suggest that NaHS attenuates $A\beta_{25-35}$-induced neurotoxicity by upregulating the expression of PPAR-α and PPAR-γ, but it does not affect the protein level of PPAR-β.

NaHS blocks the degradation of IκB-α and suppresses NF-κB p65 phosphorylation. To further explore the molecular mechanisms underlying the agonistic effects of NaHS, the protein levels of IκB-α and NF-κB p65 phosphorylation were examined by western blot analysis. There was a marked decrease in IκB-α protein expression after the $A\beta_{25-35}$ injection, whereas treatment with NaHS induced a significant increase in the protein expression of IκB-α (Fig. 6A and B), a primary member of the IκB family. It was found that $A\beta_{25-35}$ injection into the hippocampus markedly enhanced the level of phosphorylated NF-κB p65 (P<0.01). However, treatment with NaHS significantly decreased the $A\beta_{25-35}$-induced NF-κB p65 phosphorylation (P<0.05; Fig. 6A and C). On the whole, these findings demonstrate that NaHS blocks IκB-α degradation and the activation of NF-κB p65 induced by $A\beta_{25-35}$.

Discussion

It is widely recognized that the formation and deposition of Aβ is one of the main typical pathological characteristics of AD the brain. Aβ is a 40-42 amino acid peptide fragment derived by proteolysis from the integral membrane protein known as Aβ precursor protein (39). The neurotoxicity of Aβ, including different Aβ fragments, has been widely reported. $A\beta_{25-35}$ is the shorter toxic fragment corresponding to amino acids 25-35, which encompasses the β sheet of the full protein (40). In the

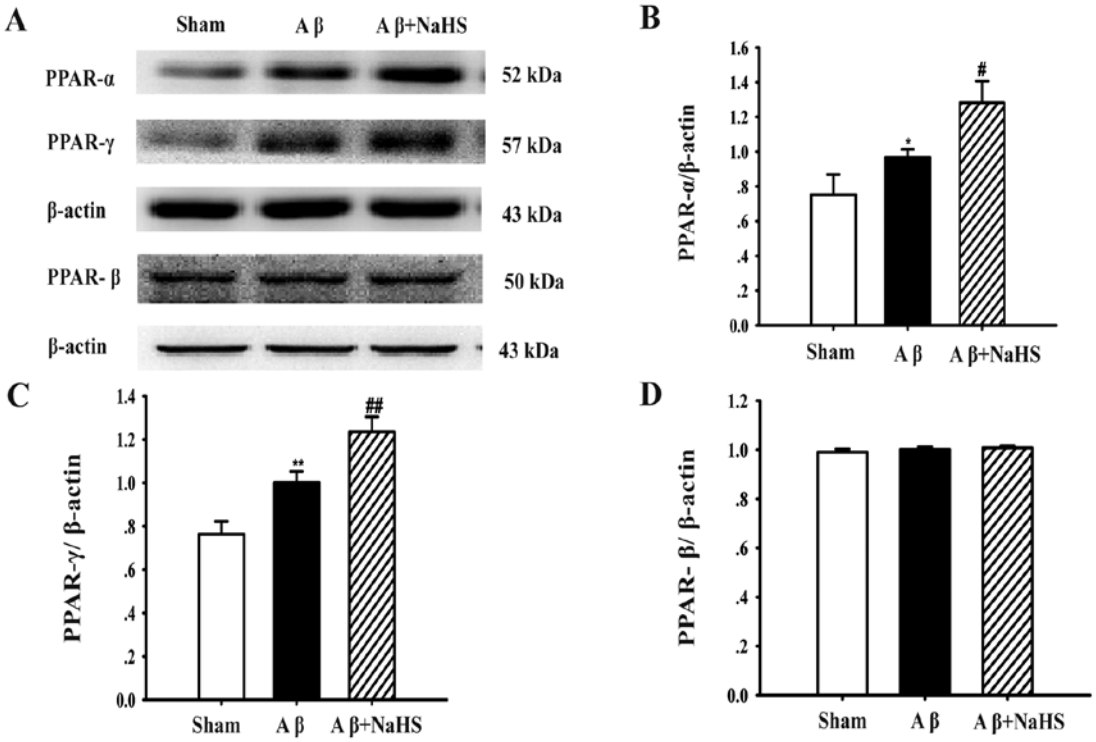

Figure 5. Effects of sodium hydrosulfide (NaHS) on peroxisome proliferator activated receptor-α (PPAR-α), PPAR-β and PPAR-γ protein levels in the hippocampus after the injection of $A\beta_{25\text{-}35}$ for 15 days. (A) Protein contents were determined by western blot analysis for the sham-operated, $A\beta_{25\text{-}35}$ and $A\beta_{25\text{-}35}$ + NaHS groups, respectively. (B) Quantification of PPAR-α protein. (C) Quantification of PPAR-γ protein. (D) Quantification of PPAR-β protein. The relative optical density was normalized to β-actin. Data are presented as the means ± SEM (n=3 rats in each group, for triplicate experiments). $^{*}P<0.05$ or $^{**}P<0.01$ vs. sham-operated (sham) group; $^{\#}P<0.05$ or $^{\#\#}P<0.01$ vs. $A\beta_{25\text{-}35}$ group.

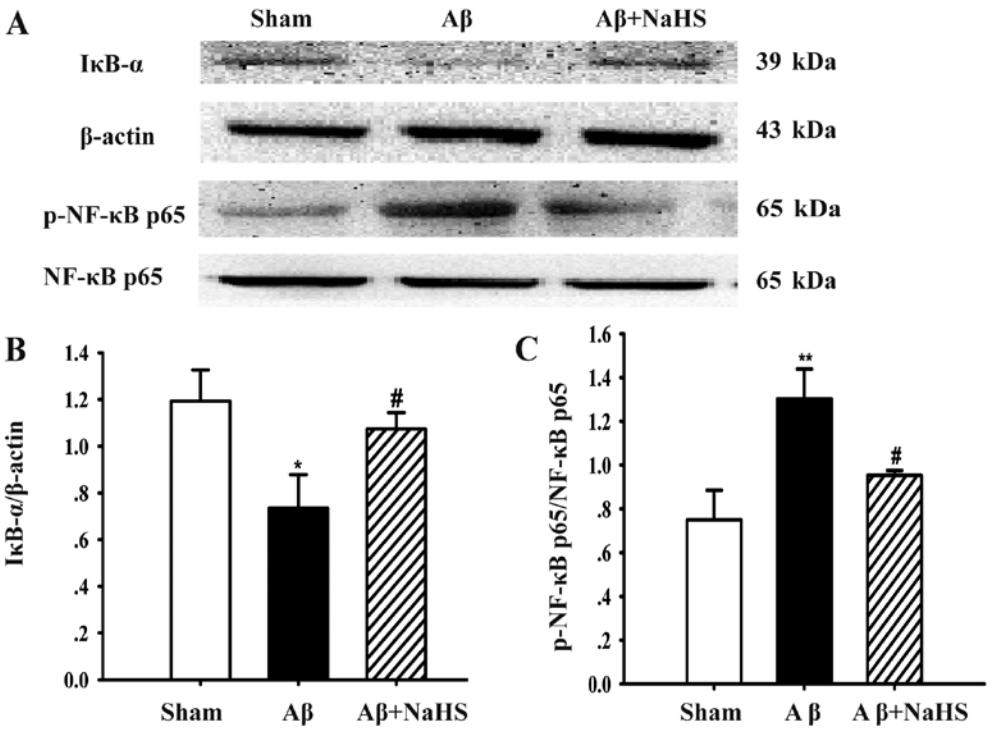

Figure 6. Effects of sodium hydrosulfide (NaHS) on IκB-α degradation and nuclear factor-κB (NF-κB) p65 phosphorylation. (A) Western blot analysis of various protein contents for IκB-α and NF-κB p65 phosphorylation. Protein contents for the sham-operated, $A\beta_{25\text{-}35}$ and $A\beta_{25\text{-}35}$ + NaHS groups, respectively. (B) Quantification of IκB-α protein; (C) Quantification of NF-κB p65 phosphorylation protein, β-actin protein was used as an internal control. Data are presented as the means ± SEM (n=3 rats in each group, for triplicate experiments). $^{**}P<0.01$ vs. sham-operated (sham) group; $^{\#}P<0.05$ vs. $A\beta_{25\text{-}35}$ group.

present study, we injected $A\beta_{25\text{-}35}$ into the hippocampus of the rats to induce neurodegenerative changes and neurotoxicity. The rats in the $A\beta_{25\text{-}35}$ + NaHS group were treated with NaHS at the dose of 5 mg/kg once daily intraperitoneally as previous

reported (35,41). Nissl staining was applied to observe the neurons in the hippocampus and Nissl bodies are one of the characteristic structures of neurons. Our results revealed that the $A\beta_{25-35}$ injection induced neuronal cell death, whereas treatment with NaHS significantly diminished neuronal cell death. Apoptosis is a means of neuronal death; thus, in present study, TUNEL staining was adapted to confirm the neuronal cell death induced by $A\beta_{25-35}$. There were many apoptotic cells which were stained dark brown in the $A\beta_{25-35}$ group, whereas in the NaHS group, the number of apoptotic cells was decreased. These results indicate that NaHS suppresses $A\beta_{25-35}$-induced apoptosis. Apoptosis is a basic physiological process in different biological systems. Previous studies have shown that neuronal apoptosis is a critical factor leading to neuronal loss, and neuronal loss in AD is intimately linked with apoptosis (42). Aβ is the core component of senile plaques (SP) in the AD-affected brain. The abnormal deposition of Aβ is an important cause of AD. As an initiation factor of apoptosis, Aβ can induce mitochondrial dysfunction caused by the extrinsic pathway of apoptosis. It has been shown in *in vitro* experiments that Aβ activates caspases and then induces apoptosis only in the presence of the functional electron transport chain of the mitochondria (43).

The caspase family plays a very important role in mediating the process of apoptosis, where caspase-3 is responsible for the proteolytic cleavage of many major proteins in a number of apoptotic signaling pathways. Normally, caspase-3 acts as a zymogen (pro-caspase-3, 32 kDa) that is active and presents in the cytoplasm. Pro-caspase-3 can be activated by the Fas/FasL pathway (44) and also through the activation of the granzyme B pathway in the cytotoxic effects of CTL cells (45). The active enzyme is shown to consist of two subunits of 17 and 12 kDa, originated from the precursor protein by cleavage at Asp-28-Ser-29 and Asp-175-Ser-176 by using electrospray MS and N-terminal sequence analysis (46). The two subunits comprise the active-caspase-3. Thus, in this study, the protein levels of pro-caspase-3 and active-caspase-3 were examined to further investigate the protective effects of NaHS against $A\beta_{25-35}$-induced apoptosis. Indeed, in the present study, $A\beta_{25-35}$ increased the levels of active-caspase-3, whereas treatment with NaHS decreased the protein expression of active-caspase-3. However, the protein expression of pro-caspase-3 is contrary to active-caspase-3. It is confirmed that $A\beta_{25-35}$ induces the apoptosis of hippocampal neuronal cells by an enhanced caspase signaling pathway. On the other hand, treatment with NaHS reversese these apoptotic changes.

ELISA was applied to determine whether H_2S attenuates memory impairment by inhibiting PDE5 in the central nervous system (CNS). In our study, the expression of PDE5 was significantly increased after the $A\beta_{25-35}$ injection, whereas treatment with NaHS decreased the level of PDE5 in the hippocampus. Evidence suggests that endogenous H_2S can act both as a vasodilator and a vasoconstrictor according to its concentration, and it also has a promoting effect on erectile function (47,48). The occurrence of erectile dysfunction in aged rats is related to the disruption of the H_2S pathway and the deficiency of androgen *in vivo* (49). It is widely accepted that PDE5 inhibitors, such as vardenafil, sildenafil and tadalafil, are appropriate for the treatment of erectile dysfunction. A number of studies have demonstrated that PDE5 inhibitors can restore memory impairment in different models of AD. For example, sildenafil has been shown to decrease beta-secretase 1 (BACE1) and cathepsin B levels and reduce APP amyloidogenic processing in SAMP8 mice (50) and to attenuate the age-related impairment of synaptic plasticity and memory by restoring CREB phosphorylation (51). Overall, PDE5 inhibitors can attenuate age-related memory impairment and cognitive dysfunction in physiological animal models of AD through a variety of central and peripheral mechanisms. Of note, H_2S has also been identified as an endogenous inhibitor of PDE5, able to enhance cGMP and cAMP levels in vessels (52). This suggests that NaHS may be a PDE5 inhibitor although its underlying mechanisms of action remain to be elucidated.

There is evidence to indicate that type 2 diabetes mellitus (T2DM) enhances the risk of developing AD (53-55). PPARs belong to the family of ligand-dependent nuclear hormone receptor transcription factors. Three isotypes have been identified, including PPAR-α, PPAR-β/δ and PPAR-γ in various species. The present study also demonstrated the effect of NaHS on the expression of PPAR-α, PPAR-β and PPAR-γ in the hippocampus of rats with neurotoxicity induced by $A\beta_{25-35}$. Our results demonstrated that NaHS enhanced the expression of PPAR-α and PPAR-γ in the hippocampus of both the sham-operated and Aβ-treated animals. Consistent with the PPAR-γ elevation in the AD-affected brain, our data revealed an increase in both the expression and transcriptional activity of PPAR-α and PPAR-γ in the hippocampus of Aβ-inoculated rats compared with the sham-operated group. Since PPAR-γ is a transcription factor with well-established neuroprotective features (56), its activation may serve as an adaptive response to protect neurons against the deleterious effects of Aβ. Our results were consistent with those of another study which demonstrated that WIN55212-2 exerts neuroprotective and anti-inflammatory effects against Aβ-induced damage by increasing the PPAR-γ level (57). It has been shown that both a PPAR-γ agonist (ciglitazone) and a PPAR-α agonist (WY 14.643) are able to protect neurons by modulating mitochondrial fusion and fission, leading to a better response of neurons to oxidative stress in neurodegenerative disorders, such as AD (58). However, it is not possible to determine whether NaHS acts as a PPAR agonist by observing the upregulation of PPAR-α and PPAR-γ.

The inflammatory reaction induced by Aβ deposition, leading to the activation of microglia and astroglia, and the subsequent release of inflammatory cytokines (IL-β, TNF-α and COX-2 and so on), plays a significant role in the pathological processing of AD. PPAR-γ has been shown to inhibit the expression of IL-1β, TNF-α and other inflammation-related mediators (59), although the potential mechanisms responsible for these effects are not yet fully understood. These factors may be situated downstream of the NF-κB signaling pathway; as a result, the suppressive effect on the pro-inflammatory genes of PPAR-γ is through the antagonism of the actions of NF-κB (60). NF-κB is well known as a key regulator that upregulates the expression of many pro-inflammatory cytokines and inducible effector enzymes linked to the inflammatory process. NF-κB remains inactivated by being coupled with an inhibitory protein, IκB. NF-κB p65 is widely studied among its several protein subtypes. The degradation of IκB is followed by the translocation of NF-κB p65 and subsequent liberation (61).

In this study, it was found that the degradation of IκB-α and NF-κB p65 phosphorylation were enhanced after the $A\beta_{25-35}$ injection. However, treatment with NaHS decreased the degradation of IκB-α and restrained NF-κB p65 phosphorylation in rats with $A\beta_{25-35}$-induced neurotoxicity. Therefore, this study suggests that NaHS may act as an anti-inflammatory mediator. These findings are consistent with those of a previous study showing that NF-κB and its nuclear translocation can prevent Aβ-induced toxicity and apoptosis (62).

In conclusion, the present study demonstrated that NaHS attenuated $A\beta_{25-35}$-induced neuronal death and suppressed apoptosis in the rat hippocampus. The underlying mechanisms are, at least partly due to the inhibition of the protein content of PDE5 and the upregulation of PPAR-α and PPAR-γ expression. Hence, NaHS may prove to be beneficial in the treatment of AD.

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