Research Paper

Hydrogen sulfide protects against amyloid beta-peptide induced neuronal injury via attenuating inflammatory responses in a rat model

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Abstract

Neuroinflammation has been recognized to play a critical role in the pathogenesis of Alzheimer´s disease (AD), which is pathologically characterized by the accumulation of senile plaques containing activated microglia and amyloid β-peptides (Aβ). In the present study, we examined the neuroprotective effects of hydrogen sulfide (H_2S) on neuroinflammation in rats with Aβ1-40 hippocampal injection. We found that Aβ-induced rats exhibited a disorder of pyramidal cell layer arrangement, and a decrease of mean pyramidal cell number in the CA1 hippoc–ampal region compared with those in sham operated rats. NaHS (a donor of H_2S, 5.6 mg/kg/d, i.p.) treatment for 3 weeks rescued neuronal cell death significantly. Moreover, we found that H_2S dramatically suppressed the release of TNF-α, IL-1β and IL-6 in the hippocampus. Consistently, both immunohistochemistry and Western blotting assays showed that H_2S inhibited the upregulation of COX-2 and the activation of NF-κB in the hippocampus. In conclusion, our data indicate that H_2S suppresses neuroinflammation via inhibition of the NF-κB activation path–way in the Aβ-induced rat model and has potential value for AD therapy.

Keywords: Alzheimer´s disease, hydrogen sulfide, cyclooxygenase-2, nuclear factor-κB (NF-κB), amyloid

INTRODUCTION

Alzheimer´s disease (AD) is a late-onset, progres–sive neurodegenerative disorder pathologically char–acterized by the presence of senile plaques and neu–rofibrillary tangles in the brain. In particular, the senile plaques are extracellular aggregates of the amyloid β-peptide (Aβ) that is cleaved from the amyloid pre–cursor protein (APP)[1]. Previous studies have reported that chronic inflammation is a common mechanism for the pathogenesis of AD[2,3,4,5]. Neuroinflammation involves neural immune interactions that activate im–mune cells, glial cells and neurons[6].

Hydrogen sulfide (H_2S) has recently been regarded as an endogenous gaseous mediator, which has multi–ple functions in the central nervous system and other systems[7,8,9]. Recent studies revealed that H_2S exerts anti-neuroinflammatory[10], anti-oxidant[11], anti-apop–totic effects[12], and protective effects on Aβ-induced cellular injury[13] . In primary cultured microglia and astrocytes from humans and rats, or murine im–mortalized BV2 microglial cells, NaHS attenuated

lipopolysaccharide (LPS)-induced production and release of nitric oxide (NO) and tumor necrosis factor (TNF)-α[10]. However, a possible role for H_2S as an anti-inflammatory agent in a rat model of AD has not been extensively evaluated [14, 15]. In this study, we investigated the effect of H_2S on rats by intrahippocampal Aβ1-40 injection.

MATERIALS AND METHODS

Rats and treatment

Male Wistar rats (7 weeks old, Vital River China, Beijing, China) weighing 250 ± 10 g at the beginning of experiments were used in the present study. They were housed 3 per cage with free access to food and water under controlled laboratory conditions (a 12 hour light/dark cycle with lights on at 09:00 am, $23 \pm 0.5°C$, and $50 \pm 0.5\%$ humidity).

Rats were randomly divided into four groups: the sham-operated group, the sham+NaHS group, the Aβ group and the NaHS+Aβ group. Rats in the Aβ group were slowly injected with 5 μg Aβ in 2 μL into the bilateral hippocampal CA1 region (bregma: -3.0 mm, lateral: -2.2mm and ventral: -3.0mm), via a microsyringe mounted on the stereotaxic holder, under deep anesthesia (10% chloral hydrate, 3.5 mL/kg, intraperitoneal injection). The injection was performed over 10 minutes to minimize reflux along the injecting track. Aβ1-40 (Sigma) was dissolved (2.5 μg/μL) in sterile 0.1 M phosphate-buffered saline (PBS), pH 7.4, and prepared at 37°C for at least 7 days. Rats in the NaHS+Aβ group received hippocampal injection the same as the Aβ group in the first day, then were administered with NaHS (Sigma) by intraperitoneal injection at a dose of 5.6 mg/kg from the second day once daily for 3 weeks[16]. Rats in the sham+NaHS group received PBS, followed with NaHS 5.6 mg/kg once daily for 3 weeks. NaHS was freshly dissolved with 0.9% saline. The sham operated group was injected with an equivalent volume of PBS. All the rats were killed after 3 weeks under deep anesthesia.

The study protocol was approved by the Committee for Animal Experiments at the author´s affiliated institution, and all experiments were carried out in accordance with the National Institutes of Health Guidelines for the use of experimental animals. Every effort was made to minimize animal use and suffering during experiment.

Nissl staining

The rats were anesthetized and killed by intracardiac perfusion with 120 mL normal saline, followed by 110 mL ice-cold 4% paraformaldehyde in 0.1 M PBS.

Brains were carefully removed, post-fixed in the same fixative for 24 hours, then were paraffin-enbedded for examination. Four-micrometer-thick tissue sections were used with chemical stain and immunohistochemistry. Sections were stained with toluidine blue (Sigma) according to Nissl technique. Neurons were counted as previously described[17]. Counts of neuronal cells were made by means of an automatic image analyser (KS300, Zeiss-Kontron, Munich, Germany). Cell counting over area of CA1 was performed by using five equally spaced coronal sections passing through the hippocampus for each brain.

Immunohistochemistry

Sections were deparaffined and then washed (3×5 minutes) in PBS, and endogenous peroxidase was inactivated with 3% H_2O_2 in 70% methanol. Sections were subjected to microwave antigen retrieval (in citric acid, pH6, 10 mM) prior to blocking with PBS containing 5% bovine serum albumin (BSA) and 0.2% Triton X-100 for 1 hour. Afterwards, samples were incubated overnight at 4°C with polyclonal rabbit anti-COX-2 and anti-NF-κB p65 antibodies (both from Abcam). After incubation with primary antibody, sections were rinsed in PBS and incubated for 1 hour with biotinylated secondary antibodies. The immunoreaction was visualized with 3,3´-diaminobenzidine. In negative controls, the primary antibody was omitted. The percentage of tissue area that was immunoreactive was measured by quantitative image analysis.

Western blotting assays

Rats were sacrificed by decapitation and the brain was removed and immediately placed in an ice-cold dish. The hippocampus was dissected according to the atlas and quickly frozen in liquid nitrogen and stored at -80°C. The hippocampus was lysed at 4°C in a lysis buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L NaF, 10 mmol/L EDTA, 10% NP-40, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium diphosphate decahydrate, 0.5 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride (PMSF), 4 mg/mL pepstatin, 4 mg/mL aprotinin and 4 mg/mL leupeptin (pH 7.4). The lysate was centrifuged at 8,000 g for 10 minutes at 4°C. The protein concentration of supernatant was determined with an Enhanced BCA Protein Assay Kit (P0010S, Beyotime, China). For preparation of nuclear extracts, the hippocampus was separately homogenized in five volumes of a buffer containing 15 mmol/L HEPES (pH 7.2), 0.25 mol/L sucrose, 60 mmol/L KCl, 10 mmol/L NaCl, 1 mmol/L PMSF and 2 mmol/L NaF. The cells were pelletized at 2,000 g for 10 minutes. To lyse the cells, the pel-

let was incubated in 5 volumes of a buffer containing 10 mmol/L HEPES (pH 7.2), 1.5 mmol/L MgCl$_2$, 10 mmol/L KCl, 1 mmol/L PMSF and 2 mmol/L NaF for 5 minutes. The nuclei were pelletized at 4,000 *g* for 10 minutes. The nuclei were lysed in one bed volume of 100 mmol/L HEPES (pH 7.2), 1.5 mmol/L MgCl$_2$, 1 mmol/L EDTA, 0.8 mmol/L NaCl, 25% glycerol, 2 mmol/L NaF and 1 mmol/L PMSF. Cell debris and genomic DNA were removed by centrifugation at 14,000 *g* for 30 minutes. The supernatant solution was used as nuclear extracts. Samples (50 μg/lane) were loaded on a 10% SDS-polyacrylamide gel, and elec−troblotted onto a PVDF membrane from the gel by a wet blotting apparatus (Tanon, Shanghai, China). The membranes were blocked for 1 hour in TBS contain−ing 0.1% Tween-20 and 5% dry milk, and then incu−bated overnight with primary antibodies anti-COX-2 and anti-NF-κB antibodies, followed by incubation with the corresponding secondary antibodies for 1 hour at room temperature and detected by the en−hanced chemiluminescence detection kit. The optical densities of the antibody-specific bands were analyzed by a Luminescent Image Analyzer, LAS-3000 (Fuji Film, Tokyo, Japan).

Measurement of TNF-α, IL-1β and IL-6

TNF-α and IL-6 concentrations were measured in the supernatant of the hippocampus using a com−
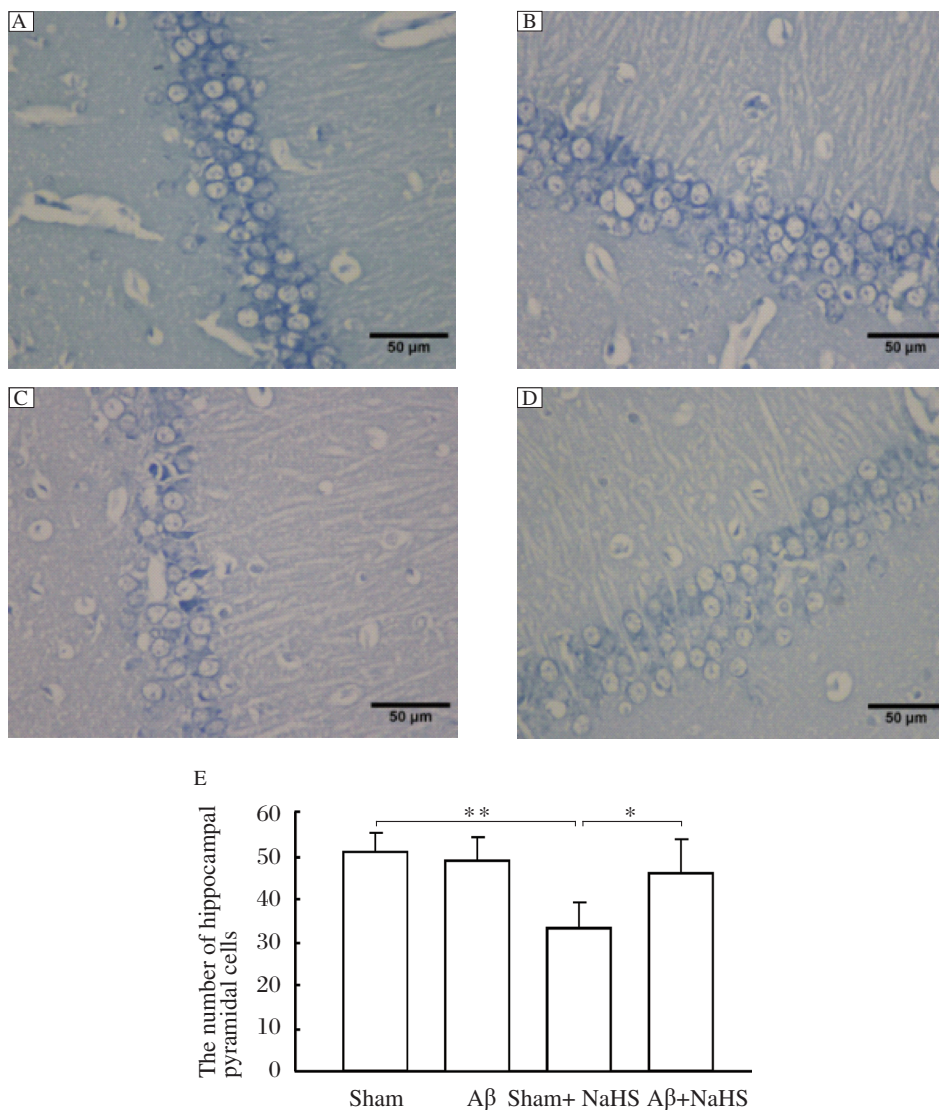
Fig. 1 **Toluidine blue staining and the mean number of pyramidal cells of the CA1 hippocampal region in the sham, sham+Aβ, Aβ-induced and Aβ+NaHS treated rats**. Rats in the sham (A) and sham+NaHS (B) groups displayed a healthy cellular and structural morphol−ogy in cell layers. Aβ-induced rats (C) displayed derangement in pyramidal cell layers and the mean number of pyramidal cells in the CA1 hippoc−ampal region (E) was significantly decreased compared with sham operated rats. Treatment with NaHS (5.6 mg/kg/day) for 3 weeks in the Aβ+NaHS group (D) partially inhibited neuronal cell death. $^{**}P < 0.01$, $^{*}P < 0.05$
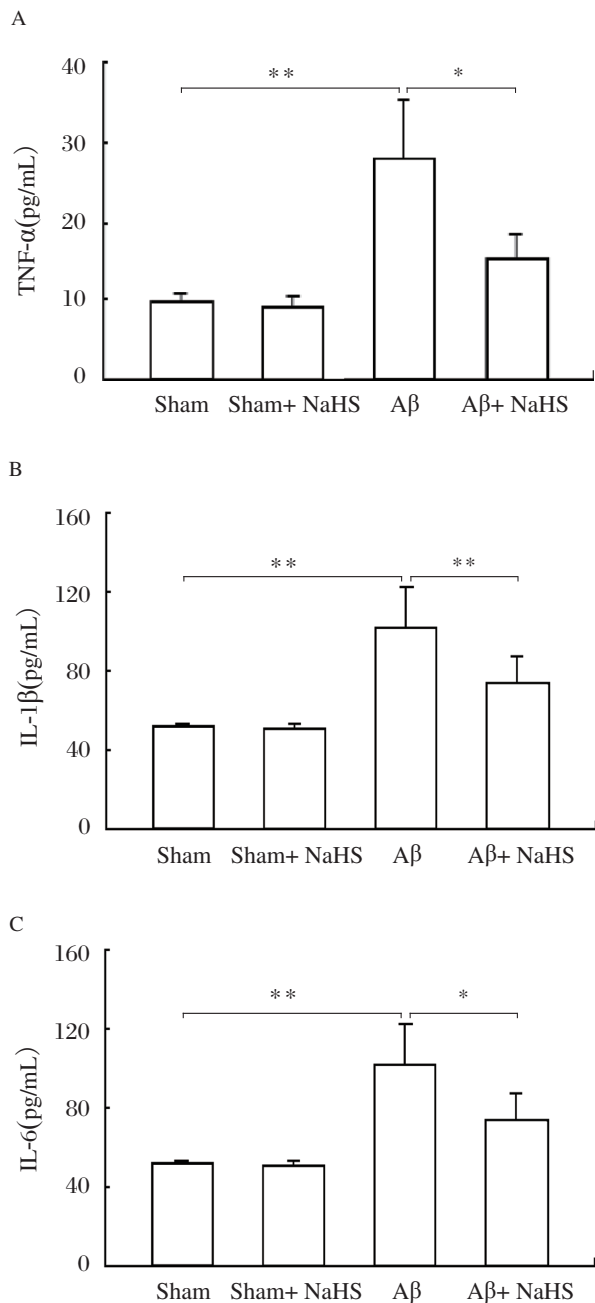
Fig. 2 **Effect of NaHS on the production of cytokines in the hippocampus**. The levels of TNF-α (A), IL-1β (B) and IL-6 (C) in the hippocampus were examined by ELISA, $^{**}P < 0.01$, $^{*}P < 0.05$.

mercial enzyme-linked immunosorbent assay kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer´s instructions. IL-1β contents were measured using a commercial kit (ENZO life sciences, ADI-900-131). Absorbance was read at 450 nm using a microplate absorbance reader (SunriseTM, TECAN, Switzerland).

Statistical analysis

The data were presented as mean±SEM and sta−tistically analyzed by one-way ANOVA with post hoc comparisons. Optic density data from immunohisto−chemistry and Western blotting assays were analyzed by ANOVA followed by Duncan´s multigroup tests. For all tests, $P < 0.05$ was considered to be significant.

RESULTS

### NaHS attenuated neuronal death in the hip−pocampus of rats injected with Aβ

Toluidine blue staining was used to evaluate the morphology of the hippocampus of each group ($n = 5$). In sham-operated rats, structural integrity and sub−field cell layers were well preserved in all subfields. Hippocampal pyramidal cells appeared healthy with intact nuclei and chromatin density (**Fig. 1A**). Rats in the sham +NaHS group displayed no distinct changes compared with the sham operated rats (**Fig. 1B**). Rats injected with Aβ displayed a significant increase in the number of pyknotic nuclei and derangement in pyramidal cell layers (**Fig. 1C**). Treatment with NaHS for 3 weeks reduced the number of pyknotic cells (**Fig. 1D**). As shown in **Fig. 1E**, the mean number of cells in the CA1 hippocampal region of the Aβ group was significantly decreased compared to that of the sham operated ($P < 0.01$). The number of pyramidal cells of the NaHS+Aβ group was higher than that of the Aβ group ($P < 0.05$, **Fig. 1E**). These results indicated that NaHS attenuated neuronal death in the hippocampus of rats injected with Aβ.

### NaHS suppressed the production of cytokines in the hippocampus

Proinflammatory cytokines, including TNF-α, IL-1β and IL-6, have been considered to play a central role in inflammation. To elucidate the potential ef−fects of NaHS on cytokine production, we examined the levels of TNF-α, IL-1β and IL-6 in the rats treated with or without NaHS ($n = 5$). While TNF-α, IL-1β and IL-6 were expressed at low levels in both the sham-operated and sham+NaHS group, the expression of these cytokines was signficantly upregulated after hippocampal injection with Aβ1-40 ($P < 0.01$, **Fig. 2**). The Aβ1-40-stimulated levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) were markedly reduced by NaHS ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively), suggesting that NaHS negatively regu−lated the production of TNF-α, IL-1β and IL-6.

NaHS inhibited the upregulation of COX-2 in the hippocampus

It is known that increased expression of cyclooxy−genase-2 (COX-2) plays an important role in the in−flammatory process. Previous report has also shown
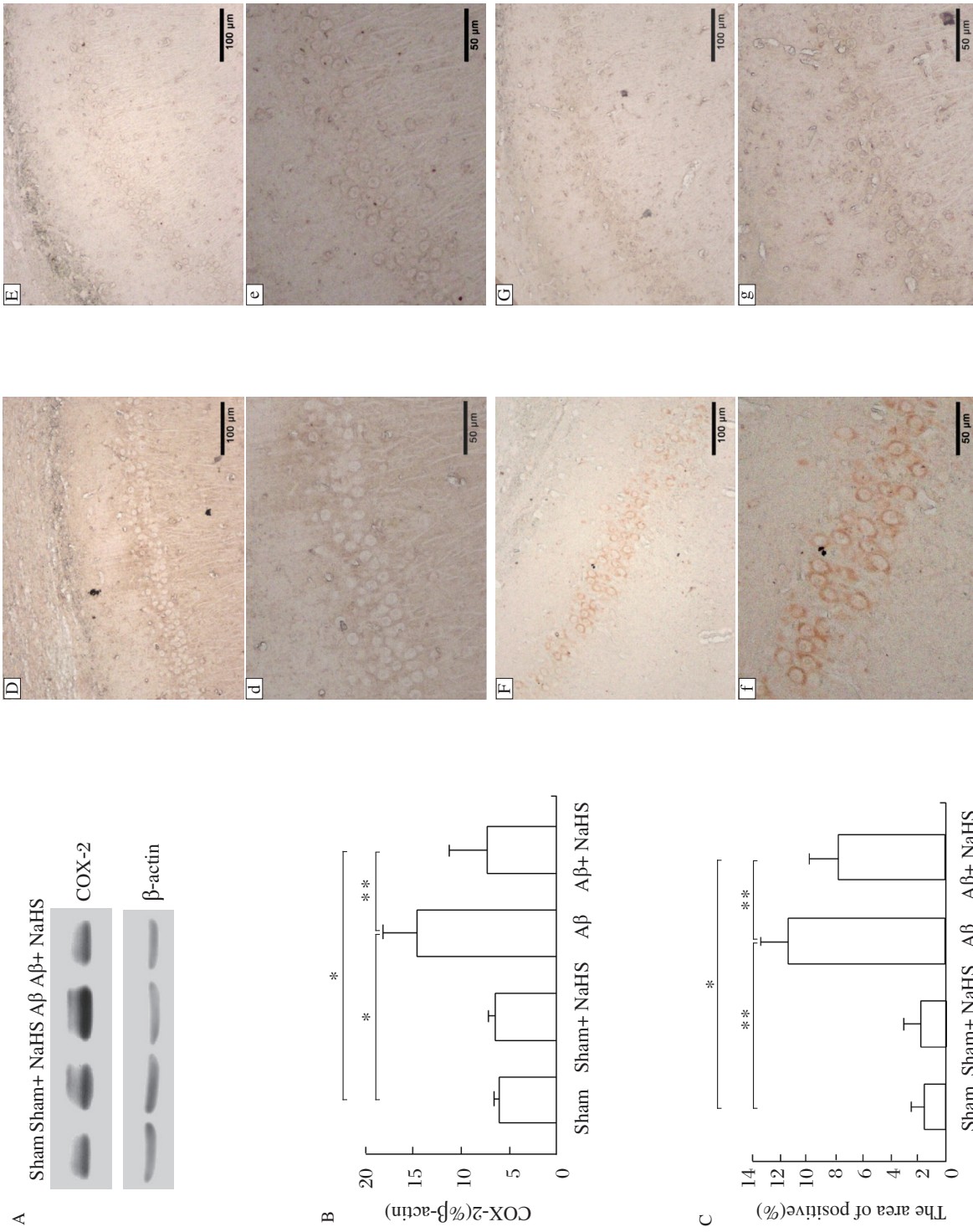
Fig. 3 Effects of NaHS on COX-2 expression in the hippocampus. Western blotting assays (A) and statistical data (B) show that NaHS reversed Aβ-induced upregulation of COX-2. Similar findings were seen in immunohistochemistry. (D & d): the sham operated group, (E & e): the sham+NaHS group, (F & f): the Aβ group, and (G & g): the Aβ+NaHS group. The rate of positive tissue area was measured by quantitative image analysis (C). $^{**}P < 0.01$ vs. the sham operated group, $^{*}P < 0.05$, $^{**}P < 0.01$.
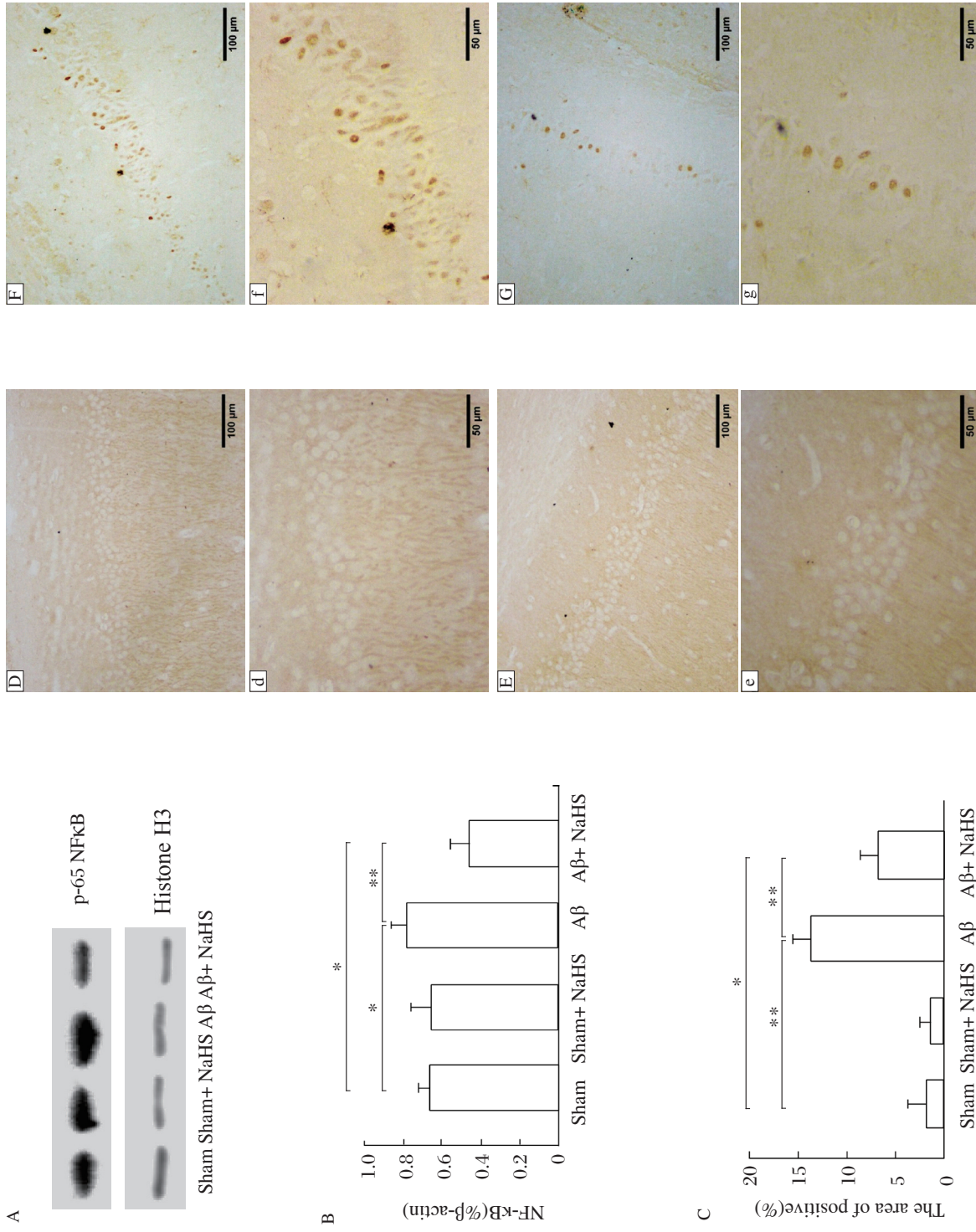
Fig. 4 Effects of NaHS on NF-κB expression in the hippocampus. Western blotting assays (A) and statistical data (B) show that NaHS reversed Aβ-induced upregulation of p65 NF-κB. NF-κB expression was also examined by immunohistochemistry. (D & d): the sham operated group; (E & e): the sham+NaHS group; (F & f): the Aβ group; and (G & g): the Aβ+NaHS group. The positive area of NF-κB expression was measured by quantitative image analysis (C) *P < 0.05, **P < 0.01.

that H_2S reduces Aβ-upregulated COX-2 expression in BV-2 microglia[13]. We thus investigated the effects of H_2S on the expression of COX-2 in Aβ1-40-induced rats. As shown in **Fig. 2A** and **2B**, Aβ1-40 significantly increased the protein expression of COX-2 in the Aβ group than that in the sham operated ($P < 0.01$), which was markedly suppressed by NaHS ($P < 0.05$, **Fig. 3A** and **3B**). The improvement was also demonstrated by immunohistochemistry (**Fig. 3C, D** & **d, E** & **e, F** & **f, G** & **g**). These findings demonstrated that NaHS in‐hibited the upregulation of COX-2 in hippocampus.

NaHS inhibited NF-κB (p65) activation in the hippocampus

To study the underlying signaling mechanism, we detected the activities of NF-κB in the hippocampus of each group ($n = 5$). NF-κB has been demonstrated to play an essential role in LPS-induced expres‐sion of both COX-2 and proinflammatory cytokine genes. Therefore, to further elucidate the underlying mechanism whereby NaHS exerted its effect in rats injected with Aβ, we examined the effects of NaHS on the transcription factor, NF-κB (p65). NF-κB activ‐ity was evaluated by the nuclear translocation of p65 subunit of NF-κB. As shown in **Fig. 4**, Aβ enhanced the nuclear translocation of p65 subunit NF-κB com‐pared with the sham operated group ($P < 0.05$, **Fig. 4 A** and **4B**). NaHS (5.6 mg/kg) treatment for 3 weeks significantly reversed Aβ-stimulated NF-κB expres‐sion ($P < 0.01$, **Fig. 4A** and **4B**) in the Aβ+NaHS group. Immunohistochemistry also displayed high levels of p65 NF-κB in the Aβ group, while NaHS suppressed the tendency (**Fig. 4C, D** & **d, E** & **e, G** & **g**). These results implied a potential role of H_2S, through the inhibition of NF-κB in suppressing the expressions of COX-2 and pro-inflammatory cytokines TNF-α, IL-1β and IL-6.

DISCUSSION

It has been proposed that Aβ1-40 aggregates play an important role in the pathogenesis of AD[18]. Numerous reports have showed that the injection of Aβ1-40 into rat hippocampus provides an effective model to mimic some of the pathologic and behavioral changes of AD[19, 20, 21]. In our study, we demonstrated that Aβ1-40 injec‐tion could induce neural injury. Although the specific causes of sporadic AD are still unknown, factors un‐der study include aging, mitochondrial defects, neuron apoptosis, oxidative stress, and neuroinflammation.[22]. LPS-induced-neuroinflammation increases intracellular accumulation of Aβ and Aβ precursor protein (PP) as well as exacerbates tau pathology in a transgenic model of AD[23,24,25]. In particular, chronic neuroinflammation

appears to be a prominent feature of AD, as supported by findings such as astrocytic and microglial activation as well as the upregulation of many proinflammatory cytokines[26,27,28]. These data further indicate that neu‐roinflammation is involved in AD.

In the brains of AD rats, the production of IL-1β and TNF-α play an important role in augmenting in‐flammatory reaction and formation of Aβ[29]. Several reports have provided evidence for a role for IL-1β in the etiology of AD based largely on the finding that IL-1β was expressed in different brain areas in AD and also in the cerebrospinal fluid of AD patients[30,31]. In the present study, we found that the levels of TNF-α, IL-1β and IL-6 were high predominantly in the hippocampus in Aβ-stimulated rats, indicating that proinflammatory cytokines were involved in the in‐flammatory response in AD rats.

It is well known that inflammatory processes asso‐ciated with increased expression of COX-2 occur in a variety of neurodegenerative diseases, including AD[32]. In normal conditions, the constitutive form of COX-2 has low expression or is undetectable in most glia cells of oligodendrocytes and microglia[33]. COX-2 expres‐sion that dramatically increases in some pathological conditions was proposed to be an inducible form of COX-2[34]. In the current study, we found that COX-2 was overexpressed in Aβ-stimulated rats compared with the sham operated rats, indicating that COX-2 was involved in the inflammatory response in AD rats.

In the present study, we tested the effect of H_2S on proinflammatory cytokine production in Aβ-stimulated rats. The dose of NaHS (5.6 mg/kg/day for 3 weeks) used in this study was similar to that for Parkinson´s disease[16], which is a neurodegenerative disease. We found that H_2S suppressed the levels of TNF-α, IL-1β and L-6 in the Aβ-stimulated rats. This is consistent with the results by Xuan[14] reported in AD rats. These indicated that the H_2S may be an ef‐fective anti-inflammatory agent. We further found that Aβ induced upregulation of COX-2, which was reversed by NaHS treatment. The study of Liu et al.[13] reported that NaHS suppressed the upregulation of protein expression of COX-2 in Aβ1-40 induced BV-2 microglial cells. These data suggest that the anti-in‐flammatory effect of H_2S contributes to its neuropro‐tective effects.

To further understand the molecular mechanisms of the effects of NaHS on the expression of inflam‐matory factors, the expression of phospho-p65 NF-κB was analyzed by Western blotting assays and immunohistochemistry. NF-κB is an essential and ubiquitous transcription factor for the expression of many inflammation-related genes, including COX-

2, TNF-α, IL-1β and IL-6. In AD brains, p65 NF-κB immunoreactivity is greater in neurons and astrocytes surrounding amyloid plaques[35, 36]. Additionally, it has been reported that Aβ stimulation leads to p65 NF-κB activation in cultured neurons and glia[37]. Corrobo–rating these findings, the present results showed that increased hippocampal concentration of inflammatory cytokines stimulated by Aβ is accompanied by phos–phorylation of p65 NF-κB. Our results also suggest that the effects of H₂S against the released levels of pro-inflammatory cytokines may include the capabil–ity of H₂S to reduce the levels of phospho-p65 NF-κB. This could be supported by previous reports that NaHS attenuates LPS-induced inflammation by inhi–bition of p65 NF-κB in rodent and rat microglia[10, 38].

In summary, we found that the interaction of Aβ can trigger NF-kB intracellular signaling cascades, transcriptional induction of downstream proinflam–matory mediators, including COX-2 and the subse–quent release of cytokines (e.g., IL-1β and TNF-α), which are associated with neuroinflammation. How–ever, this process could be inhibited in the presence of H₂S, which may downregulate the release of pro-inflammatory factors including TNF-α, IL-1β and IL-6. Our findings indicate that H₂S may have potential therapeutic value for the treatment of AD.

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