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Exogenous Hydrogen Sulfide Attenuates Cerebral Ischemia/Reperfusion Injury by Inhibiting Autophagy in Mice

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Abstract

Hydrogen Sulfide, the third gas transmitter, has been proven to be neuroprotective in cerebral ischemia injury, but whether its effect is mediated by regulating autophagy is not yet clear. The present study was undertaken to explore the underlying mechanisms of exogenous H₂S on autophagy regulation in cerebral ischemia. The effects and its connection with autophagy of NaHS, a H₂S donor, were observed through neurological deficits and cerebral infarct volume in middle cerebral artery occlusion (MCAO) mice; autophagy related proteins and autophagy complex levels in ischemia hemisphere were detected with western blot assay. Compared to model group, NaHS significantly decreased infarct volume and improved neurological deficits; Rapamycin, an autophagy activator, abolished the effect of NaHS; NaHS decreased the expression of LC3-II and up-regulated p62 expression in ischemia cortex 24 hours after ischemia. However, NaHS did not significantly influence Beclin-1 expression. H₂S has neuroprotective effect on ischemia injury in MCAO mice, this effect is associated with its influence in down-regulating autophagosomes accumulation.

Keywords

Exogenous hydrogen sulfide; MCAO; mice; autophagy; LC3-II;

INTRODUCTION

Hydrogen sulfide (H₂S), formerly known as a toxic gas with odorous smell, has been testified as an endogenous signaling transmitter that regulates cellular function (Szabó 2007) and vascular tone (Zhao et al. 2001). The previous research has suggested that H₂S provides a protective effect for cerebral injury in rodent models and cellular models (Zhang and Bian 2014). Among these data, the effect of H₂S was partially due to inducing angiogenesis around the ischemia core (Jang et al. 2014) and promoting synaptic remodeling (Pradip et al. 2015). Moreover, its effect has been demonstrated in parallel with reduced oxidative stress, inflammation and apoptosis (Yin et al. 2013).

Recently, the protective effect of H₂S has been allegedly associated with autophagy in myocardia (Jang and Xiao 2015), in spinal cord ischemia/reperfusion (I/R) (Li and Jiang 2015) and in traumatic brain injury (Zhang and Shan 2014). However, the exact connection of autophagy and H₂S in cerebral ischemia is not yet clear.

Autophagy is a highly conserved process, by which dysfunctional organelles and selected proteins are degraded in a sequestered double-membrane vesicle known as autophagosomes (Wong et al. 2013). Under ischemia insults, energy depletion and metabolic disorder contributes to the activation of energy sensing kinases like MTOR and AMPK, resulting to the activation of autophagy (Ai and Yue 2014). It requires several molecular complexes in the process of autophagosome formation, including the ULK1 kinase complex, the beclin-1-VPS34-AMBRA1 complex, Atg12-Atg5-Atg16L complex, effector proteins like WIPI-1 and-2, and binding proteins like LC3 and p62 (Nazio et al. 2013). Among these, Beclin-1-VPS34-AMBRA1 complex, known as Beclin-1 complex, is usually considered as a vital participator in the initiation of starvation-induced autophagy. In this study, we aimed to investigate the elementary regulation of H₂S on autophagy activity in cerebral ischemia and the influence of H₂S on Beclin-1 complex.

MATERIALS AND METHODS

Chemicals and antibodies

H₂S donor, NaHS (Sodium hydrosulfide, 161527) was purchased from Sigma-Aldrich. Anti- β -actin (sc-47778, 1:1,000), Beclin-1 (sc-11427, 1:1,000) antibodies were purchased from Santa Cruz. Anti-LC3B/MAP1LC3B (NB600-1384, 1:1,000) antibody was purchased from Novus Biologicals. Anti-p62 (PM-045, 1:1,000) antibody and positive control for anti-LC3 antibody (PM036-PN) were purchased from MBL. Peroxidase-conjugated affinipure goat anti-rabbit IgG (H+L) (ZB-2301, 1:2500) was purchased from ZSGB-BIO. Goat-anti-mouse IgG (IS001, 1:10,000) was purchased from M&C Gene Technology. 3-MA (369172) and rapamycin (948477) were purchased

from J&K.

Animals

Male KM mice (22g-25g), were purchased from Laboratory Animal Center, Peking University Health Science Center. The experimental procedures and animals care administration were performed in accordance with the guidelines of Care and Use of Laboratory Animals by Beijing Committee on Animal Care and Use. The experiments were approved by the Committee on the Ethics of Animal Experiments of the Peking University Health Science Center.

MCAO model in mice

The procedures of MCAO model were adopted as previously described (Hara et al. 1996). Briefly, mice were anesthetized with chloral hydrate (500 mg/kg, ip). The body temperature was sustained at 37 °C with a heating surgery table. The right common carotid artery was exposed by a midline incision. Afterwards, right common carotid artery, internal carotid artery and external carotid artery were dissociated. A round tip 6-0 monofilament nylon suture was introduced into the internal carotid artery through an incision in the external carotid artery, and gently advanced 9-10mm until the rounded tip reached the origin of the MCA. An abrupt drop in cortical perfusion (< 25% of the baseline) indicates successfully occlusion, which was monitored by the laser Doppler flowmetry. 90 minutes later, the monofilament was gently removed, and then the external carotid arteries was permanently ligated.

Intracerebral ventricular (icv) injection

The treatment protocol was selected according to previous study (Finan et al. 2016). Mice were anesthetized with 5% chloral hydrate (500 mg/kg, ip) and placed in a small animal stereotaxic instrument (ZS-B, ZSLABL). The skull was exposed by an incision along with the sagittal suture and the bregma was revealed. Intracerebroventricular injection in the right lateral ventricle was performed according to the a location of -0.3 mm anteroposterior, 1.0 mm lateral, -2.5 mm dorsoventral relative to bregma. A flat-ended syringe (TP-0003HPLCZ, GAUGE) was used to infuse the solution into the

ventricle at a speed of 0.25 μ l per minute.

Experimental groups and treatments

Experiment 1: This experiment was performed for the observation of dose-effect relationship of H₂S protective action on cerebral ischemia. Mice were randomly divided in to sham group, model group (normal saline (NS-treatment)), and NaHS-treatment group in difference doses (1 mg/kg, 2 mg/kg, 4 mg/kg, 8 mg/kg, 16 mg/kg), NS or NaHS were intraperitoneal injected at onset of ischemia.

Experiment 2: Mice were randomly divided in to sham group, model group (normal saline), NaHS (2 mg/kg) group, NaHS (2 mg/kg) + RAP (35 pmol) group, NaHS (2 mg/kg) + RAP (70 pmol) group and 3-MA (400 nmol) group. Intracerebroventricular injection of rapamycin (35 pmol or 70 pmol) was administered ten minutes before ischemia. Rapamycin was dissolved in ethanol (5 mg/ml) and diluted with normal saline (the final ethanol concentration < 2%). The 3-MA (400 nmol) or normal saline was administered ten minutes before ischemia using icv injection. 3-MA was directly dissolved in normal saline by heating in 60 °C water bath and cooled to room temperature immediately before icv injection (Gao, et al. 2012). NaHS was administered as previously described. This experiment was performed for the observation of the association of H₂S and autophagy in cerebral ischemia.

Experiment 3: Mice were randomly divided into sham group, model group and NaHS group. NaHS was administered as previously described. This experiment was conducted for western blotting analysis of autophagy associated proteins.

Determination of neurological deficit and infarct volume

Twenty-four hours after ischemia, the neurological deficit of mice in experiment 1 was evaluated with a blinded-manner using a well-established protocol (Serge et al. 2010). Mice of experiment 1 and 2 were anesthetized with chloral hydrate (500 mg/kg, ip) and transcardially perfused with cold normal saline. The brains were dissected rapidly and cut into five coronal slices, and stained with 2% of TTC for 30 minutes. Then slices were fixed in 4% PFA overnight and photographed the slices with a digital camera

(Power-Shot G12, Cannon). The white regions were defined as infarct regions. The infarct volume was calculated with Image J software (NIH Image), by measuring the white regions of each slice across the thickness of every slice. The corrected infarct volume was calculated with the formula as previous described (Jackman et al. 2011).

Western blot

Mice were anesthetized 24 h after ischemia and perfused with precooled PBS. Then cortex and striatum were respectively isolated from right hemispheres on ice and proteins were extracted from tissue samples in cold RIPA buffer supplement with 1% DTT and 1% protease inhibitor cocktail (Sigma, P8340-1ML) (Zhang et al. 2013). Samples were separated by SDS-PAGE, and blots were incubated with rabbit anti-LC3 antibody, anti-Becn1 antibody or anti-p62 antibody overnight at 4 °C, respectively. Subsequently, blots were incubated with relevant secondary antibody at room temperature for 90 minutes. Ultimately, immunoreactivity was detected by ECL kit and quantified using the Imagelab software.

Statistical analysis

All data were represented as mean $\pm$ SEM. Statistical significance among groups was measured by One-way ANOVA, followed by *Tukey's* test. $P < 0.05$ was considered as statistically significant.

RESULTS

Neuroprotective effects of H₂S in mice

A remarkable brain infarction ($45.6\% \pm 2.6\%$) and damage in motor function (7.3 ± 0.6) were resulted in MCAO model mice, 2 mg/kg NaHS significantly reduced infarct volume ($26.0\% \pm 4.4\%$) and neurological deficits (3.7 ± 0.4) (Fig. 1). 1, 4, 8 mg/kg NaHS also showed the effect of reducing infarct volume, which were $34.6\% \pm 2.2\%$, $31.2\% \pm 3.6\%$ and $33.4\% \pm 3.0\%$, respectively (Fig. 1B). Obviously, 2 mg/kg of NaHS showed the best effect, which was used in following experiments.

Dose-dependent interruption effect of rapamycin on H₂S protection

As showed in Fig. 2, compared with model group, NaHS treatment alleviated the infarct volume from $44.8\% \pm 4.2\%$ to $21.2\% \pm 2.4\%$. Subsequently, pretreatment with an autophagy inducer rapamycin, dose-dependently attenuated the protective effects of NaHS on infarct volume, which increased from $21.2\% \pm 2.4\%$ to $32.6\% \pm 3.8\%$ and $45.4\% \pm 3.0\%$, respectively (Fig. 2B). 3-MA, an autophagy inhibitor, also decreased the infarction volume to $17.8\% \pm 2.0\%$, presenting a similar effect to H_2S . These results indicated that H_2S may act as an autophagy inhibitor contributing to the reduction of infarction.

Influence of H_2S on expression of autophagy-related proteins in cortex and striatum

To further explore the mechanisms of H_2S in autophagy, we examined the expression of LC3-II (Kimura et al. 2009) and p62, the specific biomarkers on autophagic vacuole, in ischemia cortex and striatum by western blot assay. In ischemia cortex the expression of LC3-II was significant up-regulated in model group, whereas H_2S suppressed the elevation of LC3-II (Fig. 3A, 3B). p62 was significant down-regulated in model group and was reversed with H_2S treatment (Fig. 3A, 3C). Different from the results in cortex, no evident change in NaHS pretreatment was observed in striatum (Fig. 3D-3F). Thereby, we assume that the effect of H_2S in autophagy vary from one brain region to another.

On the basis that Beclin-1 complex is vital for initiation of autophagy, we detected the expression level of Beclin-1 in ischemia cortex with western blot. The protein levels of Beclin-1 were significantly increased at 24 h after ischemia. Unexpectedly, NaHS treatment did not result in significant effect on ischemia-induced up-regulation of Beclin-1 expression (Fig. 4). Collectively, these data demonstrate that the neuroprotective effect of H_2S is closely related to its inhibition of autophagy in I/R cortex.

DISCUSSION

This study showed that NaHS, a donor of H_2S , alleviated the ischemia cerebral injury through decreasing infarct volume and reducing neurobehavioral defect score in MCAO

mice (Fig. 1). These results were in accordance with the previous research (Yin et al. 2013). Most importantly, we provided the first evidence that the neuroprotection of H₂S was associated with autophagy.

We observed that the protective effect of H₂S could be suppressed by an autophagy activator, and an autophagy inhibitor, 3-MA exhibited protective effect similar to H₂S in this study (Fig. 2B). These data first demonstrated that H₂S played a cerebral protective role through autophagy regulation. 3-MA, a PtdIns3K inhibitor, blocks the formation of autophagosomes from upstream signal pathway at the early stage by inhibiting the class III PtdIns3K (Klionsky et al. 2008). Therefore we'd like to know whether H₂S influences the formation of autophagosomes. LC3-II and p62, two specific biomarkers on autophagic vacuole were determined with western blot. The result showed that H₂S reversed the elevation of LC3-II and the reduction of p62 induced by ischemia (Fig. 3A-3C), indicating that the H₂S treatment decreased autophagosomes.

However, because decreased autophagosome does not equate with decreased autophagy, the influence of H₂S on the elevation of LC3-II and the reduction of p62 may be caused either by the reduction of autophagosome synthesis or by the acceleration of autophagosome degradation (Klionsky et al. 2008). Our results showed that H₂S did not influence the expression of Beclin-1 (Fig. 4). As the activity of Beclin-1-VPS34-AMBRA1 complex may not be precisely reflected by Beclin-1 levels (Liu et al. 2016), more definitive studies would be required to determine the role of Beclin-1 complex in the regulation of autophagy pathways for H₂S. Whether H₂S regulates the activity of other autophagy-related complexes, such as ULK1 complex, or even the fusion of autophagosomes with lysosomes has been under investigation in our lab.

In this study we found that H₂S manifested different effects on autophagy in cortex and in striatum. It has been reported that hippocampus and cerebral cortex showed different autophagy response to ischemia insults and the diversity of results may be caused by different basal autophagy levels (Diego et al. 2015). Therefore, we suggested that the diverse effect of H₂S may be partly dependent on the different levels of basal autophagy

in cortex and striatum. Additionally, the previous study has demonstrated that endogenous H₂S, produced in ischemia brain, is mainly distributed in cortex and hippocampus instead of in the striatum (Zhang et al. 2013), which may also contributed to the diversity of intracerebral effect of H₂S.

In conclusion, this study demonstrates that H₂S presents the protective effect on cerebral ischemia in mice, and this effect is associated with decreasing autophagosomes accumulation in ischemia cortex.

DECLARATION OF INTERESTS

The authors report no conflicts of interest.

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Figure Legends

Fig. 1.

Neuroprotective Effect of H₂S in Mice. Mice were treated with different dose of NaHS at onset of MCAO, and were killed 24 hours after ischemia. (A) Representatives of TTC stained brain slices. (B) Quantitative analysis of cerebral infarct volume. (C) Statistical results of neurological deficits 22 hours after ischemia. Bar represents mean $\pm$ SEM.

Fig. 2.

Dose-dependent interruption effect of rapamycin on H₂S protection. Mice were intraventricular injected with different dose of rapamycin 10 minutes before ischemia in presence of NaHS or injected with 3-MA alone. (A) Representative TTC staining of brain sections. (B) Statistical analysis of brain infarct volume. Bar represents mean $\pm$ SEM.

Fig. 3.

Effect of H₂S on expression of autophagy-related proteins in cerebral Ischemia cortex and striatum. Tissues were isolated 24 hours after ischemia. (A) Representatives western blots of LC3-II and p62 in cortex. (B-C) Protein expression of LC3-II and p62 in ischemia cortex. (D) Representatives western blots of LC3-II and p62 in striatum. (E-F) Protein expression of LC3-II and p62 in ischemia striatum. Levels of β -actin protein were used as the control. Quantitative analysis was performed with Imagelab Software. Bar represents mean $\pm$ SEM.

Fig. 4.

Effect of H₂S on Beclin-1 complex. (A) Representatives western blots of Beclin-1 in ischemia cortex. (B) Quantitative Analysis of Beclin-1 expression level. Bar represents mean $\pm$ SEM.

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Fig. 3. Effect of H2S on expression of autophagy-related proteins in cerebral Ischemia cortex and striatum. Tissues were isolated 24 hours after ischemia. (A) Representatives western blots of LC3-II and p62 in cortex. (B-C) Protein expression of LC3-II and p62 in ischemia cortex. (D) Representatives western blots of LC3-II and p62 in striatum. (E-F) Protein expression of LC3-II and p62 in ischemia striatum. Levels of β-actin protein were used as the control. Quantitative analysis was performed with Imagelab Software. Bar represents mean ± SEM.

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