Original Paper

Hydrogen Sulfide Attenuates Myocardial Hypoxia-Reoxygenation Injury by Inhibiting Autophagy via mTOR Activation

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Key Words

Autophagy • mTOR • H₂S • Hypoxia-reoxygenation • Cardiomyocyte

Abstract

Background: Autophagy plays a significant role in myocardial ischemia reperfusion (IR) injury. Hydrogen sulfide (H₂S) has been demonstrated to protect cardiomyocytes against IR injury, while whether it has anti-autophagy effect has not been known. The aim of this study was to investigate whether H₂S regulates autophagy during IR injury and its possible mechanism. **Methods and Results:** The cardiomyocytes of neonatal rats were randomized into Con, hypoxia-reoxygenation (HR) and H₂S protection groups. The severity of cell injury was evaluated by cell vitality (MTT) and lactate dehydrogenase (LDH) release assays, and autophagy level was evaluated by flow cytometry and the assessment of autophagy-related gene (Atg) expression, such as that of Beclin1 and LC3-II. In response to H₂S, Beclin1 and LC3-II protein were found to be down-regulated and p-mTOR protein was found to be up-regulated, together with an increase in cell vitality and a decrease in LDH. Furthermore, to find out whether mTOR was involved in the protective effect of H₂S, rapamycin, inhibitor of mTOR, was used with or without applying NaHS and HR. It was found that rapamycin attenuated the cardiomyocyte protective effect of H₂S. To demonstrate the effect of autophagy during HR injury, the cardiomyocytes were pre-treated with 3-MA, which is an autophagy inhibitor, cell injury was attenuated by 3-MA. **Conclusions:** H₂S plays a myocardial protective role against IR injury by regulating autophagy via mTOR activation.

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Introduction

Autophagy is a key mechanism for the maintenance of cellular homeostasis, which has been associated with the degradation of damaged or unnecessary proteins and organelles [1]. Excessive autophagy may promote cell death, which is referred to as autophagic cell death or type II programmed cell death, together with apoptosis and necrosis [2]. There are 3 main autophagic pathways, namely macroautophagy, microautophagy, and chaperone-mediated autophagy. Unless otherwise specified, the term “autophagy” refers to macroautophagy [3]. Autophagy exists at low levels under physiological conditions but is up-regulated in response to stress, such as nutrient deprivation, hypoxia, ischemia-reperfusion (IR), mitochondrial dysfunction, and infection [4]. Autophagy provides protection in times of ischemia but can be detrimental during reperfusion [5]. Thus, we hypothesized that it could be beneficial for cardiomyocytes to attenuate autophagy induced by IR.

Hydrogen sulfide (H₂S) is recognized as an important signaling molecule in mammalian systems, as nitric oxide (NO) and carbon monoxide (CO) [6]. It is endogenously synthesized by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) [7, 8]. In the heart, little CBS is present, whereas CSE is plentiful. It has been demonstrated that H₂S protects the myocardium against IR injury by inhibiting apoptosis via a mechanism that involves PI3K/Akt [9, 10]. However, it is also known that autophagy may induce cell death when apoptosis is inhibited [3].

The present study was undertaken to determine whether H₂S protects the myocardium against IR injury by inhibiting autophagy in a dose-dependent manner. Our study shows that the inhibition of autophagy induced by H₂S is mainly dependent on mTOR activation.

Material and Methods

Animal care

All animal experiments were approved by the Animal Research Ethics Committee of the Second Military Medical University, Shanghai, China, and conformed to the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health.

Cell culture and experimental protocols

Neonatal cardiomyocytes were prepared from the heart of SD rats less than 3 days old [11]. On the 4th day, the cardiomyocytes were divided to three groups: the control group (Con), in which the cells were cultured in DMEM in 5% CO₂ and 95% air for 24 h; the hypoxia-reoxygenation (HR) group, in which the cells were cultured in 1% O₂, 5% CO₂ and 94% N₂ atmosphere for 24 h, and 5% CO₂ and 95% air for 6 h; and the H₂S group, in which the cells were first treated with different concentrations of NaHS (10 μM, 30 μM, 50 μM, 100 μM) 30 min before HR and then followed the same protocol as the HR group. To determine whether mammalian target of rapamycin (mTOR) was involved in the protective effect of H₂S, different doses of rapamycin (20 nM, 50 nM, 100 nM), an inhibitor of mTOR, were added 30 min before NaHS. To evaluate whether autophagy promoted cardiomyocyte death, the cardiomyocytes were treated with different doses (1 mM, 2 mM, 5 mM) of 3-methyladenine (3-MA), an autophagy inhibitor [5], 30 min before HR. Cell vitality and LDH release were measured after HR.

MTT assay

The MTT method was used to evaluate cardiomyocyte vitality. The absorbance of the purple solution was determined at 450 nm using a microtiter plate reader (Bio-Rad, USA).

Counting of autophagic vacuoles

Cardiomyocytes (1 × 10⁴ cells per well) were incubated in 50 μM monodansylcadaverine (MDC, Sigma) at 37°C for 1 h. Cells were then washed 3 times with PBS at 37°C and fixed in 4% paraformaldehyde for 30 min. After fixation, cells were washed 3 times with PBS. Autophagic vacuoles were analyzed by flow cytometry in the presence of Indo-1 (blue) dye with excitation laser set at 355 nm and detection of the fluorescence intensity at 488 nm.

Quantitative real-time RT-PCR

The total RNA of cardiomyocytes was extracted using TRIzol reagent and reverse transcribed according to the manufacturer's instructions (Fermentas, CA). The annealing temperature of β -actin and Beclin1 was set at 58°C. The relative expression of both target (Beclin1, Atg5) and housekeeping (β -actin) genes was determined using the threshold cycle (Ct) method based on the $2^{-\Delta\Delta C_t}$ equation. The following sense and antisense primers were used: Beclin1 (accession number NM_001034117): 5'-GGCAGTGGCTCCTATT-3' and 5'-GGCGTGTGTGCTCTGAAAA-3', Atg5 (accession number NM_001014250): 5'-AGTGGAGGCAACAGAACC-3' and 5'-GACACGAACCTGGCACATT-3'.

Western blotting

Protein concentration was determined using the BCA protein assay kit according the manufacturer's protocol. Equal amounts of protein (50 μ g) from the cardiomyocytes were subjected to Western blot analysis to evaluate the expression of LC3 (Sigma), mTOR, and p-mTOR(Ser-2448) (Cell Signaling Technology Inc, USA) using the ECL detection kit (Amersham Biosciences, Piscataway, NJ). The autophagy results are presented as a ratio of LC3- II / LC3-I.

In vivo rat model and experimental protocols

SD rats (250-300 g) were anesthetized with 10% chloral hydrate (300 mg/kg, i.p.) before endotracheal intubation. IR was induced by ligating the left anterior descending artery (LAD) as previously reported [12]. Thirty rats were randomized to five groups: the control group, in which the rats underwent thoracotomy without ligation; the IR group, in which the rats were subjected to ischemia for 30 min and reperfusion for 3 h; the 10 μ M NaHS group, in which the rats were treated with 10 μ M /kg(body weight) NaHS by intraperitoneal injection 30 min before IR, and then followed the same protocol as the IR group; the 30 μ M NaHS group, in which the rats were treated with 30 μ M /kg NaHS by intraperitoneal injection 30 min before IR, and then followed the same protocol as the IR group; the 100 μ M NaHS group, in which the rats were treated with 100 μ M /kg NaHS by intraperitoneal injection 30 min before IR, and then followed the same protocol as the IR group.

Statistical analysis

Quantitative data are presented as the mean $\pm$ standard error (SEM). Statistical significance was determined by one-way ANOVA. Significance was established at the $P < 0.05$ level.

Results

Cardiomyocytes were injured by HR, but H₂S attenuated cell injury in vitro

Cardiomyocyte viability was decreased and LDH release was increased after HR. To evaluate the protective effect of H₂S, cardiomyocytes were treated with H₂S before HR. Compared with the HR group, cell vitality was increased and LDH release was decreased after HR. These effects were concentration dependent (Fig. 1).

Cardiomyocyte autophagy induced by HR was attenuated by H₂S in vitro

To assess the autophagy ratio of myocytes, the amount of autophagosome was measured by flow cytometry. It was found that autophagy induced by HR was attenuated by H₂S (Fig. 2). Furthermore, to evaluate cardiomyocyte autophagy, Beclin1 mRNA levels were determined by RT-PCR and the levels of Beclin1 and LC3 proteins, autophagosome markers, was determined by western blotting. The ratio of LC3- II / I was used to measure the relative autophagy level of cardiomyocytes. Beclin1 mRNA, Beclin1 protein and the ratio of LC3- II / I were found to be up-regulated by HR but down-regulated by H₂S. (Fig. 3).

H₂S inhibited cardiomyocyte autophagy via mTOR activation

To determine the mechanism of the anti-autophagic effect of H₂S, we evaluated the protein expression of p-mTOR(Ser-2448). It was found that H₂S activated mTOR by up-regulating p-mTOR protein phosphorylation, but the level of mTOR protein was not dysregulated. To examine the effect of mTOR on cardiomyocytes, the cells were pre-treated

Fig. 1. Results of LDH in the cell culture serum and cardiomyocytes vitality measured with MTT method (n=4). It was found that LDH was increased and cell vitality was decreased after HR, while compared with HR group, the injury induced by HR was attenuated by H₂S (n=4). (A) LDH level of culture serum (from left to right and the follows were the same. *P=0.000, 0.043, 0.011, 0.000 vs Con, # P=0.000, 0.001 vs HR); (B) Cardiomyocytes vitality measured with MTT method (*P=0.000, 0.000, 0.002, 0.000, 0.000 vs Con, # P=0.009, 0.000, 0.000, 0.004 vs HR).

Fig. 2. Results of cardiomyocyte autophagy ratio measured with flow cytometry system (n=5). It was found that cardiomyocyte autophagy ratio was increased after HR, and was decreased by H₂S. It was concentration dependent. (*P=0.000, 0.000, 0.017, 0.000 vs Con, # P=0.006, 0.000, 0.000 vs HR).

with rapamycin, a known inhibitor of mTOR, with or without HR. The ratio of LC3- II / I was increased by rapamycin, together with an increase in LDH release and a decrease in cell

Fig. 3. Results of Atg mRNA and protein expression of cardiomyocyte (n=5). It was found that Beclin1 mRNA and protein were up-regulated after HR, together with LC3-II / I ratio increased. While NaHS pre-treatment could down-regulated Beclin1 expression and LC3- II / I ratio. (A) Result of Beclin1 mRNA expression (*P=0.002, 0.013, 0.002 vs Con, # P=0.002, 0.031 vs HR). (B) Result of Beclin1 protein expression (*P=0.000, 0.012, 0.014 vs Con, # P=0.002, 0.013, 0.047 vs HR). (C) Results of LC3- I and LC3- II protein expression, and the ratio of LC3- II / I was calculated to demonstrate the autophagy level. (*P=0.000, 0.000, 0.016, 0.004, 0.000 vs Con, # P=0.033, 0.000, 0.000 vs HR).

viability. Furthermore, to demonstrate whether H₂S inhibited cardiomyocyte autophagy via mTOR, the cardiomyocytes were treated with rapamycin 30 min before NaHS pre-treatment and HR. In that experiment, LDH was increased, and cell viability was decreased, compared with the H₂S group. In addition, the ratio of LC3- II / I was increased by rapamycin, which attenuated the inhibitory effect of H₂S on autophagy (Fig. 4).

Cardiomyocyte injury was attenuated by inhibiting autophagy with 3-MA

To determine whether autophagy was one type of cardiomyocyte death induced by HR, the cardiomyocytes were treated with 3-MA 30 min before HR. Cell vitality and LDH release were measured after HR. It was found that cell vitality was increased and LDH release was decreased by 3-MA in comparison to the HR group (Fig. 5).

LC3-II protein, Atg5 and Beclin1 mRNA were down-regulated by H₂S in vivo

It was found that Atg5 and Beclin1 mRNA, which were up-regulated by IR, were down-regulated by H₂S pre-treatment. The ratio of LC3- II / LC3- I, which was increased in IR group, was also decreased in the H₂S pre-treatment groups; the ratio was optimal in the 30 μM/kg group (Fig. 6).

Fig. 4. Results of mTOR promoted autophagy and cardiomyocyte injury. (A) p-mTOR (ser2481) protein was up-regulated by H₂S, while mTOR protein was unchanged. Comparing with Con group, p-mTOR was down-regulated in HR group. But it was up-regulated by NaHS pre-treatment before HR (*P=0.020, 0.009 vs Con, # P=0.001, 0.000, 0.003, 0.001 vs HR). (B) Rapamycin promoted myocardiocytes injury. It was found that cell vitality was decreased after rapamycin treatment with or without HR (*P=0.018, 0.006, 0.001, 0.000, 0.000, 0.000, 0.000 vs Con). But there was no difference between HR and rapamycin pre-treatment groups. (C) LDH of serum was increased after rapamycin treatment with or without HR (*P=0.007, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000 vs Con). And comparing with HR group, LDH was more increased after HR with rapamycin pre-treatment (# P=0.009, 0.000, 0.000 vs HR). (D) Myocardiocytes autophagy was induced by rapamycin. (*P=0.048, 0.002, 0.000, 0.002, 0.014, 0.001, 0.000 vs Con, # P=0.021 vs HR) (E) To find whether H₂S's myocardium protection was mediated by mTOR activation, the cell was pretreatment by rapamycin 30min before NaHS, followed by HR. it was found that cell vitality was decreased by rapamycin comparing with H₂S group. (*P=0.000, 0.003, 0.001, 0.000 vs Con, # P=0.016 vs HR, ▲P=0.028 vs H₂S+HR) (F) LDH was increased by rapamycin comparing with H₂S group. (*P=0.000, 0.000, 0.000, 0.000, 0.000, 0.000 vs Con, # P=0.000, 0.000, 0.000, 0.000, 0.000 vs HR, ▲P=0.003, 0.000 vs H₂S+HR) (G) Anti-autophagy effect of H₂S was attenuated by rapamycin. Comparing with H₂S group, ratio of LC3- II / I was increased by rapamycin (*P=0.001, 0.006, 0.009, 0.000 vs Con, # P=0.032 vs HR, ▲P=0.006 vs H₂S+HR).

Fig. 5. Results of cardiomyocytes protection induced by 3-MA during HR. It was found that cell vitality was increased and LDH was decreased in 3-MA groups comparing with HR group. (A) Result of cardiomyocyte vitality measured with MTT (*P=0.000, 0.000, 0.000, 0.000 vs Con, # P=0.004 vs HR). (B) Result of LDH of serum in different groups. (*P=0.000, 0.000, 0.000, 0.000 vs Con, # P=0.008, 0.001, 0.000 vs HR, ▲ P=0.001, 0.002 vs 5mM 3-MA+HR).

Fig. 6. Results of LC3-II protein, *Atg5* and *Beclin1* mRNA expression of myocardium *in vivo* (n=6). It was found that *Atg5* and *Beclin1* mRNA and protein were up-regulated after IR, together with LC3-II / I ratio increased. While NaHS pre-treatment could down-regulated *Atg5* and *Beclin1* mRNA expression and LC3-II / I ratio. (A) Result of *Atg5* mRNA expression (*P=0.009, 0.03 vs Con, ▲ P=0.019 vs IR). (B) Result of *Beclin1* mRNA expression. (*P=0.027 vs Con, ▲ P=0.03 vs IR) (C) Results of LC3- I and LC3- II protein expression, and the ratio of LC3- II / I was calculated to demonstrate the autophagy level. (*P=0.002, 0.023 vs Con, # P=0.005, 0.015 vs IR).

Discussion

Autophagy has been demonstrated to be involved in many physiological and pathological processes. When autophagy destroys the cytosol and organelles beyond a certain threshold,

Fig. 7. The signal transduction mechanism of autophagy regulated by H₂S.

autophagic cell death will occur [13, 14]. Excessive autophagy can be stimulated by nutrient starvation and growth factor deprivation when cells are unable to take up external nutrients [15]. Furthermore, autophagy can induce cell death when apoptosis is inhibited because the early stages of autophagy are required for apoptosis [3]. Under the condition of cardiac ischemia reperfusion injury (IR injury), the process of autophagy is activated in response to energy crisis and oxidative stress [1]. Although autophagy is protective during ischemia, it is detrimental during reperfusion followed by heart failure, as demonstrated by Matsui et al. [16].

The autophagy process is regulated by the autophagy-related genes (Atg), among which Beclin1 is needed for the autophagy vesicle nucleation step of autophagy [17]. In the mouse heart, the expression of Beclin 1 in the area at risk was only slightly up-regulated by ischemia alone, but its expression was dramatically enhanced after IR. While autophagosomes were significantly attenuated in *beclin 1*^{-/-} mice, the size of myocardial infarction or the area at risk after IR was also significantly smaller [16].

The formation of autophagosomes occurs via two pathways, the Atg12-Atg5-Atg16 pathway and the Atg4-Atg7-Atg3 pathway. Conjugations led to the conversion of the soluble form of LC3 (LC3-I) to the autophagic vesicle-associated form (LC3-II), which is used as a marker of autophagy [18]. The ratio of LC3-II/LC3-I was used to evaluate the autophagy level in most studies [16, 19]. In our study, the autophagy level was determined by flow cytometry. It was found that the autophagy ratio was increased after HR, together with cell injury. Beclin1 and LC3-II, the autophagosome marker, were also up-regulated by HR. To demonstrate whether autophagy was detrimental during HR injury, 3-MA, which is the special inhibitor of autophagy, was used before HR. Cardiomyocyte vitality was found to be increased and LDH was found to be decreased by 3-MA. Therefore, the inhibition of autophagy may attenuate cardiac HR injury.

Recently, H₂S, a gasotransmitter, has attracted wide attention because of its protective effect of brain, liver, kidney, heart, lung and other organs [20-24]. Many studies have demonstrated the cytoprotective effects of H₂S in myocardial IR injury [25, 26]. However, whether it could attenuate cardiomyocyte autophagy has not been reported. We found that beclin1 and LC3-II were down-regulated by H₂S, together with a decrease in the autophagy ratio after HR. Furthermore, the cardiomyocyte vitality was increased and LDH release was decreased by H₂S during HR. These findings suggest that H₂S could attenuate not only apoptosis but also autophagy induced by IR. The same results were also found in heart injuries in rats inhaling tobacco smoke [7]. The anti-autophagy mechanism of H₂S is still uncertain.

Mammalian target of rapamycin (mTOR), the downstream effector of PI3K/Akt, plays a key role in autophagy regulation [27, 28]. mTOR negatively regulates autophagy [29], and autophagy would be activated by inhibiting mTOR activation [30, 31]. In the present study, the LC3-II protein, which was induced by HR, was down-regulated by H₂S *in vitro*. This was associated with an up-regulation of p-mTOR protein. To demonstrate whether H₂S inhibited cardiomyocyte autophagy induced by HR by activating the mTOR system,

rapamycin, a specific mTOR inhibitor, was used with or without HR. The ratio of LC3-II/LC3-I was found to be increased, together with a decrease in cell vitality and an increase in LDH release. These results indicate that both autophagy and cell injury could be induced by the inhibition of mTOR. Furthermore, the treatment of cardiomyocytes with rapamycin 30 min before NaHS followed by HR attenuated the anti-autophagy and cell protective effects of H₂S. These observations suggested that H₂S might protect cardiomyocytes against HR injury by inhibiting autophagy through the activation of the Akt/mTOR pathway.

Conclusion

In summary, our study demonstrated that H₂S played an important and protective role in cardiomyocytes by regulating autophagy against HR injury via the mTOR system (Fig. 7). Autophagy can spare cell death, or at least postpone it, because auto-digestion provides an alternative source of intracellular nutrients and clearance of destructive organelles in ischemia phase; but once autophagy over expressed, it might phagocytize more organelles and promote cell death. However, the exact function of autophagy in HR may be more complex.

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Disclosure Statement

The authors declare no conflict of interest.

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