Original Article
PTEN/PI3K/Akt pathway is involved in the protection of hydrogen sulfide against myocardium ischemic/reperfusion injury

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Abstract: Myocardial ischemia-reperfusion injury (MIRI) is one of the important reasons for the high mortality of ischemic heart disease in patients who have undergone a blood supply reconstruction. Hydrogen sulfide (H₂S) has been thought of to be just a toxic gas with a strong odor of rotten eggs for hundreds of years, but it has been demonstrated that H₂S plays a protective role in the pathogenesis and development of heart diseases, especially in MIRI. In the present study, we treated rats with H₂S after MIRI and then observed its effects on heart function parameters following in situ ischemia/reperfusion. Multiple oxidative products, myeloperoxidase and proinflammatory cytokines in the myocardium were measured to evaluate the anti-oxidative and anti-inflammatory effect of H₂S. The role of phosphatase and tensin homologue (PTEN)/PI3K/Akt pathway was examined to explore the underlying mechanism. The treatment of H₂S for seven consecutive days exhibited dramatic improvement in cardiac function, as manifested by increased LVSP and ± (dP/dt) max, and decreased LVDP. PTEN mRNA and expression were decreased by H₂S. PI3K/Akt pathway was activated by H₂S and PTEN inhibitor bisperoxovanadium (Phen) but de-activated by PI3K inhibitor LY294002. The heart function was enhanced by Phen but worsened by LY294002, suggesting the involvement of PTEN/PI3K/Akt pathway. These results revealed that H₂S may exert its cardioprotection by inactivation of PTEN, which in turn activates the PI3K/Akt signaling and increases survival in the MIRI model.

Keywords: Myocardium ischemic/reperfusion injury, hydrogen sulfide, phosphatase and tensin homologue, PI3K/Akt pathway

Introduction

Despite the rapid developments in medical care, acute myocardial infarction remains a major cause of morbidity and mortality throughout the modern world. Blood supply reconstruction, represented by PCI and thrombolysis, is still the most effective treatment of ischemic heart disease. However, data from large clinical trials show that mortality of ischemic heart disease remains high in patients who have undergone a blood supply reconstruction, and that 5-6% of patients died of complications within one month after blood supply reconstruction [1]. The proportion of patients who develop into long-term heart failure is nearly 25% [2]. Myocardial ischemia reperfusion injury (MIRI) is one of the important reasons. Reperfusion injury is initiated when blood flow returns to the ischemic tissue [3], and involves many cell injury pathways, such as membrane destabilization, intracellular calcium dysregulation, free radical production, mitochondrial injury and pro-apoptotic pathway activation [4]. Hydrogen sulfide (H₂S) has been thought of to be a toxic gas with a strong odor of rotten eggs for hundreds of years. Since in 1996 when Abe and Kimura discovered that H₂S facilitated the induction of hippocampal long-term potentiation [5], H₂S has been regarded as a novel gaseous signaling molecule, which may be involved in a multitude of pathophysiologic processes, such as oxidative stress, inflammation, apoptosis, and angiogenesis [6]. In recent years, it has been showed that H₂S plays a protective role in the pathogenesis and development of heart diseases [7].

For ischemia reperfusion (I/R) injury, a current research focus is reperfusion injury salvage kinase (RISK) [8], a group of signal pathways that can reduce cell death in reperfusion injury.
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[9]. Their activation can reduce the infarct size, increase myocardial contractility and improve local blood supply recovery. PI3K/Akt pathway is a representative one of RISK. It is associated with cell survival and inhibits the proapoptotic gene Bad and activates NF-kB [10, 11], then leads to transcription of downstream survival-promoting genes and reduces cell apoptosis. It also reduces beclin-1 (a regulator that activates autophagy) [12] and activates mammalian target of rapamycin (mTOR), thus inhibiting autophagy. In addition, activation of Akt can inhibit Caspase 3/9 [13] and GSK-3β [14], enhance the activity of endothelial nitric oxide synthase [14] and murine double minute 2 [15]. The gene of phosphatase and tensin homologue (PTEN), a natural inhibitor of PI3K, participates in growth, apoptosis, adhesion, invasion, and migration [16]. It has been reported that PTEN increases apoptosis in cardiomyocytes, whereas the inactivation of PTEN activates the Akt signaling, reduces apoptosis, and increases survival [17, 18]. The role of PTEN/PI3K/Akt pathway in the cardioprotection of H$_2$S needs clarification.

Hence, the present work was performed to explore the protective effect of H$_2$S against MIRI and its underlying mechanism. We treated rats with H$_2$S after MIRI and then observed its effects on heart function parameters following in situ I/R. Multiple oxidative products, myeloperoxidase (MPO) and proinflammatory cytokines in the myocardium were measured to evaluate the anti-oxidative and anti-inflammatory effect of H$_2$S. Next, the role of PTEN/PI3K/Akt pathway was further examined to explore the underlying mechanism of the protective effects of H$_2$S.

Materials and methods

Animals

Male Wistar rats, weighing from 200 g to 220 g, were purchased from the Beijing Laboratory Animal Center (Beijing, China) and used in this study. The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Edition. The animals were housed in the Beijing Anzhen hospital animal center, under conditions of 22°C-25°C temperature, 38%-45% relative humidity, and 12-/12-hour light/dark cycles, with free access to water and food. All the protocols were approved by the institutional animal care and use committee of the Capital Medical University.

Experimental protocol

In the first part of study, rats were randomly assigned to four groups: Sham, MIRI, MIRI+H$_2$S and H$_2$S. In the second part of study, rats were randomly assigned to five groups: Sham, MIRI, MIRI+H$_2$S, MIRI+H$_2$S+bisperoxovanadium (Phen), and MIRI+H$_2$S+LY294002. Rats in the Sham group received a sham myocardial I/R surgery. Rats in the MIRI group received a myocardial I/R surgery. Rats in the MIRI+H$_2$S group received a myocardial I/R surgery, followed by low concentration H$_2$S inhalation 8 hour/day for 7 days. Rats in the H$_2$S group received only low concentration H$_2$S inhalation 8 hour/day for 7 days. There were 18 rat in each group: 10 rats were used for functional studies; 8 rats were used for biomarker measurements. PTEN inhibitor Phen was purchased from EMD Chemicals (Gibbstown, NJ, United States) and intraperitoneal injected at a dose of 0.2 mg/kg/day during H$_2$S exposure. PI3K inhibitor LY294002 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and intraperitoneal injected at a dose of 100 mg/kg/day during H$_2$S exposure.

Myocardial I/R procedure

The myocardial I/R procedure was similar to Yin et al [19]. Briefly, rats were anesthetized by intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg). A middle cervical incision was made to expose the trachea, then a section of tubing was passed through the exposed trachea until the tip was 3 mm below the larynx. After the pericardium was opened, the left coronary artery was located and a 3-0 silk black braided suture was inserted around the artery near its origin. A snare was created by passing both ends of the suture through the tip of an angiocatheter that could then be tightened and released by sliding a Voss clip down the angiocatheter. After 60 minutes of ischemia, the occlusive snare was released for reperfusion. Sham rats underwent the same surgical procedures except that the suture was not snared.

Hydrogen sulfide inhalation

Rats were kept in a 30-L plastic chamber and breathed air alone or air mixed with H$_2$S at 40 ppm for 8 h each day for 7 days. The inhalation procedure was previously described by Kida et al [20]. Rats breathed H$_2$S from 10 AM to 6 PM on each of 7 consecutive days after they received the myocardial I/R surgery. The H$_2$S
gas/air mixture was flowing through the chamber to obtain a stable gas concentration (40 ppm).

**Surgical procedures for evaluating hemodynamic parameters**

The measurement of hemodynamic parameters was similar to Yin et al [19]. After the seven days of H\textsubscript{2}S treatment was finished, rats were anesthetized, a small incision was made on the right side of the neck. The external right carotid artery was exposed and a microtip pressure transducer catheter (Millar Instruments, Houston, USA) was inserted into the artery. The proximal end of the catheter was connected to an electrostatic chart recorder (Gould, Cleveland, USA). The inserted tip of this catheter was then advanced until it reached the left ventricular lumen. The left ventricular systolic pressure (LVSP), left ventricular developed pressure (LVDP), and ± ventricular contractility (dP/dt max) measurements were obtained from the left ventricular pressure (LVP) signal.

**Measurement of oxidative products, MPO and proinflammatory cytokines in myocardial tissues**

Transmural tissue from the area at risk was homogenized in 2 mL of 10 mM phosphate buffer (pH 7.4). After centrifugation at 10,000 g for 30 min, MDA and protein carbonyl content determined using commercial kits (Beyotime Biotechnology, Jiangsu, China). All procedures were performed according to the manufacturer’s instructions. For the 8-OHdG assay, DNA was extracted from the tissue using a DNA Extraction Kit (Wako Chemical, Osaka, Japan), then added to plate wells pre-coated with anti-8-OHdG antibody (Nikken SEIL Co., Fukuuroi, Japan) and incubated for 45 min at 37°C. The wells were then sequentially treated with IgG, Streptavidin-Horseradish Peroxidase, and 3, 3',5,5'-tetramethylbenzidine. After incubation for 15 min, the reaction was terminated by sulfuric acid and the absorbance was read at 450 nm. MPO, proinflammatory cytokines TNF-α, and IL-1β levels were measured using ELISA kits, according to the manufacturer’s instructions (Sigma, St. Louis, USA). Absorbance was measured with a microplate reader to obtain the MPO, TNF-α, and IL-1β levels. The protein concentration was determined using a standard BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China).

**Real-time PCR of PTEN**

RT-qPCR was performed with the CFX Connect Real-Time system (Bio-Rad, USA) using a SYBR Green PrimeScript RT kit (TaKaRa, Japan) based on the manufacturer’s instructions. The PCR conditions included predenaturation at 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 10 s and combined annealing/extension at 58°C for 30 s. The mRNA expression levels were calculated based on the comparative quantification method (2\textsuperscript{-ΔΔCT}). β-actin were used as internal controls for PTEN mRNA quantitation.

**Western blot**

Cardiac tissues were harvested and washed in ice-cold saline, and then homogenized in RIPA lysis buffer (25 mg/ml) with 1 mM phenylmethylsulfonyl fluoride on ice. All samples were centrifuged at 3000xg at 4°C for 15 min and the supernatants were collected. The protein concentration was determined using a standard BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts (50 μg) of proteins were separated and trans-
ferred to a polyvinyl difluoride membrane. After blocking with 5% nonfat dried milk, the membranes were probed with antibodies PTEN, pAkt, Akt and β-actin and incubated with either horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (1:5000, ThermoFisher Scientific, Shanghai, China). Immunoreactive proteins were visualized and then scanned. The densitometry was performed to quantify the expression using Bio-Rad Quantity One 4.4.0 software (Bio-Rad, Hercules, CA, USA).

**Statistical analysis**

The statistical analysis was performed using SPSS 17.0 with one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc test. P<0.05 was considered significant.

**Results**

**Changes in cardiac function by H$_2$S**

Figure 1A-D shows the significant changes caused by MIRI and H$_2$S on hemodynamic parameters. MIRI treatment significantly increased LVDP, and decreased LVSP and ± (dP/dt)$_{max}$ (P<0.05 compared to the Sham group). The LVDP values in the MIRI+H$_2$S group was lower than that in the MIRI group (P<0.05), while the LVSP and ± (dP/dt)$_{max}$ values were higher than those in the MIRI group (P<0.05). The treatment of H$_2$S alone had no effect on cardiac function compared to Sham.

**Changes in oxidative products and pro-inflammatory cytokines in myocardial tissues by H$_2$S**

As shown in Figure 2A-C, myocardial oxidative products (protein carbonyl, 8-OHdG and MDA) assays demonstrated that MIRI caused significant oxidative stress, but H$_2$S could significantly mitigate the oxidative stress, indicated by the dramatic decreases in the protein carbonyl, 8-OHdG and MDA levels compared to the MIRI group (P<0.05). As shown in Figure 2D-F, MIRI caused significant increases in myocardial TNF-α, IL-1β and MPO levels (P<0.05 compared to the Sham group), which were inhibited by H$_2$S treatment, as shown by the dramatic decreases in the MPO, TNF-α, and IL-1β levels (P<0.05 compared to the MIRI group). The treatment of H$_2$S alone had no effect on oxidative products, MPO and proinflammatory cytokines compared to Sham.

**Figure 2.** Changes in oxidative products, MPO and proinflammatory cytokines in myocardial tissues. It shows a significant increase in myocardial oxidative products (protein carbonyl, 8-OHdG and MDA), MPO and proinflammatory cytokines (TNF-α, and IL-1β) by MIRI and a decrease of them by H$_2$S. MIRI: myocardium ischemic/reperfusion injury; 8-OHdG: 8-hydroxy-2-deoxyguanosine; MDA: Malondialdehyde; MPO: myeloperoxidase; TNFα: Tumor necrosis factor; IL-1β: Interleukine-1 beta. Values are expressed as Mean ± SEM. #: P<0.05 compared to Sham; §: P<0.05 compared to MIRI.
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Changes in PTEN mRNA and PTEN/PI3K/Akt pathway activation

We first measured the mRNA in the presence of H\textsubscript{2}S, PTEN inhibitor Phen or PI3K/Akt pathway inhibitor LY294002. As shown in Figure 3A, PTEN mRNA was increased by MIRI but decreased by H\textsubscript{2}S; it was not changed by Phen or LY294002. Figure 3B-D show the protein expression of PTEN and the activation of Akt. PTEN expression was enhanced by MIRI but decreased by H\textsubscript{2}S and Phen, while LY294002 caused no change in PTEN expression. Akt was activated by H\textsubscript{2}S and Phen, while de-activated by LY294002.

Changes in cardiac function by inhibitors of PTEN/PI3K/Akt pathway

To evaluate the roles of PTEN/PI3K/Akt pathway in the protection mediated by H\textsubscript{2}S, we treated rats with inhibitors of PTEN/PI3K/Akt pathway and then examined the changes in cardiac function. As shown in Figure 4A-D, the LVDP was decreased by Phen but increased by LY294002 compared to the MIRI+H\textsubscript{2}S groups (P<0.05). By contrast, LVSP and ± (dP/dt) max were increased by Phen but decreased by LY294002 compared to MIRI+H\textsubscript{2}S (P<0.05).

Discussion

With the advancement of scientific technology in recent years, researchers have discovered that H\textsubscript{2}S can be produced by cystathionine-γ-lyase (CSE) in the cardiovascular system and be an endogenous gaseous mediator exerting pronounced physiological effects as the third gasotransmitter in addition to nitric oxide (NO) and carbon monoxide (CO). Accumulating evidence indicated that exogenous H\textsubscript{2}S could mediate the cardioprotective effects in myocardial ischemia model. However, the underlying mechanism still needs clarification.

Some investigators have explored the effects of H\textsubscript{2}S on MIRI, but the mechanism remains controversial. The cardioprotection induced by H\textsubscript{2}S have been studied by others, mainly on H\textsubscript{2}S preconditioning. It was found that H\textsubscript{2}S precon-
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The PTEN/PI3K/Akt pathway is involved in the protection of H₂S against MIRI conditions. The db/db diabetic mouse heart is protected against I/R injury by activating Nrf2 signaling in an Erk-dependent manner. Kang et al. reported the involvement of miR-1 in the protective effect of H₂S against cardiomyocyte apoptosis induced by I/R. A study by Pan found that H₂S preconditioning protected the heart against I/R insults by PKC activation. H₂S post-conditioning protected isolated rat hearts against I/R injury via activation of the JAK2/STAT3 signaling pathway. It is also found that H₂S infusion reduced myocardial infarct size and improved regional left ventricular function in an I/R model by suppressing cardiomyocyte apoptosis and autophagy. Consistent with previous studies, in the present study, we confirmed that MIRI impaired cardiac function and induced oxidative products including protein carbonyl, 8-OHdG, and MDA in the myocardium. It also activated MPO and pro-inflammatory factors, such as TNF-α and IL-1β. The treatment of H₂S for seven consecutive days exhibited dramatic improvement in cardiac function, as manifested by increased LVSP and ± (dP/dt) max, and decreased LVDP, as well as ameliorated oxidative stress and inflammation.

PTEN, a tumor suppressor gene, has been found to participate in growth, apoptosis, adhesion, invasion, and migration. It was reported that the increased PTEN expression by recombinant adenovirus in cultured neonatal rat primary cardiomyocytes caused cardiomyocyte apoptosis. On the other hand, the inhibition of PTEN limits myocardial infarct size and improves left ventricular function after MI. The inactivation of PTEN activates the Akt signaling, reduces apoptosis, and increases survival. To explore the underlying mechanism, we first examined the PTEN mRNA expression and the PI3K/Akt pathway activation. PTEN mRNA was increased by MIRI but decreased by H₂S and Phen. PI3K/Akt pathway was activated by H₂S and Phen but de-activated by LY294002. These results indicate that PTEN were regulated by MIRI and H₂S and could mediate the PI3K/Akt pathway in the MIRI model. Next, we re-measured the cardiac function in the presence of Phen or LY294002. The results revealed that the LVDP was decreased by Phen but increased by LY294002 compared to MIRI+H₂S. By contrast, LVSP, ± (dP/dt) max were increased by Phen but decreased by LY294002 compared to MIRI+H₂S, suggesting the involvement of PTEN/PI3K/Akt pathway.
These results revealed that H$_2$S may exert its cardioprotection by inactivation of PTEN, which in turn activates the PI3K/Akt signaling and increases survival in the MIRI model.

To conclude, the present study showed that H$_2$S could effectively protect heart against I/R injury. It may act as a strong antioxidant and protect the cardiac system through its antioxidant role. It may also restrain the extent of inflammation and limit the extent of MIRI by preventing cytokine release. These findings reveal that the PTEN/PI3K/Akt pathway plays an important role in the cardioprotection exerted by H$_2$S and suggest that exogenous elevated H$_2$S serve to protect heart against I/R injury and may serve as an important therapeutic target.

Disclosure of conflict of interest

None.

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