Original Article

H_2S Protecting against Lung Injury following Limb Ischemia-reperfusion by Alleviating Inflammation and Water Transport Abnormality in Rats*

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Abstract

Objective To investigate the effect of H_2S on lower limb ischemia-reperfusion (LIR) induced lung injury and explore the underlying mechanism.

Methods Wistar rats were randomly divided into control group, IR group, IR+ Sodium Hydrosulphide (NaHS) group and IR+ DL-propargylglycine (PPG) group. IR group as lung injury model induced by LIR were given 4 h reperfusion following 4 h ischemia of bilateral hindlimbs with rubber bands. NaHS (0.78 mg/kg) as exogenous H_2S donor and PPG (60 mg/kg) which can suppress endogenous H_2S production were administrated before LIR, respectively. The lungs were removed for histologic analysis, the determination of wet-to-dry weight ratios and the measurement of mRNA and protein levels of aquaporin-1 (AQP_1), aquaporin-5 (AQP_5) as indexes of water transport abnormality, and mRNA and protein levels of Toll-like receptor 4 (TLR_4), myeloid differentiation primary-response gene 88 (MyD88) and p-NF-κB as indexes of inflammation.

Results LIR induced lung injury was accompanied with upregulation of TLR_4-Myd88-NF-κB pathway and downregulation of AQP_1/AQP_5. NaHS pre-treatment reduced lung injury with increasing AQP_1/AQP_5 expression and inhibition of TLR_4-Myd88-NF-κB pathway, but PPG adjusted AQP_1/AQP_5 and TLR_4 pathway to the opposite side and exacerbated lung injury.

Conclusion Endogenous H_2S, TLR_4-Myd88-NF-κB pathway and AQP_1/AQP_5 were involved in LIR induced lung injury. Increased H_2S would alleviate lung injury and the effect is at least partially depend on the adjustment of TLR_4-Myd88-NF-κB pathway and AQP_1/AQP_5 expression to reduce inflammatory reaction and lessen pulmonary edema.

Key words: Hydrogen sulfide; Limb Ischemia-reperfusion; Toll-like receptors; Nuclear Factor-κB; Aquaporin-1; Aquaporin-5

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INTRODUCTION

Acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome (ARDS), remains a leading cause for morbidity and mortality in critically ill patients. The lung injury may be resulted from direct factors, such as pneumonia, aspiration and mechanical ventilation, or indirect factors, such as trauma and major operations[1]. Previous studies have demonstrated that lower limb ischemia-reperfusion (LIR) may result in remote organ injury or even multiple organ dysfunction syndromes, and the lung is one of the most susceptible organs. A

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growing body of evidence supports that pulmonary injury resulting from LIR might lead to pulmonary dysfunction characterized by systemic inflammatory, intertistial edema, oxidative response[2-3] and depression of cystathionine gamma-lyase (CSE)/ hydrogen sulphide (H_2S) system[4].

H_2S has emerged as an endogenous gaseous signaling transmitter just as nitric oxide (NO) and carbon monoxide (CO). It is endogenously synthesized mainly by CSE in the respiratory system[5]. H_2S is reported to possess potent anti-oxidant, anti-inflammatory and other physiological regulatory functions and may serve as an important biological agent for ischemia reperfusion (I/R) injury of the lung, liver, kidney, heart[6-8], and so on. Notably, our previous study and some other recent reports have indicated that exogenous H_2S gas inhalation or H_2S donor administration might offers both local protection for I/R tissues and systemic protection for the remote organs[9-10]. However, there have been few reports about the effect of H_2S pathway on LIR induced lung injury. In the present study, we explored the association between H_2S pathway and the inflammation and water transport abnormality in a rat model of LIR induced lung injury and evaluated the protective effect of H_2S pathway.

It is well known that inflammation and water transport abnormality causing lung edema are essential changes involving in the pathophysiology of LIR induced lung injury. Toll-like receptors (TLRs) are the major microbial receptors on immune and nonimmune cells and have been recently recognized as one of the main contributors to pathogen and injury induced inflammation. To date, 11 human TLRs have been identified, and most of them are dependent on the expression of Myeloid differentiation primary-response gene 88 (MyD88) for their signaling. As a member of this family, Toll-like receptor 4 (TLR_4) have been shown to initiate an inflammatory damage of many organs and tissues induced by ischemia and reperfusion (I/R). TLR_4 may stimulate the production of the pro-inflammatory nuclear factor κB (NF-κB) and lead to the expression of proinflammatory cytokines such as IL-6, IL-1, and TNF-α[11-12]. These proinflammatory cytokines are required for remote lung injury[13], and the inhibition of their production may lead to reduced remote lung injury following LIR as reported[14].

Aquaporins (AQPs) are small, integral transmembrane proteins that facilitate water transport across cell plasma membranes in response to osmotic gradients. There are 13 mammalian aquaporins, which are widely distributed in specific cell types in many organs and tissues. AQP_1 and AQP_5 are the main distribution of AQPs in the lower respiratory tract. They are demonstrated to play important roles in water clearance in bronchial tree and alveolar space and to be biological markers in the development of lung edema and lung injury[15-16]. However, it hasn't been determined that the alteration of AQP_1 and AQP_5 might involve in the process of lung injury and edema resulting from LIR.

Given these evidence, we hypothesized that endogenous H_2S would alleviate lung injury and the protective effect is at least partially depend on the adjustment of TLR_4-Myd88-NF-κB pathway and AQP_1/AQP_5 expression. This study was aimed to explore the potential effects of H_2S on lung injury and the underlying mechanism involving TLR_4-NF-κB pathway and water transport ability mediated by AQP_1/AQP_5 in a rat model of limb ischemic reperfusion.

MATERIALS AND METHODS

Animals and Surgeries

Thirty two male Wistar rats, weighing 280-330 g each, were obtained from the Animal Department of Academy of Military Medical Science, Beijing, China. All animal care and experimental protocols were in accordance with the Guidelines of the National Institutes of Health for Care and Use of Laboratory Animals and the Guide for the Care and Use of the Laboratory Animals of the People's Liberation Army General Hospital. The rats were randomly divided into 4 groups: control group (n=8), IR group (n=8), IR+NaHS group (n=8), and IR+PPG group (n=8).

The rats were deprived of food but not water for 12 h before the procedure. LIR model was established by 4 h reperfusion following 4 h ischemia of bilateral hindlimbs with rubber bands applied[17]. Reperfusion was initiated by removing the bands. Sodium hydrosulphide (NaHS, Sigma), as exogenous H_2S donor, was administrated at dosage of 0.78 mg/kg intraperitoneally to the rats in IR+NaHS group before ischemia and reperfusion. DL-propargylglycine (PPG, sigma), which can suppress endogenous H_2S production as a specific inhibitor of CSE, was given at dosage of 60 mg/kg intraperitoneally to the rats in IR+PPG group. Stock solution of NaHS (0.1%) and PPG (2.7%) was freshly

prepared before use. All the rats were anesthetized with amobarbital sodium (80 mg/kg, im injection) prior to the experiment, and were sacrificed at the end of the 4 h reperfusion. The lungs were removed and fixed with 10% formalin for histopathological analysis or frozen in liquid nitrogen immediately for the measurement of protein and mRNA expression levels of target genes. The right lower lobe of were used for the determination of the lung wet-to-dry weight (W/D) ratio.

Histological Evaluation

Rat pulmonary tissues was fixed in 10% formalin and then embedded with paraffin. After fixation, the tissue blocks were embedded in paraffin and statined with hematoxylin-erosin (H&E) for characterizing lung injury by microscopic evaluation.

W/D Ratio

To quantitatively assess pulmonary edema after ischemia and reperfusion of limb, lung W/D ratios were determined. The lungs were dissected and weighed to obtain the 'wet' weight, and then were dried in a 80 °C oven for 72 h to obtain the 'dry' weight. The W/D ratio was calculated by dividing the wet weight by the dry weight.

Immunohistochemistry Analysis

Immunohistochemistry test was performed to analyze the expression of AQP_1 and AQP_5. Tissue sections (5 mm) were dewaxed, hydrateds, and mounted on slides. Following antigen retrieval with citrate buffer (10 mmol/L, pH 6.0), tissue sections were preincubated with goat serum for 20 min. After washing thrice with PBS, immunochemistry staining of the sections was carried out by using polyclonal antibody against AQP_1 (Abcam, 1:500) and monoclonal antibody against to AQP_5 (Abcam, 1:200). After washing with PBS, the sections were incubated with goat anti-mouse IgG and anti-rabbit IgG antibodies conjugated to horseradish peroxidase (1:250) for 45 min as second antibodies, and then diaminobenzidine (DAB) was used for visualization.

Western Blotting Analysis

The lung tissues were removed, perfused with pH7.4 PBS to remove the red blood cells, and then frozen with liquid nitrogen. After homogenization, the total protein of lung tissues was extracted. Protein preparations were separated in 10% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose membrane, and incubated overnight with target primary antibody for 1 h at room temperature. β-actin in corresponding samples was used as an internal standard. The antibodies included polyclonal antibody to AQP_5 (Abcam, 1:100), monoclonal antibody to AQP_1 (Abcam, 1:100), monoclonal antibody to TLR_4 (Abcam, 1:100), monoclonal antibody to p-NF-κB (CST, 1:100), and monoclonal antibody to β-actin (Sigma, 1:10 000). The membrane was then washed and incubated with horseradish peroxidase (HRP) conjugated antibody. The bands were detected by the way of enhanced chemiluminescence, and quantified by scanning densitometry with Image-Pro Plus 5.0 software.

Semei-quantitative Real-time PCR

The lung tissues for the detection of AQP_1, AQP_5, TLR_4, and MyD88 mRNA level were frozen in liquid nitrogen immediately after collection and homogenization in TRIzol reagent (Invitrogen). Total RNA was extracted according to the manufacturer's instructions of TRIzol. RNA integrity was electrophoretically confirmed by ethidium bromide staining and the concentration was determined by standard spectrophotometric analysis. Single cDNA was synthesized from 1 μg total RNA using M-MLV Reverse Transcriptase (Promega). Relative quantification of real-time PCR was performed on the Eco™ Real-Time PCR system.

The following sense and antisense oligonucleotide primers of each gene were designed by Primer 5.0 (direction 5' to 3') (Table 1). Thermal cycling conditions included 3 min preincubation at 95 °C, followed by 45 cycles of 95 °C for 10 s and 59 °C for 15 s. The appropriate annealing temperature for each gene is 59 °C. For real-time PCR analysis, the mRNA expression levels were normalized using the rat GAPDH gene as an internal standard and then presented as the mean fold difference compared with the control group. The data were analyzed with Eco™ Software v3.1.7.0. from three independent experiments.

Table 1. Primers Used for Real-time PCR

Gene	Sense primer (5'-3')	anti-sense primer (5'-3')
AQP_1	gacaatgtgaaggtgtcactg	cacactgggcgatgatataca
TLR_4	gaatctggtggctgtggagac	gaaagatccacatgttctagg
Myd88	ctgaaggaccgcatcgaggag	cagtagcagatgaaggcgtcg
AQP_5	gccacatcaatccagccattac	attgagaggcgccagccagtac
GAPDH	caagattgtcagcaatgcatcc	atcacgccacagctttccagag

Statistical Analysis

The parameters of W/D ratios and Western-blot density ratios were expressed as mean±SE, and the parameters of relative mRNA expression from real-time PCR were natural logarithm transformed for homogeneity of variance. Analysis of variance (ANOVA) procedures with LSD multi-comparisons were used to compare the differences among groups. Statistical analyses were performed with statistical analysis software (SPSS version 15.0). *P* value of <0.05 was considered statistically significant.

RESULTS

Histological Assessment of Damage

H&E staining was used to evaluate histopathological changes of lung injury including edema, inflammatory cell infiltration, congestion and preservation of the alveolar septum (Figure 1). Compared with control group, bilateral limbs ischemia and reperfusion induced alveolar congestion, breakdown of alveoli architecture, alveolar wall thickening and inflammatory cell infiltration. The histological lesions of IR+NaHS group improved somehow with less histological changes. In contrast, IR+PPG group showed more prominent and diffuse lung injuries.

Assessment of Edema Formation

We determined the lung W/D ratio to quantitatively evaluate the formation of pulmonary edema (Figure 2). The W/D ratio was significantly increased after bilateral limbs ischemia reperfusion (*P*<0.05). Compared with IR group, the administration of PPG led to an even higher level of W/D ratio (*P*<0.05), and NaHS induced a significant reduction in lung edema (*P*<0.05).

Immunochemistry Analysis for AQP_1 and AQP_5

The expression of AQP_1 and AQP_5 decreased significantly and the distribution of AQPs positive cells was less wider for the rats in IR group (Figure 3). Compared with IR group, the administration of NaHS strikingly increased AQP_1 and AQP_5 protein levels in lung tissues, while the administration of PPG decreased AQPs protein expression (Figure 3).

Semi-quantitative Western-blot for AQP_1, AQP_5, TLR_4, and p-NF-κB

The expression of AQP_1 (28 kd), AQP_5 (29 kd), TLR_4 (110 kd), and p-NF-κBp65 (65 kd) in the lung tissues of rats in different groups was detected by Western-Blot (Figure 4). The amount of protein was normalized against the expression of β-actin (42 kd) in corresponding samples as an internal standard. Data from semi-quantitative western blotting further confirmed the above results from immunohistochemistry staining. Both AQP_1 and AQP_5 protein expression in the LIR group showed a significant trend of depression compared with control group (*P*<0.05). The administration of NaHS resulted in upregulation of AQP_1 and AQP_5 protein expression in lungs (*P*<0.05), while PPG resulted in even less expression of AQP_1 after limb ischemia reperfusion (*P*<0.05). We also found a decreasing trend of AQP_5 expression in IR+PPG group compared with IR group although the difference has no statistical significance (*P*>0.05). The reason for this finding might be related with the small sample size. On the other hand, the amount of TLR_4 and p-NF-κBp65 of IR group increased significantly compared with control group (*P*<0.05). The administration of NaHS downregulated the TLR_4 and Myd88 protein levels, while the administration of PPG upregulated the levels for LIR subjects (*P*<0.05).

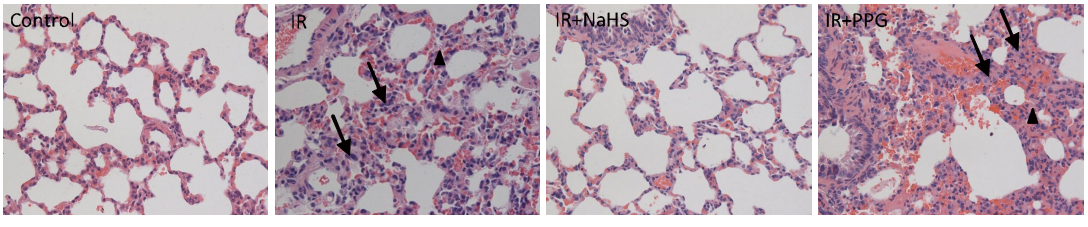

Figure 1. H&E staining (×40) was used to evaluated the effects of NaHS and PPG on LIR induced histopathological changes of lung tissues. The arrows depicted infiltration of cells and the arrowheads depicted congestion of the alveolar septum.

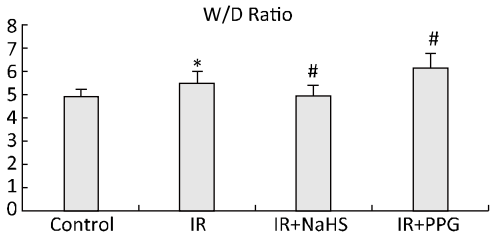

Figure 2. Pulmonary W/D ratio in different groups. The data are expressed as mean±SE. $^{*}P<0.05$ *vs*. control group. $^{\#}P<0.05$ *vs.* IR group. There are eight rats in each group.

Real-time PCR for the mRNA Expression of AQP_1, AQP_5, TLR_4, and MyD88

The expression levels of AQP_1, AQP_5, TLR_4, and Myd88 mRNA in lung tissues of the rats were determined by real-time PCR. As shown in Figure 5, the mRNA levels of AQP_1 and AQP_5 significantly decreased after LIR model establishment ($P<0.05$). The levels increased in IR+NaHS group and decreased in IR+PPG group compared with IR group significantly ($P<0.05$). The reactive pattern of TLR_4 and Myd88 mRNA levels was similar to the changes of their protein levels. Namely, the TLR_4 and Myd88

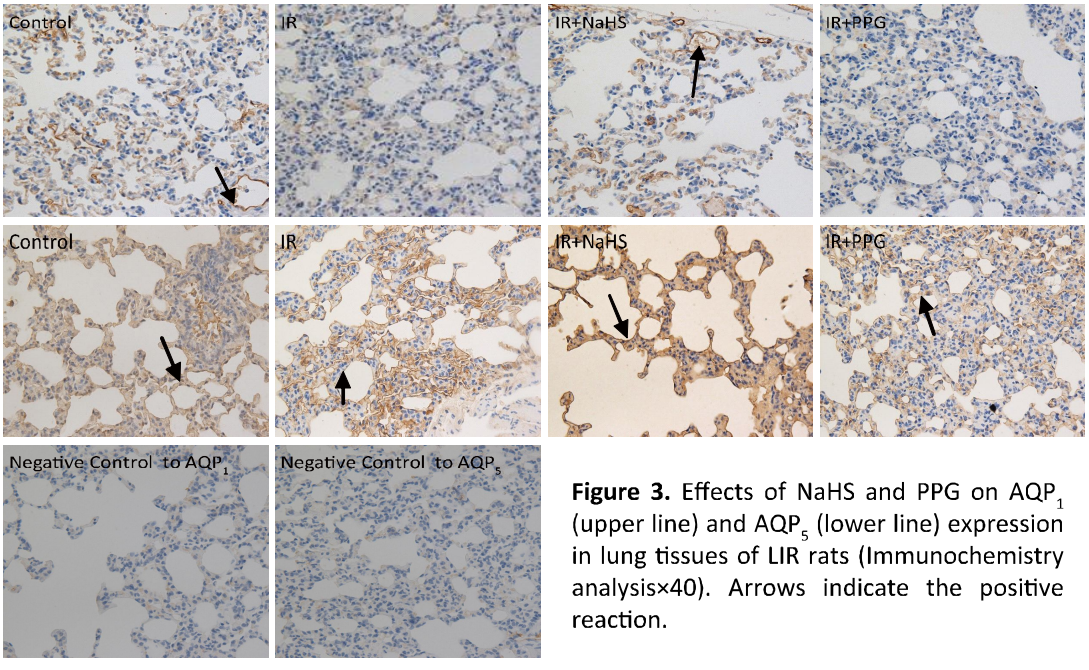

Figure 3. Effects of NaHS and PPG on AQP_1 (upper line) and AQP_5 (lower line) expression in lung tissues of LIR rats (Immunochemistry analysis×40). Arrows indicate the positive reaction.

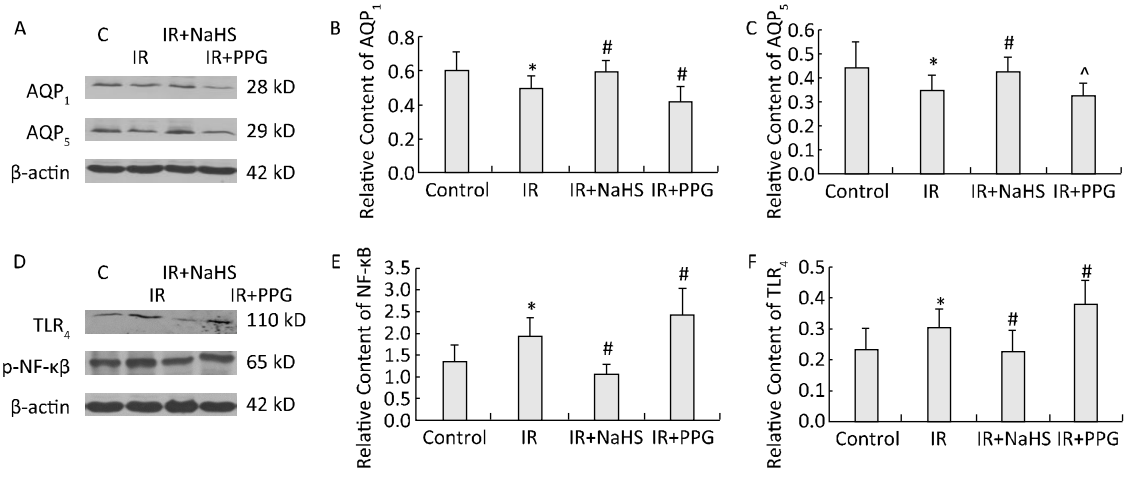

Figure 4. Western-blotting images (A, D) and relative content of AQP_1 (B), AQP_5 (C), p-NF-Kb (E), and TLR_4 (F) in different groups. The data are expressed as mean±SE. $^{*}P<0.05$ *vs.* control group. $^{\#}P<0.05$ *vs.* IR group. $^{\wedge}P>0.05$ *vs.* IR group.

mRNA levels of IR group increased significantly compared with control group. NaHS treatment downregulated the TLR_4 and Myd88 mRNA levels (P<0.05), while PPG treatment upregulated the levels in LIR subjects (P<0.05).

DISCUSSION

Prolonged limb ischemia followed by re-establishing reperfusion may worsen the initial ischemic injury, which is known as 'limb ischemia/reperfusion injury, LIRI', and paradoxically cause systemic complications and unexpected mortality. LIRI is a common clinical issue resulting from thrombotic occlusion, embolism, long-time application of tourniquet in extremity operation, trauma, etc. The fact that the lungs are more susceptible to IR of the lower limbs than other remote organs (such as heart, kidney, liver, etc.) has stimulated researchers' interest, however, the underlying mechanism remains to be unclear. The present study was designed to evaluate the potential effect of endogenous H_2S on acute lung injury resulting from LIR in rats. It was hypothesized that increased endogenous H_2S would alleviate lung injury and the protective effect is at least partially depend on the adjustment of TLR_4-Myd88-NFκB pathway and AQP_1/AQP_5 expression to reduce inflammatory reaction and pulmonary edema.

It is known that LIR triggers an intense inflammatory response accompanied with increased pro-inflammatory cytokines that may precipitate remote lung injury[13]. TLRs have been recently recognized as one of the main contributors to inflammation induced by pathogen or injury. To date, 11 human TLRs have been identified. TLR_4, as an receptor to LPS, HSPs, HMGB1, some viral proteins and hyaluronic acid[18], has recently been demonstrated to play a central role in early recognition of endogenous proteins released from damaged tissues in I/R injuries [19]. After recognition, TLR_4 stimulates the MyD88-NF-κB pathway and leads to the direct induction of IL-1, TNF-α, and IL-6, which result in inflammatory injury[11-12]. TLR_4 antagonists seem to be beneficial in cerebral, myocardial, renal, and hepatic I/R injury[18]. Recent studies have also suggested that TLR_4 and NF-κB activation could play a key role in lung injury resulting from many reasons, as well as intestinal I/R induced lung injury[18,20]. But the role of TLR_4-Myd88-NF-κB signal pathway in acute lung injury following LIR is poorly understood.

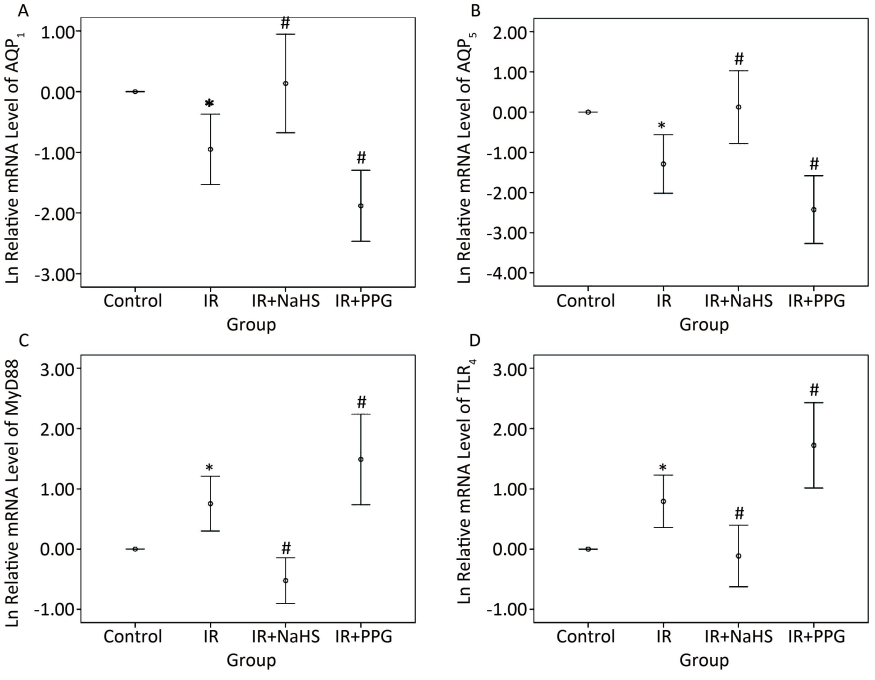

Figure 5. Error bar plots of real-time PCR analysis of mRNA expression for AQP_1(A), AQP_5(B), Myd88(C), and TLR_4(D) in rat lung tissue (mean and 95% confidence interval). The data were based on 3 independent experiments, expressed as the relative quantification values in natural logarithm scale. $^*P$<0.05 *vs*. control group. $^\#P$<0.05 *vs*. IR group.

There are 6 mammalian AQPs expressed widely in various cell types of the lung and airways[21]. AQP_1 is expressed mainly in the capillary endothelium and AQP_5 at the apical membrane of alveolar epithelial cells[22]. They are the main distribution of AQPs in the lower respiratory tract. In line with their distribution, AQP_1 and AQP_5 have been described as important mediators for removal of pulmonary edema fluid from pulmonary interstitium and the alveolar space, respectively[23-24]. The osmotic water permeability between the airspace/capillary barrier is reduced (10-fold) by deletion of AQP_1 or AQP_5, and reduced even more (>30-fold) by the deletion of AQP_1 and AQP_5 together[25-26]. Increased expression of AQP_1 and AQP_5 has been reported to be related with improved alveolar fluid reabsorption, oxygenation and lung compliance[21]. Several previous reports also support that the decreased expression of AQP_1 and AQP_5 could play a vital role in the pathogenesis of lung edema and injury resulting from adenoviral infection[27], lipopolysaccharide administration[28], or mechanical ventilation[15-16]. However, it is still unknown whether the alteration of AQP_1 and AQP_5 might be involved in the process of lung injury resulting from LIR.

This study showed significantly decreased expression of AQP_1 and AQP_5 and the upregulation of TLR_4-Myd88-NF-κB pathway in the mRNA and protein level of the lung tissue after LIR model were established, which was accompanied with increased W/D ratio, as well as the histological alterations. The results suggested that the upregulation of TLR_4-Myd88-NF-κB pathway might aggravate the severity of pulmonary inflammation and thus worsen remote lung injury, and the significantly decreased expression of AQP_1 and AQP_5 might reduce the removing of edematous fluid from alveolar space and pulmonary interstitium and cause alveolar and interstitial edema. All the evidence indicate indirectly that the alteration of local TLR_4-Myd88-NFκB pathway and AQP_1/AQP_5 are involved in the process of LIR-induced acute lung injury.

H_2S has been reported to possess potent anti-oxidant, anti-inflammatory and other functions and may serve as an important biological agent for lung injury as mentioned above. In the present study, NaHS was given at dosage of 0.78 mg/kg and PPG was given at dosage of 60 mg/kg according to our previous reports[9]. This dosage injection was proved to be safe with little toxicity[29-30]. NaHS has been used as exogenous H_2S donor to increase H_2S level, and PPG as a CSE inhibitor to decrease the production of H_2S as reported[31]. Our previous study showed also that the administration of NaHS or PPG would change endogenouse level of many tissues[9,32]. In this study, the pretreatment of NaHS as exogenous H_2S donor was shown to significantly downregulate TLR_4-Myd88-NF-κB pathway and upregulate the expression of AQP_1 and AQP_5 compared with LIR model group. These changes were accompanied with the decrease of the pulmonary water content and histological improvement. Blockage CES activity with a CSE inhibitor, PPG, which could decrease the production of H_2S, was shown to exacerbate lung injury and edema, further stimulate TLR_4-Myd88-NF-κB pathway and downregulate the AQP_1 and AQP_5 expression in both mRNA and protein levels. All the alterations were of statistical significance except the alteration of AQP_5 protein level between IR group and IR+PPG group. The reason for this finding is unclear, but it might be related with the sample size. It is indicated that the administration of PPG might worsen the inflammatory reaction and water transport abnormality, and exacerbate lung injury following LIR.

In conclusion, our study with the model of lung injury following bilateral limbs ischemia reperfusion suggests that LIR could induce remote lung injury and edema accompanied with upregulation of TLR_4-Myd88-NF-κB as well as downregulation of AQP_1/AQP_5. Increased endogenous H_2S level may have protective effect in the pathogenesis of LIR induced lung injury at least partially by upregulation of AQP_1/AQP_5 expression and downregulation of TLR_4-Myd88-NF-κB pathway, but decreased H_2S level may adjust AQP_1/AQP_5 and TLR_4 pathway to the opposite side and exacerbate lung injury and edema. The altered expression of endogenous H_2S, TLR_4-Myd88-NF-κB pathway and AQP_1/AQP_5 were all involved in the process of LIR induced lung injury and there might be some kind of interrelation among them, which remains to be elucidated.

On the one hand, recent studies have demonstrated that the activation of TLR4-Myd88-NF-κB pathway triggers the expression of some inflammatory mediators, such as TNF-α, IL-1, IL-8, etc. As reported in human salivary gland acinar cells, TNF-α inhibits the expression levels of AQP_5 mRNA and protein[33]. It was also reported that TNF-α might have the ability to decrease the expression of AQP_1/AQP_5 and aggravate lung edema[34-35]. On the other hand, a growing number of studies have showed that H_2S exerts

anti-inflammatory, anti-oxidative effects and play a pivotal role in I/R injury of many organs. Based on the previous evidence and our results, we presume that it might be the underlying mechanism for the protective effect of H_2S that increased level of endogenous H_2S downregulate TLR_4-Myd88-NF-κB pathway and reduced inflammatory levels, and less inflammatory factors lead to increasing AQP_1 and AQP_5 expression. Although AQPs family also transport glycerol or carbon dioxide as selective transporters[36-37], it has been reported that H_2S transports through epithelial and endothelial barriers by simple diffusion and does not require facilitation by membrane channels such as AQPs[38].

Further studies are needed to explore the mechanisms involved in the complex regulatory process of LIR induced remote lung injury. The dose-response of NaHS or PPG and the effect of some direct inhibitors for AQPs and TLR_4 signal pathway were not involved in our study. The clear interrelationship among H_2S pathway, TLR_4-Myd88-NF-κB and AQP_1/AQP_5 needs to be further studied. Whether H_2S adjusted TLR_4-Myd88-NF-κB pathway and AQP_1/AQP_5 expression in a time- and dose-dependent manner needs also to be determined.

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