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Original Article

Hydrogen-rich saline prevents the down regulation of claudin-5 protein in septic rat lung via the PI3K/Akt signaling pathway

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Abstract: Background: Acute lung injury (ALI) is characterized by capillary leak and increased pulmonary permeability with high mortality. Hydrogen-rich saline, which has therapeutic anti-inflammatory and anti-apoptotic activity, can attenuate pulmonary edema in sepsis-related lung injury. However, the mechanisms of the protective effect are not completely clear. Thus, we investigated the effects and mechanisms of hydrogen-rich saline on the expression of claudin-5 protein which is associated with endothelial paracellular permeability in lipopolysaccharide (LPS)-induced ALI. Methods: Sixty male Sprague-Dawley rats, which were randomly divided into six groups (n = 10, in each group): control group, LPS group, LPS+H_2 group, H_2 group, LPS+H_2+LY294002 group and LPS+LY294002 group, received intratracheal administration of LPS followed by intraperitoneal injection of hydrogen-rich saline or intravenous injection of PI3K inhibitor LY294002. The severity of pulmonary edema was assessed by wet-to-dry rate and Evans blue infiltration; the expression of claudin-5 protein was examined by immunofluorescence double-labeling staining and western blot; the level of phospho-Akt was detected by immunohistochemistry staining and western blot. Results: Pretreatment with hydrogen-rich saline significantly alleviated pulmonary edema and attenuated the deterioration of claudin-5 protein induced by LPS in rat lung. Moreover, Hydrogen-rich saline enhanced LPS-induced activation of the PI3K/Akt pathway, which is associated with the regulation of claudin-5 expression. However, the protective effects of hydrogen-rich saline were partly suppressed by LY294002. Conclusion: Hydrogen-rich saline ameliorates LPS-induced ALI through reducing disruption of claudin-5 protein, which may be associated with the enhanced activation of the PI3K/Akt pathway.

Keywords: Acute lung injury, hydrogen, claudin-5, PI3K/Akt

Introduction

Acute lung injury, a common complication of sepsis, is a severe clinical problem with 30~50% mortality rate [1]. It is characterized with increased alveolar-capillary permeability and amounting inflammatory cytokines, which subsequently resulted in pulmonary edema and acute respiratory distress syndrome [2, 3]. The root cause is that the integrity of the alveolar membrane damages. Tight junction proteins are required to maintain the integrity of lung epithelial barrier, a key component of structural and functional lung defense [4]. Claudin-5, an integral membrane protein, is a critical component of tight junctions of vascular endothelial cells and the downregulation of its expression is associated with an increase in endothelial paracellular permeability [5, 6].

Recently, majority of studies have found that hydrogen-rich saline exerts an effective therapeutic role on many disorders such as ischemic reperfusion injury, stroke, sepsis, atherosclerosis, organ transplantations via reducing oxidative stress, inflammation and apoptosis. Furthermore, accumulated evidences show that administration of hydrogen-rich saline can improve the survival rate and ameliorate lung damage in septic mice in concentration and time dependent manner [7]. The beneficial effect of hydrogen-rich saline on sepsis was

associated with the deceased levels of oxidative stress and inflammatory cytokines in serum and tissues [8]. It has been approved that hydrogen-rich saline can decrease the vascular permeability and reduce pulmonary edema in lungs of septic rat. However, the effect of hydrogen-rich saline on the tight junction protein claudin-5 remains unknown.

The expression of claudin-5 protein is regulated through the activation of the PI3K/Akt pathway in endothelia cells, which plays a vital role survival/dead way [9]. PI3K is essential for activation of Akt, which is significant in inducing claudin-5 upregulation by phosphorlation of several downstream macromolecules. In this study, we hypothesize that hydrogen-rich saline attenuates permeable edema in septic rat lungs through reversing down-regulation of claudin-5 protein via the PI3K/Akt signaling pathway.

Methods

Animals

Male Sprague-Dawley rats (8~10 weeks) weighing between 200~250 g were provided by the Experimental Animal Center of Xuzhou Central Hospital. The care and handling of the laboratory animals were in accordance with the Institutional Animal Ethics Committee of Xuzhou Central Hospital guidelines for ethical animal research. Rates were housed in a controlled environment and provided with standard rodent chow and water ad libitum.

Acute lung injury model

As previously described [10], acute lung injury was induced by intratracheal administration of lipopolysaccharide (LPS). In brief, animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Rats were orally intubated with a sterile plastic catheter and intratracheally given a single dose of aerosolized LPS (50 μg/rat, dissolved in 100 μL of phosphate-buffered saline [PBS]). The mice in control group were administrated with 100 μL of sterile PBS.

Preparation of hydrogen-rich saline

Hydrogen-rich saline was prepared as in previous studies [11, 12]. Hydrogen gas was dissolved in normal saline for 6 hours under high pressure (0.4 MPa) to a supersaturated level. The saturated hydrogen-rich saline was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume and was sterilized by gamma radiation. To make sure the hydrogen level in the saline was at least 0.6 mmol/L, gas chromatography was performed following the method described by Ohsawa [13].

Experimental protocols

Sixty rats were randomly divided into six groups (n = 10, in each group): control group, LPS group, LPS+H_2 group, H_2 group, LPS+H_2+LY294002 group and LPS+LY294002 group. Hydrogen-rich saline (5 mL/kg) or equal volume of saline was intraperitoneally injected at 1 h and 4 h after LPS administration. The dosage and time point of hydrogen-rich saline were based on the previous studies [14]. The PI3K inhibitor, LY294002 (50 μM in 25% dimethyl sulfoxide and PBS), was injected though caudal vein 30 minutes before LPS administration. Mice were humanely killed at 8 h after LPS challenge to collect lung tissues for further analysis. Another 40 rats were randomly assigned to the following groups (n = 10 per group): control group, LPS group, LPS+H_2 group and H_2 group. Each group was injected Evans blue dye (EB; 2%; 5 ml/kg) for permeability analysis.

Hematoxylin-eosin (H&E) staining

Lung samples were collected at 24 h after LPS administration. Tissues were fixed in 4% buffered paraformaldehyde and subsequently embedded in paraffin. Sections were stained with hematoxylin-eosin using a standard protocol and analyzed by light microscopy.

Wet to dry (W/D) lung weight ratio

To quantify the magnitude of pulmonary edema, we evaluated lung W/D weight ratio. The harvested wet lung was weighed, and then placed in an oven for 24 hours at 80°C and weighed when it was dried. The ratio of wet lung to dry lung was calculated.

Evans blue staining

To further assess lung permeability, Evans blue was dissolved in 0.9% saline at a final concentration of 5 mg/ml. Animals were anesthetized,

weighed, and injected with 20 mg/kg Evans blue in the vein. After 30 min, the animals were killed and the lungs perfused with 1 ml PBS containing 5 mM EDTA. The lungs were collected and frozen in liquid N_2. The frozen lungs were homogenized in 2 ml PBS. The homogenate was diluted with two volume of formamide and incubated at 60°C for 2 h, followed by centrifugation at 5000 g for 30 min. The supernatant was collected and absorbance was measured at 650 nm in a spectrophotometer. The Evans blue concentration was determined from the standard absorbance curves evaluated in parallel. Correction for contaminating heme dye was calculated as described earlier [15].

Immunofluorescence microscopy

Rats were anesthetized and sacrificed by perfusion with 4% paraformaldehyde 8 hours after LPS administration. After washing with PBS (pH 7.4) for 5 min × 3, the sections were then blocked with 10% goat serum for 30 min at 37°C. Slides were incubated with primary antibody (anti-claudin-5 [1:100, Invitrogen], anti-NeuN [1:200, Abcam]) at 4°C overnight and then the second antibody with biotin for 30 min at room temperature. Before any step, there must be sufficient washes with PBS for 3 min × 3, and all the incubation must be done in wet box. Images were captured by confocal laser scanning microscopy (Eclipse 80i, Nikon, Japan).

Western blot

The proteins of samples were prepared according to the method described by the protein extract kit. Proteins concentrations were determined by the BCA protein assay kit (Piece Biotechnology). Protein extracts (50 mg) were separated by electrophoresis on 10% polyacrylamide sodium dodecyl sulfate gels and transferred to PVDF membranes. The membranes were blocked for 1 h at room temperature with a blocking solution (5% non-fat milk in Tris-buffered saline with Tween 20) and then incubated overnight at 4C with primary polyclonal antibodies: claudin-5 (1:500; Invitrogen), p-Akt (1:500; Cell signaling Technology) and β-actin (1:1000, Santa Cruz). After washing three times, membranes were incubated with a goat anti-rabbit or goat anti-mouse secondary antibody (1:2000; Sigma) for 1 h at room temperature. Chemiluminescence reagent was used to investigate the signal intensities. The protein bands were analyzed by Image Lab software.

Real-time PCR

Total RNA was extracted from the lung tissues with TRIzol reagent. RNA samples were reverse transcribed into complementary DNA using an RT-PCR kit, according to the manufacturer's instructions. Quantitative RT-PCR was performed using ABI PRISM® 7500 Sequence Detection System. The sequence of primers were as follows: claudin-5 forward primer 5'-CACAGAGAGGGGTCGTTGAT-3', claudin-5 reverse primer 5'-ACTGTTAGCGGCAGTTTGGT-3', β-actin forward primer 5'-CGCGAGTACAACCTTCTTGC-3', β-actin reverse primer 5'-CGTCATCCATGGCGAACTGG-3'. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data were expressed as means ± standard deviation (SD). The inter-group differences of the rest data were tested by one-way ANOVA followed by LSD-*t* Test for multiple comparisons. The statistical analysis was performed with *SPSS* 16.0 software. Values of $P < 0.05$ were considered statistically significant.

Results

Hydrogen-rich saline attenuated LPS-induced acute lung injury in rats

In the present study, we investigated the effects of hydrogen-rich saline on lung histopathology and function in rats with either LPS or PBS challenge. H&E staining was used to show pathological changes. There was no significant damage observed in the control and H_2 group. In contrast, rats in LPS group exhibited acute lung injury characterized by alveolar wall thickening, infiltration of neutrophils into lung interstitium and alveolar space, consolidation and alveolar hemorrhage. Hydrogen-rich saline attenuated the histologic damage and had a normal alveolar structure (**Figure 1A**).

Moreover, the lung wet/dry ratios and extravasation of Evans blue were used to evaluate the

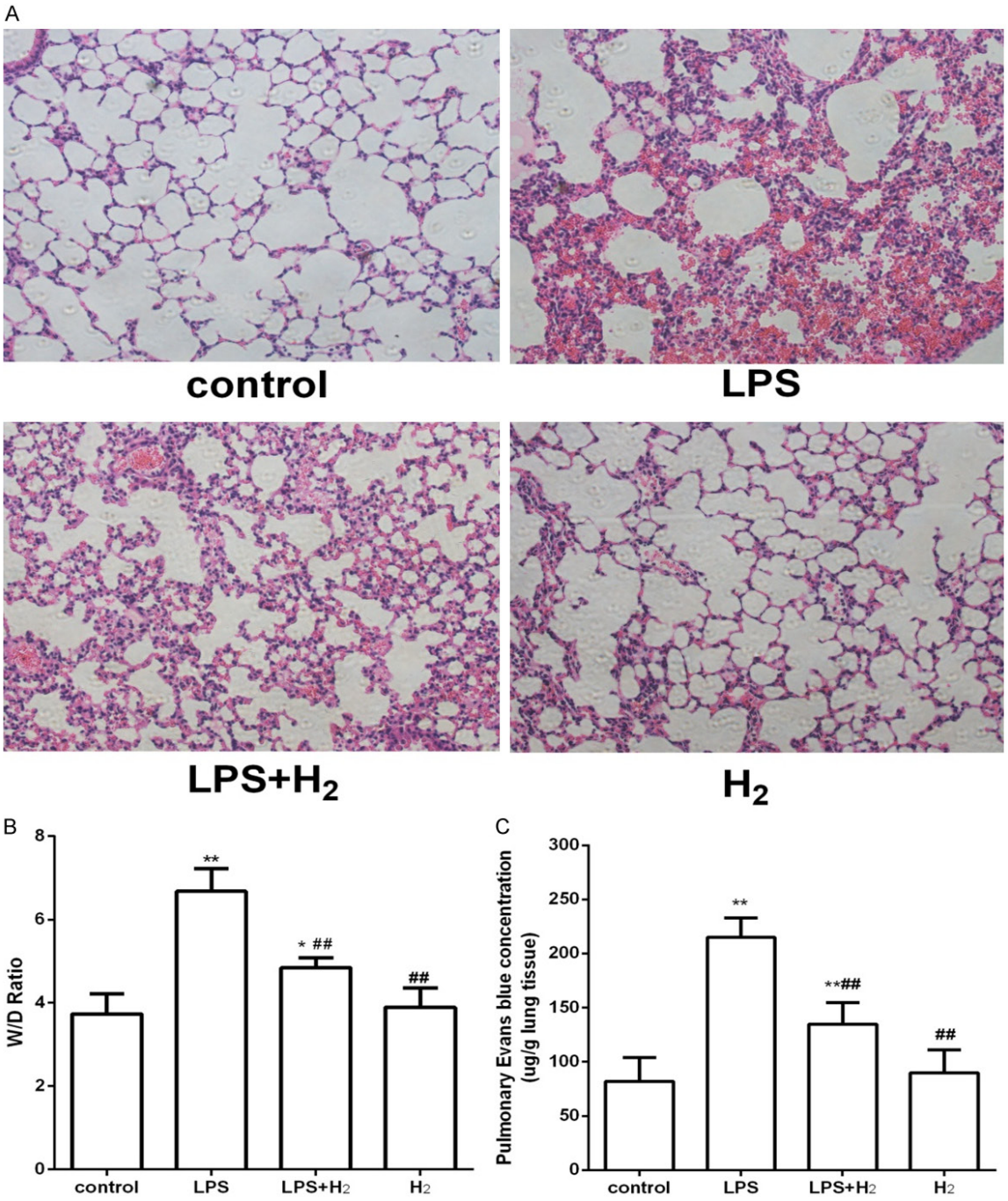

Figure 1. Hydrogen-rich saline ameliorated the lung histopathological changes and pulmonary edema in LPS-challenged rats. A. Representative photographs under light microscopy showing the pulmonary histology (hematoxylin and eosin, magnification × 200). B. Lung tissue wet to dry weight ratio (W/D ratio). C. Evans blue concentration in the alveolar was measured to evaluate the pulmonary endothelial permeability. Results shown were means ± SD (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group; $^{\#\#}P < 0.01$ versus LPS group.

severity of pulmonary edema in endotoxemia rats. As shown in **Figure 1B** and **1C**, the W/D ratio and extravasation of Evans blue increased dramatically in the LPS group compared with that in the control group, suggesting that LPS induced significant disruption of the pulmonary capillary barrier and elevation of pulmonary capillaries permeability. This increase was reduced in the LPS+H_2 group, showing that hydrogen-rich saline treatment can effectively attenuate pulmonary capillaries permeability in LPS-induced rats.

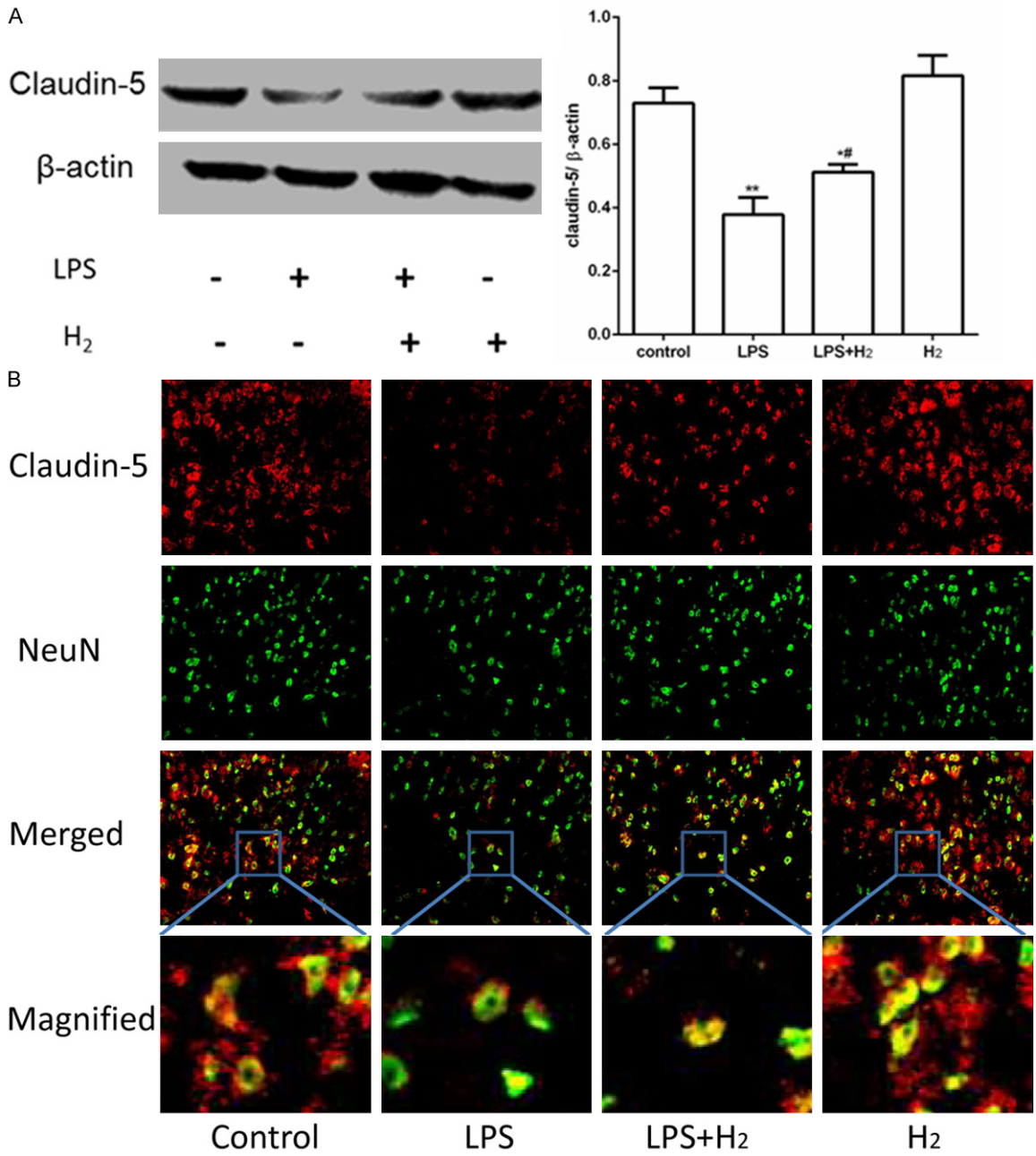

Figure 2. Hydrogen-rich saline prevented LPS-induced down regulation of claudin-5 protein in the lung. A. The expression of claudin-5 protein in lung tissue was detected by Western blotting. Claudin-5/β-actin was used to show the relative expression. Data were presented as means ± SD (n = 10). $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus LPS group. B. Representative double immunofluorescence staining for claudin-5 and NeuN in the lung. Claudin-5-positive cells (red) colocalized mainly with neurons (green). When there is co-localization, the color will turn into yellow. Scale bar = 50 mm. The high magnification pictures from merged were also displayed to show co-localization. Scale bar = 200 mm.

Hydrogen-rich saline prevented LPS-induced downregulation of claudin-5 protein in the lung

To determine whether the claudin-5 protein were affected in LPS-challenged rats, its expression and location was detected by Western blotting and immunofluorescence staining, respectively. Our results found that exposure to LPS markedly reduced the protein expression of claudin-5 in the lung tissue compared with control group, which significantly attenuated by hydrogen-rich saline (**Figure 2A**). As shown in

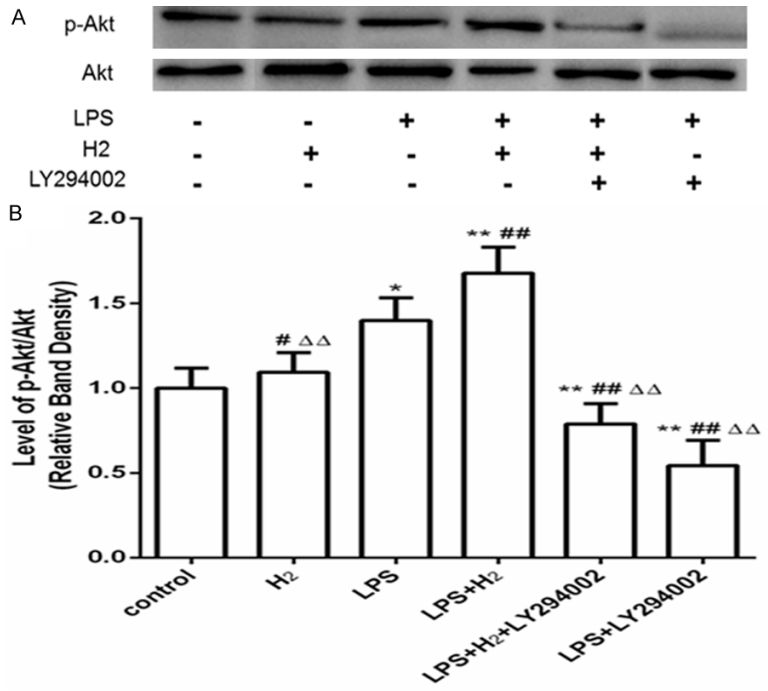

Figure 3. Effect of hydrogen-rich saline on the expression of p-Akt (ser-473) in lung tissues. Rats were pretreated with or without LY294002 (the PI3K inhibitor) 30 min before hydrogen-rich saline or vehicle followed by LPS administration. Western blotting analysis of p-Akt expression was showed. The total Akt expression was similar among all groups. Level of p-Akt/Akt was used to show the relative expression. Data were presented as mean ± SD (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LPS group, $^{\Delta\Delta}P < 0.01$ versus LPS+H_2 group.

Figure 2B, double immunofluorescent for claudin-5 and NeuN was performed and we found claudin-5 positive cells colocalized mainly with neurons in the lung tissues. In addition, the variation tendency of claudin-5 expression paralleled the Western blotting results.

Hydrogen-rich saline enhanced LPS-induced activation of PI3K/Akt pathway

To test whether PI3K/Akt signaling pathway was involved in the protective effects of hydrogen-rich saline, LY294002, a highly selective inhibitor of PI3K, was administered before treatment with hydrogen-rich saline. The protein level of p-Akt was increased after treatment with LPS, which was enhanced by administration of hydrogen-rich saline. However, pretreatment with LY294002 blocked the activation of p-Akt induced by LPS (**Figure 3**). In addition, only administration of hydrogen-rich saline without LPS did not result in any significant change of p-Akt level in lung tissue. It demonstrated that hydrogen-rich saline treatment could greatly increase phosphorylation of Akt following LPS challenge, and when LY294002 is administered, the phosphorylation of Akt is significantly suppressed.

Blocking phosphorylation-activated Akt reversed the protection of hydrogen-rich saline on LPS-induced lung injury

As shown in **Figure 4A**, hydrogen-rich saline treatment significantly ameliorated the lung pathohistologic change. W/D ratio and extravasation of Evans blue in LPS-challenged rats, which was abolished by LY294002 (as seen in **Figure 4B** and **4C**). Moreover, compared with LPS group, the pulmonary edema and endothelial permeability was aggravated in LPS+LY294002 group. These results suggested that the activation of PI3K/Akt signal pathway might play an important role in the protection of hydrogen-rich saline on pulmonary injury induced by LPS.

PI3K/Akt-mediated hydrogen-rich saline protection on LPS-induced deterioration of claudin-5

To determine whether phosphorylated Akt contribute to the protection of hydrogen-rich saline on deterioration of claudin-5 induced by LPS, we used western blotting analysis and Real-Time PCR to detect the expression of claudin-5 at protein and mRNA level, respectively. Results showed that LY294002 can significantly suppressed the effect of hydrogen-rich saline on protecting claudin-5 from disruption (**Figure 5**), suggesting that hydrogen-rich saline prevents LPS-induced deterioration of claudin-5 via the PI3K/Akt signaling pathway.

Discussion

The administration of lipopolysaccharide (LPS), a gram-negative bacterial endotoxin, was used as a model of sepsis-related lung injury in the present study [16]. Our study showed that LPS resulted in severe interstitial and alveolar pulmonary edema, hypoxemia, overproduction of

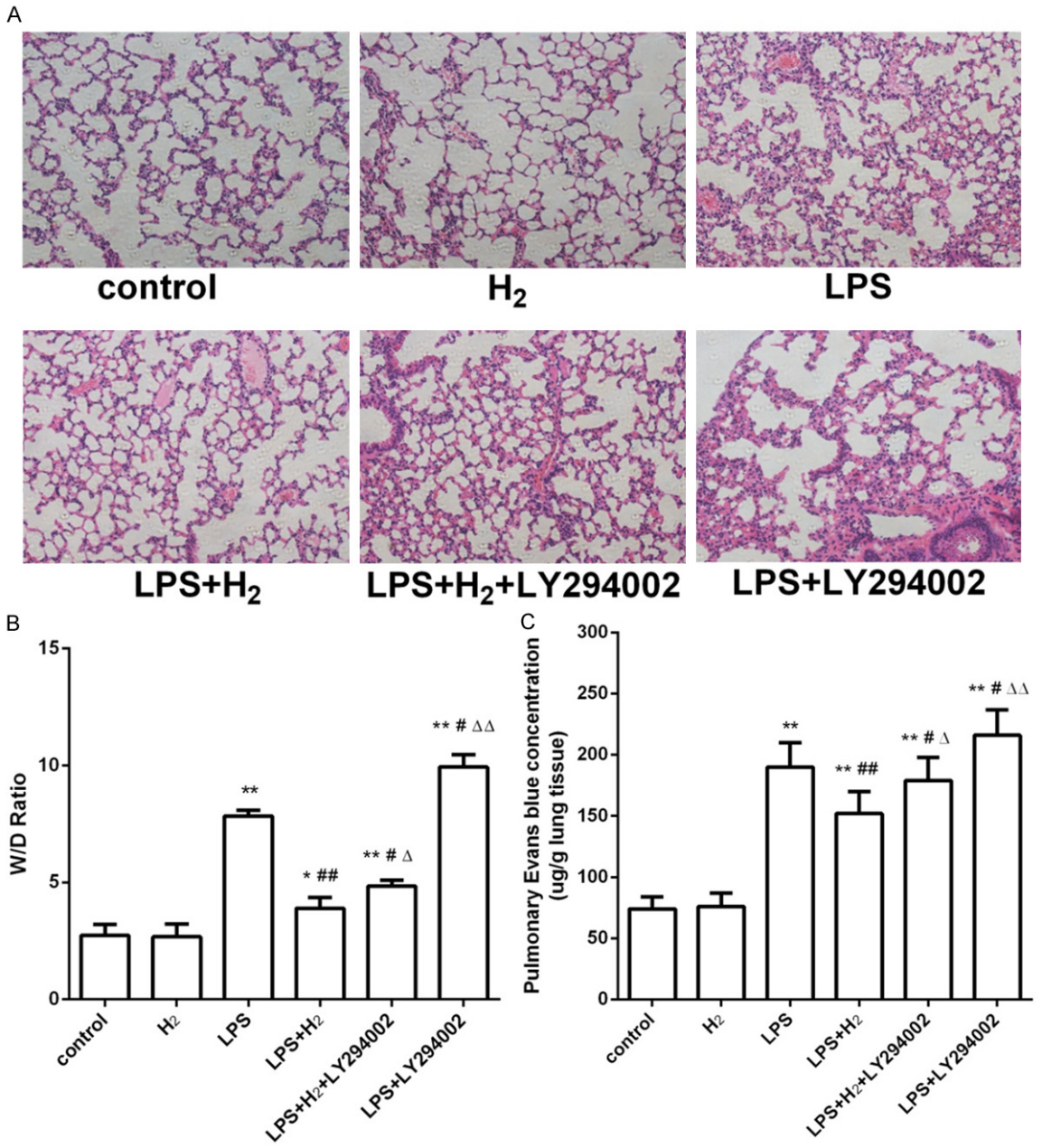

Figure 4. Effect of LY294002 on the lung pathology changes and pulmonary edema in LPS-challenged rats. A. The histopathological changes in the lung (hematoxylin and eosin, magnification × 200). B. Lung tissue wet to dry weight ratio (W/D ratio). C. Evans blue concentration in the alveolar was measured to evaluate the pulmonary endothelial permeability. Results shown were means ± SD (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LPS group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus LPS+H_2 group.

cytokines after LPS intratracheal injection, which indicated that LPS successfully induced ALI model in rats.

Lung-tissue edema is considered to be a critical stage in the pathophysiology of ALI. In this study, the lung wet/dry ratios and extravasation of Evans blue, which are used to evaluate the severity of pulmonary edema, were increased in LPS group as compared with that in control group. As reported in previous studies [17], the intraperitoneal injection of hydrogen-rich saline improved lung function and lung edema. We also found that LPS downregulated the expression of claudin-5 protein, which was prevented by hydrogen-rich saline treatment.

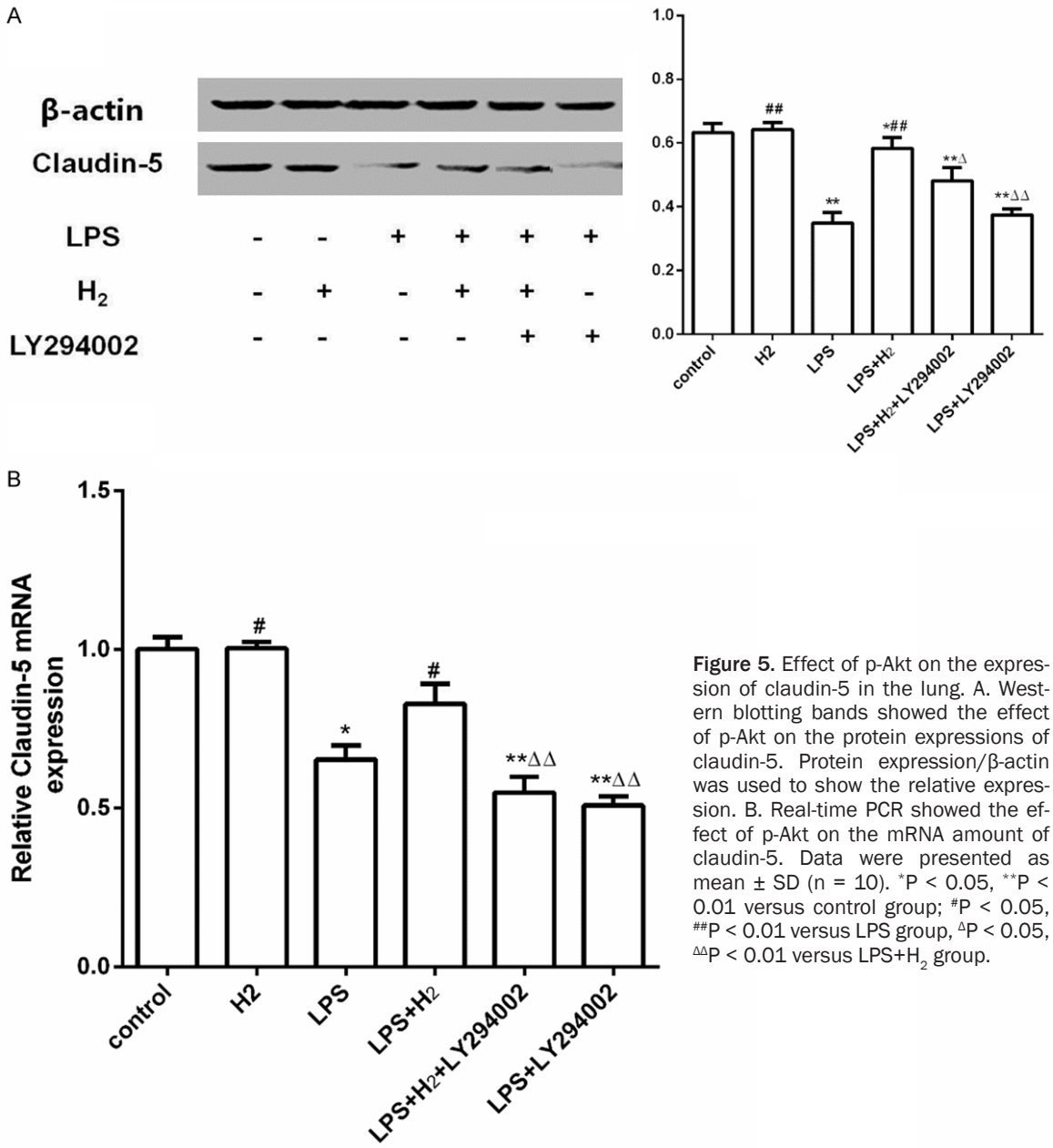

Figure 5. Effect of p-Akt on the expression of claudin-5 in the lung. A. Western blotting bands showed the effect of p-Akt on the protein expressions of claudin-5. Protein expression/β-actin was used to show the relative expression. B. Real-time PCR showed the effect of p-Akt on the mRNA amount of claudin-5. Data were presented as mean ± SD (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LPS group, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus LPS+H_2 group.

This is the first study to show that the protective effect of hydrogen-rich saline can be traced to its protective effect on the expression of claudin-5.

In the normal lung, claudin-5 is expressed strongly in endothelium and is considered a major contributor to the formation of endothelial tight junctions which control pericellular permeability [18]. Tight junctions located in the apicolateral membranes of epithelia form a barrier between adjacent cells and regulate the movement of ions and solutes across the paracellular space [19]. An important consequence of acute lung injury is the disruption of paracellular alveolar permeability barrier [20]. In addition to these components, the importance of claudins in pulmonary barrier function is underscored by the viability of occluding-deficient mice [21]. Inducing claudin-5 expression in leaky rat lung endothelial cells can help to restore paracellular barrier function [22]. In the present study, hydrogen-rich saline increased the expression of claudin-5 and alleviated the

lung morphological damage, indicating that claudin-5 may play a role in the development of pulmonary edema.

But how is the expression of claudin-5 regulated by LPS or hydrogen-rich saline?

In endothelia cells, the expression of claudin-5 protein is regulated through the activation of the PI3K/Akt pathway [9]. In the present study, we investigated whether hydrogen-rich saline treatment induced lung-protection is mediated, at least, partially via the PI3K/Akt pathway in the LPS-challenged rats. Our data showed that the expression of p-Akt increased in the LPS group rats. When treated with hydrogen-rich saline, the expression of p-Akt level upregulated to a higher level. However, the inhibitor of PI3K, LY294002, significantly suppressed the favorable effects of hydrogen-rich saline. And the pathology revealed that lung injury became more serious after LY294002 was injected in LPS+LY294002 group. Besides, most important is that the expression of claudin-5 in the LPS+H_2 group reduced markedly by pretreatment with LY294002, but the claudin-5 protein in the LPS+LY294002 group did not show much difference compared with the LPS group. Therefore, the results proved that LY294002 itself cannot change the expression of claudin-5, and the changes attributed to the expression of p-Akt. Our results showed that hydrogen-rich saline could protect claudin-5 protein from disruption via the PI3K/Akt signaling pathway.

LPS is known to enhance the formation of reactive oxygen species (ROS), inflammatory mediators, and promotes oxidative stress [23]. ROS and inflammatory mediators triggers significant disruption of tight junction proteins resulting in increased pulmonary permeability [24, 25]. Oxidative stress increases PI3K reaction products, which moderately triggers Akt phosphorylation within several cell types and animal models [26-28], but excessive oxidative stress, such as after LPS challenge, may lead to dephosphorylation of Akt [29]. In the present study, we concluded that hydrogen may markedly eliminate LPS-induced oxidative stress resulting in a decreased dephosphorylation of Akt, which mediates the protective effect of hydrogen-rich saline on the LPS-induced ALI. However, we could not conclude that PI3K/Akt pathway is the only signaling pathway involved in the beneficial of hydrogen. Further studies focused on exploring other signaling pathway will be needed.

Conclusion

In summary, our findings demonstrated that hydrogen-rich saline alleviated pulmonary edema through preventing the downregulation of claudin-5 in septic rat lungs, which may be at least partially mediated by the PI3K/Akt pathway. This is the first study to investigate the mechanism of hydrogen-induced protective effect on claudin-5 expression in LPS-induced ALI. Our study provides a potential new therapeutic target for septic lung injury.

Disclosure of conflict of interest

None.

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