EXPERIMENTAL STUDY

Effect of emodin on Aquaporin 5 expression in rats with sepsis-induced acute lung injury

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Abstract

Objective: To investigate the effects of emodin on aquaporin 5 (AQP5) expression in rats with sepsis-induced acute lung injury.

Methods: We divided 60 adult male Sprague-Dawley rats, weighing 200-230 g, into four groups: control, sham surgery, model and emodin groups (n = 15 for each). We created a sepsis model with cecal ligation and puncture; the sham surgery group had their cecums replaced after exposure outside the abdominal cavity. Each group was further divided into three subgroups (n = 5 for each) and expressions of AQP5 mRNA and proteins in lung tissue were measured by real-time fluorescence polymerase chain reaction and western blot at 6, 12 and 24 h after surgery.

Results: AQP5 expression did not change over time in the control group and sham surgery group, but decreased over time in the model group. The lowest expression was found in 12-h subgroup, which significantly differed from the 6-h subgroup (P < 0.01). Compared with the model group, AQP5 expression in the emodin group was significantly higher in all the subgroups (all P < 0.01). Expressions in the 12-h subgroup were the highest, and significantly differed from the other subgroups. We found that lung tissue damage, such as pulmonary edema, alveolar damage and the exudation of red blood cells in pulmonary interstitium and alveolar, was significantly milder in the emodin group under light microscope than the model group.

Conclusion: AQP5 expression was significantly down-regulated in rats with sepsis-induced acute lung injury induced by cecal ligation and puncture. Early prophylactic use of emodin can significantly enhance the AQP5 expression, thus effectively reducing the degree of pulmonary edema in septic rats.

Key words: Emodin; Sepsis; Acute lung injury; Aquaporin 5

Introduction

The lung is the most vulnerable target organ in sepsis and pulmonary injuries usually occur early during sepsis. Acute lung injury (ALI) is initiated by increased lung microvascular permeability, and characterized by pulmonary edema and atelectasis. Clinical manifestations of ALI are characterized by progressive hypox-
emilia and respiratory distress syndrome. The incidence of ALI in patients with severe sepsis is 25%-50%. Despite aggressive treatments, the mortality of ALI remains as high as 70%-90%, which indicates the severity of the disease. The 2012 International Severe Sepsis and Septic Shock Treatment Guidelines have improved management of the disease, however, treatment of sepsis remains a prominent challenge in critical care medicine. Therefore, it might be meaningful to explore potential traditional Chinese medicine (TCM) interventions.

TCM believes that the lung communicates with the large intestine and is the source of water, which affects the metabolism of water. Accordingly, we speculate that phlegm is associated with water metabolism of the lung during sepsis. However, studies in this area are relatively scarce. The sepsis animal model uses a classical cecal ligation and puncture (CLP) method, to investigate effects of emodin on aquaporin 5 (AQP5) expression in lung tissues of rats with sepsis-induced ALI; and to provide an experimental basis for clinical treatment with TCM.

**Materials and methods**

Animals and grouping: sixty male SD rats, weighing 200-230 g (Shanghai Super-B&K Laboratory Animal Corp., Ltd.), were fed a normal diet for a week. The animals were divided into four groups using random numbers table: control group (a), sham surgery group (b), model group (c) and emodin pre-treated group (d) (n = 15 for each). In the following 5 days, Group D received emodin at 5 mg/mL. Group B was pre-treated with 2.5 mg/kg sodium carboxymethyl cellulose solution by gavage. Group C received the same amount of 0.5% sodium carboxymethyl cellulose solution. Group A did not receive any intervention. Anesthesia, the cecum was exposed from the abdominal cavity and replaced 2 min later before closure of the abdomen. Group A did not receive any intervention. Animals were sacrificed at 6, 12 and 24 h after modeling (1 cm in length) was made after the rats were anesthetized, the cecum was exposed from the abdominal domen. Group A did not receive any intervention. Animals were divided into four groups using random numbers table: control group (a), sham surgery group (b), model group (c) and emodin pre-treated group (d) (n = 15 for each). In the following 5 days, Group D received emodin in sodium carboxymethyl cellulose suspension by gavage (35 mg·kg⁻¹·d⁻¹). Groups A, B and C received the same amount of 0.5% sodium carboxymethyl cellulose solution by gavage. Group B was prepared as follows 2 h after the last gavage: a midline incision (1 cm in length) was made after the rats were anesthetized, the cecum was exposed from the abdominal cavity and replaced 2 min later before closure of the abdomen. Group A did not receive any intervention. Animals were sacrificed at 6, 12 and 24 h after modeling in each group, each time point with 5 rats. Dead animals were replenished in time during the observation period.

Medications: emodin and sodium carboxymethyl cellulose were purchased from Shanghai Bo Gu Biotechnology Company (Shanghai, China). The emodin (2.5 g) was dissolved into 0.5% sodium carboxymethyl cellulose solution (500 mL) to prepare a suspension of emodin at 5 mg/mL.

Establishment of the animal model: the septic rat model was created with classical method of cecal ligation and puncture (CLP) first adopted by Chaudry et al. with procedures as follows. After 12 h of preoperative fasting, rats were anesthetized with intraperitoneal urethane injection (1250 mg/kg). A 1-cm incision was made along the abdominal midline and the mesentery and the cecum were dissociated before ligation of the cecum with 4-0 silk ring roots. A puncture was made at the end of the ligation with an 18-gauge needle before the bowels were replaced. The peritoneum and skin were then sutured.

Real-time fluorescence PCR was used to detect AQP5 mRNA in lung tissue: the right main bronchus was ligated; tissues in the mid-right lung were taken and placed immediately into liquid nitrogen tanks (−180 °C). After the tissue grinding, total tissue RNA extraction was done with Trizol; cDNA was synthesized with reverse transcription kit according to the pre-existing manual (Takara, Code No. RR036A). The 20-μL reaction system was established by referring to SYBRPremix Ex Taq kit instructions, in which 2 μL cDNA was amplified as a template and β-actin used as an internal reference in the PCR instrument, with the gene and primer sequences in Table 1. Reaction conditions were denaturation at 95 °C for 30 s, at 95 °C for 5 s and at 60 °C for 31 s with a total of 40 cycles. Three replicating tubes were set for each internal reference and sample tubes. After completion of the reaction, the fluorescence signal was automatically analyzed with ABI7300 SDS Software and converted into a Ct value. The final Ct value is the average of three replicating tubes. The value of △Ct indicates the difference in Ct value between the target gene AQP5 and internal reference. The 2-△△Ct method was used to calculate the ratio of fluorescence between the target gene and internal reference gene. Total RNA extraction was performed referring to the TRIPure Reagent protocols. After the purity and integrity of RNA extractions were measured by UV spectrophotometer and agarose gel electrophoresis, 1 μg RNA extraction was used to synthesize cDNA with the Prime Script RT Reagent Kit, with 2.5 μL cDNA as a template and β-actin as internal reference.

| Table 1 Gene and primer sequences |
|-----------------------------|-----------------------------|-----------------------------|
| Gene | Primer and probe | Synthesized sequence |
| β-actin | Forward primer | AAGGGAAATCGTGCCGTGAC |
| | Reverse primer | CGCTCATTTGCGGATGTC |
| | Probe | FAM-CTGTGCTATGTTGCCCTAGACTTC-TAMRA |
| AQP5 | Forward primer | GCATCTTTCTCTCCACCACGAC |
| | Reverse primer | TGACAGACACAGCCAATGGATAAG |
| | Probe | FAM-ACCAGGCCCTGTGGGCTCC-TAMRA |

Notes: AQP5: Aquaporin 5.
The 20 μL reaction system was established following SYBR Premix Ex Taq kit instructions and amplified in a thermocycler. The fluorescence intensity of the AQP1 target gene and expression of internal standard β-actin were detected; to compare the effects of interventions on target gene expression, the fluorescence ratio of the target gene and the internal standard gene were calculated with the 2-ΔΔCt method.

**Expression of AQP5 protein in lung tissue**
The mid-right lung tissue of the rats was homogenated to extract the proteins. The protein concentration of sample was assayed by BCA Protein Assay Kit, with each sample containing 50 μg of total protein sample solution. SDS-PAGE electrophoresis was used for protein separation. Membranes were transferred and incubated at room temperature with 5% bovine serum albumin (BSA) solution for 1 h before being irrigated with Tris Buffered Saline with Tween-20 (TBS/T). It was then incubated with the antibody overnight according to the instructions, before incubating with the reaction solution at room temperature and X-ray exposure. The images were scanned and saved as computer files; the gray value of each specific band was digitalized with the semi-quantitative ratio of AQP-5/B-actin, using image analysis software.

**Statistical analysis**
All results are shown as mean ± standard deviation (\(\bar{x} \pm s\)). Analysis of variance and multiple sample mean pairwise comparisons with Q tests were performed on SPSS13.0 Software (SAS Institute, Chicago, IL, USA). \(P < 0.05\) was considered significant.

**RESULTS**

**Pathological changes in the lung tissue**
Six hours later after modeling, rats presented with restlessness, piloerection, diarrhea, dyspnea and claw cyanosis; these symptoms deteriorated with time. A rat model was considered successfully established when bloody or purulent peritoneal exudates with malodorous smell, as well as flatulence, visceral congestion and edema were present after rats were sacrificed.

Lung tissues in Group C appeared larger with increased lung volume, swelling of cut surface, decreased flexibility and hemorrhage. Compared with Group D, lung tissues in Group C had mild edema and tiny spots of hemorrhage. Lung tissues in Group C also had varying degrees of widened alveolar walls, pulmonary interstitial edema, alveolar lumen narrowing and alveolar damage. Red blood cells effusions were seen in the alveolar and the interstitium under light microscope at 12 and 24 h in Groups C and D. Damage to lung tissues in Group D was significantly milder than in Group C at the same time point, with small amounts of red blood cells, compared with large effusion of red blood cells in Group C (Figure 1).

**Comparison of AQP5 mRNA in rat lung tissues of each group**
In Groups A and B, expression of AQP5 mRNA did not change in a timely manner, but decreased over time in Group C, which reached the lowest level in the 12-h subgroup, and was significantly lower than in the 6-h subgroup (\(P < 0.01\)). Expression in the 24-h subgroup rebounded slightly, but did not significantly differ from the 12-h subgroup.

Expression of AQP5 mRNA in Group D was significantly higher than that in Group C (\(P < 0.01\)) at all time points and reached the highest at 12 h time point (\(P < 0.01\) vs 6 h; \(P < 0.01\) vs 24 h; Table 2, Figure 2).

**Comparison of AQP5 protein expressions among groups**
In Groups A and B, expression of AQP5 protein did not change over time. In Group C, AQP5 protein expression declined by varying degrees and was lowest in the 12-h subgroup, which differed significantly from the 6-h subgroup (\(P < 0.01\)), but expression rebounded slightly for the 24-h subgroup. AQP5 protein expression in Group D was significantly higher than Group C (\(P < 0.01\)) at all time points, which was the highest in the 12-h subgroup in all subgroups and differed significantly from the 6-h subgroup (\(P < 0.01\)) and the 24-h subgroup (\(P < 0.01\); Table 3, Figure 3).
DISCUSSION

Abundant inflammatory mediators and lipid metabolites are produced during sepsis, which recruit and activate inflammatory cells in lung tissues and cause an inflammatory reaction cascade by further production of cytokines, chemokines, oxygen free radicals and proteases. These reactions would not only damage and increase the permeability of alveolar and vascular epithelial cells, but also affect the sodium-water transport system and lead to production of surface-active substances. Protein-rich and cellular-rich fluids would penetrate into the lung tissue at faster rates, thus predisposing the tissue to develop acute pulmonary edema, leading to the formation of transparent membrane and alveolar collapse accompanied with pulmonary interstitial fibrosis. Previous understanding of transmembrane transport of water molecules as a form of simple diffusion failed to explain the phenomenon that water could rapidly get through the lipid bilayer membrane. The discovery of aquaporins in 1991 changed the traditional theory about the transmembrane mechanism of water molecules.

Table 2 Comparison of Aquaporin-5 mRNA expression in each group rats ($\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>1.01±0.04a</td>
<td>0.99±0.12a</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>0.96±0.05b</td>
<td>0.96±0.04b</td>
<td>1.06±0.21</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>0.68±0.13ab</td>
<td>0.34±0.07bd</td>
<td>0.47±0.12ab</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>1.46±0.11abc</td>
<td>4.97±0.47abc</td>
<td>3.35±0.89abc</td>
</tr>
</tbody>
</table>

Notes: A: control group treated with the same amount of 0.5% sodium carboxymethyl cellulose solution 5 days by gavage; B: sham group treated with exposing the cecum from the abdominal cavity and restoring it before closing the abdomen; C: model group treated with classical method of cecal ligation and puncture; D: emodin pre-treated group treated with emodin sodium carboxymethyl cellulose suspension 35 mg·kg$^{-1}·$d$^{-1}$ 5 days by gavage before surgery.

Table 3 Comparison of Aquaporin-5 protein expression in each group rats ($\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>0.60±0.084a</td>
<td>0.61±0.063a</td>
<td>0.62±0.093a</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>0.62±0.081b</td>
<td>0.62±0.051b</td>
<td>0.62±0.111b</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>0.39±0.072ab</td>
<td>0.24±0.041bd</td>
<td>0.32±0.023bd</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>0.86±0.124abc</td>
<td>1.89±0.352abc</td>
<td>1.25±0.104abc</td>
</tr>
</tbody>
</table>

Notes: A: control group treated with the same amount of 0.5% sodium carboxymethyl cellulose solution 5 days by gavage; B: sham group treated with exposing the cecum from the abdominal cavity and restoring it before closing the abdomen; C: model group treated with classical method of cecal ligation and puncture; D: emodin pre-treated group treated with emodin sodium carboxymethyl cellulose suspension 35 mg·kg$^{-1}·$d$^{-1}$ 5 days by gavage before surgery. *P < 0.01, compared with the model group; ’P < 0.01, compared with the control group; ’’P < 0.01, compared with the same group at 6 h; ’’’P < 0.01, compared with the same group at 12 h.
Two main ways of water transportation are seen in the alveoli: secondary transported accompanying active transportation of sodium; or transported through the AQPs in the alveolar epithelium. AQPs, the general term for a large family of homologous proteins, is a type of water channel subject to gene regulations. It is responsible for transport of liquid under physiological conditions and may be associated with the imbalance of fluid transport in pathological conditions. AQPs identified in mammals have 11 subtypes (AQP0-AQP10), with the lung containing mostly AQP1 and AQP5. AQP1 is located mainly in the mesothelial cells of capillaries, lymphatic tissues and visceral pleura of the respiratory tract, some are found with small amounts in the apical membrane of type II alveolar epithelial cells. AQP5 is found predominantly in the apical membrane type I alveolar epithelial cells and endothelial cells in the airway. The specific functions of AQP1 and AQP5 are closely related to their distributions. AQP1 affects transport of liquid in lung interstitium, and AQP5 is important for liquid reabsorption in the alveolar space. Reportedly, water permeation through the alveolar capillary in AQP5 knockout mice was ~10% that in wild-type mice. Furthermore, removing AQP1 and AQP5 at the same time led to water permeability of only 1/25 to 1/30 that of a normal mouse. Several studies have shown that expression of AQP5 was decreased in many animal models of lung injury, such as adenovirus infection, lipopolysaccharide (LPS) induction and hyperoxia stimulation. In acute pancreatitis-induced acute lung injury, AQP5 expression was significantly reduced in the first 4-12 h after the injury, but increased 12 h after the injury with alleviation of pulmonary edema. Moreover, AQP5 expression was found to significantly increase in hypertonic environments, such as in seawater-induced ALI model. In addition, in bleomycin-induced ALI model, AQP5 expression was also increased. Both results show that AQP5 expression is related to the ALI is induced. In the sepsis-induced ALI of the present study, we found that expression of AQP5 mRNA decreased over time (0.34 ± 0.07 at 12 h versus 0.68 ± 0.13 at 6 h, P < 0.01), with a similar trend for AQP5 protein expression. Despite the various causes, a hallmark of ALI is reduced AQP5 expression in damaged lung tissues. Rhubarb is a representative of "dismount" drugs in TCM. Modern medical research has found various pharmacological effects of rhubarb. It could inhibit systemic inflammatory responses, protect the intestinal mucosal barrier, effectively alleviate the toxic intestinal paralysis and improve tolerance to enteral nutrition in critically patients. Recent studies also found rhubarb scavenged oxygen free radicals and alleviated intestine-induced lung injury. Emodin, as one of the main components of rhubarb, can reduce Ca²⁺ influx by blocking calcium channels in inflammatory cells, thereby inhibiting uncontrolled release of NF-kB and blocking MAPKs signal transduction pathway to inhibit produc- tion and release of various cytokines, such as TNF-a, IL-2, IL-6, IL-8 and IL-10. It could also prevent activation of neutrophils and the amplification of inflammatory mediators. Recent animal studies showed the classical function of rhubarb (purgative and diuretic) was associated with down-regulation of AQP4 in colonic mucosal epithelial cells and expression of AQP2 in normal rat kidney cells.

In this experiment, AQP5 protein expression in the Emodin group was significantly higher than the model group (P < 0.01) for all subgroups. Expression in the 12-h subgroup was significantly higher than 6- and 24-h subgroups (P < 0.01). Our results indicate that the expression of AQP5 in lung tissues significantly increased in rats with sepsis-induced ALI after emodin pretreatment. Many current studies have focused on the treatment of ALI with Salvia. Some studies showed that Tanshinone could alleviate pulmonary edema and vascular leakage in rats with seawater-induced ALI, increasing expression of AQP1 and AQP5 in the lung tissue; Others found that salvia acid B pretreatment can improve lung microcirculation and subsequent ALI caused by LPS. However, studies of the effects of emodin on expression of AQPs in sepsis-induced ALI lung tissues are limited. Clinical trials have shown shorter duration of ventilator support and lower mortality in patients with high lung water clearance rate. Therefore, enhancing water clearance by increasing AQP5 expression is a key issue to a better prognosis in critically-ill patients and might be a potentially effective treatment for sepsis-induced ALI.

REFERENCES


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