Emodin enhances alveolar epithelial barrier function in rats with experimental acute pancreatitis

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Abstract

AIM: To investigate the effect of emodin on expression of claudin-4, claudin-5 and occludin, as well as the alveolar epithelial barrier in rats with pancreatitis induced by sodium taurocholate.

METHODS: Experimental pancreatitis was induced by retrograde injection of 5% sodium taurocholate into the biliopancreatic duct. Emodin was injected via the external jugular vein 3 h after induction of acute pancreatitis. Rats from sham operation group and acute pancreatitis group were injected with normal saline (an equivalent volume as emodin) at the same time point. Samples of lung and serum were obtained 6 h after drug administration. Pulmonary morphology was examined with HE staining. Pulmonary edema was estimated by measuring water content in lung tissue samples. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels, and wet/dry ratio were decreased in rats after treatment with emodin. Immunostaining of claudin-4, claudin-5 and occludin was detected in lung tissue samples from rats in sham operation group, which was distributed in alveolar epithelium, vascular endothelium, and bronchial epithelium, respectively. The mRNA and protein expression levels of claudin-4, claudin-5 and occludin in lung tissue samples were markedly decreased, the expression level of claudin-4, claudin-5 and occluding was increased, and the pulmonary dye extravasation was reduced in lung tissue samples from rats with acute pancreatitis after treatment with emodin.

CONCLUSION: Emodin attenuates pulmonary edema and inflammation, enhances alveolar epithelial barrier function, and promotes expression of claudin-4, claudin-5 and occludin in lung tissue samples from rats with acute pancreatitis.

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Key words: Acute pancreatitis; Emodin; Lung injury; Claudin; Occludin; Epithelial barrier

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INTRODUCTION

Acute pancreatitis is a common disease with a considerable morbidity and mortality of 20%-30%. Its mortality is attributed to inflammation-related complications, such as pancreaticitis-associated lung injury, clinically presenting as adult respiratory distress syndrome (ARDS). Intervention can reduce its morbidity and mortality, although its mechanism remains unclear.

Pancreatitis-associated lung injury is characterized by significant pulmonary edema, hyperemia and inflammatory infiltration in alveoli. It has been established that pulmonary edema is related to increased permeability and loss of barrier function. Although elevated levels of pancreatic enzymes and pro-inflammatory cytokines are attributed to pulmonary vasculature damage and increased endothelial permeability, the molecular basis for these damages remains largely undefined. Tight junctions are intimately involved in epithelial and endothelial permeability. Fernandez et al. recently demonstrated that claudins, the key components of tight junctions, restrict the paracellular movement of water, proteins, and solutes across cellular barriers including alveolar epithelium. In mammals, the claudin family includes at least 24 members. With small interfering RNA and a blocking peptide, Wray et al. described that inhibition of claudin-4 decreases transepithelial electrical resistance in primary rat and human epithelial cells, as well as air space fluid clearance, resulting in pulmonary edema in mice, suggesting that claudin-4 plays an important role in alveolar epithelial barrier function. Moreover, claudin-5 and occludin are also decreased in models of acute lung injury accompanying increased paracellular permeability, indicating that claudin-5 and occludin may also play a role in alveolar epithelial barrier function. However, the relation between expression of claudin-4, claudin-5, and occludin in lung tissues of patients with acute pancreatitis and pancreatitis-associated lung injury remains largely undefined.

It was reported that emodin (1,3,8-trihydroxy-6-methyl-anthaquinone), an anthraquinone derivative from the Chinese herb Radix et Rhizoma Rhei, inhibits the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α). Our previous study demonstrated that emodin significantly reduces serum TNF-α and interleukin-6 (IL-6) levels, thus attenuating lung injury in rats with acute pancreatitis. The effect of emodin on pulmonary tight junction expression and alveolar epithelial barrier function, however, needs to be further defined.

In the present study, the effect of emodin on pancreatitis-associated lung injury and alveolar epithelial barrier function was assessed by examining pulmonary morphology, myeloperoxidase (MPO) activity (indicator of inflammatory infiltration), expression of claudin-4, claudin-5 and occludin, as well as dye extravasation, in lung tissue samples from rats with acute pancreatitis.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawely rats, weighing 200-250 g, obtained from Animal Facility of Jinling Hospital (Nanjing, China), were housed under controlled temperature and humidity in a day-night cycle, with free access to standard laboratory food and water. The study was approved by Animal Studies Ethics Committee of Jinling Hospital.

Experiment model

Acute pancreatitis was induced as previously described. Briefly, animals were anesthetized with intraperitoneal ketamine (80 mg/kg) and acepromazine (2.5 mg/kg). The biliopancreatic duct was cannulated through the duodenum, and the hepatic duct was closed with a small bulldog clamp. Pancreatitis was induced by retrograde injection of 5% sodium taurocholate (Sigma, St. Louis, MO, USA) into the biliopancreatic duct (1 mL/kg body weight), at a constant infusion pressure of 20 mmHg. Rats in sham operation group received retrograde sterile saline infusion.

Experiment design

Effect of emodin on expression of claudin-4, claudin-5 and occludin, as well as on pulmonary dye extravasation, a marker to evaluate alveolar epithelial barrier, was detected in rats with acute pancreatitis. Time course of pulmonary edema and inflammation was recorded. Rats with acute pancreatitis were randomly allocated into pancreatitis group and emodin treatment group. Rats in pancreatitis group were injected with emodin (2.5 mg/kg body weight) via the external jugular vein 3 h after sodium taurocholate infusion. Rats in sham operation group were injected with normal saline (equivalent volume as emodin) at the same time point and served as a control group.

Lung tissue samples were obtained 6 h after emodin injection, and maintained at -80°C until assay. Blood samples were obtained from the inferior cava vein by direct puncture. Lung tissue samples were fixed in 4% neutral phosphate-buffered formalin and embedded in paraffin wax for histology examination. Serum amylase activity was detected to confirm the appropriate induction of pancreatitis.

Measurement of serum amylase level

Serum amylase level was measured by incubating serum with 4,6-ethylidene (G7)-p-nitrophenyl (G1)-1-D-maltoheptoside for 2 min at 37°C, with its absorbance detected once a minute for 2 min at 405 nm by high through universal microplate assay (BMG Lab Technologies, Germany).
Western blotting analysis was performed as previously de-
scribed[19]. Total protein (20 μg) was separated from each sample by electrophoresis on a 4%-20% SDS-polyacryl-
amide gel and electroblotted onto polyvinylidene difluo-
ride membranes. Membranes were blocked in a blocking
solution, incubated overnight with primary antibodies,
and developed with a horseradish peroxidase-conjugated
secondary antibody (Kangchen Biotech, Shanghai, China)
diluted at 1:1000. Primary antibody (Zymed Laboratories,
South San Francisco, CA, USA) was diluted as follows: claudin-4 at 1:100, claudin-5 at 1:100, and occludin at 1:300.
The immune complexes were then visualized on X-ray
film using chemiluminecent HRP substrate (Millipore,
Boston, MA, USA). Additional immunoblots were per-
formed using GAPDH antibody (Abcam, OFW, UK) as
the primary antibody to evaluate equal loading.

**Immunohistological analysis**

Lung tissue sections (4 μm) were dewaxed in graded alco-
hols, and washed with tap water. Endogenous peroxidase
activity was blocked with 3% (v/v) H2O2, and antigen was
retrieved with microwave in 0.01 mol/L citrate buffer.
The sections were then washed with phosphate-buffered
saline (PBS, 0.1 mol/L). Mouse anti-rat claudin-4 and
claudin-5, and rabbit anti-rat occludin polyclonal antibod-
ies (Zymed Laboratories, South San Francisco, CA, USA)
diluted at 1:100 and incubated overnight at 4°C. The
sections were washed 4 times with PBS, 5 min once. Pow-
er vision two-step histostaining reagent (ImmunoVision
Technologies, Norwell, MA, USA) was used for detection
of claudin and occludin expression. All sections were de-
veloped using diaminobenzidine and counterstained with
hematoxylin.

**Quantitative reverse transcriptase-polymerase chain
reaction analysis**

Total RNA was extracted with a TRIzol kit (Invitrogen
Carlsbad, CA, USA) and converted to cDNA with a
first strand cDNA synthesis kit (Fermentas, Burlington,
Canada). Quantitative reverse transcriptase-polymerase
chain reaction (RT-PCR) was performed using SYBR
Green SuperMix-UDG (Invitrogen Carlsbad, CA, USA).
The primer sequences used for PCR are as follows: clau-
din-4 (forward 5'-CCITTTCCCATAGGCTTGTGCT-3',
reverse 5'-CCGGTACCTTCCACAGACTG-3'), claudin-5
(forward 5'-TCTCAGCAGCCAAGGCAACCAC-3',
reverse 5'-GGGGCTTCCCACAGCTGGT-3'), occludin
(forward 5'-AGTACATTGGCTGCTGATG-3', reverse
5'-CCCACCATTCTCCTGATG-3'), GAPDH
(forward 5'-CAGTCCAGCCTCGTCGCTCATA-3', reverse
5'-TGCGCTGGTAGATGTCATA-3'). Amplifica-
tion was performed at 50°C for 2 min (UDG incubation),
at 95°C for 2 min, followed by 40 cycles of denaturing at
95°C for 15 s and annealing at 60°C for 30 s. All reactions
were performed in triplicate. Melting curve analysis was
performed to ensure the specificity of quantitative PCR.
Data analysis was performed as previously described[20],
with GAPDH used as a reference gene.
**RESULTS**

**Emodin attenuated pulmonary edema and inflammation in rats with acute pancreatitis**

The appropriate induction of pancreatitis-associated lung injury was demonstrated by histology and elevated serum amylase activity (Figure 1 and Table 1). Lung injury was characterized by pulmonary edema, leukocyte infiltration, and alveolar collapse. Pulmonary pathological scores and serum amylase activity were significantly lower after treatment with emodin.

Pulmonary edema was evaluated by measuring the water content in lung tissue samples and expressed as wet/dry ratio, which was significantly decreased after treatment with emodin (Table 1).

In the present study, the effect of emodin on pulmonary inflammation and MPO activity was evaluated. The TNF-α and IL-6 levels and MPO activity were decreased after treatment with emodin (Table 2).

**Emodin promoted expression of claudin-4, claudin-5 and occludin in rats with acute pancreatitis**

The expression levels of claudin-4, claudin-5, and occludin were markedly lower in experimental group than in control group (data not shown). Immunolocalization of claudin-4, claudin-5 and occludin in lung tissue samples was investigated with immunohistochemical staining. Moderate immunostaining of claudin-4, claudin-5 and occludin in control group, which was distributed in alveolar epithelium, vascular endothelium, and bronchial epithelium, respectively (Figure 2A, D and G). Immunostaining of claudin-4, claudin-5, and occludin was markedly decreased in experimental group (Figure 2B, E and H), and moderately elevated after treatment with emodin (Figure 2C, F and I).

RT-PCR analysis showed that emodin could increase the expression levels of claudin-4, claudin-5, and occludin mRNA in rats with acute pancreatitis (Figure 3A).

Western blotting analysis showed that the expression levels of claudin-4, claudin-5, and occludin were significantly higher after treatment with emodin in each experimental group than in control group, which was investigated with immunoblotting analysis. In the present study, we identified the down-regulation of claudin-4, claudin-5, and occludin in rats with acute pancreatitis induced by sodium taurocholate. Intravenous administration of emodin promoted the down-regulation of tight junctions, enhanced alveolar epithelial barrier function, attenuated pulmonary edema and inflammatory infiltration in rats with acute pancreatitis.

**Emodin enhanced alveolar epithelial barrier function in rats with acute pancreatitis**

The pulmonary dye extravasation, as a marker of local paracellular permeability, was significantly reduced in rats with acute pancreatitis after treatment with emodin, indicating that emodin can augment alveolar epithelial barrier function (Figure 4).

**DISCUSSION**

In the present study, we identified the down-regulation of claudin-4, claudin-5, and occludin in rats with acute pancreatitis induced by sodium taurocholate. Intravenous administration of emodin promoted the down-regulation of tight junctions, enhanced alveolar epithelial barrier function, attenuated pulmonary edema and inflammatory infiltration in rats with acute pancreatitis.

Among the systemic complications of severe acute pancreatitis, pulmonary complication, also known as pancreatitis-associated lung injury, is the most frequent and serious[3]. Pancreatitis-associated lung injury is characterized by significant pulmonary edema, hyperemia and inflammatory infiltration in alveoli[4]. Increased interstitial edema cuts down the transport of carbon dioxide through the alveolar barrier, causing respiratory distress syndrome. It has been recently reported that claudins, the key components of tight junctions, restrict paracellular movement of water, proteins, and solutes across cellular barriers including pulmonary vascular endothelium and alveolar epithelium[5]. Disruption of claudins impairs barrier function and increases paracellular permeability, which may allow noxious contents to enter pulmonary interstitium and alveoli, further aggravating pulmonary edema and inflammation[5].

Recently, several studies have demonstrated the localization and function of claudin-4 in pulmonary cellular barriers[6-8]. In human airway epithelia, elevated claudin-4 level is associated with increased transepithelial electrical resistance, indicating that claudin-4 plays a role in alveolar epithelial barrier function[7-9]. Although increased claudin-4 expression has been found in a mice model of acute lung injury, inhibition of claudin-4 can lead to pulmonary edema in mice by decreasing transepithelial electrical

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**Table 1** Serum amylase level and pulmonary pathological scores in different groups (mean ± SD)

| Group       | Amylase (U/mL) | Edema (wet/dry) | Pathological score |
|-------------|----------------|-----------------|--------------------|
| Sham        | 3.5 ± 0.4      | 1.7 ± 0.2       | 0.3 ± 0.1          |
| Pancreatitis| 13.8 ± 1.6     | 6.4 ± 0.5       | 7.4 ± 0.8          |
| Emodin      | 8.2 ± 1.1      | 4.1 ± 0.6       | 4.8 ± 0.7          |

**Table 2** Pulmonary cytokines and MPO in different groups (mean ± SD)

| Group    | TNF-α (pg/mg protein) | IL-6 (pg/mg protein) | MPO (U/mg protein) |
|----------|-----------------------|----------------------|-------------------|
| Sham     | 2.8 ± 0.3             | 25.8 ± 2.9           | 3.1 ± 0.3         |
| Pancreatitis | 34.1 ± 3.5           | 114.4 ± 12.6         | 25.8 ± 2.9        |
| Emodin   | 29.4 ± 2.4            | 93.0 ± 9.8           | 20.3 ± 1.9        |

Six rats were studied in each experimental group. *P < 0.05 vs sham group; †P < 0.05 vs pancreatitis group.

**References**

[1] Pancreatitis, pulmonary complication, also known as pancreatitis-associated lung injury, is the most frequent and serious.

[2] Disruption of claudins impairs barrier function and increases paracellular permeability, which may allow noxious contents to enter pulmonary interstitium and alveoli, further aggravating pulmonary edema and inflammation[5].

[3] Six rats were studied in each experimental group. *P < 0.05 vs sham group; †P < 0.05 vs pancreatitis group.

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**Statistical analysis**

Data were expressed as mean ± SD. ANOVA was used to analyze differences between experimental and control groups. Student-Newman-Kleus method was used for multiple pair-wise comparisons. All statistical analyses were carried out using the SPSS version 11.5 for Windows (Chicago, IL, USA). P < 0.05 was considered statistically significant.
resistance and air space fluid clearance, suggesting that claudin-4 plays an important role in alveolar epithelial barrier function, and early increased claudin-4 expression may represent a mechanism by which pulmonary edema is limited. Similar to claudin-4, claudin-5 also plays a role in cellular barrier function. Recombinant claudin-5 protects brain microvascular endothelial cell cultures against increased paracellular permeability induced by VEGF, showing that claudin-5 is a key determinant of blood-brain barrier function. It has been recently reported that expression of pulmonary claudin-5 is decreased in models of carrageenan-induced acute lung inflammation, associated with the decreased pulmonary paracellular permeability, suggesting that claudin-5 may play role in alveolar epithelial barrier function.

Occludin shares a very similar membrane location with claudin. Based on the staining feature of claudins and occludin along the endothelial cell borders, Persid...
In the present study, we identified the localization of claudin-4, claudin-5, and occludin in lung tissue samples from rats with acute pancreatitis, and found that claudin-4 and claudin-5 were uniformly and continuously distributed along the alveolar epithelium and vascular endothelium in normal lung tissue samples, which are consistent with the reported findings[9-12]. Furthermore, occludin was uniformly and continuously distributed along the alveolar epithelium, vascular endothelium, and bronchiolar epithelium, which is in line with the reported results[10,12]. In this study, RT-PCR and Western blotting showed that the expression of claudin-4, claudin-5 and occludin was down-regulated in lung tissue samples from rats with acute pancreatitis. Aggravated pulmonary edema and increased paracellular permeability (marked by extravasation of Evans blue) were in parallel with the down-regulation of claudin-4, claudin-5 and occludin expression, which is consistent with the findings in previous studies[9-12], suggesting that claudin-4, claudin-5 and occludin may play a role in alveolar barrier function.

In the present study, emodin significantly promoted the expression of claudin-4, claudin-5 and occludin at mRNA transcription and protein synthesis level, and decreased pulmonary edema and paracellular permeability. Based on the previous and present studies, we speculate that emodin may contribute, in part at least, to the expression of claudin-4, claudin-5 and occludin by increasing the alveolar barrier function.

Emodin has long been used for anti-inflammatory purposes. Many studies have demonstrated that emodin intervention can significantly decrease TNF-α and IL-6 levels, or MPO activity in lung tissues[13,23,24], and the mechanism of emodin underlying cytokine inhibition is involved in NF-κB activity suppression[23-26]. Moreover, emodin also has antioxidant effects, promotes generation of ATP and antioxidant components, such as glutathione, α-tocopherol, and superoxide dismutase[27,28], and exhibits a promising free radical scavenging activity[29]. It has been shown that emodin markedly reduces serum amylase, TNF-α and IL-6 levels, attenuates lung damage in rats with acute pancreatitis[14,29,30], which is in line with the present study. Considering that MPO activity is a marker of local leukocyte sequestration[30], the results of our present study suggest that emodin ameliorates pancreatitis-associated lung injury by inhibiting the production of cytokines and the infiltration of leukocytes in lungs.

**Figure 3** Effects of emodin on claudin-4 (A), claudin-5 (B), and occludin (C) mRNA transcription and protein synthesis in rats. Six rats were studied in each experimental group. Results are expressed as mean ± SD. *P < 0.05 vs sham group; †P < 0.05 vs pancreatitis group.

**Figure 4** Effects of emodin on alveolar epithelial barrier in rats. Six rats were studied in each experimental group. Results are expressed as mean ± SD. *P < 0.05 vs sham group; †P < 0.05 vs pancreatitis group.
In conclusion, emodin can attenuate pulmonary edema and inflammation, enhance alveolar epithelial barrier function, and promote expression of claudin-4, claudin-5 and occludin in lung tissues.

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