Research Article

Effects of Daphnetin on Experimental Acute Pancreatitis-Associated Acute Lung Injury in Mice

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Background and Aim. Daphnetin, an active monomer ingredient extracted from D. marginata, is proved to have anti-inflammatory and antioxidant effect. The aim of this study is to explore the effect and possible mechanism of daphnetin on acute lung injury (ALI) associated with acute pancreatitis (AP) in mice.

Methods. A total of 36 mice were randomly assigned into three groups: control group, AP group, and daphnetin group. The mouse model of AP was induced by caerulein and lipopolysaccharide. Animals were sacrificed at 6 and 12 h after daphnetin treatment, respectively. The pathological changes of lung and pancreas were determined by hematoxylin-eosin staining and the pathological scores. Levels of IL-1β, IL-6, and TNF-α in serum and lung and the activity of myeloperoxidase (MPO) in lung tissue homogenate were detected by ELISA. The protein level of toll-like receptor 4 (TLR4), phospho-nuclear factor-kappa B p65 (p-NF-κB p65), nuclear factor-kappa B p65 (NF-κB p65), and hypoxia-inducible factor 1 alpha (HIF-1α) in the lung was detected by Western blot.

Results. Results showed extensive neutrophil infiltration, hemorrhage, and edema in the pancreas tissues or lung tissues in mice with AP. The daphnetin treatment improved pathological changes in the lung tissues of AP mice. The MPO activity and the levels of inflammatory cytokines including IL-1β, TNF-α, and IL-6 of lung tissues and serum in the AP group were significantly higher than those in the control group (P < 0.05), and daphnetin intervention significantly reversed the changes (P < 0.05). Compared with the control mice, the protein levels of TLR4, p-NF-κB p65, and HIF-1α were significantly higher in the lung tissue of the AP mice (P < 0.05), while daphnetin treatment decreased these protein expression levels. No significant difference was observed in the NF-κB p65 level among control, AP, and daphnetin groups (P > 0.05). Conclusions. Daphnetin exerted a protective effect on the acute lung injury induced by SAP in mice. The mechanism may be related to the regulation of TLR4/NF-κB/HIF-1α pathway to reduce the release of inflammatory factors.

1. Introduction

Acute pancreatitis (AP) is a relatively common abdominal organ disorder, and severe acute pancreatitis (SAP) is associated with a high mortality rate of 20%–30% [1]. Acute lung injury (ALI) is a major complication of SAP, with approximately 20% of patients developing acute respiratory distress syndrome (ARDS) [2, 3]. ALI and ARDS are two types of severe pancreatitis-associated acute lung injury (PALI), which is one of the predominant causes of death during the early stages of SAP [4, 5]. Vascular endothelial cells and pulmonary alveolar epithelial cells are compromised by oxidative stress, which is the main pathophysiologic characteristic [6, 7]. However, the mechanisms of PALI are complicated and incompletely understood. Thus, greatly understanding its regulatory network is essential for the treatment of ALI induced by SAP.

SAP starts as an inflammation of local pancreatic acini [8]. Nuclear factor (NF)-κB activation is considered to be a key link in the pathogenesis of SAP [9]. TLR4 is a transmembrane protein that is mainly expressed in immune cells such as macrophages and activates NF-κB through biological signals; studies have reported that it is essential for the activation of NF-κB [10–12]. Hypoxia-inducible factor 1α (HIF-1α) is
activated by lipopolysaccharide (LPS) in a TLR4-dependent manner and promotes the production of inflammatory cytokines, including TNF-α, IL-1, and IL-6 [13, 14]. Besides, a large quantity of proinflammatory cytokines can be released as a result of NF-κB activation in the process of pancreatitis, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 [11, 15]. Inflammatory factors such as TNF-α, IL-1, and IL-6 are closely related to SAP-related acute lung injury. In the early stage of SAP inflammatory response, the damaged pancreatic tissue produces and releases a large amount of TNF-α, IL-1, and IL-6, promoting the systemic inflammatory response, leading to systemic inflammatory response syndrome (SIRS) and even multiple organ dysfunction syndrome (MODS), and lung is the most common organ involved in SAP [16, 17].

Daphnetin, an active monomer ingredient extracted from D. marginata, has been widely used to treat rheumatoid arthritis and systemic lupus erythematosus [18]. Daphnetin can decrease the TNF-α and IL-1 level of serum and is proved to have anti-inflammatory and antioxidant effect [18]. Recently, it has been shown that daphnetin can inhibit the secretion of inflammatory factors in pancreatic inflammatory response and reduce oxidative stress and inflammatory response, thereby alleviating pancreatic injury in SAP rats [19]. However, the relevant role of daphnetin in acute lung injury in mice with SAP needs to be further explored. The present study was planned to explore the effect of daphnetin on SAP-related acute lung injury and further investigate its potential mechanism.

2. Materials and Methods

2.1. Animals. Male C57BL mice, 6–8-week-old, weighing 18–20g, were purchased from SPF Biotechnology Co., Ltd. (Beijing, China), and housed in rooms that controlled temperature (21–24°C) and maintained light/dark cycle (12:12) for 1 week to acclimate to the surroundings, with free access to tap water and standard laboratory chow. The animal experiments were approved by the Animal Care and Welfare Committee of Southwest Medical University (ethical protocol code: SWMU20210418) and conducted according to the guidelines of the Local Animal Use and Care Committees of Luzhou as well as the National Animal Welfare Law of China.

2.2. Reagents. Caerulein (Cae) was obtained from MCE (China), and LPS was purchased from Sigma-Aldrich (USA). Daphnetin was supplied by Shanghai Tauto Biotech Co. (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, IL-1β, and IL-6 were purchased from ELK Biotechnology (Wuhan, China). Myeloperoxidase (MPO) kits were obtained from Jiancheng Company (Nanjing, China). Antibodies against TLR-4 (#14358), NF-κB (IL-1β), and HIF-1α (#36169) were obtained from Cell Signaling Technology (CST, USA). Antibodies for HMGB1 (ab18256) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ab8245) were obtained from Abcam (USA).

2.3. Animal Treatment. Mice were divided randomly into experimental groups as follows: control group (n = 12), AP group (n = 12), and daphnetin group (n = 12, treated with daphnetin). Each group was then randomly subdivided into two subgroups as 6 and 12h according to the time course in the experiment, with 6 rats in each subgroup. Before the induction of AP, mice were fasted for 12h, but drinking water was available ad libitum. The mice model of AP was established by Cae and LPS as follows [17, 20]: Cae (50μg/kg) was intraperitoneally injected 13 times, followed by intraperitoneal injection of LPS (15mg/kg). The control group was administered an equivalent volume of saline. In the daphnetin group, daphnetin was administered at 4mg/kg 30min after the last injection of Cae. The mice were randomly sacrificed by a lethal dose of pentobarbital at respective time points following the induction of pancreatitis. The blood, pancreas, and lung of each mouse were obtained for subsequent analysis.

2.4. Histological Assessment. Tissue samples of lung and pancreas were fixed in 4% paraformaldehyde overnight. Then, the samples were embedded in paraffin and sliced into 4μm sections. These sections were stained with hematoxylin-eosin (H-E) for morphological observation and pathological scoring. For this study, specimens were given a score according to the criteria described previously [2, 21].

2.5. Measurement of the Levels of Inflammatory Factors. ELISA was performed to detect the concentrations of TNF-α, IL-6, and IL-1β of the Lung tissue homogenate and serum according to the instructions of the ELISA kits. The values were normalized with protein contents.

2.6. Determination of the MPO Activity. Myeloperoxidase (MPO) activity was determined in the lung tissues according to the manufacturer’s instruction. The MPO activity values were normalized with protein contents. Values are expressed as MPO units per g tissue.

2.7. Western Blot Analysis. The protein expressions of HMGB1, TLR4, p-NF-κB p65, NF-κB p65, HIF-1α, and GAPDH were examined by Western blot analysis. Total protein from lung tissues was extracted by RIPA buffer, and the concentrations were determined by a BCA protein assay kit (ASPEM, Wuhan, China). Proteins (40μg) were loaded and separated in 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) membrane (Millipore, USA). The membrane was blocked with 5% fat-free milk for 1h at room temperature and further incubated at 4°C overnight with primary antibodies for NF-κB (1:3000 dilution), p-NF-κB p65 (1:500 dilution), HMGB1 (1:1000 dilution), TLR4 (1:300 dilution), HIF-1α (1:1000 dilution), and GAPDH (1:10000 dilution). The next day, the membranes were incubated with HRP-conjugated secondary antibody (ASPEM, Wuhan, China) diluted to 1:10000 at room temperature for 30 minutes. Eventually, the proteins
were visualized with a chemiluminescence kit (Millipore, USA).

2.8. Statistical Analysis. The results were depicted as mean ± standard deviation. P < 0.05 was considered to indicate a statistically significant variation. All statistical tests were performed using SPSS software program.

3. Results

3.1. Acute Lung Injury Was Induced in AP Mice. As shown in Figure 1(a), the typical pathological sections of pancreatic tissue in AP mice at 6 h after LPS injection showed acinar edema, interstitial hemorrhage, widening of interlobular space structure, and increased neutrophil exudation. Twelve hours after modeling, pancreatic tissues of AP mice showed obvious inflammatory cell infiltration, bleeding, destruction of pancreatic lobule structure, and acinar cell necrosis. Figure 1(c) showed pulmonary alveolar and interstitial edema, hemorrhage, and inflammatory cell infiltration in the lung tissues of AP group. The pathological changes of lung tissues in AP mice worsened at 12 h after LPS injection, which showed the lung tissues of AP group were characterized by aggravated pulmonary alveolar and interstitial edema, increased bleeding areas, alveolar septa thickening, and increased leukocyte infiltration. Microscopic evaluation indicated the pathological structure of pancreas and lung was normal in the control group. Pathological scores of the pancreas and lung of pancreatitis mice were assessed further, and statistical analysis showed that the pancreatic and lung tissue damage tended to worsen progressively over time (Figures 1(b) and 1(d)).

3.2. Daphnetin Prophylaxis Mitigates the Severity of AP-Induced ALI. The effects of daphnetin on the mouse model of AP were examined. Compared with untreated mice with AP, treatment with daphnetin (4 mg·kg⁻¹) clearly decreased the severity of ALI induced by AP, as shown by reduced congestion, edema, and alveolar interstitial thickening (Figure 1(c)). The pathological scores of lung in AP mice treated with daphnetin were also lower than those in the AP group (P < 0.05), indicating that the impaired lungs were relieved by daphnetin intervention (Figure 1(d)).

Myeloperoxidase is an enzyme derived from white blood cells and is present mainly in neutrophils and macrophages. It is also a marker of neutrophil activation. Then, the MPO activity in the lung homogenate was investigated. The results showed that MPO activity increased at 6 and 12 h after modeling in AP mice (P < 0.05). Compared with mice in AP group, the MPO activity was reduced in the daphnetin-treated mice (P < 0.05) (Figure 2).

3.3. Daphnetin Reduces the Levels of IL-1β, IL-6, and TNF-α. These inflammatory cytokines have been demonstrated in association with the development of systemic complications of AP in early phase. In the present study, the levels of IL-1β, IL-6, and TNF-α in lung homogenate and serum were increased significantly in the AP group than those in the control group. But the levels of IL-1β, IL-6, and TNF-α decreased at 12 h after modeling. Daphnetin treatment could reduce the levels of IL-1β, IL-6, and TNF-α in AP mice (P < 0.05), and the inhibiting effect of IL-6 and TNF-α continues till 12 h after modeling, indicating the anti-inflammatory role of daphnetin in ALI induced by AP (Figures 3 and 4).

3.4. Daphnetin Decreases the TLR4, p-NF-κB p65, NF-κB p65, and HIF-1α Expression in Lung. The effects of daphnetin on the expression of TLR4, p-NF-κB p65, and HIF-1α in AP-induced lung injury were further investigated. As shown in Figure 4, compared with the control group, the expression of TLR4, p-NF-κB p65, and HIF-1α was increased in lung tissues of AP mice, and the increase of TLR4 and HIF-1α expression was more significant at 12 h than 6 h after modeling (P < 0.05), while the TLR4, p-NF-κB p65, and HIF-1α protein expression in lungs could be suppressed by daphnetin treatment. In contrast, there was no significant difference in NF-κB p65 protein expression between control and AP mice (Figure 5).

4. Discussion

ALI is the most common and earliest distant organ complication of SAP. Unfortunately, approximately 45% of SAP patients develop respiratory failure, which is unacceptably high in morbidity and mortality and imposes a heavy economic burden on the general population [22–24]. The pathogenesis of PALI is rather complicated [25]. Studies have shown that inflammatory cytokines play an important role in the occurrence and development of PALI. During acute lung injury, the permeability of the alveolar-microvascular barrier increases; therefore neutrophils and macrophages, as the two most critical immune cells in the progression of PALI, will transfer to the lung tissue by means of inflammatory mediators. Neutrophils and macrophages then release a series of inflammatory mediators, aggravating pulmonary microvascular disorder and inflammatory response, forming a vicious circle and causing atelectasis and pulmonary edema [26–28]. The current clinical treatment strategies for acute lung injury mainly include lung protective ventilation in ventilator strategy [29], prone position to improve oxygenation [30], fluid conservative therapy in fluid management [31], nutritional support [32], and other conservative strategies. The efficacy of other pharmacological interventions such as the use of systemic corticosteroids, β-2 agonists, statins, exogenous surfactants, and inhaled vasodilators is uncertain [33]. Therefore, it is of great significance to deeply explore the pathophysiological mechanism of acute lung injury and new treatment methods.

Daphnetin is the monarch herb of Zushima-Pian, which is used for the treatment of collagen-induced arthritis and autoimmune diseases. Recent research studies have confirmed that daphnetin has analgesic, anti-inflammatory, antipyretic, and antiparasitic effects [19, 34, 35]. In an animal randomized controlled trial in SAP treatment in rats...
receiving daphnetin intervention, daphnetin was shown to inhibit pancreatic inflammatory response, reduce the production of inflammatory factors, and have a protective effect on the pancreas [36]. This study was designed to investigate the possible protective effect of daphnetin in SAP-associated lung injury and explore its underlying potential mechanisms. A mice model of PALI was established by using lipopolysaccharide and caerulein, and the experimental

**Figure 1:** Representative sections were stained with hematoxylin-eosin (H-E). The original magnification is ×200. (a) Pancreas sections of mice in control, SAP, and daphnetin groups. (b) The pathological scores of pancreas. (c) Lung tissue sections of mice in control, SAP, and daphnetin groups. (d) The pathological scores of lung. *P < 0.05, versus control group; **P < 0.05, versus control group; **** P < 0.05, versus SAP group.

**Figure 2:** The MPO activity of lung tissue. **P < 0.05, versus control group at the same time point; **** P < 0.05, versus SAP group at the same time point.
results showed that neutrophil infiltration, hemorrhage, edema, and alveolar septum thickening in the lung tissue of AP mice, while daphnetin treatment could improve the pathological lung injury induced by AP. Since MPO is a marker of tissue neutrophil infiltration, the activity of MPO in lung tissues was also examined. The results showed that MPO activity was strongly activated in the lung tissue of the mice in the AP group. In addition, it is also investigated that intervention of daphnetin could decrease the MPO activity in AP mice, suggesting that daphnetin can effectively reduce the activity of MPO in the lung tissue, reduce the infiltration of neutrophils, attenuate the inflammatory response of lung tissue in AP mice, and have a protective effect on AP-related acute lung injury.

In the early stage of SAP, the main feature is systemic inflammatory response syndrome as well as related organ failure [37, 38]. The proinflammatory cytokines including IL-1β, TNF-α, and IL-6 are considered to be determining mediators for initiating, amplifying, and perpetuating lung injury, which play a vital role in the occurrence and development of early ALI [39–41]. TNF-α and IL-6 are potent proinflammatory factors that are consistent with the severity of ALI, and IL-1β can induce monocytes and macrophages to promote the process of inflammation that plays a role in the formation of SAP [1, 2, 42]. The development of inflammation is often accompanied by the infiltration of inflammatory cells and the release of inflammatory cytokines [43]. Therefore, the levels of inflammatory mediators including IL-1β, IL-6, and TNF-α in lung tissue and serum were examined. The results showed that the IL-1β, IL-6, and TNF-α levels in the lung homogenate and serum were increased in AP mice, suggesting that the inflammatory response was induced in the early stage of AP. As we expected, the treatment of daphnetin reduced the levels of these inflammatory cytokines. These results showed that daphnetin attenuated the inflammatory response in the lung injury induced by AP, and these anti-inflammatory effects may explain the protective role of daphnetin in SAP-related acute lung injury.

TLRs play a pivotal role in the innate immune system, which may also play a central role in the initiation of signal transduction in the inflammatory reaction during SAP [11, 44]. In an animal experiment of SAP-related acute lung injury in rats, Wang et al. found that in the early stage of acute lung injury, effectively inhibiting the TLR4/NF-κB pathway could reduce the expression of inflammatory mediators in lung tissue, thereby reducing lung injury [44]. Extracellular activation of TLR4 could lead to ALI and lethal
inflammatory processes by inducing NF-κB translocation to the nucleus [42, 45]. NF-κB is a key transcription factor mediating the release of multiple proinflammatory cytokines, playing a vital role in controlling the inflammatory cascade [46]. NF-κB also mediates apoptosis, cell growth, and immune responses and controls the production of inflammatory factors such as TNF-α, IL-1β, and IL-6 [47, 48]. Previous studies have shown that selective inhibition of NF-κB activation efficiently suppresses expression levels of cytokines and reduces the degree of lung injury in laboratory murine acute pancreatitis [49, 50]. In the present study, the effect of daphnetin on TLR4 and NF-κB expression in ALI induced by AP was detected. AP can lead to the phosphorylation of NF-κB p65 and subsequent upregulation of NF-κB p65 [46]. The total NF-κB p65 protein expression did not differ significantly among the three groups. The result showed that the protein expressions of p-NF-κB p65 and TLR4 in lung tissue of mice in AP group were significantly increased compared to those in control mice. Importantly, the levels of p-NF-κB p65 and TLR4 in the lung were suppressed by daphnetin treatment.

HIF-1α can adapt to hypoxic and inflamed microenvironments during infection and is a heterodimeric protein. HIF-1α is involved in inflammation and pulmonary vascular barrier dysfunction and plays an important role in ALI, innate immunity, and host defense [13, 51]. An animal experiment has showed that LPS raises the level of HIF-1α in a TLR4-dependent fashion. Moreover, HIF-1α contributes to the production of inflammatory cytokines, including TNF-α, IL-1, and IL-6, which are vital biological mediators of sepsis [13]. Previous study has found that HIF-1α plays an important role in the pathogenesis of acute pancreatitis [52]. Qi et al. have confirmed that blocking the expression of HIF-1α can avoid the destruction of the alveolar-capillary membrane barrier and achieve the effect of alleviating SAP-related acute lung injury [53]. Western blot demonstrated that the HIF-1α expression of lung tissue was increased in AP mice, while daphnetin significantly downregulated its protein expression in this experiment. NF-κB pathway can activate HIF-1α and promote HIF-1α expression under hypoxic or inflammatory conditions [54]. The inhibition of the NF-κB/HIF-1α pathway can reduce the production of inflammatory factors such as TNF-α and IL-1β and have a protective effect on LPS-induced ALI in mice [55]. Jiang et al. believed that LPS increased the level of HIF-1α in a TLR4-dependent manner and found that the induction of HIF-1α during acute lung injury in mice requires the participation of the TLR4/NF-κB pathway [56]. In this study, the data implied that the expression of TLR4/NF-κB/HIF-1α in lung tissue was consistent with the expression of inflammatory mediators and MPO. These results suggest that daphnetin may downregulate the expression of HIF-1α through the TLR4/NF-κB pathway to inhibit the synthesis and release of inflammatory mediators during the progression of PALI, thereby reducing the pathological damage and inflammatory response of lung caused by AP.

In conclusion, the current study showed that daphnetin was identified to have a therapeutic effect on PALI. It could alleviate the pathological injury and inflammatory reactions of lung caused by PALI. The mechanism may be related to the reduction of inflammatory factor release by regulation of TLR4/NF-κB/HIF-1α. However, further studies are needed.
to elucidate the exact mechanism by which daphnetin mediates SAP-related acute lung injury and provide a theoretical basis for the future clinical application of daphnetin.

Data Availability

The data generated in the present study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

TC and OC designed and completed the study, interpreted the data, and wrote the first draft. MZ did the statistical analysis and provided manuscript design. XC designed the study and did the critical revision of the manuscript. All authors have approved the publication of this manuscript. Tao Chen and Ou Chen these authors contributed equally to this work.

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