Amentoflavone prevents sepsis-associated acute lung injury through Nrf2-GCLc-mediated upregulation of glutathione

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INTRODUCTION

Sepsis is a serious systemic inflammatory syndrome that is caused by infection, blood loss, trauma, and neoplasms (Liu et al., 2016). Sepsis affects millions of individuals worldwide and is one of the main causes of high mortality in intensive care units (ICU). To date, most of sepsis complications remain refractory, and development of novel effective therapeutic approaches is urgently needed. Although the mechanism of sepsis injury is not completely clear, oxidative stress has been believed to play a pivotal role (Bajcetic et al., 2014; Duran-Bedolla et al., 2014). It is also found that oxidative stress and final amelioration of inflammation and histological injury of lung. The data provide new therapeutic options for the treatment of sepsis-associated ALI.

Key words: Sepsis; Acute lung injury; Amentoflavone; Nrf2; Glutathione

INTRODUCTION

Sepsis is a serious systemic inflammatory syndrome that is caused by infection, blood loss, trauma, and neoplasms (Liu et al., 2016). Sepsis affects millions of individuals worldwide and is one of the main causes of high mortality in intensive care units (ICU). To date, most of sepsis complications remain refractory, and development of novel effective therapeutic approaches is urgently needed. Although the mechanism of sepsis injury is not completely clear, oxidative stress has been believed to play a pivotal role (Bajcetic et al., 2014; Duran-Bedolla et al., 2014).

Sepsis could severely result in multiple organ dysfunction, including cardiovascular system, liver, kidney and lung. It is generally considered that damage of the lung is one of the most severe outcomes of sepsis (Andrews et al., 2005). It is estimated that about fifty percent of patients with severe sepsis will develop acute lung injury (ALI) and even acute respiratory distress syndrome (ARDS), a more severe consequence of ALI (Rubenfeld et al., 2005; Maybauer et al., 2006). During the development of ALI/ARDS, oxidative stress and inflammatory responses were enhanced, leading to the failure of multiple organs (Dolinay et al., 2012).

Materials and Methods

Chemicals and reagents. AMF and BSO were obtained from Sigma-Aldrich. TNFα and IL-1β ELISA kits were purchased from R&D Systems. TBARS and SOD assay kits were obtained from Nanjing Jiancheng Company. GSH assay kit was obtained from Biovision. ELISA-based NF-κB p65 transcription factor assay kit was obtained from R&D Systems. Oxidative stress and inflammatory responses were enhanced, leading to the failure of multiple organs (Dolinay et al., 2012).
purchased from Active Motif North America. CHP assay kit was obtained from Abcam. RNA isolation and Real-time PCR reagents were purchased from TaKaRa. All other chemicals were used of analytical grade.

Animals. Animal experiments were conducted under the guidelines for the care and use of laboratory animals published by the National Institute of Health. Experimental protocols were approved by the Ethical Committee of Shaanxi Provincial People’s Hospital on Animal Care. Male Sprague-Dawley (SD) rats (220–260 g) were obtained from the Animal Center of Xi’an Jiaotong University. Rats were kept in a temperature-controlled room with a 12 h light/12 h dark cycle for 1 week before the experiment, with free access to food and water.

Animal model of sepsis. Fifty animals were randomly divided into five groups (10 rats in each group): Sham group, CLP group, AMF group (CLP + 50 mg/kg AMF), AMF + LV-shNrf2 (CLP + 50 mg/kg AMF + LV-shRNA Nrf2), AMF + BSO (CLP + 50 mg/kg AMF + 10 mg/kg buthionine sulfoximine). CLP-induced sepsis model was built in CLP, AMF, AMF + LV-shNrf2, and AMF + BSO groups as previously described (Liu et al., 2016). In brief, after fasting overnight, rats were anesthetized using 10% chloral hydrate (3 ml/kg, intraperitoneal). After sterilization, a 1.5-cm incision was cut in ventral midline of abdomen and then the cecum was gently exposed and ligated with a 3–0 silk and punctured with an 18-gauge needle at three locations. After that, the cecum was repositioned and the abdomen was closed. The same sham surgery and cecum manipulation was conducted in Sham rats without ligation and puncture. Rats in AMF + LV-shNrf2 group were treated intranasally with either 4 × 10^7 PFU’s (in 50 μl) lentivirus shRNA targeting Nrf2 (Shanghai GenePharma Co., Ltd, China) 24 hours before the surgery and rats in the other groups were treated with scramble shRNAs. Rats in AMF + BSO group were intraperitoneally injected with 10 mg/kg BSO 2 hours before the surgery and rats in the other groups were injected with vehicle. Rats in AMF, AMF + LV-shNrf2, and AMF + BSO groups were intraperitoneally administrated with 50 mg/kg AMF 6 hours before the surgery and rats in the other groups were given with vehicle (0.1% Tween 80). Saline (2 ml/100 g body weight) was given subcutaneously to the rats immediately after the operation for resuscitation. No antibiotics were administered. AMF was administrated daily for 5 consecutive days. 6 days after the surgery, rats were euthanized. Blood samples were collected. Left lung was excised for wet/dry weight ratio and then centrifuged at 3500 r/min for 10 min. The absorbance at 532 nm was measured and the results were expressed as percentage of oxygen consumption. The results were expressed as percentage of oxygen consumption.

Determination of oxidative stress. TBARS levels and SOD activities in lung homogenates were detected by commercial kits (Nanjing Jiancheng Company, China). TBARS content was determined by the TBA reaction. The reaction mixture was heated at constant temperature at 95°C for 40 min, cooled by running water and then centrifuged at 3500 r/min for 10 min. The absorbance at 532 nm was measured and the results were expressed as nmol/mg protein.

Real-time PCR. Total RNA was isolated from lung tissues using the TRIzol reagent. 500 ng total RNA was transcribed into cDNA using a commercial kit (TaKaRa, Japan) according to the manufacturer’s instructions. Real-time PCR was performed in a 20 μl reaction mixture containing 10 μl 2×SYBR green master mix, 7 μl H₂O, 2 μl primers (10 μM), and 1 μl template DNA. Cycling was performed using a quantitative PCR instrument (Biorad, USA) and a SYBR® Premix Ex Taq™ kit (TaKaRa, Japan) were used to detect target gene expressions, and β-actin was used as an internal reference. The 2^(-ΔΔCT) method was employed to determine the relative expression of target genes normalized to β-actin, and experiments were repeated in triplicate.

Histological examination. Lung tissues were fixed in 4% paraformaldehyde overnight, dehydrated, embedded, and sliced into 5-μm sections. After that, sections were stained with hematoxylin and eosin, and were observed under a light microscopy (Olympus, Tokyo, Japan).

Lung wet/dry weight ratio. Wet weight of left lung was recorded immediately after the excision. Then the lung tissues were placed in an incubator at 70°C for 48 h and the dry weight was recorded. The wet to dry weight ratio was calculated as follows: wet weight–dry weight/dry weight.

Determination of inflammation. Levels of tumor necrosis factor-alpha (TNFα) and interleukin-1β (IL-1β) in serum and lung homogenates were determined by ELISA kits according to the manufacturer’s instructions. Nuclear protein was extracted and DNA-binding activity of p65 NF-κB was determined using an ELISA-based NF-κB p65 transcription factor assay kit according to the manufacturer’s protocols. In each assay, a standard curve and positive/negative controls were included. The absorbance at 450 nm was measured using a microtiter ELISA reader (Biorad, USA). The assay sensitivity for IL-1β was <2 pg/ml and for TNF-α was 0.5–5.5 pg/ml.

Statistical analysis. Results were expressed as means ± S.E.M. Graph-Pad Prism software was used to perform the statistical analysis using one-way ANOVA fol-
RESULTS

AMF ameliorated CLP-induced histological injury in lung tissues

To evaluate the effect of AMF on CLP-induced injury in lung tissues in rats, histological examination was performed. As shown in Fig. 1, we showed that there was severe hemorrhage, alveolar congestion, thickening of alveolar wall, and infiltration and aggregation of neutrophils in alveoli and vessel walls in the lung of CLP-treated mice, indicating that CLP resulted in significant inflammatory response. The administration of AMF notably inhibited these inflammatory changes in rats. However, the treatment of letivirus of shNrf2 or BSO, a glutathione (GSH) synthesis inhibitor, markedly inhibited AMF-induced improvement of inflammatory response in lung tissues.

AMF ameliorated CLP-induced pulmonary edema

Wet/dry weights ratio of lung tissue was detected. In Fig. 2, we show that CLP induced a significant increase of wet/dry weights ratio of lung tissue, indicating that CLP resulted in a marked pulmonary edema. AMF administration markedly inhibited the increase of wet/dry weights ratio of lung tissue in CLP-treated rats. In contrast, the treatment of shNrf2 letivirus or BSO markedly suppressed AMF-induced amelioration of pulmonary edema in CLP-treated rats.

AMF ameliorated CLP-induced inflammation

To evaluate the effect of AMF on inflammation in CLP-treated rats, levels of cytokines and activity of p65 NF-κB were measured. Systemic and lung levels of TNFα and IL-1β were determined by ELISA kits and the results showed that serum and lung TNFα and IL-1β levels were significantly increased by CLP (Fig. 3A-C and D). Moreover, p65 NF-κB binding activity was markedly increased by CLP (Fig. 3E). AMF administration markedly inhibited CLP-increased levels of serum and lung TNFα and IL-1β levels and p65 NF-κB binding activity in CLP-treated rats (Fig. 3). The results indicated that AMF administration prevented CLP-increased inflammation in lung tissue which was inhibited by shNrf2 or BSO.

AMF ameliorated CLP-induced oxidative stress

To evaluate the effect of AMF on oxidative stress in rats treated by CLP, mitochondrial function and several oxidation-sensitive markers were determined. Mitochondria are main targets of oxidative injury and mitochondrial function is a sensitive indicator of oxidative stress status. In Fig. 4A, we showed that oxygen consumption rate in lung tissues treated by CLP was significantly decreased. AMF markedly increased oxygen consumption rate in lung tissues of CLP-treated rats. In contrast, the treatment of shNrf2 letivirus or BSO markedly suppressed AMF-induced amelioration of oxidative stress in CLP-treated rats.
lung tissue and shNrf2 and BSO could inhibit the antioxidative effect of AMF.

AMF prevented CLP-induced decrease of Nrf2-GCLc signaling

Considering the pivotal role of GSH in the antioxidant defense system, we further explored the mechanism of AMF-induced regulation of GSH. Expression of GCLc, the rate-limiting enzyme for GSH synthesis, was determined. As shown in Fig. 5A, mRNA expression of GCLc was significantly decreased by CLP. AMF markedly inhibited CLP-induced decrease of GCLc mRNA expression (Fig. 5A). shNrf2, but not BSO, resulted in a significant inhibition of AMF-exhibited suppression of CLP-induced decrease of GCLc mRNA expression (Fig. 5A). To further explore the possible mechanism underlying AMF-induced regulation of GCLc expression, expression and activity of Nrf2, a central transcription factor controlling redox balance, were measured. In Fig. 5B, we showed that AMF induced a significant inhibition of CLP-resulted decrease of Nrf2 mRNA expression. The effect of AMF on Nrf2 mRNA expression in CLP-treated rats was suppressed by shRNA of Nrf2, but not BSO (Fig. 5B). Moreover, CLP resulted in a notable decrease of Nrf2-binding in GCLc promoters which effect was prevented by AMF administration (Fig. 5C). shRNA of Nrf2, but not BSO, suppressed AMF-induced increase in Nrf2-binding activity in GCLc promoters (Fig. 5C). The results demonstrated that regulation of GCLc by Nrf2 may be involved in AMF-induced protective effects against CLP-resulted GSH depletion and lung injury.

DISCUSSION

CLP-induced sepsis model is a widely accepted animal model for the research of sepsis-associated mechanism and the investigation of potential therapeutic options for the treatment of sepsis. In the current study, using CLP-induced septic rats, we investigated the protective effects of AMF against sepsis-associated ALI. We found that AMF administration protected against septic ALI, as reflected by marked amelioration of histological injury in lung tissues and decrease of pulmonary edema in CLP-treated rats.

It is generally believed that inflammation is the fundamental pathological process in the pathogenesis of organ injury in sepsis (Dounousi et al., 2016; Hahn et al., 2016). Especially for ALI, inhibition of inflammation is considered to be a potent therapeutic strategy (Xie et al., 2012; Lin et al., 2015). It has been found
that proinflammatory cytokines, such as TNFα and IL-1β, are increased in the serum of septic patients (Kang et al., 2001a). NF-κB is a central transcription factor in the regulation of inflammation through controlling various proinflammatory gene expression, including TNFα, IL-1β, IL-6, and IL-8 (Cortez et al., 2013). NF-κB-mediated inflammation plays an important role in the pathogenesis of ALI and sepsis (Kang et al., 2001b). p65 is a major sub-member of NF-κB that mediates its proinflammatory effect. Consistent with previous literature, we found that CLP resulted in severe inflammation, as illustrated by increase of systemic and lung TNFα and IL-1β levels and the binding activity of NF-κB. The administration of AMF significantly inhibited these changes induced by CLP, indicating the amelioration of inflammation.

To explore the mechanism of the protective effect of AMF against CLP-induced inflammation and ALI, redox status was measured in the study. We found that AMF administration notably inhibited CLP-induced decrease of oxygen consumption, increase of TBARS content, decrease of SOD activity and GSH level, indicating that AMF inhibited CLP-induced oxidative stress. Oxidative stress is defined as imbalance between reactive oxygen species (ROS) generation and antioxidant defense (Avogaro et al., 2008). Damage of antioxidant system could result in excessive accumulation of ROS, leading to oxidative injury of various macromolecules and important organelles (Avogaro et al., 2008). Oxidative stress is closely related with inflammation and contributes to sepsis-associated injury (Zolali et al., 2015; Gerin et al., 2016). Mitochondrial function is a sensitive indicator of intracellular redox state. TBARS indicates the content of malondialdehyde (MDA), an end product of lipid peroxidation (Napierala et al., 2016). SOD is an important antioxidant enzyme catalyzing the dismutation of superoxide into hydrogen peroxide (Shi et al., 2016). GSH is one of the most important antioxidant proteins, functioning to eliminate ROS directly or indirectly under the catalysis of antioxidant enzymes (Couto et al., 2016). We should point out that the markers of oxidative stress determined in the study, including TBARS, SOD, and GSH, are indirect markers. Only the antioxidant enzyme SOD was determined to evaluate the antioxidant defense. In future study, more direct markers, such as ROS concentration, and antioxidant enzymes are needed to accurately evaluate the redox status. Even so, based on previous literature and our results, we demonstrated that the antioxidative activity of AMF was involved in the protective effects against sepsis-associated ALI.

To further explore the molecular mechanism of AMF-exhibited antioxidative activity, regulation of GSH synthesis by AMF was evaluated. GCLc is reported to be the rate-limiting enzyme for the synthesis of GSH under the transcriptional regulation of Nrf2 (Jiang et al., 2016; Takahashi et al., 2016; Kuwano et al., 2015). Activation of Nrf2 and its target gene GCLc was shown to protect against LPS-induced inflammatory response (Thimmulappa et al., 2007; Thimmulappa et al., 2006). In the present study, we showed that knockdown of Nrf2 by lentivirus and inhibition of GSH synthesis by BSO significantly suppressed AMF-induced inhibitory effect on histological injury, pulmonary edema, inflammatory response and oxidative stress in the development of ALI in septic rats. We also showed that AMF increased the binding activity of Nrf2 in the promoters of GCLc. The results demonstrated that enhancement of GSH through Nrf2-regulated GCLc was involved in the protective effects of AMF. Further studies are needed to explore whether Nrf2 is a direct target of AMF and to examine the structure-activity relationship. Since the mRNA expression of Nrf2 was altered, it was indicated that AMF could activate the upstream transcriptional regulator of Nrf2 which needed further efforts to elucidate. Moreover, the results also showed that knockdown of Nrf2 and inhibition of GSH synthesis did not completely reversed the beneficial effect of AMF, indicating that additional mechanism may be involved in the protective effect of AMF against sepsis. Furthermore, we should notice that AMF could not completely prevent sepsis-associated injury. Therefore, AMF can be used as a promising therapeutic option in combination with additional agents in the prevention and treatment of sepsis and its complications.

In conclusion, in the current study, we found that AMF protected against ALI in septic rats through upregulation of Nrf2-GCLc signaling, enhancement of GSH antioxidant defense, reduction of oxidative stress and final amelioration of inflammation and histological injury of lung. The data provide new therapeutic options for the treatment of sepsis-associated ALI.

Conflict of interest
The authors declare none.

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