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Original Research Article

Pulegone ameliorates inflammation and oxidative stress in L-arginine-induced acute pancreatitis in mice by regulating the activation of p38 MAPK pathway

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Abstract

Purpose: *To evaluate the effects of pulegone (PLG) on inflammation and oxidative stress in L-arginine-induced acute pancreatitis (AP), and determine its molecular mechanism.*
Methods: *Hematoxylin-Eosin (H & E) staining assay were performed, and histopathological score, blood glucose concentration, and serum amylase level were evaluated in order to assess the effects of PLG on pancreatic injury in L-arginine-induced AP mice. Enzyme linked immunosorbent assay (ELISA) was conducted to assess the levels of myeloperoxidase (MPO), malonaldehyde (MDA) and inflammatory factors (IL-6, TNF-α and IL-1β) in L-arginine-induced AP mice. Serum lactate dehydrogenase (LDH) and relative levels of ROS generation in L-arginine-induced AP mice were determined using an LDH kit and immunofluorescence assay, respectively. The effect of pulegone (PLG) on the activation of p38 MAPK pathway in L-arginine-induced AP mice was evaluated by Western blot.*
Results: *Significant pancreatic tissue injury occurred in L-arginine-induced AP mice was revealed. PLG alleviated the pathological injury of pancreatitis, and decreased the blood glucose concentration and serum amylase level in L-arginine-induced AP mice. In addition, PLG inhibited oxidative stress and inflammatory responses, and was enabled to inhibit the activation of p38 MAPK pathway in L-arginine-induced AP mice. Furthermore, PLG exhibited protective effect against the development of pancreatic injury in L-arginine-induced AP mice.*
Conclusion: *PLG ameliorates L-arginine-induced inflammation and oxidative stress in AP mice in vivo by inhibiting p38 MAPK pathway, and therefore, is a potential therapeutic agent for the management of acute pancreatitis.*

Keywords: *Pulegone, Inflammation, Oxidative stress, Acute pancreatitis, p38 MAPK pathway*

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INTRODUCTION

Acute pancreatitis (AP) is a serious and acute abdominal disease caused by inflammation in the pancreas. AP not only leads to local pancreatic tissue injury, it also causes systemic harm to multiple organs [1]. Patients with AP suffer not only severe abdominal pain and elevated digestive enzymes such as blood amylase, lipase and trypsinogen, but also elevated blood glucose

and pro-inflammatory cytokine release [2]. Systemic inflammation aggravates the burden on the organs, the severity of AP, and also brings secondary injury to patients [3]. In addition, oxidative stress is another factor that contributes to the initiation of inflammation and complication during AP. Reactive oxygen species (ROS) accumulated in cells can promote the progression of AP [4].

Recent studies have demonstrated that traditional Chinese medicine plays an important role in the treatment of AP. (R) - (+) - Pulegone (PLG) is a transformational product of monoterpene ketone extracted from the leaves and flowering tops of peppermint plants [5]. Studies showed PLG has obvious anti-lipase, anti-diastase, and anti-inflammatory effect [6]. In a mouse model of dermatitis induced by 2, 4-dinitrochlorobenzene, PLG suppresses allergic reaction, and inhibits the release of inflammatory factors, the activity of NLRP3 inflammasomes in THP-1 and reduce the enrichment of ROS [7]. It inhibited p38 MAPK and NF- κB pathway activity. Studies showed p38 MAPK and NF-κB in the mouse model induced by L-arginine and the inhibition of this pathway, alleviates the symptoms of AP and the damage of other organs [8]. Therefore, it is speculated that PLG was related to the down regulation of of MAPK and other pathways in the treatment of AP.

In this study, the effect of PLG on AP was investigated.

EXPERIMENTAL

Animals

Male C57BL/6 J mice (20 - 25 g, 8-week-old) purchased from Shanghai Experimental Animal Center (Shanghai, China) were used in this study. All mice were kept in a room at 25 ºC at a 12/12-h light/dark cycle with free to acquire food and water. All the procedures and protocols were approved by the Ethics Committee of Chongqing Medical University (Approval no. 2018019), and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [9].

Chemicals and reagents

Pulegone (HY-N1500) and L-arginine (HY-N0455) were obtained from MedChemExpress (Shanghai, China). p-NF-κBp65 antibody (1:1000 dilution, 3033S, CST), NF-κB antibody (1:1000 dilution, ab239882, Abcam), p-ERK antibody (1:1000 dilution, ab201015, Abcam). Furthermore, ERK antibody (1:10000 dilution, ab184699, abcam), p-p38 antibody (1:1000 dilution, ab195049, Abcam), p38 antibody (1:1000 dilution, 8690s, CST), β-actin antibody (1:2000 dilution, ab6276, Aabcam). IL-1β ELISA kit (ab255730, Abcam), IL-6 ELISA kit (ab234570, Abcam), TNF-α ELISA kit (SEKR-0009, Solarbio) were obtained from the suppliers indicated.

AP mouse model and drug treatment

The AP mouse model was constructed by intraperitoneal injection of 0.2 mL L-arginine solution (2 times with a 1-hour interval, 4 g/kg body weight). The animals were randomly divided into four groups: (1) control group (Sham group), (2) untreated AP mouse group (AP group), (3) AP group treated with low concentration of PLG (AP + PLG 0.1 g/kg group), and (4) AP group treated with high concentration of PLG (AP + PLG 0.2 g/kg group). The treatment was performed by intragastric administration with PLG at the time point of 10 h, and then continuous administration for five time (once per 10 h). The Sham group and untreated AP group were treated with PBS for 3 weeks.

Histologic evaluation

The pancreatic tissues from the anesthetized mice were collected and the tissues were cut into pieces after fixing in 4 % paraformaldehyde. Then the tissues were washed in water for 2 h, and dehydrated in 50, 70, 80 and 90 % anhydrous ethanol. Subsequently, the tissues were separately dehydrated with xylene and paraffin, embedded in paraffin and cut into slices with 4 μm thickness. Next, the slices were dewaxed and stained with hematoxylin and eosin (H & E). After staining, sections were sealed in neutral balsam and examined under an optical microscope. The histopathological score of pancreatic tissue injury was evaluated by H & E staining, and the score ranged from Sham group (score = 0) to the AP mouse model group (score = 10). The assessment was repeated 3 times, and the mean value taken.

Measurement of blood glucose and serum amylase levels

Blood was collected at different time points, and the blood glucose concentration and serum amylase levels were separately determined by the manufacturer's instructions from the kit (BC2495, BC5055, Solarbio, Beijing, China). The measurement was repeated 3 times, and the average value was taken.

Determination of MPO, MDA, ROS and LDH levels

Pancreatic tissues (20 mg) from each group were isolated and homogenized in precooling PBS immediately. After the centrifugation, the supernatant was collected and the levels of myeloperoxidase (MPO), malondialdehyde (MDA) and lactate dehydrogenase (LDH) were evaluated according to the kit manufacturer's instructions (SEKM-0118, BC0025 and BC0685, Solarbio, Beijing, China), respectively. The ROS generation levels in pancreatic tissues were evaluated using dihydroethidium (DHE) fluorescent probe.

Enzyme linked immunosorbent assay (ELISA)

ELISA assay was performed to determine the secretion levels of inflammatory factors (IL-1β, IL-6, and TNF-α) in serum samples from L-arginine-induced AP mice. The primary antibody was diluted with carbonate coated buffer (pH = 9.6) until the protein content reached 10 μg/mL; 100 μL of the diluted antibody was added into 96-well plates (Corning® 9018), and the samples incubated at 4 °C overnight. After 24 h, the plates were washed three times with > 250 μL/well PBS washing buffer (pH = 7.4). Subsequently, 100 μL of diluted rat serum samples (1:5 ratio) were added to the wells. After 2 h incubation, the plates were washed three times with > 250 μL of 0.5 % Tween-20 in PBS, and then horseradish peroxidase (HRP)-conjugated antibody (Abcam, Cambridge, USA) was added and incubated at 37 ^{0}C for 1 h. After the addition of 100 μL of TMB substrate (Innoreagents, Hangzhou, China), incubation was continued at 37 ℃ for 30 min. When the reaction was terminated, the absorbance was measured at wavelength of 450 nm using a microreader.

Western blot

Radio Immunoprecipitation Assay (RIPA) Buffer (9800, Cell Signaling, Danvers, MA) was used to lyse and extract total proteins from the tissues. Protein was quantified using the BCA method, and 20 μg protein was added, and electrophoresed using 15 % SDS-PAGE. Subsequently, samples were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5 % milk for 2 h at 37 °C. Then the incubation of the PVDF membrane with anti-p-NF-κBp65, anti-NF-κB, anti-p-ERK, anti-ERK, anti-p-p38, anti-p38, and anti-β-actin antibody was performed at 4^{0}c overnight. Then the PVDF membranes were washed at least 3-5 times and co-incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Enhanced chemiluminescence (ECL) detection reagents purchased from Amersham Pharmacia Biotech, (Tokyo, Japan) were used to determine the visualized blots. Image Pro software was used in this assay to calculate the intensity of each blot.

Statistical analysis

All experiments performed were repeated three times, and the data were presented as mean ± SD. Data were analyzed by one-way ANOVA followed by Tukey's test. GraphPad Prism 8.0 software was used for data analysis. A value of $p < 0.05$ was taken to indicate significant difference.

RESULTS

PLG alleviated pancreatic histopathological injury and decreased blood glucose and serum amylase levels in L-arginine-induced AP

H & E staining outcome (Figure 1 A) and histopathological score of the degree of pancreatic tissue injury (Figure 1 B) were used to evaluate the effects of PLG on the pathological injury are shown below. We found L-arginine 0.1 g / kg of PLG treatment (injury score = 5) effectively repaired the pancreatic tissues injury in L-arginine-induced AP mice ($p < 0.05$). Moreover, 0.2 g/kg of PLG (injury score = 3) treatment significantly inhibited the pancreatic tissues injury in L-arginine-induced AP mice ($p < 0.001$). In addition, blood glucose and serum amylase levels were also determined in L-arginine-induced AP mice before and after PLG treatment. The data showed that PLG treatment could significantly decrease the blood glucose concentration in a time and concentration-dependent manner (Figure 1 C), but can also reduce the secretion of serum amylase levels (Figure 1 D, $p < 0.001$). Therefore, PLG attenuated pancreatic tissue injury in L-arginine-induced AP.

PLG relieved oxidative stress injury in pancreatic tissue in L-arginine-induced AP

The levels of MPO, MDA, LDH and ROS generation increased after L-arginine induction, whereas PLG treatment significantly decreased the levels of MPO and MDA (Figure 2 A), ROS generation (Figure 2 B and C) and LDH (Figure 2 D). Therefore, PLG inhibited the oxidative stress injury in L-arginine-induced AP.

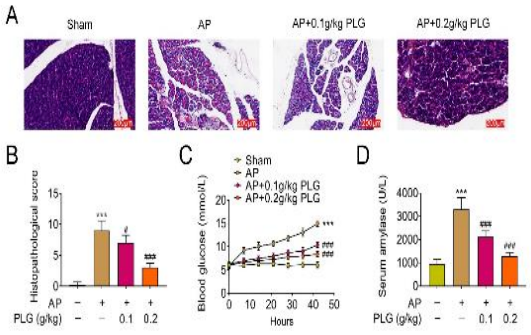

Figure 1: PLG alleviated pancreatic tissue injury in L-arginine-induced AP mice**.** H & E staining (A) and histopathological score of pancreatic tissue injury (B) in L-arginine-induced AP mice. Blood glucose concentration (C) and serum amylase level (D) of L-arginine-induced AP mice. Results are presented as mean ± SD, AP group vs Sham group, $^{***}p < 0.001$. AP group vs AP + PLG group (0.1 g / kg, 0.2 g / kg), $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$

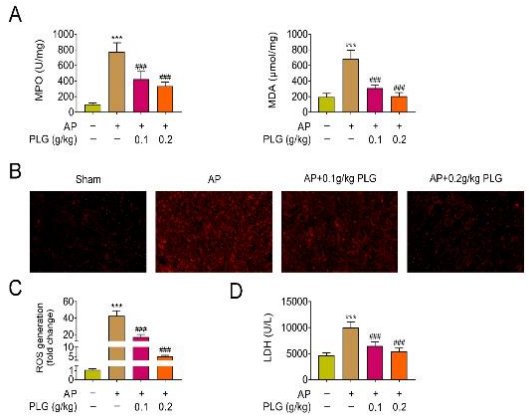

Figure 2: PLG inhibited oxidative stress in L-arginine-induced AP mice. (A) Effects of PLG on the expression of MPO and MDA in pancreatic tissue of L-arginine-induced AP mice. (B and C) relative levels of ROS generation in pancreatic tissue of L-arginine-induced AP mice. (D) Release level of LDH in serum of L-arginine-induced AP mice. Results are presented as mean ± SD, AP group vs Sham group, $^{***}p < 0.001$. AP group vs AP + PLG group (0.1 and 0.2 g/kg), $^{\#\#\#}p < 0.001$

PLG ameliorated inflammatory responses in L-arginine-induced AP

The results showed that L-arginine effectively increased the expression levels of IL-1β, IL-6 and TNF-α, as well as the p-NF-κBp65/NF-κBp65 ratio ($p < 0.001$), and PLG treatment (0.1 or 0.2 g/kg) significantly decreased the expression levels of IL-1β, IL-6 and TNF-α (Figure 3 A), and the p-NF-κBp65 / NF-κBp65 ratio ($p < 0.001$) (Figure 3B). Therefore, PLG suppressed the inflammatory responses, and inhibited the activation of NF-κB in L-arginine-induced AP.

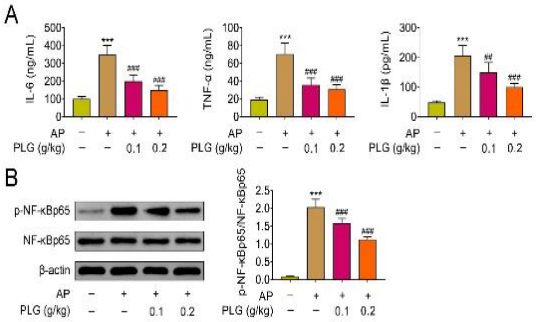

Figure 3: PLG suppressed the inflammatory responses in L-arginine-induced AP mice. (A) Effect of PLG on the secretion of inflammatory factors (IL-6, TNF-α and IL-1β) in L-arginine-induced AP mice. (B) Effects of PLG on the expression of p-NF-κBp65 and NF-κBp65 in L-arginine-induced AP mice. Results are presented as mean ± SD, AP group vs Sham group, $^{***}p < 0.001$. AP group vs AP + PLG group (0.1 and 0.2 g/kg), $^{\#\#\#}p < 0.001$

PLG blocked the activation of p38 MAPK pathway in L-arginine-induced AP

L-arginine could effectively up-regulate the ratio of p-ERK/ERK and p-p38/p38 ($p < 0.001$), whereas PLG treatment (0.1 or 0.2 g/kg) significantly decreased the ratio of p-ERK / ERK and p-p38 / p38 ($p < 0.001$) (Figure 4).

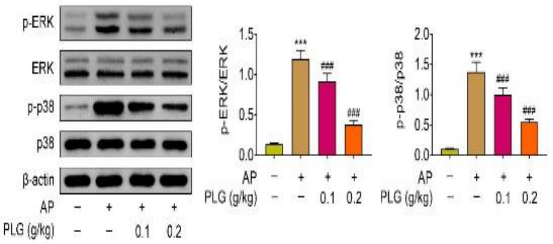

Figure 4: PLG inhibited the activation of p38 MAPK signaling pathway in L-arginine-induced AP mice. Expression of p-ERK, ERK, p-p38 and p38 in L-arginine-induced AP mice. All experiments were repeated three times. Data are presented as mean ± SD. AP group vs Sham group, $^{***}p < 0.001$. AP group vs AP + PLG group (0.1 and 0.2 g/kg), $^{\#\#\#}p < 0.001$.

DISCUSSION

As a serious acute non-infectious disease in clinics, AP has high morbidity. In recent times, the incidence of AP has increased significantly. Epidemiological studies showed that AP was caused by the abnormal activation of inflammation and oxidative stress [10]. The

abnormal regulation of blood glucose and various enzymes (e.g., serum amylase) in pancreas leads to the abnormal activation and secretion of pro-inflammatory factors such as IL-6, IL-1β and TNF-α, resulting in the initiation and progression of AP.

Currently, pancreatic secretory trypsin inhibitor (e.g., atropine, meperidine, ulinastatin) and anti-inflammatory and anti- antioxidant drugs such as ciprofloxacin and aztreonam are the main clinical drugs used to treat AP. Whereas long-term treatment with these drugs causes serious side effects and drug resistance, and lack of safety in patients with complications [11]. increasing evidence has revealed that traditional Chinese medicines which have low toxicity may be a promising agent in the treatment of AP [12]. In this study, PLG had the potential to inhibit inflammation and oxidative stress, and exerted the protective effects in alleviating L-arginine induced AP.

High blood glucose and overexpression of serum amylase are the highest risk factors for AP, which are closely associated with the pathological injury of AP [13]. L-arginine significantly induced the pancreatic tissue injury. PLG alleviated the pathological injury of AP in L-arginine-treated mice, and its protective effect was achieved in a concentration-dependent manner. The results indicate that the PLG treatment can result in not only a remarkable alleviation of pancreatic tissue injury, but also a significant reduction in high blood glucose and serum amylase expression.

Previous studies have reported that the secretion of pro-inflammatory factors as well as the extensive production of oxidative stress chemicals play key roles in the induction of pancreatic tissue injury during the development of AP [14]. PLG exerts multiple pharmacological effects, including anti-inflammation, anti-oxidation and hypoglycemic activities [7]. Hence, the effects of PLG on the oxidative stress in L-arginine-induced AP were investigated.

Since higher expression levels of MPO, MDA, LDH and ROS generation were observed in L-arginine-treated mice, it follows that PLG has stronger anti-oxidative stress effects and decreased the expression levels of MPO, MDA, LDH and ROS generation in mice caused by L-arginine stimulation. Increasing evidence has it that L-arginine induces the abnormal activation of NF-κB and results in the extensive secretion of pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α, and block the NF-κB pathway enabled to alleviate this state [15]. In this study, the results revealed that PLG suppressed the activity of NF-κB, and down-regulated the expressions of IL-6, IL-1β and TNF-α in a concentration-dependent manner in L-arginine-induced AP mice. However, the precise inhibitory mechanism needs to be further investigated.

The p38 MAPK pathway plays a critical role in different physiological and pathophysiological processes, and this pathway is activated by various factors such as inflammation and oxidative stress [16]. Recently, p38 MAPK pathway was directly involved in the development of AP, and L-arginine stimulation leads to the activation and signal transduction of intracellular p38 MAPK pathway and the regulation of inflammatory cytokines such as IL-6, IL-1β and TNF-α. Therefore, suppression of the activation of p38 MAPK-mediated inflammatory pathways may contribute to the treatment of AP. For example, Li *et al* reported that p38 MAPK pathway was involved in the pathogenesis of AP-related acute lung injury and its down-regulation by Naringin suppressed the inflammatory response and oxidative stress [17]. Zhang *et al* found active diterpene quinone tanshinone IIA protect against L-arginine induced AP in mice by inhibiting inflammation and oxidative stress through the regulation of the Nrf2 / ROS pathway [18].

CONCLUSION

This study indicates that PLG down-regulated the expression of p-ERK, ERK, p-p38 and p38, and displayed an inhibitory effect on the activation of p38 MAPK pathway in L-arginine induced AP mice, which is consistent with the results FROM previous studies. Therefore, PLG Is a promising agent for the inhibition of the activation of p38 MAPK pathway, and thus may serve as a potential therapeutic agent for the treatment of L-arginine-induced AP.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Qinpan Xiong and Chao Du designed the experiments and Wei Xia carried them out. Kang Tang analyzed and interpreted the data, and Chao Du prepared the manuscript with contributions from all co-authors.

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