Original Article
Protective effect of asiatic acid in an experimental cerulein-induced model of acute pancreatitis in mice

Wenqin Xiao1, Weiliang Jiang2, Kai Li2, Yangyang Hu2, Sisi Li2, Li Zhou2, Rong Wan1,2

1Department of Gastroenterology, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, 301 Yanchang Road, Jingan District, Shanghai 200072, People’s Republic of China; 2Department of Gastroenterology, Shanghai First People’s Hospital, School of Medicine, Shanghai Jiao tong University, 100 Haining Road, Hongkou District, Shanghai 200080, People’s Republic of China

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Abstract: Asiatic acid (AA), a triterpenoid derived from the medicinal plant Centella asiatica, is considered to have anti-inflammatory, anti-fibrotic and anti-tumor effects, but its effects in acute pancreatitis (AP) are unknown. Our purpose of this study was to investigate the effects of AA in a mouse model of cerulein-induced pancreatitis. We evaluated AA in an experimental model of AP induced in mice by six hourly intraperitoneal injections of cerulein 50 µg/kg. Mice were pretreated with vehicle or AA 50 mg/kg 2 h before the first cerulein injection. The severity of AP was evaluated histologically and by biochemistry, myeloperoxidase activity, proinflammatory cytokine production, and nuclear factor (NF)-κB activity. Administration of AA significantly reduced the severity of AP, and was associated with reduction of serum amylase and lipase levels, decreased pancreatic histological damage, and decreased myeloperoxidase activity. The serum levels and mRNA expression of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and NF-κB activity were reduced. AA also significantly improved the in vitro viability of pancreatic acinar cells induced by cholecystokinin (CCK) and suppressed NF-κB activity. AA protected against experimental AP, possibly by reducing production of proinflammatory cytokines via suppression NF-κB activation.

Keywords: Acute pancreatitis, inflammatory cytokines, asiatic acid, NF-κB

Introduction

Acute pancreatitis (AP) is a common inflammatory disease with high morbidity and mortality, and its incidence has been increasing [1, 2]. Several lines of evidence show that both the development and severity of pancreatitis are determined by initial events that occur in pancreatic acinar cells, including activation of zymogens and production of proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. The subsequent recruitment of a variety of inflammatory cells and inflammatory mediators leads to further injury and inflammation [3-5]. The necrosis of acinar cells is another key pathological feature of AP, and leads to a series of inflammatory responses. The critical involvement of inflammation in AP, highlights the importance of effective measures to prevent this severe response. A nuclear transcription factor, NF-κB, a key molecule of the NF-κB signaling pathway, is activated early in the development of AP and is involved in its progression [6]. NF-κB regulates the transcription of various factors involved in inflammation and immune responses. In the cytoplasm, inhibitory κB (IκBα), an inhibitory protein in the IκB family, binds to NF-κB transcription factors and prevents them from entering the nucleus [7]. Previously, Chen et al. reported that the activation of NF-κB in pancreatic and systemic inflammatory response [8], and Huang et al. showed that activation of NF-κB in acinar cells increased the severity of pancreatitis in mice [9]. The evidence thus suggests that inactivation of NF-κB or suppression of the NF-κB signaling pathway might be effective in treating AP by preventing inflammation associated with the release of cytokines. Despite increasing knowledge of the pathogenesis of AP, the mechanisms remain unclear, and there is no specific medical treat-
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Most therapy aims to improve hydration, reduce abdominal pain, maintain vital signs, and administer proper nutrition and antibiotics [10]. Therapeutic agents that are effective for treatment of AP are needed.

Asiatic acid (AA) is a pentacyclic triterpenoid extracted from Centella asiatica, a well-known medicinal plant. It has a wide range of therapeutic effects including inhibition of liver, kidney and pulmonary fibrosis, and may be a potential treatment of other diseases with fibrotic components [11-13]. A number of studies indicate its therapeutic potential. Chao et al. showed that AA inhibited apoptosis and reduced inflammatory stress in the striatum of MPTP-treated mice, and suggested that it might be a useful nutraceutical agent to slow the progression of Parkinson’s disease [14]. Nataraj et al. demonstrated the neuroprotective effect of AA on rotenone-induced mitochondrial dysfunction and oxidative stress-mediated apoptosis in differentiated SH-SY5Y cells [15]. Chen et al. indicated AA has anti-inflammation effects in lipopolysaccharide-induced human corneal epithelial cells [16]. AA has also been shown to suppress an inflammatory response in a mouse model of paw edema that was related to expression of NF-κB [17], adding to the evidence linking AA activity to inhibition of NF-κB.

We used a mouse model of cerulein-induced pancreatitis, which included early activation of NF-κB, production of proinflammatory cytokines, and histological damage similar to human pancreatitis [18, 19]. We aimed to determine whether AA could slow progression and reduce the severity of AP by decreasing the production of proinflammatory cytokines and the expression of NF-κB.

**Materials and methods**

*Ethics statement*

All animal-related procedures were approved by the Animal Care and Use Committee of The Tenth People’s Hospital of Shanghai, Tongji University (ID: SYXK 2011-0111). Mice were maintained under 12 h light-dark cycles at 22°C, given water ad libitum, fed standard laboratory chow, and allowed to acclimatize for a minimum of 1 week. The environment was maintained at a relative humidity of 30-70%.

**Animals and materials**

Male BALB/c mice were purchased from Shanghai Laboratory Animal Co Ltd (SLAC, Shanghai, China). Mice weighing 20 ± 2 g were randomly assigned to control or experimental groups. All mice were fasted for 12 h before the induction of AP. Purified AA (≥97%) was purchased from PureOne Biotechnology (Shanghai, China). Cerulein, dimethylsulfoxide (DMSO), eosin and hematoxylin, and β-actin were acquired from Sigma Chemical (Sigma-Aldrich, St. Louis, MO), and antibodies against NF-κB p65, IκB-α, IκB-β, and Lamin-A from Abcam (Abcam, Cambridge, MA, USA). Unless stated otherwise, all other chemicals were purchased from Sigma.

**Experimental design**

AP was induced by hourly (6 times) intraperitoneal injections of 50 µg/kg cerulein, meanwhile control mice received a comparable amount of saline [20]. AA was dissolved in vehicle (2% DMSO). A preliminary study was performed to obtain an optimal effective dose of AA for preventing AP. 25 mice were randomly divided into 5 groups: group 1, normal control; group 2, vehicle-treated; groups 3, 4 and 5, AA-treated (25, 50 and 75 mg/kg, per os (p.o)), respectively. Pretreatment with vehicle or AA was performed 2 h before the first cerulein injection. Mice were sacrificed at 6 h after injection of the first saline or cerulein. Blood samples were collected to examine the levels of serum amylase and lipase, two biochemical markers closely related to pancreatic damage during AP. The optimal dose of AA was consequently established as 50 mg/kg and used for the following experiments. 45 mice were divided into 3 groups randomly: group 1, normal control; group 2, cerulein + vehicle-treated; group 3, cerulein + AA-treated. The induction of AP and pretreatment with vehicle or AA was performed as same as in the preliminary study. Mice were sacrificed at 6, 9 and 12 h after the first cerulein injection, 5 mice at every time-point in each group. The pancreatic tissues were rapidly removed from each mouse, a portion fixed in 4% paraformaldehyde buffered with phosphate-buffered saline (PBS) overnight at 4°C, and embedded in paraffin wax or frozen immediately at -80°C. The remaining portion was quickly ground into liquid nitrogen and frozen at -80°C. Blood samples were collected at room temperature for 2 h.
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before centrifugation for 3000 \( g \) at 4°C for 15 min, and serum stored at -80°C.

**Isolation of pancreatic acinar cells**

Acinar cells were isolated from mice using a collagenase digestion as described previously [21]. Freshly acinar cells were incubated at 37°C, 5% CO\(_2\) in Dulbecco’s modified Eagle’s medium/Ham F-12 (DMEM/F12; Gibco BRL, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (PS; Gibco) with or without CCK, AA at different doses (0, 10, 25 and 50 \( \mu \)mol/l), and other agents as described for the relevant figures.

**Determination of serum amylase, lipase and proinflammatory cytokines**

The levels of serum amylase and lipase were measured via enzyme dynamics chemistry using commercial kits on a Roche/Hitachi modular analytics system (Roche, Mannheim, Germany), and a commercial enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems, Minneapolis, MN, USA) was used for measuring the levels of serum IL-1β, IL-6 and TNF-\( \alpha \), according to the manufacturer’s protocol.

**Histological analysis**

The histology of mouse pancreas, heart, liver, lung and kidney was examined by Hematoxylin and eosin (H&E). Tissues were fixed in 4% phosphate-buffered formaldehyde in 24 h, dehydrated via a graduated ethanol series, and embedded in paraffin blocks. 5 \( \mu \)m thick tissue sections were dewaxed in xylene, hydrated through an upgraded ethanol series, and stained with H&E. Morphological changes were examined under a light microscope by three pathologists who were unaware of the origin of the specimens. In brief, the severity of AP was evaluated using a semiquantitative graded score: acinar edema (0-3), cell vacuolization (0-3), inflammation (0-3), and acinar cell necrosis (0-3).

**Measurement of myeloperoxidase activity**

Neutrophil sequestration in the pancreas was quantified by measuring tissue myeloperoxidase (MPO) activity as described previously [22]. The tissue samples were thawed, homogenized in phosphate buffer (20 mmol/l, pH 7.4), and centrifuged for 10,000 \( g \) at 4°C for 10 min. The pellet was resuspended in phosphate buffer (50 mmol/l, pH 6), containing 0.5% hexadecyltrimethyl ammonium bromide (HETAB). The suspension was subjected to four cycles of freezing and thawing, and further disrupted by sonication for 40 sec. Then the sample was centrifuged for 12,000 \( g \) at 4°C for 5 min, the supernatant was used for the MPO assay. The reaction mixture contained the supernatant, tetramethylbenzidine (1.6 mmol/l) and hydrogen peroxide (0.3 mmol/l), which were prepared in sodium phosphate buffer (80 mmol/l, pH 5.4). After incubation at 37°C for 110 sec, the reaction was terminated with H\(_2\)SO\(_4\) (2 mol/l), and absorbance measured at 450 nm for 5 min using a Beckman spectrophotometer (Beckman DU 640B, CA, USA). One unit of MPO activity was defined as that degrading peroxide (1 mmol/l) at 25°C per min. Activity was expressed in units per milligram of tissue.

**Real-time quantitative PCR**

Total RNA was extracted from pancreas and mouse acinar cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions, and subjected to reverse transcription using the PrimeScript RT reagent Kit (TaKaRa, Japan). Quantitative real-time PCR (qRT-PCR) was performed in triplicate for each gene of interest using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA), according to the SYBR Premix EX Taqmanual (TaKaRa). GAPDH was used as a separate endogenous control to which the gene of interest was normalized, and fold changes for gene expression calculated using the comparative CT (2\(^{-\Delta\DeltaCT}\)) method. Primer sequences for biomarkers were designed with software as shown in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Primer sequences used in qRT-PCR assays</th>
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<tbody>
<tr>
<td>Gene</td>
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</tbody>
</table>
| IL-1β | Forward: TTGACGGACCCAAAAAGAT  
Reverse: GAAGCTGGAT6CTCTCATCG |
| IL-6 | Forward: TTCCCTCTTTGCTCTGAATTAGA  
Reverse: GCTGACCTTTAGCTCAATACTCT |
| TNF-α | Forward: TCTCCTCAAGGGCAAGGCTG  
Reverse: ATAGCAAAATCGGCTGACGCT |
| GAPDH | Forward: GTTCGGTGTTGAACCGATTTG  
Reverse: TGTAGACCATGTAATTTAGGTC |

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Western blotting analysis

For western blotting, mouse pancreas were retrieved from storage and rapidly ground in liquid nitrogen. The resulting powder or isolated acinar cells were lysed in using a nuclear and cytoplasmic protein extract kit (Beyotime), following the manufacturer's protocol, for preparation of nuclear and cytoplasmic proteins (Pierce, CA, USA). The concentrations of nuclear and cytoplasmic proteins were determined using the BCA method (Pierce, Rockford, LA, USA). A 80 µg aliquot of protein or equal proportion of concentrated supernatant was subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE Bio-Rad, CA, USA) and transferred to nitrocellulose/PVDF membrane following the standard method. Non-specific binding blocked with 5% non-fat milk at room temperature for 1 h in a covered container. Membranes were incubated overnight at 4°C with rabbit polyclonal anti-NF-κB p65 antibody (1:1000), rabbit polyclonal anti-IκB-α antibody (1:500), rabbit polyclonal anti-IκB-β antibody (1:500 dilution), rabbit polyclonal anti-Lamin-A antibody (1:1000 dilution), and mouse monoclonal anti-β-actin antibody (1:1000 dilution) in 5% bovine serum albumin (BSA). Lamin-A and β-actin were used as the internal references for nuclear and cytoplasmic proteins respectively. Membranes were washed with TBST and incubated with a secondary goat anti-rabbit IgG-horseradish peroxidase (HRP) antibody (1:2000) or goat anti-mouse IgG-HRP antibody (1:2000) (Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature. Finally, membranes were washed and developed using the ECL detection system (Santa Cruz Biotechnology).

Immunohistochemical analysis

Formalin-fixed, paraffin-embedded samples were cut into 5 µm thick sections. Tissue sections were deparaffinized and rehydrated with upgraded ethanol. For antigen retrieval, slides were boiled in EDTA (1 mmol/l, pH 8.0) for 15 min in a microwave oven. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide solution for 10 min at room temperature. After rinsing with PBS, slides were blocked with BSA in PBS for 30 min. Slides were subsequently incubated with a polyclonal antibody against NF-κB p65 (1:100) overnight at 4°C. Antibody binding was detected with an Envision Detection Kit, Peroxidase/DAB, Rabbit/Mouse (Gene Tech, Shanghai, China). Sections were counterstained with hematoxylin. For NF-κB p65 in control status, the cytoplasm of positive cells was stained, and translocation of positive cells to nuclei from the cytoplasm indicated activation of NF-κB p65. Positive areas stained with NF-κB p65 were observed in all specimens under a microscope at a magnification of ×400 by three pathologists who were unaware of specimen origins (CTR 6000; Leica, Wetzlar, Germany).

Quantification of acinar cell viability

The proliferation of acinar cells was examined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), and the viability of acinar cell was detected by measuring ATP depletion using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Statistical analysis

Results were expressed as means ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls (SNK) as a post hoc test. The Kruskal-Wallis test was used to evaluate the differences in categorical values, followed by Mann-Whitney U tests as a post hoc test. P<0.05 was considered statistically significant differences.

Results

Preliminary study

It was previously shown in a mouse model that AA protected against liver damage and had a significant therapeutic effect in mice when given at doses of 25, 50, and 100 mg/kg [23]. Thus, to choose an optimal dose, we evaluated three doses below 100 mg/kg (25, 50, and 75 mg/kg) in a preliminary study. As shown in Figure 1A-C, the higher doses of AA (50, 75 mg/kg) resulted in significantly lower serum amylase and lipase, and significantly less damage to pancreas histology compared with the lower dose (25 mg/kg) at 6 h after the first cerulein injection. As there were no significant differences in the effects of 50 and 75 mg/kg AA, we selected 50 mg/kg as the optimal dose
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for use in the experimental procedures. Moreover, there is no significant histological differences in heart, liver, lung and kidney in control, vehicle and 50 mg/kg AA groups (Figure 1D), indicating that 50 mg/kg is a safe dose in our present study.

Effect of AA on pancreas histology, enzyme production, IL-1β, IL-6 and TNF-α in cerulein-induced AP

We used a mouse model of cerulein-induced AP to determine the therapeutic effects of AA
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Mice were given vehicle or AA 2 h before the first cerulein injection; serum and pancreas tissues were collected 6, 9, and 12 h after the first cerulein injection. As shown in Figure 2A and Figure 2B, 2C. Effect of AA on pancreas histology and enzyme production of cerulein-induced AP in vivo. Mice were given 6 hourly injections of cerulein 50 μg/kg. Vehicle or AA 50 mg/kg was administered 2 h before the first cerulein injection. The control group was given saline instead of cerulein. Five mice were sacrificed at 6, 9, and 12 h after the first cerulein injection. A. Pancreatic tissues were examined by H&E staining (magnification ×200). B, C. Blood samples were collected for assay of serum amylase and lipase. D. MPO activity at 6, 9, and 12 h after the first cerulein injection. Results are means ± SD of three independent experiments. *P<0.05, vs. controls; #P<0.05, vs. cerulein and vehicle-treatment.

Table 2. Effect of AA on pancreas pathology scores in cerulein-induced AP

<table>
<thead>
<tr>
<th>Group</th>
<th>Edema</th>
<th>Vacuolization</th>
<th>Inflammation</th>
<th>Necrosis</th>
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<tbody>
<tr>
<td>Control</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Cerulein + Vehicle</td>
<td>2.75 ± 0.43 *</td>
<td>1.12 ± 0.47 *</td>
<td>2.12 ± 0.37 *</td>
<td>1.46 ± 0.43 *</td>
</tr>
<tr>
<td>Cerulein + AA</td>
<td>1.36 ± 0.27 *</td>
<td>0.42 ± 0.29 *</td>
<td>0.98 ± 0.41 *</td>
<td>0.69 ± 0.25 *</td>
</tr>
</tbody>
</table>

Results are mean ± SD of three independent assays. *P<0.05, vs. controls; #P<0.05, vs. cerulein and vehicle.
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Table 2, AA significantly reduced histological damage of the pancreas and inhibited MPO activity at 6, 9, and 12 h (Figure 2D). Serum amylase and lipase are the most frequently used biochemical markers of AP, and we used them to assess the severity of pancreatitis in this experimental model. As shown in Figure 2B and 2C, AA significantly reduced the levels of serum amylase and lipase in cerulein-treated mice.

AP is an inflammatory disease that is associated with the production of many inflammatory cytokines and mediators, including IL-1β, IL-6 and TNF-α. Analysis of protein expression by ELISA and mRNA expression by qRT-PCR revealed that AA significantly reduced both protein (Figure 3A) and mRNA (Figure 3B) expression of the markers that were tested.

Effect of AA on NF-κB activity in cerulein-induced AP

NF-κB activation plays a vital role in inflammatory and immune responses, with nuclear translocation of NF-κB preceded by the degradation of IκB-α and IκB-β in the cytoplasm. To determine whether AA affected NF-κB activity, we investigated the nuclear translocation of NF-κB p65 and the expression of IκB-α and IκB-β by western blotting. As shown in Figure 4A, AA up-regulated the expression of IκB-β and IκB-β down-regulated the expression of nuclear NF-κB p65. Immunohistochemical analysis further confirmed that AA blocked the nuclear translocation of NF-κB p65 (Figure 4B).

Effect of AA on CCK-induced AP in vitro

To extend the in vivo finding that AA alleviated damage to the pancreas in cerulein-induced AP, we evaluated the in vitro protective effects of AA on CCK-induced cell death in freshly isolated acinar cells. We used a Cell Counting Kit-8 and a sensitive colorimetric test (CellTiter-Glo Luminescent Cell Viability Kit), to assay acinar cells viability and ATP depletion associated with necrosis of acinar cells incubated with or without CCK (200 nmol/l) and AA at 0, 10, 25, or 50 μmol/l. As shown in Figure 5A and 5B, AA significantly improved the in vitro viability of acinar cells, suppressed NF-κB activity by decreasing the protein expression of nuclear NF-κB.
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Figure 4. Effect of AA on NF-κB activity in cerulein-induced AP in vivo. A. Nuclear NF-κB p65, IκB-α and IκB-β protein levels were assayed in western blots with Lamin-A and β-actin as internal references for nuclear proteins and cytoplasmic proteins, respectively. B. Immunohistochemical staining of NF-κB p65 detect nuclear translocation (magnification ×400). Results are means ± SD of three independent experiments.

p65 protein, as well as the degradation of IκB-α, and IκB-β (Figure 5C). As our in vivo results demonstrated that AA significantly down-regulated the production of inflammatory cytokines, we further detected the production of IL-1β, IL-6 and TNF-α in CCK-induced AP in vitro. As shown in Figure 5D, AA obviously decreased the release of inflammatory cytokines in CCK-induced AP, suggesting that AA has anti-inflammation effects in experimental pancreatitis.
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Discussion

Most AP patients experience mild disease with a low complication rate, but several develop acute respiratory distress syndrome, or multiple organ dysfunction, and eventually die due to the lack of specific therapy [4, 24]. AP is characterized by a complex cascade of events, including pancreatic inflammation, destruction of pancreatic tissue, and systemic inflammation [8]. The complex molecular events that underlie these changes remain largely unknown and there are no therapeutic agents to treat AP. AA has a variety of potent pharmacological effects, and a role in AP is yet to be established, but we found it to have a protective effect in a mouse model of cerulein-induced AP.

Expression of the proinflammatory cytokines IL-1β, IL-6 and TNF-α, which are involved in the development of pancreatitis, are up-regulated in experimental AP models, and their blockade has been shown to slow the progression of pancreatitis [25]. Previously, Norman et al. showed that IL-1, IL-6, and TNF-α secretion increased with the presence of inflammatory macrophages in cerulein-induced AP [26], and Rau et al. found that inhibition of secretion of those cytokines reduced the severity of AP [27, 28]. The number of neutrophils infiltrating the pancreas can be estimated by assaying tissue MPO activity [29]. A decrease of serum amylase and lipase concentrations, improved histological features, reduced MPO activity, and decreased IL-1β, IL-6 and TNF-α serum protein and mRNA expression in vivo and in vitro all indicate that AA attenuated the severity of pancreatitis induced in the mice by cerulein.

Recent studies show that AA can inhibit the stimulation of IL-1β, TNF-α and other inflammatory cytokines to reduce functional damage and fibrosis of liver cells [11, 30]. Huang et al. showed that the anti-inflammatory mechanisms of AA might be related to a decrease of iNOS and NF-κB via increases of catalase,
superoxide dismutase, and glutathione peroxidase activity [17]. Pakdeechote et al. reported that AA alleviated hemodynamic and metabolic alterations by restoring eNOS/NO expression and reducing oxidative stress and inflammation in rats with diet-induced metabolic syndrome [31]. The transcription factor NF-κB is activated early in AP, and promotes inflammation by regulating the expression of inflammatory mediators. In this process, cytoplasmic NF-κB is released from its association with inhibitory proteins in the IκB family, such as IκB-α and IκB-β. This allows NF-κB to translocate into the nucleus and activate the expression of specific target genes [8, 32]. Previous studies have shown a correlation between NF-κB activation and experimental AP [6, 9], and NF-κB p65 has been identified as the key transcription factor involved in pancreatitis [33]. These findings have led to interest in investigating NF-κB inhibition as a novel approach in treating AP. As activation of NF-κB is an early event in AP, we investigated NF-κB activity at 6, 9 and 12 h after the first cerulein injection. The western blots revealed that pretreatment with AA markedly reduced the degradation of IκB-α and IκB-β, resulting in down-regulation of NF-κB p65 expression in the nucleus. The nuclear translocation of NF-κB p65 was subsequently confirmed by immunohistochemistry, and the results showed a significant reduction in the staining intensity of nuclear NF-κB p65 in mice treated with AA. AA greatly increased the in vivo viability of acinar cells from mice with AP, and suppressed expression of NF-κB. The results warrant further investigation of the therapeutic effect of AA in AP.

The mechanism of AP is complex and remains unclear. In this experimental mouse model, AA alleviated the severity of AP, reduced pancreatic tissue damage, decreased proinflammatory cytokine production and MPO activity, and increased the viability of acinar cells. The suppression of NF-κB activation was involved in the mechanism of AA effects on pancreatitis. AA deserves further experimental and clinical evaluation as a potential treatment of AP.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Rong Wan, Department of Gastroenterology, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, China. Tel: +86-021-6630-0588; Fax: +86-021-5677-3983; E-mail: wanrong1970@163.com

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