Icariin attenuates the severity of cerulein-induced acute pancreatitis by inhibiting p38 activation in mice

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Received January 18, 2019; Accepted July 8, 2019

DOI: 10.3892/ijmm.2019.4312

Abstract. Acute pancreatitis (AP) is an inflammatory disease of the pancreas. Icariin (ICA), a flavonoid glycoside, has been reported to have several pharmacological effects; however, the anti-inflammatory effects of ICA against AP require further study. Therefore, we aimed to investigate the effect of ICA on cerulein-induced AP. In the present study, AP was induced by intraperitoneally administering a supramaximal concentration of cerulein (50 μg/kg/h) for 6 h. ICA was also administered intraperitoneally, and mice were sacrificed 6 h after the final cerulein injection. Blood samples were collected to determine serum amylase and lipase levels. The pancreas and lung were rapidly removed for histological examination, and the analysis of myeloperoxidase activity. In addition, reverse transcription-quantitative polymerase chain reaction was conducted to analyze the expression of inflammatory cytokines in pancreatic tissues. Our results revealed that the administration of ICA prevented an increase in the pancreas weight/body weight ratio of mice and serum digestive enzyme levels. ICA treatment also inhibited cerulein-induced histological injury and neutrophil infiltration of the pancreas and lung. In addition, ICA suppressed the production of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6 and tumor necrosis factor-α in the pancreas. Furthermore, ICA administration was observed to inhibit p38 activation during cerulein-induced AP. Inhibition of p38 activation resulted in alleviated pancreatitis. Collectively, our results suggested that ICA exhibits anti-inflammatory effects in cerulein-induced AP via the inhibition of p38.

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Key words: acute pancreatitis, cerulein, cytokines, icariin, p38

Introduction

Acute pancreatitis (AP) is a sudden inflammation of the pancreas and the most common reason for hospitalization among non-malignant gastrointestinal disorders in the United States (1,2). AP is commonly triggered by various risk factors, such as alcohol abuse, duct obstruction, obesity, smoking, drug toxicity and metabolic disorders (3,4). This condition arises from pancreatic acinar cell injury by the activation of proteolytic pancreatic enzymes, including amylase, lipase, and trypsin (5). Injured acinar cells produce pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α (6). These cytokines damage the acinar cell, causing systemic inflammatory response syndrome, which is a subset of cytokine storm (5-8). Although AP is a critical disease, few studies have investigated effective therapeutic treatments for AP.

Icariin (ICA) is a natural flavonoid glycoside isolated from plants in the *Epimedium* genus (Yin-Yang-Huo) of the Berberidaceae family and a well-known traditional Chinese medicine for treating male sexual dysfunctions, such as erectile dysfunction and premature ejaculation (9,10). ICA, the major and active compound of *Epimedium*, has been associated with many biological and pharmacological effects, such as anti-osteoporotic (11), antioxidative (12), anti-inflammatory (13), anti-cancer (14), anti-depressant (15) and neuro-protective effects (16). Previous studies have reported that ICA ameliorates various diseases, including atherosclerosis, asthma and erectile dysfunction (17-19); however, the therapeutic effects of ICA in acute pancreatitis require further investigation.

The purpose of this study was to investigate the effects of ICA against cerulein-induced AP. Pancreas weight/body weight (PW/BW) ratio, serum amylase and lipase levels, histological appearance of the pancreas and lung, myeloperoxidase (MPO) activity and pro-inflammatory cytokine levels were evaluated to determine the attenuating effects of ICA on the severity of cerulein-induced AP. Furthermore, we evaluated the activation of mitogen activated protein kinases (MAPKs, indicated by MAPKs phosphorylation) and nuclear factor κB (NF-κB, indicated by Iκ-Bα degradation) to determine the inhibitory mechanisms of ICA on AP.

Materials and methods

Chemicals and reagents. ICA, dimethyl sulfoxide (DMSO), cerulein, NaCl, hexadecyl-trimethyl-ammonium bromide (HTAB), and Tris-HCl were purchased from Sigma-Aldrich (Merck KGaA). ICA stock solutions were using DMSO. Easy-Blue™ total RNA extraction kit was purchased from iNtRON Biotechnology. Anti-phosphorylated extracellular signal-regulated kinase (ERK)1/2 (1:1,000; cat. no. 9101L), phosphorylated c-Jun N-terminal kinase (JNK 1/3; 1:1,000; cat. no. 9251L), and p38 (1:1,000; cat. no. 9211L) antibodies and SB203580 (p38 inhibitor) were purchased from Cell signaling Technology, Inc. Total MAPK antibodies against ERK 1/2 (1:1,000; sc-93), JNK 1/3 (1:1,000; sc-474) and p38 (1:1,000; sc-535), inhibitory κ-Bα (Iκ-Bα; 1:1,000; sc-371), and β-actin antibody (1:1,000; sc-1615) were obtained from Santa Cruz Biotechnology, Inc. In addition, horseradish peroxidase (HRP)-conjugated secondary antibodies, including chicken anti-rabbit IgG-HRP (1:5.000; sc-516087) and chicken anti-goat IgG-HRP (1:10.000; sc-516086) were purchased from Santa Cruz Biotechnology, Inc.

Ethical aspects. All experiments were performed in accordance with the animal care regulations of Wonkwang University set forth and approved by the Wonkwang University Animal Ethics Committee.

Animal models. All experiments were performed according to the protocols approved by the Animal Care Committee of Wonkwang University. C57BL/6 mice (n=324, 6-8 weeks old, female, weighing 15-20 g), were purchased from Orient Bio. All animals were bred and housed in standard shoebox cages in a climate-controlled room with an ambient temperature of 23±2˚C under a 12 h light/dark cycle for 7 days. Animals were fed standard laboratory chow, and water *ad libitum*. Mice were randomly assigned to control or experimental groups.

Experimental design. AP was induced via six intraperitoneal injections (i.p.) of cerulein (50 μg/kg) at intervals of 1 h. Control animals were administered DMSO under the same conditions. In the pre-treatment groups, ICA (5, 10 or 20 mg/kg, i.p.), SB203580 (1 mg/kg i.p., n=6) or DMSO (i.p.) were administered 1 h before the first cerulein injection, in the experimental (n=6) and the control group (n=6) respectively. Mice were sacrificed 6 h after the last cerulein injection. In the post-treatment groups, ICA (20 mg/kg, n=6) or DMSO (control group, n=6) were administered 1, 3 and 5 h after the first cerulein injection. Mice were sacrificed 6 h after the last cerulein injection. Blood (withdrawn from the heart), the pancreas, and the lung were rapidly removed and stored at -80˚C for further analysis. For the detection of MAPKs and NF-κB, mice were pretreated with ICA (10 or 20 mg/kg) or DMSO 1 h before the intraperitoneal injection of cerulein (50 μg/kg), and sacrificed at 0, 15, 30 and 60 min after cerulein injection, respectively. For immunofluorescence, mice were pretreated with ICA (20 mg/kg) or DMSO 1 h before the intraperitoneal injection of cerulein (50 μg/kg), and sacrificed at 30 min after cerulein injection. Mice were sacrificed via CO_2 asphyxiation. The CO_2 flow rate displaced 20% of the cage volume per minute. To ensure death following CO_2 asphyxiation, cervical dislocation was performed. The pancreas was rapidly removed and stored at -80˚C for further analysis.

Histological analysis. The appearance of the entire pancreas and the semi-quantitative index based on edema and inflammation were examined from each treatment group. The pancreas and lung tissues were fixed in 4% formalin solution at room temperature overnight, embedded in paraffin, cut into 4 μm sections, stained with hematoxylin for 8 min and eosin for 2 min overnight. Samples were examined under a light microscope. Tissue sections from each sample, representing a minimum of 100 fields were examined and scored on a scale of 0-3 (0 corresponding to normal appearance and 3 corresponding to severe disease), based on the presence of interstitial edema and interstitial inflammation. In the assessment of the lung, the sections were examined for the presence of wall thickening and inflammation. The levels of edema, and pro-inflammatory cell infiltration were scored by pathologists in a blinded manner who were unware of the study design; scores were set based on a scale of 0 (normal) to 3 (severe) (20). In brief, a score of 0 presented the absence of edema and inflammatory cells, 1 indicated focal edema between lobules and 10 inflammatory cells; 2 suggested diffuse edema between lobules and 10-20 inflammatory cells, while a score of 3 reflected diffuse edema between acinar cells and >20 inflammatory cells.

Measurement of serum amylase and lipase levels. Blood samples for the determination of serum amylase and lipase levels were obtained 6 h after the last injection of cerulein. Mice were sacrificed via CO_2 asphyxiation followed by cervical dislocation; blood samples were then withdrawn from the heart. Amylase and lipase activities were determined by an assay kit of Bio Assay Systems.

MPO activity. Sequestration of neutrophils within the pancreas and lung was evaluated by measuring the MPO activity within tissues. Briefly, tissue samples were weighed, homogenized with 20 mM phosphate buffer (pH 7.4), and centrifuged at 10,000 x g for 10 min, 4˚C. The pellets were then re-suspended in 50 mM phosphate buffer (pH 6.0) containing 0.5 % HTAB. The samples were centrifuged at 10,000 x g for 5 min, 4˚C, and mixed with 80 mM sodium phosphate buffer (pH 5.4) containing 1.6 mM TMB. The mixture was incubated at 37˚C for 110 sec, and the reaction was terminated with 2 M H_2SO_4. Tissue MPO activity was determined by measuring the absorbance at 450 nm with a Versa Max microplate reader (Molecular Devices, LLC) and was expressed as U/mg protein.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). mRNA transcripts of mouse pancreatic tissues were analyzed by RT-qPCR. Total RNA was isolated from the mouse pancreas using Easy-Blue™ and subjected to RT using ABI cDNA synthesis kit according to the manufacturer's protocols (Applied Biosystems; Thermo Fisher Scientific, Inc.). For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for the expression of the gene of interest, and the results were normalized to those of the 'housekeeping' hypoxanthine phosphoribosyltransferase (HPRT) mRNA. qPCR was performed using a standard protocol from the TaqMan™ Universal Master Mix II, no UNG (Applied Biosystems; Thermo Fisher Scientific, Inc.) on StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed at 50˚C for 2 min and

95˚C for 10 min, followed by 60 cycles of amplification at 95˚C for 10 sec and 60˚C for 30 sec. Forward, reverse, and probe oligonucleotide primers for multiplex real-time TaqMan PCR were purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.). The forward, reverse and probe oligonucleotide primers for multiplex real-time TaqMan PCR were as follows: For mouse TNF-α forward, 5'-TCTCTTCAAGGGACAAGG CTG-3', reverse, 5'-ATAGCAAATCGGCTGACGGT-3'; mouse IL-1β forward, 5'-TTGACGGACCCCAAAAGA T-3', reverse, 5'-GAAGCTGGATGCTCTCATCTG-3'; mouse IL-6 forward, 5'-TTCATTCTCTTTGCTCTTGAATTAGA-3', reverse, 5'-GTCTGACCTTTAGCTTCAAATCCT-3'; mouse HPRT (forward, 5'-GACCGGTCCCGTCATGC-3'; reverse, 5'-CAT AACCTGGTTCATCATCGCTAA-3').

Western blotting. Pancreatic tissues were homogenized and lysed on ice with radioimmunoprecipitation assay lysis buffer (iNtRON Biotechnology). Then, the lysates were boiled in 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 20% glycerol, and 10% 2-mercaptoethanol. Proteins were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in phosphate-buffered saline with Tween-20 for 2 h at room temperature, followed by incubation with primary antibodies overnight at 4˚C. After washing three times, the membrane was incubated with secondary antibodies for 1 h at room temperature. The proteins were visualized using an enhanced chemiluminescence detection system (GE Healthcare) according to the manufacturer's protocols. The bands were detected and quantified by using Quantity One software (version 4.5.2; Bio-Rad Laboratories, Inc.).

Immunofluorescence. Immunofluorescence for the analysis of phosphorylated p38 was performed on pancreas tissue. The pancreas tissues were embedded in frozen section compound, cut into 9 μm sections, fixed in 100% methanol at -20˚C for 5 min The tissues were incubated with the primary antibodies against phosphorylated p38 (1:250) at 4˚C overnight followed by the fluorescence labeled secondary antibodies Alexa Fluor® 488 goat anti rabbit (1:2,000; A27034; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 2 h. Nuclei were counter stained with DAPI (5 ng/ml) at room temperature for 5 min. Stained sections were visualized using a confocal laser microscope (Olympus Corporation).

Statistical analysis. Data were expressed as the mean ± standard error of the mean. Statistical significance of intergroup differences was evaluated using two-way analysis of variance, with time and dose as variables, followed by a Duncan's post-hoc test. All statistical analyses were performed using SPSS version 10.0 statistical analysis software (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference. All experiments were conducted in triplicate.

Results

Effects of ICA on the severity of cerulein-induced AP. To evaluate the prophylactic effects of ICA against cerulein-induced AP, histological examination of the pancreas and lung was carried out. In the DMSO-treated mice, histological features of the pancreas and lung showed typical normal architecture. In the DMSO-treated mice with AP, histological features of damaged pancreas tissue were observed, as characterized by interstitial edema and inflammatory cell infiltration. However, treatment with ICA ameliorated the pancreatic histological damage in a dose-dependent manner (Fig. 1B). In addition, histological features of the lung in cerulein-induced AP were observed; lung injury was characterized by alveolar wall thickening and inflammatory cell infiltration. Nevertheless, lung injury was significantly reduced by treatment with ICA compared with the control (Fig. 1D and E). In addition, to assess the effects of ICA on the severity of cerulein-induced AP, PW/BW ratio, serum amylase and lipase levels were measured. In the cerulein-induced AP mice, the PW/BW ratio was increased than in the control mice due to pancreatic edema; however, cerulein-induced increases in the PW/BW ratio was inhibited by ICA treatment in a dose-dependent manner (Fig. 1F). Furthermore, the levels of serum digestive enzymes, including amylase and lipase were significantly increased in the cerulein-induced AP mice than in the control mice; the levels of both enzymes were decreased by ICA treatment (Fig. 1G and H).

Effects of ICA on MPO activity and pancreatic cytokine production in cerulein-induced AP. Neutrophil migration into target tissue is increased when inflammation occurs (21). Therefore, we examined MPO activity in the pancreas and lung after the induction of AP, in order to measure neutrophil infiltration. As shown in Fig. 2A, MPO activity was significantly higher in cerulein-induced AP mice than in control mice; however, the increase of MPO activity was inhibited by ICA treatment in the pancreas and the lung in a dose dependent manner. Of note, the production of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α were determined to increase during cerulein-induced AP (22). Thus, we examined the pancreatic mRNA levels of IL-1β, IL-6 and TNF-α. As shown in Fig. 2B, the expression levels of these pro-inflammatory cytokines significantly increased in cerulein-induced AP mice compared with the control; however, ICA treatment inhibited IL-1β, IL-6 and TNF-α expression in a dose-dependent manner.

Effects of ICA on the activation of MAPK and NF-κB in cerulein-induced AP. To investigate the inhibitory mechanisms of ICA on AP, the activation of MAPKs and NF-κB was examined in the pancreas. Mice were administered ICA (20 mg/kg) or DMSO for 1 h and then stimulated with cerulein for 0, 15, 30 and 60 min. Cerulein treatment triggered the phosphorylation of MAPKs and the degradation of Iκ-Bα. However, administration of ICA inhibited the phosphorylation of p38, but not of ERK 1/2, JNK, and degradation of Iκ-Bα (Fig. 3A). Consistent with Fig. 3A, p38 phosphorylation was also inhibited by ICA as determined via immunofluorescence staining (Fig. 3C). To determine whether the inhibition of p38 activation was responsible for the effects of ICA, we administered ICA at different concentrations (10 or 20 mg/kg). As shown in Fig. 3D and E, the phosphorylation of p38 was inhibited by ICA in a dose-dependent manner.

Effects of P38 on the severity in cerulein-induced AP. To determine whether inhibition of p38 could affect the reduction of

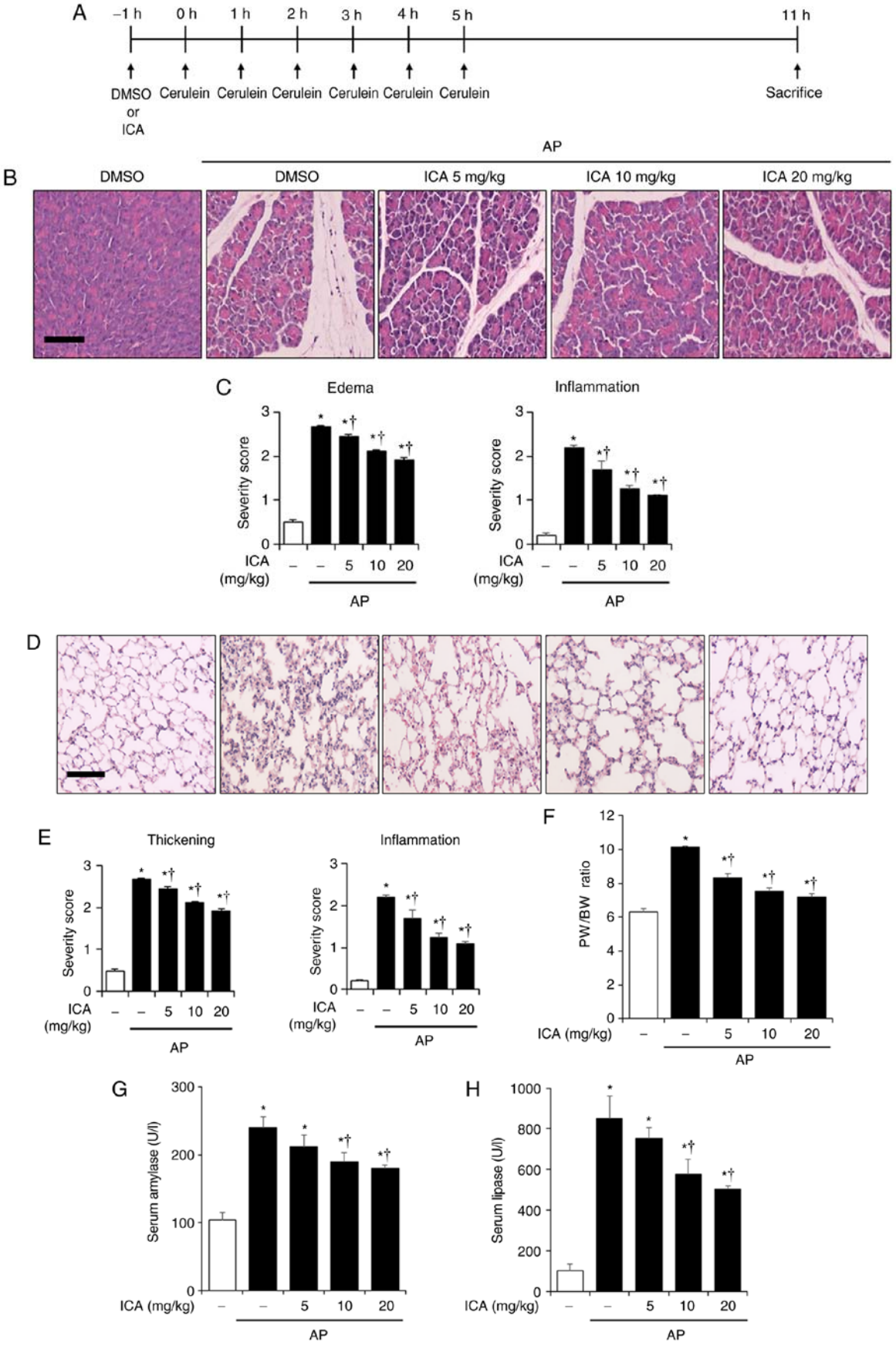

Figure 1. Effects of ICA on the severity of cerulein-induced AP. (A) Scheme for pretreatment experiment. Mice were pretreated with ICA (5, 10 or 20 mg/kg) or DMSO 1 h prior to the induction of AP with cerulein (50 μg/kg). Mice were sacrificed 6 h after the last cerulein injection. Representative H&E-stained sections of the (B) pancreas and (D) lung (original magnification, x200). Histological scores for (C) pancreatic edema and inflammation, (E) pulmonary wall thickening and inflammation. (F) PW/BW ratio, serum levels of (G) amylase and (H) lipase. The groups were treated as indicated in the experimental protocol. Data are presented as the mean ± standard error of the mean, n=6. Results are representative of three experiments. *P<0.05 vs. DMSO treatment alone; †P<0.05 vs. cerulein treatment alone. Scale bar, 20 μm. AP, acute pancreatitis; DMSO, dimethyl sulfoxide; ICA, icariin; PW/BW ratio, pancreas weight/body weight ratio.

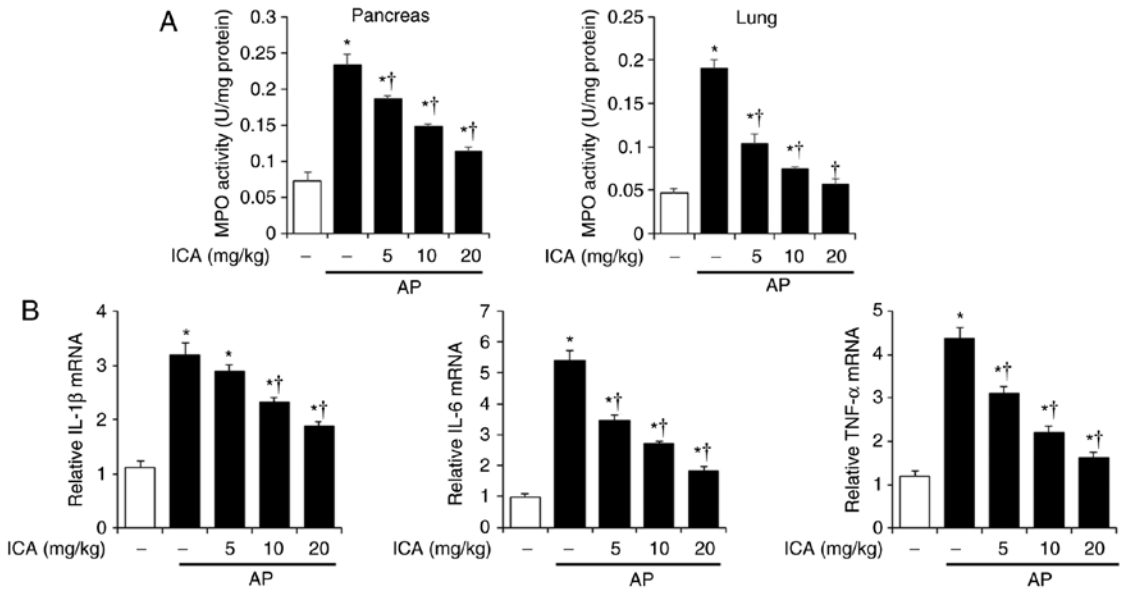

Figure 2. Effects of ICA on MPO activity and the pancreatic cytokines production in cerulein-induced AP. Mice were pretreated with ICA (5, 10 or 20 mg/kg) or DMSO 1 h prior to the induction of AP with cerulein (50 μg/kg). Mice were sacrificed 6 h after the last cerulein injection. (A) Pancreas and lung MPO activity was measured 6 h after the last injection of cerulein. (B) mRNA expression levels of pancreatic IL-1β, IL-6 and TNF-α were quantified by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard error of the mean, n=6. Results are representative of three experiments. *P<0.05 vs. DMSO treatment alone; †P<0.05 vs. cerulein treatment alone. AP, acute pancreatitis; ICA, icariin; IL-1β, interleukin-1β; IL-6, interleukin-6; MPO, myeloperoxidase; TNF-α, tumor necrosis factor-α.

inflammatory responses, a p38 inhibitor (SB203580, 1 mg/kg) was employed, after which the severity of AP was evaluated. As presented in Fig. 4, inhibition of p38 by SB203580 suppressed the histological damage to the pancreas and lung, and reduced the PW/BW ratio, and the levels of amylase and lipase compared with AP mice (Fig. 4). These observations were similar to the effects of ICA (Fig. 1). Additionally, administration of SB203580 significantly reduced MPO activity, and the mRNA levels of IL-1β, IL-6 and TNF-α compared with in AP mice (Fig. 5).

Therapeutic effects of ICA in cerulein-induced AP. To examine the therapeutic effects of ICA in cerulein-induced AP, we applied ICA after the onset of AP. Post-treatment of ICA at 1 h but not 3 and 5 h after first cerulein injection significantly reduced the PW/BW ratio, serum amylase and lipase activities, and histological injury of the pancreas and lung compared with the AP mice, which suggests that ICA could exhibit therapeutic properties in the early phase of AP (Fig. 6).

Discussion

AP is an acute inflammatory disease that occurs in the pancreas, and its incidence is ~300 in 1 million (23). The symptoms of AP vary from mild inflammation of the pancreas to severe inflammatory reactions with multiple organ failure (24). The mortality rate of mild AP is ~10% and most of them are self-healing without complications (25). The mortality rate of severe AP is about 20-30%, which is caused by complications of multiple organ failure, such as liver dysfunction and pulmonary dysfunction (26). In general, AP is treated via temporary symptomatic relief, including antibiotics or analgesics, and in severe cases, the necrotic pancreatic tissue is removed through surgery. However, current therapies have limitations such as AP-associated complications and high mortality (27). Therefore, agents for the prevention and treatment of AP are urgently required.

The diagnosis of AP is based on the increasing levels of serum amylase and lipase. This is due to the fact that the levels of these digestive enzymes, which increase during AP, contribute to acinar cell injury, further promoting the inflammatory process, including the release of cytokines and chemokines (28,29). Serum amylase concentration increases at least three times in comparison with the upper limit of the normal range (30). It starts to rise within a few hours after the onset of symptoms and reverts to the normal level within 3-5 days. Due to these features (serum amylase elevation in early AP and short half-life of amylase), the measurement of serum amylase level may be a suitable diagnosis criterion in early presentation. Additionally, serum lipase concentration is increased at least four times of the normal level in cerulein-induced AP (31). It remains high for a longer period of time than the levels of amylase at 8-14 days (31). Therefore, serum lipase analysis may have an advantage over serum amylase levels as of its delayed presentation. In the present study, serum amylase and lipase levels increased in mice with cerulein-induced AP; however, ICA treatment inhibited the elevation of serum amylase and lipase levels, which suggests the therapeutic effect of ICA on AP was mediated by the reduction in the levels of these enzymes.

Neutrophils have a central role in the development of AP by mediating local tissue damage and remote organ injury (32). They are recruited to sites of infection or inflammation, and in regions of pancreatitis (32). Infiltration and activated neutrophils prolong lifespan of neutrophils by several days and release inflammatory mediators, which further worsen the severity of AP (33,34). As inflammation continues, neutrophil accumulation increases in which

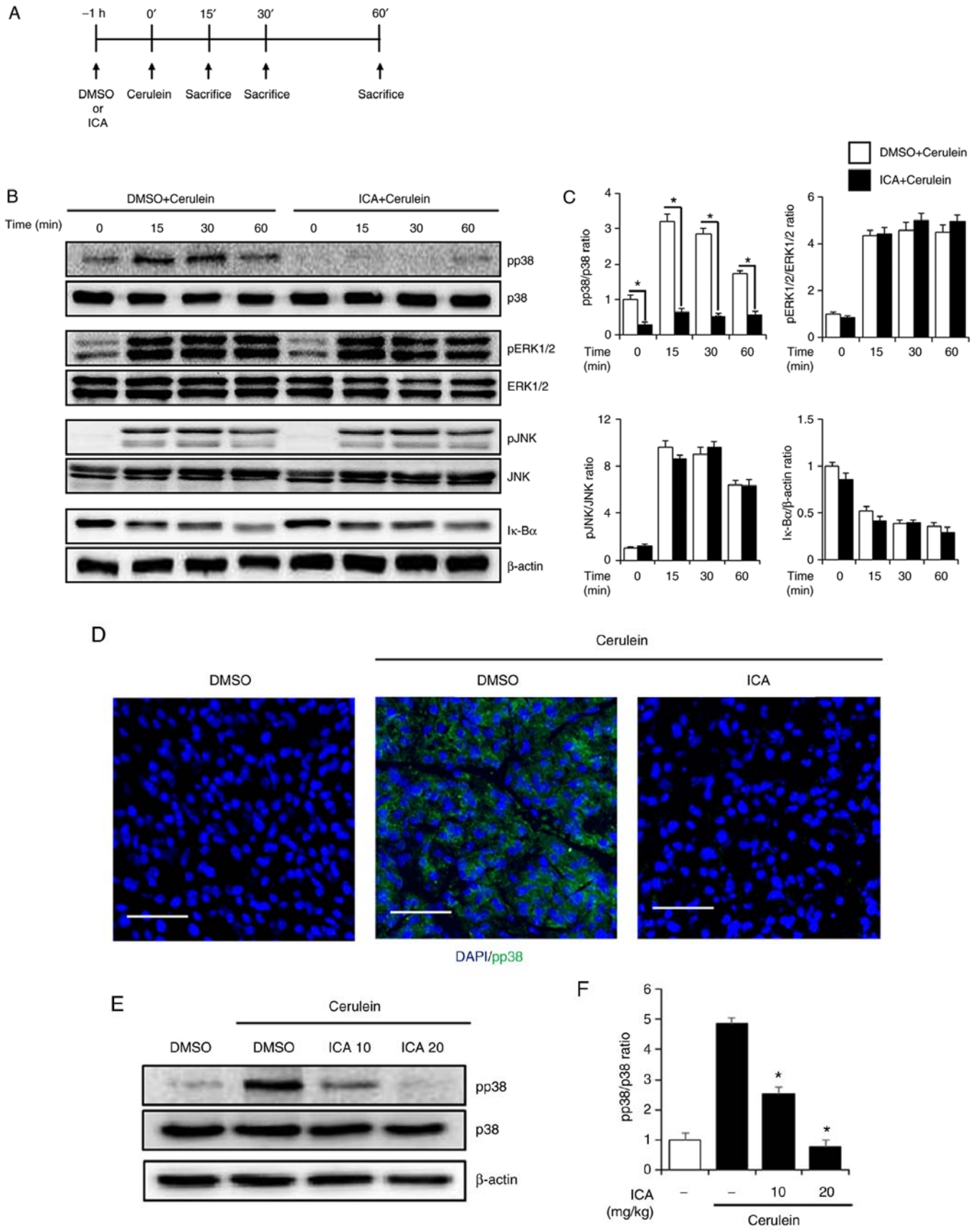

Figure 3. Effects of ICA on the activation of MAPK. (A) Scheme for applied in the study. Mice were pretreated with ICA (20 mg/kg) or DMSO (control) 1 h prior to the induction of AP with cerulein (50 μg/kg). Mice were sacrificed at 0, 15, 30 and 60 min after cerulein treatment. Pancreatic tissues were harvested for western blot analysis to detect (B) phosphorylation of MAPKs and degradation of Iκ-Bα. β-actin was used as a loading control. (C) Quantitative analysis of western blot. (D) Phosphorylation of p38 at 30 min after cerulein treatment was detect by immunofluorescence staining. (E) After 30 min of cerulein treatment, phosphorylation of p38 in different concentrations of ICA (10 or 20 mg/kg) was detected by western blot analysis. (F) Quantitative analysis of western blot. Results are representative of three experiments. *P<0.05 vs. DMSO + cerulein treatment. Scale bar, 40 μm. ERK, extracellular signal-regulated kinase; ICA, icariin; IF, immunofluorescence; Iκ-Bα, inhibitory κ-Bα; JNK, c-Jun N-terminal kinase; p, phosphorylated.

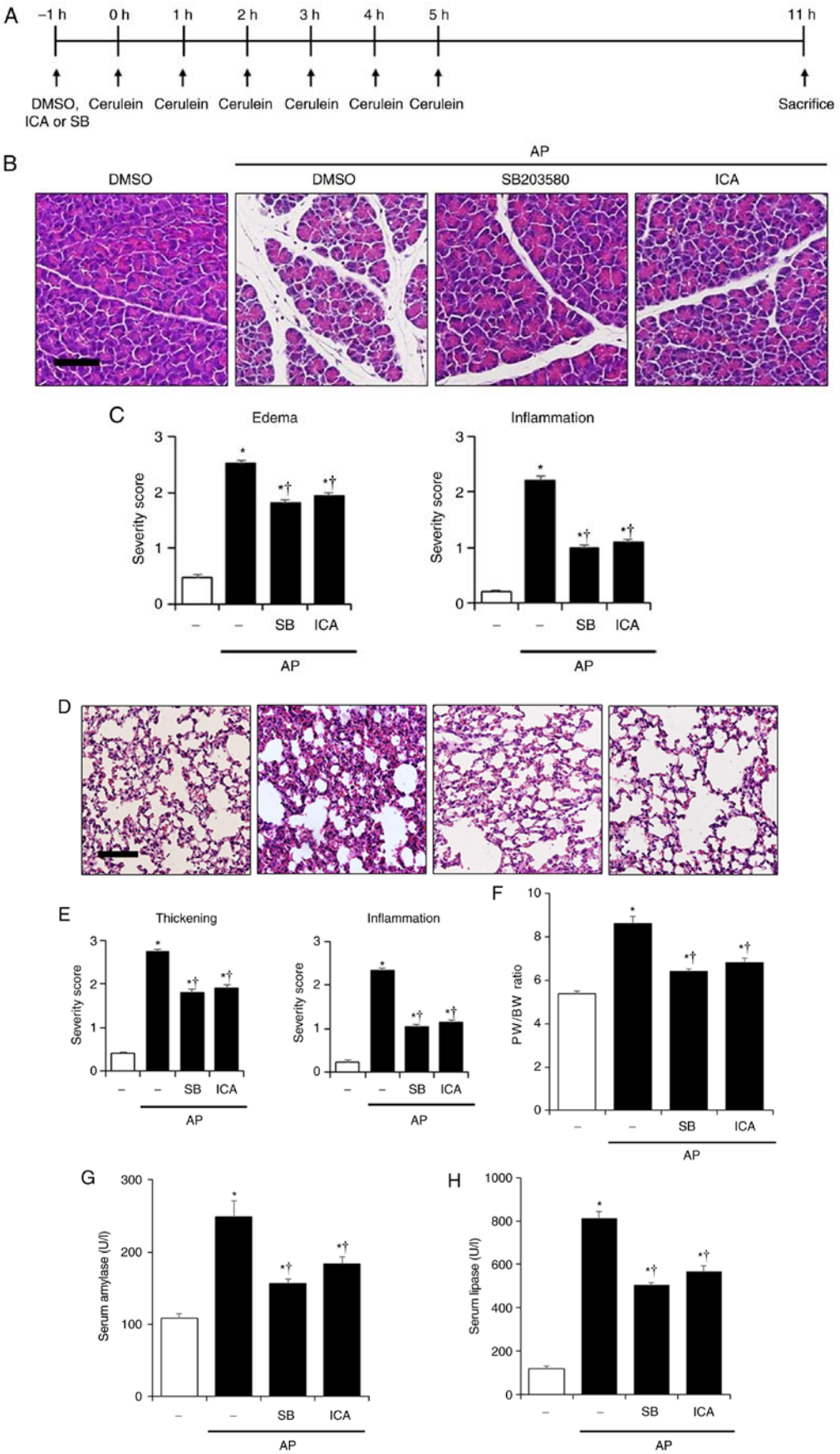

Figure 4. Effects of p38 inhibition by SB203580 on the severity of cerulein-induced AP. (A) Scheme for p38 inhibitor experiment. Mice were pretreated with ICA (20 mg/kg), SB203580 (1 mg/kg) or DMSO 1 h prior to the induction of AP with cerulein (50 μg/kg). Mice were sacrificed 6 h after the last cerulein injection. H&E-stained sections of the (B) pancreas and (D) lung (original magnification, x200). Histological scores for (C) pancreatic edema and inflammation, (E) pulmonary wall thickening and inflammation. (F) PW/BW ratio, and serum levels of (G) amylase and (H) lipase. Data are presented as the mean ± standard error of the mean, n=6. Results are representative of three experiments. *P<0.05 vs. DMSO treatment alone; †P<0.05 vs. cerulein treatment alone. Scale bar, 20 μm. AP, acute pancreatitis; DMSO, dimethyl sulfoxide; ICA, icariin; PW/BW ratio, pancreas weight/body weight ratio.

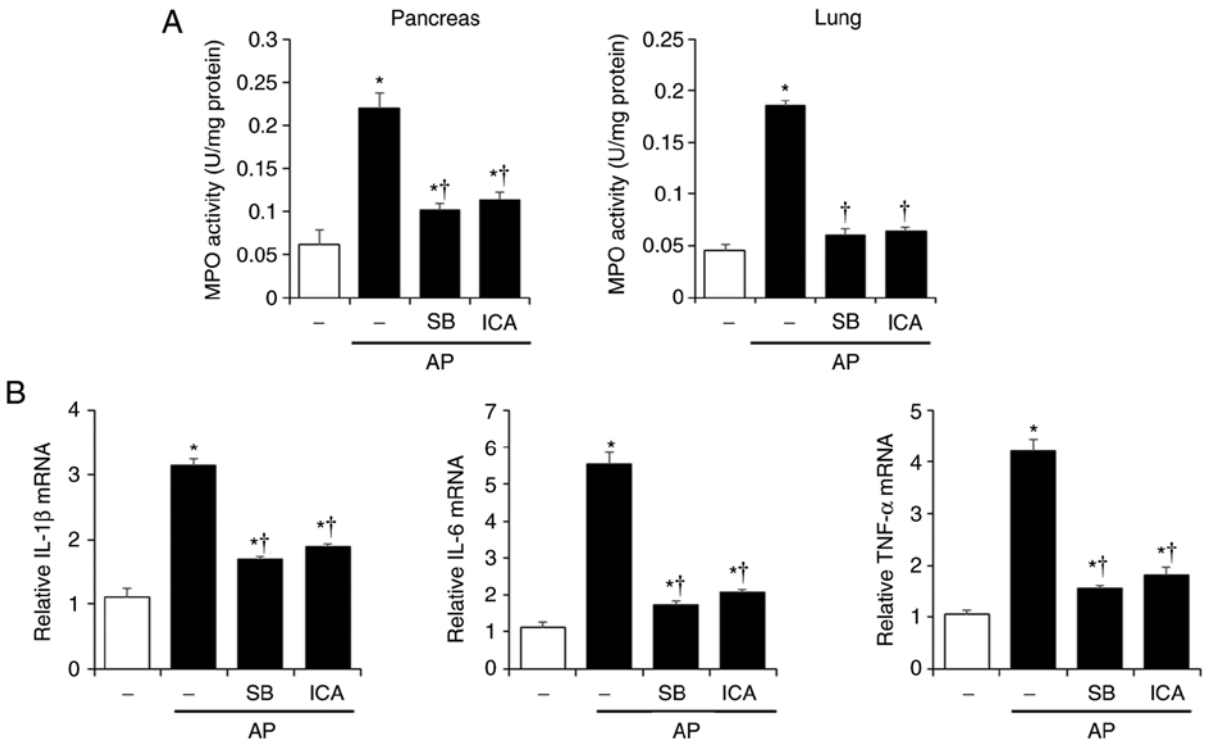

Figure 5. Effects of p38 inhibition by SB203580 on MPO activity and pancreatic cytokine production in cerulein-induced AP. Mice were pretreated with ICA (20 mg/kg), SB203580 (1 mg/kg) or DMSO 1 h prior to the induction of AP with cerulein (50 μg/kg). Mice were sacrificed 6 h after the last cerulein injection. (A) Pancreas and lung MPO activity was measured 6 h after the last injection of cerulein. (B) mRNA levels of pancreatic IL-1β, IL-6 and TNF-α were quantified by reverse transcription-quantitative polymerase chain reaction. Data are presented as the means ± standard error of the mean, n=6. Results are representative of three experiments. *P<0.05 vs. DMSO treatment alone, †P<0.05 vs. cerulein treatment alone. AP, acute pancreatitis; ICA, icariin; IL-1β, interleukin-1β; IL-6, interleukin-6; MPO, myeloperoxidase; TNF-α, tumor necrosis factor-α.

'neutrophil swarmimg' occurs; this promotes the progression of local pancreatic inflammation to multiple organ dysfunction syndrome (MODS) by overwhelming inflammatory responses (35). Neutrophil depletion by anti-neutrophil serum was reported to inhibit the severity of cerulein-induced AP (36). Thus, the measurement of neutrophil count may be regarded as a diagnosis index of AP. In this study, we assessed neutrophil infiltration into the pancreas and lung during cerulein-induced AP by measuring MPO activity. ICA treatment was determined to inhibit the activity of MPO in the pancreas and lung in AP. This suggested that the inhibitory effects of ICA on neutrophil infiltration could contribute to the amelioration of AP and AP-associated lung injury.

Severe AP often leads to MODS, and the most common and earliest target organ is the lung (37). Additionally, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) the more severe form of ALI, are the common complications of AP (38). ALI occurred in 10-25% of AP patients and has been linked with ≤60% of AP-associated mortalities, particularly in elderly patients (39,40). Generally, AP-associated mild lung injury recovers rapidly; however, secondary lung infection, which leads to the release of lung-derived inflammatory mediators across the injured epithelial barrier into the circulation, gives rise to ARDS and mortality (41-43). The variety of effectors that originate from the inflammatory effects of AP are involved in the pathogenesis of secondary lung infection (44-46). Pancreatic mediators, such as pro-inflammatory cytokines and inflammatory cells, are representative of secondary lung infection pathogenesis (44-48). In the present study, our results showed that ICA treatment inhibited the elevation of pancreatic cytokines, which could be promote ALI. In addition, histological lung injury and lung MPO activity were decreased by ICA during AP in our study. Overall, ICA was proposed to exhibit protective activities in mice against AP-associated ALI via the inhibition of pancreatic mediators, such as pro-inflammatory cytokines and neutrophil infiltration.

In regards to the anti-inflammatory activities of ICA, which were determined to be mediated by MAPKs and the NF-κB pathway in a mouse model (49), we investigated the effects of ICA on the activation of MAPKs and NF-κB in cerulein-induced AP. The important signaling pathways, MAPKs and NF-κB, are known to involve various inflammatory regulators in the pancreas (50). In the present study, cerulein activated MAPK and NF-κB in the pancreas as expected, although, only cerulein-induced activation of p38 was inhibited by ICA treatment. These results are in line with that of a previous study where the anti-inflammatory activity of ICA was mainly mediated by the deactivation of p38 MAPK; however, the model of inflammation differed (13). In addition, we reported that the administration of SB203580, a p38 inhibitor, improved the severity of AP similar to ICA. These results suggest that the administration of ICA could reduce the severity of cerulein-induced AP via inhibition of p38.

In the present study, we have demonstrated that ICA exhibited protective and therapeutic effects against cerulein-induced AP and AP-associated lung injury. Furthermore, ICA inhibited the activation of p38 MAPK in cerulein-induced

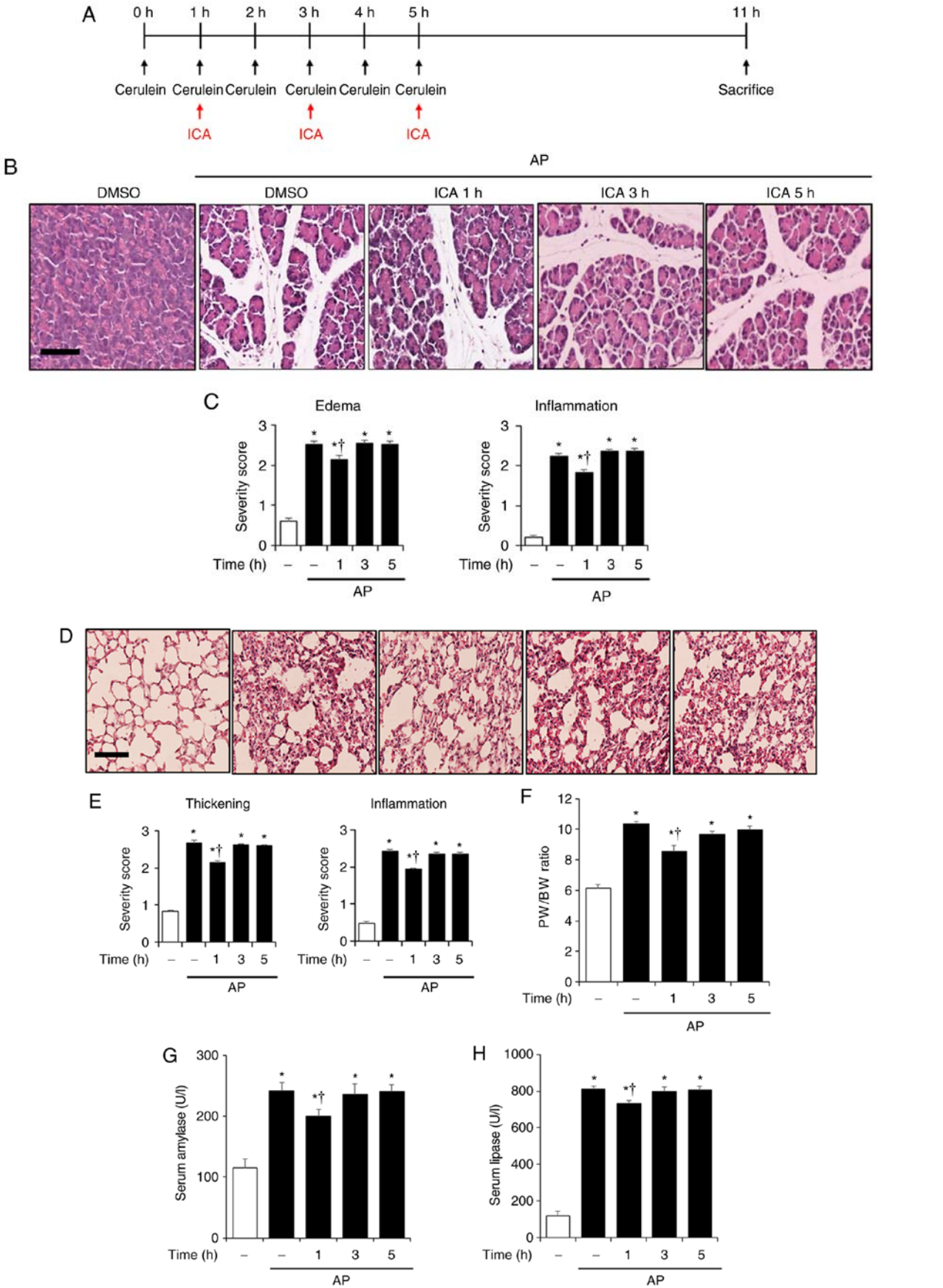

Figure 6. Therapeutic effects of ICA against cerulein-induced AP. (A) Scheme for posttreatment experiment. Mice were treated with ICA (20 mg/kg) or DMSO 1, 3 or 5 h after the first cerulein injection. Mice were sacrificed 6 h after the last cerulein injection. Representative H&E-stained sections of the (B) pancreas and (D) lung (original magnification, x200). Histological scores for (C) pancreatic edema and inflammation, (E) pulmonary wall thickening and inflammation. (F) PW/BW ratio, and serum levels of (G) amylase and (H) lipase. Data are presented as the mean ± standard deviation, n=6. Results are representative of three experiments. *P<0.05 vs. DMSO treatment alone; †P<0.05 vs. cerulein treatment alone. Scale bar, 20 μm. AP, acute pancreatitis; DMSO, dimethyl sulfoxide; ICA, icariin; PW/BW ratio, pancreas weight/body weight ratio.

AP. Collectively, our findings suggest that ICA is a potential therapeutic agent for the treatment of AP.

Acknowledgements

Not applicable.

Funding

This study was supported by the National Research Foundation of Korea grant funded by the Korea government (MEST; grant nos. NRF-2017R1C1B2010031, NRF-2017R1D1A1B03032371 and NRF-2017R1A5A2015805).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DUK, GSB, HJS and SJP made substantial contributions to the design of the study. DUK, GSB, MJK, JWC and DGK performed the experiments. MJK and DGK contributed to the statistical analysis of data. DUK and GSB wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Ethics approval and consent to participate

All experiments were carried out in accordance with the animal care regulations set forth and approved by the Wonkwang University Animal Ethics Committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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