Emodin attenuates adenosine triphosphate-induced pancreatic ductal cell injury *in vitro* via the inhibition of the P2X7/NLRP3 signaling pathway

QINGKAI ZHANG[1,2*], FENGLIN HU[3*], FANGYUE GUO[3*], QI ZHOU[3], HONG XIANG[2] and DONG SHANG[1,3]

[1]Department of General Surgery, and [2]Clinical Laboratory of Integrative Medicine, First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116011; [3]Institute (College) of Integrative Medicine, Dalian Medical University, Dalian, Liaoning 116044, P.R. China

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Abstract. Acute pancreatitis (AP) is an inflammatory disease with high morbidity and mortality rates. Pancreatic ductal cells are the most susceptible of all cell types that are exposed to the noxious stimuli of pancreatitis. Our previous studies demonstrated that emodin, a natural product extracted from *Rheum palmatum* L., protected pancreatic acinar cells from injutyh due to its anti-inflammatory activity. In the present study, in order to investigate the protective effects and molecular mechanisms of action of emodin on injured pancreatic ductal cells, an adenosine triphosphate (ATP)-induced model of cell injury was established using the human pancreatic ductal epithelial cell line, HPDE6-C7. The results revealed that emodin attenuated ATP-induced HPDE6-C7 cell injury by decreasing the levels of inflammatory factors, including interleukin (IL)-1β and IL-18. Furthermore, emodin significantly downregulated the protein levels of purinergic receptor P2X, ligand-gated ion channel, 7 (P2X7), NOD-like receptor protein 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1 in the injured HPDE6-C7 cells. The results also indicated that emodin attenuated HPDE6-C7 cell injury at least partially through the inhibition of the P2X7/NLRP3 signaling pathway. The protective effects of emodin were abrogated upon pre-treatment with P2X7 overexpression plasmid, which further confirmed that the P2X7 signaling pathway is the drug target of the effects of emodin against ATP-induced pancreatic ductal cell injury. Collectively, the findings of this study demonstrate that emodin attenuates ATP-induced pancreatic ductal cell injury in AP mainly through the inhibition of the P2X7/NLRP3 signaling pathway. This study suggests that emodin may be further developed for its application in future medical therapy.

Correspondence to: Professor Dong Shang, Department of General Surgery, First Affiliated Hospital of Dalian Medical University, 222 Zhongshan Road, Dalian, Liaoning 116011, P.R. China
E-mail: shangdong@dmu.edu.cn

Dr Hong Xiang, Clinical Laboratory of Integrative Medicine, First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116011, P.R. China
E-mail: xianghong0806@163.com

*Contributed equally

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Introduction

Acute pancreatitis (AP) is associated with an annual incidence ranging from 13 to 45 per 100,000 individuals in the USA (1-3). Thus, the attenuation of the increasing prevalence of AP is a major challenge in the medical field (4). In clinical situations of pancreatitis, including endoscopic retrograde pancreatography and gallstone passing, the pancreatic ductal epithelium would appear to be the most exposed to noxious stimuli (5). Pancreatic ductal epithelial cells and their secreted mucus constitute a pancreatic duct mucosal barrier, which prevents bile and trypsin from flowing back into the pancreas in a physiological state and has the barrier function of protecting pancreatic tissue from damage by internal and external substances (6). It has been reported that transient hypertension in the pancreatic duct triggers a pancreatic inflammatory cascade and destroys tight junction integrity in a mouse model (7,8). The inappropriate activation of pancreatic zymogen in the pancreas results in exocrine gland parenchymal cell damage and necrosis, which eventually causes AP (9-11). Furthermore, injured pancreatic ductal cells can produce and release inflammatory factors, including interleukin (IL)-6 and IL-8 (5,12). Thus, pancreatic ductal cells play critical roles in injury in AP. However, previous studies on AP have focused on pancreatic acinar cells (13-15), while studies on pancreatic ductal cells are limited (7).

Several recent studies have indicated that adenosine triphosphate (ATP), mainly released by acinar cells in the pancreas, can regulate pancreatic duct function via P2Y and P2X purinoceptor, and ATP-activated purinergic receptors may play important roles in pancreas pathophysiology (16,17).

The study by Hoque *et al* also demonstrated that ATP released from upstream pancreatic acinar cells induced the activation of inflammatory responses (18). As regards the underlying molecular mechanisms, purinergic receptor P2X, ligand-gated ion channel, 7 (P2X7), is a member of the P2X family of ATP-gated cation channels, and it can trigger diverse downstream signaling cascades, mainly including mitogen-activated protein kinases (MAPKs), reactive oxygen species (ROS) and nuclear factor-κB (NF-κB) transcription through coupling the ATP channels (19-23). Previous studies have demonstrated that P2X7 respectively cleaves pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 via the recruitment of the NOD-like receptor protein 3 (NLRP3) inflammasome, which is composed of NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and the effector protein caspase-1. IL-1β and IL-18-dependent pathways further cause pancreatic injury (19,24). Thus, the ATP activated-P2X7/NLRP3 signaling pathway may be a potent therapeutic target for AP insult.

In China, *Rheum palmatum* L. has been utilized in the treatment of AP for a number of years as a separate or principal component in traditional formulas (e.g., Da-Cheng-Qi decoction). Emodin, also known as 1,3,8-trihydroxy-6-methylanthraquinone, is major component of *Rheum palmatum* L. (25). Over the past decades, significant progress has been made to evaluate the pharmacological activities of emodin *in vivo* and *in vitro*. Our previous study demonstrated that emodin exerts significant protective effects on sodium taurocholate-treated pancreatic acinar cells, although its effects on the injury of pancreatic ductal cells have not been reported to date, at least to the best of our knowledge (26). The present study evaluated the effects and potential mechanisms of action of emodin on the injury of pancreatic ductal cells in AP using an ATP-induced pancreatic ductal cell injury model.

Materials and methods

Cells and cell culture. The pancreatic human duct cell line, HPDE6-C7, was purchased from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone) supplemented with high glucose and 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific) at 37˚C in a 5% CO_2 incubator.

ATP-induced cell injury. Cell viability assay using the MTT method was carried out to determine the most suitable damaging concentration of ATP (Solarbio) *in vitro*. In brief, HPDE6-C7 cells ($1x10^5$ cells/ml) were treated with various concentrations of ATP (40, 20, 10, 5, 2.5 and 1.25 mM) for 16 h after 24 h of culture in 96-well plates. Subsequently, 10 μl MTT solution (5 mg/ml) were added into each well followed by 4 h of incubation at 37˚C. Finally, the formazan crystals were dissolved with 100 μl dimethyl sulfoxide (DMSO) and measured with a microplate reader (BioTek) at a wavelength of 490 nm.

Emodin cytotoxicity. The HPDE6-C7 cells ($1x10^5$ cells/ml) were treated with gradient concentrations of emodin (180, 90, 45, 22.5, 11.25 and 5.625 μM) (Solarbio) for 24 h in a humidified atmosphere of 5% CO_2 at 37˚C. Emodin was dissolved in DMSO to a final concentration of <0.1%. Finally, the MTT method was used to evaluate cell viability as described above.

Cell viability and morphology. To evaluate the effects of emodin on ATP-induced HPDE6-C7 cell injury, the cells were divided 5 five groups as follows: i) The control group; ii) ATP group; and iii) low-, iv) medium- and v) high-dose emodin groups. The cells in the control group were cultured under normal conditions without being subjected to any other treatment. The cells in the ATP group were pre-treated with 40 mM ATP for 16 h, while the cells in the emodin groups were respectively first treated with 11.25, 22.5 and 45 μM emodin for 24 h prior to a 16 h-incubation with ATP (40 mM). Cell viability was then assessed using the aforementioned MTT method. To observe the effects of emodin on the morphology of the HPDE6-C7 cells, the cells were grown in 6-well plates and divided into 3 groups as follows: i) The control group; ii) ATP group; and iii) high-dose emodin (45 μM) group. The cells were pre-treated as described above, and then imaged using a phase contrast microscope (Olympus Corp.).

Immunofluorescence detection of P2X7. Following culture in 6-well plates for 24 h at 37˚C, the HPDE6-C7 cells were subjected to different treatments. The cells were then fixed in 4% paraformaldehyde and incubated with diluted anti-P2X7 antibody (1:100; cat. no. ab48871; Abcam) at 4˚C overnight. The cells were then incubated with FITC-conjugated goat anti-rabbit IgG (1:50; cat. no. SA00003-2; ProteinTech Group) for 1 h and re-stained with DAPI (1 μg/ml; ProteinTech Group) for 5 min in the dark at 37˚C. Immunohistochemical images (x100 magnification) were obtained using an Olympus BX63 fluorescence microscope (Olympus Corp.).

Overexpression of the P2X7 gene. The HPDE6-C7 cells ($1x10^5$ cells/ml) were seeded into 6-well plates for 24 h, and then transfected with a P2X7 overexpression plasmid using Lipofectamine 2000 according to the manufacturer's instructions (Thermo Fisher Scientific). The P2X7 overexpression plasmid, also termed pEX-3-P2X7 cDNA (cat. no. Y5251), was purchased from GenePharma Co., Ltd. Briefly, pEX-3-P2X7 cDNA (2 μg) was diluted in 200 μl of DMEM without serum, and then Lipofectamine 2000 (4 μl) was diluted in 200 μl of DMEM. Following a 5-min incubation at room temperature, the diluted DNA was combined with the diluted Lipofectamine 2000. This was followed by gentle mixing and incubation for 20 min at room temperature to allow the DNA-Lipofectamine 2000 complexes to form. Finally, 400 μl of the complexes were added to each well, and the wells were replenished with 1.6 ml of DMEM. Following 24 h of transfection, the cells were treated with emodin (45 μM) and ATP (40 mM). Finally, the P2X7, NLRP3, ASC and caspase-1 protein expression levels, and the contents of IL-1β and IL-18 in the cell supernatant were measured by western blot analysis and ELISA, respectively.

ELISA. According to the manufacturer's instructions, the IL-1β and IL-18 contents in the cell supernatant were evaluated using commercially available human ELISA kits (Boster Bio). The OD values in each well were measured using a multifunctional microplate reader (BioTek) at a wavelength of 450 nm.

Western blot assay. Total proteins from the HPDE6-C7 cells were extracted using a protein extraction kit (cat. no. KGP2100; KeyGen Biotech), and the protein

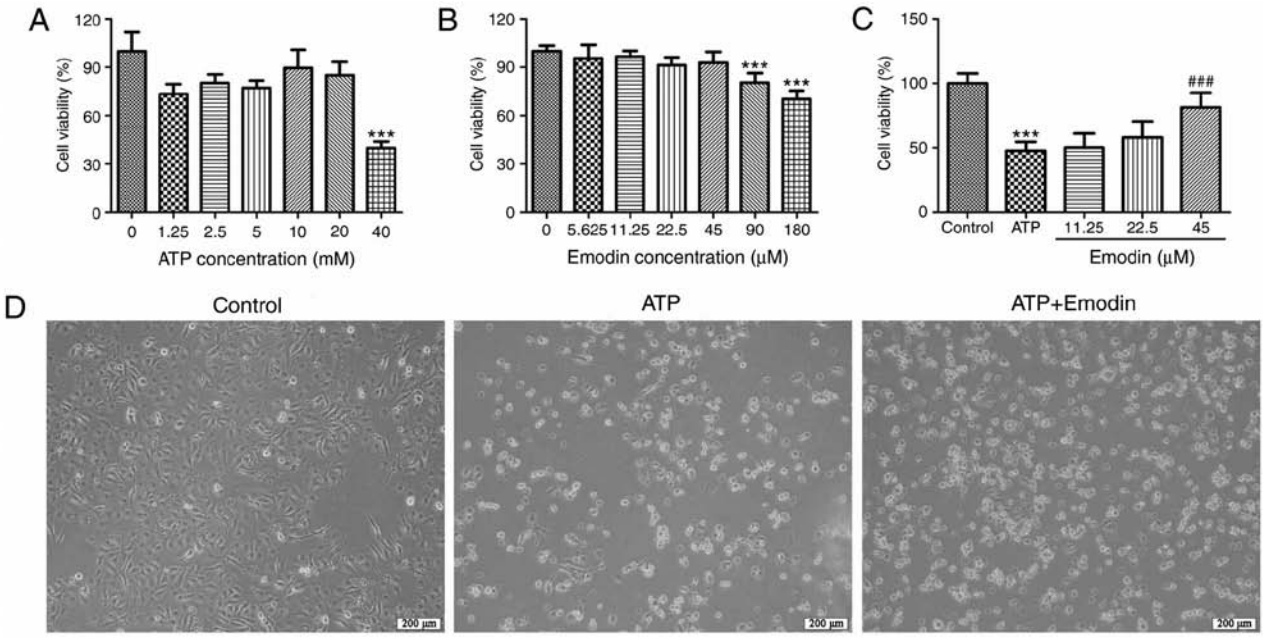

Figure 1. Emodin attenuates ATP-induced HPDE6-C7 cell injury. (A) Effects of various concentrations doses of ATP (1.25, 2.5, 5, 10, 20 and 40 mM) on HPDE6-C7 cell viability following 16 h of pre treatment. (B) Cytotoxicity of emodin (5.625, 11.25, 22.5, 45 and 90 μM) on HPDE6-C7 cells following 24 h of pre-treatment. (C) Effects of emodin (11.25, 22.5 and 45 μM) on the viability of ATP (40 mM)-treated HPDE6-C7 cells. (D) Representative images of HPDE6-C7 cell morphology in the different groups (magnification, x100); ATP, 40 mM; emodin, 45 μM (n=6; 'n' refers to the number of samples). ***P<0.001 vs. control group, $^{###}$P<0.001 vs. ATP group. ATP, adenosine triphosphate.

concentrations were determined using a BCA protein assay kit (cat. no. KGP902; KeyGen Biotech). Protein samples were separated by SDS-PAGE (10-12%) and then transferred onto PVDF membranes (EMD Millipore). The membranes were then blocked and incubated with primary antibodies against P2X7 (cat. no. ab48871), NLRP3 (cat. no. ab214185), ASC (cat. no. ab155970) and caspase-1 (cat. no. ab1872) (all from Abcam and 1:800 dilution in TBST) overnight at 4˚C. Following incubation with the secondary antibody [cat. no. SA00001-2; HRP-conjugated Affinipure goat anti-rabbit IgG (H+L) ProteinTech Group] (1:2,000 dilution in TBST) at room temperature for 2 h, the protein expression in the membranes was visualized by enhanced ECL using Tanon-5200 Multi Gel Imaging System (Tanon Science and Technology). β-actin (cat. no. ab179467; Abcam) was used as the internal control. Quantitative analysis was performed using Gel-Pro analyzer 4.0 software (Media Cybernetics).

Statistical analysis. Data are expressed as the means ± standard deviation (SD) and analyzed using SPSS 17.0 software (SPSS, Inc.). Differences between multiple groups were analyzed using one-way analysis of variance with Tukey's post-hoc test. Comparisons between 2 groups were made using an unpaired Student's t-test. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Emodin attenuates ATP-induced HPDE6-C7 cell injury. In order to establish a model of ATP-induced injury using normal human pancreatic ductal epithelial cells, we adopted various concentrations of ATP to stimulate the HPDE6-C7 cells. As shown in Fig. 1A, ATP at a concentration of 40 mM significantly decreased the viability of the HPDE6-C7 cells. Thus, 40 mM was selected as the stimulus dose for the *in vitro* model in the subsequent experiments. Furthermore, to identify the safe concentration of emodin, the cytotoxicity of emodin against the HPDE6-C7 cells was evaluated by MTT assay. As shown in Fig. 1B, the results indicated that treatment with 90 μM emodin exerted marked cellular cytotoxicity (P<0.001), which demonstrated that 45 μM was the maximum safe concentration of emodin. In addition, as shown in Fig. 1C, emodin (45 μM) notably increased cell viability compared with the ATP model group. Furthermore, the cell morphology images presented in Fig. 1D indicated that ATP significantly induced HPDE6-C7 cell death, and that this was attenuated by emodin (45 μM). Therefore, these results suggested that emodin exerted a potent protective effect against ATP-induced HPDE6-C7 cell injury.

Emodin suppresses the expression of proteins associated with the P2X7/NLRP3 signaling pathway in ATP-stimulated HPDE6-C7 cells. In order to examine the effects of emodin on P2X7 protein expression, we performed a P2X7 immunofluorescence assay. As shown in Figs. 2 and S1, the number of P2X7-positive cells exhibiting green fluorescence was significantly increased in the ATP group; however, treatment with emodin (45 μM) markedly decreased this green fluorescence. Subsequently, we evaluated whether the P2X7/NLRP3 signaling pathway is involved in the protective effects of emodin against ATP-induced HPDE6-C7 cell injury. The results shown in Fig. 3 revealed that the expression of proteins associated with the P2X7/NLRP3 signaling pathway,

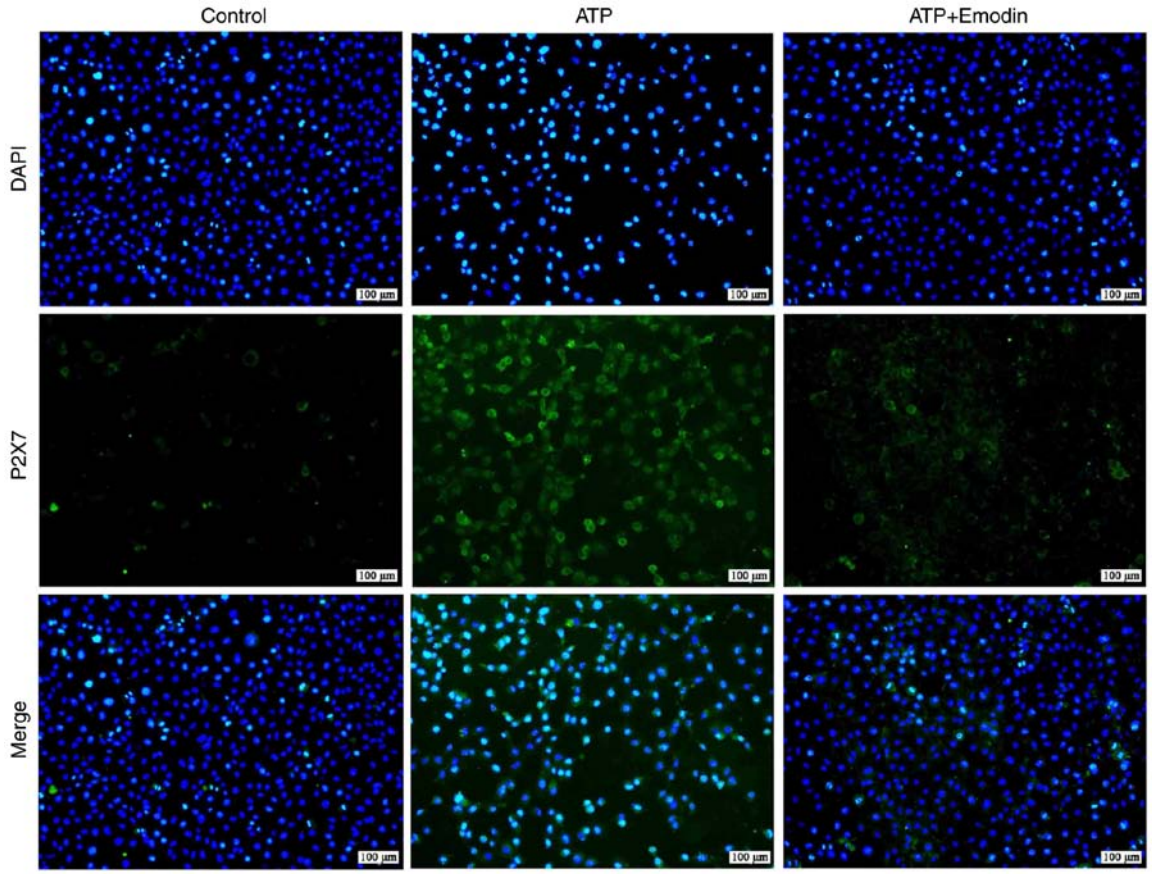

Figure 2. Emodin (45 μM) downregulates P2X7 protein expression in ATP-treated HPDE6-C7 cells based on the results of an immunofluorescence assay (magnification, x200). P2X7, purinergic receptor P2X, ligand-gated ion channel, 7.

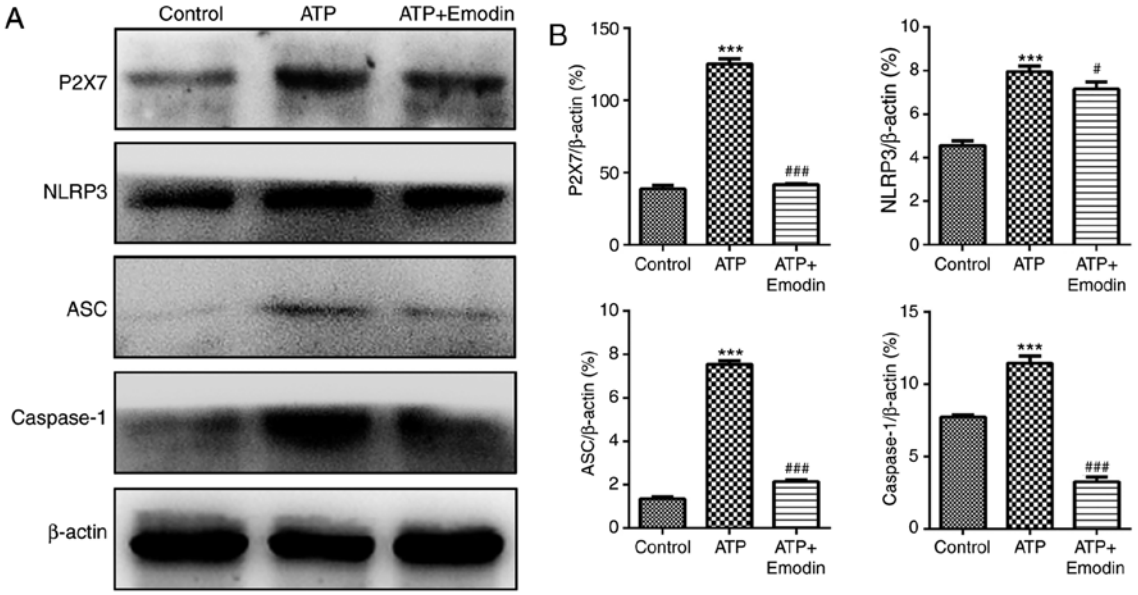

Figure 3. Emodin inhibits the expression of proteins associated with the P2X7/NLRP3 signaling pathway in ATP-stimulated HPDE6-C7 cells. (A) Effects of emodin (45 μM) on the protein expression of P2X7, NLRP3, ASC and caspase-1 in ATP-stimulated HPDE6-C7 cells. (B) Statistical analysis of the effects of emodin on protein expression (n=3; 'n' refers to the number of repeats). ***P<0.001 vs. control group, $^{\#}$P<0.05 vs. ATP group, $^{\#\#\#}$P<0.001 vs. ATP group. ATP, adenosine triphosphate; P2X7, purinergic receptor P2X, ligand-gated ion channel, 7; NLRP3, NOD-like receptor protein 3; ASC, apoptosis-associated speck-like protein containing a CARD.

including P2X7, NLRP3, ASC and caspase-1 in the ATP group was significantly increased compared with the control group. Treatment with emodin (45 μM) markedly inhibited the levels of these proteins. Therefore, emodin can attenuate HPDE6-C7

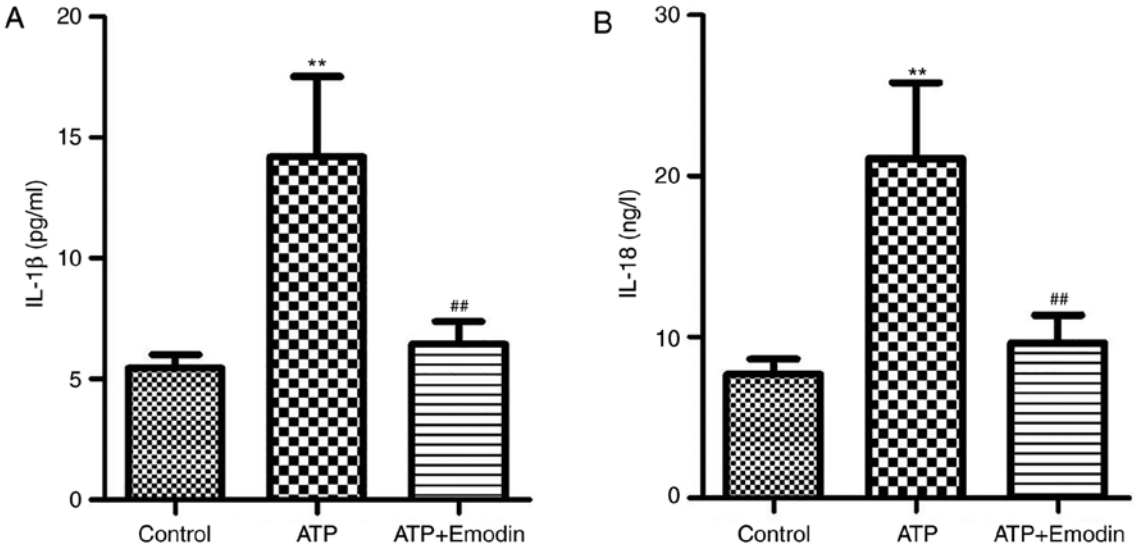

Figure 4. Emodin reduces the contents of (A) IL-1β and (B) IL-18 in the cell supernatant (n=6; 'n' refers to the number of samples). **P<0.01 vs. control group, ##P<0.01 vs. ATP group. ATP, adenosine triphosphate; IL, interleukin.

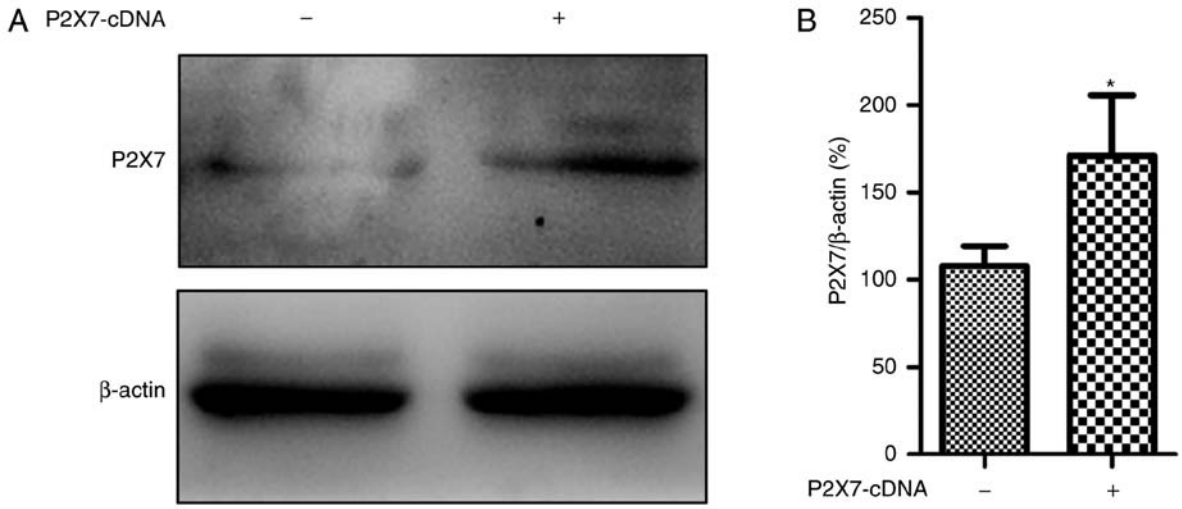

Figure 5. P2X7 expression is significantly increased following transfection with a P2X7 plasmid (pEX-3-P2X7 cDNA). (A) Effects of P2X7 overexpression on the protein expression of P2X7 in HPDE6-C7 cells. (B) Statistical analysis of P2X7 protein expression (n=3; 'n' refers to the number of repeats). *P<0.05 vs. negative control group. P2X7, purinergic receptor P2X, ligand-gated ion channel, 7.

cell injury induced by ATP through the inhibition of the P2X7/NLRP3 signaling pathway.

Emodin reduces the contents of IL-1β and IL-18 in the supernatant of ATP-stimulated HPDE6-C7 cells. The present study examined the effects of emodin on the conents of IL-1β and IL-18 in ATP-stimulated HPDE6-C7 cells. The results shown in Fig. 4 indicated that the contents of IL-1β and IL-18 in the cell supernatant were significantly increased in the model group, and that these effects were reversed by treatment with emodin (45 μM).

Overexpression of P2X7 abrogates the protective effects of emodin on ATP-stimulated HPDE6-C7 cells. In order to explore the role of P2X7 on the protective effects of emodin against ATP-induced HPDE6-C7 cell injury, a P2X7-cDNA plasmid was used to overexpress the P2X7 gene. As shown in Fig. 5, the protein level of P2X7 was notably increased following transfection with the P2X7 plasmid compared with the negative control group. Furthermore, as shown in Figs. 6 and S2, the morphological damage and the number of P2X7-positive cells in the P2X7 overexpression group were significantly increased compared with the negative control group. However, these effects were not attenuated by treatment with emodin (45 μM). Therefore, P2X7 overexpression reversed the protective effects of emodin against ATP-induced HPDE6-C7 cell injury.

In addition, as shown in Fig. 7, following transfection with the P2X7 overexpression plasmid, regardless of the presence of emodin, the expression levels of P2X7, NLRP3, ASC and caspase-1 were all notably upregulated compared with the control group. In addition, as shown in Fig. 8, similar results were also observed regarding the release of IL-1β and IL-18 in the cell supernatant. Overall, these results suggested that P2X7

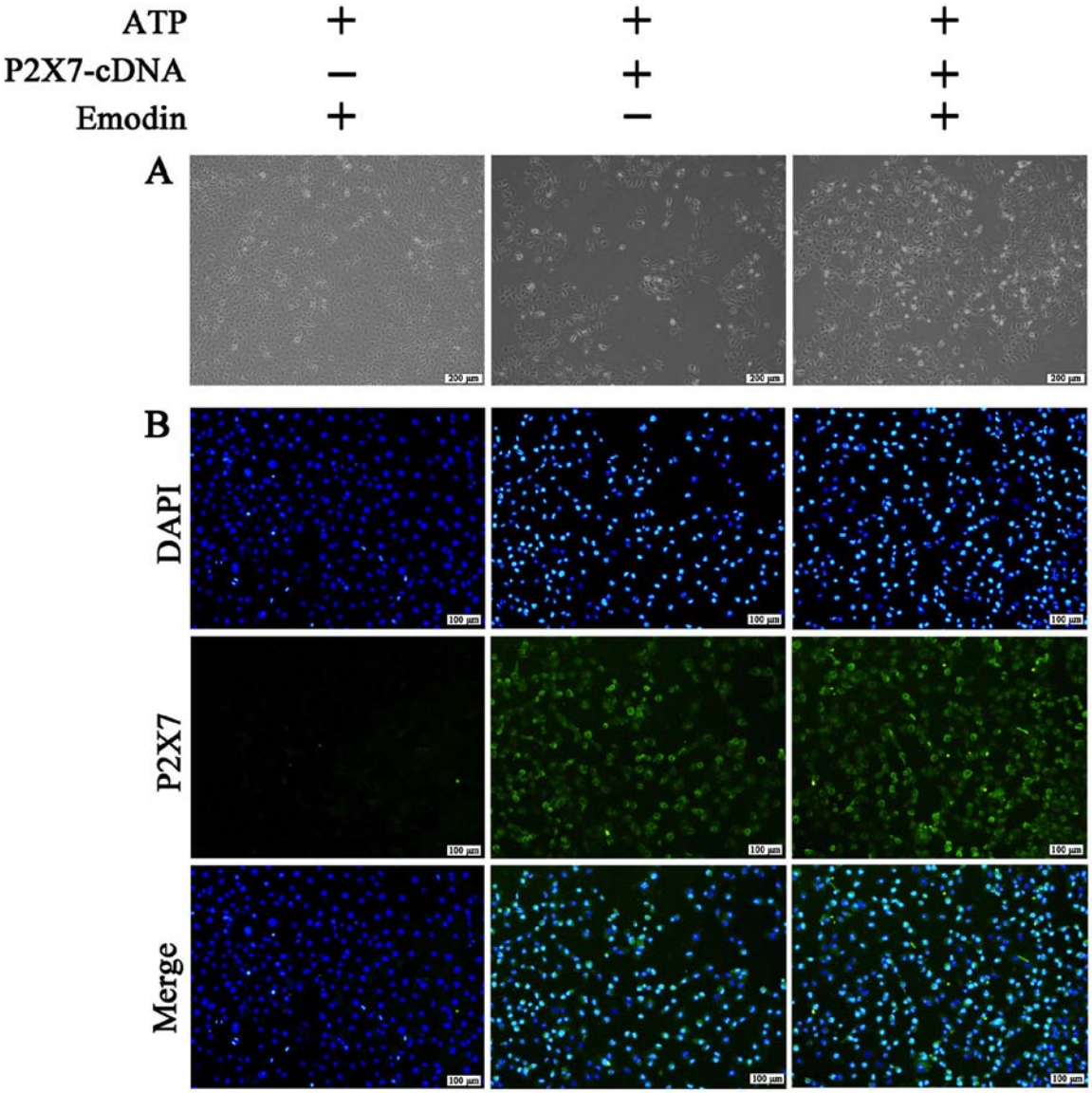

Figure 6. The protective effects of emodin on ATP-injured HPDE6-C7 cells are abrogated by P2X7 overexpression performed by transfection with pEX-3-P2X7 cDNA. (A) Effects of emodin and P2X7 overexpression on the morphology of HPDE6-C7 cells (magnification, x100). (B) Effects of emodin and P2X7 overexpression on P2X7 protein expression based on the results of an immunofluorescence assay (magnification, x200). ATP, adenosine triphosphate; P2X7, purinergic receptor P2X, ligand-gated ion channel, 7.

overexpression abrogated the regulation of the P2X7/NLRP3 signaling pathway mediated by emodin, which further demonstrated that the protective actions of emodin on ATP-induced pancreatic ductal cell injury were mainly due to the inhibition of the P2X7/NLRP3 signaling pathway.

Discussion

The pancreatic duct is mainly formed by epithelial cells, since it is linked by centro-acinar cells interfaced with pancreatic acini at the terminal end (27). A previous study reported that pancreatic acini can release ATP into the ductal lumen in response to various stimuli, thus activating P2Y and P2X receptors to regulate the epithelial secretion of the pancreatic duct (28). Ductal occlusion and injury have been postulated to lead to focal pancreatic inflammation, and the time of ductal occlusion determines the severity of AP. Thus, the present study established a model of ATP-induced injury using normal human pancreatic ductal epithelial cells (HPDE6-C7) and revealed that ATP at a concentration of 40 mM markedly decreased cell viability. Furthermore, to examine the protective effects of emodin against ATP-induced HPDE6-C7 cell injury, the cytotoxicity of emodin on HPDE6-C7 cells was evaluated, and it was observed that 45 μM was the maximum safe concentration for emodin. Emodin at this safe concentration markedly increased cell viability, and attenuated the adverse morphological changes in the HPDE6-C7 cells stimulated with ATP. Therefore, the results of the present study indicated that emodin exerted a potent effect against ATP-induced pancreatic ductal epithelial cell injury.

P2X7 is primarily expressed in rodent duct cells in the pancreas and is a member of the P2X family of ATP-gated cation channels (29,30). The activation of P2X7 can effectively stimulate NLRP3, which is a member of the NOD-like receptor family, and can respond to a series of intrinsic stimuli and external stress signals (31). Activated NLRP3 protein can

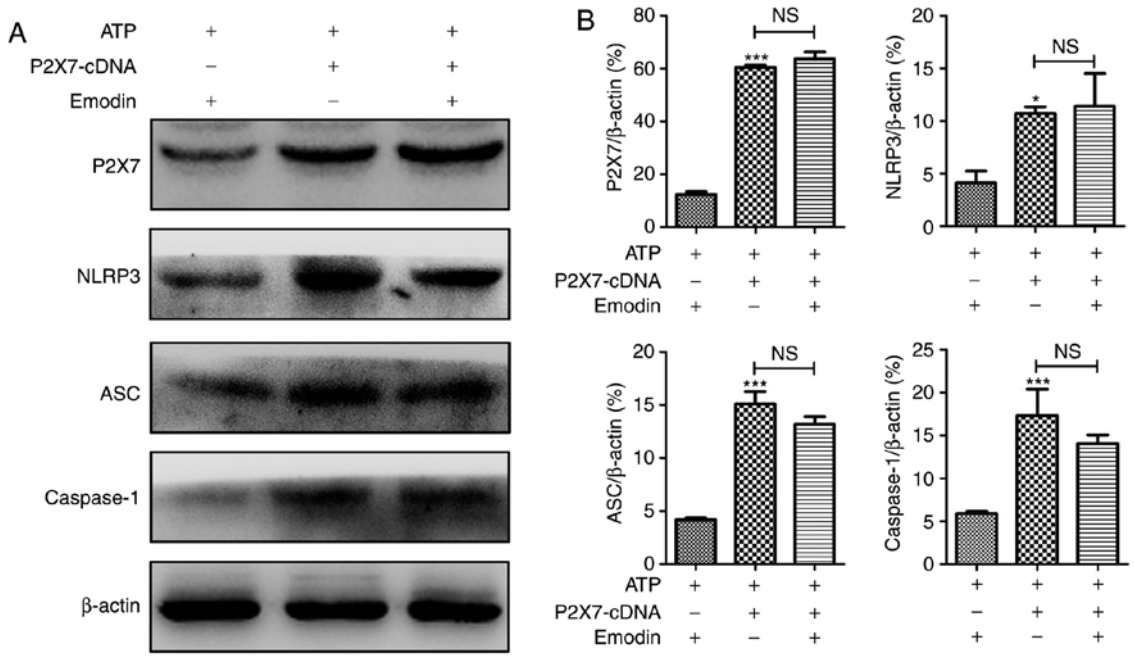

Figure 7. The inhibitory effects of emodin on the P2X7/NLRP3 pathway are abrogated by P2X7 overexpression performed by transfection with pEX-3-P2X7 cDNA. (A) Effects of emodin and P2X7 overexpression on P2X7, NLRP3, ASC and caspase-1 expression levels in adenosine triphosphate-treated HPDE6-C7 cells based on the results of western blot analysis. (B) Statistical analysis of the effects of emodin and P2X7 overexpression on protein expressions levels (n=3; 'n' refers to the number of repeats). *P<0.05 vs. control group, ***P<0.001 vs. control group, NS, not significant; ATP, adenosine triphosphate; P2X7, purinergic receptor P2X, ligand-gated ion channel, 7; NLRP3, NOD-like receptor protein 3; ASC, apoptosis-associated speck-like protein containing a CARD.

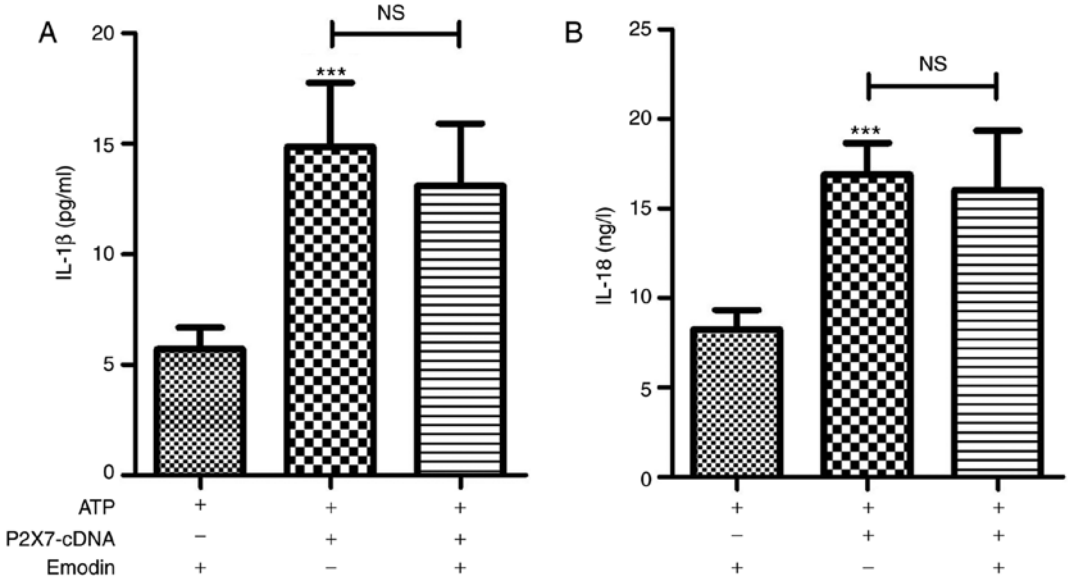

Figure 8. The inhibitory effects of emodin on (A) IL-1β and (B) IL-18 in the cell supernatant are abrogated by P2X7 overexpression performed by pEX-3-P2X7 cDNA based on the results of ELISA (n=6; n' refers to the number of samples). ***P<0.001 vs. control group, NS, not significant; P2X7, purinergic receptor P2X, ligand-gated ion channel, 7; IL, interleukin.

recruit ASC and caspase-1 proteins to form the NLRP3 inflammasome. The activated NLRP3 inflammasome promotes the caspase-1-dependent cleavage of pro-IL-1β and pro-IL-18 into active IL-1β and IL-18, respectively, and increases their subsequent release from the cell (32). The study by Hoque *et al* reported that the genetic deletion of *Nlrp3* reduced pro-IL-1β expression, inflammatory responses and pancreatic edema in caerulein-induced pancreatitis (18). Therefore, the ATP-dependent P2X7/NLRP3 signaling pathway may be a potent therapeutic target for pancreatic ductal epithelial cell injury in AP. The present study detected the expression levels of the proteins involved in the P2X7/NLRP3 signaling pathway, and observed that emodin markedly downregulated the protein expression of P2X7, NLRP3, ASC and caspase-1.

Mature IL-1β can modify and activate pro-inflammatory cytokines, including IL-8, IL-17 and tumor necrosis factor-α by binding to IL-1β receptors. Thus, IL-1β is an early and potent pro-inflammatory factor in response to infections and tissue

damage (33,34). IL-18 has a similar structure and function to IL-1β, and plays an important role in regulating innate immunity and inflammation in multiple inflammatory diseases, thus acting as a novel pro-inflammatory cytokine (24). The findings of this study demonstrated that emodin notably decreased the levels of IL-1β and IL-18.

In addition, in this study, P2X7 overexpression by the P2X7 plasmid abrogated the protective effects of emodin on pancreatic ductal cells and its inhibition of the P2X7/NLRP3 signaling pathway. Therefore, P2X7 may be a drug target of emodin against ATP-induced pancreatic ductal cell injury. These results further confirmed that emodin exerts potent effects against ATP-induced pancreatic ductal cell injury by inhibiting the P2X7/NLRP3 signaling pathway and the subsequent inflammatory reaction.

In conclusion, P2X7 acts as a potent drug target of emodin for preventing the spontaneous activation of ATP-dependent P2X7/NLRP3 signaling, which has important implications in pancreatic ductal cell injury in AP pathologies. These findings may contribute to further advancements in the development of emodin for medical interventions in patients with AP.

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Availability of data and materials

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

Authors' contributions

HX and DS conceived and designed the experiments, and drafted the manuscript. QZhang, FH, and FG performed the cell culture experiments and gene transfection experiments. QZhang and FH performed the western blot analyses, and QZhou performed the ELISA. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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