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Original Article

Interleukin-1β induces autophagy by affecting calcium homeostasis and trypsinogen activation in pancreatic acinar cells

Bin Xu[1*], Bin Bai[1*], Sumei Sha[1*], Pengfei Yu[1], Yanxin An[1], Shiqi Wang[1], Xiangyun Kong[2], Chaoxu Liu[1], Ni Wei[1], Quanxin Feng[1], Qingchuan Zhao[1]

[1]State Key Laboratory of Cancer Biology & Xijing Hospital of Digestive Diseases, The Fourth Military Medical University, 127 Changle Western Road, Xi'an, Shaanxi Province 710032, China; [2]Department of Gastroenterology, Xi'an No.1 Hospital, Xi'an, Shaanxi Province 710002, China. [*]Equal contributors.

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Abstract: The strong up-regulation of inflammatory mediators has been reported to play a key role in acute pancreatitis (AP). Elevated serum levels of interleukin-1β (IL-1β) are associated with the development of AP. However, the precise effect and mechanism of IL-1β in AP remains obscure. In this study, we investigated the potential role and mechanism of IL-1β in AP. We measured autophagy activation in response to IL-1β in AR42J cells. The disrupting effects of IL-1β on cellular Ca^{2+} were observed. To determine whether the disruption of Ca^{2+} signaling has protective effects *in vivo* during AP, male C57BL/6 mice were treated with cerulein to induce AP. We found that the treatment of AR42J cells with IL-1β triggered autophagy and that the autophagic flux was impaired. In addition, IL-1β induced Ca^{2+} release from the ER. Furthermore, the expression of the ER stress markers GRP78 and IRE1 also increased. 2APB, an antagonist of the $InsP_3$ receptor, inhibited increased expression of autophagy markers. Subsequent biochemical assays revealed that co-culture with IL-1β could induce the activation of trypsinogen to trypsin and reduce the viability of acinar cells. Pathological changes of the pancreas were also observed *in vivo*. We found that the pathological injuries of the pancreas were significantly alleviated in mice co-treated with 2APB. Taken together, our results indicate that IL-1β can induce trypsin activation and decrease cellular viability in pancreatic acinar cells. These effects depend on impaired autophagy via intracellular calcium changes. Ca^{2+} signaling may become a promising therapeutic target in the treatment of pancreatitis.

Keywords: IL-1β, autophagy, calcium, pancreatitis

Introduction

Acute pancreatitis (AP) is an autodigestive illness with a high mortality rate and an incidence of 28-31 in 100,000 per year [1]. During the past decade, significant progress has been achieved in our understanding of the pathogenesis of AP. The intra-acinar cell activation of trypsinogen to trypsin is generally believed to be an early and critical event in AP [2-4]. This progression is considered to play a central role in AP because trypsin is a "switch" in the digestive enzyme cascade [5]. However, the underlying mechanism is not fully understood.

Autophagy is a highly evolutionarily conserved homoeostatic mechanism for the degradation and recycling of cytosolic constituents, including long-lived macromolecules, damaged organelles and intracellular pathogens [6, 7]. Autophagosomes are formed in response to a number of environmental stimuli, especially in response to inflammatory or stress conditions [8, 9]. However, if cellular stress leads to excessively or continuously induced autophagy, cell injury may ensue. Autophagy also plays a role in many diseases, and mounting evidence has shown that impaired autophagy is involved in the pathogenesis of AP, which is associated with the generation of intracellular trypsin [10-12].

A considerable body of evidence has shown a key role for interleukin-1β (IL-1β) in AP [13-15];

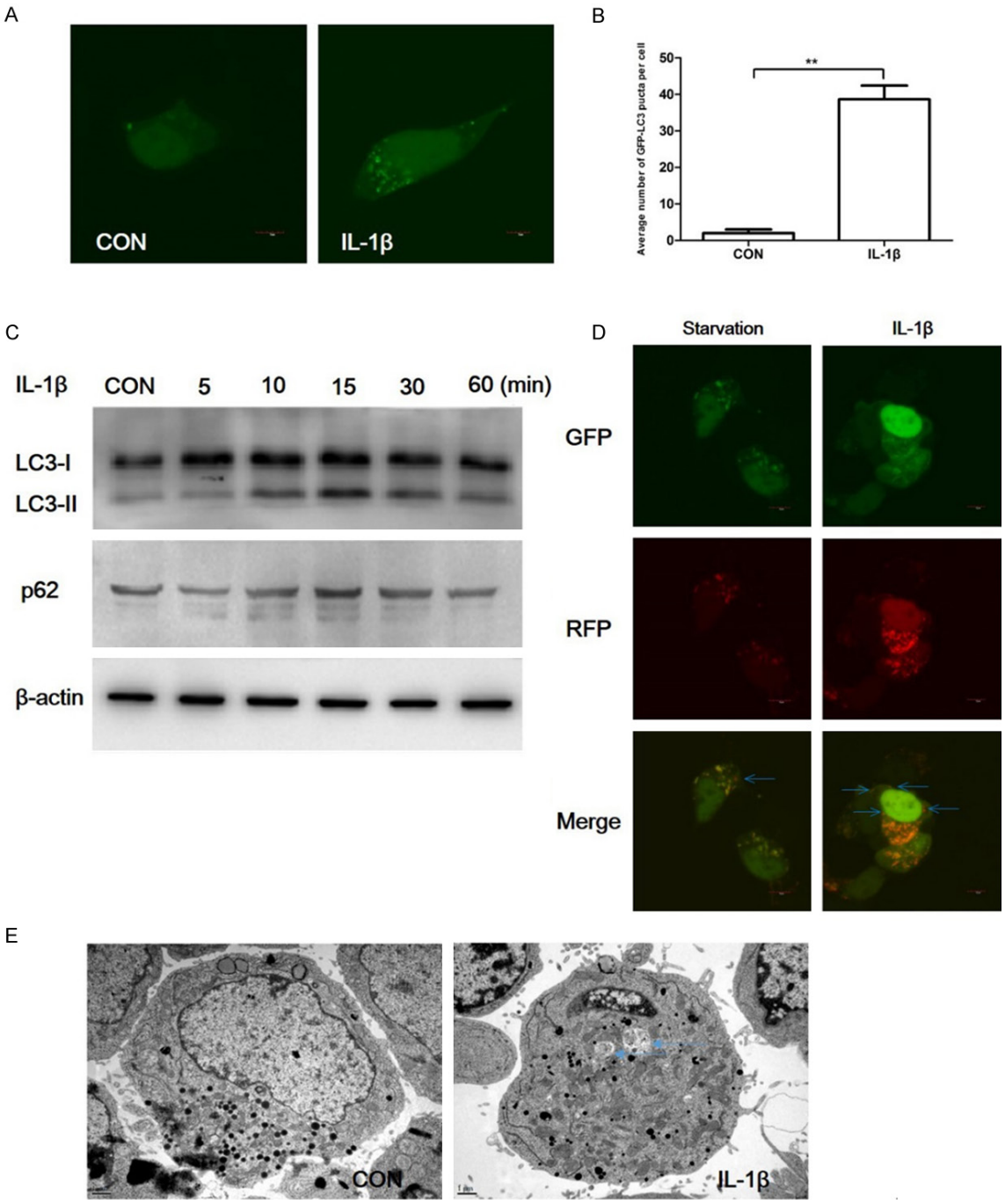

Figure 1. IL-1β induce impaired autophagy in AR42J cells. Representative fluorescent images of GFP-LC3B labeled puncta in cells treated with or without 2 ng/ml IL-1β (A). The results showed that after stimulation with IL-1β, a large number of GFP-LC3B puncta formed in the cytoplasm. Scale bar: 5 μm. The average number of GFP-LC3B puncta per cell were quantified and showed in histogram (B). The expression levels of LC3 and p62 were assessed by western blot using anti LC3 and p62 antibodies after incubation with or without IL-1β at different time points (C). β-actin was used as loading control. Representative fluorescent images of AR42J cells transiently transfected with RFP-GFP-LC3 treated with IL-1β or EBSS. The red puncta in cells were indicated by blue arrow. Scale bar: 5 μm (D). Representative electron micrographs of AR42J cells treated with or without IL-1β (E). Autophagic vacuoles was indicated by blue arrows. Scale bar: 1 μm.

this molecule is up-regulated at the early stages of the disease and is directly correlated with many deleterious systemic inflammation events, both locally in the pancreas and in dis-

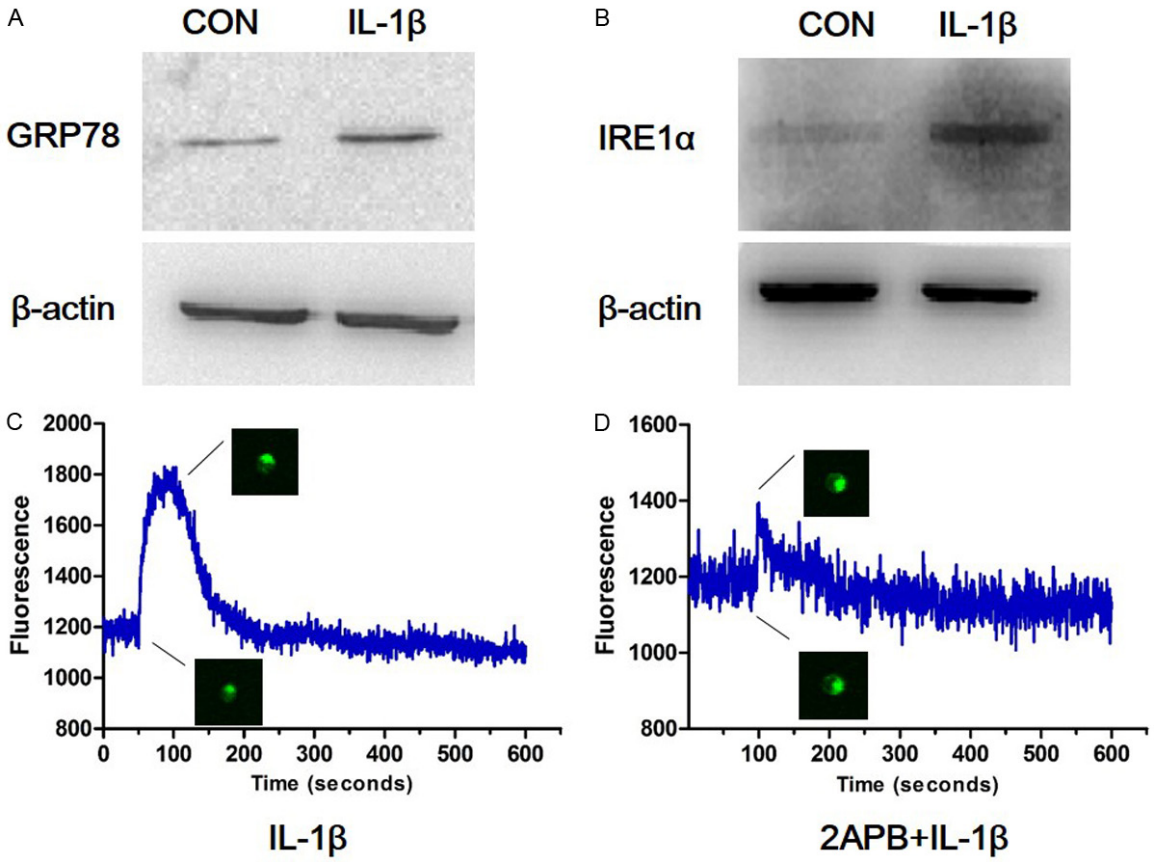

Figure 2. IL-1β induces ER stress and increases Ca^{2+} release from the ER in AR42J cells. AR42J cells were treated with or without IL-1β. The ER stress markers GRP78 (A) and IRE1α (B) expression levels were quantified by western blot analysis. Representative trace shows the effect of IL-1β on inducing [Ca^{2+}] oscillation (C). The data were obtained from at least 5 separate experiments. Representative trace shows the effect of 2APB on IL-1β induced Ca^{2+} release (D).

tant organs (such as acute lung injury and liver failure). Although the key role of IL-1β in the process of inflammation in AP is known, whether IL-1β can induce trypsinogen activation remains unknown. IL-1β can stimulate autophagy in human and murine macrophages and induce endoplasmic reticulum (ER) stress [16-19]. The ER is an important organelle for calcium storage and can regulate intracellular Ca^{2+} homeostasis. ER stress may cause the ER to release calcium into the cytosol [20]. Ca^{2+} has long been considered to play a major role in several aspects of the cellular processes involved in pancreatitis [21]. Studies have also suggested a relationship between intracellular Ca^{2+} signaling and autophagy, indicating that elevations of free cytosolic Ca^{2+} can lead to the activation of autophagy [22, 23]. Based on these various observations, we hypothesized that IL-1β causes ER stress, resulting in Ca^{2+} release into the cytosol and the subsequent activation of trypsinogen via impaired autophagy in AP.

In this study, we investigated the role of autophagy in AR42J cells after stimulation by IL-1β to determine whether changes in intracellular Ca^{2+} are essential for IL-1β-induced autophagy and whether suppressing Ca^{2+} release from the ER can play a role in protecting acinar cells during AP.

Materials and methods

Materials

Anti-LC3 antibodies and chloroquine (10 μM) were obtained from Sigma. Recombinant IL-1β was obtained from R&D Systems. The substrate Rhodamine 110, bis-(CBZ-L-isoleucyl-L-

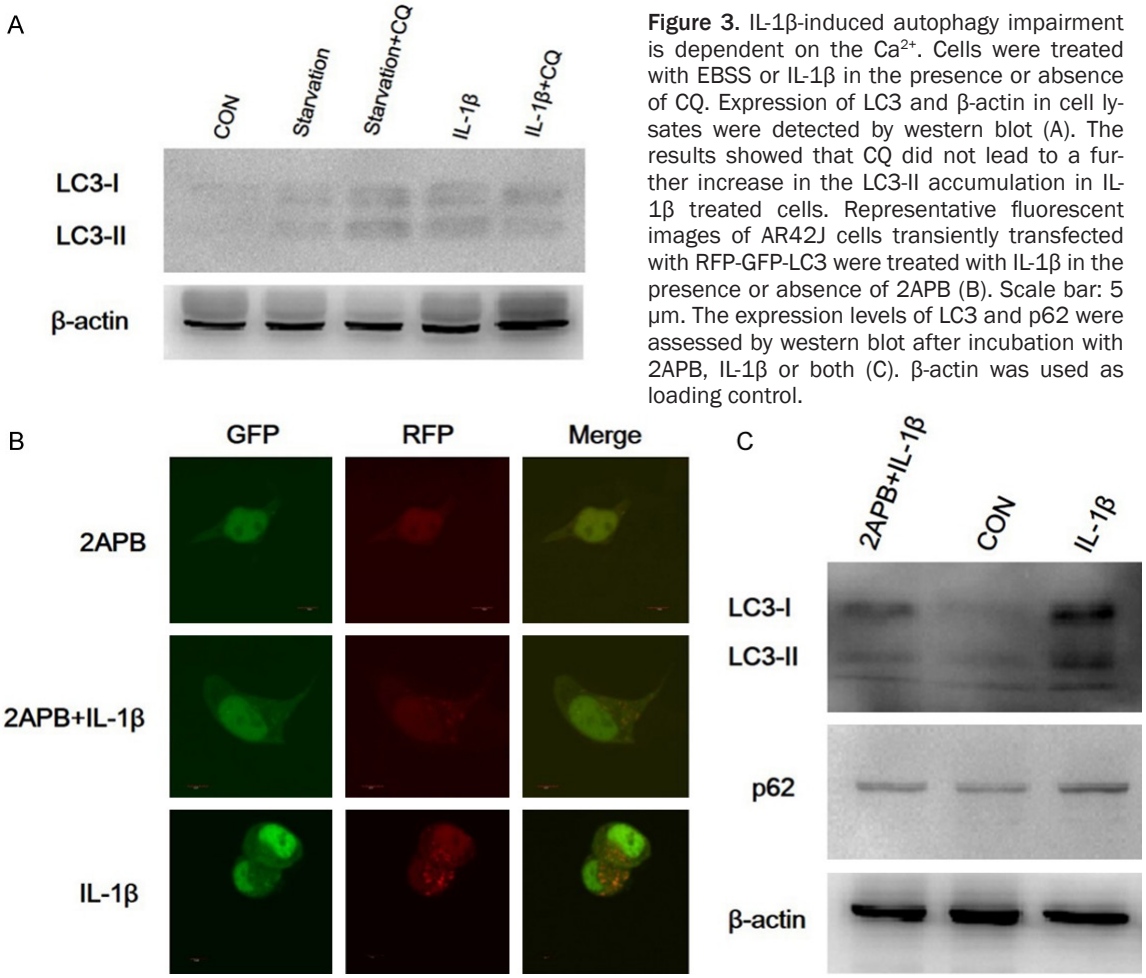

Figure 3. IL-1β-induced autophagy impairment is dependent on the Ca^{2+}. Cells were treated with EBSS or IL-1β in the presence or absence of CQ. Expression of LC3 and β-actin in cell lysates were detected by western blot (A). The results showed that CQ did not lead to a further increase in the LC3-II accumulation in IL-1β treated cells. Representative fluorescent images of AR42J cells transiently transfected with RFP-GFP-LC3 were treated with IL-1β in the presence or absence of 2APB (B). Scale bar: 5 μm. The expression levels of LC3 and p62 were assessed by western blot after incubation with 2APB, IL-1β or both (C). β-actin was used as loading control.

prolyl-L-arginine amide), dihydrochloride and Fluo-4-AM were obtained from Invitrogen (Eugene, Oregon, USA). Medium F-12K, and fetal bovine serum were obtained from Gibco Invitrogen Corporation (Grand Island, NY). 2ABP was obtained from Santa Cruz Biotechnology.

Cell culture

The rat pancreatic acinar cell line AR42J was obtained from ATCC and cultured in F-12K medium with 20% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37°C in the presence of 5% CO_2. For adenoviral infection, RFP-GFP-LC3- (Hanbio Biotechnology, Shanghai, China) and GFP-LC3B- (Invitrogen Eugene, OR, USA) were used following the manufacturer's instructions. The transfection efficiency was determined by fluorescence microscopy.

Autophagy determination

RFP-GFP-LC3- and GFP-LC3B -transfected cells were visualized by confocal microscopy. To quantify the number of puncta, RFP-GFP-LC3- or GFP-LC3B -transfected cells were seeded in a dish one day before the treatment. The cells were then analyzed by confocal microscopy. The number of puncta per cell was determined using Image-Pro plus 6.0. More than 10 cells were analyzed for each condition, and a cell was considered autophagic if more than 10 puncta were present in that cell.

Western blot analysis

Western blots were performed to determine the expression levels of LC3, p62 and β-actin. Briefly, AR42J cells were homogenized in T-per buffer (Thermo Fisher) with a protease inhibitor cocktail (Thermo Fisher). Lysates were centrifuged at 16,000 g for 15 min, and then, the

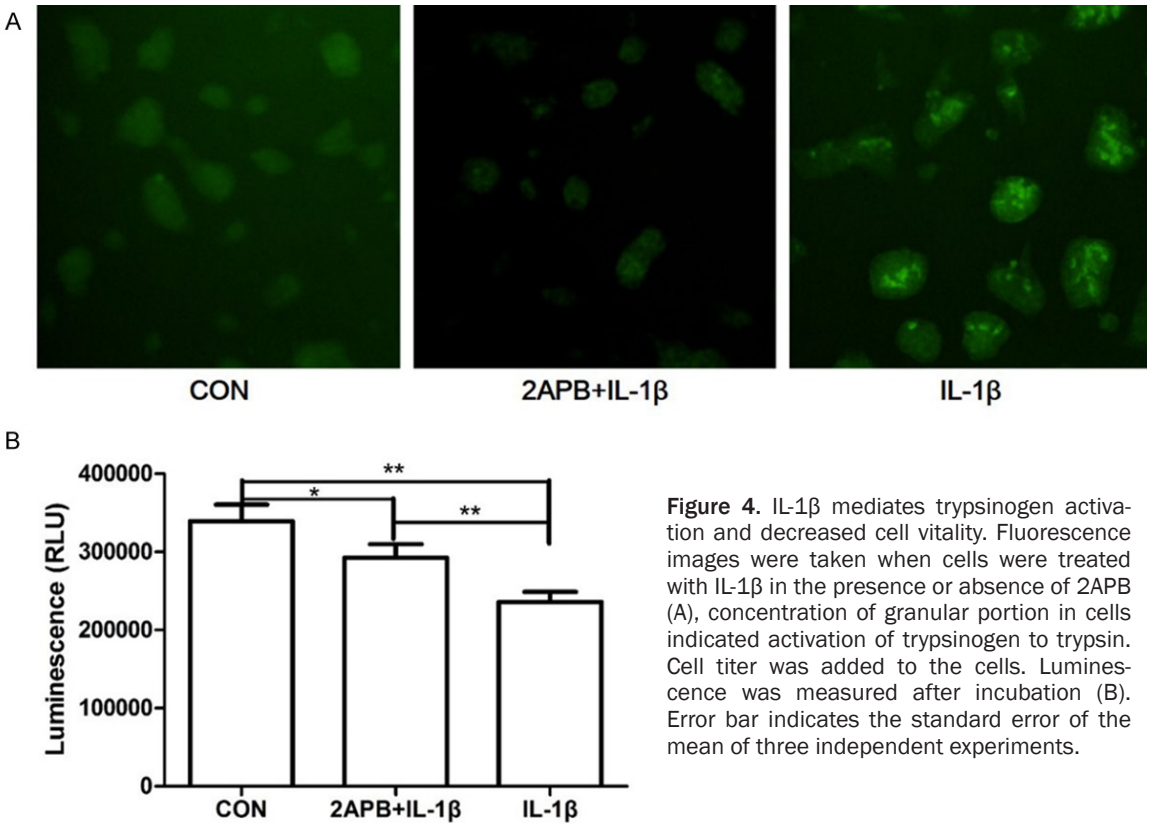

Figure 4. IL-1β mediates trypsinogen activation and decreased cell vitality. Fluorescence images were taken when cells were treated with IL-1β in the presence or absence of 2APB (A), concentration of granular portion in cells indicated activation of trypsinogen to trypsin. Cell titer was added to the cells. Luminescence was measured after incubation (B). Error bar indicates the standard error of the mean of three independent experiments.

samples were boiled in loading buffer before western blot analysis. The protein content was determined by the BCA assay. The western blot was performed according to standard procedures. The protein bands were detected by a ChemiDOC XRS imaging system (Bio-Rad, USA), and β-actin served as a loading control.

Confocal Ca^{2+} measurement in intact cells

Intracellular Ca^{2+} was monitored by the fluorescent dye Fluo-4-AM. Confocal imaging for free cytosolic Ca^{2+} was performed using confocal microscopy (Olympus, JP). Briefly, AR42J cells were seeded in glass-bottomed plate at a density of approximately 30% and investigated one day after seeding. The cells were loaded with 5 μM Fluo-4-AM (excitation 495 nm; emission 518 nm) at 37°C for 40 min in complete culture medium for dye loading. After loading, the cells were washed with HBSS to remove any dye and then incubated for a further 30 min. Images were acquired for 0.5 s per image using the confocal system. The recorded images were analyzed using FV10-ASW 3.0 (Olympus, JP), and the fluorescence intensity of the cells was measured by selecting a region surrounding the cell.

Cell viability

CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) was used to investigate cell viability. Cell-Titer was added to each well of a 96-well plate containing AR42J cells that had been treated with 2APB or 2APB plus IL-1β following the manufacturer's instructions. The cells were mixed for 2 min on an orbital shaker to induce cell lysis after incubation and then incubated for another 10 min to stabilize the luminescent signal. The luminescent signal of the sample was read after incubation.

Trypsin activity

To measure trypsin activity in acinar cells, rhodamine 110, bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide), dihydrochloride was used as a substrate. This is a specific substrate for the serine protease trypsin, and becomes fluorescent after cleavage of the oligopeptide side chain. The fluorescence was determined after

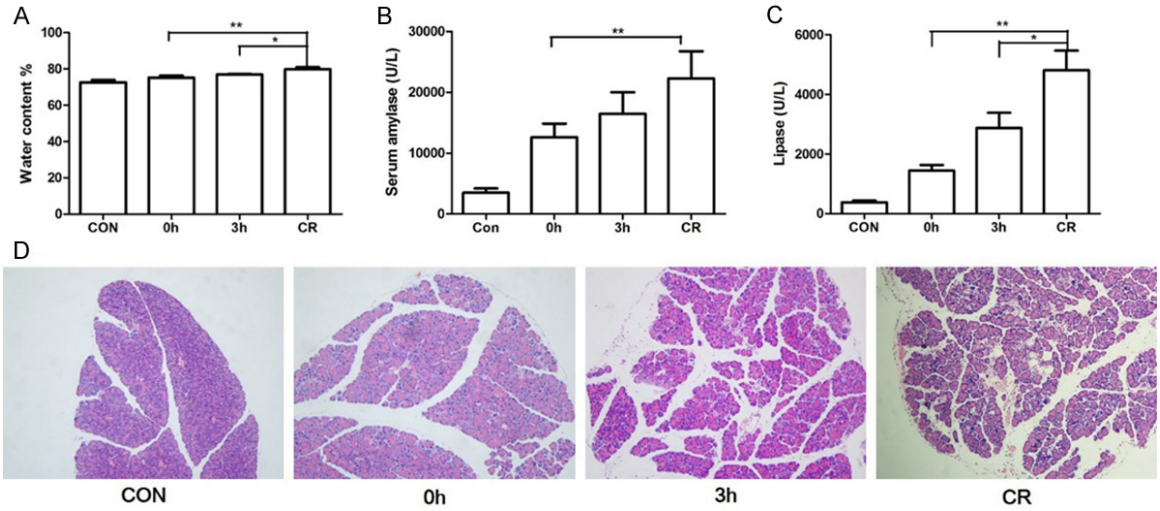

Figure 5. Water content (A), serum amylase (B), and lipase (C) were measured after the induction of AP. Pathologic changes in the pancreatic gland were also observed (D). Normal pancreatic tissue had integrated lobules, the spaces were not obviously broadened and there was no significant inflammatory infiltration. After injection of cerulein, the intervals were broadened and inflammatory infiltration was observed.

incubation by fluorescence microscope (Olympus, JP).

Animal experimental design

Male C57B1/6 mice (6-8 weeks old) were purchased from the Animal Experimental Center of The Fourth Military Medical University (Xi'an, China) and were housed at 25°C under a 12-h light/12-h dark cycle with free access to water and food. For the induction of AP, the mice were given 7 hourly injections of cerulein, as described previously [24]. To investigate the protective effect of 2APB on AP, the mice were divided into 4 groups. The AP group included 5 mice. An additional 5 mice were injected with 2APB (20 mg/kg) at 0, 3, and 6 h after the induction of AP. Another 5 mice were injected with 2APB at 3 and 6 h after the induction of AP. The control group received equal volumes of saline. The mice were sacrificed 1 h after the final cerulein injection of AP induction, and the venous blood, and pancreas were collected.

Transmission electron microscopy

Fresh pancreatic tissue samples were fixed with 2.5% glutaral at 4°C overnight. After post-fixation, the samples were dehydrated and embedded in an epoxy resin. The embedded tissue was then processed for transmission electron microscopy following standard procedures. Finally, ultrathin sections were examined with a JEM-1230 electron microscope.

Results

IL-1β induces autophagy in AR42J cells

The conversion of the ATG protein microtubule-associated protein 1 light chain 3 (LC3) from its soluble form, LC3-I, to its PE-conjugated form, LC3-II, is considered to be a hallmark of autophagy. This conversion can be detected by examining the accumulation of the LC3-II form [25]. To determine the effect of IL-1β on autophagy in pancreatic acinar cells, AR42J cells were incubated with IL-1β, and the expression of LC3 was evaluated by western blot. As expected, the results showed that the level of LC3-II protein was significantly higher after treatment with IL-1β (2 ng/ml) compared with the untreated control. Furthermore, this increase showed a time-dependent pattern (**Figure 1C**). The expression of LC3-II increased to its maximum at 15 min and then returned to its basal level at 1 h.

To further confirm that the treated acinar cells underwent autophagy, we employed a GFP-tagged version of LC3B. GFP-LC3B displays a diffuse cytoplasmic distribution in the absence of autophagy. In contrast, after the induction of autophagy, LCB forms autophagic membranes and can be observed as visible puncta representing autophagic vacuoles. After transfection with GFP-LC3B for 48 h, the cells were examined, and the number of GFP-LC3B puncta was

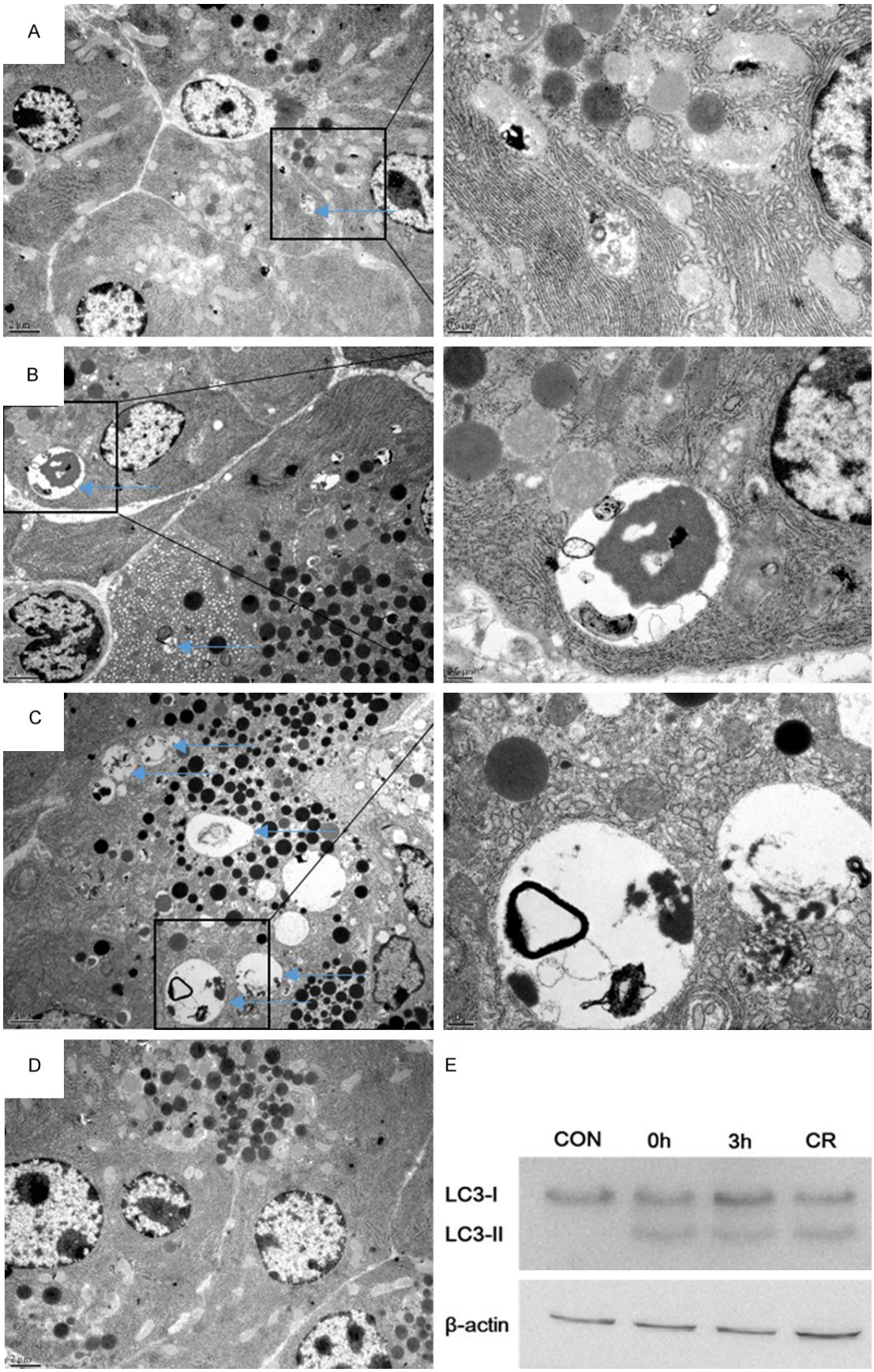
A
B
C
D
E
CON
0h
3h
CR
LC3-I
LC3-II
β-actin

Figure 6. Representative electron microscopy images of tissue sections from 0h 2APB injection group (A), 3 h 2APB injection group (B), AP group (C) and control pancreas (D), cerulein results in autolysosome containing intracellular organelles. 2APB decreases the number of autolysosme. Autophagic vacuoles was indicated by blue arrows. The expression levels of LC3 was assessed by western blot (E). β-actin was used as loading control.

observed by confocal microscopy. In the baseline condition, most transfected cells displayed a diffuse distribution of GFP-LC3B and had few GFP-LC3B puncta, with only a few cells having more than 3 GFP-LC3B puncta. Consistent with the western blot results, after stimulation with IL-1β, a large number of GFP-LC3B puncta formed in the cytoplasm (**Figure 1A**). The analysis of the GFP-LC3B distribution confirmed that the average number of GFP-LC3 puncta per cell was significantly increased in the IL-1β treated group compared with the untreated group (**Figure 1B**). This result is consist with electron micrographs founding (**Figure 1E**), indicating that autophagy was induced by IL-1β.

To examine the autophagic flux status, we transfected cells with RFP-GFP-LC3. The GFP signal can be quenched in a lysosomal environment; in contrast, the RFP signal is more stable in an acidic environment [26]. Therefore, autophagosomes and autolysosomes are labeled with a yellow (green and red) or red color, respectively. By detecting and analyzing the two different fluorescent signals, the autophagic flux can be monitored. After culturing for 48 h, the cells were incubated with IL-1β or starvation for 4 h, and numerous yellow puncta were observed in both groups, indicating that both of them increase autophagy. Meanwhile, more red puncta were found in IL-1β group than starvation group (**Figure 1D**). This phenomenon suggests that the autophagic flux may retard at autolysosome stage after treating with IL-1β. This finding was also confirmed by the detection of p62, a cargo protein degraded inside autolysosomes. When we evaluated the expression of p62, the results showed that p62 was increased, which indicated that autophagy was impaired in autolysosome degradation. These results clearly demonstrate that IL-1β could induce impaired autophagy in AR42J cells.

IL-1β induces ER stress and increases Ca^{2+} release from the ER in AR42J cells

Previous studies have shown that endoplasmic reticulum (ER) stress alters ER Ca^{2+} homeostasis by inducing Ca^{2+} release into the cytoplasm. In this study, we explored the effects of IL-1β in cultured AR42J cells. After the application of IL-1β, the ER stress markers GRP78 and IRE1 were significantly increased (**Figure 2A** and **2B**). Then, we tested whether IL-1β would also induce Ca^{2+} release from the ER. After stimulation with IL-1β, a typical cytoplasmic calcium response (a short silent period followed by a short but significant increase and a subsequent decrease) was observed in responsive cells via fluorescent Ca^{2+} imaging (**Figure 2C**). The transient Ca^{2+} amplitude was significantly increased in the treated AR42J cells compared with the control cells, which can be quantified by the fluorescence intensity profile.

Then, we investigated, using an $InsP_3$ receptor antagonist, 2-aminoethoxydiphenyl borate (2APB), whether IL-1β mobilizes calcium ions predominantly from ER stores through the $InsP_3$ signaling pathway. We observed that 2APB could completely block the Ca^{2+} increase in IL-1β treated cells (**Figure 2D**). These results indicate that $InsP_3R$ activation contributes to Ca^{2+} spikes and modulates the late sustained Ca^{2+} increase.

IL-1β-induced autophagy impairment is dependent on the Ca^{2+}

The turnover rate of LC3 (LC3-I to LC3-II) is usually used to monitor the autophagic process in cells. To measure autophagic flux, the lysosomotropic agent chloroquine (CQ) was employed to determine whether the IL-1β-induced LC3-II accumulation was due to enhanced synthesis or reduced degradation. We compared the LC3-II expression in IL-1β-treated cells in the presence or absence of CQ. Our results showed that the treatment with lysosomotropic agent did not lead to a further increase in the LC3-II accumulation (**Figure 3A**), suggesting that IL-1β causes an impaired autophagic flux.

We found that IL-1β could induce autophagy based on the increased biochemical signs of the conversion of LC3-I to LC3-II. To determine whether the IL-1β effect on autophagy relied on the $[Ca^{2+}]$ changes, we examined the LC3, LC3-

II and p62 expression levels with and without the addition of 2APB. The formation of fluorescent puncta and LC3-II in AR42J cells treated with 2APB and stimulated with IL-1β was decreased compared with those cells not treated with 2APB. Notably, the p62 level was also reduced in the cells treated with 2APB (**Figure 3B** and **3C**).

Impaired autophagy mediates trypsinogen activation and decreased cell vitality

To determine whether IL-1β can induce the activation of trypsinogen to trypsin, rhodamine 110-based substrates was employed. When IL-1β was added, the fluorescence intensity was significantly increased compared with the control cells. However, when the cells were pretreated with 2APB, the fluorescence intensity was decreased (**Figure 4A**), indicating that blocking [Ca^{2+}] flux with 2APB could partially prevent the trypsinogen activation induced by IL-1β in AR42J cells.

There is increasing evidence that trypsinogen activation can induce a decrease in cell vitality that may then promote trypsinogen activation and cell death. We next assessed cell vitality in treated cells. After 30 min of incubation, cell vitality was reduced by approximately 30% compared with the unstimulated cells (**Figure 4B**). This effect was partially inhibited by pretreatment with 2APB in AR42J cells. These results suggest that IL-1β affects cell vitality through a mechanism that depends on autophagy. Ca^{2+} was also involved in this process, and inhibiting the [Ca^{2+}] flux may protect the acinar cells in AP.

2APB attenuates AP induction in vivo

To explore the role of Ca^{2+} signaling in AP, we examined the effects of 2APB *in vivo* in mice treated with cerulein, which induces AP. Treatment with 2APB at the same time as the cerulein injection resulted in a protective effect. The mice with 2APB presented with reductions in the pancreas water content, serum amylase levels, lipase levels and pathological changes in the pancreatic gland compared with the mice that received the cerulein injection alone (**Figure 5A-D**). In the delayed 2APB injection group, this protective effect still occurred but was limited. These data confirmed the effective protection of 2APB against cerulein-induced AP in mice when given near the time of the cerulein injection. Moreover, the electronic microscopy and western blot results showed that co-treatment with 2APB and cerulein reduced the number and size of autophagosomes and the accumulation of LC3 (**Figure 6A-E**). Our data indicated that inhibiting the [Ca^{2+}] flux might have protective effects by blocking autophagy.

Discussion

Recently, with the increasing understanding of autophagy, the importance of autophagy in pancreatitis is being realized. There are two possible explanations for the crucial role of autophagy in trypsinogen activation in pancreatitis [27]. One is the colocalization hypothesis [28], which states that the explanation is the colocalization of digestive enzymes and lysosomal hydrolases. This hypothesis is based on cathepsin B, which can activate trypsinogen, and the observation that both lysosomal and digestive enzymes are present within cytoplasmic vacuoles in various experimental forms of pancreatitis. The second hypothesis is that the auto-activation of trypsinogen occurs optimally at pH 5.0, which is similar to the pH of acinar cell vacuoles [29].

IL-1β plays a crucial role in the inflammatory responses and functions of pancreatic acinar cells during AP. Many studies have shown that IL-1β can trigger autophagy. Considering this observation, whether IL-1β can induce trypsinogen activation though an autophagy pathway in pancreatic acinar cells needs to be clarified. Therefore, we performed a detailed characterization of the effects of IL-1β on pancreatic acinar cells. In this study, we showed that IL-1β causes impaired autophagy via an alteration of intracellular Ca^{2+} homeostasis that may result in trypsinogen activation. An increase in intracellular Ca^{2+} mediates IL-1β-induced autophagy in pancreatic acinar cells. Impaired autophagy is responsible for IL-1β-induced trypsinogen activation, and the inhibition of the intracellular Ca^{2+} increase could suppress this process in pancreatic acinar cells and maintain cell vitality.

Numerous studies have demonstrated the interaction between autophagy and IL-1β. IL-1β can stimulate autophagy in human macrophages. Exogenous IL-1β could induce endogenous IL-1β mRNA expression and protein pro-

duction. Lee et al [30] proposed that autophagy inhibits IL-1β signaling by down-regulating the expression of p62, which is an important scaffold in the IL-1β pathway whose increased expression promotes IL-1β production. Studies have shown that the p62 protein is accumulated in impaired autophagy and that impaired autophagy contributes to trypsinogen activation. The continuation of this cycle in existing cells will prevent patients with AP from recovering. However, approximately 80% of AP cases will be resolved without serious morbidity [31]. In this study, our data showed that IL-1β could induce impaired autophagy in acinar cells and that this effect was time dependent. From 0 to 15 minutes, the level of the autophagy marker LC3-II increased gradually, and the expression level of LC3-II increased to its maximum. From 15 to 60 minutes, the LC3-II expression level decreased gradually, and the LC3-II expression was reduced almost to its basal level at 60 min. As the main function of autophagy in cells is to maintain homeostasis during cellular stress, this phenomenon indicated that autophagy has a certain self-limited course in pancreatitis. This observation may help acinar cells to resist cytokine stimulation during the early stage of pancreatitis. However, this protective effect may be limited, and when that balance is broken, autophagy plays a hazardous role.

Pathogenic interactions between altered cellular Ca^{2+} signaling and AP have been extensively studied for years. Abnormal (global and sustained) increases in pancreatic acinar cell cytosolic Ca^{2+} are a key pathological signal associated with AP. Elevations in cytosolic Ca^{2+} are believed to mediate trypsinogen activation and other pathological responses of pancreatitis [32]. The ER is a major intracellular organelle that releases Ca^{2+} into the cytosol and has a high concentration of the IP_3 receptor [33]. This study found that IL-1β could elicit transiently oscillatory $InsP_3$-mediated Ca^{2+} signaling that increased cytosolic Ca^{2+} levels in acinar cells. We also showed that after inhibiting the Ca^{2+} signal, the expressions of LC3-II and trypsinogen activation were both reduced. However, we didn't determine whether IL-1β activates IP_3Rs directly or indirectly.

In a mouse model of AP, we found that 2APB has a protective effect against pancreatic injury and that early treatment in particular had a more beneficial effect than late treatment. Ultrastructural examination also identified fewer autophagic vacuoles in the co-treated group. Our data are consistent with the previous finding that a sustained increase in cytosolic Ca^{2+} will result in vacuole formation and trypsinogen activation [34], which are thought to be a constant feature of the early pathological changes in pancreatic acinar cells [35]. The cytoplasmic vacuoles in the acinar cells in AP have been demonstrated to have an autophagic origin. The pathological mechanism responsible for the increased cytosolic Ca^{2+} is not well understood. Our study has an important implication for understanding the mechanism of the inflammatory response in AP. Our results imply that the initial stress of IL-1β increases the intracellular Ca^{2+} level, resulting in a series of events that include impaired autophagy and trypsinogen activation. We believe that if this activation event cannot be effectively controlled, the progression of pancreatitis is aggravated.

In conclusion, our work provides new insight into the role of IL-1β in AP. We show that IL-1β can induce impaired autophagy and trypsinogen activation within 15 min of stimulation. Our results show for the first time that the ER can release Ca^{2+} into the cytosol in response to IL-1β stimulation and that the inhibition of IP_3Rs could decrease the amplitude of the response and the impaired autophagy. We conclude that autophagy plays an important role in helping acinar cells resist external inflammatory mediators during early pancreatitis. This finding may provide a new strategy in pancreatitis therapy.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Quanxin Feng or Dr. Qingchuan Zhao, State Key Laboratory of Cancer Biology and Xijing Hospital of Digestive Diseases, The Fourth Military Medical University, 127 Changle Western Road, Xi'an, Shaanxi Province 710032, China. Tel: +86-029-84771503; Fax: +86-29-

82539041; E-mail: fengqx@fmmu.edu.cn (QXF); zhaoqc@fmmu.edu.cn (QCZ)

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