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Original Article

ER stress is associated with pancreatic acinar cell injury induced by nicardipine

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Abstract: Premature trypsinogen activation (TA) induced pancreatic acinar cell injury is an important initiator for acute pancreatitis, a life threatening disease in severe cases. However, the mechanisms underlying TA have not been fully understood. Calcium overload and impaired autophagy leading to intracellular TA are contained in the early events of acute pancreatitis. In our previous research the L-type calcium channel blocker nicardipine was found to induce TA. In this study, the mechanism underlying TA triggered by nicardipine was investigated. The results show that nicardipine induced impaired autophagy both in vitro and in vivo which accompanied pancreatic acinar cell injury. Furthermore, nicardipine induced pancreatic acinar cell injury as well as impaired autophagy was reduced by ER stress inhibitor. In conclusion, the axis of ER stress/impaired autophagy might play a role in nicardipine-induced pancreatic acinar cell injury.

Keywords: Pancreatic acinar cell injury, impaired autophagy, ER stress, nicardipine

Introduction

Pancreatic cell injury is one of the major causes for acute pancreatitis (AP) which is a life threatening disease in severe cases [1, 2]. Duct obstruction, alcohol, or drugs lead to premature of intracellular digestive enzyme release and result in acinar cell injury [3-6]. Therefore, digestive enzyme activity is very important in the initiation of AP.

As an inducer for many other digestive enzyme, trypsin is very important [7]. It had been reported that intracellular trypsinogen activation was associated with calcium overload [8, 9]. However, the role of calcium channel blockers in trypsinogen activation has been inconsistently described [10-12]. Previously, we found that L-type calcium channel blocker nicardipine induced trypsinogen activation *in vitro*, but the details were not fully understood.

Accumulative autophagosome could be observed in the early stage of AP where trypsinogen was activated [13, 14]. Autophagy with the molecular indicators like LC3II, p62 and p62 marked insoluble ubiquitinated protein is responsible for damaged organelle or misfolded protein degradation [15]. Increased LC3II, p62 and p62 marked insoluble ubiquitinated protein upregulation was associated with autophagy flux blockage [15, 16]. When autophagosome blockage established as impaired autophagy occurred, trypsin leaked into cytosol leading to the digestion of acinar cells [17]. Inhibition of impaired autophagy could protect trypsinogen from activation and ameliorate the pathology of acute pancreatitis [9]. Calcium channel blockers are known to induce autophagy in brain cells but how they work in pancreatic acinar cells is unknown [18].

Calcium channel blockers have been reported recently to induce ER stress which is a potential stimulus for autophagy [19]. However, calcium channel blocker induced impaired autophagy related to ER stress has not been reported, especially in pancreatic acinar cells.

In this study, the mechanism underlying nicardipine-induced trypsinogen activation as well as pancreatic acinar cell injury *in vitro* and *in vivo* was studied. It was found that impaired autophagy might be associated with nicardipine-induced acinar cell injury. Furthermore, ER stress inhibitor reduced impaired autophagy under nicardipine treatment.

Materials and methods

Chemicals and antibodies

The following commercial antibodies were used: anti-LC3 (rabbit, Sigma), anti-p62 (rabbit, PTG), anti-Ubiquitin (rabbit, PTG), anti-Tubulin (rabbit, PTG).

Cell culture

AR42J, a rat pancreatic cell line, purchased from ATCC was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Solabal). All cultures were maintained in a 37°C incubator with 5% CO_2.

Trypsin activity assay

AR42J cells were lysed, each sample is mixed with butoxycarbonyl-Gln-Ala-Arg-7-amido-4-methylcou-marin hydrochloride (Sigma), in trypsin reaction buffer (10 mM Tris, 20 mM $CaCl_2$, pH 7.4), and then incubated 30 minutes at 37°C. The fluorescence intensity from trypsin substrate was measured at 450 nm in a fluorescence microplate reader under excitation at 380 nm [9].

Western blotting

The lysates from AR42J cells or animal pancreas were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Millipore). After probing with indicated primary antibodies and then anti-rabbit or anti-mouse IgG secondary antibodies (PTG), the reactions were visualized by chemiluminescence reagents.

The ubiquitination analysis of protein

1% Triton X-100 insoluble fractions from culture cells and mice pancreatic tissues were dissolved in 2% SDS separately, and then were analyzed by Western blotting with anti-ubiquitin antibody.

Animals and treatment

Female C57BL/6 mice from the Shanghai Slac Laboratory Animal Co. weighting 20-25 g were used. Mice were kept in clean environment and randomly divided into three groups (n = 14). All animal care and experimental procedures complied with the guidelines for the Animal Care and Use of Laboratory Animals (Eighth Edition), and were approved by the Ethical Committee on Animal Experiments at Youjiang Medical University for Nationalities. Mice from one group were intravenously (I.V.) injected saline; ones from the other groups were I.V. injected with 5 mg/kg nicardipine hydrochloride (MedChemExpress) with or without intraperitoneal injection of sodium 4-phenylbutyrate (MedChemExpress, 1 mg/kg) one time a day for two weeks. Animals were euthanized and then blood and pancreas tissue were collected to investigate pancreatic acinar cell injury. One mouse in the nicardipine group died before sacrifice.

Blood chemistry

Serum amylase and lipase levels were determined using the commercial assay kits (Bioassay) according to the manufacturer's instructions.

Histological analysis

Formalin-fixed pancreas samples were processed, and 4-μm thick paraffin sections were stained with hematoxylin and eosin (H&E).

Statistical analysis

Error bars for microscopy were presented as the standard deviation of triplicate samples. Error bars for Western blotting analysis represent the standard deviation of densitometry data from 3 unique experiments. Student's *t* test in GraphPad Prism was used for statistical analysis.

Results

Nicardipine induces trypsinogen activation in pancreatic acinar cells

In previous research, nicardipine induced trypsin activation. Here, nicardipine increased the activity of trypsin in a dose-dependent manner below 10 μM (**Figure 1A**). Furthermore short time treatment of nicardipine resulted in tryp-

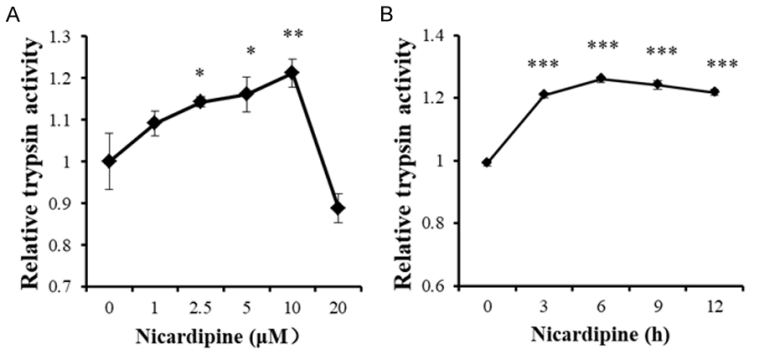

Figure 1. Nicardipine induces trypsinogen activation in pancreatic acinar cells. AR42J cells were treated with nicardipine at indicated concentrations for 6 h (A), or with 10 μM nicardipine for 0, 3, 6, 9 and 12 hours (B) and then cells were lysed. Cell lysates were subjected to trypsin activity assays. Error bars for enzyme activity were presented as the standard deviation of triplicate samples. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. vs without nicardipine treatment.

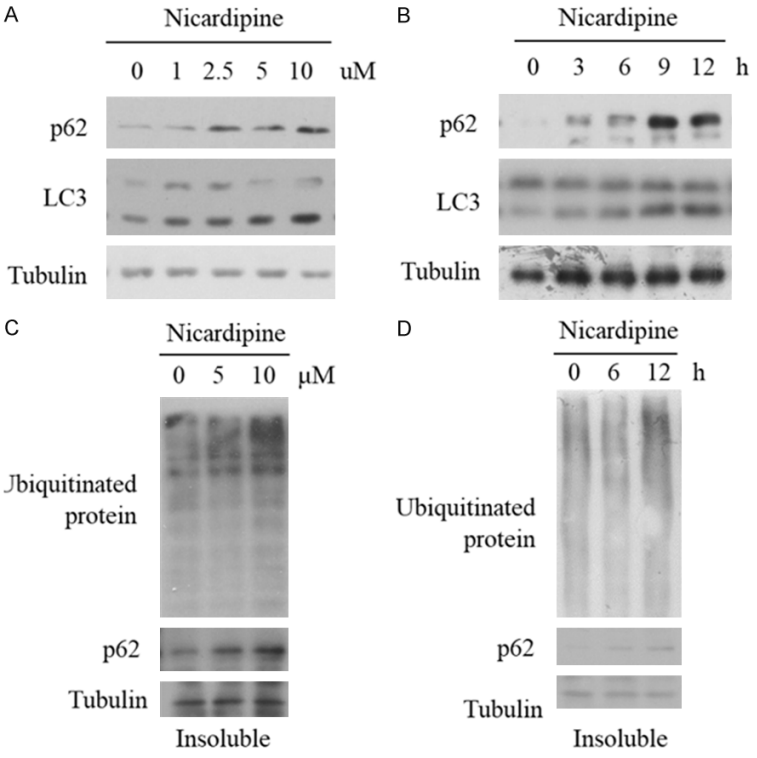

Figure 2. Nicardipine induces impaired autophagy in pancreatic acinar cells. AR42J cells were treated with nicardipine at indicated concentrations for 6 hours (A, C), or with 10 μM nicardipine for indicated durations (B, D). Total cell lysate (A, B), or Triton X-100 insoluble fractions (C, D) were subjected to Western blotting with indicated antibodies.

sinogen activation (**Figure 1B**). While, nicardipine with high dose or long time exposure had the opposite effect (**Figure 1**). These results suggest that nicardipine had a dual role in trypinogen activation which was consistent with our previous research.

Nicardipine induces impaired autophagy in pancreatic acinar cells

Calcium channel blockers have been reported to induce autophagy [18]. Furthermore, autophagosome accumulation has been established as impaired autophagy and occurred in the early stage of acute pancreatitis [13]. Therefore, the effect of nicardipine on autophagy in pancreatic acinar cells line AR42J was detected. Surprisingly, nicardipine increased the total protein level of p62, LC3II in a dose and time dependent manner (**Figure 2A** and **2B**). Furthermore, the level of insoluble ubiquitinated protein and p62 also increased (**Figure 2C** and **2D**). Additionally, increased LC3II, p62 and insoluble p62 marked ubiquitinated protein predict autophagy flux blockage. Taken together, these results indicate that autophagy was blocked and nicardipine activated trypsinogen may be through impaired autophagy.

Nicardipine induces impaired autophagy as well as trypsinogen activation is reduced by ER stress inhibitor

Calcium channel blocker was reported to induce ER stress which was an important stimulus of autophagy [19]. Therefore nicardipine had the potential to induce ER stress and inhibition of ER stress might ameliorate the autophagy blockage induced by nicardipine. When AR42J cells were treated with nicardipine in the presence of ER stress inhibitor 4-phenylbutyric Acid (4-PBA), the level of LC3II, p62, insoluble uibiq-

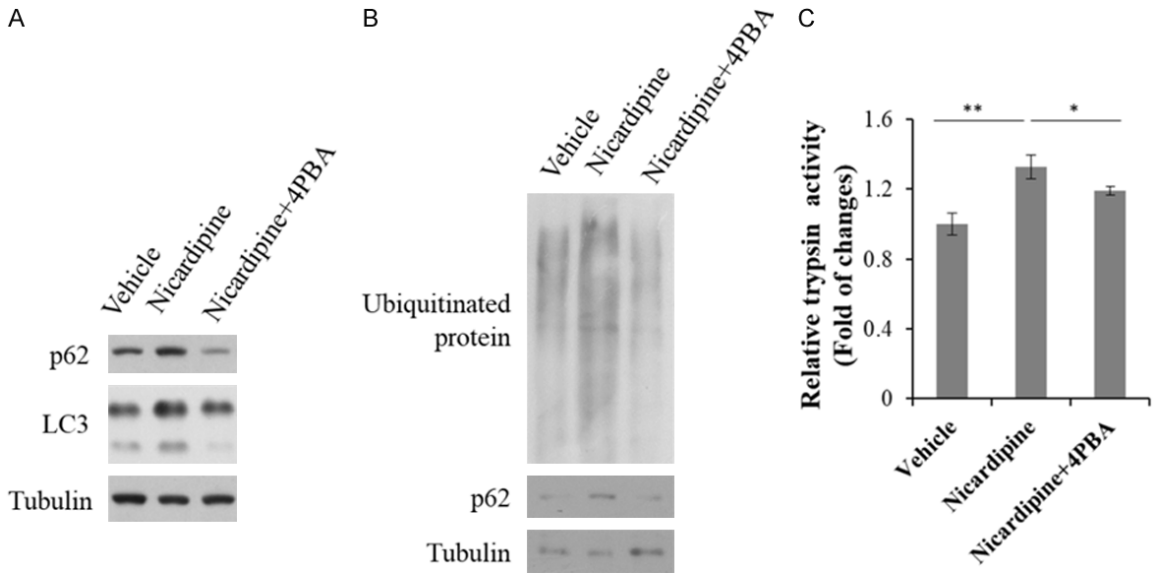

Figure 3. Nicardipine induces impaired autophagy as well as trypsinogen activation is reduced by ER stress inhibitor. AR42J cells were treated control solvent or 10 μM nicardipine for 6 hours during which 5 mM 4-PBA was added for 1 hour. One part of total cell lysate (A), or Triton X-100 insoluble fractions (B) were subjected to Western blotting with indicated antibodies. Trypsin activity in the other part of total cell lysate was analyzed (C). The graph represents the mean ± SD from three independent experiments. Student's *t* test were used to analyze the difference between indicated two groups. $^{*}P < 0.05$, $^{**}P < 0.01$.

uitinated protein and p62 decreased (**Figure 3A** and **3B**). Furthermore, intracellular trypsinogen activation induced by nicardipine was inhibited in the presence of 4-PBA (**Figure 3C**). These results indicate that impaired autophagy and trypsinogen activation caused by nicardipine may be through ER stress.

Continuous injection with nicardipine induces pancreatic acinar injury through ER stress

Since nicardipine activated trypsinogen activation *in vitro*, we questioned whether it could cause damage to acinar cell injury *in vivo*. C57BL/6 mice were used to investigate the toxicity of nicardipine. Mice were continuously intravenously injected with nicardipine in the presence or absence of 4-PBA for two weeks. Changes of serum biomarkers as well as pathology in pancreas were detected. Nicardipine significantly induced serum amylase and lipase (**Figure 4A** and **4B**). Additionally, mice injected with nicardipine showed obvious edema in pancreas acinar (**Figure 4C**). However, 4-PBA treatment decreased serum amylase and lipase activity (**Figure 4A** and **4B**) induced by nicardipine. Moreover, edema under nicardipine treatment can be ameliorated by ER stress inhibitor (**Figure 4C**). Therefore, nicardipine caused pancreatic acinar injury *in vivo* is probably through ER stress.

Impaired autophagy caused by ER stress in pancreas

The above results indicated that nicardipine induced trypsinogen activation and impaired autophagy *in vitro* was related to ER stress. We wondered whether impaired autophagy caused by ER stress could be observed *in vivo*. As expected, nicardipine treated mice showed increased p62 and LC3II protein as well as insoluble ubiquitinated protein or p62 (**Figure 5A** and **5B**). Furthermore, upregulation in the levels of the indicated proteins was inhibited by 4-PBA (**Figure 5**). These results suggest that in mice nicardipine caused trypsinogen activation is related to impaired autophagy which may be through ER stress.

Discussion

In the present study, our results demonstrate that nicardipine has a dual role in trypsinogen activation. Nicardipine induced pancreatic acinar cell injury *in vivo* and *in vitro*. Additionally, trypsinogen activation and impaired autophagy under nicardipine treatment is reduced by ER

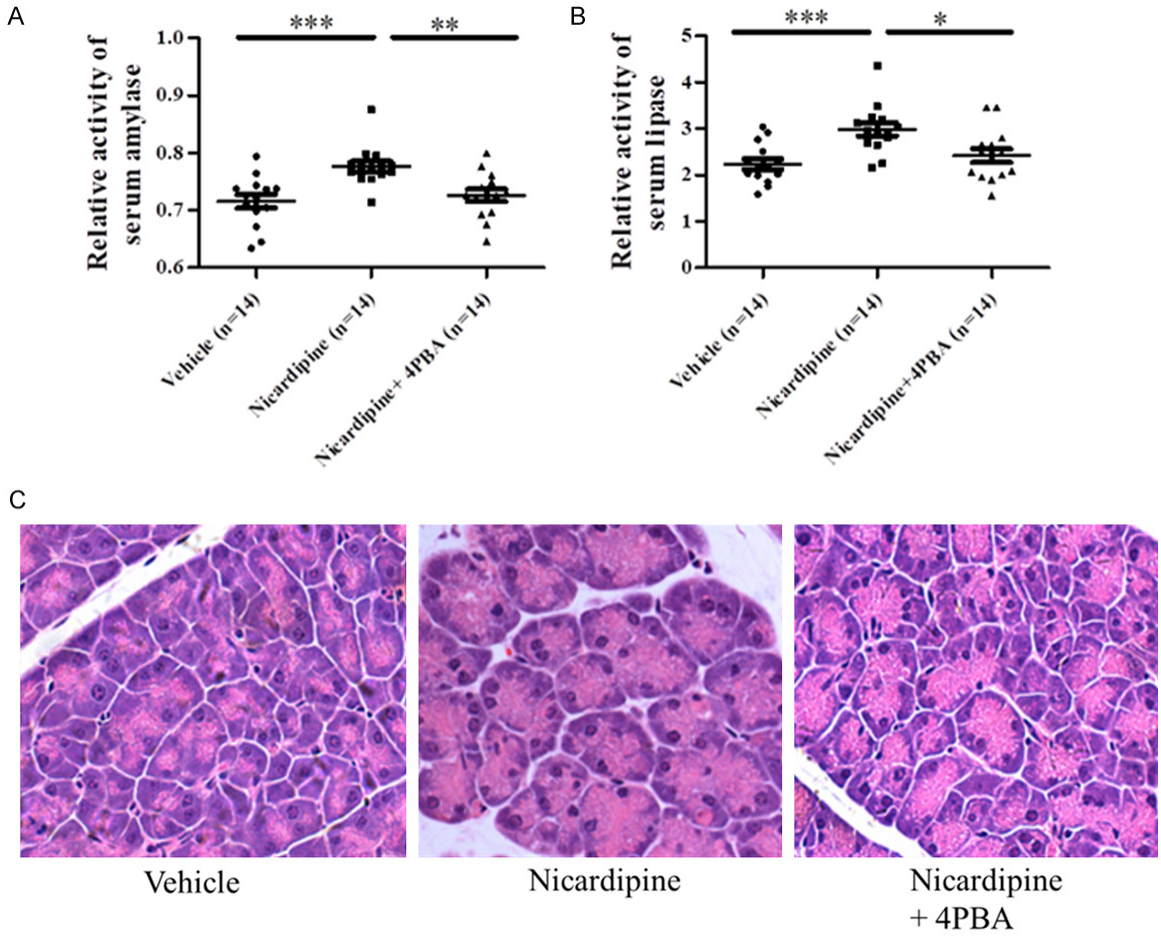

Figure 4. Continuous injection with nicardipine induces pancreatic acinar injury through ER stress. The mice under indicated treatments were euthanized for analyzing serum amylase (A), lipase (B) levels, histopathological change (C). The data from serum analyses were tested using Student *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

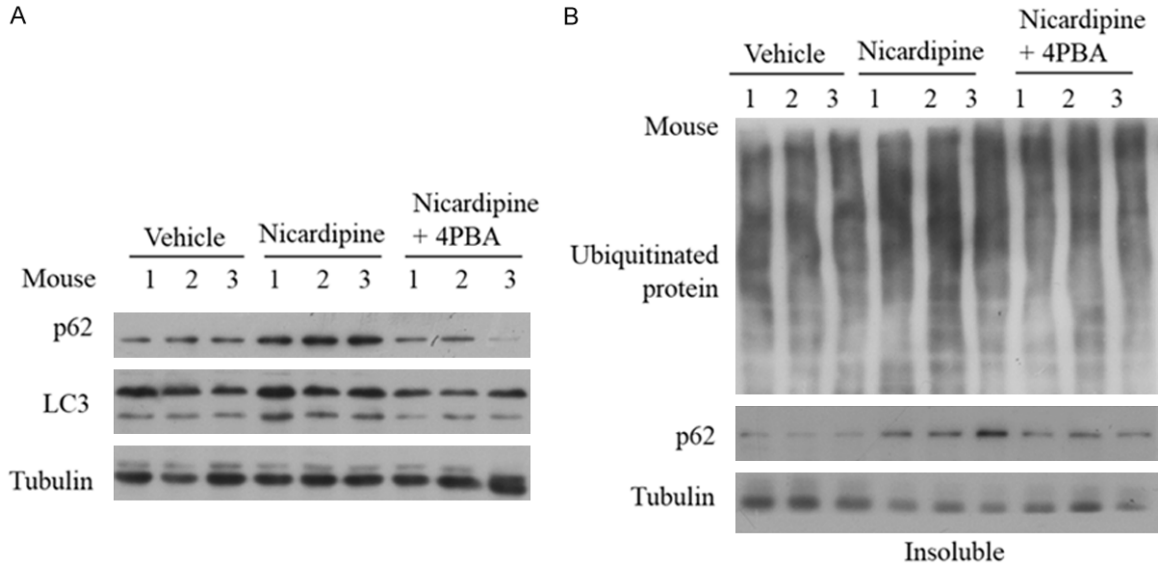

Figure 5. Impaired autophagy caused by ER stress in pancreas. The mice under indicated treatments were sacrificed for analyzing the levels of LC3II (A), p62 (A, B) and Ub (B) in the pancreatic tissues. Representative samples are shown.

stress inhibitor. As impaired autophagy is well known to induce trypsinogen activation, ER stress/impaired autophagy/trypsinogen activation may be present in the mechanism underlying pancreatic acinar cell injury caused by nicardipine.

According to the results in this manuscript, low dose or short time treatment of nicardipine activated trypsinogen but high dose or long time treatment of the compound worked the opposite way. This dual effect might be the reason for the inconsistent phenomenon observed in mice under calcium channel blockers treatment [10-12]. Whether nicardipine ameliorated acute pancreatitis depended on its dosage. Continuous injection with nicardipine mice showed acinar cell injury in pancreas which was similar to the previous report [12]. However, prolonged treatment of nicardipine inhibited trypsin *in vitro*. It might be the presence of unknown targets of nicardipine *in vivo* resulting in the opposite effect.

Nicardipine is now used in the short-term treatment of hypertension [20]. While in this study intravenous nicardipine induced pancreatic acincar injury. Therefore nicardipine might not be a best choice for hypertension patients with the history of acute pancreatitis.

Nicardipine had been reported to induce autophagy resulting in the increased LC3II [21]. According to the guideline in autophagy nowadays, both autophagy flux upregulation and impaired blockage lead to the elevation in LC3II [15]. Therefore autophagy flux induced by nicardipine in the previous report might actually was blocked. Of note, the effect of nicardipine on autophagy might be different among various types of cells. Additionally, insoluble ubiquitinated protein was considered to be the marker of acute pancreatitis which was confirmed in this manuscript [9]. It seemed that insoluble ubiquitinated protein was associated with acinar cell injury in various cases.

Mitochondrial dysfunction induced impaired autophagy following by ER stress has been recently reported to occur in the development of acute pancreatitis [22]. While, impaired autophagy in nicardipine caused pancreatic acinar cell injury was reduced by ER stress inhibitor. Taken together, it could be inferred that positive feedback loop between impaired autophagy and ER stress might be involved in the mechanism that underlies acute pancreatitis.

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Disclosure of conflict of interest

None.

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