Cav 1.2 and Cav 2.2 expression is regulated by different endogenous ghrelin levels in pancreatic acinar cells during acute pancreatitis

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Abstract. Ghrelin influences pancreatic endocrine and exocrine functions, regulates intracellular calcium $[Ca^{2+}]_i$ levels, and has an anti-inflammatory role in acute pancreatitis. This study investigated the role of endogenous ghrelin in the expression of Cav 1.2 (L-type of Ca^{2+} channel) and Cav 2.2 (N-type of Ca^{2+} channel) in acute pancreatitis. For this purpose, acute edematous pancreatitis (AEP) and acute necrotizing pancreatitis (ANP) rat models were established. Cav 1.2 and Cav 2.2 expression was assessed by immunohistochemistry in the pancreatic tissues of rats; ghrelin, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) serum levels were detected using ELISA. Next, in AR42J cells with either knock-out or overexpression of ghrelin, Cav 1.2 and Cav 2.2 expression was examined using western blot analysis, and intracellular calcium $[Ca^{2+}]_i$ was detected with confocal microscopy. In this study, the ghrelin serum level was highest in the ANP group and was higher in the AEP group than the normal group. Expression of Cav 1.2 and Cav 2.2 in the ANP and AEP groups was higher than in the respective control groups. The serum IL-1β and TNF-α levels were significantly higher in the ANP group compared to the other groups. Cav 1.2 and Cav 2.2 expression and $[Ca^{2+}]_i$ decreased in ghrelin knockdown AR42J cells but increased in ghrelin overexpressing cells. In conclusion, Cav 1.2 and Cav 2.2 expression increased in ANP. The $[Ca^{2+}]_i$ level, which is mediated by Cav 1.2 and Cav 2.2 expression, is directly regulated by ghrelin in pancreatic acinar cells, and serum ghrelin levels may be involved in the severity of acute pancreatitis.

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Introduction

Acute pancreatitis (AP) is an inflammatory disorder of the pancreas, with an incidence of 13 to 45/100,000 people (1). AP has been reported as one of the most frequent principal gastrointestinal discharge diagnoses in the USA (2). According to the 2012 Atlanta classification for acute pancreatitis, there are three types of AP: mild acute pancreatitis (MAP), moderately acute pancreatitis and severe acute pancreatitis (SAP), which are defined based on the extent of organ failure and its duration (3). MAP is self-limiting, but SAP is a life-threatening condition with a high mortality rate and rapid progression that is associated with many complications (4). Several mechanisms of pancreatic damage have been proposed in recent studies, such as pancreatic duct obstruction, trypsinogen activation, pancreatic microcirculation malfunction (5), calcium (Ca^{2+}) overload (6) and the activation of inflammatory pathways (7).

Under normal physiological conditions, Ca^{2+} signals are transient and localized in granules at the apical pole; however, the sustained elevation of cytosolic Ca^{2+} concentrations is fatal (8,9). Ca^{2+} cell entry is mediated by voltage-dependent Ca^{2+} channels (principally L-type Ca^{2+} channels) and is involved in a variety of Ca^{2+}-dependent processes, including muscle contraction, hormone or neurotransmitter release and gene expression (10). In recent years, Ca^{2+} overload has received increasing attention, and its role is being extensively investigated in the context of pancreatic acinar cells injury (11). Digestive enzymes have been reported to be produced by pancreatic acinar cells and packaged in zymogen granules in the apical pole (12). When Ca^{2+} overload occurs, it activates several signaling pathways, including mitogen-activated protein kinases, phosphoinositide 3-kinase, and nuclear factor-κB (NF-κB) cascades, which leads to the induction of several proinflammatory mediators (13). However, Ca^{2+} overload causes intracellular trypsin activation, vacuolization and necrosis (14-16), which aggravates subsequent cell injury and increases mortality in human acute pancreatitis (17). Voltage-dependent Ca^{2+} channels have recently been demonstrated to be regulated by growth hormone secretagogue receptor type 1a (GHSR1a). Ghrelin-dependent GHSR1a inhibition is reversible and involves the altered function of Ca^{2+} channels

via $G_{i/o}$ or G_q signaling pathways (18). Voltage-gated ion channels are widely known to be involved in the control of growth hormone (GH) synthesis and release (19). The molecular basis of these regulatory actions has not been determined.

Ghrelin is a novel GH-releasing peptide that was initially isolated from gastric X/A-like cells and is a natural ligand for GHSR (20). Acute treatment with ghrelin increases intracellular calcium $[Ca^{2+}]_i$ (21). In somatotropes, this may upregulate voltage-activated Ca^{2+} influx in a larger time scale through activated L-type Ca^{2+} channels (22). Several studies showed that ghrelin has an anti-inflammatory role in acute pancreatitis due to its involvement in NF-κB inhibition, an increase in pancreatic blood flow and DNA synthesis, anti-oxidation, and the stimulation of pancreatic cell proliferation (23-25). However, the molecular mechanism of endogenous ghrelin calcium channel regulation in pancreatic acinar cells in acute pancreatitis remains unclear. Therefore, this study examined the level of serum ghrelin in acute edematous pancreatitis (AEP) and acute necrotizing pancreatitis (ANP) rat models. Additionally, Cav 1.2 (L-type of Ca^{2+} channel) and Cav 2.2 (N-type of Ca^{2+} channel) expression were examined in rat pancreatic tissues and transfected AR42J cells with ghrelin overexpression and knockdown.

Materials and methods

Antibodies and reagents. Antibody against ghrelin (cat. no. ab134978) was purchased from Abcam PLC (Cambridge, MA, USA). Antibodies against Cav 1.2 (cat. no. sc-16229-R) and Cav 2.2 (cat. no. sc-20129) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (cat. no. 2118) was purchased from Cell Signaling Technology (Beverly, MA, USA). Pierce ECL Western Blotting Substrate (cat. no. 32209) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Blasticidin (cat. no. 203351) was purchased from EMD Millipore (Darmstadt, Germany). Fluo-4/AM (cat. no. F14217) was purchased from Invitrogen (Grand Island, NY, USA). The kit for enzyme-linked immunosorbent assay (ELISA) ghrelin detection (cat. no. EIA-GHR) was purchased from RayBiotech, Inc. (Norcross, GA, USA). Interleukin-1β (IL-1β) (cat. no. ERC007.96) and TNF-α (cat. no. ERC102a.96) ELISA kits were purchased from Neobioscience Biotechnology (Shenzhen, China). Caerulein and sodium taurocholate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Animal groups. For the *in vivo* experiments, 40 healthy male Sprague-Dawley rats weighing 200-250 g were purchased from the Guangxi Medical University Animal Experimentation Center, China (certificate no. SCXK GUI 2009-0002). All rats were maintained in an environment with controlled temperature (20-24˚C) and humidity (55-58%), a 12-h light/dark cycle and fed with standard pellet rat food (210 kcal/100 g/day). Before each experiment, the animals were fasted overnight but allowed free access to water. The following day, the rats were randomly divided into five groups: the normal group, an acute edematous pancreatitis (AEP) group, an AEP-control group, an acute necrotizing pancreatitis (ANP) group and an ANP-control group.

Acute pancreatitis rat models and pathological scores of pancreatic tissues. In this study, AEP was induced by the administration of 50 μg/kg of caerulein with intraperitoneal injections five times per day in 1-h intervals, and the same volume of normal saline was injected intraperitoneally in the AEP control rats. ANP was induced by the injection of 1 ml/kg of 5% sodium taurocholate into a biliopancreatic duct for 5 min, following the closure of the surgical incision with a double layer of stitching. After the operation, the rats were subcutaneously injected with 30 ml/kg of the same volume of normal saline. The pancreas and duodenum treatments were switched as in the ANP control group. After surgery, the rats were provided with water *ad libitum*. Next, all rats were anesthetized and blood samples (2 ml) were collected from the inferior vena cava 24 h following surgery or from the last intraperitoneal injection. All animals were checked daily to monitor their health. They were finally euthanized by cervical dislocation. All animal care and studies were conducted in accordance with the approval of the Medical Ethics Committee of the First Affiliated Hospital of Guangxi Medical University for Ethical Approval for Research Involving Animals (Nanning, China, permit no. KY-113). All surgeries were performed under 10% chloral hydrate, and all efforts were made to minimize suffering. Pancreatic tissue was excised and fixed in 10% formalin and embedded in paraffin. For pathological observation, tissue blocks were cut into sections and stained with hematoxylin and eosin. A double-blind microscopic analysis was performed by two senior pathologists. Pathological scores for pancreatic tissues on a scale from 0 to 4 were determined with regard to the degree of edema, inflammation, hemorrhage and necrosis according to the method described by Kusske *et al* (26).

Cell culture and transfection. For the *in vitro* experiments, the rat pancreatic exocrine cell line AR42J was obtained from American Type Culture Collection (Manassas, VA, USA) and used for stable ghrelin overexpression or knockdown transfections. In brief, AR42J cells were grown in high glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml) in an atmosphere of 5% CO_2 at 37˚C. In this study, cells were transfected with ghrelin-overexpressing vector, knockdown ghrelin short hairpin RNA (shRNA) vector or blank vector [negative control (NC)]. Lentiviral vector encoding human ghrelin was generated by cloning ghrelin PCR fragments (full sequence) into a pcDNA3.1-GFP vector (Invitrogen) through *Eco*RI/*Xho*I digestion sites. The ghrelin shRNA (3'-AGAAAGGAATCCAAGAAGCCACC-5', 5'-TGCCAACA TCGAAGGGAGC-3') was cloned into a pcDNA6.2-EGFP-ghrelin-miR vector (Invitrogen). For the lentiviral infection of cells, cells were cultured in medium and inoculated with lentivirus at a multiplicity of infection (MOI) of 10 for 48 h, and the percentage of cells that became infected at this MOI was ~95%. Blasticidin S (0.2 μg/ml) was added into the medium for 2 weeks followed by another 2 weeks at 0.1 μg/ml. Stable ghrelin overexpression or knockdown cell lines were then isolated via fluorescence-activated cell sorting and verified using western blot analysis. Finally, stably transfected AR42J cell clones with ghrelin overexpression or knockdown were chosen for subsequent experiments.

Western blot analysis. In this study, the protein expression of ghrelin, Cav 1.2, and Cav 2.2 in AR42J cells was examined using western blot analysis. For western blot analysis, cells were lysed in Triton X-100-based lysis buffer. The protein concentration in the supernatant was determined using Bradford colorimetry. Next, 40 μg of protein from each sample was denatured and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Following blocking in 5% non-fat milk in TBST for 1 h, the membranes were incubated overnight at 4˚C with appropriate antibodies as follows: ghrelin (diluted 1:250), Cav 1.2 (diluted 1:300), Cav 2.2 (diluted 1:300) and GAPDH (diluted 1:3,000). After washing with phosphate-buffered saline (PBS), PVDF membranes were incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000) (Santa Cruz Biotechnology, Inc.) for 2 h. Protein signals were visualized using enhanced chemiluminescence reagents according to the manufacturer's instructions. Optical density of the imaged bands was normalized using a GAPDH signal obtained on the same blot. The data were summarized as the means ± SD of three independent experiments.

Immunohistochemistry. All pancreatic tissue samples were fixed with 4% paraformaldehyde for 12 h, embedded in paraffin and cut into 4-μm sections. For the immunohistochemical analysis, sections were deparaffinized, rehydrated, and endogenous peroxidases were blocked in methanol with 3% H_2O_2 for 10 min. After antigen retrieval induced by heat in a microwave at 93˚C for 30 min, sections were blocked in 10% normal goat serum for 1 h and incubated with primary antibody (Cav 1.2, diluted 1:100; Cav 2.2, diluted 1:100) for 4 h. Next, biotinylated secondary antibody (Santa Cruz Biotechnology, Inc.) was applied for 30 min. The immunohistochemical reaction was visualized using 0.01% DAB chromogen (Santa Cruz Biotechnology, Inc.) for 2 min. All slides were evaluated by two pathologists. Evaluation of the staining reaction was performed in accordance with the immunoreactive score (IRS) (27): IRS = SI (staining intensity) x PP (percentage of positive cells). SI was defined as 0, negative; 1, weak; 2, moderate; and 3, strong. PP was defined as 0, no positive cells present; 1, 10% positive cells; 2, 11-50% positive cells; 3, 51-80% positive cells; and 4, >80% positive cells. Ten visual fields from different areas of each tissue were used for IRS evaluation. Pancreatic tissue slides with at least 3 IRS points in this study were classified as immunoreactive.

$[Ca^{2+}]_i$ imaging in the AR42J cells. For $[Ca^{2+}]_i$ imaging, AR42J cells were seeded in a 24-well culture plate containing glass coverslips for 24 h and fixed with 4% formaldehyde. Next, the cells were washed three times with PBS and incubated in 5 μM fluo-4/AM (Invitrogen) for 30 min at room temperature. The cells were then washed five times with PBS, antifade mounting medium was added and cells were examined under fluorescence microscope (IX83 system; Olympus, Tokyo, Japan). Fluo-4 was excited at 495 nm, and fluorescence emissions were separately collected at 510 nm. $[Ca^{2+}]_i$ was quantified from fluo-4 levels (red fluorescence). Each sample was analyzed three times.

Enzyme-linked immunosorbent assay (ELISA). Levels of ghrelin, IL-1β and TNF-α in rat serum were measured using commercially available ELISA kits according to the manufacturer's instructions. In brief, supernatants were collected at the 24 h time point and centrifuged at 1,500 rpm for 20 min. Next, a 100-μl aliquot of supernatant, standard sample, or positive control sample was added into a 96-well plate and incubated for 1 h at 37˚C. Then, 100 μl of enzyme-linked antibodies were added, and the plate was incubated for 30 min at 4˚C. After washing nine times with washing buffer and incubation for 30 min at 37˚C, 2 M H_2SO_4 was added to terminate the reaction. Absorbance at 450 nm was determined using a microplate reader (Thermo Fisher Scientific). Each sample was analyzed three times.

Statistical analysis. The data are presented as the means ± SD. The statistical significance of differences between the means was evaluated using the one-way analysis of variance test. Statistical analysis was performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). A value of $p<0.05$ was considered significant.

Results

Histopathological scores of pancreatic tissues, serum ghrelin, IL-1β and TNF-α in AEP and ANP rats. In this study, no obvious pathological changes were observed in the normal group, the AEP-control group or ANP-control group of animals (Fig. 1A-C). When pancreatic tissues of AEP rats were examined, fewer foci were observed. Additionally, hemorrhagic ascites in the pancreas and saponifying spots in the mesentery or the greater omentum were not observed in AEP rats. Under light microscope, edema and inflammatory cells infiltrating the pancreatic stroma were observed; nevertheless, diffuse bleeding and piecemeal necrosis did not appear in the pancreas of AEP rats (Fig. 1D).

However, hemorrhagic ascites, necrosis foci in the pancreas and several saponifying spots in the mesentery and greater omentum were observed in rats with ANP. Infiltrating inflammatory cells in the pancreatic stroma and glandular lobule, as well as diffuse bleeding and necrosis were also observed under light microscope in these rats (Fig. 1E). Pathohistological scores of pancreatic tissues in the ANP group were significantly higher than in the other groups ($p<0.05$). Additionally, these scores were also higher in the AEP group compared with the AEP-control and normal rats (Table I).

Furthermore, ghrelin serum levels were significantly increased in the ANP group compared with those in the other groups ($p<0.05$). Additionally, ghrelin serum levels in the AEP group were higher than the normal group and AEP-control group ($p<0.05$). Finally, IL-1β and TNF-α serum levels were significantly higher in the ANP group compared with the other groups ($p<0.05$) (Table I).

Expression of calcium channels in the pancreas of AEP and ANP rats. In this study, Cav 1.2 and Cav 2.2 expression in the pancreas of AEP and ANP rats were examined using immunohistochemistry. The IRS of Cav 1.2 and Cav 2.2 were higher in the ANP group compared with the ANP-control group ($p<0.05$). IRS scores in the AEP rats were higher than those obtained for the AEP-control and normal rats (Fig. 2 and Table I).

Table I. Pathohistological scores of pancreatic tissues, serum levels of ghrelin, IL-1β and TNF-α, and the IRS of Cav 1.2 and Cav 2.2 in AEP and ANP rats.

Group	N	Pathohistological score	Ghrelin (pg/ml)	IL-1β (pg/ml)	TNF-α (pg/ml)	Cav 1.2 IRS	Cav 2.2 IRS
Normal	6	0.33±0.52	71.15±6.28	40.45±7.05	7.98±1.29	1.76±0.57	1.74±0.47
AEP-control	6	1.67±1.03	74.94±11.95	46.07±27.81	9.34±3.15	1.76±0.36	1.74±0.33
AEP	6	4.50±1.64[a,b]	98.96±9.06[a,b]	67.52±25.38	28.02±11.60	3.69±0.52[a,b]	2.89±0.51[a,b]
ANP-control	6	2.83±1.72	87.11±7.90	60.80±21.58	14.70±5.47	3.06±0.29	3.56±0.58
ANP	6	10.83±2.04[a-d]	291.37±57.35[a-d]	182.82±65.28[a-d]	54.59±16.60[a,b,d]	5.74±1.04[a-d]	5.74±1.04[a-d]

[a]vs. normal, p<0.05; [b]vs. AEP control, p<0.05; [c]vs. AEP, p<0.05; [d]vs. ANP control, p<0.05. IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IRS, immunoreactive score; AEP, acute edematous pancreatitis; ANP, acute necrotizing pancreatitis.

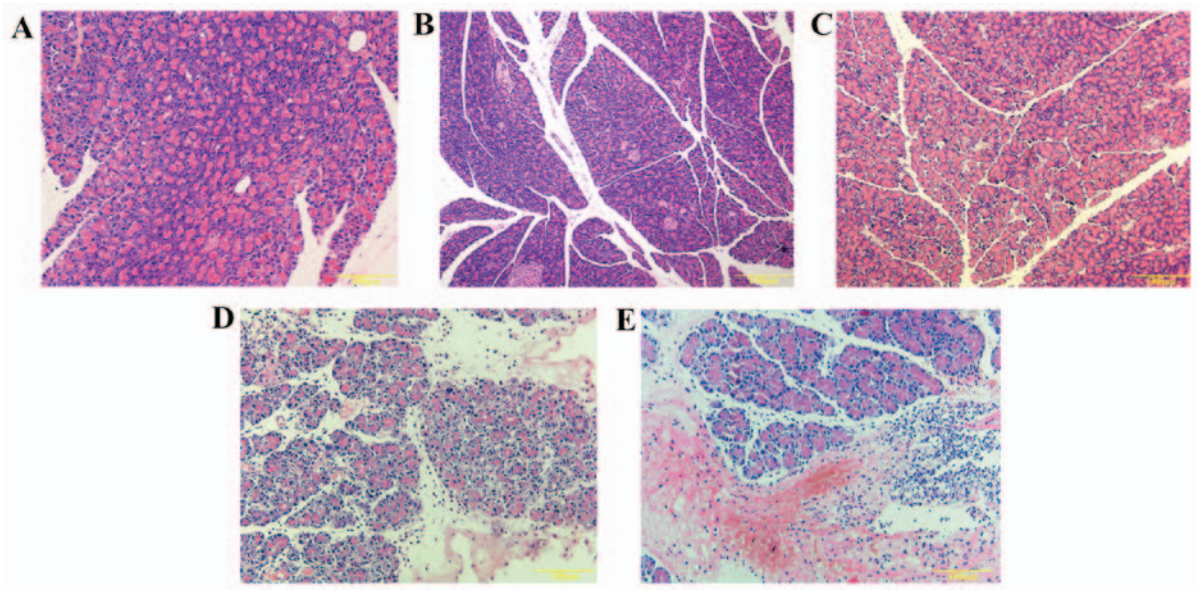

Figure 1. Histopathological analysis of pancreatic tissues in acute edematous pancreatitis (AEP) and acute necrotizing pancreatitis (ANP) rats. (A) Normal group. (B) AEP-control group. (C) ANP-control group. (D) AEP group: edema and inflammatory cells infiltrating the pancreatic stroma are visible, but diffuse bleeding and piecemeal necrosis are not present. (E) ANP group: infiltrating inflammatory cells in the pancreatic stroma and glandular lobule and diffuse bleeding and necrosis are detected.

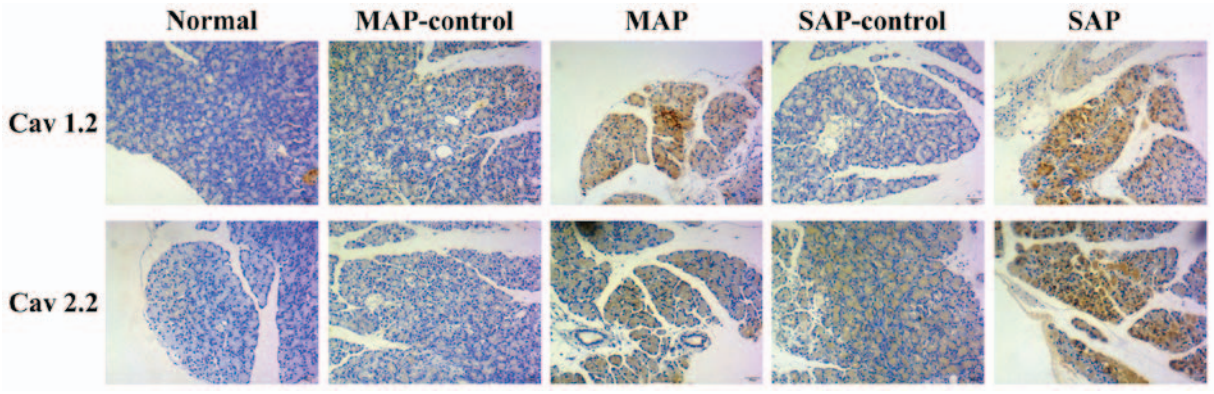

Figure 2. Expression of calcium channels in the pancreas of acute edematous pancreatitis (AEP) and acute necrotizing pancreatitis (ANP) rats. Expression of Cav 1.2 and Cav 2.2 was higher in the ANP rats compared with other groups. Additionally, the IRS scores of the AEP rats were higher than those obtained for the AEP-control and normal rats.

The expression of calcium channels in AR42J cells with endogenous ghrelin. In this study, stable ghrelin knockdown in AR42J cells resulted in low ghrelin protein expression, whereas cells transfected with an empty vector (ghrelin

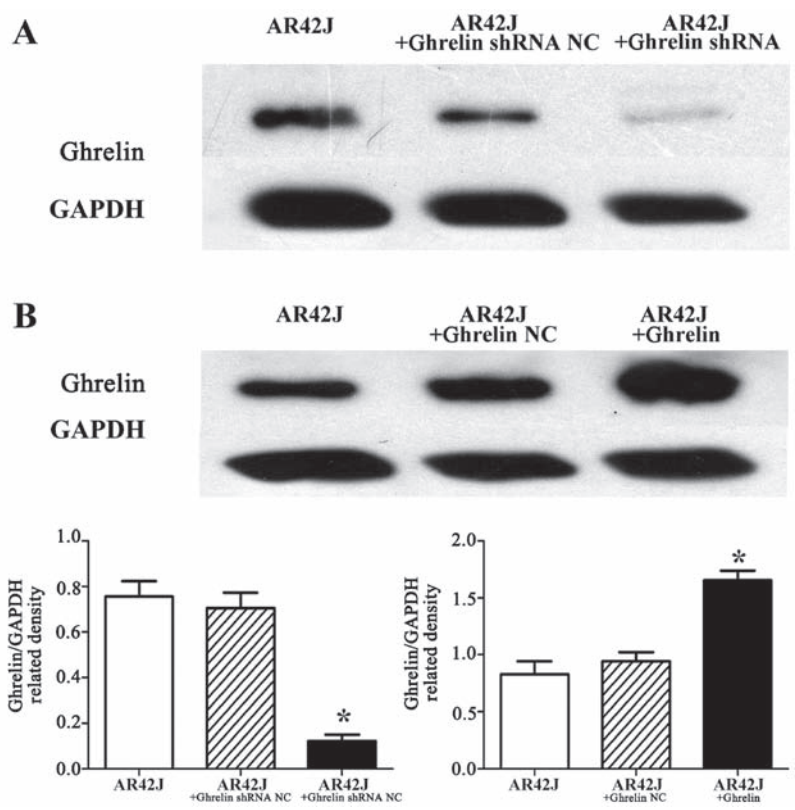

Figure 3. Lentivirus-mediated transfection of AR42J cells. (A) Expression of ghrelin in AR42J cells with ghrelin knockdown. (B) Expression of ghrelin in AR42J cells with ghrelin overexpression. AR42J cells transfected with an empty vector (NC) had ghrelin expression similar to the control untransfected AR42J cells, as determined with western blot analysis. AR42J transfected vs. untransfected AR42J, *p<0.05.

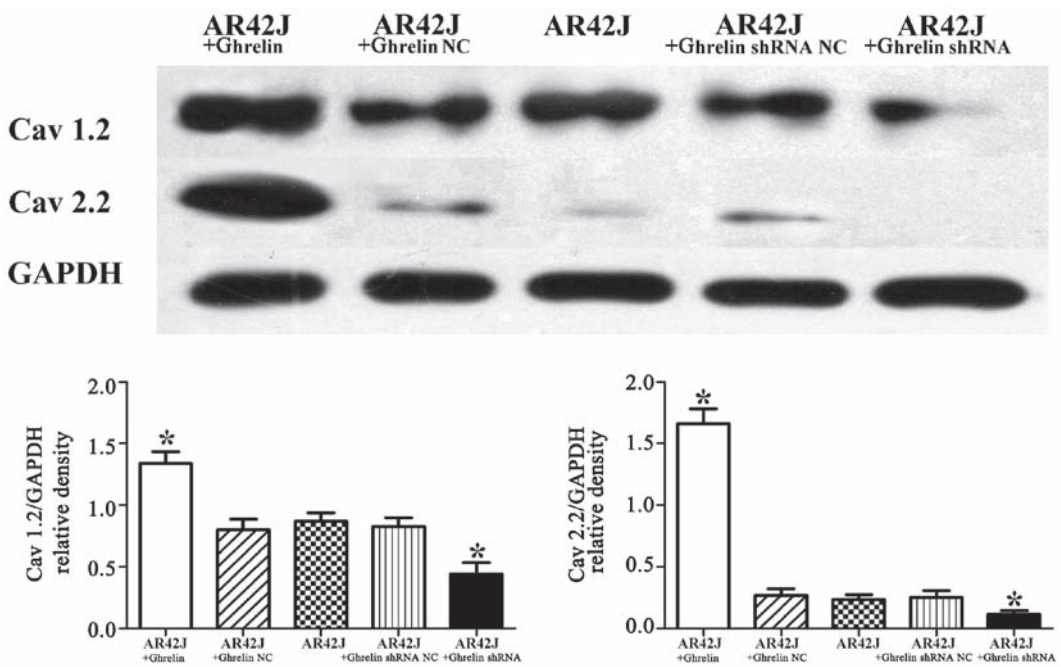

Figure 4. Endogenous ghrelin regulates the expression of calcium channels in AR42J cells. Control untransfected AR42J cells showed low Cav 1.2. and Cav 2.2 expression. Compared with control untransfected AR42J cells, Cav 1.2 and Cav 2.2 expression decreased in cells with ghrelin knockdown, whereas the expression of these two calcium channels increased in cells with ghrelin overexpression, as determined using western blot analysis. AR42J transfected vs. untransfected AR42J, *p<0.05.

shRNA NC) had similar ghrelin expression as that detected for control untransfected AR42J cells (p<0.05) (Fig. 3A). Additionally, the stable ghrelin overexpression in AR42J cells resulted in high ghrelin expression, whereas the cells transfected with an empty vector (ghrelin NC) had a low ghrelin expression that was similar to the control untransfected

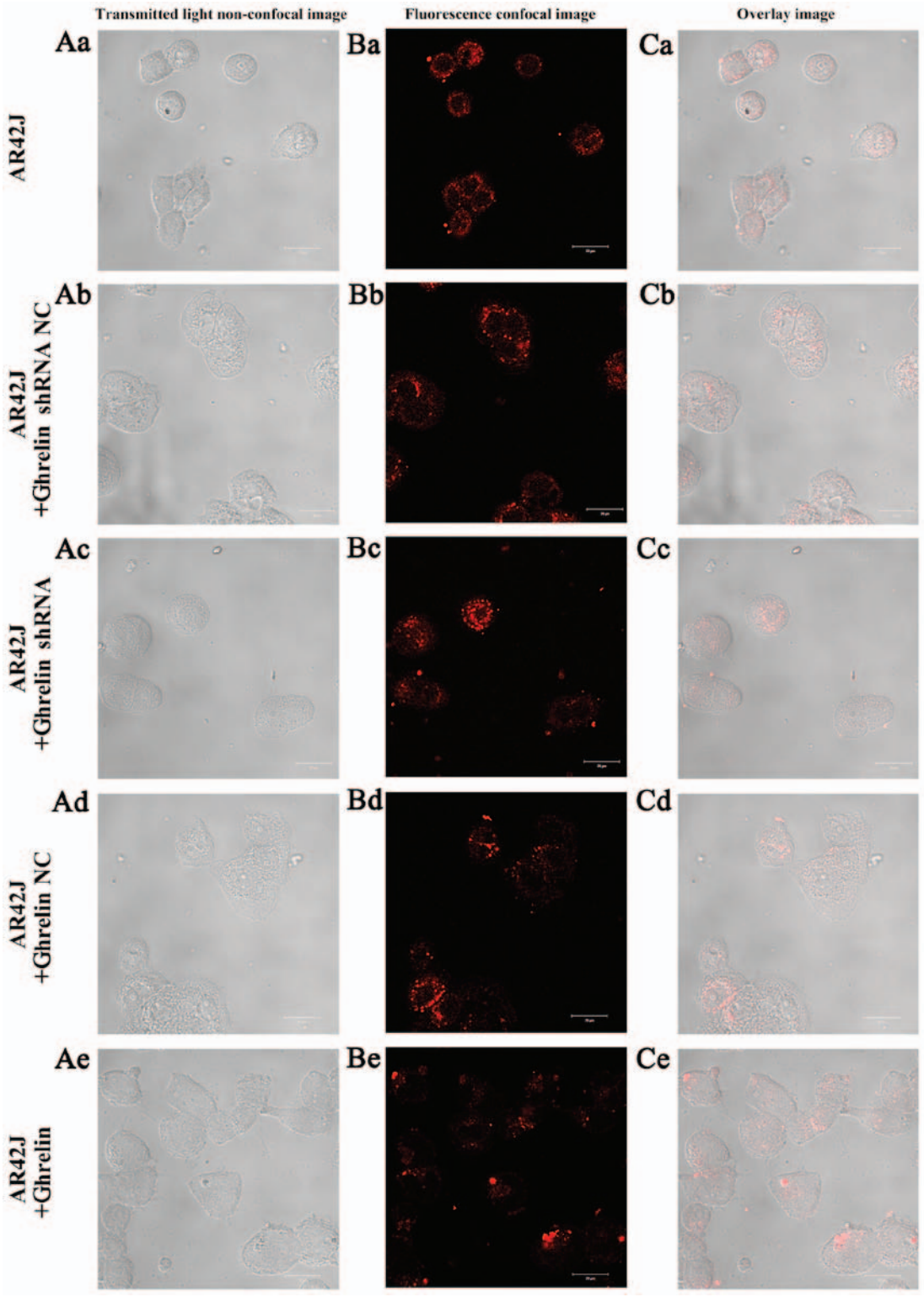

Figure 5. The $[Ca^{2+}]_i$ imaging in AR42J cells with ghrelin overexpression and knockdown. (Aa-e) A transmitted light non-confocal image of the acinar cell doublet. (Ba-e) Fluorescence confocal image of cells revealing red fluorescence accumulated in vacuoles. (Ca-e) Overlay of the transmitted light non-confocal image and fluorescence confocal image.

AR42J cells ($p<0.05$) (Fig. 3B). Furthermore, in this study, control untransfected AR42J cells had low Cav 2.2 expression. Compared with the control untransfected AR42J cells, Cav 1.2 and Cav 2.2 expression decreased in AR42J cells with ghrelin knockdown, whereas the expression of these calcium channels was increased in AR42J cells with ghrelin overexpression ($p<0.05$) (Fig. 4). Collectively, these results indicate that endogenous ghrelin changes the expression of calcium channels in AR42J cells.

The $[Ca^{2+}]_i$ imaging in AR42J cells with ghrelin overexpression and knockdown. The $[Ca^{2+}]_i$ imaging of AR42J cells showed that red fluorescence was similar between the untransfected AR42J cells and two groups of NC transfected cells (Fig. 5).

Red fluorescence was weakened in the ghrelin knockdown AR42J cells but increased in cells with ghrelin overexpression. These results collectively suggest that a difference in ghrelin expression could affect the $[Ca^{2+}]_i$ in AR42J cells.

Discussion

AP is a relatively common inflammatory disorder of the pancreas. Though most cases of AP are of the MAP type, which is a self-limiting disease, SAP accounts for substantial additional morbidity, with mortality rates as high as 10-20% (28).

Many molecular signaling pathways, such as intra-acinar trypsinogen activation, local inflammation, systemic inflammatory response, intra-acinar NF-κB activation, abnormal intracellular calcium $[Ca^{2+}]$i, mitochondrial dysfunction, autophagy, ER stress and oxidative stress, have been proposed to play a role in the etiology of pancreatic cellular injury in acute pancreatitis (29). Among these possible pathways, Ca^{2+} overload induced by abnormal $[Ca^{2+}]_i$ is receiving increasing attention as an important molecular change in the pathogenesis of acute pancreatitis (11). Ca^{2+} entry pathway was previously described to be provided by voltage-dependent Ca^{2+} channels, including L-, N-, T-, P- and R-type Ca^{2+} channels (30). Of these channels, L-type calcium channels may play a critical role in enhancing the selectivity and regulating specific targets via complexes with G protein-coupled receptors; N-type Ca^{2+} channels are thought to directly interact with proteins of the synaptic vesicle docking and fusion machinery (31). As proposed by Gerasimenko *et al* (32), a Ca^{2+} channel blocker has been proven useful in preventing the premature digestive enzyme activation, vacuolization, skeletal disruption and pancreatic acinar cell necrosis induced by Ca^{2+} overload (33).

The aim of this study was to examine the role of endogenous ghrelin in the expression of Cav 1.2 (L-type of Ca^{2+} channel) and Cav 2.2 (N-type of Ca^{2+} channel) in acute pancreatitis. For this purpose, we established AEP and ANP rat models, which were induced by caerulein and sodium taurocholate, respectively. In this study, the expression of Cav 1.2 was higher in ANP rats compared with other groups; however, the expression of Cav 2.2 showed no difference between the groups. These results indicate that Cav 1.2 has a potential role in the Ca^{2+} overload in acute pancreatitis. Additionally, in this study, ghrelin serum levels in ANP rats were higher than those in other groups, as were the IL-1β and TNF-α serum levels. Ghrelin serum levels in AEP rats were also higher than control and normal rats. Collectively, these results indicate that endogenous ghrelin is involved in acute pancreatitis development and may influence the severity of pancreatitis.

In clinical studies, the ghrelin serum level was not found to be a predictor of the severity of disease; however, its combination with the Gastroparesis Cardinal Symptom Index improved its predictive accuracy (34,35). Other studies reported that ghrelin could be implicated in the natural protection of the pancreatic tissue through the activation of the innate immune system to prevent the development of the inflammatory process in the pancreas. This protective pancreatic effect appears to be indirect and depends on the release of GH and insulin-like growth factor-1 by ghrelin (23,36,37). Our study used AR42J cells, which have many characteristics of normal pancreatic acinar cells and have been used as an *in vitro* model to study pancreatic acinar cellular secretion, proliferation, and apoptosis (38,39). Previous study showed that ghrelin increases $[Ca^{2+}]_i$ through activated L-type Ca^{2+} channel expression (22). This study performed the knockdown and overexpression of ghrelin in AR42J cells, which retained many characteristics of normal pancreatic acinar cells, such as the synthesis and secretion of digestive enzymes. The expression of Cav 1.2 and Cav 2.2 decreased following ghrelin knockdown; however, the expression of these two calcium channels increased in the ghrelin-overexpressing AR42J cells. Additionally, $[Ca^{2+}]_i$ showed the same trend as ghrelin expression in AR42J cells.

In conclusion, our results suggest that Cav 1.2 and Cav 2.2 expression are increased in ANP rats and that serum ghrelin levels may be involved in the severity of acute pancreatitis. Additionally, the $[Ca^{2+}]_i$ levels mediated by Cav 1.2 and Cav 2.2 expression are regulated by ghrelin expression in pancreatic acinar cells, at least in part. Nevertheless, the molecular implications of ghrelin-mediated $[Ca^{2+}]_i$ regulation in the acute pancreatitis remain to be elucidated.

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Availability of data and material

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

Authors' contributions

MQ and GT conceived and designed the study. HW, JH, HF, HS performed the animal experiments. JZ, MQ and ZL performed the cell experiments. JZ and MQ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal care and studies were conducted in accordance with the approval of the Medical Ethics Committee of the First Affiliated Hospital of Guangxi Medical University for Ethical Approval for Research Involving Animals (Nanning, China; permit no. KY-113)

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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