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

JIE ZHOU\(^1\), MENGBIN QIN\(^1\), HUILIN WANG, JIAPING HE, HONGZONG FU, HUIRONG SHI, ZHIHAI LIANG and GUODU TANG

Department of Gastroenterology, The First Affiliated Hospital, Guangxi Medical University, Nanning, Guangxi 530021, P.R. China

Received September 21, 2016; Accepted February 7, 2018

DOI: 10.3892/ijmm.2018.3490

Abstract. Ghrelin influences pancreatic endocrine and exocrine functions, regulates intracellular calcium \([\text{Ca}^{2+}]_i\) 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 \(\text{Ca}^{2+}\) channel) and Cav 2.2 (N-type of \(\text{Ca}^{2+}\) channel) in acute pancreatitis. For this purpose, acute edematous pancreatitis (ANP) 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 \([\text{Ca}^{2+}]_i\) 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 respective control groups. The serum IL-1β and TNF-α levels were significantly higher in the ANP group than in the respective control groups. Cav 1.2 and Cav 2.2 expression was assessed by confocal microscopy in the AEP groups. Cav 1.2 and Cav 2.2 expression and \([\text{Ca}^{2+}]_i\) levels were significantly higher in the ANP group compared to the other groups. Cav 1.2 and Cav 2.2 expression and \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) 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.

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 (\(\text{Ca}^{2+}\)) overload (6) and the activation of inflammatory pathways (7).

Under normal physiological conditions, \(\text{Ca}^{2+}\) signals are transient and localized in granules at the apical pole; however, the sustained elevation of cytosolic \(\text{Ca}^{2+}\) concentrations is fatal (8,9). \(\text{Ca}^{2+}\) cell entry is mediated by voltage-dependent \(\text{Ca}^{2+}\) channels (principally L-type \(\text{Ca}^{2+}\) channels) and is involved in a variety of \(\text{Ca}^{2+}\)-dependent processes, including muscle contraction, hormone or neurotransmitter release and gene expression (10). In recent years, \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) 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, \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) channels.
via $G_{i}$ or $G_{o}$ 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 [$Ca^{2+}$] (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-$\kappa$B 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 Thermo Fisher Scientific (Waltham, MA, USA). Blasticidin (cat. no. sc-16229-R) and TNF-$\alpha$ (cat. no. ERC102a.96) ELISA kits were purchased from Abcam PLC (Cambridge, MA, USA). Antibodies against Cav 1.2 and Cav 2.2 (N-type of $Ca^{2+}$ channel) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture and transfection. AR42J cells were grown in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. In brief, AR42J 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 EcoRI/Xhol digestion sites. The ghrelin shRNA (3′-AGAAAGGAATCCAGAGGCACC-5′, 5′-TGCCACACTGAGGGGAC-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.

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 H2SO4 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).
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

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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pathohistological score</th>
<th>Ghrelin (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>Cav 1.2 IRS</th>
<th>Cav 2.2 IRS</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>6</td>
<td>0.33±0.52</td>
<td>71.15±6.28</td>
<td>40.45±7.05</td>
<td>7.98±1.29</td>
<td>1.76±0.57</td>
</tr>
<tr>
<td>AEP-control</td>
<td>6</td>
<td>1.67±1.03</td>
<td>74.94±11.95</td>
<td>46.07±27.81</td>
<td>9.34±3.15</td>
<td>1.76±0.36</td>
</tr>
<tr>
<td>AEP</td>
<td>6</td>
<td>4.50±1.64&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>98.96±9.06&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>67.52±25.38</td>
<td>28.02±11.60</td>
<td>3.69±0.52&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ANP-control</td>
<td>6</td>
<td>2.83±1.72</td>
<td>87.11±7.90</td>
<td>60.80±21.58</td>
<td>14.70±5.47</td>
<td>3.06±0.29</td>
</tr>
<tr>
<td>ANP</td>
<td>6</td>
<td>10.83±2.04&lt;sup&gt;a-d&lt;/sup&gt;</td>
<td>291.37±57.35&lt;sup&gt;d&lt;/sup&gt;</td>
<td>182.82±65.28&lt;sup&gt;d&lt;/sup&gt;</td>
<td>54.59±16.60&lt;sup&gt;a,b,d&lt;/sup&gt;</td>
<td>5.74±1.04&lt;sup&gt;a-d&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> vs. normal, p<0.05; <sup>b</sup> vs. AEP control, p<0.05; <sup>c</sup> vs. AEP, p<0.05; <sup>d</sup> 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.
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 AR42J cells, as determined with western blot analysis. AR42J transfected vs. untransfected AR42J, *p<0.05.

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.
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\textsuperscript{2+}]\textsubscript{i} imaging in AR42J cells with ghrelin overexpression and knockdown. The [Ca\textsuperscript{2+}]\textsubscript{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 \([\text{Ca}^{2+}]\) 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-kB activation, abnormal intracellular calcium \([\text{Ca}^{2+}]\), 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, \(\text{Ca}^{2+}\) overload induced by abnormal \([\text{Ca}^{2+}]\), is receiving increasing attention as an important molecular change in the pathogenesis of acute pancreatitis (11). \(\text{Ca}^{2+}\) entry pathway was previously described to be provided by voltage-dependent \(\text{Ca}^{2+}\) channels, including L-, N-, T-, P- and R-type \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) channel blocker has been proven useful in preventing the premature digestive enzyme activation, vacuolization, skeletal disruption and pancreatic acinar cell necrosis induced by \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) channel) and Cav 2.2 (N-type of \(\text{Ca}^{2+}\) 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 these two calcium channels increased in the ghrelin-overexpressing AR42J cells. Additionally, \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), regulation in the acute pancreatitis remain to be elucidated.

**Acknowledgements**

Not applicable.

**Funding**

This study was supported by grants from the National Natural Science Foundation of China (nos. 81060043 and 81260087) and Self-Raised Topic of the Guangxi Zhuang Autonomous Region Health Department (no. Z2012105).

**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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