Cholecystokinin Acts as an Essential Factor in the Exacerbation of Pancreatic Bile Duct Ligation-Induced Rat Pancreatitis Model Under Non-fasting Condition

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ABSTRACT—We examined the influence of 2 gut hormones involved in the enhancement of pancreatic exocrine secretion, secretin and cholecystokinin (CCK), in the exacerbation of pancreatitis. We also examined the role of the vagal system, which was considered to be a transmission route for these hormones. Our model of pancreatitis in the rat was prepared by pancreatic bile duct ligation (PBDL), which simultaneously ligated the pancreatic duct and the common bile duct. Serum amylase activity and histopathological changes in the pancreas were used as indices of pancreatitis. We also measured the volume of pancreatic juice, as well as the amylase activity and protein level of the pancreatic juice, as indices of increased pancreatic exocrine secretion. Two gut hormones were given 6 times at 1-h intervals. Administration of secretin (1–3 μg/kg, s.c.) did not influence serum amylase activity in rats with PBDL-induced pancreatitis. However, food stimulation and administration of CCK-8 (1 μg/kg, s.c.) increased serum amylase activity and promoted vacuolation of the pancreatic acinar cells in rats with PBDL-induced pancreatitis. Administration of atropine (3 mg/kg, s.c.) or a CCK₁-receptor antagonist, Z-203 (0.1 mg/kg, i.v.), inhibited food-stimulated or CCK-8-induced (1 μg/kg, s.c.) enhancement of pancreatic exocrine secretion and exacerbation after the development of PBDL-induced pancreatitis. These results suggest that not secretin, which regulates the volume of pancreatic juice, but CCK, which regulates the secretion of pancreatic enzymes via the vagal system, plays an essential role in food-stimulated exacerbation after the development of pancreatitis.

Keywords: Pancreatic bile duct ligation (PBDL), Pancreatitis, Cholecystokinin

Acute pancreatitis causes autodigestion of the pancreas by pancreatic enzymes that escape to the periacinar space. It is known that food-stimulated enhancement of pancreatic exocrine secretion causes exacerbation of pancreatitis (1). Previous studies using a pancreatic bile duct ligation (PBDL) model (2–7), which is prepared by simultaneously ligating the pancreatic duct and bile duct, and a pancreatic duct ligation (PDL) model (8–10) have reported that food stimuli exacerbate pancreatitis. To explain the mechanism behind this finding, the pancreatic duct obstruction and secretion theory (11–13) has been proposed: simple obstruction of the pancreatic duct very slightly increases pancreatic duct pressure, but obstruction with enhancement of pancreatic exocrine secretion increases the pancreatic duct pressure further, and pancreatic enzymes activation, causing exacerbation after the development of pancreatitis.

Cholecystokinin (CCK) and secretin are gut hormones that enhance food-stimulation-related pancreatic exocrine secretion. It has been suggested that these hormones are involved in the exacerbation after the development of pancreatitis (5, 12). In recent years, it has been shown that pancreatic exocrine secretion related to the presence of these gut hormones is mediated by the vagal system (14–19). It has been speculated that vagal stimuli are involved in the exacerbation of PBDL-induced pancreatitis, but the mechanism remains unclear. We examined the influence of 2 gut hormones involved in pancreatic exocrine secretion, secretin and CCK, after the development of PBDL-induced pancreatitis. We also examined the role of the vagal system, which could be a transmission route for these hormones. The ultimate aim is to clarify the mechanisms involved in exacerbation after the development of pancreatitis.

MATERIALS AND METHODS

Animals

Male CD:SD (IGS) rats (age: 7–10 weeks of age; Japan
Charles River, Kanagawa) were used. The rats were acclimatized in the Laboratory Animal Institute of our laboratory under the following conditions: temperature, 23 ± 3°C; humidity, 55 ± 10%; ventilation, 80% return system; and lighting cycle, 7:00 – 19:00. Polycarbonate cages (270 × 422 × 185 mm; Natsume Seisakusho, Tokyo) and beta-chip floor mats (Northeastern Product Corp., Warrensburg, NY, USA) were used. Solid food (CRF-1, Japan Charles River) and tap water were given ad libitum. The experiments in this study were conducted in accordance with “The Guidelines of the Zeria pharmaceutical Animal Care and Use Committee”.

**Agents**

Z-203, sodium (5)-3-[1-(2-fluorophenyl)-2,3-dihydro-3-[3-isoquinolinyl]-carbonyl]-amino-6-methoxy-2-oxo-1H-indole] propanoate, (MW 521.48) (20 – 22), solution (1%, dissolved in physiological saline and synthesized at our company) was diluted with physiological saline before use. Furthermore, atropine (Sigma Chemicals, St. Louis, MO, USA), CCK-8 sulfate (CCK-8) and secretin (derived from pigs) (Peptide Institute, Osaka) were dissolved in physiological saline before use.

**Preparation of an advanced pancreatitis model with the enhancement of pancreatic exocrine secretion**

Influence of non-fasting conditions and gut hormones on pancreatic exocrine secretion: Non-fasted and fasted rats were anesthetized with urethane (Sigma Chemicals, 1.3 g·5 ml·1·kg⁻¹, i.p.). We performed a midline laparotomy and cannulated the pancreatic duct on the side of the duodenal orifice. The hepatic side of the common bile duct was then ligated. At 1-h intervals, CCK-8 (0.1 – 3 µg·2 ml·1·kg⁻¹) or secretin (0.3 – 3 µg·2 ml·1·kg⁻¹) was given subcutaneously to the fasted rats. At 1-h intervals, pancreatic juice was collected in a centrifuge tube (volume: 1.5 ml) (Treff Inc., Degersheim, Switzerland). The volume of pancreatic juice, as well as the level of protein and the amylase activity in the pancreatic juice was measured in the fasted and non-fasted rats. The protein levels were measured with BCA reactive reagent (Pierce, Rockford, IL, USA). Amylase activity was measured with an amylase B-test Wako kit (Wako Pure Chemical Industry, Osaka).

CCK-8 (1 µg·2 ml·1·kg⁻¹, s.c., 1-h intervals) was given to the fasted rats. At the same time, atropine (3 mg·2 ml·1·kg⁻¹, s.c., 1-h intervals) or the CCK₂-receptor antagonist Z-203 (0.1 mg·ml⁻¹·kg⁻¹, i.v.) was given in order to examine the effects of these drugs on pancreatic exocrine hypersecretion.

**Preparation of a non-fasted PBDL-induced pancreatitis model**: Two groups of rats: fasted group and non-fasted group were established. In the fasted group, animals were fasted for 16 h before the experiment began, while water was given ad libitum. Laparotomy was performed under ether anesthesia, and the common bile duct on the hepatic side and the pancreatic duct on the duodenal orifice side were completely ligated with 5-0 braided silk (Matsuda Medical Industry, Tokyo). The abdomen was then sutured. Six hours after ligation, blood was collected, and the pancreas was extirpated. The serum amylase activity was measured (amylase B test Wako). The extirpated pancreas was fixed in formalin. Sections were prepared and stained with hematoxylin and eosin (HE) for microscopy. The pancreatic head was examined under a light microscope. Any evidence of edema, autolysis, necrosis of acinar cells, hemorrhage and vacuolation of acinar cells was noted. Each parameter was evaluated by 3 grades: no change (−), moderate (+), and severe (+++).

**Preparation of a PBDL-induced pancreatitis model loaded with gut hormones**: The PBDL treatment described above in rats that had been fasted was performed. CCK-8 (0.1 – 3 µg·2 ml·1·kg⁻¹, s.c.) or secretin (0.3 – 3 µg·2 ml·1·kg⁻¹, s.c.) was administered 6 times at 1-h intervals. Six hours after the ligation, the serum amylase activity was measured. The extirpated pancreas was examined histopathologically, as described above.

**Influence of agents on non-fasted or CCK-8 loaded model of PBDL-induced pancreatitis**: Atropine (3 mg·2 ml·1·kg⁻¹, s.c., 6 times at 1-h intervals) or Z-203 (0.1 mg·ml⁻¹·kg⁻¹, i.v.) was administered immediately after PBDL treatment under non-fasting conditions. We also administered CCK-8 (1 µg·2 ml·1·kg⁻¹, s.c., 6 times at 1-h intervals) with atropine (0.3 – 3 µg·2 ml·1·kg⁻¹, s.c.) and CCK-8 (1 µg·2 ml·1·kg⁻¹, s.c., 6 times at 1-h intervals) with Z-203 (0.01 – 0.1 mg·ml⁻¹·kg⁻¹, i.v.).

**Statistical analyses**

All data were expressed as the means ± S.E.M. Super ANOVA v1.11 (Abacus Concepts Inc., Berkeley, CA, USA) software for statistical analysis was used. The significance of the differences was evaluated by Dunnett’s test for multiple group comparisons and Student’s t-test for comparison between two groups. *P<0.05* was regarded as significant.

**RESULTS**

**Influence of non-fasting conditions and gut hormones on pancreatic exocrine secretion**

Pancreatic exocrine secretion in non-fasted rats and fasted rats was compared. The volume of pancreatic juice, the pancreatic juice amylase activity and pancreatic juice protein levels were significantly higher in non-fasted rats than in fasted rats, suggesting enhancement of pancreatic exocrine secretion. In non-fasted rats, administration of atropine (3 mg·2 ml·1·kg⁻¹, s.c., 1-h intervals) significantly in-
Table 1. Effects of atropine and CCK-8-receptor antagonist administration on pancreatic exocrine secretion under non-fasting conditions

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Weight (mg/h)</th>
<th>P-Amylase (IU/h)</th>
<th>Protein (μg/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting + Vehicle</td>
<td>28 ± 3</td>
<td>129 ± 16</td>
<td>291 ± 35</td>
<td></td>
</tr>
<tr>
<td>Non-fasting + Vehicle</td>
<td>42 ± 5**</td>
<td>476 ± 174**</td>
<td>1021 ± 309**</td>
<td></td>
</tr>
<tr>
<td>Non-fasting + Atropine</td>
<td>3.0</td>
<td>22 ± 6*</td>
<td>112 ± 20**</td>
<td>349 ± 89**</td>
</tr>
<tr>
<td>Non-fasting + Z-203</td>
<td>0.1</td>
<td>34 ± 3</td>
<td>159 ± 30*</td>
<td>391 ± 43**</td>
</tr>
</tbody>
</table>

Results are expressed as the means ± S.E.M. of 10 to 12 rats in each group. *P<0.05; **P<0.01: vs Fasting-Vehicle group with Student’s t-test. *P<0.05, **P<0.01: vs Non-fasting-Vehicle group with Dunnett’s test. P-Amylase: pancreatic juice amylase activity.

Table 2. Effects of gut hormone-stimulated pancreatic exocrine secretion in fasted rat

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (μg/kg)</th>
<th>Weight (mg/h)</th>
<th>P-Amylase (IU/h)</th>
<th>Protein (μg/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>28 ± 3</td>
<td>101 ± 20</td>
<td>288 ± 41</td>
<td></td>
</tr>
<tr>
<td>Fasting + CCK-8</td>
<td>34 ± 3</td>
<td>103 ± 39</td>
<td>339 ± 81</td>
<td></td>
</tr>
<tr>
<td>+ CCK-8</td>
<td>57 ± 13*</td>
<td>468 ± 205*</td>
<td>1350 ± 576*</td>
<td></td>
</tr>
<tr>
<td>+ Secretin</td>
<td>47 ± 6</td>
<td>458 ± 124*</td>
<td>1241 ± 297*</td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>37 ± 10</td>
<td>46 ± 15</td>
<td>326 ± 108</td>
<td></td>
</tr>
<tr>
<td>Fasting + Secretin</td>
<td>73 ± 8</td>
<td>62 ± 11</td>
<td>545 ± 93</td>
<td></td>
</tr>
<tr>
<td>+ Atropine</td>
<td>89 ± 17**</td>
<td>87 ± 33</td>
<td>986 ± 458</td>
<td></td>
</tr>
<tr>
<td>+ Z-203</td>
<td>103 ± 16**</td>
<td>69 ± 4</td>
<td>459 ± 83</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as the means ± S.E.M. of 6 to 10 rats in each group. *P<0.05, **P<0.01: vs Fasting group with Dunnett’s test. P-Amylase: pancreatic juice amylase activity.

Table 3. Effects of atropine and CCK-8-receptor antagonist administration on CCK-8 stimulated pancreatic exocrine secretion in fasted rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Weight (mg/h)</th>
<th>P-Amylase (IU/h)</th>
<th>Protein (μg/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting + Vehicle</td>
<td>32 ± 4</td>
<td>141 ± 17</td>
<td>281 ± 30</td>
<td></td>
</tr>
<tr>
<td>Fasting + CCK-8</td>
<td>63 ± 9**</td>
<td>1117 ± 289**</td>
<td>2257 ± 718**</td>
<td></td>
</tr>
<tr>
<td>+ Atropine</td>
<td>3.0</td>
<td>504 ± 114*</td>
<td>1002 ± 201</td>
<td></td>
</tr>
<tr>
<td>+ Z-203</td>
<td>0.1</td>
<td>267 ± 43**</td>
<td>692 ± 186*</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as the means ± S.E.M. of 10 to 12 rats in each group. *P<0.01: vs Fasting-Vehicle group with Student’s t-test. *P<0.05, **P<0.01: vs CCK-8-Vehicle group with Dunnett’s test. P-Amylase: pancreatic juice amylase activity.

Exacerbation of pancreatitis in non-fasting PBDL-induced pancreatitis model

The serum amylase activity of the rats was measured 6 h after PBDL treatment under fasting and non-fasting conditions. In the PBDL group, the serum amylase activity was 10.5 times higher than that in the control group (Sham). In the PBDL group, we observed pathological changes in the pancreas, including edema and necrosis of the pancreatic acinar cells. In addition to these findings, vacuolation of the pancreatic acinar cells was noted under non-fasting conditions. Administration of atropine (3 mg·2 ml⁻¹·kg⁻¹, s.c., 6 times at 1-h intervals) or Z-203 (0.1 mg·ml⁻¹·kg⁻¹, i.v.) inhibited the necrosis and vacuolation of the pancreatic acinar cells. Atropine significantly inhibited increase in serum amylase activity. The Z-203 also significantly inhibited serum amylase activity (Table 4).
**Table 4.** Serum amylase activity and histopathology findings after atropine or CCK\(_\text{r}\)-antagonist administration on food-stimulated PBDL pancreatitis model

<table>
<thead>
<tr>
<th>Group</th>
<th>S-amylase (IU/ml)</th>
<th>Edema</th>
<th>Autolysis</th>
<th>Acinar necrosis</th>
<th>Hemorrhage</th>
<th>Vacuolization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Sham)</td>
<td>7.59 ± 0.24</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Fasting + Vehicle</td>
<td>38.85 ± 8.41</td>
<td>1/5</td>
<td>1/5</td>
<td>5/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Non-fasting + Vehicle</td>
<td>79.51 ± 7.52**</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>1/5</td>
<td>4/5 0/5 (3/1 + 1)</td>
</tr>
<tr>
<td>Non-fasting + Atropine</td>
<td>43.20 ± 3.65**</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Non-fasting + Z-203</td>
<td>24.26 ± 1.16**</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

After PBDL treatment under the non-fasting condition, atropine (3 mg/kg, 6 times, s.c.) or Z-203 (0.1 mg/kg, i.v.) was administered. Serum amylase activity (S-amylase) is expressed as the means ± S.E.M. of 5 rats in each group. **P<0.01: vs Fasting-Vehicle group with Student's t-test. **P<0.01: vs Non-fasting-Vehicle group with Dunnett's test. Histopathological findings were examined 6 h after ligation. Pancreatic head site was examined under a light microscope. Each parameter was evaluated using 3 grades: no change (−), moderate (+), severe (++) Figures show the number of histopathological findings per total number of animals examined.

**CCK-8 loading-related exacerbation of on PBDL-induced pancreatitis**

The serum amylase activity was measured 6 h after PBDL treatment under fasting conditions. The serum amylase activity in the fasted PBDL group was 3.5 times higher than that in the control group. CCK-8 (1 μg·2 ml\(^{-1}\)·kg\(^{-1}\), s.c.) loading was performed at 1-h intervals after PBDL treatment. CCK-8 loading further increased serum amylase activity to 11.5 times that of the control group (Fig. 1A). On the other hand, secretin (0.3 - 3 μg·2 ml\(^{-1}\)·kg\(^{-1}\), s.c.) loading did not increase serum amylase activity after PBDL treatment (Fig. 1B). In rats with PBDL-induced pancreatitis, atropine (3 mg·2 ml\(^{-1}\)·kg\(^{-1}\), s.c., 6 times at 1-h intervals) (Fig. 2A) or Z-203 (0.1 mg·ml\(^{-1}\)·kg\(^{-1}\), i.v.) significantly inhibited increases in serum amylase activity in a dose-dependent manner after CCK-8 loading (Fig. 2B). Histopathological examination of the pancreas after PBDL treatment under fasting conditions showed edema, autolysis and necrosis of the pancreatic acinar cells. CCK-8 loading induced vacuolation of the pancreatic acinar cells (Table 5).

**DISCUSSION**

We examined the influence of 2 gut hormones involved in pancreatic exocrine secretion (secretin and CCK) and the vagal system, which could be a transmission route for these hormones, in PBDL-induced pancreatitis. Serum amylase

![Graph A](image1.png)

**Fig. 1.** Effect of CCK-8 (A) or secretin (B) administration on serum amylase activity (S-amylase) in the PBDL group under fasting conditions. The data represent the means ± S.E.M. of 4 to 6 rats. **P<0.01: significantly different from the PBDL-Vehicle group.
Fig. 2. Effect of atropine (A) and CCK₁-receptor antagonist, Z-203 (B), treatment on serum amylase activity (S-amylase) in the CCK-8 stimulated PBDL group under fasting conditions. The data represent the means ± S.E.M. of 6 rats. **P<0.01: significantly different from the PBDL-Vehicle group (Student’s t-test); *P<0.05, **P<0.01: significantly different from the CCK-8 stimulated PBDL group.

Table 5. Serum amylase activity and histopathology findings after gut hormone-stimulation in the PBDL pancreatitis model

<table>
<thead>
<tr>
<th>Group</th>
<th>S-amylase (IU/ml)</th>
<th>Edema</th>
<th>Autolysis</th>
<th>Acinar necrosis</th>
<th>Hemorrhage</th>
<th>Vacuolization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>49.16 ± 14.23</td>
<td>4/4</td>
<td>3/4</td>
<td>4/4 (1/4 *)</td>
<td>2/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Fasting + CCK-8</td>
<td>79.66 ± 4.23</td>
<td>4/4</td>
<td>1/5</td>
<td>3/4 (1/4 *)</td>
<td>1/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Fasting + Secrein</td>
<td>45.32 ± 4.56</td>
<td>4/5</td>
<td>2/5</td>
<td>5/5 (2/5 +)</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

After PBDL treatment under the fasting condition, CCK-8 (1 µg/kg, 6 times, s.c.) or secretin (1 µg/kg, 6 times, s.c.) was administered. Serum amylase activity (S-amylase) is expressed as the means ± S.E.M. of 4 or 5 rats in each group. Histopathological findings were examined 6 h after ligation. Pancreatic head site was examined under a light microscope. Each parameter was evaluated using 3 grades: no change (−), moderate (+), severe (++) figures show the number of histopathological findings per total number of animals examined.

activity and histopathological changes in the pancreas were used as indices.

Among the rats with PBDL-induced pancreatitis, serum amylase activity differed between the non-fasted group and the fasted group. Vacuolation of the pancreatic acinar cells was a characteristic pathological feature in the non-fasted group. Administration of either atropine or a CCK₁-receptor antagonist, Z-203 (20–22), which inhibits the enhancement of pancreatic exocrine secretion under non-fasting conditions, inhibited both PBDL-related increases in serum amylase activity and vacuolation of the pancreatic acinar cells under non-fasting conditions. These differences may therefore reflect an exacerbation after the development of pancreatitis related to enhancement of pancreatic exocrine secretion.

The relationship between the enhancement of pancreatic exocrine secretion and vacuolation of the pancreatic acinar cells is unclear. However, in a pancreatitis model prepared by administration of a supramaximal dose of cerulein and in an ethionine-induced pancreatitis model, it has been reported that vacuolation is caused by impaired segregation of zymogen granules and lysosomal hydrolases (23–25). These findings suggested that the similar impairment of segregation may be developed in this PBDL-induced model.

It is speculated that CCK, which increases the secretion
of pancreatic enzymes, and secretin, which increases the volume of pancreatic juice secretion, are physiologically involved in the enhancement of pancreatic exocrine secretion induced by food stimuli. However, we compared the actions of these hormones with respect to their influence on pancreatic exocrine secretion and PBDL-induced pancreatitis. These results suggested that they had different effects. When CCK-8 was given at a dose (1–3 μg·2 ml⁻¹·kg⁻¹, s.c., 6 times at 1-h intervals) that does not influence serum amylase activity in normal rats to rats with PBDL-induced pancreatitis under fasting conditions (data were not shown), it increased the serum amylase activity and induced vacuolation of the pancreatic acinar cells after PBDL-induced pancreatitis. Moreover, it has been reported that fasting lessens the severity of supramaximal cerulein-induced acute pancreatitis by reducing endogenous CCK release (26). Endogenous CCK may contribute to worsening for pancreatitis observed in the fed ad libitum rats. However, secretin did not have these effects, suggesting that CCK-related secretion of pancreatic enzymes is exacerbated after the development of pancreatitis. It has been reported that secretin inhibits CCK binding to acinar cells and exerts a preventive effect on supramaximal cerulein-induced acute pancreatitis in dogs and rats (27, 28). Therefore, secretin is thought to be not harmful in PBDL-induced pancreatitis.

In previous studies, because administration of food or CCK-8 loading at physiological concentration exacerbated acute pancreatitis, CCK was suggested to play an important permissive or contributory role in the development of acute pancreatitis (29, 30). The exacerbation of PBDL-induced pancreatitis under non-fasting conditions resembled CCK-8 stimulation-related exacerbation of PBDL-induced pancreatitis under fasting conditions. Exacerbation of the pancreatitis by the addition of food or CCK stimulation in these models was inhibited by the administration of atropine, which inhibits the secretion of pancreatic enzymes, and by a CCK-receptor antagonist.

In conclusion, we suggest that exacerbation of pancreatitis under non-fasting conditions is associated with CCK-related secretion of pancreatic enzymes via the vagal system. Therefore, inhibition of CCK stimulation may be useful for controlling food-stimulus-related exacerbation after the development of pancreatitis.

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