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EFFECT OF SENSORY NERVES AND CGRP ON THE
DEVELOPMENT OF CAERULEIN-INDUCED PANCREATITIS AND
PANCREATIC RECOVERY

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The function of primary sensory neurons is to receive and transmit information from
external environment and these neurons are able to release neuromediators from the
activated peripheral endings. The aim of this study was to determine the influence of
sensory nerves and administration of their mediator — calcitonin gene related peptide
(CGRP) on the course of acute pancreatitis (AP). Ablation of sensory nerves was
performed by neurotoxic dose of capsaicin (100 mg/kg). Single or repeated episodes
of AP were induced by caerulein infusion (10 μg/kg/h for 5 h). Five repeated AP were
performed once a week. Capsaicin at the dose which stimulates sensory nerves (0.5
mg/kg/dose) or CGRP (10 μg/kg/dose) was administrated before and during or after
single induction of AP, as well as, after each induction of repeated AP. Rats were
killed at the time 0, 3 or 9 h after single induction of AP or two weeks after last
induction of repeated AP. Ablation of sensory nerves aggravated pancreatic damage in
cærulein-induced AP. Treatment with stimulatory doses of capsaicin or CGRP before
and during single induction of AP attenuated the pancreatic damage in morphological
examination. This effect was also manifested by partial reversion of AP evoked drop
in DNA synthesis and pancreatic blood flow (PBF). Administration of CGRP after
single AP induction aggravated histologically manifested pancreatic damage. The
further decrease in PBF and DNA synthesis was also observed. Animals with five
episodes of AP showed almost full pancreatic recovery two weeks after last
induction of AP concerning all parameters tested. In stimulatory doses of capsaicin treated rats,
we observed the decrease in pancreatic amylase and fecal chymotrypsin activity, as
well as, the drop in DNA synthesis. Similar but less pronounced effects were observed
after treatment with CGRP. We conclude that effect of sensory nerves and CGRP on
AP is two-phase and time dependent. Stimulation of sensory nerves or the
administration of CGRP during development of AP exhibits protective effects against
pancreatic damage induced by caerulein overstimulation. After induction of AP,
persistent activity of sensory nerves and presence of CGRP aggravate pancreatic
damage and lead to functional insufficiency typical for chronic pancreatitis.

Key words: capsaicin, acute pancreatitis, CGRP, pancreatic regeneration, pancreatic
functional insufficiency

INTRODUCTION

Primary sensory neurons serve for conduction of nociceptive information to
the central nervous system, but also activation of these neurons causes the
release of neuromediators from peripheral endings (1). Sensory fibers have a special sensitivity to capsaicin (2). Low doses of capsaicin result in the stimulation of sensory nerves accompanied with the release of CGRP and other neuromediators (3, 4), whereas high doses of capsaicin lead to ablation of sensory nerves with the decrease in plasma and tissue level of CGRP (5, 6). CGRP is widely distributed throughout the central, peripheral and enteric nervous systems (7, 8). Within the enteric nervous system, CGRP-containing nerves have been found in large numbers among others in the stomach (9, 10), the intestine (8, 10) and the pancreas (9—12). CGRP is identified as a major mediator of thin, unmyelinated capsaicin-sensitive sensory neurons (3, 13). Capsaicin-sensitive primary afferent neurons and CGRP are involved in different aspects of the stomach pathology. The stimulation of sensory fibers, as well as, administration of exogenous CGRP was found to exert a protective effect in different experimental models of gastric ulcers (14—16), whereas the ablation of sensory nerves aggravates gastric mucosal lesions induced by various ulcerogenic factors (17, 18), inhibits the gastric mucosal growth (19) and prolongs gastric ulcer healing (20).

Beneficial effect of afferent nerve stimulation and CGRP administration before ulcer induction has been attributed, at least in part, to improvement of mucosal circulation (21, 22). In the pancreas, vascular mechanism was shown to play an important role in exocrine secretion (23) and organ integrity. The development of acute pancreatitis is associated with the decrease in pancreatic blood flow. Clinical and experimental studies have shown that pancreatic ischemia may initiate acute pancreatitis and always aggravates pancreatic damage (24—26). The severity of such experimental pancreatitis is closely correlated with tissue ischemia (27). Moderate and severe pancreatitis was found to be accompanied with progressive decrease in pancreatic blood perfusion (27).

On the other hand, there is a growing number of evidence suggesting that CGRP released from unmyelinated, afferent capsaicin-sensitive sensory nerves may contribute to the chronic inflammatory response (28). Alterations of intrapancreatic nerves including edema of nerve bundles and increased density of peptidergic nerves with an intensification of immunostaining for CGRP have been seen in surgical specimens from patients and animals with chronic pancreatitis (29—31). Moreover, activation of these nerves may produce the neurogenic inflammation described as the local vasodilatation and plasma extravasation (32).

The aim of this study was to determine: (a) the effect of sensory nerves and pretreatment with CGRP on development of acute caerulein-induced pancreatitis, (b) the influence of CGRP administration after induction of acute pancreatitis on development of pancreatic damage, (c) the effect of prolonged stimulation of sensory nerves with low doses of capsaicin or CGRP administration on pancreatic repair after repeated episodes of acute pancreatitis.
MATERIALS AND METHODS

Animals and treatment

Studies were performed on male Wistar rats weighing 160—190 g (single induction of pancreatitis) or 120—140 g (repeated induction of pancreatitis) at the start of experiments. The animals were housed in cages with wire mesh bottoms, with normal room temperature and a 12-hour light-dark cycle. Drinking of water and food were available ad libitum. The study was conducted following the experimental protocol approved by the Committee for Research and Animal Ethics of the Jagiellonian University.

Experiments were carried out in three separate series.

First series of experiments were performed to determine the effect of sensory nerves and pretreatment with CGRP on the development of single episode of acute pancreatitis. The following groups of animals were used in this series of the experiment: (1) rats infused with saline (0.9% NaCl) s.c. to serve as the control group; (2) rats infused with caerulein (caerulein-induced pancreatitis); (3) rats with stimulation of sensory nerves (capsaicin 2 × 0.5 mg/kg s.c., first injection 30 min before the start of saline infusion, second 3 h later) and infused with saline; (4) rats with ablation of sensory nerves (capsaicin 100 mg/kg s.c.) and infused with saline; (5) rats treated with CGRP (2 × 10 μg/kg s.c., first injection 30 min before the start of saline infusion, second 3 h later) and infused with saline; (6) rats with ablation of sensory nerves, treated with CGRP (2 × 10 μg/kg s.c., first injection 30 min before the start of saline infusion, second 3 h later) and infused with saline; (7) rats with stimulation of sensory nerves (capsaicin 2 × 0.5 mg/kg s.c., first injection 30 min before the start of caerulein infusion, second 3 h later) and infused with caerulein; (8) rats with ablation of sensory nerves and infused with caerulein; (9) rats treated with CGRP (2 × 10 μg/kg s.c., first injection 30 min before the start of caerulein infusion, second 3 h later) and infused with caerulein; (10) rats with ablation of sensory nerves treated with CGRP (2 × 10 μg/kg s.c., first injection 30 min before the start of caerulein infusion, second 3 h later) and infused with caerulein.

Ablation of afferent sensory nerves was induced by pretreatment with capsaicin (Fluka, Buchs, Switzerland) in a total dose of 100 mg/kg, which was given in six injections (2.5 + 10 + 12.5 + 25 + 25 + 25 mg/kg s.c.) over 3 consecutive days. Two injections per day were performed in rats under ether anesthesia and a recovery period of 10 days was allowed before further experiments. To assess the effectiveness of sensory denervation, the day before the induction of pancreatitis, a drop of capsaicin (0.33 mM) was instilled into rat eye, and animals showing any wiping movements were excluded from the study.
Acute edematous pancreatitis was induced by caerulein infusion in rats kept in individual cages. Caerulein (Takus, Pharmacia & Upjohn GmbH, Erlangen, Germany) was diluted in saline and infused s.c. for 5 h at a dose 10 µg/kg/h and at a rate of 1.0 ml/h.

Rat, synthetic CGRP-I was obtained from Sigma Chemical Co, St. Louis, MO, USA. Animals from the first series of experiments were sacrificed after 5 h of infusion with saline or caerulein.

Second series of experiments were performed to evaluate the influence of CGRP administration after single induction of acute pancreatitis on development of pancreatic damage. The following groups of animals were used in this series of experiments: (1) control (intact rats infused with saline only); (2) caerulein induced pancreatitis; (3) caerulein induced pancreatitis + CGRP (20 µg/kg of CGRP given s.c. in two doses: first 10 µg/kg 30 min prior to caerulein infusion and second 10 µg/kg 3 h later, during caerulein infusion); (4) caerulein induced pancreatitis + CGRP given at the time 1 h, 4 h, and 7 h after the end of caerulein infusion (10 µg of CGRP per dose).

Acute edematous pancreatitis was induced by caerulein as in the first series of experiments. Rats were sacrificed at the time 0 h, 3 h or 9 h after cessation of caerulein infusion.

Third series of experiments was performed to determine the effect of prolonged activity of sensory nerves or CGRP administration on the pancreatic repair after repeated episodes of acute pancreatitis. The following groups of animals were used in this series of experiment: (1) rats infused with saline s.c. to serve as the control group; (2) rats with repeated induction of acute pancreatitis and treated with saline s.c. after each induction of acute pancreatitis; (3) rats with repeated induction of acute pancreatitis and stimulation of sensory nerves by capsaicin after each induction of pancreatitis; (4) rats with repeated induction of acute pancreatitis and treated with CGRP after each induction of pancreatitis.

Five episodes of acute pancreatitis were performed by caerulein infusion at weekly intervals. After each induction of acute pancreatitis, saline, capsaicin or CGRP were administrated s.c. three times daily starting 1 h after termination of caerulein infusion. Capsaicin was used at the sensory nerve stimulatory dose: 0.5 mg/kg/dose. CGRP was administrated at the dose 10 µg/kg/injection. Two weeks after last induction of acute pancreatitis rats were sacrificed.

**Determination of pancreatic blood flow**

At the end of each series of experiments, animals were anesthetized with ether, weighed and the abdominal cavity was opened. The pancreas was exposed for measurement of blood flow in the pancreatic tissue by laser Doppler
flowmeter using PeriFlux 4001 Master monitor (Perimed AB, Jrflla, Sweden). Blood flow was measured in five different portions of the pancreas. The pancreatic blood flow was presented as percent change from control value obtained in rats infused with saline.

Determination of plasma amylase activity and plasma interleukin 1-β concentration

Immediately after measurement of pancreatic blood flow the abdominal aorta was exposed and blood was taken for plasma amylase and interleukin-1β (IL-1β) determination. Plasma amylase activity was determined by an enzymatic method [Amylase reagent set (kinetic), Alpha Diagnostic sp. z o.o., Warsaw, Poland]. The values were expressed as units/liter (U/L) or units/pancreas. Plasma IL-1β was measured in duplicate using the BioSource Cytoscreen rat IL-1β kit based on a solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) (BioSource International, Camarillo, California, USA). Concentration of plasma IL-1β was expressed as pg/ml.

Determination of DNA synthesis and amylase activity in the pancreas

After the blood withdrawal, the pancreas was carefully dissected out from its attachment to the stomach, the duodenum, and the spleen. Fat and excess tissue were trimmed away. Samples of pancreatic tissue from each series of experiment were taken for study of pancreatic DNA synthesis and morphological examination. Additionally, samples of pancreatic tissue from the third series of experiment were taken for study of pancreatic amylase activity. The rate of DNA synthesis in the portion of minced pancreatic tissue was determined by incubating the tissue at 37°C for 45 min in 2 ml of medium containing 8 μCi/ml of [3H]thymidine ([6-3H]-thymidine, 20—30 Ci/mmol; Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic). The reaction was stopped with 0.4 N perchloric acid containing carrier thymidine (5 mM). Tissue samples were centrifuged and the precipitate washed twice in cold 0.2 N perchloric acid and recentrifuged. RNA was hydrolyzed in 0.3 M KOH incubated for 90 min at 37°C. DNA and protein were reprecipitated with 10% perchloric acid. After standing for 10 min on ice, the tubes were centrifuged and the supernatant was discarded. DNA in the residual pellets was solubilized in 10% perchloric acid by heating at 70°C for 20 min. Denaturated protein was removed by centrifugation for 20 min. Using calf thymus as a standard, the DNA concentration was determined by Giles and Myers procedure (33). The incorporation of [3H]thymidine into DNA was determined by counting 0.5 ml DNA-containing supernatant in a liquid scintillation system. DNA synthesis was
expressed as \([^{3}H]\)thymidine disintegrations per minute per microgram DNA (dpm/\(\mu\)g DNA).

The pancreatic amylase activity was determined in the portion of pancreatic tissue which was weighed and placed in 2 ml pH 7.4 sodium — potassium phosphate buffer containing 0.2 mg of trypsin inhibitor (Type I-S, Sigma, St. Louis, USA). Pancreatic tissues were homogenized, sonicated and centrifuged ad 30,000 g for 10 min. Amylase activity in aliquot from the supernatant was determined by the same enzymatic method as in plasma samples. The pancreatic amylase activity was expressed as units per pancreas.

*Determinaton of fecal chymotrypsin*

Fecal chymotrypsin activity in the third series of experiment was measured by colorimetric method with a test kit Chymo (Boehringer, Mannheim, Germany). Values were expressed as U/g of stool.

*Histological examination*

Samples of pancreatic tissue were excised, fixed in 10% formalin, embedded in paraffin and sections were stained with hematoxylin and eosin. The slides were examined histologically by two experienced pathologists without the knowledge of treatment given. The histological grading of edema was made using a scale ranging from 0 to 3; 0 = no edema, 1 = interlobular edema, 2 = interlobular and moderate intralobular edema, and 3 = severe interlobular and intralobular edema. Leukocyte infiltration was graded from 0 (absent) to 3 for maximal alterations (diffuse infiltration in the entire pancreatic gland) Grading of vacuolization was based on the percentage of cells involved: 0 = absent, 1 = less than 25%, 2 = 25—50% and 3 = more than 50%.

Additionally, samples from the third series of experiments were examined for histological signs of chronic pancreatitis such as acinar cell necrosis, atrophy, fibrosis and tubular complexes. Findings of acinar necrosis, atrophy and fibrosis were evaluated as lesion size score: 0 = absent, 1 = a lesion slightly shown in the lobule or intralobular region (less than one-half), 2 = a lesion widely shown in the intralobular region (more than one-half), and 3 = a lesion shown across lobules and intralobular regions or with destruction of lobular architecture. Tubular complexes were expressed as: 0 = lack or 1 = presence of these lesions.

*Statistical analysis*

Results are expressed as means ± S.E.M. and were analyzed by analysis of variance and t-Student test for unpaired values, with p < 0.05 considered significant.
RESULTS

First series of experiments

Subcutaneous administration of caerulein at a dose 10 μg/kg/h for 5 h induced the acute edematous pancreatitis in all tested rats. Pancreata appeared grossly swollen and enlarged with visible collection of edematous fluid. Pancreatic DNA synthesis was deeply decreased to 52% of control value (Fig. 1), the pancreatic blood flow was reduced by about 50% (Fig. 2). The plasma amylase activity (Fig. 3) and the plasma IL-1β level (Fig. 4) were increased eight and twofold, respectively. Histological changes were closely correlated with the biochemical findings (Table 1). There was moderate interlobular and intralobular edema, and in about half of rats severe intralobular edema. The edema was accompanied by perivascular infiltration by neutrophils and the presence of vacuolization in about half of the acinar cells.

Fig. 1. Effect of saline (control), stimulatory (0.5 mg/kg) and neurotoxic (100 mg/kg) doses of capsaicin and CGRP given alone or in their combination with single saline or caerulein infusion on pancreatic DNA synthesis. Mean ± S.E.M. of 8—14 observations. *P < 0.05 compared with control, †P < 0.05 compared with caerulein given alone, ‡P < 0.05 compared with the neurotoxic dose of capsaicin given in combination with caerulein.
Stimulatory doses of capsaicin (0.5 mg/kg) given alone were without any significant effect on the pancreatic DNA synthesis (Fig. 1), plasma amylase activity (Fig. 3), plasma IL-1 (Fig. 4) or pancreatic histology. Pancreatic blood flow was increased by 29% (Fig. 2).

Ablation of sensory nerves by high dose of capsaicin (100 mg/kg) caused a significant decrease in pancreatic DNA synthesis and pancreatic blood flow by 18 and 30%, respectively (Fig. 1 and 2). Plasma amylase activity showed a small but significant increase (Fig. 3). Plasma IL-1β concentration was increased by 58% (Fig. 4). Histological examination has shown that ablation of sensory nerves produced slight leukocyte infiltration and lack or slight interlobular edema without vacuolization of acinar cells (Table 1).

CGRP given alone caused a significant increase in pancreatic blood flow by 20% over the control value (Fig. 2), whereas other parameters were not significantly affected. Treatment with CGRP in combination with ablatory doses of capsaicin (100 mg/kg) partly reversed the capsaicin-induced reduction

![Graph](image_url)

**Fig. 2.** Effect of saline (control), stimulatory (0.5 mg/kg) and neurotoxic (100 mg/kg) doses of capsaicin and CGRP given alone or in their combination with single caerulein infusion on pancreatic blood flow. Mean ± S.E.M. of 8—14 observations. ∗P < 0.05 compared with control, ∗∗P < 0.05 compared with caerulein given alone, ∗∗P < 0.05 compared with the neurotoxic dose of capsaicin given in combination with caerulein, ∗∗∗P < 0.05 compared with the neurotoxic dose of capsaicin given alone.
in pancreatic DNA synthesis (Fig. 1) and pancreatic blood flow (Fig. 2). In this group of animals, plasma amylase activity was above the control value or value observed after CGRP or neurotoxic dose of capsaicin given separately (Fig 3). CGRP partly reduced the plasma IL-1β concentration in animals with ablation of sensory nerves (Fig. 4). Morphological features (Table 1) have shown that CGRP prevented against perivascular leukocyte infiltration induced by neurotoxic dose of capsaicin.

Administration of the stimulatory dose of capsaicin with caerulein infusion reduced the severity of caerulein-induced pancreatitis. Pancreatic DNA synthesis (Fig. 1) and pancreatic blood flow (Fig. 2) were higher, whereas an increase in plasma amylase activity (Fig. 3) was smaller compared to caerulein infusion alone. Also histological examination showed a reduction in pancreatic damage (Table 1). Edema was limited to interlobular space in most cases, only in a few cases moderate intralobular edema was observed. Leukocyte infiltration and acinar cells vacuolization were decreased. Moreover, an

![Graph]

Fig. 3. Effect of saline (control), stimulatory (0.5 mg/kg) and neurotoxic (100 mg/kg) doses of capsaicin and CGRP given alone or in their combination with single caerulein infusion on plasma amylase activity. Mean ± S.E.M. of 8—14 observations. aP < 0.05 compared with control, bP < 0.05 compared with caerulein given alone, cP < 0.05 compared with the neurotoxic dose of capsaicin given in combination with caerulein, dP < 0.05 compared with the neurotoxic dose of capsaicin given alone.
increase in plasma IL-1β concentration (Fig. 4) caused by caerulein was partially reversed by stimulatory doses of capsaicin, but this effect was statistically insignificant.

Capsaicin-induced deactivation of sensory nerves (100 mg/kg) prior to caerulein infusion aggravated pancreatic damage created by caerulein, which was manifested by an additional decrease in pancreatic DNA synthesis (Fig. 1) and a increase in plasma IL-1β concentration (Fig. 4). Alterations of pancreatic blood flow and plasma amylase activity were not statistically significant (Fig. 2 and 3). Histological examination revealed severe interlobular and intralobular edema in all animals. Leukocytic infiltration and vacuolization of acinar cells were more pronounced than after caerulein given alone.

Table 1. Effect of saline (control), stimulatory (0.5 mg/kg) and neurotoxic (100 mg/kg) doses of capsaicin and CGRP given alone or in their combination with single caerulein infusion on histological signs of pancreatic damage.

<table>
<thead>
<tr>
<th></th>
<th>EDEMA (0—3)</th>
<th>INFLTRATION (0—3)</th>
<th>VACUOLIZATION (0—3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (control)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Caerulein</td>
<td>2/3</td>
<td>1/2</td>
<td>2</td>
</tr>
<tr>
<td>Capsaicin 0.5 mg/kg</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Capsaicin 100 mg/kg</td>
<td>0/1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CGRP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Capsaicin 100 mg/kg + CGRP</td>
<td>0/1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Capsaicin 0.5 mg/kg + Caerulein</td>
<td>1/2</td>
<td>0/1</td>
<td>1</td>
</tr>
<tr>
<td>Capsaicin 100 mg/kg + Caerulein</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>CGRP + Caerulein</td>
<td>1/2</td>
<td>0/1</td>
<td>1/2</td>
</tr>
<tr>
<td>Capsaicin 100 mg/kg + CGRP + Caerulein</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Numbers represent the predominant histological grading in each group.

Treatment with CGRP during caerulein infusion attenuated the severity of acute pancreatitis. Caerulein-induced a reduction in pancreatic DNA synthesis (Fig. 1) and pancreatic blood flow (Fig. 2) was partly, but significantly reversed. Also morphological features showed improvement of pancreatic histology, edema was limited to the interlobular space in most cases, leukocytic infiltration and acinar cells vacuolization were strongly reduced. Plasma amylase activity (Fig. 3) and plasma IL-1β concentration (Fig. 4) were not altered significantly.

Deleterious effect of the ablation of sensory nerves on caerulein induced pancreatitis was completely reversed by CGRP administration. Pancreatic DNA
synthesis (Fig. 1) and pancreatic blood flow (Fig. 2) were increased, whereas plasma amylase activity (Fig. 3) and plasma IL-1β concentration (Fig. 4) were decreased. These changes were statistically significant. Histologically, pancreatic damage was reduced. Pancreatic edema and leukocytic infiltration were even smaller than after caerulein given alone (Table 1).

![Graph showing plasma interleukin-1β concentration](image)

Fig. 4. Effect of saline (control), stimulatory (0.5 mg/kg) and neurotoxic (100 mg/kg) doses of capsaicin and CGRP given alone or in their combination with single caerulein infusion on plasma interleukin-1β concentration. Mean ± S.E.M. of 8—14 observations. *P < 0.05 compared with control, †P < 0.05 compared with caerulein given alone, ‡P < 0.05 compared with the neurotoxic dose of capsaicin given in combination with caerulein, §P < 0.05 compared with the neurotoxic dose given alone.

**Second series of experiments**

Like in first series of experiments, subcutaneous infusion of caerulein resulted in the formation of acute pancreatitis in all tested rats. Pancreatic DNA synthesis was decreased by 42% at the time 0 h after the end of caerulein infusion (Fig. 5) and an additional inhibition of DNA synthesis was observed after 3 and 9 h. At the time 0, 3 and 9 h after the end of caerulein infusion, pancreatic blood flow was reduced by 30, 31 and 51%, respectively (Fig. 6). At the time 0 h after caerulein infusion, plasma amylase activity (Fig. 7) and
Table 2. Effect of CGRP administration before and during or after single caerulein infusion on histological signs of pancreatic damage.

<table>
<thead>
<tr>
<th>TIME AFTER CAERULEIN INFUSION</th>
<th>HISTOLOGY</th>
<th>EDEMA (0—3)</th>
<th>INFILTRATION (0—3)</th>
<th>VACUOLIZATION (0—3)</th>
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<tbody>
<tr>
<td><strong>0 h after caerulein infusion</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Saline infusion (control)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Caerulein alone</td>
<td></td>
<td>2/3</td>
<td>1/2</td>
<td>2</td>
</tr>
<tr>
<td>CGRP before and during caerulein</td>
<td></td>
<td>1/2</td>
<td>0</td>
<td>1/2</td>
</tr>
<tr>
<td><strong>3 h after caerulein infusion</strong></td>
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<td></td>
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<tr>
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<td>2</td>
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<tr>
<td>CGRP before and during caerulein</td>
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<td>1</td>
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<tr>
<td>CGRP after caerulein</td>
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<td><strong>9 h after caerulein infusion</strong></td>
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<tr>
<td>Caerulein alone</td>
<td></td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CGRP before and during caerulein</td>
<td></td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CGRP after caerulein</td>
<td></td>
<td>2</td>
<td>1/2</td>
<td>1</td>
</tr>
</tbody>
</table>

Numbers represent the predominant histological grading in each group.

Fig. 5. Effect of CGRP given before and during or after single caerulein infusion on pancreatic DNA synthesis. Mean ± S.E.M. of 8—10 observations. \(^aP < 0.05\) compared with control, \(^bP < 0.05\) compared with caerulein given alone at the same time of observation, \(^cP < 0.05\) compared with animals treated with CGRP before and during caerulein infusion at the same time of observation.
plasma IL-1β concentration (Fig. 8) were increased about nine- and threefold, respectively and these parameters trended to an additional increase at the time 3 and 9 h. Morphological examination of pancreata at the time 0 h revealed the interlobular and moderate intralobular edema in half of the animals treated with caerulein, the rest of animals treated with caerulein, revealed severe interlobular and severe intralobular edema (Table 2). The same feature of edema was observed at the time 3 h after cessation of caerulein infusion, whereas at the time 9 h the edema was in most cases limited to the interlobular space. Edema was accompanied by perivascular infiltration by leukocytes and the presence of acinar cells vacuolization. The maximum infiltration by leukocytes and vacuolization were observed at the time 0 and 3 h, whereas at the time 9 h were less pronounced (Table 2).

Treatment with CGRP before and during caerulein infusion attenuated the severity of pancreatitis. The pancreatic edema was permanently reduced at the time 0, 3 and 9 h after caerulein infusion (Table 2). Caerulein-induced reduction in pancreatic DNA synthesis was partly reversed by pretreatment with CGRP (Fig. 5) and this effect was statistically significant at the time 0, 3 and 9 h after

![Fig. 6. Effect of CGRP given before and during or after single caerulein infusion on pancreatic blood flow. Mean ± S.E.M. of 8—10 observations. *P < 0.05 compared with control, †P < 0.05 compared with animals treated with CGRP before and during caerulein infusion at the same time of observation.](image-url)
cessation of caerulein-infusion. Caerulein evoked fall in pancreatic blood flow (Fig. 6), it was partly, but permanently and significantly reversed by pretreatment with CGRP. Administration of CGRP before and during caerulein infusion was without significant effect on caerulein evoked increase in plasma amylase activity at the time 0 and 3 h after the end of caerulein infusion (Fig. 7), whereas at the time 9 h, a significant reduction in this parameter was observed. Plasma IL-1β concentration in the group treated with CGRP before and during caerulein infusion obtained the same value as in the caerulein alone treated group (Fig. 8) at the time 0 h after cessation of caerulein infusion, but later, plasma IL-1β concentration decreased reaching at the time 9 h a significance lower level, than in the caerulein treated group. Also morphological features showed improvement of pancreatic histology when CGRP was administered before and during caerulein infusion (Table 2). Pancreatic edema, leukocytic infiltration and acinar cells vacuolization were strongly reduced at the time 0, 3 and 9 h after the end of caerulein infusion.

Fig. 7. Effect of CGRP given before and during or after single caerulein infusion on plasma amylase activity. Mean ± S.E.M. of 8—10 observations. aP < 0.05 compared with control, bP < 0.05 compared with caerulein given alone at the same time of observation, cP < 0.05 compared with animals treated with CGRP before and during caerulein infusion at the same time of observation.
Treatment with CGRP after caerulein infusion aggravated pancreatic damage created by caerulein. Pancreatic DNA synthesis (Fig. 5) and pancreatic blood flow (Fig. 6) were decreased additionally and significantly reaching the lowest values at the time 9 h after cessation of caerulein infusion. Plasma amylase activity (Fig. 7) was higher than in the caerulein alone treated group, but this difference was not statistically significant. A significant difference in plasma amylase activity was observed between animals treated with CGRP before and during caerulein infusion and animals treated with CGRP after caerulein infusion at the time 9 h after cessation of caerulein infusion. Administration of CGRP after caerulein infusion strongly increased the plasma IL-1β concentration (Fig. 8). At the time 3 h after cessation of caerulein infusion, histological examination revealed that CGRP given after caerulein, leads to severe inter-and intralobular edema in all cases (Table 2). Also, at the time 9 h after caerulein infusion, pancreatic edema was strongly expressed in this group of animals. In the group of animals treated with CGRP after caerulein infusion, leukocytic infiltration and vacuolization of acinar cells were

Fig. 8. Effect of CGRP given before and during or after single caerulein infusion on plasma interleukin 1β concentration. Mean ± S.E.M. of 8—10 observations. *P < 0.05 compared with control, †P < 0.05 compared with caerulein given alone at the same time of observation, ‡P < 0.05 compared with animals treated with CGRP before and during caerulein infusion at the same time of observation.
more pronounced than in caerulein alone treated group or in the group of animals treated with CGRP before and during caerulein infusion.

Third series of experiments

Two weeks after last episode of repeated acute pancreatitis, pancreata of animals treated with placebo (saline) showed almost full recovery. Pancreatic DNA synthesis was not significantly different when compared with values obtained from intact rats without induction of pancreatitis (Fig. 9), whereas pancreatic blood flow was significantly increased by 26% (Fig. 9). Pancreatic amylase activity and fecal chymotrypsin activity reached 90 and 88% of control value, respectively (Fig. 10). Plasma amylase activity and plasma IL-1β concentration were similar to control values (Fig. 11). Morphological examination of pancreata obtained from spontaneously healed animals revealed slight interlobular edema and slight or lack of leukocytic infiltration and acinar cells vacuolization (Table 3). Acinar cell necrosis, atrophy, fibrosis or tubular complexes were not observed in pancreata obtained from animals with repeated induction of acute pancreatitis and treated with saline.

Treatment with capsaicin after each repeated episodes of acute pancreatitis caused a delay in pancreata healing. In this group of animals, pancreatic DNA synthesis (Fig. 9), pancreatic amylase activity (Fig. 10) and fecal chymotrypsin activity (Fig. 10) were significantly lower than in the control group, as well as, in animals treated with saline after each induction of acute pancreatitis. Contrary to these findings, pancreatic blood flow was increased by 72% above control value (Fig. 9) and by 36% when compared with animals treated with saline after each induction of acute pancreatitis. Also, administration of capsaicin in animals with repeated induction of pancreatitis caused an increase in plasma IL-1β concentration above control value and value observed for animals treated with saline after each induction of acute pancreatitis (Fig. 11). Reduction in plasma amylase activity was insignificant (Fig. 11). Morphological features showed interlobular and moderate intralobular edema and a mild stage of leukocytic infiltration, and acinar cells vacuolization in animals treated with capsaicin (Table 3), but in most cases the acinar cell necrosis, atrophy, fibrosis or tubular complexes were not observed.

Treatment with CGRP after each induction of pancreatitis produced marked reduction in pancreatic amylase activity and fecal chymotrypsin activity to the level as observed in animals treated with capsaicin (Fig. 10). Influence of CGRP on pancreatic DNA synthesis (Fig. 9), pancreatic blood flow (Fig. 9), plasma IL-1β concentration and plasma amylase activity (Fig. 11) was similar to that produced by capsaicin, but changes evoked by CGRP were less pronounced. Histological examination of pancreata obtained from rats treated
Fig. 9. Effect of five episodes of caerulein-induced pancreatitis combined with administration of capsaicin or CGRP on histological signs of pancreatic damage.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL (without pancreatitis)</th>
<th>REPEATED PANCREATITIS + SALINE</th>
<th>REPEATED PANCREATITIS + CAPSAICIN</th>
<th>REPEATED PANCREATITIS + CGRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>edema</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1/2</td>
</tr>
<tr>
<td>leukocyte infiltration</td>
<td>0</td>
<td>0/1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>vacuolization</td>
<td>0</td>
<td>0/1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>acinar cell necrosis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>acinar cell atrophy</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>fibrosis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>tubular complexes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Numbers represent the predominant histological grading in each group.

Table 3. Effect of five episodes of caerulein-induced pancreatitis combined with administration of capsaicin or CGRP on histological signs of pancreatic damage.
Fig. 10. Effect of five episodes of caerulein-induced pancreatitis combined with administration of capsaicin (stimulatory doses) or CGRP on the pancreatic amylase activity and fecal chymotrypsin activity. Mean ± S.E.M. of 8—12 observations. aP < 0.05 compared with control, bP < 0.05 compared with repeated caerulein-induced pancreatitis + saline.

Fig. 11. Effect of five episodes of caerulein-induced pancreatitis combined with administration of capsaicin (stimulatory doses) or CGRP on the plasma amylase activity and interleukin-1β concentration. Mean ± S.E.M. of 8—12 observations. aP < 0.05 compared with control, bP < 0.05 compared with repeated caerulein-induced pancreatitis + saline.
with CGRP showed only interlobular edema or interlobular and moderate
intralobular edema (Table 3). Leukocytic infiltration was slight and the
vacuolization was found in less than 25% of acinar cells. Acinar cell necrosis,
atrophy, fibrosis or tubular complexes were not found in any cases of animals
treated with CGRP after repeated induction of acute pancreatitis.

DISCUSSION

The present study confirms and extends our previous findings, that
stimulation of sensory nerves (34, 35) and administration of CGRP (36) before
and during induction of pancreatitis reduces pancreatic damage evoked by the
overdose of caerulein. Morphological features and the increase in pancreatic
blood flow and pancreatic DNA synthesis have shown an improvement of
pancreatic tissue condition. Stimulation of sensory nerves or CGRP
administration reduced leukocyte infiltration of pancreatic tissue but both
factors were without significant effect on plasma IL-1β concentration. Also,
treatment with CGRP did not affect plasma amylase activity significantly.
These results suggest that protective effect of stimulation of sensory nerves and
administration of CGRP is mainly dependent on the improvement of pancreatic
blood flow. Vasodilatation evoked by CGRP and an increase in pancreatic
blood flow before vascular damage, allow for removal of active digestive
enzymes and mediators of inflammation from pancreatic tissue and attenuate
the pancreatic damage in pancreatitis. For the same reason plasma amylase
activity and plasma IL-1β concentration remained increased.

Another beneficial mechanism of CGRP action can be dependent on the
inhibition of exocrine pancreatic secretion (37, 38). Debas et al. (39) suggested
that CGRP evoked inhibition of exocrine pancreatic secretion is indirect,
neurally mediated and may be explained by the release of somatostatin. The
protective effect of sensory nerve stimulation and administration of CGRP
against caerulein-induced pancreatitis can be also dependent on the release of
nitric oxide (NO). Interaction between release and action of CGRP and NO is
unclear. Some previous reports have suggested that release of CGRP is NO
dependent (39, 40), others have suggested that CGRP acts by NO release (41,
42). Moreover, it has been shown that CGRP stimulates NO liberation by
acinar cells in the course of acute pancreatitis (43) and increases the activity of
NO synthase (NOS) (44). Stimulation of afferent sensory nerves results in the
release of endogenous CGRP (3) and NO (45), where both factors: CGRP and
NO are strong vasodilators (23, 46). Furthermore, a reduction in NO synthesis
by an inhibition of NOS aggravates the damage of the pancreas created by
caeulein (47) and the degree of this injury is almost the same as after sensory
nerve ablation in combination with caerulein. Addition of L-arginine, a substrate for NOS, reverses deleterious effect of NOS inhibition (47).

On the other hand, excessive amounts of NO cause the oxidative injury and contribute to multiorgan oxidative stress in pancreatitis (48), and NO may also reduce the antioxidant capacity of injured organs by binding the SH group (49). Moreover, NO can induce pancreatitis by itself (50). These findings have shown that the level of NO should to be within an appropriate range. Either excess or lack of NO can exhibit deleterious effect on pancreatic tissue. As initially reported for constitutive NOS, also inducible NOS activity may be associated to reduced leukocyte-endothelium interaction and platelet aggregation, as well as, protection of microcirculation (51). It is possible, that the stimulation of NO release by CGRP allows maintenance of a physiological amount of NO.

Protective effect of sensory nerve stimulation or pretreatment with CGRP against development of pancreatic damage in caerulein-induced pancreatitis was seen immediately after the termination of caerulein infusion, as well as, 3 and 9 h later. This observation indicates that it is a permanent protective effect.

The present study has shown that ablation of sensory nerves increases the severity of caerulein-induced pancreatitis and this effect has been completely reversed by exogenous CGRP. This observation and the information that low doses of capsaicin stimulate the release of CGRP (5) demonstrate that protective effects of sensory nerve stimulation in the pancreas could be attributed to CGRP release. Additional support for this hypothesis is the finding that administration of a neurotoxic dose of capsaicin causes the persistent decrease in tissue CGRP-like immunoreactivity (1, 52).

Another finding of our present study is observation that administration of CGRP after induction of pancreatitis increases the pancreatic damage. This deleterious effect of CGRP administration is in agreement with our previous report (52) and with results obtained during induction of gastric lesions by Lopez-Belmonte and Whittle (53). They showed that CGRP can exert both anti- and pro-inflammatory action leading, respectively to reduction and augmentation of gastric mucosal injury. This effect has been dependent on the time of CGRP administration. Pretreatment with CGRP before induction of gastric lesions protected gastric mucosa against injury, whereas administration of CGRP after application of endothelin-1 exacerbated the mucosal damage. This effect of CGRP on the stomach, as well as, on the pancreas seems to be dependent on the degree of tissue and vascular damage. Induction of gastric ulcer or induction of pancreatitis led to tissue and vascular damage and for this reason addition of a vasodilator, such as CGRP increased the plasma protein leakage from injured vessels to pancreatic tissue leading to tissue edema and a maximal decrease in pancreatic blood flow. This hypothesis is in agreement with studies performed by Cambridge et al. (54) and Newbold et al. (55) who found that treatment with CGRP promotes increasing vascular permeability
leading to the production of edema. Moreover, there is a growing number of evidence suggesting that CGRP released from unmyelinated, afferent capsaicin-sensitive sensory nerves may contribute to the chronic inflammatory response (28). Activation of these nerves may produce the neurogenic inflammation described as the local vasodilatation and plasma extravasation (32). Furthermore, CGRP and substance P may promote neutrophil adherence to endothelium (57). It is well known that adhesion of leukocytes to microvascular endothelium is an early and rate limiting step in the inflammatory response (57) leading, among others, to induction of cytokines production. In our present study, we found that treatment with CGRP after induction of pancreatitis, increases the plasma IL-1β concentration to a higher extent than in the group treated with caerulein alone and aggravates the pancreatic damage. This observation is consistent with findings that IL-1β plays the crucial role in the induction of cytokine cascade and development of pancreatitis (58). The use of IL-1 naturally occurring receptor antagonist, almost completely attenuates a rise in serum IL-6 and TNF-α, as well as, decreases the severity of acute pancreatitis as was shown in the study performed by Norman et al. (58).

The mechanisms that induce chronic pancreatitis remain unclear (59) and the pathogenesis of this disease is under much debate as to whether it is a new process or a result of single attack or recurrent episodes of severe acute pancreatitis (60, 61). In our present study repeated induction of acute edematous pancreatitis by caerulein failed to induce chronic pancreatitis. Two weeks after the last episode of acute pancreatitis, pancreata from animals treated with saline showed almost full functional and morphological recovery. This result is in agreement with our previous observations (62, 63) and data obtained by others (64—66) that repeated induction of acute pancreatitis alone is insufficient to induce chronic pancreatitis.

Sensory neurons play a role in regulation of the inflammatory and immune responses in peripheral tissue. The peripheral localized inflammation induces increase in the synthesis and transport of neuropeptides in sensory nerves innervating inflamed tissue (67), as well as, the activation of these nerves may produce neurogenic inflammation described as the local vasodilatation and plasma extravasation (32). On the other hand, clinical (29, 30) and experimental (31) studies in chronic pancreatitis have shown an increase in the number and diameter of intralobular and interlobular nerve bundles and the intensification of immunostaining for sensory nerve mediators. In our present study, treatment with capsaicin was performed after each induction of acute pancreatitis leading to continuous stimulation of sensory nerves. Long lasting activity of sensory nerves led to reduction in digestive enzyme amount in the pancreas causing the decrease in pancreatic and fecal digestive enzyme activity. The treatment with capsaicin inhibited the pancreatic regeneration as proven by the decrease in
pancreatic nucleic acid content (63) and DNA synthesis. This pancreatic functional insufficiency is typical for chronic pancreatitis, however, the histological examination did not reveal signs of chronic pancreatitis, such as acinar cell necrosis, atrophy, fibrosis or tubular complexes. In rats treated with CGRP pancreatic and fecal activity of digestive enzymes, as well as, morphological features were similar to noticed in the capsaicin treated group but less pronounced.

Finally, our present data demonstrate that the physiological role of capsaicin-sensitive sensory nerves and their mediator — CGRP is dependent on local protection against damage if noxious agent acts on intact tissue. The prolonged activity of sensory neurons induced by multiple injection of stimulatory dose of capsaicin, as well as, the prolonged administration of CGRP on damaged tissue aggravate tissue lesions and lead to enzymatic insufficiency typical for chronic pancreatitis.

REFERENCES


