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THE INFLUENCE OF SENSORY NERVES AND CGRP ON THE PANCREATIC REGENERATION AFTER REPEATED EPISODES OF ACUTE PANCREATITIS IN RATS.

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Stimulation of capsaicin sensitive nerves or administration of calcitonin gene-related peptide (CGRP) before induction of acute pancreatitis (AP) attenuates pancreatic damage, whereas CGRP administration after development of AP aggravates lesion of pancreatic tissue. The aim of this study was to determine the effect of prolonged activity of sensory nerves or CGRP administration on the pancreatic repair after repeated episodes of AP. Five episodes of acute caerulein-induced pancreatitis (10 μg/kg/h for 5 h s.c.) were performed at weekly intervals in rats receiving either vehicle or capsaicin at the sensory nerve stimulatory dose (0.5 mg/kg, 3 times daily), or CGRP (10 μg/kg, 3 times daily). Two weeks after the last induction of AP morphological signs of pancreatic damage, pancreatic blood flow (PBF), serum and pancreatic amylase activity, fecal chymotrypsin activity, pancreatic weight, pancreatic RNA and DNA content, as well as, serum interleukin-1β (Il-1β) were assessed. Pancreata of animals receiving vehicle alone showed almost full recovery within two weeks after last episode of pancreatitis induction. In capsaicin-treated group of rats, we observed the increase in PBF by 44% and in serum Il-1β concentration by 91%. The pancreatic amylase activity, fecal activity of chymotrypsin, pancreatic nucleic acids content and DNA synthesis were decreased. In rats treated with CGRP the alterations in PBF, serum Il-1β concentration, as well as, in pancreatic and fecal activity of enzymes were similar to capsaicin treated group but less pronounced. We conclude that prolonged activity of capsaicin-sensitive sensory nerves and the presence of their main mediator-CGRP during pancreatic regeneration after AP leads to pancreatic functional insufficiency typical for chronic pancreatitis.

Key words: *sensory nerves, CGRP, repeated acute pancreatitis, pancreatic regeneration, chronic pancreatitis, interleukin-1β*

INTRODUCTION

The function of primary unmyelinated sensory neurons is to receive and transmit information from the external and internal environment and thereby contribute to maintenance of homeostasis. The excitation of these neurons is

followed not only by conduction of nerve activity to the central nervous system but also activation of peripheral endings of these nerves causes the release of neuromediators, and this process is a basic for local "axon reflex" (1). Thin, unmyelinated sensory fibers have a special sensitivity to capsaicin (1). Low doses of capsaicin result in the stimulation of sensory nerves accompanied with the release of calcitonin gene-related peptide (CGRP) and other neuromediators (2—4), whereas high doses of capsaicin lead to ablation of sensory nerves with the decrease in plasma and tissue levels of CGRP (5—6). The stimulation of sensory fibers or administration of exogenous CGRP was found to exert a protective effect in different experimental models of gastric ulcers (7—9), whereas the ablation of sensory nerves aggravates gastric mucosal lesions induced by various ulcerogenic factors (10—11), as well as, prolongs the gastric ulcer healing (12). The similar influence of capsaicin and CGRP on tissue damage was found in the pancreas. Activation of sensory nerves (13) or treatment with CGRP (14) prior to induction of acute pancreatitis by caerulein attenuates the pancreatic damage, whereas deactivation of sensory nerves contributed to the enhancement of acute pancreatitis severity (13, 15). On the second hand, administration of CGRP after induction of gastric mucosal injury (16), as well as, after development of acute pancreatitis (17) was found to aggravate tissue damage.

Chronic pancreatitis is characterized by recurrent or persistent abdominal pain with destruction and permanent loss of exocrine pancreatic parenchyma (18). Histological examination reveals regressive changes in acinar tissue, pancreatic fibrosis and foci of chronic inflammatory cells (19). Some of these foci are closely associated with nerves causing damage of perineurium and removing the barrier that separates inner compartment of the nerve from surrounding tissue (19). Moreover, in chronic pancreatitis, the remarkable increase in the density and staining intensity of CGRP, VIP and NPY immunoreactive fibers is observed in the clinical (20), as well as, in experimental (21) studies. These observations suggests that sensory nerves may be involved in the pathophysiology of chronic pancreatitis.

The aim of this study was to determine the effect of prolonged activity of sensory nerves or CGRP administration on the pancreatic repair after repeated episodes of acute pancreatitis.

MATERIALS AND METHODS

Animals and treatment

Studies were performed on male Wistar rats weighing 120—140 g at the start of experiments. The animals were housed in cages with wire mesh bottoms at room temperature with a 12 hour light, dark cycle. Water and food were available ad libitum.

The following groups of animals were used: [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 at weekly intervals. Each episode of acute pancreatitis was induced by s.c. infusion of caerulein (Takus, Pharmacia & Upjohn GmbH, Erlangen, Germany). Caerulein was diluted in saline and infused in conscious rats for 5 h at the dose 10 μg/kg/h and at a rate 1 ml/h. After each induction of acute pancreatitis the saline, capsaicin or CGRP (CGRP-I, rat, synthetic, Sigma Chemical CO., St. Louis, MO, USA) 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

Two weeks after last infusion of caerulein, the animals were anesthetized with ether, weighed and the abdominal cavity was opened. The pancreas was exposed for the measurement of the blood flow in the pancreatic tissue by laser Doppler flowmeter using PeriFlux 4001 Master monitor (Perimed AB, Järfälla, 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 serum amylase activity and interleukin 1-β concentration

Immediately after measurement of pancreatic blood flow the abdominal aorta was exposed and blood was taken for serum amylase and interleukin 1β determination. Serum amylase activity was determined by an enzymatic method [Amylase reagent set (kinetic), Alpha Diagnostic sp. z o.o., Warszawa, Poland]. The values were expressed as units/liter. Serum 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 was expressed as pg/ml. The ELISA detection limit of Il-1β was 3 pg/ml.

Determination of DNA synthesis, RNA and DNA content, and amylase activity in the pancreas

After blood withdrawal the pancreas was carefully dissected from its attachment to the stomach, the duodenum and the spleen. Fat and excess tissue were trimmed away. The pancreas was rinsed with saline, blotted on paper and weighed. 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 [^{3}H]-thymidine ([6-^{3}H]-thymidine, 20—30 Ci/mmol, Institute for Research, Production and Application of Radioisotopes, Prague, The 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 RNA content of the supernatant was measured using orcinol reaction (22). 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 content of the samples was determined by Giles and Myers procedure (23). The

incorporation of [^{3}H]-thymidine into DNA was determined by counting 0.5 ml DNA-containing supernatant in a liquid scintillation system. RNA and DNA contents were expressed as milligrams per pancreas. DNA synthesis was expressed as [^{3}H]-thymidine disintegrations per minute per microgram DNA (dpm/μg 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.

Determination of fecal chymotrypsin

Fecal chymotrypsin activity 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 the 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 = interlobular edema and severe intralobular edema. Leukocytic 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%. 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

Pancreata of animals treated with placebo (saline) showed almost full recovery two weeks after last episode of repeated acute pancreatitis The pancreatic weight and pancreatic nucleic acids content were not significantly different when compared with values obtained from intact rats without induction of pancreatitis (*Table 1*). At the end of experiments pancreatic amylase activity reached 90% of control value (4607 ± 258 v. 5084 ± 207 U/pancreas) (*Fig. 1*). Also, almost full recovery was observed in

DNA synthesis (36.42±1.58 v. 41.93±2.08 dpm/μg DNA) (*Fig. 2*) and fecal chymotrypsin activity (12.53±0.62 v. 14.17±0.72 U/g of stool) (*Fig. 3*). The pancreatic blood flow was significantly increased by 26% (*Fig. 4*), whereas changes of serum amylase activity (*Fig. 5*) and serum Il-1β concentration (*Fig. 6*) were unmarked. Morphological examination of pancreata obtained from spontaneously healed animals revealed slight interlobular edema and slight or lack of leukocytic infiltration and cellular vacuolization (*Table 2*). The lack of acinar cell necrosis, atrophy, fibrosis or tubular complexes was observed.

Table 1. Effects of five episodes of caerulein induced pancreatitis combined with administration of capsaicin of CGRP on the pancreatic weight, RNA and DNA content. (Observation two weeks after last induction of acute pancreatitis).

	Pancreatic weight (mg)	RNA content (mg/pancreas)	DNA content (mg/pancreas)
Control (without pancreatitis)	1675±92	13.38±0.69	9.44±0.61
Pancreatitis+saline	1520±97	11.84±0.72	8.69±0.69
Pancreatitis+capsaicin	1782±85	10.66±0.52[a]	5.16±0.37[a,b]
Pancreatitis+CGRP	1823±77	12.02±0.48	7.34±0.43[a]

Mean±SEM of 10—12 rats. [a] $P < 0.05$ compared with control [b] $P < 0.05$ compared with pancreatitis+saline.

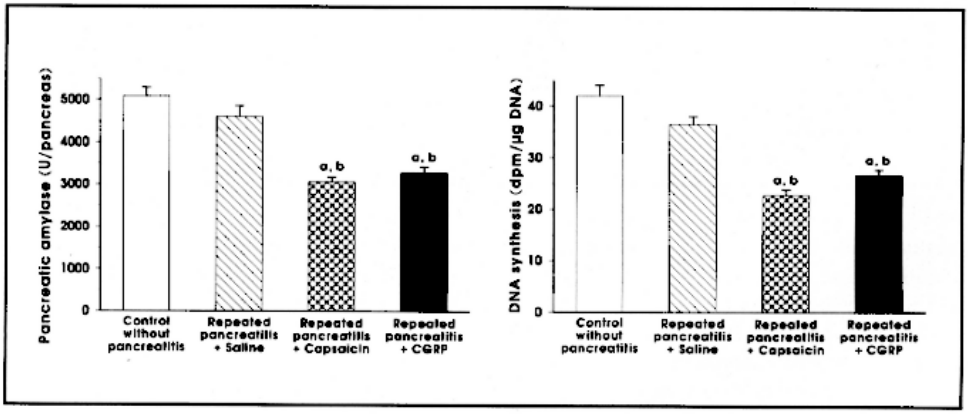

Fig. 1. Effect of five episodes of caerulein-induced pancreatitis combined with administration of capsaicin or CGRP on the pancreatic amylase activity. Mean±S.E.M. of 8—10 observations. [a] $P<0.05$ compared with control, [b] $P<0.05$ compared with repeated pancreatitis+saline.

Fig. 2. Effect of five episodes of caerulein-induced pancreatitis combined with administration of capsaicin or CGRP on the pancreatic DNA synthesis. Mean±S.E.M. of 8—10 observations. [a] $P<0.05$ compared with control, [b] $P<0.05$ compared with repeated pancreatitis+saline.

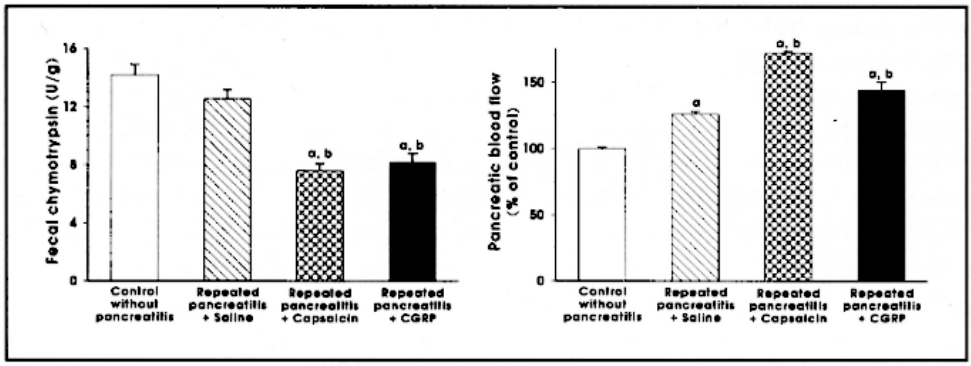

Fig. 3. Effect of five episodes of caerulein-induced pancreatitis combined with administration of capsaicin or CGRP on the fecal chymotrypsin activity. Mean ± S.E.M. of 8—10 observations. [a] $P<0.05$ compared with control, [b] $P<0.05$ compared with repeated pancreatitis + saline.

Fig. 4. Effect of five episodes of caerulein-induced pancreatitis combined with administration of capsaicin or CGRP on the pancreatic blood flow. Mean ± S.E.M. of 8—10 observations. [a] $P<0.05$ compared with control, [b] $P<0.05$ compared with repeated pancreatitis + saline.

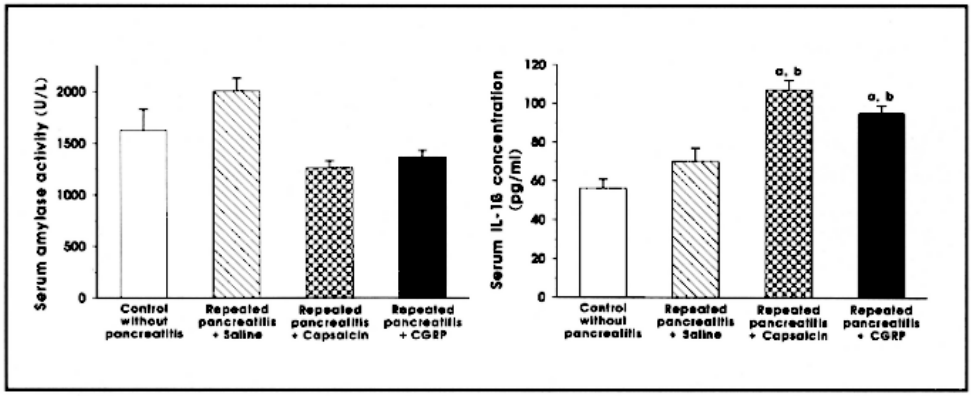

Fig. 5. Effect of five episodes of caerulein-induced pancreatitis combined with administration of capsaicin or CGRP on the serum amylase activity. Mean ± S.E.M. of 8—10 observations. [a] $P<0.05$ compared with control, [b] $P<0.05$ compared with repeated pancreatitis + saline.

Fig. 6. Effect of five episodes of caerulein-induced pancreatitis combined with administration of capsaicin or CGRP on the serum interleukin-1β concentration. Mean ± S.E.M. of 8—10 observations. [a] $P<0.05$ compared with control, [b] $P<0.05$ compared with repeated pancreatitis + saline.

Treatment with capsaicin after repeated episodes of acute pancreatitis caused delay in the spontaneous healing of pancreata. In this group of animals the pancreatic RNA content and DNA content were significantly lower than in control intact group (*Table 1*), moreover the pancreatic DNA content was significantly lower than in animals treated with saline after each induction of

acute pancreatitis. Administration of capsaicin reduced pancreatic amylase activity (*Fig. 1*), DNA synthesis (*Fig. 2*) and fecal chymotrypsin by 40, 46 and 46% respectively when compared with control group. These values were also significantly lower than observed in animals treated with saline after each development of acute pancreatitis. Capsaicin produced the increase in pancreatic blood flow above control value by 72% (*Fig. 4*) and the slight reduction in serum amylase activity without statistical significance (*Fig. 5*). Serum Il-1β concentration was significantly increased in capsaicin treated rats with pancreatitis when compared to control (107.2 ± 5.1 v. 56.3 ± 4.6 pg/ml), as well as, when compared to rats with pancreatitis alone (70.3 ± 6.9 pg/ml) (*Fig. 6*). Morphological features showed interlobular and moderate intralobular edema and mild stage of leukocytic infiltration, and vacuolization in animals treated with capsaicin (*Table 2*), but in the most cases the acinar cell necrosis, atrophy, fibrosis or tubular complexes were not observed.

Table 2. Effects of five episodes of caerulein induced pancreatitis combined with adminiatration of capsaicin or CGRP on histological signs of pancreatic damage.

	Control (without pancreatitis)	Repeated pancreatitis	Repeated pancreatitis +capsaicin	Repeated pancreatitis +CGRP
Edema	0	1	2	1/2
Leukocyte infiltration	0	0/1	1	1
Vacuolization	0	0/1	1	1
Acinar cell necrosis	0	0	0	0
Acinar cell necrosis	0	0	0	0
Acinar cell atrophy	0	0	0	0
Fibrosis	0	0	0	
Tubular complexes	0	0	0	0

Numbers represent the predominant histological grading in each group.

Treatment with CGRP after each induction of pancreatitis produced marked reduction in the pancreatic DNA content when compared to control group (*Table 1*), but in contrast RNA content in animals treated with capsaicin was not statistically different when compared to animals with pancreatitis alone. In CGRP treated group with pancreatitis the pancreatic amylase content was decreased to the level observed in animals treated with capsaicin (*Fig. 1*). Also the influence of CGRP on DNA synthesis (*Fig. 2*), fecal chymotrypsin (*Fig. 3*) and pancreatic blood flow (*Fig. 4*) was similar to observed in capsaicin treated group, however changes evoked by CGRP were less pronounced.

CGRP failed to affect significantly serum amylase activity (*Fig. 5*), whereas serum Il-1β concentration was significantly increased in CGRP treated group when compared with control or saline treated group with repeated pancreatitis (95.2 ± 4.1 v. 56.3 ± 4.6 and 70.3 ± 6.9 pg/ml) (*Fig. 6*). Histological examination of pancreata obtained from rats treated with CGRP showed interlobular and in half of cases moderate intralobular edema (*Table 2*). 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

Chronic pancreatitis is a protracted inflammation of the pancreas characterized by a irreversible alteration of the basic anatomical structure and thus frequent functional deficiency, even if the cause is eliminated. In advanced stages the main features of chronic pancreatitis are exocrine and endocrine pancreatic insufficiency, and pain. It generally accepted that prolonged ingestion of large amounts of alcohol is a major risk factor for development of chronic pancreatitis (18). The mechanisms that induce chronic pancreatitis remain unclear (18) 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 (24, 25). 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 observation (26) and data obtained by others (27—29) that repeated induction of acute pancreatitis alone is insufficient to induce chronic pancreatitis.

Several experimental studies have reported the temporal appearance of chronic pancreatitis-like lesions after induction of acute pancreatitis (27, 30—32). During pancreatic regeneration, the transient atrophy of acini, fibrosis, increase in deposition of collagen fibers and fibronectin in pancreatic extracellular matrix, the increase in pancreatic hydroxyproline content were observed. But some days or weeks later, all lesions were cleared and the exocrine pancreas had been restored to normal. Also in our present study, two weeks after last induction of repeated pancreatitis, we did not observe morphological features typical for chronic pancreatitis. These data suggest that even severe chronic pancreatitis lesion evoked by recurrent acute pancreatitis are reversible in the absence of primary causative factor.

Sensory neurons play a role in regulation of the inflammatory and immune responses in peripherial tissue. The peripherial localized inflammation induces

the increase in the synthesis and transport of neuropeptides in sensory nerves innervating inflamed tissue (33), as well as, the activation of these nerves may produce neurogenic inflammation described as the local vasodilatation and plasma extravasation (34). On the other hand, clinical (20) and experimental (21) studies of chronic pancreatitis have shown the 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 continuos stimulation of sensory nerves. Long lasting activity of sensory nerves led to the reduction of digestive enzyme amount in the pancreas causing the decrease in pancreatic and fecal digestive enzyme activity. The treatment with capsaicin inhibited the pancreatic regeneration what was found as the decrease in pancreatic nucleic acids content and DNA synthesis. This pancreatic functional insufficiency is typical for chronic pancreatitis, however the histological examination did not reveal the 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 observed in capsaicin treated group but less pronounced.

The reason for the reduction of pancreatic enzyme and inhibition of pancreatic regeneration after sensory nerves stimulation and CGRP administration remains unclear. In our present study, treatment with capsaicin or CGRP caused the potent increase in pancreatic blood flow and this effect seems to be dependent on vasodilatation evoked by CGRP (35). Vasodilatation and an increase in pancreatic blood flow before vascular damage allow the removal of active digestive enzymes and mediators of inflammation from pancreatic tissue and attenuate the pancreatic damage in pancreatitis (14). In our present study, capsaicin or CGRP were administered after induction of pancreatitis when the blood vessels were damaged. For this reason vasodilatation promoted the pancreatic edema and increased the pancreatic damage. This observation is consistent with studies performed by Cambridge *et al.* (36) and Newbold *et al.* (37) who reported that treatment with CGRP potentates the effect of increasing vascular permeability factors leading to production of edema.

Another possible mechanism of deleterious effect of sensory nerves stimulation and CGRP administration could be related to activation of leukocytes. Study performed by Zimmerman *et al.* (38) has shown that Substance P and CGRP (both are mediators of sensory nerves) promotes neutrophil adherence to endothelial monolayers. Activation of leukocytes plays an important role in the development of acute pancreatitis (39) and in the development of multiple organ failure (40), whereas neutrophil depletion reduces the acute pancreatitis-associated lung injury (40). The mechanism of

deleterious effects of leukocyte activation is strongly dependent on production and liberation of interleukins. In our present study, two weeks after last induction of pancreatitis, we have found normalization of serum Il-1β concentration and almost full pancreatic recovery in rats treated with saline. In contrast, in rats treated with capsaicin or CGRP the serum Il-1β level and pancreatic damage were high. Il-1 plays the crucial role in the induction of cytokine cascade and inflammatory process (41). The role of Il-1 in the development of acute pancreatitis has been shown by Norman *et al.* (42) who have found that the use of Il-1 receptor antagonist decreases the serum level of Il-6 and tumor necrosis factor (TNF), and reduces the severity of acute pancreatitis.

The development of chronic pancreatitis may be dependent on the activity of transforming growth factor (TGF). Clinical (43—45) and experimental studies (46, 47) have shown overexpression of transforming growth factor β1 (TGF-β1) in human chronic pancreatitis, as well as, in the rat pancreas during regeneration after acute pancreatitis. TGF-β1 is a regulator of extracellular matrix remodeling in the pancreas during tissue repair and it was postulated to play the important role, as a promoting factor, in the pathogenesis of chronic pancreatitis. This hypothesis is supported by data obtained by Van Laethem *et al.* (28). They have shown that application of recombinant TGF-β1 after recurrent episodes of acute pancreatitis promotes pancreatic fibrosis, inflammation and acinar atrophy (28). Unfortunately, their study were finished one week after last induction of pancreatitis and no information was given whether it is a temporal or permanent effect. On the second hand, studies with rat dorsal root ganglion neurons has shown that TGF-β1 and TGF-β2 increase survival of sensory nerves in culture and increase the level of neuromediators in these nerves (48). This effect is synergistic with effect of nerve growth factor (NGF) which exhibits trophic activity on nerves (48). Moreover, Buchman *et al.* (49) have reported that TGF-β isoforms can increase the level of NGF. These data indicates relationship between sensory nerves and TGF-β.

Finally, our present data demonstrate that prolonged activity of sensory nerves, as well as, the presence of CGRP during pancreatic regeneration after acute pancreatitis lead to pancreatic exocrine insufficiency and delay in pancreatic regeneration.

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