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CALCITONIN GENE-RELATED PEPTIDE CAN ATTENUATE OR AUGMENT PANCREATIC DAMAGE IN CAERULEIN-INDUCED PANCREATITIS IN RATS

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We have recently shown that treatment with calcitonin gene-related peptide (CGRP) before and during induction of acute pancreatitis exhibits a protective effect against pancreatic damage evoked by overdose of caerulein. Studies in the stomach have shown that administration of CGRP exhibits dual action on gastric mucosa, CGRP administration before induction of gastric lesions, protects gastric mucosa against damage, whereas treatment with this peptide after development of gastric ulcer exacerbates mucosal injury. These observations prompt us to determine the influence of CGRP administrated before and after induction of pancreatitis on development and evolution of pancreatic tissue damage. Methods: Acute pancreatitis was induced by s.c. infusion of caerulein (10 µg/kg/h) for 5 h. CGRP was administrated (10 µg/kg s.c. per dose) 30 min prior to caerulein infusion and 3 h later during caerulein infusion or at the time 1 h, 4 h and 7 h after the end of caerulein infusion. Rats were sacrificed at the time 0 h, 3 h or 9 h after cessation of caerulein administration. The pancreatic blood flow (PBF), plasma activity of amylase, plasma interleukin-1β concentration, cell proliferation, biochemical and morphological signs of pancreatitis were examined. Results: Caerulein-induced pancreatitis (CIP) led to 42% decrease in DNA synthesis, 30% inhibition of PBF, as well as, a significant increase in pancreatic weight, plasma amylase activity, plasma interleukin-1β concentration, and development of the histological signs of pancreatic damage (edema, leukocyte infiltration and vacuolization). Treatment with CGRP prior and during induction of CIP attenuated the pancreatic damage what was manifested by partial reversion of the drop in DNA synthesis (40.9+1.7 v. 34.2+2.0 dpm/µg DNA) and PBF (83+3% v. 70+3%). Increases in pancreatic weight and plasma interleukin-1β were reduced. Morphology showed improvement of pancreatic integrity. Administration of CGRP after induction of CIP aggravated pancreatic damage what was manifested by additional decrease in PBF and DNA synthesis. Also pancreatic weight as well as histological signs of pancreatic damage were increased Conclusions: (1) Administration of CGRP before and during induction of pancreatitis protects pancreas against pancreatic damage. (2) Treatment with CGRP after development of CIP aggravates pancreatic damage.

Key words: CGRP, pancreatitis, pancreatic blood flow, DNA synthesis, interleukin-1β

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is widely distributed within the central, peripherial and enteric nervous system (1). In the gut, CGRP immunoreactivity is localized in the nerve fibres inervating the gastrointestinal tract and the pancreas (2, 3). CGRP is identified as a major mediator of thin, unmyelinated, capsaicin-sensitive sensory fibers (4). CGRP affects gastrointestinal tract causing, among others, the vasodilatation (5) and the inhibition of gastric (6, 7) and pancreatic secretion (7, 8). Either activation of sensory fibers leading to the release of endogenous CGRP (9) or administration of exogenous CGRP was found to exert a protective effect on different experimental models of gastric ulcers (10—13). Ablation of sensory nerves by systemic administration of high dose of capsaicin leads to a depletion of CGRP content and aggravates gastric mucosal lesions induced by various ulcerogenic factors (14, 15), as well as, prolongs the gastric ulcer healing (16). Sensory nerves stimulation by low doses of capsaicin or administration of CGRP was found to affect also the pancreas. Activation of sensory nerves (17) or pretreatment with CGRP (18) attenuates the pancreatic damage in caerulein-induced pancreatitis, whereas deactivation of sensory nerves contributed to the enhances severity of pancreatitis (17).

On the second hand, CGRP can also increase the tissue damage. It is known as a potent vasodilator (5) and administration of CGRP was shown to promote the effect of increasing vascular permeability mediators leading to potentation of vascular leakage and induction of edema (19, 20), whereas administration of CGRP receptor antagonist $CGRP_{8-37}$ attenuated the histamine-induced vascular leakage and edema formation (21). Moreover, study performed by Lopez-Belmonte and Whittle (22) has shown that CGRP can exert both anti- and pro-inflammatory action leading to reduction or augmentation of gastric mucosal injury evoked by local intra-arterial infusion of endothelin-1. This effect has been dependent on the time of CGRP administration. Pretreatment with CGRP before induction of gastric lesions has protected gastric mucosa, whereas administration of CGRP after application of endothelin has exacerbated mucosa damage.

In the previous study, we have found that pretreatment with CGRP protects the pancreas against caerulein induced pancreatitis in rats (8) but clinically patients are normally seen hours after onset of acute pancreatitis and there is no possibility to introduce treatment before development of this disease. Therefore, the aim of this study was to examine whether administration of CGRP after induction of acute pancreatitis, what more corresponds with clinical conditions, exhibits any beneficial effect on the course of caerulein-induced pancreatitis.

MATERIALS AND METHODS

Animals and treatment

Studies were performed on male Wistar rats weighing 160—200 g. 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.

Several series of experiments were carried out including: (1) control (intact rats treated 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 caerulein infusion (10 µg of CGRP per dose). Rats were sacrificed at the time 0 h, 3 h or 9 h after cessation of caerulein administration.

Caerulein, used for induction of acute pancreatitis, was diluted in saline and infused subcutaneously for 5 h in conscious animals at a dose 10 µg/kg/h and at a rate 1ml/h.

Rat, synthetic CGRP-I was obtained from Sigma Chemical Co, St. Louis, MO, USA. Caerulein (Takus) was obtained from Pharmacia GmbH, Erlangen, Germany.

Determination of pancreatic blood flow

After infusion of caerulein for 5 h, 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 plasma amylase activity and interleukin 1-β concentration

Immediately after measurement of pancreatic blood flow the abdominal aorta was exposed and blood was taken for plasma amylase determination. Plasma amylase 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. 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 was expressed as pg/ml. The ELISA detection limit of Il-1β was 3 pg/ml.

Determination of DNA synthesis, RNA and DNA content

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

Histological examination

Samples of pancreatic tissue were excised, fixed in 10 % formalin, embedded in paraffin and sections were stained with hematoxilin 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 raging 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 also graded from 0 (absent) to 3 for maximal alterations (diffuse infiltration in the entire pancreatic gland) Grading of vacuolization was based on the appropriate percentage of cells involved: 0 = absent, 1 = less than 25%, 2 = 25—50% and 3 = more than 50%.

Statistical analysis

Results are expressed as means ± S.E.M. and were analyzed by analysis of variance and Student's t test for unpaired values, with $p < 0.05$ considered significant.

RESULTS

Subcutaneous infusion of caerulein at a dose 10 µg/kg/h for 5 hours resulted in the formation of acute pancreatitis in all tested rats. The pancreas was swollen and enlarged with visible collection of edematous fluid. The weight of the pancreas at the time 0 h after the end of caerulein infusion was increased by 38% (*Table*). After next 3 and 9 h the pancreatic weight rose additionally reaching 150 and 159% of control value, respectively. DNA synthesis was decreased by 42% at the time 0 h after induction of pancreatitis (*Fig. 1*) and an additional inhibition of DNA synthesis was observed after 3 and 9 h. At the time 0 h after caerulein infusion the plasma amylase activity (*Fig. 2*) and the plasma interleukin-1β concentration (*Fig. 3*) were about nine and three fold increased, respectively, and these parameters tended to an additional increase at the time 3 and 9 h. At the time 0, 3 and 9 h after the end of caerulein infusion, the pancreatic blood flow was reduced by 30, 31 and 51%, respectively (*Fig. 4*). Morphological examination of pancreata at the time 0 h revealed the interlobular and moderate intralobular edema in all animals treated with caerulein and in almost all cases severe intralobular edema (*Table*). 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 the most cases limited to interlobular space. The edema was accompanied by perivascular infiltration by leukocytes and the presence of 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*). Administration of caerulein, as well as, CGRP were without any significant effect on pancreatic RNA and DNA content at any time of observation (data not shown).

Table. Effect of CGRP administration before and during or after caerulein infusion on pancreatic weight and histological signs of pancreatis such as edema, leukocyte infiltration and vacuolization.

	Pancreatic Weight (mg)	HISTOLOGY		
		Edema (0-3)	Infiltration (0-3)	Vacuolization (0-3)
		0 h after caerulein infusion		
Saline infusion (control)	845 ± 34	0.3 ± 0.2	0	0
caerulein alone	1182 ± 40^a	2.7 ± 0.2^a	1.7 ± 0.2^a	2.0 ± 0.0^a
CGRP before and during caerulein	$994 \pm 31^{a,b}$	$1.4 \pm 0.1^{a,b}$	$0.8 \pm 0.1^{a,b}$	$1.4 \pm 0.1^{a,b}$
		3 h after caerulein infucion		
caerulein alone	1280 ± 39^a	2.7 ± 0.2^a	1.7 ± 0.2^a	2.0 ± 0.0^a
CGRP before and during caerulein	$1016 \pm 33^{a,b}$	$1.2 \pm 0.1^{a,b}$	1.0 ± 0.2^a	$1.0 \pm 0.2^{a,b}$
CGRP after caerulein caerulein	$1430 \pm 31^{a,b,c}$	3.0 ± 0.0^a	1.4 ± 0.2^a	$0.8 \pm 0.2^{a,b}$
		9 h after caerulein infusion		
caerulein alone	1346 ± 40^a	1.3 ± 0.2^a	0.8 ± 0.1	1.0 ± 0.0
CGRP before and during caerulein	$1152 \pm 39^{a,b}$	1.4 ± 0.1^a	0.3 ± 0.2	0.2 ± 0.1
CGRP after caerulein	$1520 \pm 38^{a,b,c}$	2.0 ± 0.2^a	1.4 ± 0.2	0.8 ± 0.1

Mean $\pm$ SEM of 8—10 observations. [a]$P < 0.05$ compared with control group. [b]$P < 0.05$ compared with caerulein alone at the same time of observation. [c]$P < 0.05$ compared with animals treated with CGRP before and during caerulein infusion at the same time of observation.

Treatment with CGRP before and during caerulein infusion attenuated the severity of pancreatitis. The increase of pancreatic weight as a effect of pancreatic edema was permanently and markedly reduced at the time 0, 3 and 9 h after caerulein infusion (*Table*). Caerulein-induced drop of DNA synthesis was partly, but significantly reversed by pretreatment with CGRP (*Fig. 1*). The administration of CGRP before and during caerulein infusion was without significant effect on caerulein evoked increase in plasma amylase activity at the
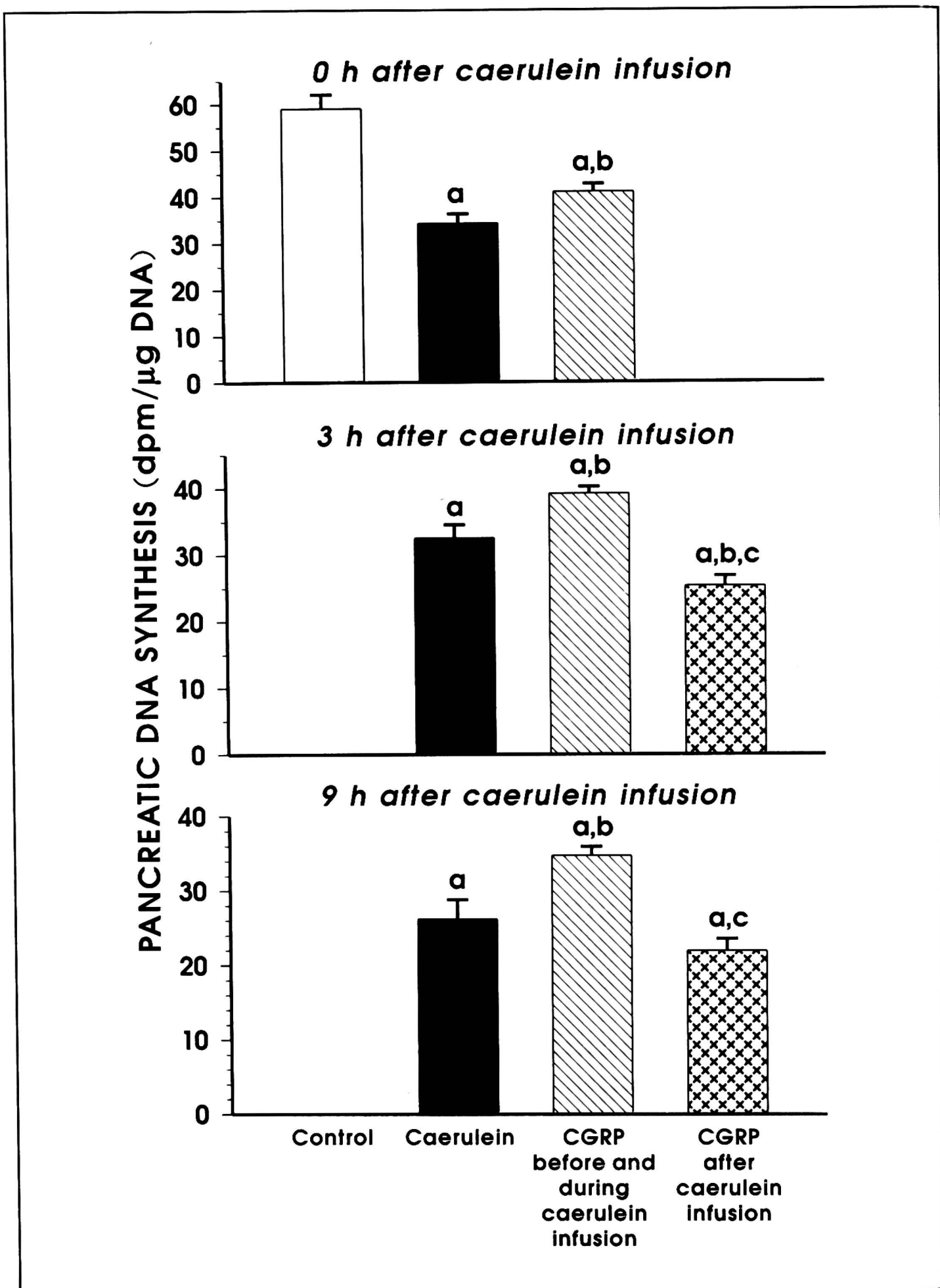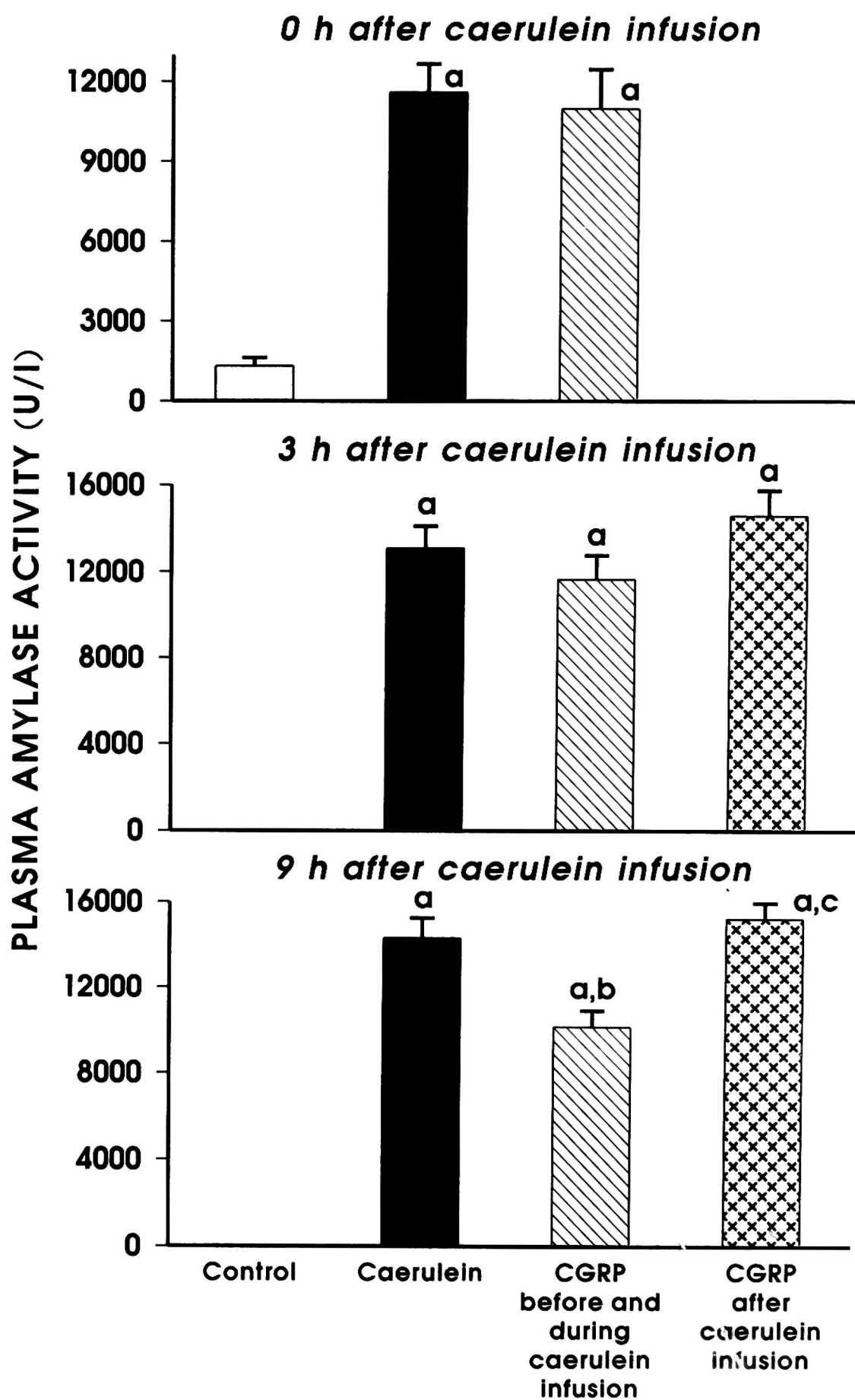
Fig. 1. Effect of CGRP given before and during or after caerulein infusion on DNA synthesis in the pancreas. Mean ± S.E.M. of 8—10 observations. $^aP < 0.05$ compared with control, $^bP < 0.05$ compared with caerulein given alone at the same time of observation, $^cP < 0.05$ compared with animals treated with CGRP before and during caerulein infusion at the same time of observation.

Fig. 2. Effect of CGRP given before and during or after caerulein infusion on plasma amylase activity. Mean ± S.E.M. of 8—10 observations. [a]P < 0.05 compared with control. [b]P < 0.05 compared with caerulein given alone at the same time of observation. [c]P < 0.05 compared with animals treated with CGRP before and during caerulein infusion at the same time of observation.
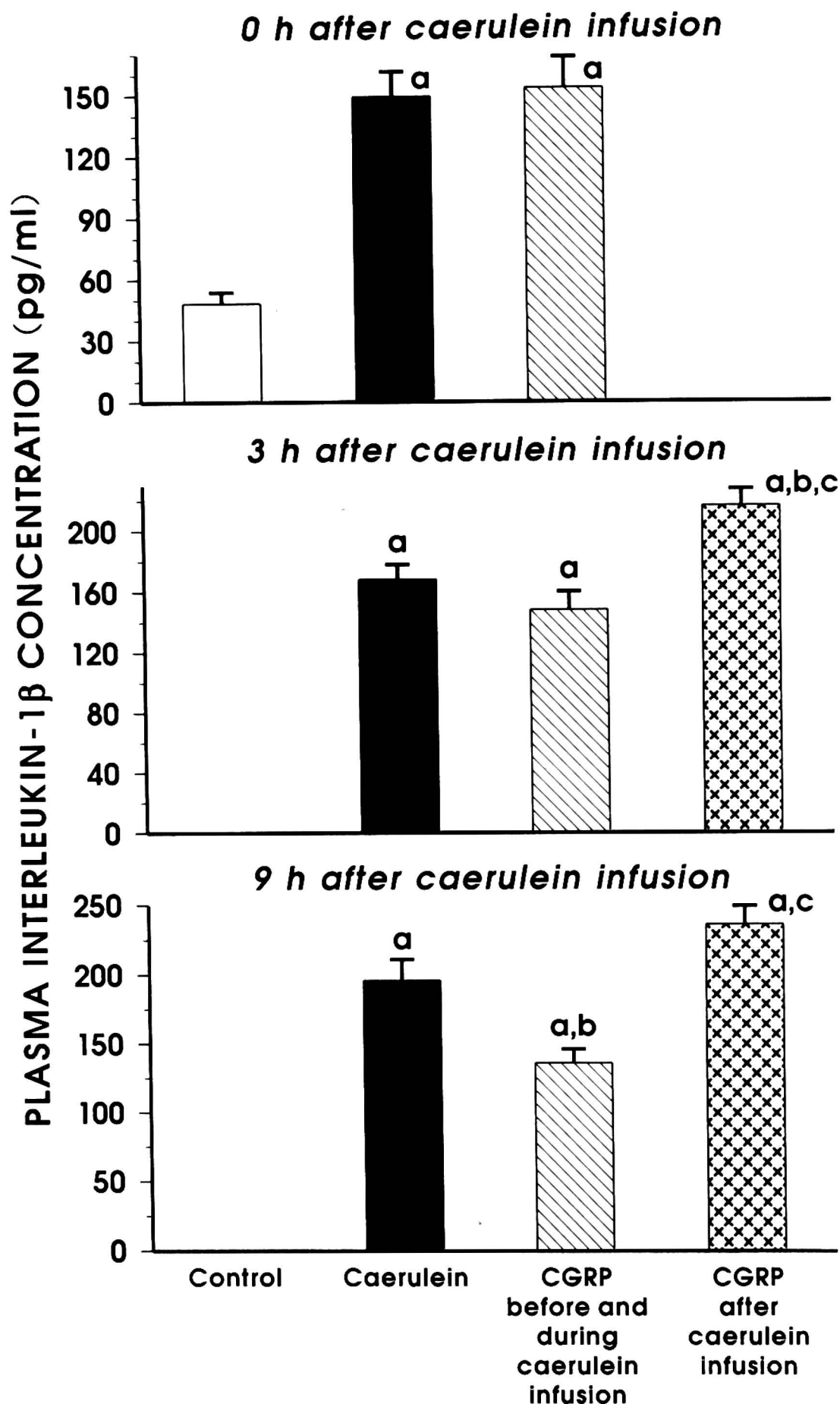
Fig. 3. Effect of CGRP given before and during or after caerulein infusion on plasma interleukin-1β concentration. Mean ± S.E.M. of 8—10 observations. [a]P < 0.05 compared with control, [b]P < 0.05 compared with caerulein given alone at the same time of observation, [c]P < 0.05 compared with animals treated with CGRP before and during caerulein infusion at the same time of observation.
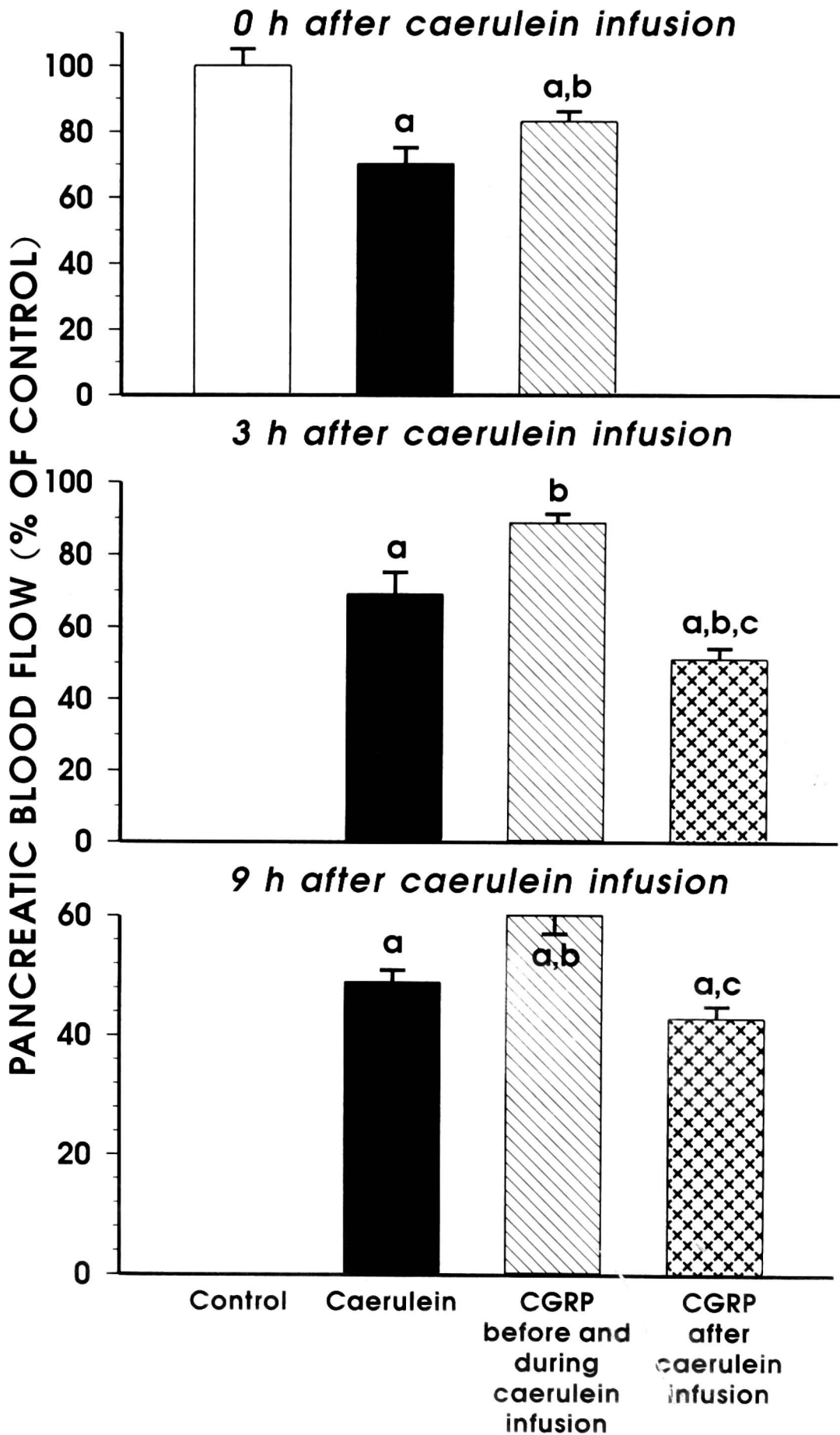
Fig. 4. Effect of CGRP given before and during or after caerulein infusion on pancreatic blood flow. Mean ± S.E.M. of 8—10 observations. [a]P < 0.05 compared with control, [b]P < 0.05 compared with caerulein given alone at the same time of observation, [c]P < 0.05 compared with animals treated with CGRP before and during caerulein infusion at the same time of observation.

time 0 and 3 h (*Fig. 2*), whereas at the time 9 h the significant reduction in this parameter was observed ($10,147 \pm 773$ v. $14,311 \pm 994$ U/L). At the time 0 h, the plasma interleukin-1β concentration in the group treated with CGRP before and during caerulein infusion obtained the same value like in caerulein alone treated group (*Fig. 3*) but later the plasma interleukin-1β concentration progressively came down reaching at the time 9 h the significant lower level then in caerulein treated group (136 ± 10 v. 196 ± 15 pg/ml). The caerulein evoked fall in pancreatic blood flow (*Fig. 4*) was partly, but constantly and significantly reversed by pretreatment with CGRP. Also morphological features showed improvement of pancreatic histology when CGRP was administrated before and during caerulein infusion (*Table*). The edema was limited to interlobular space in most cases and leukocytic infiltration was strongly reduced at the time 0 h. The similar effect was observed at the time 3 and 9 h.

Treatment with CGRP after caerulein infusion aggravated pancreatic damage created by caerulein, what was manifested by an additional and significant decrease in DNA synthesis (*Fig. 1*). The pancreatic weight (*Table*) was maximally increased. The level of plasma amylase activity (*Fig. 2*) was higher than in caerulein alone treated group but this difference was not statistically significant. Moreover, administration of CGRP after caerulein infusion strongly increased the plasma interleukin-1β concentration (*Fig. 3*). The pancreatic blood flow was deeply decreased in this group of animals reaching the lowest level (*Fig. 4*). Histological examination (*Table*), at the time 3 h after cessation of caerulein infusion, revealed that CGRP given after caerulein leads to severe inter-and intralobular edema all cases. At the time 9 h after caerulein infusion the pancreatic edema was still the most strongly expressed in the group treated with CGRP after caerulein infusion, also leukocytic infiltration was the highest in this group but these effects were not statistically significant when compared to caerulein alone treated group or group of animals treated with CGRP before and during caerulein infusion.

DISCUSSION

The present study confirms and extends our previous findings, (18) that administration of CGRP before and during induction of pancreatitis exerts protective effects against caerulein evoked pancreatic damage. Functional, biochemical and histological parameters have shown an improvement of pancreatic tissue condition. This effect was observed at the time 0 h, as well as, 3 and 9 hours after caerulein infusion.

The new findings of the present study is observation that administration of CGRP after induction of pancreatitis augments the pancreatic damage. This dual effect of CGRP on the course of caerulein-induced pancreatitis seems to

be mainly dependent on CGRP evoked vascular dilatation and severity of microvascular damage. The mechanism of caerulein induced pancreatitis is not clear, but an alteration of pancreatic blood flow and insufficient oxygen supply seem to play an important role in this case. In the beginning, the hyperstimulation of pancreas by overdose of caerulein increases the pancreatic exocrine secretion (25) and pancreatic metobolic rate leading to pancreatic arterial hyperemia (26). The pancreatic blood flow is initially increased, but the most likely the pancreas suffers from ischemia. The situation seems to be similar to that observed in the heart with coronary insufficiency during exertion or stress. In spite of arterial hyperemia the pancreatic oxygen need exceed the oxygen supply. Pancreatic cell ischemia with subcellular damage is able to activate the intrapancreatic lysosomal and exocrine enzymes (27) leading to pancreatic autodigestion, tissue damage and the induction or aggravation of pancreatitis. The convincing evidence of an important role of pancreatic ischemia in development of acute pancreatitis provides the study performed by Redha et al. (28). They have shown that intraarterial injection of microspheres induces acute, hemorrhagic and necrotizing pancreatitis within 24 h. Moreover, the severity of pancreatitis was closely correlated with degree of tissue hypoxia (26). Active hyperemia observed initially during caerulein infusion (26) is followed by severe reduction in pancreatic blood flow, what was previously found by us (25, 29, 30) and others (31) and is confirmed by present study.

Changes of blood flow and intrapancreatic activation of digestive enzymes lead to induction of acute pancreatitis manifested in our present study by pancreatic edema, enhanced plasma amylase activity and plasma Il-1β concentration, acinar cells vacuolization, as well as, pancreatic infiltration by leukocytes. Moreover the acute pancreatitis led to a decrease in pancreatic DNA synthesis, as was demonstrated in our study, what is additional evidence of tissue damage.

CGRP is known as the potent vasodilator (5, 32—33) and its administration, in our study, before and during caerulein infusion prevented the decrease in pancreatic blood flow. The improvement of pancreatic circulation before vascular damaged allowed the removal of active digestive enzymes and mediators of inflammation from the pancreatic tissue and protected the pancreas against damage caused by these factors. For this reason, the plasma amylase activity, the most accepted index of pancreatic damage, was not significantly affected by pretreatment with CGRP at the time 0 and 3 h after caerulein infusion. At the time 9 h, the better condition of pancreatic tissue led to smaller liberation of pancreatic enzymes into blood, therefore, plasma amylase activity was significantly lower. The same changes were observed with plasma Il-1β concentration.

In our present study, administration of CGRP after induction of pancreatitis augmented pancreatic damage evoked by caerulein. Caerulein-induced

pancreatitis led to tissue and vascular damage and for this reason addition of vasodilator such as CGRP increased the plasma protein leakage from injured vessels to pancreatic tissue and maximally decreased the pancreatic blood flow. These findings are in agreement with studies performed by Cambridge *et al.* (19) and Newbold *et al.* (20) who found that treatment with CGRP promotes the effect of increasing vascular permeability mediators 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 (34). Activation of these nerves may produce the neurogenic inflammation described as the local vasodilatation and plasma extravasation (35), as well as, CGRP and substance P may promote neutrophil adherence to endothelium (36). It is well known that adhesion of leukocytes to microvascular endothelium is an early and rate limiting step in an inflammatory response (36) leading, among others, to induction of cytokines. In our present study, we found that treatment with CGRP after induction of pancreatitis increases the plasma interleukin-1β concentration to the higher extend 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 (37). The use of Il-1 naturally occurring receptor antagonist almost completely attenuated rise in serum Il-6 and TNF-α, as well as, decreased the severity of acute pancreatitis in the study performed by Norman *et al.* (37).

A common component in the pancreas of patients with chronic pancreatitis are foci of chronic inflammatory cells. Some of them are closely associated with nerves causing the damage of perineurium and removing the barrier that separates the inner compartment of the nerve from surrounding tissue (38). This leads to chronic stimulation of these nerves and may be responsible for chronic pain and local liberation of neuropeptides from sensory nerves. Moreover, morphological features of chronic pancreatitis shows the greater density of nerves and these nerves have a mean diameter greater than normal (38).

These data indicates that physiological role of capsaicin-sensitive sensory nerves and their mediator — CGRP is dependent on local protection against damage if noxious agent acts on undamaged tissue. The prolonged action of CGRP on damaged tissue aggravates tissue lesion or even may be responsible for induction of chronic inflammation.

In conclusion, the results of this study demonstrate that only pretreatment with CGRP before the onset of acute pancreatitis can attenuate the acute pancreatic damage and for this reason CGRP seems to be useless in the eventual therapy of acute pancreatitis.

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