Vasoactive mediators and the progression from oedematous to necrotising experimental acute pancreatitis

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Abstract
Little is known about the pathophysiological factors that determine the clinical severity of acute pancreatitis. Because impairment of pancreatic circulation and oxygenation is associated with greater disease severity and morphological damage in experimental pancreatitis it has been suggested that various vasoactive mediators might participate in the progression from the oedematous to the necrotising variety of the disease. This study used an animal model of acute pancreatitis induced by intravenous caerulein (10 μg/kg/h for up to six hours), which does not entail either haemorrhage or significant necrosis of the pancreas. This study considered whether the administration or the inhibition of either nitric oxide, bradykinin, or adrenergic mediators can convert this mild variety into haemorrhagic and necrotising pancreatitis. Neither nitric oxide nor catecholamines were involved in the progression from oedematous to haemorrhagic pancreatitis. Their substitution, activation, and inhibition all failed to change the severity of the disease process. Bradykinin alone seemed to be critically involved in the pathogenesis of pancreatic haemorrhage and necrosis. However, the inhibition of bradykinin and not its activation or substitution increased the severity of the disease.

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Most patients with acute pancreatitis suffer from a mild, self limiting disease and quickly recover without specific treatment. Fifteen to 20% of patients, however, develop haemorrhage and necrosis of the pancreas and this severe variety of the disease is associated with a significant mortality despite intensive care treatment or surgical intervention. Since the end of the last century ischaemia and impaired oxygenation of the pancreas have been known to be critically involved in determining the severity of pancreatitis, and a large body of experimental evidence suggests that all experimental models of pancreatitis are to a greater or lesser degree associated with microcirculatory changes in the pancreas. These vascular and circulatory changes, however, have been seen in mild as well as in morphologically severe varieties of the disease. It is still unclear which factor determines whether oedematous or haemorrhagic necrotising pancreatitis results in a given experimental or clinical situation and the potential vasoactive mediators responsible for the progression of the disease severity have largely remained subject to speculation and debate. We have selected three probable candidates that have been implicated in the past. The finding that stress and shock can convert oedematous to haemorrhagic experimental pancreatitis would suggest that adrenergic mediators might participate in this process. The activation of the kinin system and the depletion of kinin degrading enzymes during pancreatitis would suggest bradykinin as a potential candidate. And finally, the potent regulator of the pancreatic blood supply, nitric oxide (NO), could represent the critical vasoactive compound responsible for the conversion of oedematous to haemorrhagic pancreatitis.

We have chosen an animal model of acute pancreatitis that involves hyperamylasaemia, pancreatic oedema, and a number of cell biological events that characterise pancreatitis. but is not associated with either haemorrhage or significant acinar tissue necrosis. We have used this model to either administer analogues of these vasoactive mediators or to administer potent inhibitors of their actions. Our results show that, among these agents, bradykinin is the only mediator involved in the development of pancreatic haemorrhage and necrosis, and, that the inhibition of bradykinin is responsible for this effect.

Methods

Materials
Caerulein was obtained from Farmitalia (Freiburg, Germany), Mercox resin from Ladd (Burlington, VT, USA), and HOE-140 was a kind gift from Hoechst AG (Frankfurt, Germany). All other chemicals were of the highest purity commercially available and were purchased from either Sigma (Deisenhofen, Germany) or Polysciences (Eppelheim, Germany).

Animals and ethical approval
Animals were obtained from Charles River Breeding Laboratories (Sulzbach, Germany) and the breeding colony of Ulm University Animal Facilities. They were housed in nalgene shoebox cages under a 12 hour
light/dark cycle and were allowed to adapt after transportation for a minimum of one week before the experiments. All animal experiments were begun after an overnight fast and were conducted according to the guidelines of the local animal use and care committee and the guiding principles of the American Physiological Society.

Experimental pancreatitis
In vivo supramaximal stimulation of the pancreas with the cholecystokinin analogue caerulein rapidly induces a mild, self limiting, form of pancreatitis.14 This experimental model of pancreatitis is associated with the formation of considerable interstitial oedema, hyperamylasaemia, and a number of cell biological events that are considered characteristic for the disease process.16 It does not, however, cause pancreatic haemorrhage or significant acinar tissue necrosis.14 15 Male Wistar rats (280–320 g) were equipped with jugular vein catheters (PE 10) under general anaesthesia (Pentobarbital 60 mg/kg/intraperitoneally) and were allowed to recover for 12 hours with tap water ad libitum but no chow. Animals were then randomly divided into treatment groups of six or more animals and received either an infusion of caerulein (10 μg/kg/h in normal saline) or an equal volume of normal saline (controls) for up to six hours.

Vasoactive mediators
Administration of all vasoactive compounds was begun five minutes before the start of the caerulein infusion and continued until the end of the experiments. The range of concentrations for these therapeutic agents was chosen to include the lowest dose that was known to have a significant biological effect when given in vivo, and the highest doses that was either equivalent to the LD50 in rats or found to be associated with pharmacological toxicity unrelated to pancreatitis. All compounds and concentrations were investigated in control animals to exclude a potential induction of pancreatitis before they were used in caerulein pancreatitis animals. NO was substituted exogenously by administration of nitroglycerin (1 mg–10 mg/kg/h intravenously) and endogenously by infusing the natural substrate for NO synthetase L-arginine (50 mg-1000 mg/kg/h intravenously). NO generation was inhibited with the competitive inhibitor of N-synthase N–Nitro-L-Arginine methyl ester hydrochloride (L-NAME, 100 mg–900 mg/kg/h intravenously).17 To mimic adrenergic stimulation we used the catecholamine analogue phenylephrine (300 μg–10 mg/kg/h intravenously), and to antagonise the catecholamine effect we used the combined α-adrenergic and β-adrenergic blocker labetalol (5–50 mg/kg/h intravenously). To substitute kinins we infused active lys-bradykinin (18–180 mg/kg/h intravenously) and to antagonise their effect we used the potent and long acting bradykinin antagonist D-Arg[Hyp³, Thr⁵, D-Tic⁷, Oic⁹]-BK n CH₃COOH (HOE-140, 0.1–10 mg/kg/h subcutaneously).18

Morphology
For histological examination the pancreas was rapidly removed and pieces from the head, body, and tail of the organ were fixed in 10% neutral phosphate buffered formalin (4°C) and embedded in paraffin wax. Sections (5 μm) were stained with haematoxylin and eosin and graded as previously reported by an observer familiar with pancreatic pathology but unaware of the treatment groups.19 For morphometric evaluation of the extent of necrosis, sections from the head, body, and tail of the pancreas were photographed at a fixed magnification (×40), printed on 8 by 10 bonded paper, and the tissue affected measured on a graphic tablet and expressed as per cent of total tissue area (Tektronix 4933, IBM-PC).

For transmission electron microscopy strips of pancreas no larger than 2 mm were immersed in iced 2% glutaraldehyde/2% formaldehyde solution at pH 7.4 with 0.1 M cacodylate buffer. Blocks were postfixed in 1% osmiumtetroxide and embedded in Epon. Silver thin sections were contrasted with uranyl and lead and viewed on a Zeiss EM 10 electron microscope (Oberkochen, Germany).

Vascular resin casts
To obtain resin casts of the pancreatic microvasculature animals were anaesthetised again at the end of the experiment. The abdomen was opened through a midline celiotomy and the aorta ligated above the caeliac artery and below the superior mesenteric artery. Into this closed aortic segment Krebs-Ringer buffer was perfused through a 25 G cannula until venous return to the vena cava was clear. The subsequent perfusion medium used contained 1% paraformaldehyde and 1% glutaraldehyde and was immediately followed by low viscosity resin (Mercox). The resin was allowed to harden for 30 minutes before the entire pancreas was removed.6 The tissue was subsequently digested in concentrated potassium hydroxide at 60°C for four to five weeks with weekly changes of solution. After complete dissolution of the pancreatic tissue the casts were air dried, sputtered with gold/palladium, and mounted for scanning electron microscopy. A Zeiss SEM (Oberkochen, Germany) at magnifications ranging from 20 to 2000 was used to examine the microvascular anatomy.

Assays
An increase in serum amylase activity was regarded as an indicator for acute pancreatitis and measured as described by Pierre et al using maltotetraose as a substrate.20 An increase in pancreatic water content was considered as an indicator for the formation of pancreatic oedema. After the rats were killed, the pancreas was rapidly removed, blotted dry on filter paper, weighed and then dried again after desiccation (48 hours, 160°C). The wet weight/dry weight ratio was expressed in per cent.15
of inducing acute pancreatitis by itself. The intravenous administration of a supramaximal concentration of the synthetic cholecystokinin analogue caerulein resulted in hyperamylasaemia, pancreatic oedema, and the formation of intra-acinar cell vacuoles in all animals. These phenomena are known to characterise this mild model of pancreatitis.

Serum amylase
The hyperamylasaemia induced by supramaximal caerulein stimulation was not changed by NO substitution (nitroglycerin and L-Arginine) nor by NO inhibition (L-NAME) irrespective of the concentration given (Fig 1). The same failure to reduce hyperamylasaemia was seen when catecholamines were infused (phenylephrine) or their action antagonised (labetalol). Infusion of bradykinin reduced serum amylase activity already at the lowest concentration tested (18 ng/kg/h). No additional reduction of circulating serum amylase activity was seen with higher concentrations of bradykinin. The inhibition of bradykinin had no significant effect on hyperamylasaemia at six hours.

Statistical analysis
The results are the mean (SEM) of six or more experiments in each treatment and control group. We used analysis of variance (ANOVA) to evaluate differences between experimental groups and the Tukey method \(^{21}\) as a post hoc test. Significant differences were defined as those with an error probability \(<0.05\) and are shown with asterisks in the Figures.

Results
The inhibition or substitution of none of the vasoactive compounds alone resulted in either hyperamylasaemia, pancreatic oedema, or tissue haemorrhage and necrosis regardless of the concentration used for infusion. We concluded that, not even at toxic concentrations, was any of the agonists and antagonists capable

Pancreatic oedema
Caerulein infusion induced gross pancreatic oedema as reflected by an increase in pancreatic water content to up to 90% (Fig 2). Neither NO substitution nor NO inhibition had any effect on the formation of oedema at three hours and six hours after the start of the secretagogue infusion. This failure to reduce oedema was irrespective of the concentration given. Similarly, phenylephrine, labetalol, and bradykinin had no significant effect on pancreatic water content at either three hours or six hours (Fig 2) of pancreatitis. Bradykinin inhibition with its antagonist HOE-140 significantly reduced pancreatic oedema at three hours and six hours of pancreatitis at the lowest concentration tested (0.1 mg/kg/h). Increased concentrations of HOE-140 did not further reduce pancreatic water content and could not completely prevent the formation of pancreatic oedema at six hours.

Morphology
Supramaximal stimulation with caerulein resulted in interstitial oedema and the formation of large intracellular vacuoles in pancreatic acinar cells. On light or electron microscopic examination caerulein infusion was not seen to be associated with either significant necrosis of acinar tissue or haemorrhage from pancreatic blood vessels (Fig 3A). Neither the substitution of NO nor the inhibition of NO synthase changed the morphological appearance of caerulein induced pancreatitis (Fig 3A). The same absence of morphological differences to caerulein pancreatitis was found for phenylephrine, labetalol, and bradykinin. None of these compounds prevented the interstitial oedema and intracellular vacuolisation and none induced haemorrhage and necrosis in the pancreas.
three hours massive haemorrhage was apparent at the end of the observation period (Fig 3B). Necrotic pancreatic tissue was only found in areas of haemorrhage or its immediate vicinity suggesting that cellular necrosis was secondary to vascular damage. In all groups that had received caerulein the morphometric extent of necrotic acinar cells never exceeded 7% whereas in animals with coadministration of HOE-140 the area affected by tissue necrosis could be as much as 36% (Fig 4).

**Transmission electron microscopy**

On ultrastructural examination of tissue from pancreatitis animals large cytoplasmic vacuoles were easily identified in acinar cells of the pancreas (Fig 5). Microvascular endothelial cells were found to contain occasional small vacuoles and form intraluminal protrusions but the integrity of capillaries and venules remained invariably intact and no bleeding was seen (Fig 5A). None of the mediators and their antagonists with the exception of HOE-140 changed the appearance of either the acinar tissue or the small vessels of the pancreas. Administration of the bradykinin antagonist was found to be associated with a resolution of the limiting membranes of endothelial cells in capillaries and venules as early as three hours after the start of pancreatitis (Fig 5B). It is important to note that administration of HOE-140 alone in the absence of caerulein infusion had no effect on acinar cell integrity and vascular morphology of the pancreas. At six hours of caerulein induced pancreatitis many venules and capillaries, but not arteries, had completely lost part of their endothelial circumference (Fig 5C) and multiple erythrocytes and leucocytes had infiltrated the interstitial tissue surrounding these vascular leaks. Necrotic pancreatic acinar cells were found in great numbers only in these areas of haemorrhage. These phenomena were unique for the experimental group that had received caerulein infusion together with the bradykinin antagonist and was not seen with any of the other mediators or with caerulein infusion alone.

**Scanning electron microscopy**

In the normal rat pancreas microvessels and capillaries formed multiple anastomoses resulting in a continuous acinar network of small blood vessels (Fig 6A). Caerulein induced pancreatitis was not associated with any gross changes of the vessel wall morphology but with a significant reduction in anastomoses (Fig 6B) and many capillaries were found to end blindly. This appearance of the capillary network was neither improved nor worsened by either nitroglycerin, L-arginine, L-NAME, phenylephrine, labetalol, or bradykinin. The vascular morphology was found to differ significantly after administration of the bradykinin antagonist HOE-140. After three hours the resin cast of small venules and capillaries were covered with multiple blebs showing that low viscosity resin could protrude from the vessel towards the surrounding tissue (Fig 6C).
part in the development and outcome of pancreatitis.\textsuperscript{22,25} Often these suggestions were based on the finding that circulating serum concentrations of a given mediator were either greatly increased or rapidly consumed during the disease process.\textsuperscript{26,27} Evidence, however, that either the presence or absence of one of these hormones and peptides directly participated in the transition from mild to severe pancreatitis remained sparse. We have studied three potential candidates that have previously been implicated in this context. The finding that extreme stress\textsuperscript{7} can convert oedematous experimental pancreatitis into a necrotising variety has led others to assume that adrenal hormones, and catecholamines in particular, could represent the critical vasoactive mediator. Another candidate would be NO. NO is not only a very potent regulator of the pancreatic blood supply\textsuperscript{11} but has also been implicated in the severity of acute pancreatitis.\textsuperscript{28} The third vasoactive mediator we studied was bradykinin. The activation of kinins is a consistent finding in experimental and clinical pancreatitis\textsuperscript{26,27} and it has been suggested that the inhibition of bradykinin could have a beneficial effect on the course of the disease.\textsuperscript{29}

We have used an animal model of acute pancreatitis, which is characterised by massive oedema, hyperamylasaemia, and a number of cell biological events that are thought to be critical for the initiation of the disease.\textsuperscript{13,16} This caerulein induced variety is not associated with either haemorrhage or significant necrosis of the gland.\textsuperscript{14,15} Although, as in other models of the disease, the microvascular perfusion is impaired,\textsuperscript{6} the vascular permeability is increased,\textsuperscript{30} and the endothelial integrity is affected,\textsuperscript{5} all pancreatitis animals recover rapidly once the secretagogue infusion is stopped after six or 12 hours and neither tissue necrosis nor chronic pancreatitis result.\textsuperscript{31} We have investigated whether one of the above mentioned mediators could convert this mild and self limiting form of the disease into a haemorrhagic and necrotising variety. To this end we used compounds that either liberate or activate these mediators or that can effectively inhibit their actions. Alternative experimental models of acute pancreatitis such as intraduodenal taurocholate injection or feeding a choline deficient and ethionine supplemented diet were considered unsuitable for this investigation because they are in themselves associated with severe tissue haemorrhage and necrosis and a mortality of 100% is an inherent part of the protocol. Therefore these severe models can be used to test whether a given experimental therapy prevents the deleterious sequelae of pancreatitis but they seem ill suited to investigate the pathophysiological factors that determine the progression from a mild to a severe disease variety.

Our data show that the release of catecholamines seems to be unrelated to the development of pancreatic haemorrhage and necrosis in acute pancreatitis. This finding suggests that the progression from mild to severe pancreatitis under water immersion stress\textsuperscript{7} is

Discussion

A large body of experimental and clinical studies\textsuperscript{2,4,5,22,23} suggest that an impairment of the pancreatic blood supply, microcirculation, and tissue oxygenation can contribute greatly to the severity of acute pancreatitis. While the most common aetiological factors, ethanol and gall stones,\textsuperscript{24} are not known to determine the ultimate severity of the disease\textsuperscript{1} a number of studies have suggested that the liberation or activation of vasoactive mediators may play a
unlikely to be accounted for by adrenal catecholamines. It is, however, reassuring to know that catecholamines do not contribute to greater disease severity because they are frequently used drugs in patients with pancreatitis during intensive care. Although NO has been identified as a very potent regulator of the pancreatic blood supply,\textsuperscript{11} as well as of vascular permeability,\textsuperscript{32} we found that neither the endogenous and exogenous substitution of NO nor its inhibition could induce haemorrhagic or necrotising pancreatitis. It is therefore unlikely that NO plays a pathophysiological part in the mechanisms that determine the severity of pancreatitis.

Bradykinin is a potent bioactive peptide that is thought to play a part in the circulatory changes associated with stress and shock.\textsuperscript{33} In our experiments the administration of active bradykinin reduced the amount of circulating serum amylase activity. Rather than interpret this effect as an indicator of decreased cellular damage in the pancreas we suggest that bradykinin has increased the vascular permeability for amylase because this is a known property of the peptide\textsuperscript{18} and no other parameter of pancreatitis was changed by the treatment with bradykinin. Haemorrhage and necrosis of the pancreas were not seen after bradykinin application irrespective of the concentration given. The inhibition of bradykinin reduced the pancreatitis induced rise in pancreatic water content, as a reflection of pancreatic oedema formation, by roughly 70%. This, again, supports the assumption that bradykinin is critically involved in the vascular permeability changes during pancreatitis. In parallel with the reduction of oedema the inhibition of bradykinin was associated with significant haemorrhage and necrosis of the pancreas. Even minimally effective concentrations of the potent, long acting bradykinin antagonist HOE-140 were able to induce endothelial damage in pancreatic capillaries and venules. The extent of necrosis in pancreatic acinar tissue seemed to be directly related to the appearance and localisation of haemorrhage from these damaged vessels. It is highly unlikely that a pharmacological action other than the kinin inhibiting effect of HOE-140 accounts for the biochemical and morphological changes seen during pancreatitis. The antagonist is a synthetic decapeptide structurally derived from bradykinin but contains two iminoacids (D-Tic and Dic) at position 7 and 8. It has been shown in a number of studies to be very specific in antagonising bradykinin induced effects in vivo and in vitro and it has a high affinity to the bradykinin receptor.\textsuperscript{18} Interestingly neither bradykinin nor its antagonist had any effect on the pancreas when given to control animals whereas during pancreatitis the changes seen were dramatic.

It has been suggested that the activation of bradykinin could represent a critical step in the pathophysiology of pancreatitis and this hypothesis has become textbook knowledge.\textsuperscript{35} Accordingly the inhibition of bradykinin was proposed as a potential treatment modality for acute pancreatitis.\textsuperscript{29} Our data suggest that the opposite is, in fact, the case. The inhibition of bradykinin, rather than its activation or administration, was seen to have a deleterious effect on the development and severity of the disease. Although findings from experimental studies should only be applied to clinical pancreatitis with great caution our results may have several pathophysiological implications. If inhibition of bradykinin can greatly increase disease severity then an impaired release and activation of bradykinin or, alternatively, a rapid degradation of circulating bradykinin by carboxypeptidase and angiotensin converting enzyme could participate in the progression from oedematous to necrotising pancreatitis in humans. As far as therapeutic aspects are concerned our data suggest that an inhibition of bradykinin degrading enzymes (that is, with angiotensin converting enzyme blockers) could
represent a much more promising treatment regimen for acute pancreatitis than antagonists of bradykinin.

We have investigated whether the activation or inhibition of NO, catecholamines or bradykinin can convert a mild, oedematous model of pancreatitis into a necrotising variety of the disease. At a range of concentrations limited by pharmacological toxicity neither the administration nor the inhibition of NO and catecholamines had any significant effect on the severity of acute pancreatitis. The administration of bradykinin, a peptide known to influence vascular permeability, reduced hyperamylasemia. The inhibition of bradykinin reduced pancreatic oedema and simultaneously converted the oedematous pancreatitis into haemorrhagic and necrotising pancreatitis by a process that entailed endothelial damage to pancreatic capillaries and venules. These experimental findings may reflect pathophysiological events that participate in the severity and outcome of human acute pancreatitis. Inadequate activation of bradykinin or consumption of circulating bradykinin might represent a critical event that allows for the progression from oedematous to haemorrhagic necrotising pancreatitis.

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