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RESEARCH PAPER

Kallikrein inhibitors limit kinin B_2 antagonist-induced progression of oedematous to haemorrhagic pancreatitis in rats

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Background and purpose: Exocrine hyperstimulation with caerulein is an established model for oedematous acute pancreatitis. Prevention of oedema formation by bradykinin B_2 receptor antagonists induces a progression to a haemorrhagic course in this model. We have investigated whether increased kallikrein activity in the pancreas is responsible for vascular damage and whether this could be prevented by selective kallikrein inhibitors.
Experimental approach: Caerulein was infused i.v. and vascular damage was assessed by histological evaluation and determination of haemoglobin accumulation in the tissue. In addition, oedema formation, tissue and plasma kallikrein (PK) activities and the endogenous kallikrein inhibitors α_1-antitrypsin (α_1-AT) and α_2-macroglobulin (α_2-M) were measured.
Key results: Haemorrhagic lesions induced by icatibant in caerulein-induced pancreatitis were associated with a reduction in α_1-AT and α_2-M in the pancreas and a concomitant augmentation of tissue kallikrein (TK) activity. The TK inhibitor VA999024 (previously FE999024), or its combination with the PK inhibitor VA999026 (previously FE999026), inhibited oedema formation to the same extent but did not induce vascular damage. Furthermore, VA999024 inhibited TK activity. When icatibant was combined with VA999024 and VA999026, progression from oedematous to haemorrhagic pancreatitis was abolished.
Conclusions and implications: Reduced oedema formation by B_2 antagonists prevented influx of endogenous kallikrein inhibitors and led to an excessive activity of kallikrein in the pancreas leading to vascular damage. This can be prevented by a combined inhibition of both tissue-type and plasma-type kallikrein. Kallikrein inhibitors thus should be further evaluated for their therapeutic potential in preventing haemorrhagic lesions in acute pancreatitis.
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Keywords: pancreatitis; vascular damage; kallikreins; kallikrein inhibitors

Abbreviations: α_1-AT, α_1-antitrypsin; α_2-M, α_2-macroglobulin; PK, plasma kallikrein; TK, tissue kallikrein

Introduction

Acute pancreatitis is a disease with increasing incidence in the Western world (Corfield *et al*., 1985; Wilson and Imrie, 1990), even in children (Nydegger *et al*., 2007). The disease can take two different courses, either the oedematous course, which is self-limiting, or the haemorrhagic-necrotizing course, which is associated with considerable mortality (Büchler, 1991). Invariably, the disease is associated with the development of a massive oedema of the gland and also pain symptoms that are among the most severe to be experienced by patients. Because the mammalian kinins, bradykinin and kallidin, produce all classical signs of inflammation when given exogenously and are also among the most painful endogenous algogenic substances (Armstrong *et al*., 1957; Juan and Lembeck, 1974), the development of potent and selective bradykinin receptor antagonists such as icatibant (Hock *et al*., 1991; Lembeck *et al*., 1991; Wirth *et al*., 1991) had opened the possibility to test whether endogenous kinins contribute to the pathophysiology of pancreatitis. Indeed, previous experimental studies have shown that the inflammatory oedema is due, at least to a great part, to the endogenous release of kinins acting on B_2 receptors (Griesbacher and Lembeck, 1992; Griesbacher *et al*., 1993).

Although kinins do not participate in the induction of acute pancreatitis by caerulein (Griesbacher and Lembeck, 1992; Weidenbach *et al*., 1995), the application of B_2 antagonists not only prevented oedema formation but as a consequence also improved the removal of activated diges-

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tive enzymes from the interstitial compartment of the gland in the early stages of the inflammatory disease without apparent negative consequences on pancreatic exocrine and endocrine or liver functions (Griesbacher *et al*., 1995). Furthermore, in other, even necrotizing, models of pancreatitis, icatibant was shown to potently prevent the development of necroses (Hoffmann *et al*., 1996, 1997) and also to reduce the mortality rate significantly (Hoffmann *et al*., 1996; Yekebas *et al*., 2000). However, early blockade of B_2 receptors apparently not only has beneficial effects but can also lead to tissue damage (Weidenbach *et al*., 1995, 1996).

One interesting fact about the action of B_2 antagonists in acute oedematous pancreatitis is that such a treatment not only prevents increases in vascular permeability but also induces a massive augmentation of the increases in tissue kallikrein (TK) activity in the pancreas, most likely by preventing the influx, through the inflammatory oedema, of endogenous protease inhibitors into the inflamed tissue (Griesbacher *et al*., 2003). Kinins are released in acute pancreatitis mainly by the action of TK (human kallikrein 1), whereas plasma kallikrein (PK) seems to be of lesser importance (Griesbacher *et al*., 2002). In addition to their properties of being specific kinin-releasing enzymes, kallikreins also have a number of other, non-kininogenase, functions in physiological and pathophysiological conditions (Bhoola *et al*., 1992). Particularly, both TK and PK can activate a number of other enzymes that are involved in tissue injury and repair, such as metalloproteinases (Tschesche *et al*., 1989; Saunders *et al*., 2005).

The progression from an interstitial-oedematous to a haemorrhagic-necrotizing course is a serious event in acute pancreatitis as this drastically worsens the prognosis of the disease. Experimental studies on the mechanisms that occur in this disease are limited by the fact that all standard animal models reproduce only either of the two principal disease courses, but cannot be used for investigations on mechanisms leading to the progression from mild to severe forms. Combining caerulein-induced hyperstimulation of the exocrine function of the pancreas, the standard model for oedematous pancreatitis, with retrograde injections of bile acids and trypsin (Yamaguchi *et al*., 1990) or the combination of the caerulein model with water immersion stress (Schmidt *et al*., 1992) have been proposed to mimic progression towards an increased tissue damage, but these procedures have the disadvantage of being more invasive than standard models or involve experimentation in conscious animals. The finding that a bradykinin B_2 receptor antagonist causes a progression from oedematous to haemorrhagic pancreatitis in the caerulein model (Weidenbach *et al*., 1995, 1996) provides a much more suitable experimental procedure as it is much less invasive. At present, however, nothing is known about the possible mechanisms that might be involved in the development of increased tissue damage in this model.

The aim of the current study was, therefore, to study whether selective inhibitors of TK and PK could be used to block the kallikrein–kinin system in acute pancreatitis as an alternative to the use of a B_2 receptor antagonist, without the adverse consequence of vascular damage, and, second, whether kallikrein inhibitors could in fact even prevent the vascular damage induced by a B_2 antagonist.

Materials and methods

Surgical procedure

All animal experiments followed the Principles of Laboratory Animal Care (National Institutes of Health) and the Austrian Law on Experimentation in Living Animals. Permission for the experiments was granted by the Commission for Animal Experiments of the Austrian Ministry of Science.

Female Sprague–Dawley rats (200–250 g; Department of Laboratory Animal Sciences and Genetics, Medical University of Vienna, Vienna, Austria) were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}; i.p.) and phenobarbitone sodium (20 mg kg^{-1}; i.p.). A jugular vein was exposed by a longitudinal incision of the ventromedial skin of the neck and was cannulated with a polyethylene tubing. The cholecystokinin analogue caerulein was infused at a rate of 4 nmol kg^{-1} h^{-1} over a period of 2 h; control animals were infused with an appropriate volume of phosphate-buffered saline (8 mL kg^{-1} h^{-1}) instead. The depth of anaesthesia was monitored by assessing palpebral, corneal and toe pinch reflexes at regular intervals throughout the experiment. If needed, anaesthesia was prolonged by an additional s.c. injection of phenobarbitone sodium (<5 mg kg^{-1}).

Pretreatments with the bradykinin B_2 receptor antagonist icatibant (100 nmol kg^{-1}), the selective TK inhibitor VA999024 (Evans *et al*., 1996a) (20 μmol kg^{-1}) and/or the selective PK inhibitor VA999026 (Evans *et al*., 1996b) (20 μmol kg^{-1}) were given i.p. 30 min before the start of the i.v. infusion of caerulein. All injections were repeated twice at 2-h intervals using half the dose of the initial injection, that is, 50 nmol kg^{-1} of icatibant, and 10 μmol kg^{-1} of VA999024 and VA999026. Control animals were injected with the vehicle, phosphate-buffered saline. At 6 h after the beginning of the 2 h infusion of caerulein or saline, the animals were killed by i.p. injection of an overdose of pentobarbitone sodium.

Assays

Tissue samples of the pancreas were excised and weighed for the determination of wet weight. Samples were immersed in 2 mL 154 mmol L^{-1} NaCl solution and centrifuged at 20 400 *g* at 4 °C; supernatants were then stored at −80 °C until assayed. Dry weight of tissue samples was determined after 24 h drying in a vacuum centrifuge. The difference between wet and dry weight was taken as fluid weight, and the water content of the tissue samples was calculated as fluid weight per dry weight of tissue as a measure for inflammatory oedema formation.

Activities of TK and PK were determined by photometrical measurement using the chromogenic substrates S-2266 (D-Val-Leu-Arg-*p*-nitroanilide) (Amundsen *et al*., 1979) and S-2302 (D-Pro-Phe-Arg-*p*-nitroanilide) (Ito and Statland, 1981), respectively. All measurements were carried out in duplicate. Purified porcine kallikrein preparations were used to control the performance of the system. Values are given as

pkat g^{-1} (pmol s^{-1} g^{-1}) dry weight of tissue. The inhibitory activities in the pancreatic tissue of α_1-antitrypsin (α_1-AT) and α_2-macroglobulin (α_2-M) were measured by chromogenic substrate tests (Unitest α_1-AT assay, Unitest α_2-M assay; Unicorn Diagnostics, London, UK). The activities of α_1-AT and α_2-M were then calculated using standard plasmas containing known amounts of α_1-AT (0.95 U mL^{-1}) and α_2-M (1.06 U mL^{-1}). Activities of α_1-AT and α_2-M in blood plasma were associated with very large variations (unpublished data), so that plasma measurements could not be used for comparisons between experimental groups.

For the quantification of haemorrhagic lesions by the determination of haemoglobin in the pancreatic tissue (see below), the body of the animal was perfused through the aorta after exsanguination with 40 mL of a 154 mmol L^{-1} NaCl solution to remove blood from the intravascular compartment. Tissue samples were then incubated for 24 h in 2 mL distilled water at 4 °C for disruption of erythrocyte cell membranes before centrifugation at 20 400 *g*. Haemoglobin was quantified in the supernatant after chromogenic reaction with tetramethylbenzidine using scanning spectrophotometry (Kahn *et al.*, 1981). The amount of haemoglobin present in the tissue is given as micrograms of haemoglobin per milligram dry weight of tissue. Histological sections (1 μm) of the pancreas were taken at the end of the experiments and were stained with haematoxylin and eosin. Representative photomicrographs showing blood vessels were taken at a magnification of × 400.

Statistical analysis

Because the variances of data obtained in caerulein-induced pancreatitis differed greatly from those obtained in control animals without pancreatitis and data frequently showed significant deviations from normal distributions, comparisons between different treatment groups were made using non-parametric analysis of variance (Kruskal–Wallis *H* test) and multiple non-parametric comparisons for independent data (Dunn test). Probability values of $P<0.05$ were considered significant. All values presented are arithmetical means with s.e.mean.

Materials

VA999024 ((2*S*,2′*R*)-2-(2′-amino-3′-(4″chlorophenyl)propanoylamino)-*N*-(3-guanidinopropyl)-3-(1-naphthyl)propanoamide; previous names CH-2856 and FE999024) and VA999026 ((2′*S*, 2″*R*)-4-(2′-(2″-(carboxymethylamino)-3″-cyclohexyl-propanoylamino)-3′-phenyl-propanoylamino)piperidine-1-carboxamidine; previous names CH-4215 and FE999026) were synthesized by Vantia Ltd (Southampton Science Park, Southampton, UK) and were dissolved in 154 mmol L^{-1} NaCl solution at a concentration of 20 μmol mL^{-1}. Caerulein (Sigma Chemical Co., St Louis, MO, USA) was dissolved in phosphate-buffered saline; stock solutions were prepared at a concentration of 50 μmol L^{-1} and further dilutions were made with phosphate-buffered saline (composition in mmol L^{-1}): NaCl 136.9, KCl 2.7, KH_2PO_4 1.5, Na_2HPO_4 7.7; pH 7.4). All salts were of analytical grade and were obtained from Merck (Darmstadt, Germany). Other materials were pentobarbitone sodium (Nembutal; Sanofi Santé Animale, Libourne, France), phenobarbitone sodium (Vetanarcol; Veterinaria AG, Zurich, Switzerland), S-2266 (COA-Chrom Diagnostica, Vienna, Austria) and S-2302 (Quadratech, Epsom, UK).

Nomenclature

Nomenclature of bradykinin B_2 receptors follows the BJP's revised Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Pancreatic oedema formation

In the first set of experiments, the selective TK inhibitor VA999024 and the selective PK inhibitor VA999026 were compared with the bradykinin B_2 receptor antagonist icatibant with respect to their ability to inhibit the formation of inflammatory oedema during caerulein-induced pancreatitis (Figure 1a). Water content measured 6 h after the beginning of the experiment, that is, 4 h after the end of the caerulein infusion, was about fourfold higher than that obtained in animals infused with saline instead of caerulein. Icatibant was given as a pretreatment (100 nmol kg^{-1}; s.c.) 30 min before caerulein and was repeated twice at 2-h intervals at a dose of 50 nmol kg^{-1}. This treatment reduced oedema formation at 6 h to about half of that seen with caerulein alone. VA999024 and VA999026 were given at doses of 20 μmol kg^{-1} for the first dose and 10 μmol kg^{-1} for the two subsequent doses. VA999024 given alone inhibited oedema formation to the same extent as icatibant. VA999026 had no significant inhibitory effect on oedema formation. A combined treatment with both kallikrein inhibitors was not more effective than the treatment with VA999024 alone.

Vascular damage in the pancreas

For quantification of vascular damage, haemoglobin was extracted from the extracellular compartment of the pancreatic tissue (Figure 1b). The difference in haemoglobin values in caerulein-induced pancreatitis compared with animals without pancreatitis did not reach the predefined probability level of 0.05. Pretreatment with the B_2 antagonist icatibant in caerulein-treated rats caused a dramatic increase in haemoglobin accumulation in the tissue. In contrast to this, neither VA999024 or VA999026 given alone, nor their combined application, had any effect on haemoglobin accumulation in the tissue.

During caerulein-induced pancreatitis, blood vessels showed no signs of damage, that is, no extravasation of red blood cells could be found in the histological sections of the pancreatic tissue (Figure 1c). When rats with caerulein-induced pancreatitis were pretreated with the B_2 antagonist icatibant, most pancreatic blood vessels seemed undamaged but a number of pancreatic venules showed considerable accumulation of erythrocytes in the extravascular space. Necrotic elements were not found. When animals with caerulein-induced pancreatitis were pretreated with the TK inhibitor VA999024 or with the PK inhibitor VA999026

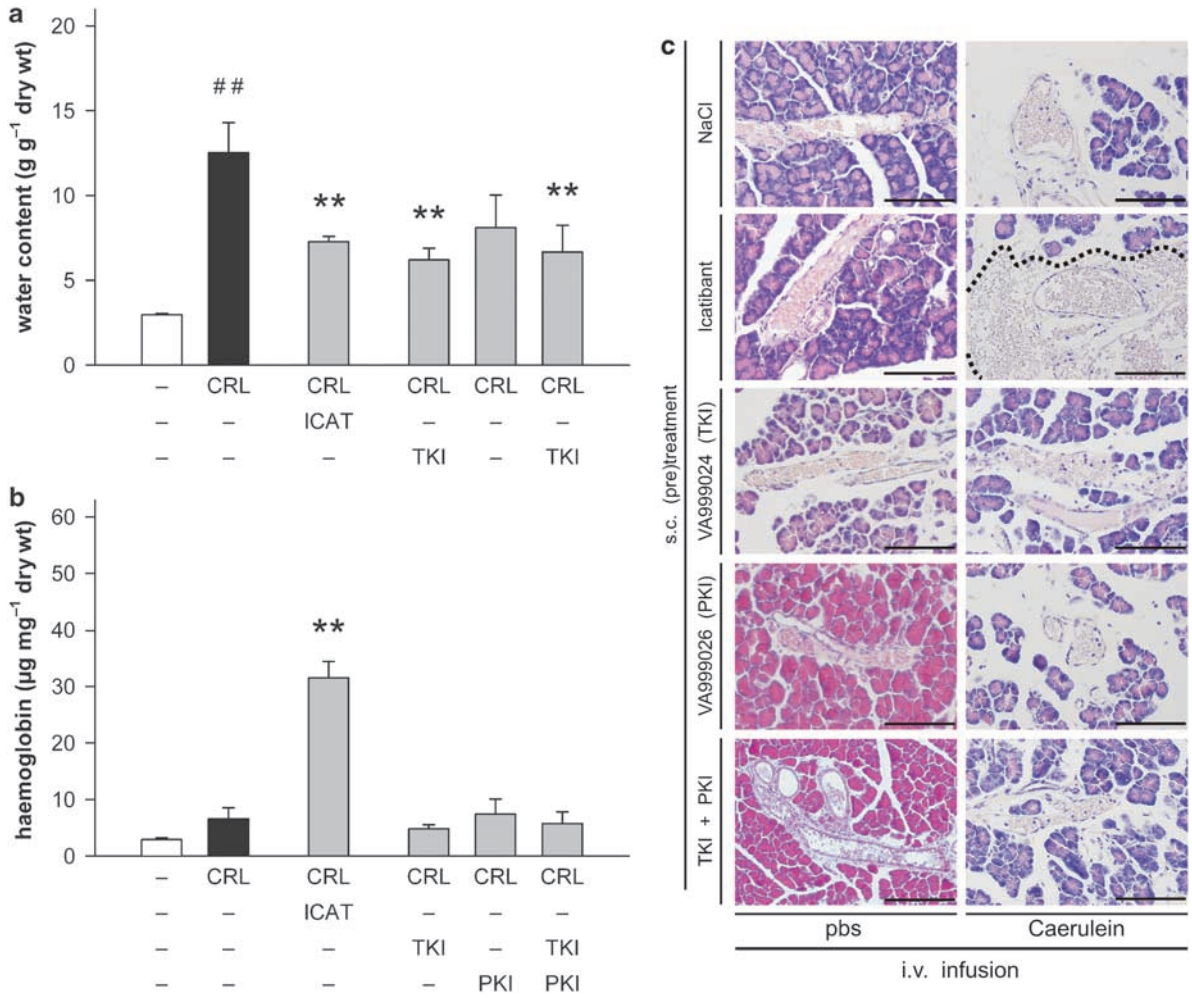

Figure 1 Effects of the B_2 antagonist icatibant (ICAT), the TK inhibitor VA999024 (TKI) and the PK inhibitor VA999026 (PKI) in caerulein (CRL)-induced pancreatitis. (**a**) Oedema formation and (**b**) haemoglobin accumulation in the pancreas: CRL or phosphate-buffered saline (PBS) was infused i.v; icatibant (100 nmol kg^{-1}), VA999024 (20 µmol kg^{-1}) and/or VA999026 (20 µmol kg^{-1}) were injected i.p. at −30 min. Control animals were injected with saline (NaCl). All treatments were repeated twice at 2-h intervals using half of the initial dose. Values are means + s.e.mean ($n = 5$–10). $^{\#\#}P < 0.01$ vs controls without CRL; $^{**}P < 0.05$ vs CRL + ICAT. (**c**) Photomicrographs of pancreatic blood vessels at 6 h. Dashed lines delineate areas of dense extravascular erythrocyte accumulation (haematoxylin and eosin stain; scale bar: 100 µm).

instead of icatibant, the pancreatic blood vessels appeared normal. A combined pretreatment with VA999024 and VA999026 also left pancreatic blood vessels unaltered.

Kallikrein activities in the pancreatic tissue

In rats with caerulein-induced pancreatitis, TK-like activity in the pancreas increased about 3- to 4-fold over values found in control animals without pancreatitis (Figure 2a). The treatment with icatibant caused a further, significant, augmentation of the TK activity in the pancreas. In contrast, the TK inhibitor VA999024 significantly reduced the TK activity to values also seen in control animals that had not received caerulein. The treatment with the PK inhibitor VA999026 did not alter increased TK activity during pancreatitis. The combination of both kallikrein inhibitors was as effective as VA999024 alone.

PK-like activity in the pancreas (Figure 2b) was also significantly increased during caerulein-induced pancreatitis. Unlike its effect on the TK activity, icatibant had no consistent effect on PK activity in the pancreas. In six out of eight animals, PK activity was completely absent from the tissue, whereas in other two animals, PK activity was much larger than that observed in untreated pancreatitis. PK-like activity was also reduced by the TK inhibitor VA999024. The PK inhibitor VA999026, however, inhibited PK activity even further; values in this experimental group were even lower than those observed in control animals without caerulein pancreatitis. The combined application of VA999024 and VA999026 together also completely prevented any increase in PK activity in the pancreatic tissue.

Activities of endogenous kallikrein inhibitors in the pancreas

α_1-AT and α_2-M were measured as two examples out of a number of endogenous protease inhibitors that are capable of inhibiting kallikreins (McConnell and Loeb, 1974; Habal *et al*., 1976; Hirano *et al*., 1984). The inhibitory activities of

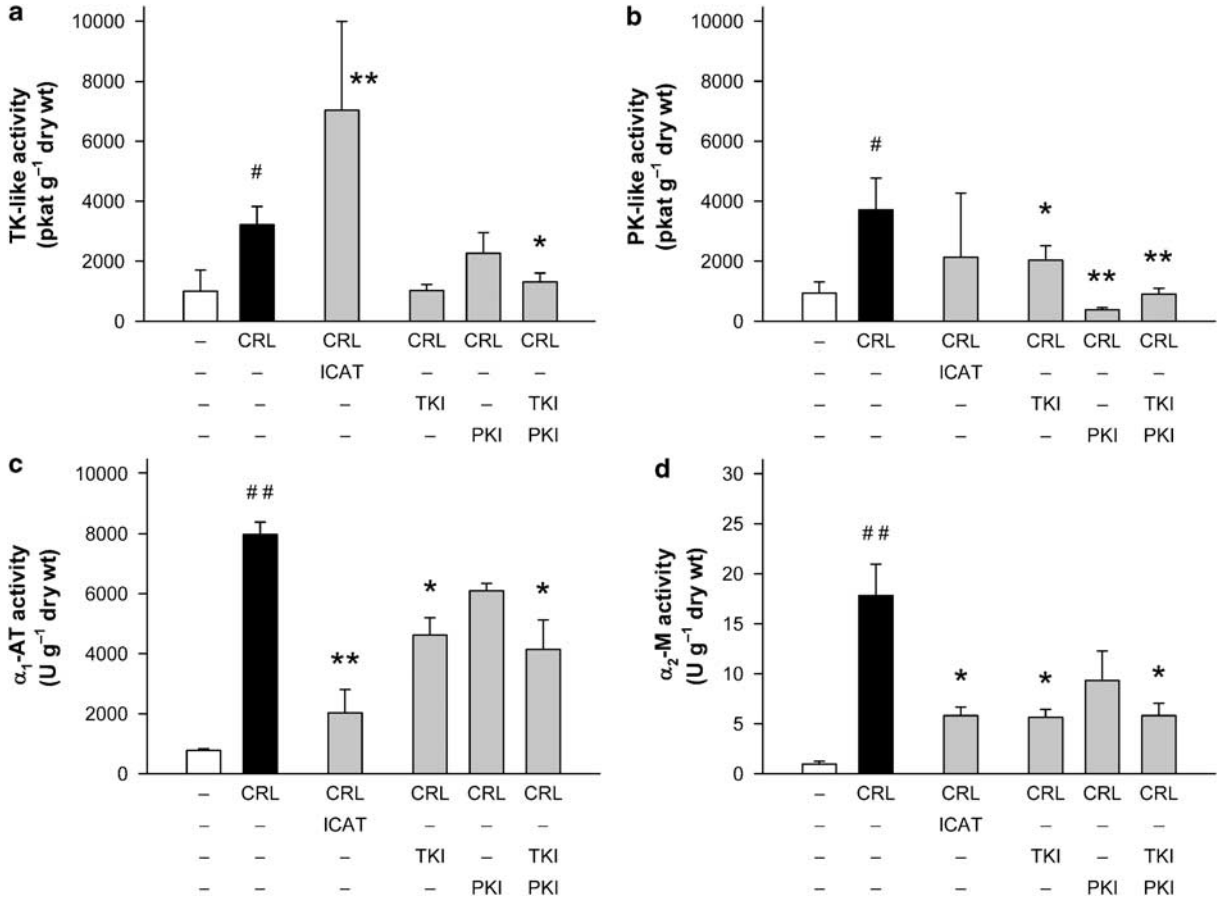

Figure 2 Catalytic activities of (**a**) tissue kallikrein (TK) and (**b**) plasma kallikrein (PK), and inhibitory activities of (**c**) α_1-antitrypsin (α_1-AT) and (**d**) α_2-macroglobulin (α_2-M) in the pancreatic tissue during caerulein-induced pancreatitis. Pretreatments with the kinin B_2 receptor antagonist icatibant (ICAT; 100 nmol kg^{-1}), the TK inhibitor VA999024 (TKI; 20 μmol kg^{-1}) and/or the PK inhibitor VA999026 (PKI; 20 μmol kg^{-1}) were given i.p. 30 min before caerulein; all i.p. injections were repeated twice at half doses at 2-h intervals; control animals were injected i.p with 154 mmol L^{-1} NaCl. Measurements were taken at 6 h. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs controls without CRL; $^{*}P<0.05$, $^{**}P<0.01$ vs CRL alone. Values are means + s.e.mean; $n=5$–10.

α_1-AT (Figure 2c) and of α_2-M (Figure 2d) increased about 10- and 20-fold, respectively, in the pancreatic tissue during caerulein-induced pancreatitis when compared with animals without inflammation. The greater part of the increased amounts of active α_1-AT and α_2-M in the pancreas was prevented by pretreatment with icatibant. The TK inhibitor VA999024 had a similar effect, although its effect on α_1-AT seemed smaller than its effect on α_2-M. Although the activities of α_1-AT and α_2-M activities in the pancreas seemed to be lower following treatment with the PK inhibitor VA999026 as compared with untreated pancreatitis, the difference was not statistically significant. The effect of VA999024 could not be further increased by a combination with VA999026.

Effect of kallikrein inhibitors on icatibant-induced vascular lesions

In the second set of experiments, we examined the effects of the kallikrein inhibitors given in addition to the B_2 antagonist icatibant (Figure 3). Neither the TK inhibitor VA999024 nor the PK inhibitor VA999026 nor their combination was able to reduce the effect of icatibant on oedema formation any further (Figure 3a).

Neither VA999024 nor VA999026 was able to affect the elevated haemoglobin accumulation caused by the B_2 antagonist (Figure 3b). If, however, the inhibitors of TK and PK were combined, the effect of the B_2 antagonist was significantly inhibited. Haemoglobin values in this group seemed to be higher than in animals without pancreatitis (treatment with icatibant alone), but the significance of difference did not reach the pre-set value of 0.05. The administration of icatibant to animals without pancreatitis did not alter basal haemoglobin values in the pancreas. Histological evaluation of tissue sections obtained from animals that had received icatibant together with VA999024 and/or VA999026 (Figure 3c) confirmed the quantitative data shown in Figure 3b. Both treatments were still associated with the extravasation of red blood cells from pancreatic venules. In contrast, pancreatic blood vessels remained intact in animals with caerulein-induced pancreatitis where icatibant was combined with both kallikrein inhibitors. The administration of icatibant in animals without caerulein-induced pancreatitis was not associated with any kind of tissue damage (see Figure 1c).

When VA999024 was administered to animals that had received icatibant, the greatly augmented TK activities

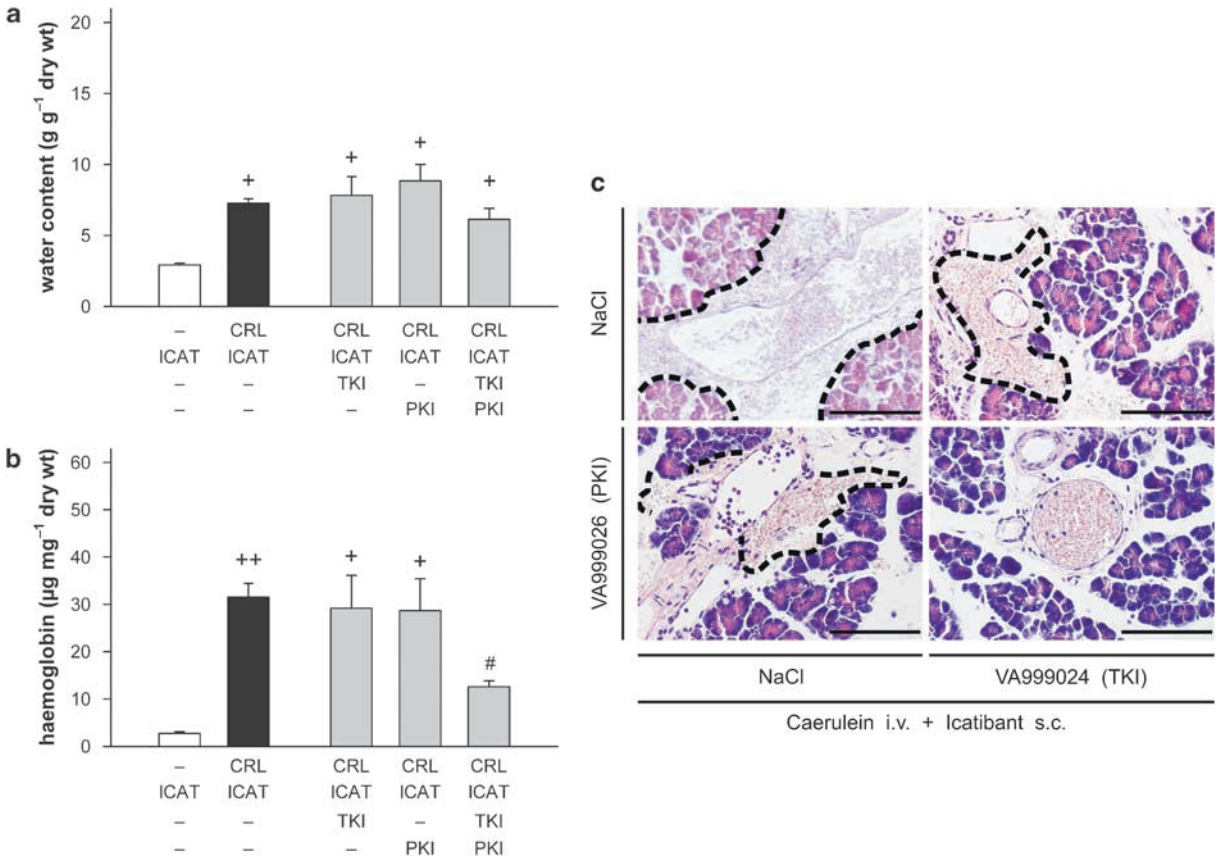

Figure 3 Effects of the tissue kallikrein (TK) inhibitor VA999024 (TKI) and the plasma kallikrein (PK) inhibitor VA999026 (PKI) in caerulein (CRL)-induced pancreatitis after treatment with the B_2 antagonist icatibant (ICAT). (**a**) Oedema formation and (**b**) haemoglobin accumulation in the pancreatic tissue: CRL or phosphate-buffered saline (PBS) was infused i.v. for 2 h. Pretreatments with icatibant (100 nmol kg^{-1}), VA999024 (20 µmol kg^{-1}) and/or VA999026 (20 µmol kg^{-1}) were given i.p. at −30 min. Control animals were injected with saline (NaCl). All treatments were repeated twice at 2-h intervals using half of the initial dose. Values are means + s.e.mean ($n = 5$–10). $^{+}P<0.05$, $^{++}P<0.01$ vs controls ICAT alone; $^{\#}P<0.05$ vs CRL + ICAT. (**c**) Photomicrographs of pancreatic blood vessels taken at 6 h. Dashed lines delineate areas of dense extravascular erythrocyte accumulation (haematoxylin and eosin stain; scale bar: 100 µm).

during caerulein-induced pancreatitis were completely inhibited ($P<0.01$) by the TK inhibitor (Figure 4a). In contrast, the PK inhibitor VA999026 had no effect on the TK activity in the pancreatic tissue. As the TK activity was already almost abolished by VA999024, the additional administration of VA999026 could not further enhance the inhibitory effect of VA999024. With respect to PK activity in the pancreatic tissue (Figure 4b), this activity was abolished by VA999026, whereas the TK inhibitor VA999024 did not alter this parameter. The combination of VA999026 and VA999024 was not more effective than VA999026 alone.

The residual activities of the endogenous protease inhibitors α_1-AT (Figure 4c) and α_2-M (Figure 4d) in the pancreas, which were observed after icatibant treatment in acute pancreatitis, were not affected by a concomitant treatment with either VA999024 or VA999026 or their combination.

Discussion

The direct comparison of the B_2 receptor antagonist icatibant with the selective TK inhibitor VA999024 and the PK inhibitor VA999026 (compare Figure 1) showed that VA999024 inhibited oedema formation to an extent similar to that seen with icatibant. However, neither icatibant nor VA999024 could completely abolish oedema formation. A previous investigation has shown that the dose of 100 nmol kg^{-1} is fully effective; higher doses were not able to induce a stronger inhibitory effect (Griesbacher and Legat, 2000). The effect of the PK inhibitor VA999026 did not reach the required level of significance, although it must be mentioned that VA999026 did have a limited, but significant, inhibitory effect in experiments performed for a different project (unpublished data). Therefore, it should be concluded that PK, if at all, only has a minor function in the inflammatory increases of vascular permeability. Given the fact that under normal conditions, almost no PK-like activity is present in the pancreatic tissue, activation of TK in the pancreas certainly will be sufficient to induce the inflammatory oedema. The doses of VA999024 and VA999026 had been found to be optimal in previous investigations (Griesbacher *et al*., 2002).

When the caerulein model of acute oedematous pancreatitis is combined with pretreatment with a B_2 receptor antagonist such as icatibant (Weidenbach *et al*., 1995), haemorrhagic lesions begin to develop in the pancreatic

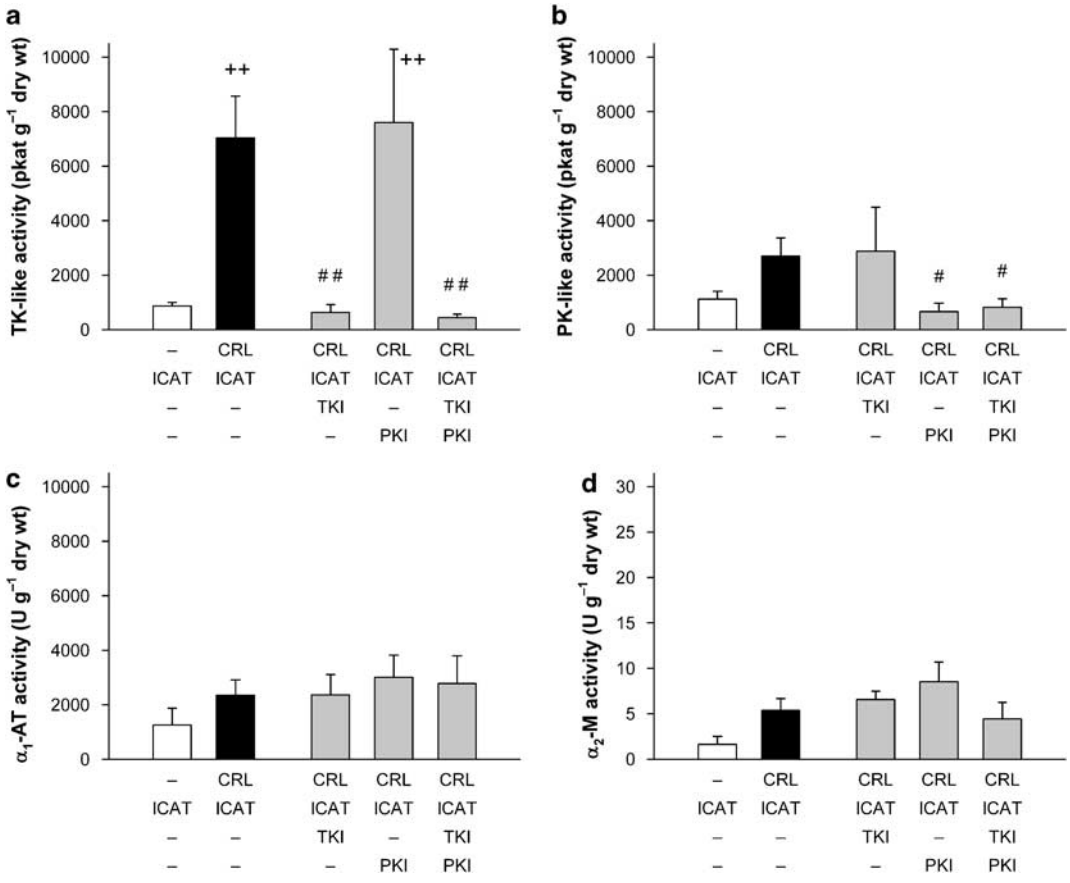

Figure 4 Catalytic activities of (**a**) tissue kallikrein (TK) and (**b**) plasma kallikrein (PK), and inhibitory activities of (**c**) α_1-antitrypsin (α_1-AT) and (**d**) α_2-macroglobulin (α_2-M) in the pancreatic tissue during caerulein-induced pancreatitis after treatment with the B_2 antagonist icatibant. The TK inhibitor VA999024 (TKI; 20 μmol kg^{-1}) and/or the PK inhibitor VA999026 (PKI; 20 μmol kg^{-1}) were given i.p. 30 min before caerulein; all i.p. injections were repeated twice at half doses at 2-h intervals; control animals were injected i.p with 154 mmol L^{-1} NaCl. Measurements were taken at 6 h. $^{++}P<0.01$ vs controls ICAT alone; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs CRL + ICAT. Values are means + s.e.mean; $n = 5$–9.

tissue about 4 h after the beginning of the experiment. We have chosen the time point of 6 h to investigate the mechanisms that might be involved at the very beginning of the development of these vascular lesions. To assess the magnitude of vascular damage and haemorrhagic lesions, we chose the measurement of haemoglobin accumulation in the pancreatic tissue as it represents a quantitative parameter. Haemorrhagic lesions following pretreatment with icatibant were observed in some, but not all, vessels that could be observed in the histological sections, which is in line with the descriptive reports published on this model (Weidenbach *et al*., 1995, 1996). No such vascular lesions were observed with VA999024 or VA999026 or their combination. Haemoglobin levels in the tissue confirm this observation in a quantitative manner. Necrotic lesions were not seen in the present investigation, but it was reported earlier that necrosis in this model seems to be secondary to, or dependent on, haemorrhage (Weidenbach *et al*., 1995), so that necrosis may not yet have developed in the experiments described here.

It is, of course, not surprising that the PK inhibitor did not induce vascular damage, because unlike icatibant it does not inhibit the inflammatory oedema to a significant extent. The effect of the TK inhibitor VA999024, however, is in contrast to the effect of icatibant, as haemoglobin levels in the tissue were not different from control animals, indicating the absence of haemorrhagic lesions. Given the similarity of the inhibitory effects of icatibant and VA999024 with respect to oedema formation, the striking difference in their effects on vascular integrity requires explanation. First of all, it must be considered that icatibant might just have had an unspecific 'toxic' effect. This, however, can be ruled out by two observations. First of all, icatibant did not induce any signs of vascular damage in animals that did not have pancreatitis, and second, identical haemorrhagic lesions could be induced in acute pancreatitis by the non-peptide B_2 antagonist FR173657, that is, by an agent of completely different chemical nature (data not shown). This confirms that the progression towards haemorrhagic lesions induced by icatibant in caerulein-induced pancreatitis is a class effect of B_2 receptor antagonists.

However, another striking difference in the consequences of icatibant and VA999026 treatment, respectively, is their discrepancy in the effects on the TK activity (compare Figure 2a). The differential effects of a B_2 receptor antagonist and the selective kallikrein inhibitors that are discussed below are also summarized in a scheme presented in Figure 5. The B_2 antagonist icatibant induced a significant augmentation of the TK activity in the pancreas, a fact that can be

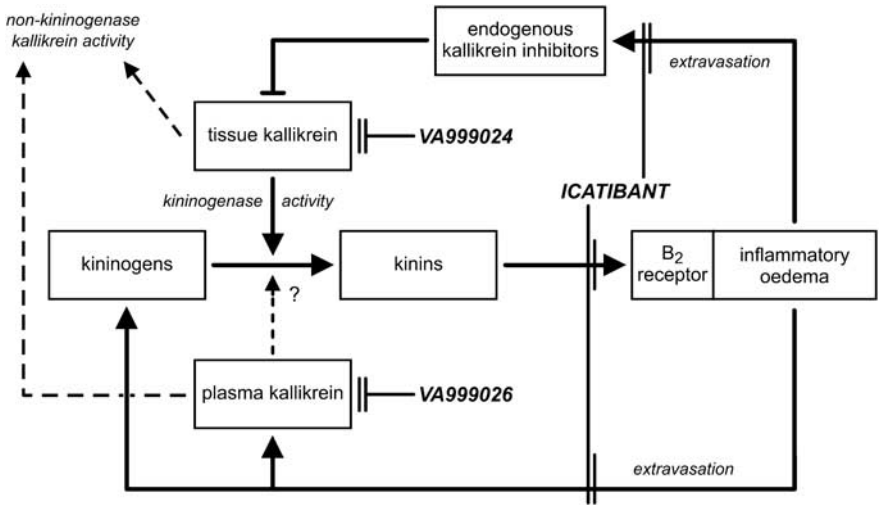

Figure 5 Proposed mechanism of action of the kallikrein–kinin system in acute pancreatitis and differential inhibitory effects of the B_2 receptor antagonist icatibant, the selective tissue kallikrein inhibitor VA99904 and the selective plasma kallikrein inhibitor VA999026. Prevention of oedema formation by icatibant reduces plasma protein extravasation and thus attenuates the presence of endogenous kallikrein inhibitors in the pancreatic tissue, which results in pronounced augmentation of the enzymatic activity of tissue kallikrein in the pancreatic tissue. Combined treatment with the selective kallikrein inhibitors VA999024 and VA999026 also reduces plasma protein extravasation by inhibition of kinin generation, but the lack of endogenous kallikrein inhibitors in the tissue caused by this treatment is compensated by the direct inhibition of kallikreins by VA999026 and VA999026.

explained by the prevention of extravasation of endogenous kallikrein inhibitors from the blood into the pancreatic tissue (compare Figures 2c and d). This protective aspect of the kinin-induced oedema was already determined in an earlier investigation (Griesbacher *et al*., 2003). Conversely, VA999024, being a specific TK inhibitor, reduced the TK activity in the pancreas to values comparable to those of sham control animals. With respect to PK (compare Figure 2b), the two agents had similar effects, both leading to an attenuation of the activity of the enzyme in the pancreatic tissue due to a reduced influx of PK from the bloodstream into the pancreas under conditions of reduced plasma protein extravasation. The PK inhibitor VA999026, on the other hand, only attenuated PK activity by direct inhibition of the enzyme, but had no apparent effect on the TK activity. Taken together, these data support our hypothesis that TK might be involved in the development of the vascular lesions.

The inhibitory activities of endogenous kallikrein inhibitors in the pancreas (compare Figures 2c and d) are not only attenuated by icatibant but also by VA999024. Reduction in the levels of protease inhibitor activity, of course, represents reduced extravasation of α_1-AT and α_2-M because neither icatibant nor VA999024 have a direct inhibitory effect on these protease inhibitors. The TK inhibitor seemed to be somewhat less effective in reducing extravasation of α_1-AT when compared with icatibant, but had a practically identical effect in reducing α_2-M extravasation. Although the reduced supply with endogenous kallikrein inhibitors, of course, leads to augmentation of the TK activity following icatibant treatment, this does not occur with VA999024 because TK is directly inhibited by the TK inhibitor taking the place of the endogenous inhibitors.

Whether or not icatibant-induced increases in kallikrein activity in the pancreas are causally related to vascular lesions was investigated by the combination of the icatibant applications with concomitant applications of VA999024 and/or VA999026 (compare Figure 3). Neither the TK inhibitor nor the PK inhibitor was able to affect icatibant-induced lesions, but the combination of both kallikrein inhibitors strongly inhibited the effect. This first of all shows that kallikreins are indeed involved in the progression towards haemorrhagic lesions and also shows that both enzymes apparently act independently of each other, so that either enzyme can take the place of the other under conditions when only one of the two types of kallikrein is inhibited. Therefore, it seems justified to conclude that combined inhibition of TK and PK can effectively prevent the development of vascular lesions.

The measurements of kallikrein activities and endogenous kallikrein inhibitors in icatibant-treated animals (compare Figure 4) strongly support these conclusions. Although the synthetic kallikrein inhibitors VA999024 and VA999026 do not affect the reduced amount of α_1-AT or α_2-M activity in the pancreas, their own inhibitory actions on the kallikreins fully compensate for the lack of endogenous inhibitors. It might be argued that vascular lesions occurring following icatibant treatment in caerulein-induced pancreatitis might also allow endogenous inhibitors such as α_1-AT or α_2-M to extravasate into the tissue. However, such an extravasation of protease inhibitors could occur only locally where haemorrhage occurs. Overall, it cannot affect TK activities as measured in the tissue as a whole.

A link between the activity of endogenous kallikrein inhibitors and the severity of acute pancreatitis had already been suggested by Bläckberg *et al*. (1999), who had shown that patients with low plasma levels of kallistatin, another endogenous kallikrein inhibitor, are more likely to develop more severe forms of pancreatitis. Certainly, these measurements had only been made in the blood plasma and not in

the pancreatic tissue. The present investigation clearly confirms this concept as it shows that haemorrhagic lesions occur only when the tissue levels of endogenous kallikrein inhibitors are decreased. Furthermore, we show that the vascular lesions can be prevented by the administration of synthetic kallikrein inhibitors.

Although our earlier measurements of components of the kallikrein–kinin system have led us to propose a modification of the conventional view of the system to include both a positive feedback function of kinin action by supplying PK and further substrate to the tissue, and a negative feedback action of B_2 receptors by supplying endogenous inhibitors that limit the kininogenase activity of kallikreins in the tissue, the current data also show that the negative feedback function of kinin action also has an important function in protecting the tissue by limiting kallikrein activity in the tissue. TK, and very likely also PK acting in support of TK, seems to have a central function in the development of vascular lesions occurring in situations where kallikrein activities are not restricted enough by endogenous inhibitors. Therefore, selective kallikrein inhibitors should be further investigated for their potential to prevent the progression of oedematous to haemorrhagic pancreatitis.

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Conflict of interests

This study was supported by a research grant from Vantia Ltd, UK, covering material costs. The study sponsor did not have any role in the study design, in the collection, analysis and interpretation of data, in the writing of the report or in the decision to submit the report for publication. The authors certify that they do not have any other potential conflict of interest.

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