Chymotrypsin-like activity in rat tissues in experimental acute pancreatitis

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Abstract

Purpose: Increase in intracellular chymotrypsin activity was reported during acute pancreatitis. Beside chymotrypsin, there are at least two enzymes with chymotrypsin-like activity: proteasome and lysosomal cathepsin A. Until now it is not known whether and to what extent they contribute to increases in chymotrypsin activity in acute pancreatitis. Our aim was to study organ chymotrypsin-like activities during experimental acute pancreatitis.

Material and methods: Rat cerulein model of acute pancreatitis was used. The chymotrypsin-like activities were assessed in pancreas, liver, lung, heart, spleen and kidney using highly selective synthetic substrates of the proteasome and the cathepsin A, at neutral and acidic pH. Determinations after addition of selective inhibitor were also performed.

Results: During acute pancreatitis we found in the pancreas an increase only in neutral chymotrypsin-like activity, as compared to the control animals. In other organs neutral chymotrypsin-like activity did not increase, and in kidney it even decreased. There were no changes in acidic chymotrypsin-like activity in any of organs studied. The studies using the inhibitor of the proteasome showed that the neutral chymotrypsin-like activity in the pancreas of the rats with acute pancreatitis should not be attributed to the proteasome activity, but rather to the chymotrypsin.

Conclusions: Our results did not confirm any significant contribution of proteasome or cathepsin A to increased chymotrypsin-like activity in acute pancreatitis. We showed a decrease in neutral chymotrypsin-like activity of proteasome in the kidney, but the significance of this finding remains to be established.

Key words: chymotrypsin-like proteases, acute pancreatitis, cerulein, rats.

Introduction

Acute pancreatitis is associated with an increase in the activity of pancreatic enzymes in the pancreas and body fluids, as well as multi organ dysfunction [1]. The pathomechanism of the disease remains unclear. Some authors [2-6] reported premature intracellular activation of trypsinogen and chymotrypsinogen as a result of instability of membranes of cell organelles, comprising also lysosomal membranes. In cerulein pancreatitis [2] subcellular fractions of secretory granules and vacuoles showed significant amounts of free chymotrypsin activities. However, there are at least two enzymes other than chymotrypsin, that exhibit chymotrypsin-like activity: proteasome and lysosomal cathepsin A. Until now it has not been reported whether and to what extent they contribute to increases in chymotrypsin activity in acute pancreatitis.

The proteasome is a large, multicatalytic proteinase complex (MPC). It exhibits at least 5 proteolytic activities responsible for the degradation of numerous cellular proteins including those mediating inflammatory and neoplastic conditions [7]. The chymotrypsin-like enzyme, which cleaves peptide bonds next to large hydrophobic residues, is the major component of the proteasome. It has been reported that the potent selective inhibitors of this enzyme such as microbial products lactacystin/ /β-lactone and epoxomicin and synthetic peptide analogues are apparently effective anti-tumor and anti-inflammatory agents [8]. Moreover, it was shown that proteasome activity may be affected by acute pancreatitis [9,10].

Cathepsin A (EC 3.4.16.1) is a multicatalytic lysosomal serine protease. At acidic pH it exhibits mainly the activity of carboxypeptidase. However, at neutral pH it acts predominantly

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as amidase. It preferentially hydrolyzes N-blocked hydrophobic peptides having phenylalanine and other hydrophobic amino acids at C-terminus, both synthetic (Cbz-Phe-Ala) and naturally occurring (angiotensin I, endothelin-1, substance P or bradykinin) [11]. The main function of this enzyme is a co-operation with other cathepsins, mainly cathepsin D, in protein degradation within the lysosomes. However, it is also postulated that after secretion from the lysosomes, cathepsin A may play an important role in local metabolism of bioactive peptides [12]. Beside catalytic function, cathepsin A was found to protect some enzymes from intralysosomal proteolysis by forming a multienzyme complex, especially with β-galactosidase and neuraminidase [11]. Earlier studies have shown that carboxypeptidase activity of cathepsin A is strongly inhibited by cell-permeable proteasome inhibitor lactacystin/β-lactone [13,14], and its amidase activity is inhibited by a potent antihypertensive agent ebelactone B [12]. The activity of lysosomal cathepsin A increases in muscular dystrophy [12] and neoplasms of the skin [13].

In acute pancreatitis proteolytic enzymes play a crucial role by inducing pancreas autodigestion, production of cytokines stimulating systemic inflammatory response syndrome (SIRS) and in consequence developement of multi organ failure (MOF). The importance of proteasome in the disease has recently been raised and for technical reasons its activity had to be measured together with cathepsin A. As cerulein acute pancreatitis in rats is a good equivalent of an oedematous form of the disease in humans, in the present study we investigated whether there is a change in the neutral and acidic chymotrypsin-like activities in the organs of rats with cerulein acute pancreatitis.

Material and methods

Chemicals

Cbz-Phe-Ala, ninhydrin reagent solution, Bradford reagent were purchased from Sigma Chemical Co, St. Louis, M, USA; Suc-Leu-Leu-Val-Tyr-AMC, lactacystin/β-lactone were purchased from Affinity research Product, UK.

Acute pancreatitis

Acute pancreatitis was induced in male Wistar rats weighing 180-200 g as described previously [15]. In brief, two intraperitoneal injections of 40 micrograms/kg of body weight of cerulein (analogue of cholecystokinin) were given at interval of 1 h. Control animals were injected similarily with 0.9% NaCl in the same volume. All animals were kept in single cages in 12 hours light/darkness cycle and were fed standard laboratory diet and water ad libitum. 24 hours after the first injection animals were sacrificed under anesthetic (intraperitoneal pentobarbital 40 mg/kg of body weight) after collection of blood and studied organs. The blood was taken from the right ventricle. 7.2 ml of blood was poured into a tube containing 0.8 ml of 3.8% sodium citrate. Immediately after sampling, blood was centrifuged at 3.500 rpm for 10 minutes at 4°C. The plasma was separated and stored at -70°C until it was assayed later. The following rat organs were taken for analyses: pancreas, liver, spleen, kidney, lung and heart. All organs were stored at -70°C until processing. To confirm changes typical of acute pancreatitis, amylase activity was determined in plasma, a portion of pancreas was examined histologically and pancreas wet/dry ratio was measured. The experiment was conducted in accordance with permission of the local ethical committee.

Amylase

Plasma amylase activity was measured using a quantitative colorimetric assay (Phadebas Amylase Test, Pharmacia & Upjohn, Sweden).

Histologic examination

A 3-5 mm^3 portion of pancreas head was fixed in 10% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 5 μm thickness and stained with hematoxylin and eosin. Assessment comprised edema, inflammatory infiltration, fat necrosis, parenchymal necrosis and hemorrhage.

Pancreatic edema

Pancreas water content was assessed by the tissue wet/dry ratio. Pieces of pancreas were weighed immediately after harvest to obtain the wet weight and again after desiccation to obtain the dry weight. Desiccation was achieved in an oven at 120°C for 24 hours.

Preparation of tissue homogenates

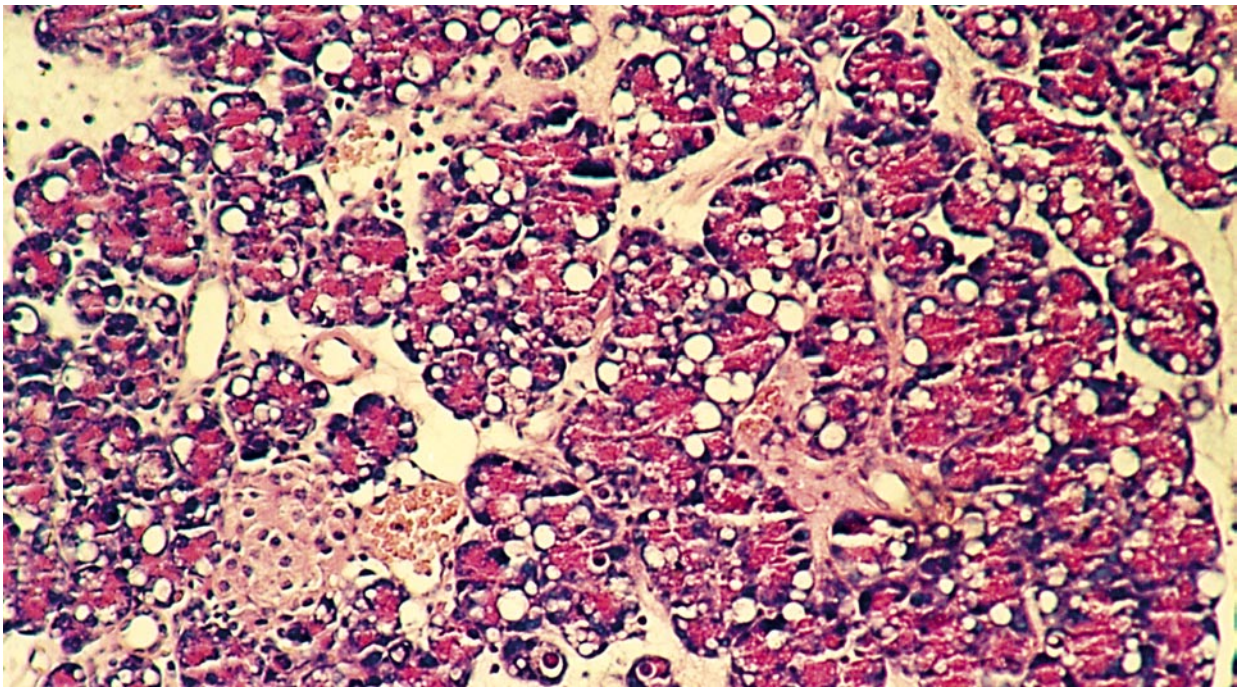
10% homogenates (w/v) were prepared using a knife homogenizer Combest X-120 (3x30 sec, 0°C) in 50 mmol/L Tris/HCl buffer at pH 8,0 containing 10% glycerol for assessment of Suc-Leu-Leu-Val-Tyr-AMC hydrolysis [16] or in 50 mmol/L sodium acetate buffer at pH 5.5 containing 0.1 mol/L NaCl, 0.1 mol/L sucrose and Triton X-100 (0.5%) for assessment of Cbz-Phe-Ala hydrolysis. During processing tissues were cooled with ice. The homogenates were then centrifuged at 15000 g for 30 minutes at a temperature +4°C, and the supernatants were used for experiments.

Biochemical Assays

The neutral chymotrypsin-like activity was assayed at pH 8.0 using Suc-Leu-Leu-Val-Tyr-AMC according to Farout et al. [16]. Incubation mixture (total volume of 200 μL) contained homogenate (10-100 μg of protein), 40 μmol/L of substrate and 50 mmol/L Tris/HCl/1mM DTT buffer at pH 8.0. After 30 minutes of incubation at 37°C, the reaction was stopped by adding 800 μL of 100 mM monochloracetic acid in 30 mM of sodium acetate, and the samples were centrifuged to obtain supernatants. The amount of AMC released from the substrate was determined spectrofluorometrically (Ex 370/Em 420). One unit of the neutral chymotrypsin-like activity was designated to 1 μmol of liberated AMC during 1 minute. Specific activity was expressed as units/mg of protein.

The acidic chymotrypsin-like activity was assayed at pH 5.5 using Cbz-Phe-Ala as described by Ostrowska et al. [14]. Briefly, the reaction mixtures (500 μL) contained homogenate (100-200 μg of protein), 5 mmol/L substrate and 50 mmol/L sodium acetate buffer at pH 5.5 containing 0.1 mmol/L NaCl, 0.1 mmol/L sucrose and 1 mmol/L EDTA. After 30 minutes of incubation at 37°C the reaction was stopped by the addition

Figure 1. Histology of pancreata of rats with oedematous acute pancreatitis 24 hours after the first injection of cerulein (H&E, original magnification x200).



of 500 µL of 10% TCA, and the amount of liberated alanine was determined by ninhydrin method [17]. One unit (U) of the activity was defined as the µmoles of alanine released per minute. Specific activity was expressed in units/mg of protein.

Lactacystin/β-lactone in a standard dose of 10 µmol/L inhibits Suc-Leu-Leu-Val-Tyr-AMC and Cbz-Phe-Ala hydrolysis in cell lysates by up to 90% [13,14]. The inhibitor was immediately added to the supernatants and reactions were started by the addition of the synthetic substrate.

All buffers contained the inhibitors of cysteine, aspartyl and metalloproteinases (2.5 mmol/L pepstatin, 1 mmol/L EDTA, 1 mmol/L EGTA and 50 nmol/L E64) [16].

Protein determination

The protein concentration in supernatants was determined by the method of Bradford [18] using Sigma reagent with bovine serum albumin as a standard.

Statistical analysis

All groups were tested with Kruskal-Wallis Anova test and inter-groups comparisons were made with Mann Whitney test. The threshold of significance was $p<0.05$.

Results

Histologic assessment (*Fig. 1*), amylase activity measurement in plasma and wet/dry weight ratio (*Tab. 1*) have confirmed the presence of eclematous acute pancreatitis in studied animals. Significant increase in plasma amylase activity was seen in the rats with acute pancreatitis 24 hours after the onset of disease, as compared to the control group ($p<0.05$) (*Tab. 1*). Also pancreas wet/dry ratio was significantly greater in rats with acute pancreatitis than in the control group ($p<0.05$) (*Tab. 1*).

Table 1. Amylase activity in plasma and wet/dry ratio of pancreas samples.

Group:	Control	Acute pancreatitis – 24h
Amylase (IU)	523 ± 81	1581 ± 207 *
Wet/dry ratio	2.13 ± 0.11	6.31 ± 0.72 *

Data represent the mean ± SEM; *-$p<0.05$; **$p<0.01$ vs. control

A comparison of Suc-Leu-Leu-Val-Tyr-AMC hydrolysis at pH 8.0 and Cbz-Phe-Ala hydrolysis at pH 5.5 in rat organs from healthy and acute pancreatitis rats is presented in *Tab. 2*. Suc-Leu-Leu-Val-Tyr-AMC was hydrolyzed with the highest rate in the pancreas among all organs studied. This chymotrypsin-like activity increased about 3-fold in the pancreas of rats with experimental acute pancreatitis (25.2 µmol/min/mg) as compared to the control animals (8.4 µmol/min/mg). In contrast, the hydrolysis of Cbz-Phe-Ala at pH 5.5 in the pancreas of acute pancreatitis rats was low (0.78 µmol/min/mg) and did not differ from this activity in the pancreas of healthy rats (0.88 µmol/min/mg). In the kidney we found a decrease in chymotrypsin-like activity assayed using Suc-Leu-Leu-Val-Tyr-AMC at pH 8.0 after 24 hours of induction of acute pancreatitis. In other organs, there were virtually no differences in comparison to the control group. The acidic chymotrypsin-like activity did not

Table 2. Comparison of Suc-Leu-Leu-Val-Tyr-AMC hydrolysis at pH 8.0 and Cbz-Phe-Ala hydrolysis at pH 5.5 in organs of control rats and 24 hours after induction of acute pancreatitis.

Organ	Suc-LLVT-AMC hydrolysis at pH 8.0μmol/min/mg	Suc-LLVT-AMC hydrolysis at pH 8.0μmol/min/mg	Cbz-Phe-Ala hydrolysis at pH 5.5μmol/min/mg	Cbz-Phe-Ala hydrolysis at pH 5.5μmol/min/mg
	Control	acute pancreatitis	Control	acute pancreatitis
Pancreas	8.4±1.4	25.2±6.3 **	0.88±0.1	0.78±0.08
Kidney	1.1±0.13	0.74±0.021 **	16. 2±2.8	12.6±1.8
Liver	0.1 ± 0.024	0.09±0.03	3.5±0.2	2.6±0.1
Spleen	0.028±0.002	0.02±0.06	2.4±0.6	2.1±0.4
Lung	0.025±0.004	0.022±0.008	1.64±0.44	1.54±0.5
Heart	0.029±0.0024	0.032 ±0.009	0.88±0.15	0.72±0.5

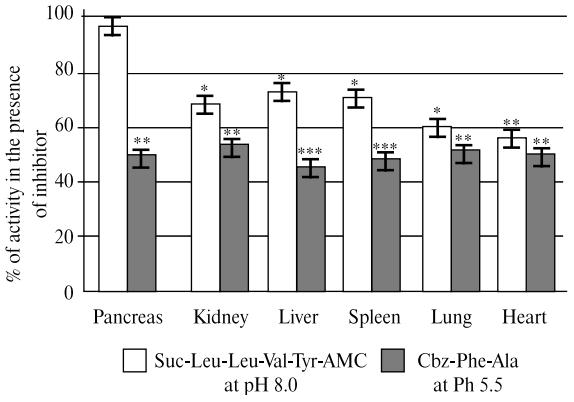
Mean values from 3 measurements for each organ of rats (n=5)±standard deviation; Statistically signi Figure description; ** p<0.01

change in any of organs studied during acute pancreatitis, as compared to the control group.

The studies using 10μM lactacystin/β-lactone, a potent inhibitor of the proteasome, showed that Suc-Leu-Leu-Val--Tyr-AMC hydrolysis was not inhibited in the pancreas (*Fig. 2*). In other organs of acute pancreatitis rats, lactacystin/β-lactone decreased the neutral chymotrypsin-like activity by about 30-40% indicating that also other proteases were involved in Suc-Leu-Leu-Val-Tyr-AMC hydrolysis.

In contrast, 10μM lactacystin/β-lactone decreased the hydrolysis of Cbz-Phe-Ala at pH 5.5 by about 50-60% in all organs studied, indicating that cathepsin A was mainly responsible for this activity.

Figure 2. Effect of lactacystin/β-lactone on Suc-Leu-Leu-Val-Tyr--AMC hydrolysis at pH8.0 and Cbz-Phe-Ala hydrolysis at pH5.5 in the organs of rat with experimental acute pancreatitis 24 hours after its induction. Relative chymotrypsin-like activities are shown as % of the activity in the absence of the inhibitor.



Mean values from 3 measurements for each organ of rats (n=5); *p<0.05; **p<0.01; ***p<0.001.

Discussion

Activation of zymogens within the pancreatic acinar cell is an early feature of acute pancreatitis [19]. Activity of several enzymes has been studied so far [20,21]. It has been speculated that the intracellular activation of trypsinogen is an early step in the development of acute pancreatitis [15]. However, it has been reported that chymotrypsin caused marked cell damage at micromolar concentrations, similarily to lipase, whereas even milimolar concentrations of trypsin failed to cause significant damage. Is has been concluded that activation of trypsin might be the trigger to start the activation cascade in acute pancreatitis, however, trypsin itself is markedly less noxious to acinar cells when compared with other digestive enzymes. It has also been suggested that studies analyzing therapeutic effects of protease inhibitors should evaluate not only the inhibitory potential against trypsin but also that against other digestive enzymes [22].

In our study we have shown the highest neutral chymotrypsin-like activity in pancreas among all organs studied. This activity increased about 3-fold in the pancreas of rats with experimental acute pancreatitis as compared to the control animals. However, the studies with a potent inhibitor of the proteasome, showed that Suc-Leu-Leu-Val-Tyr-AMC hydrolysis was not inhibited in the pancreas. These results indicate that the neutral chymotrypsin-like activity in the pancreas should not be attributed to the proteasome activity, but rather to the chymotrypsin generated by abnormal activation of chymotrypsinogen by cellular proteases in the pancreas of rats with acute pancreatitis. In other organs of rats injected with cerulein, lactacystin/β-lactone decreased the Suc-Leu-Leu-Val-Tyr-AMC hydrolysis by about 30-40% indicating that also other proteases were involved in neutral chymotrypsin-like activity.

The acidic chymotrypsin-like activity, typical of cathepsin A, was low in the pancreas of rats with acute pancreatitis and did not differ from this activity in the pancreas of healthy rats. There were no changes in neutral nor acidic chymotrypsin-like activity in liver, heart, lung or spleen.

In the kidney the neutral chymotrypsin-like activity clearly decreased after 24 hours of induction of acute pancreatitis. The significant decrease in enzymatic chymotrypsin-like activity in the kidneys is of interest in view of frequent renal function impairment during acute pancreatitis [23]. Also other enzymatic activities have been reported to be reduced in the kidneys during acute pancreatitis. Lakowska et al. [24] have found a decrease in the activity of lysosomal cathepsin B, D, L. The authors have concluded that the monitoring of lysosomal

enzyme activity can be used to evaluate the impariment of kidney function in the course of experimentally induced acute pancreatitis.

The decrease in active enzymes content in the kidneys during acute pancreatitis is not fully explained. Hypothetically this may result from inactivation of proteases or their release to the bloodstream and/or urine because of cell membranes damage.

Acute renal failure during acute pancreatitis has been explained on the basis of hypovolemia and hypotension and a role of renin-angiotensin alterations in acute pancreatitis as mediators of renal failure was postulated [23]. Levy et al. have reported that decrement in renal perfusion found in experimental acute pancreatitis in dogs could be duplicated by the infusion of chymotrypsin into normal dogs. The authors speculate, that this was due to a decline in plasma volume, associated with a rising hematocrit and declining plasma protein concentration [25].

Conclusions

In conclusion, our results indicate that induction of acute pancreatitis in rats leads to a significant increase in the neutral chymotrypsin-like activity in pancreas during acute pancreatitis. However this should be attributed to activated chymotrypsin rather than proteasome, because of lack of inhibition of Suc-Leu-Leu-Val-Tyr-AMC by a potent proteasome inhibitor lactacystin/β-lactone at the concentration suppressing the proteasome activity in cell cultures. There were no changes in neutral chymotrypsin-like activity (typical of proteasome) in liver, heart, lung or spleen during acute pancreatitis. The decrease in neutral chymotrypsin-like activity of proteasome in kidney needs further exploration. The acidic chymotrypsin-like activity typical of cathepsin A was not altered in any of organs studied. To clarify the significance of the cytosolic proteasome in acute pancreatitis, immunochemical studies would be of value. So far, to our knowledge, no commercial immunochemical kit is available.

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