Hiroshima Journal of Medical Sciences
Vol. 31, No. 1, March, 1982
MIJM 31-8

Increase in Content and Release of Kallikreinogen in Acute Experimental Pancreatitis in Rats*)

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(Received December 25, 1981)

Key words: *Kallikrein, Acute pancreatitis, Closed-loop method*

ABSTRACT

The content of kallikreinogen in the pancreas isolated from rats with acute experimental pancreatitis rose to over ten times the level in the control group. In the isolated and perfused pancreata prepared from the pancreatitis group; the "resting" leak of kallikreinogen into the portal perfusate was 8 times that in the control group, and it was further increased by stimulation with CCK (5 mU/ml). The "resting" amylase level in the portal perfusate was 5 times that in the control group, but stimulation with CCK caused no further increase. The results suggest that kallikrein formation may be an important step in the development of acute pancreatitis.

INTRODUCTION

Acute pancreatitis, especially acute hemorrhagic and necrotizing pancreatitis, is a serious disease in which the mortality rate is very high[5]. The reason for this high mortality rate is because the exocrine pancreas contains various digestive enzymes and zymogens. When the zymogens in the pancreatic acinar cells are activated, serious autodigestion of the cells may occur[4]. The autodigestion and the resultant leakage of enzymes into the blood stream may be responsible for the following clinical features of acute pancreatitis; abdominal pain, bleeding, shock, and toxicity[18].

Recent studies have demonstrated the presence of pancreatic enzymes, such as trypsin, elastase, and chymotrypsin, in the ascites fluid and plasma from patients with acute pancreatitis[1,3,6]. Takasugi et al.[17] recently reported that pancreatic kallikrein may leak into the blood stream during acute human pancreatitis, that almost all of the kallikrein leaked immediately combined with α2-macroglobulin, and that the bound form of kallikrein preserved its biological activity.

The present experiments showed that the amount of kallikreinogen in the pancreas of rats with acute experimental pancreatitis increased more than 10 times that in the control group. Furthermore, they showed that a considerable amount of kallikreinogen was present in the perfusate collected from the portal vein of the perfused pancreas isolated from rats with acute experimental pancreatitis.

METHODS

Animals

Male rats of the Wistar strain weighing about 200 g were used. The animals received no nourishment other than water ad libitum for a period of at least 24 hrs before the experiments.

Operative procedures

The rats were divided at random into three groups. Acute pancreatitis group: Rats were laparotomized in the midline under ether anesthesia and a closed-loop was made by ligating the duodenum at two points on both sides of

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the outlet of the common duct[14], the abdomen was then closed, and caerulein (1 ng/g, body weight) was injected subcutaneously. Sham operation group: Rats were laparotomized, the abdomen was then closed, and caerulein (1 ng/g, body weight) was injected subcutaneously. Control group: The animals were fasted without any treatment.

Prepartion of homogenates

The acute pancreatitis and sham operation rats were laparotomized again 18 hrs after the initial operation. The control rats were laparotomized 36 hrs after fasting. To remove blood from the vessels, the pancreas was flushed through the superior mesenteric and celiac arteries with a modified Krebs-Henseleit solution (1 ml/min) until it became pale (usually after about 2 min). After this procedure, the pancreas was removed and placed in a test tube which contained 2.5 ml of 0.1 M phosphate buffer solution (pH 7.4). The tissue was immediately homogenized for 30 sec with a homogenizer (Type PT10/35, Kinematica, Luzern, Switzerland), and centrifuged at 6000 rpm for 15 min at 4°C after which the supernatant was stored at 4°C. The pancreas from the acute pancreatitis group was fixed overnight with 10% formalin, then dehydrated with ethyl alcohol and embedded in paraffin for histological examination. Sections, 7 μm thick, were stained with haematoxylin-eosin, and observed with a light microscope.

Isolation and perfusion of the pancreas

Details of the procedures used to isolate and perfuse the whole pancreas have been given in a previous paper[11]. In brief, the pancreas was prepared for perfusion, collection of samples and isolation under ether anesthesia as follows. The mesenteric and celiac arteries and portal vein were cannulated and perfused with the aid of a roller pump at 1 ml/min. After ligating the hepatic end, the duodenal end of the pancreatic duct was cannulated so that the rate of pancreatic juice flow and amylase output could be measured simultaneously. The blood supply to the stomach, liver and spleen was stopped by ligating the appropriate vessels. The mesentery with the embedded whole pancreas and attached duodenum was then removed into a lucite chamber, which contained 20 ml of a modified Krebs-Henseleit solution. The temperature of the preparation and the perfusing solution were maintained at 37°C by immersing the apparatus in a water bath.

Hormone and solution

The composition of the standard Krebs-Henseleit solution used for perfusing and bathing the preparation was as follows (mM): NaCl, 131; KCl, 5.6; $CaCl_2$, 2.5; $MgCl_2$, 1.0; $NaHCO_3$, 25; NaH_2PO_4, 1.0; and glucose, 2.5. Dextran T-70 (Pharmacia Fine Chemicals Inc., Uppsala) was added to the perfusing solution to a final concentration of 5% (Wt/vol). The solution was equilibrated with 5% CO_2 in O_2 and had a pH of 7.4. Five mU/ml [expressed in Ivy dog units[9]] of pure natural cholecystokinin-pancreozymin (CCK; 99% pure, 3500 U/ml, Gastrointestinal Hormone Research Unit, Karolinska Institute, Stockholm) was added to the solution perfusing the isolated pancreas.

Estimation of amylase, total esterolytic activity, and Kallikrein activity

Amylase activity was assayed by a modified method of Bernfeld[2] as described previously[10]. One unit of amylase activity was defined as the amount of enzyme which produced 1 mg of maltose during 5 min incubation at 37°C.

Esterolytic activities of each sample on TAMe (p-N-tosyl-arginine metyl ester) (Foundation for Promotion of Protein Research, Institute for

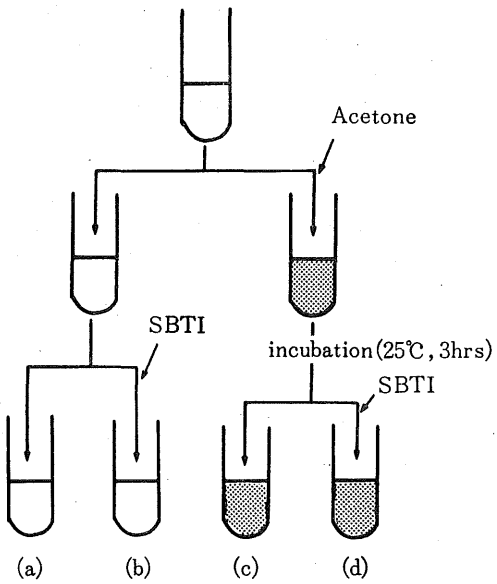

Fig. 1. A schematic representation of the four different methods for measuring in esterolytic and kallikrein activity. For details refer to the text

Protein Research, Osaka) were measured by Hestrin's method[7] as previously modified[16]. The final concentration of TAMe used in the present study was 10 mM. One unit of esterolytic activity was defined as the amount of enzyme which can hydrolyze 1 μmole of TAMe per hr at 37°C by Hestrin's method. The esterolytic activities of stored supernatant were estimated after four different types of pretreatment (Fig. 1). (a) The esterolytic activity of a part of the supernatant was estimated without adding any agent and expressed as the native esterolytic activity. (b) The activity of the other part of the supernatant was estimated after adding 200 μg of soy bean trypsin inhibitor (SBTI; Sigma Chemical Co., St. Louis, Mo) to 0.4 ml of an appropriately diluted sample, and expressed as native kallikrein activity. (c) Acetone (0.2 ml) was added to 0.8 ml of a non-diluted sample and incubated for 3 hrs at 25°C, then the activity was estimated (acetone-activated esterolytic activity). (d) Acetone (0.2 ml) was added to 0.8 ml of a non-diluted sample, and incubated for 3 hrs at 25°C, then 200 μg SBTI was added to 0.4 ml of an appropriately diluted samle. The estimated activity was expressed as acetone-activated kallikrein activity. Since the addition of acetone activates several kinds of zymogens, such as kallikreinogen and trypsinogen, to produce proteolytic enzymes, which in turn hydrolyze TAMe, the trypsin activity was inhibited by adding a sufficient amount of SBTI. The residual TAMe hydrolytic activity after incubation with SBTI was thus regarded as kallikrein activity.

Statistics

Results are expressed as mean±S.E., and are analyzed by Student's t test.

RESULTS

In the pancreatitis group, the mortality ratio was about 18% at 18 hrs after the operation. Pathological findings of the dead rats consisted of severe hemorrhagic pancreatitis with much bloody ascites. The macroscopic findings in the surviving rats in the pancreatitis group consisted of pancreatic edema, occasional hemorrhages especially in the head of the pancreas, fat necrosis in the peripancreatic fat, and brown or serious bloody ascites. The microscopic examination of the pancreata removed 18 hrs after the operation showed the following signs of

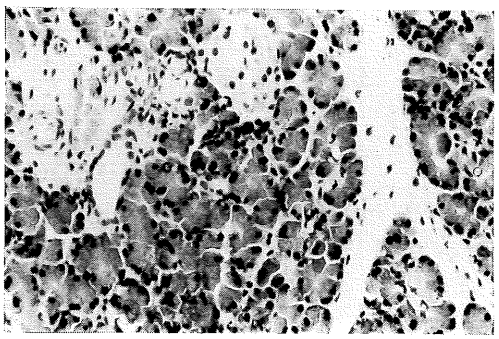

Fig. 2. Micrograph of pancreas prepared from rats with acute experimental pancreatitis. The preparation was fixed with formalin and stained with hematoxylin-eosin 18 hrs after procedures which produce acute pancreatitis. The micrograph shows severe edma and dffuse infiltration of inflammatory cells in and around pancreatic lobules (×150).

Table 1. Wet weight, total TAMe hydrolytic and kallikreinogen activities in homogenates of rat pancreata

Groups	Wet weigh (mg)	Total esterolytic activity (U) (native) (a)	(acetone-activated) (c)	Kallikrein activity (U) (native) (b)	(acetone-activateo) (d)
Control	473.5±49.1	14.35±8.52	133.45± 17.33	2.60± 2.55*	78.28± 5.55**
Sham operation	518.6±28.1	17.55±9.76	100.28± 10.78	2.95± 2.56	56.65± 8.56
Acute pancreatitis	778.1±23.9	3318.50±51.86	3435.55±134.00	847.73±51.59*	840.65±59.29**

1 U=1 μmole of TAMe hydrolyzed/hr/g wet weight
(*,**; $p<0.01$)

The values represent the mean±S.E. of 4 experiments each in the control, sham operation, and acute pancreatitis groups.

Symbols; (a), (b), (c), and (d) represent different pretreatments show by the corresponding symbols in Fig. 1.

acute pancreatitis; edema of varying degree, inflammatory cell filtration, occasional interstitial hemorrhage, and vacuolization of acinar cells (Fig. 2). In the pancreata from rats in the control and sham operation groups, however, no signs of acute pancreatitis were detected by both macroscopic and microscopic examinations.

The wet weights of pancreas with or without the treatment are summarized in Table 1, the weight in the pancreatitis group being about 1.6 times heavier than that in the control group. There was no difference between the organ weight of the control group and that of the sham operation group, indicating that caerulein alone produced no effect on the wet weight at 18 hrs after the operation.

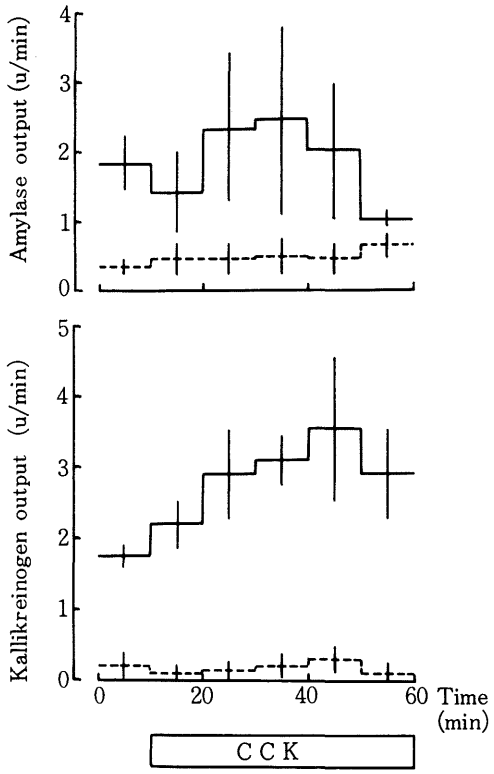

Fig. 3. Time course of the change in level of amylase and kallikreinogen before and during continuous stimulation with CCK (5 mU/ml) in the perfused pancreas of rats. The thick solid line represents amylase and kallikreinogen levels in the portal vein of perfused pancreas isolated from acute pancreatitis group. The broken line represents amylase and kallikreinogen levels in the portal vein of the pancres of the control group. In all cases CCK (5 mU/ml) was added during the period shown by the horizontal bar. Each value represents the mean±S. E. of 10 min collection periods in 5 experiments.

The most prominent biochemical change in acute experimental pancreatitis detected in the present experiment was a marked increase in the arginine esterolytic and kallikrein activities of the pancreatic tissue, because the enzyme activities in the pancreatitis group were more than ten times those in the control or sham operation group. It is noteworthy that the enzyme activities of the pancreas isolated from the pancreatitis group had already been activated during the course of isolation and homogenization prior to the activation of kallikreinogen with acetone.

To obtain an answer to the question of whether the enzymes contained in the pancreatic tissue are released into the blood stream. we used a perfused preparation from the isolated pancreas, which was first perfused for 20 min with standard Krebs-Henseleit solution to stabilize the "resting" secretory responses of the exocrine pancreas. This perfusion was then switched from the standard solution to a solution to a solution that contained 5 mU/ml CCK, while perfusion was continued for an additional 50 min (Fig. 3). As expected, in preparations isolated from rats in the control group, CCK induced an increase in pancreatic amylase output as well as juice flow into the common duct, but no increase in amylase and kallikreinogen outputs into the portal vein. In the preparation isolated from rats in the pancreatitis group, the "resting" concentration of kallikreinogen in the perfusate of the portal vein was significantly higher than that in the control group. A gradual rise in kallikreinogen output into the portal perfusate was detected after continuous stimulation with the same concentration of CCK (5 mU/ml): the total kallikreinogen output into the portal vein collected over a period of 50 min from the beginning of the CCK stimulation was 144.8±30.0 U and 7.9±3.0 U in the pancreatitis and control groups respectively. The "resting" and CCK-induced amylase outputs in preparations isolated from rats in the pancreatitis group were higher than those in the control group. Thus, the total amylase output into the portal vein collected for 50 min from the beginning of stimulation with CCK was 95.8± 45.8 U and 24.4±9.4 U in the pancreatitis and control groups respectively.

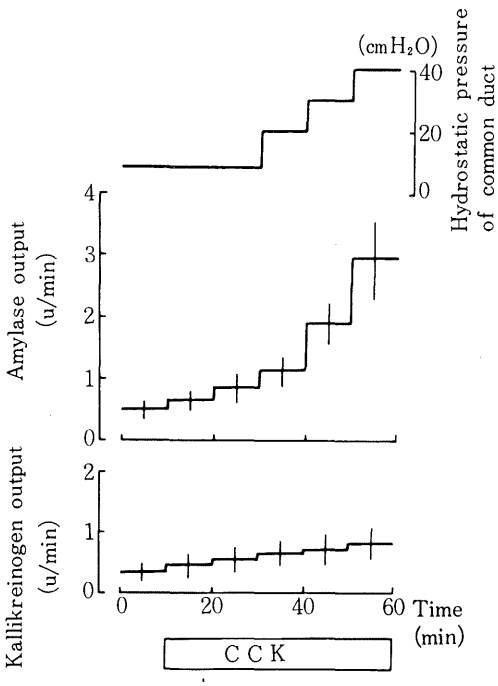

Fig. 4. Time course of the change in level of amylase and kallikreinogen before and during continuous stimulation with CCK (5 mU/ml) followed by step-wise increase in hydrostatic pressure of common duct in the isolated perfused rat pancreas. CCK was added to the perfusing solution during the period shown by the horizontal bar. Each value represents the mean±S. E. of 10 min collection periods in 5 experiments in the control group.

The following experiments confirm previous results[15] that continuous stimulation with CCK during increasing hydrostatic pressure of the common duct induced a rise in amylase output into the perfusate of the portal vein (Fig. 4). The experiments also showed that continuous CCK stimulation caused no increase in kallikreinogen output into the portal perfusate even when there was a rise in amylase output (Fig. 4). Similar results were also obtained when the isolated pancreas was stimulated with a high concentration (1 U/ml) of CCK which is known to induce partition of pancreatic enzymes[15].

DISCUSSION

Kallikreinogen content in the pancreas. The present experiments clearly demonstrate that in pancreata isolated from rats with acute pancreatitis, the amount of kallikreinogen was ten times that in the control; kallikreinogen can easily be activated during the course of isolation and homogenization of the pancreatic tissue; and the rise in kallikeinogen was not associated with an increase in other pancreatic enzymes such as amylase. Thus, the present findings provide experimental support of the synopsis proposed by Creutzfeldt and Schmidt[4], in which they proposed that one of the possible pathogenetic factors of pancreatitis might be the development of four steps; (a) injury of the acinar cells, (b) liberation or activation of small amounts of trypsin, (c) activation of kallikreinogen, and (d) appearance of edema and shock. The initial step of experimentally induced acute pancreatitis may take place in the acinar lumen because in the present experiment, acute pancreatitis was induced by the modified clsed-loop technique described by Nevalainen et al.[14], in which the duodenum on the both sides of the outlet of the common duct is obstructed, resulting in reflux of the duodenal fluid containing enterokinase[13]. It is highly possible that enterokinase triggers the series of processes which activate zymogen proteases in the pancreatic duct and acinar lumen[12]. The activated proteases may then act on the luminal membrane of the pancreatic acinar cells to increase pancreatic kallikreinogen inside the cells. Morphological changes in the initial step of experimentally induced acute pancreatitis in the dog has recently been elucidated with light and electron microscopes[18].

Increase in pancreatic secretagogues alone seems to be insufficient to produce acute pancreatitis because caerulein failed to induce acute pancreatitis in the absence of duodenal obstruction. The intermediate process between increase in activated proteases in the acinar lumen and increase in the intracellular kallikreinogen content remains to be fully defined. It may be that activated proteases act on the luminal surface resulting in increased cellular permeability.

Release of kallikreinogen into the portal vein. The present experiment showed that in the pancreata isolated from rats suffering with acute pancreatitis, the "resting" concentration as well as CCK evoked release of kallikreinogen into the perfusate of the portal vein were both significantly higher than the corresponding control values. A similar tendency was detected in amylase. These results show that as kallikreinogen rises in the pancreatic acinar cells, it may leak into the blood stream during acute

pancreatitis. An extraordinary increase in cellular permeability may be responsible for this leakage. In a previous paper, it was reported that continuous stimulation with CCK at a high concentration caused a marked elevation in amylass output into the portal vein in contrast to a small increase in the output into the duct[19]. The present study confirms this result in the control isolated perfused rat pancreas and also demonstrates that the increase in amylase output into the portal vein is not associated with an increase in kallikrein output. Furthermore, similar results were obtained in the control group when the rats were stimulated continuously with CCK (5 mU/ml) followed by step-wise increases in hydrostatic pressure of the common duct. In fact, a concomitant increase in amylase and kallikreinogen in the portal perfusate was not detected in preparations isolated from control rats, but was in those isolated from rats with acute pancreatitis. Thus, the increase in kallikreinogen content in the portal vein appears to be a clear sign of acute pancreatitis, and it may be responsible for the clinical signs and symptoms of acute pancreatitis.

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