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Increased Intrapancreatic Trypsinogen Activation in Ischemia-Induced Experimental Pancreatitis

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Objective
The potential of pancreatic ischemia to cause acute pancreatitis as indicated by morphologic changes and ectopic trypsinogen activation was investigated.

Background
Experimental evidence has shown that pancreatic ischemia is important in the evolution of severe pancreatitis, but whether ischemia can initiate pancreatitis has been disputed.

Methods
Pancreatic ischemia was induced in rats by hemorrhagic hypotension (30 mm Hg for 30 min; n = 64). Changes of pancreatic microcirculatory perfusion were studied using diffuse reflectance spectroscopy. Serum amylase, trypsinogen activation peptide (TAP) in serum and pancreatic tissue, wet/dry weight ratio, and histology were determined over 24 hours and compared with sham-operated control subjects (n = 35).

Results
In control animals, serum amylase (47.9 ± 2.1 units/L), serum (7.9 ± 0.7 nmol/L) and tissue TAP (63.0 ± 5.4 nmol/L × g), wet/dry weight ratio (2.8 ± 0.1), and histology remained unchanged. Temporary hypotension markedly decreased pancreatic perfusion with incomplete recovery after reperfusion. Pancreatic isoamylase activity increased within 1 hour (110 ± 5 units/L, $p < 0.05$) and further to 151 ± 18 units/L at 24 hours. Tissue TAP was elevated at 1 hour (134 ± 16 nmol/L × g, $p < 0.05$)and increased to 341 ± 43 nmol/L × g ($p < 0.001$) after 24 hours, whereas serum TAP remained unchanged (8.3 ± 0.5 nmol/L). Morphologic alterations included elevated wet/dry weight ratio (4.1 ± 0.3, $p < 0.01$) and increased histologic scores for edema ($p < 0.05$) and acinar necrosis ($p < 0.05$) at 24 hours. Trypsinogen activation preceded the development of pancreatic necrosis.

Conclusions
In addition to its potentiating role, severe pancreatic ischemia can play a pathogenetic role in the initiation of acute pancreatitis.

The pathogenesis of acute pancreatitis is not fully understood and appears to be a product of multiple factors, including disturbances of pancreatic blood flow, which recently have gained increasing attention. Impairment of microcirculatory blood flow is a characteristic of moderate and severe acute pancreatitis and correlates with the severity of the disease.[1-5] Laboratory studies have shown repeatedly that superimposing ischemia on reversible edematous pancreatitis leads to the development of irreversible necrotizing disease.[6-8] Studies directed at microcirculatory support or enhancement have demonstrated the ability to reduce the degree of pancreatic injury.[9,10] These observations clearly indicate the relevance of ischemia in potentiating tissue injury during the evolution of acute pancreatitis. Nevertheless, the ability of ischemia alone to initiate this disease still is controversial. That hypoperfusion of the pancreas can trigger pancreatitis has been suggested by the occurrence of acute pancreatitis after temporary ischemia during cardiopulmonary bypass,[11-15] evidence of acute pancreatitis in up to 25% of autopsies of patients dying after shock,[14-17] and the production of severe pancreatitis by experimental obstruction of terminal pancreatic arterioles.[18,19] However, the pathophysiologic basis of the pancreatic susceptibility to ischemic insults is not known, and some investigators have questioned whether pancreatic ischemia can initiate acute pancreatitis.[8,20,21] Because premature activation of pancreatic proteases is believed to be a very early and critical pathophysiologic event in the development of acute pancreatitis,[22] we asked whether ischemia alone might induce intrapancreatic trypsinogen activation along with the morphologic indices of acute pancreatitis. This study, in demonstrating that temporary pancreatic hypoperfusion leads to an early increase of ectopic trypsinogen activation, hyperamylasemia, and subsequent tissue injury characteristic of acute pancreatitis in the rat pancreas, suggests that ischemia may act as an initiating factor in the pathogenesis of acute pancreatitis.

MATERIALS AND METHODS

Experimental Protocol

The study was approved by the Subcommittee on Research Animal Care at our institution and performed according to the NIH guidelines for the care and use of laboratory animals. Experiments were performed on 89 male Sprague-Dawley rats (290–350 g) obtained from Charles River Laboratories (Wilmington, MA). Anesthesia was initiated with vaporized ether and maintained by injections of pentobarbital (20 mg/kg i.v., Anpro Pharmaceuticals, Arcadia, CA) and ketamine (40 mg/kg i.m., Ketalar, Parke-Davis, Morris Plains, NJ). On the day before the experiment, a polyethylene catheter (Intramedic, ID 58mm, Clay Adams, Parsipanny, NJ) was introduced into the left internal carotid artery and advanced into the aorta for blood sampling, infusions, and monitoring of arterial pressure. The catheter was tunneled subcutaneously to the suprascapular region and brought out through a steel tether, permitting unrestrained activity of the conscious animal. Animals then were allowed to stabilize and fasted overnight, with unlimited access to water. On the next day, animals were randomly allocated to a control group receiving no further treatment (n = 35) or an ischemia group, in which pancreatic ischemia was induced by reduction of mean arterial pressure (MAP) to 30 mm Hg for 30 minutes by controlled hemorrhage via the arterial line (n = 64). This decrease of MAP has been shown to cause a severe impairment of pancreatic blood flow and capillary perfusion[8,23,24] and was chosen as an optimal level for induction of pancreatic injury with an acceptable survival of animals for the duration of the experiment.[8] The withdrawn blood was anticoagulated with heparin-sodium (Elkins-Sinn, Cherry Hill, NJ) and incubated at 37 C. Mean arterial pressure and heart rate were continuously monitored using an Electrodyne ST-219 transducer (Becton-Dickinson, Parsippany, NJ) and carefully maintained by removal or re-infusion of small amounts of blood. After completion of the 30-minute period, the blood was completely re-infused over 5 minutes.

Assays

Concentrations of serum amylase were determined at baseline, 20 minutes, 6 hours, and 24 hours, according to the method of Ceska et al.,[25] using the Phadebas amylase test (Pharmacia Diagnostics, Uppsala, Sweden).

Electrophoretic separation of serum amylase isoenzymes was performed in 7% polyacrylamide gel with Tris-glycine buffer (pH 8.3) in specially designed glass tubes (105 × 3-mm, Metalloglass Co., Cambridge, MA). Ten microliters of serum sample were mixed with 10 μL 25% sucrose containing Bromphenol blue and applied to the gels. Serum samples were compared with secretions obtained by direct aspiration from the pancreatic and salivary ducts of control animals. Control serum was included in every experiment, and all specimens were run in duplicate. After completion of electrophoresis, 70 sequential segments of each 7.5-cm gel were extruded into

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separate test tubes, and each segment then was assayed for amylase activity by a direct saccharogenic technique using 3′, 5′-dinitrosalicylic acid.[26]

For measurement of trypsinogen activation peptide (TAP) serum levels, blood samples were collected at baseline, 20 minutes, 6 hours and 24 hours in ethylene diaminetetra-acetic acid (10 mM, pH 7.0). Samples were centrifuged, and the supernatant was stored at −20 C. For measurement of TAP concentrations in pancreatic tissue, animals were killed at the designated time points, the pancreas was resected, and two tissue portions (0.1–0.25 g) were excised. Specimens were immersed in 0.2 M Tris-HCl buffer (pH 7.3) containing 20 mM ethylene diaminetetra-acetic acid and immediately boiled (at 100 C) for 15 minutes to denaturate remaining protease activity. Each sample then was homogenized in a Brinkman Polytron (Brinkman Instruments, Westbury, NY) for 30 seconds. After subsequent centrifugation (1500 rpm, 10 min, 4 C), the resulting supernatants were coded and stored at −20 C until assayed. Aliquots of serum and tissue samples were assayed simultaneously for free TAP by a competitive enzyme-linked immunosorbent assay using purified rabbit anti-TAP antibodies.[27]

Wet-/Dry-Weight Ratio

At the designated time points (baseline, 1 hour, 6 hours, 24 hours), animals were killed by pentobarbital overdose (200 mg/kg i.v.), and the entire gland was resected carefully in a standardized fashion by the same investigator, trimmed of fat, dried, and weighed. Pancreatic water content was determined by calculating the ratio of the initial weight of the pancreatic specimen (wet weight) to its weight after incubation at 210 C for 12 hours (dry weight).

Histopathologic Analysis

Specimens of pancreatic tissue from each time point were fixed in 10% phosphate-buffered formalin (pH 7.5), embedded in paraffin, and stained with hematoxylin and eosin for light microscopic evaluation. The extent of interstitial edema formation, acinar cell necrosis, parenchymal hemorrhage, and inflammatory infiltration was quantitated separately for the pancreatic head and tail by a pathologist unaware of the identity of the specimens, using a previously described scoring system (range 0–4).[28]

Reflectance Spectroscopy

In a separate set of experiments designed to evaluate changes of tissue perfusion, pancreatic hemoglobin content and oxygen saturation of hemoglobin (ISO_2) were measured using diffuse reflectance spectroscopy as previously described.[4,5,29] Briefly, the abdomen was opened, and a probe head (2.5-mm diameter) of a modified diode array spectrophotometer (HP 84523, Hewlett Packard, CA) emitting and receiving light was placed on the pancreatic surface. Spectral changes in the reflected light were analyzed in a connected personal computer (Vectra CS, Hewlett Packard, CA), and ISO_2 was calculated from the absorbance at 577 nm (absorbance maximum of oxygenated hemoglobin) and 555 nm (absorbance maximum of deoxygenated hemoglobin) plotted against pancreatic hemoglobin content, determined from the absorbance at the isosbestic point (586 nm). Measurements were performed in four animals at six randomly selected sites in the duodenal and splenic portion of the gland at baseline, during hypotension, and reperfusion.

Statistical Analysis

The results of the present study represent the mean ± SEM of at least four different experiments in each group at each time point. Statistical significance of changes from baseline values within the experimental or control group was tested with paired Student's t test. Differences between the experimental group and the comparable control at each time point were statistically analyzed by Mann-Whitney *U* test for unpaired variables. The differences were considered significant if p values < 0.05 were obtained.

RESULTS

Circulatory Parameters and Mortality

Mean arterial pressure of all animals before hemorrhage averaged 123 ± 4 mm Hg without significant differences between the experimental groups. The average volume of blood that was withdrawn to achieve 30 mm Hg was 9.9 ± 0.3 mL (52 ± 0.1% of estimated circulating blood volume). After reinfusion of the withdrawn blood, MAP returned to 114 ± 13 mm Hg after 20 minutes. Although all control animals survived the course of the experiments, a mortality of 17% was observed in the ischemia group (11/64), indicating moderately severe ischemia.

Reflectance Spectroscopy

Pancreatic perfusion significantly decreased during hypotension, as evidenced by a reduction of total hemoglobin absorbance in the pancreas from 0.30 ± 0.02 units at baseline to 0.18 ± 0.01 units during hypotension ($p < 0.001$) (Fig. 1). Concomitantly, oxygen saturation de-

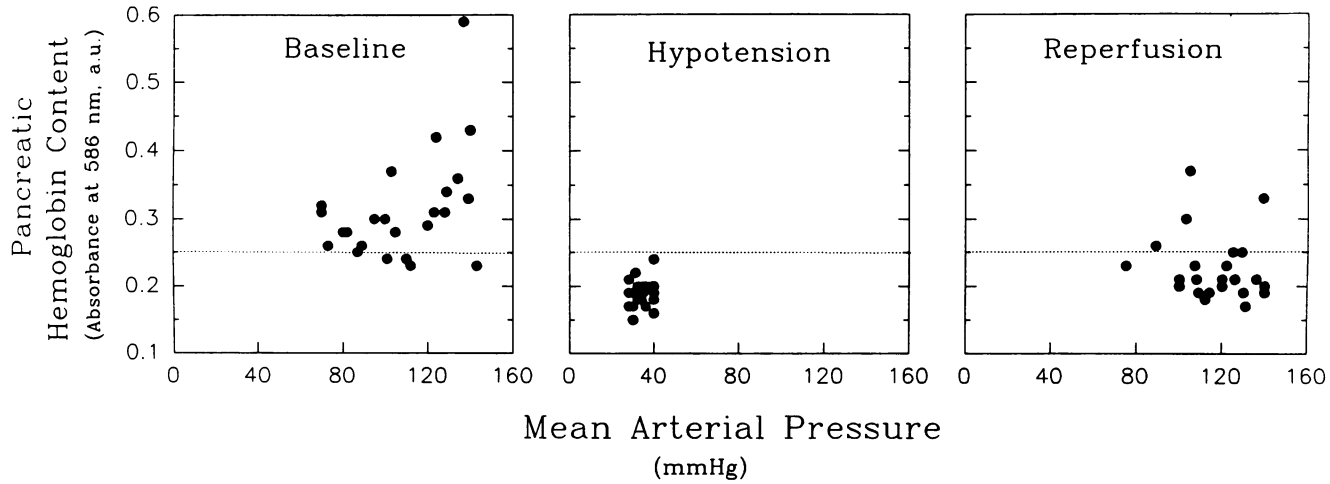

Figure 1. Changes of pancreatic hemoglobin content as a function of changes of mean arterial pressure at baseline, during controlled hemorrhagic hypotension, and reperfusion assessed by diffuse reflectance spectroscopy. Hemoglobin specific absorbance is reduced significantly during hypotension, and its failure to return to prehemorrhage levels after reperfusion indicates incomplete recovery of tissue perfusion.

creased from 51 ± 1% to 21 ± 2% ($p < 0.001$) (Fig. 2). During the subsequent reperfusion period, pancreatic hemoglobin content increased only to 0.23 ± 0.02 units ($p < 0.001$), whereas ISO_2 rose to 33 ± 3% ($p < 0.001$).

Serum Amylase and Isoamylase Pattern

Serum amylase levels of sham-operated animals showed no significant changes during the 24-hour experimental period (48.0 ± 2.1 units/L). In contrast, hyperamylasemia was observed in ischemic animals after 20 minutes of hemorrhage (110 ± 5.5 units/L, $p < 0.05$ *vs.* baseline). From these elevated levels, a second significant increase of serum amylase concentrations occurred between 6 and 24 hours (151.3 ± 18.4 units/L after 24 hours, $p < 0.001$ *vs.* 6 hours) (Fig. 3A). Electrophoretic analysis of the serum amylase isoenzyme pattern in five animals subjected to ischemia showed that the observed increase of serum amylase activity was caused primarily by isoenzymes of pancreatic origin (Fig. 3B).

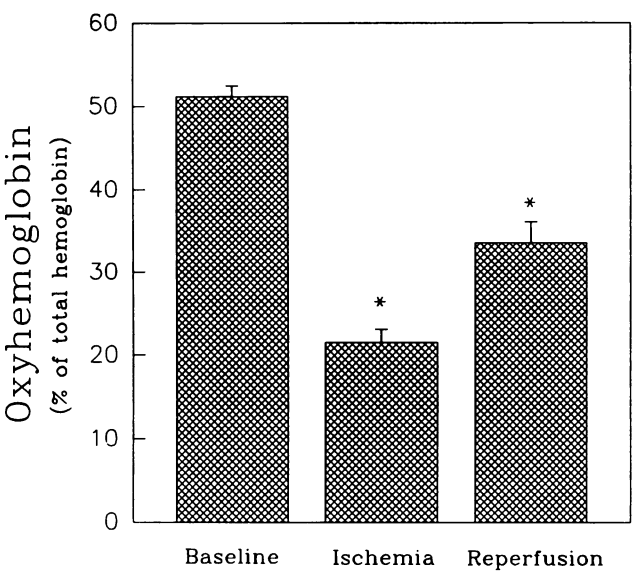

Figure 2. Changes of pancreatic hemoglobin oxygenation during pancreatic ischemia and reperfusion. Values are mean ± SEM (*$p < 0.001$).

Trypsinogen Activation Peptide

In control animals, neither TAP serum (7.92 ± 0.7 nM/L) nor tissue levels (63.0 ± 5.4 nM/L × g) changed significantly throughout the experiments. In the ischemia group of animals, a transient decrease of serum TAP concentrations (5.92 ± 0.8 nM/L, $p < 0.05$) was observed during the hypotensive period, subsequently returning to baseline values during the remaining observation period (Fig. 4A). In contrast, TAP concentrations in post-ischemic pancreatic tissue homogenates were significantly increased at 1 hour (134 ± 16 nM/L × g, $p = 0.02$) and increased to 341 ± 65 nM/L × g ($p = 0.002$) after 24 hours (Fig. 4B).

Morphologic and Histopathologic Changes

Although macroscopic edema formation was absent in control preparations (wet/dry weight ratio 2.5 ± 0.1 after 24 hours), development of pancreatic edema was visibly evident in the ischemia group within 1 hour after reinfusion of the withdrawn blood, as reflected by an increase of wet/dry weight ratio to 3.6 ± 0.1 after 1 hour ($p <$

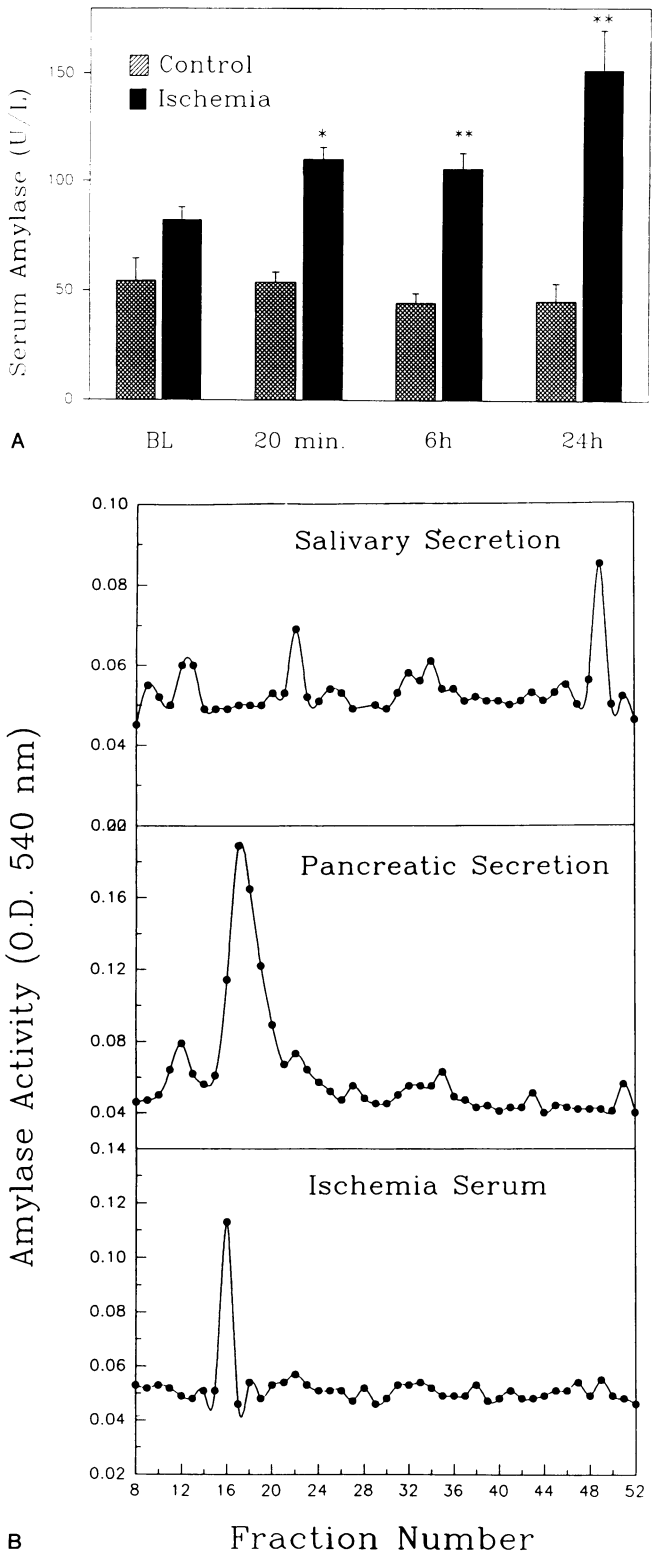

Figure 3. (A) Serum amylase activity in ischemia group of animals (solid) increases within 20 minutes after the ischemic insult (*$p < 0.05$) followed by a further increase between 6 and 24 hours (**$p < 0.001$), whereas controls (hatched) remain unchanged. (B) Representative electrophoretic patterns of amylase activity in serum of the ischemia group compared with rat pancreatic secretions and saliva depict the origin of the increased rat serum amylase as pancreatic isoamylase.

0.001) and 4.1 ± 0.3 at 24 hours ($p < 0.001$) (Fig. 5). Light microscopic analysis also revealed no significant histologic changes in the sham-operated animals, whereas ischemia induced early formation of interstitial edema at 1 hour (1.6 ± 0.2 score points *vs.* 0.6 ± 0.2 at baseline, $p < 0.001$), which persisted throughout the experiments (1.2 ± 0.1 at 24 hours, $p < 0.05$). Although no histologic evidence of pancreatic necrosis was obtained within the first 6 hours after hemorrhage, significant acinar cell necrosis was obtained at 24 hours, resulting in a significant increase of histologic necrosis score (0.3 ± 0.03 score points, $p < 0.05$) (Fig. 6). Inflammatory infiltration of the pancreas was evident in 29% of animals, but it did not reach significant levels 24 hours after the ischemic insult.

DISCUSSION

The potential role of pancreatic ischemia as an etiologic factor for acute pancreatitis is gaining increasing attention. Clinical and experimental studies have repeatedly shown the association of this factor with the development of acute pancreatitis,[6-8,11-19] however, the mechanism and significance of this pancreatic susceptibility to ischemia is still controversial.[20,21] The main debate revolves around whether ischemia alone is sufficient enough to produce acute pancreatitis or simply serves as a cofactor that converts an initial insult to the pancreas to necrotizing pancreatitis.[1,6-8]

In this study, temporary ischemia caused an acute elevation of pancreas-specific amylase in serum after 20 minutes. This increase was accompanied by significant formation of pancreatic edema as assessed by increased wet/dry weight ratio and histologic edema score. A comparable effect of temporary ischemia on amylase levels and morphology was described by other investigators using an *ex vivo* perfused pancreas model.[30,31] They attributed their findings to the generation of oxygen-derived free radicals during reperfusion because the development of these morphologic changes was inhibited by the administration of superoxide dismutase and catalase.[31] In the current study, a second increase of serum amylase levels was noted between 6 and 24 hours. This additional increase may have resulted from a release of amylase from irreversibly damaged cells as necrosis develops. Development of significant acinar cell necrosis was observed in this study between 6 and 24 hours after the initial ischemic insult. Using a similar technique for induction of pancreatic ischemia, other investigators failed to demonstrate significant necrosis formation.[8] This dis-

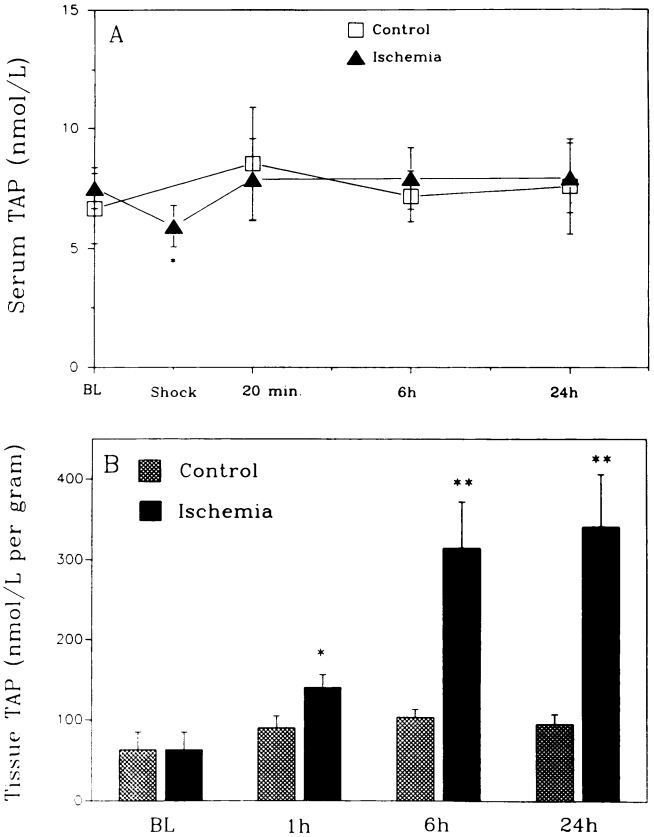

Figure 4. (A) Compared with sham operation □, animals subjected to temporary ischemia (▲) show only a transient decrease of trypsinogen activation peptide (TAP) serum levels during the hypotensive period (*$p < 0.05$) returning to baseline values thereafter. (B) In comparison, TAP in rat pancreatic tissue homogenates of animals undergoing reduction of MAP to 30 mm Hg for 30 minutes (solid) increases within the first hour after the ischemic challenge, whereas controls (hatched) are unchanged (**$p < 0.01$, *$p < 0.05$, t test).

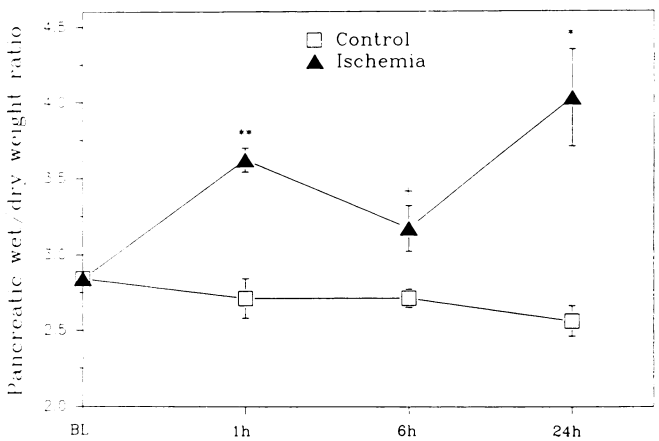

Figure 5. Development of pancreatic wet/dry weight ratio as a quantitative measure of pancreatic edema formation. Controls (□) are compared to ischemia animals (▲). Data points represent mean ± SEM of five different experiments for each time point. (**$p < 0.001$, *$p < 0.01$, +$p < 0.05$, ischemia *vs.* control, Student's t test).

crepancy may result from the detailed analysis and precision of the histologic scoring system used in the present study.

Premature extraluminal activation of trypsinogen is thought to represent a very early event in the pathogenesis of acute pancreatitis.[22] An increase of trypsin-like activity has been reported after reperfusion in pancreatic allografts after 24 hours of cold ischemia.[32] However, because trypsin-like activity mainly represented immunoreactive trypsinogen without evidence of trypsinogen activation, and similar biochemical changes also were induced by storage solutions and rejection,[33] those findings must be considered inconclusive. Investigation of premature protease activation in the present study was performed by measurement of TAP. Trypsinogen activation peptides are small peptides consisting of 5 to 8 amino acids that are physiologically cleaved from the amino-terminus of trypsinogen during its intraintestinal activation by enterokinase.[34] Normally, TAP are metabolized rapidly by mucosal peptidases, but in case of pathologic extraintestinal trypsinogen activation, they can be detected in serum, ascites, and urine in equimolar quantity to the number of generated active trypsin molecules.[35,36] Thus, they provide a quantitative measure of trypsinogen activation that is independent of antiprotease activity and other routes of trypsin degradation or clearance. We have previously used measurement of TAP to quantify trypsinogen activation in various models of acute pancreatitis and demonstrated that TAP reflected the severity of the disease.[35-37] To characterize the effect of the ischemic insult, we investigated changes of TAP concentrations in serum and pancreatic tissue homogenates. A small amount of TAP was found in both pancreatic tissue and serum at baseline. These levels remained unchanged in

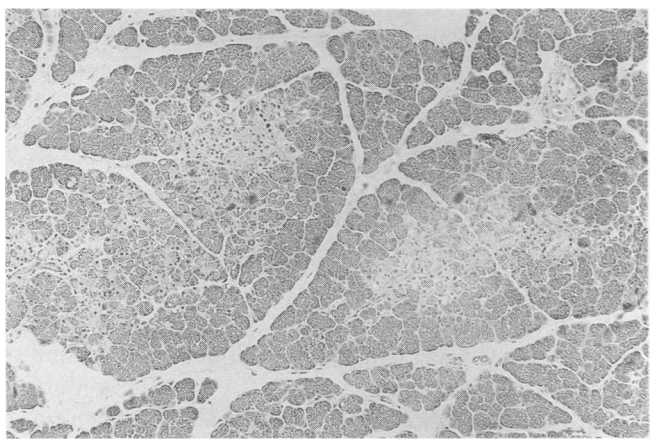

Figure 6. Histopathologic analysis of a rat pancreas 24 hours after severe temporary hypotension demonstrates moderate edematous expansion of interlobular septa, significant acinar cell necrosis, and mild inflammatory infiltration (H & E, ×80).

control animals throughout the observation period and likely reflect the small amount of physiologic autoactivation that has been described in the normal pancreas[38,39] and perhaps, some background activity of the assay. In contrast, ischemia caused a significant increase of TAP concentrations in pancreatic tissue homogenates after only 1 hour and continuing to 24 hours. In preceding development of significant necrotic changes, this early occurrence of increased TAP generation indicates that this phenomenon is a primary event in the ischemia-induced pancreatic changes, rather than a secondary effect caused by activation of proteases in necrotic cells. Simultaneous serum levels did not reflect the rise of TAP in pancreatic tissue. These findings are in accordance with other studies[20,21] that failed to show a significant increase of tryptic activity in pancreatic exudates after cross-clamping the pancreatoduodenal artery in dogs for 5 hours. The observed discrepancy between serum and tissue TAP most likely resulted from a reduced washout of TAP from the pancreas because pancreatic perfusion remained impaired even after reinfusion of the withdrawn blood, as indicated by the persistent reduction of pancreatic hemoglobin content and ISO_2. We previously have demonstrated severe reduction of perfused pancreatic exchange capillaries, limited tendency for reperfusion, and evidence for functional microcirculatory shunting by means of pancreatic intravital microscopy during hemorrhagic hypotension.[23] The observed decrease of serum TAP concentrations during the ischemic period further support our hypothesis.

Another possible explanation for the discrepancy between the observed changes of serum and tissue TAP concentrations might relate to the fact that TAP is generated in an intracellular compartment. The following two mechanisms have been proposed to initiate proteolytic conversion of zymogens within the acinar cell: 1) enzymatic activation by cathepsin B after a colocalization of this lysosomal enzyme with inactive protease precursors (crinophagy)[40] and 2) trypsinogen autoactivation.[22] Studies on the subcellular changes in the acinar cells after hemorrhagic shock demonstrated marked lysosomal vacuolization, increased lysosomal size, and elevated lysosomal fragility and disruption.[24,41] In comparison, Printz et al.[42] did not observe subcellular redistribution of cathepsin B after hemorrhagic shock. However, their experimental protocol included only a 30% reduction of MAP, which might not cause the same severity of cellular changes as the 70% to 75% reduction used by those other investigators and in the present study. Cellular acidosis represents a pathophysiologic event in all ischemic cells.[43] Decreased intracellular pH would increase the capability of cathepsin B to activate trypsinogen, which is optimal at pH 3.8,[39] and therefore, promote intracellular enzymatic activation of trypsinogen.

Elevation of cytosolic free calcium concentration is another characteristic phenomenon of ischemic cell injury and is further increased by reperfusion.[44] Because calcium is an essential cofactor in trypsinogen autoactivation[39] and increased calcium concentrations have been shown to increase autoactivation *in vitro,*[45] ischemia-induced elevation of free cytosolic calcium could result in an increase of trypsinogen autoactivation in the acinar cell, the second proposed mechanism for intracellular protease activation.[22]

Pancreatic ischemia in the rat causes significant microcirculatory changes, hyperamylasemia, pancreatic edema, and acinar cell necrosis. The morphologic changes preceded the increased intrapancreatic activation of trypsinogen. This finding for the first time conclusively shows that temporary pancreatic ischemia per se increases inappropriate protease activation, and supports the hypothesis that ischemia may initiate acute pancreatitis in addition to its known role in propagating this disease. The findings suggest a possible role for treatment with antiproteases or microcirculatory enhancement in clinical circumstances associated with pancreatic ischemic injury, such as during cardiopulmonary bypass.[11,13-16]

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