Cathepsin B inhibition prevents trypsinogen activation and reduces pancreatitis severity

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OVER 100 YEARS AGO, Chiari suggested that acute pancreatitis is an autodigestive disease in which the pancreas is injured by enzymes that it normally synthesizes and secretes. Under physiological conditions, most of the potentially harmful digestive enzymes produced by pancreatic acinar cells are synthesized and secreted as inactivezymogens that become activated only after they reach the duodenum. Considerable interest, therefore, has been focused on the mechanisms by which those enzymes might become activated, prematurely, within the pancreas and cause pancreatic injury. Our own studies have suggested that intrapancreatic activation of trypsinogen, and presumably otherzymogens, occurs within acinar cells and that it is mediated by the lysosomal hydrolase cathepsin B (19). Although controversial, several recent publications have supported this proposal. In one, genetic deletion of cathepsin B was shown to partially protect mice from pancreatitis and to reduce intrapancreatic activation of trypsinogen, which occurs during pancreatitis (8). In another study, one of the mutated forms of cationic trypsinogen shown to be associated with hereditary pancreatitis was noted to display increased sensitivity to cathepsin B-mediated activation (22).

We (19) have previously shown that, under in vitro conditions, the cell permeant cathepsin B inhibitor E64d can prevent the intra-acinar cell activation of trypsinogen, which follows exposure of freshly isolated pancreatic acini to a supramaximally stimulating concentration of the cholecystokinin analog cerulein. The present studies were designed to examine the role of cathepsin B in the in vivo rather than in vitro intrapancreatic activation of trypsinogen and the importance of this phenomenon in two dissimilar models of pancreatitis. We did not use E64d for these studies because of its relatively short half-life in vivo (23). Instead, we used another cathepsin B inhibitor, [1-3-trans-(propylcarbamoyl)oxirane-2-carbonyl]-l-isoleucyl-l-proline methyl ester (CA-074me), which is known to be highly soluble, cell permeant, and effective for prolonged periods. CA-074me was tested in two dissimilar models of pancreatitis, the secretagogue and the intrapancreatic activation model, and in both, it was found to prevent intrapancreatic activation of trypsinogen as well as to markedly reduce the severity of pancreatitis. These studies suggest that pharmacological methods of inhibiting cathepsin B activity may offer promise in the prevention and/or treatment of clinical pancreatitis.
MATERIALS AND METHODS

All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center. Male Wistar rats (100–125 g), obtained from Charles River Laboratories (Wilmington, MA) and male CD-1 mice (18–20 g) obtained from Taconic (Germantown, NY) were housed in temperature-controlled (23 ± 2 °C) rooms with a 12:12-h light-dark cycle. They were fed standard laboratory chow, given water ad libitum, fasted overnight before each experiment, and randomly assigned to control or experimental groups. Cerulein, the decapetide analog of the potent pancreatic secretagogue cholecystokinin, and the cathepsin B substrate were purchased from Research Plus (Bayonne, NJ). Boc-Glu-Ala-Arg-4-methylcoumaryl-7-amide (MCA; the trypsin substrate) and CA-074me were from Peptides International (Louisville, KY). Anti-rabbit trypsinogen activation peptide (TAP) antibody was a kind gift from D. McNally (Abbott Laboratories, North Chicago, IL), and biotinylated anti-rabbit IgG and streptavidin alkaline phosphatase were purchased from Vector Laboratories (Burlingame, CA). Ketamine and xylazine were obtained from Abbott Laboratories and Bayer, respectively. Na+-taurocholate, as well as other chemicals and reagents, were purchased from Sigma (St. Louis, MO).

Pretreatment of animals with CA-074me. Stock solutions of the cathepsin B inhibitor CA-074me were made at a concentration of 10 mg/ml in dimethyl sulfoxide. This was diluted 1:10 in saline and administered at a dose of 10 mg/kg body wt by tail vein injection 1 h before the induction of pancreatitis. Control animals received vehicle alone.

Induction of pancreatitis. CD-1 mice were given hourly (×6) intraperitoneal injections of cerulein (50 µg/kg) to elicit secretagogue-induced pancreatitis, whereas control animals received intraperitoneal injections of a comparable amount of saline. At selected times, animals were killed by CO2 asphyxia and tissue samples were obtained for study. To elicit duct infusion-induced pancreatitis, male Wistar rats were anesthetized with ketamine and xylazine and subjected to midline laparotomy. A blunt needle was introduced transduodenally into the common biliopancreatic duct by the method of Heinkel (11) and secured in place with a ligature around the duct. The hepatic duct was closed at the hilum of the liver with a bulldog clamp. Sodium taurocholate (5% in saline) was infused using a compact infusion pump (model 975; Harvard Apparatus) at a rate of 0.052 ml/min, and each rat received a total volume of 1 ml/kg body wt. The needle, ligature, and clamp were then removed, and the laparotomy incision was closed. Control animals underwent sham operation, which, under an identical anesthesia, consisted of laparotomy and puncture of the duodenum. Six hours after duct infusion or sham operation, animals were killed by CO2 asphyxia and samples were taken for study.

Trypsin activity in pancreas homogenates. Preliminary studies were done to decide the time at which trypsin activity peaked in the pancreas during each form of experimental pancreatitis. In the secretagogue-induced model, this was found to be 30 min after the start of cerulein administration, after which time the levels declined. In the duct infusion model, trypsin activity peaked 45 min after taurocholate infusion and remained elevated to the same extent until 6 h after duct infusion. After tissue harvest, the pancreas samples were homogenized in cold (4°C) MOPS buffer, pH 7.0 (250 mM sucrose, 5 mM MOPS, 1 mM MgSO4) using a motorized glass-Teflon homogenizer. The resulting homogenate was centrifuged (50, 5 min), and the supernatant was taken for assay. Trypsin activity was measured fluorometrically using Boc-Glu-Ala-Arg-MCA as the substrate according to the method of Kawabata et al. (14). DNA was measured using Hoechst dye 33258, according to the method of Labarca and Paigen (17). The trypsin results were expressed as trypsin activity per microgram of DNA in the homogenate, as described previously (12).

TAP levels. Immediately after death, the pancreas was removed and a portion was resuspended in TAP assay buffer, pH 7.4 (20 mM Triz-HCl, 5 mM EDTA, 150 mM NaCl). The samples were boiled for 10 min, homogenized in the same buffer, and centrifuged; the supernatant was frozen and stored for later TAP measurement. TAP levels were determined by direct competitive ELISA using affinity-purified rabbit polyclonal anti-TAP antibodies, according to the method of Gudgeon et al. (7). TAP levels were expressed as picograms of TAP per microgram of DNA.

Serum amylase. Harvested blood was allowed to clot and was centrifuged, and the serum obtained was used for measurement of amylase activity. Serum amylase activity was measured using 4,6-ethylidene (G7)–nitrophenyl (G 1)–naphthylamide as the substrate. Cathepsin B activity was determined according to the method of McDonald and Ellis (18), with minor modifications, using CBZ-arginyllarginine-β-naphthylamide as the substrate. Cathepsin B results were expressed as cathepsin B activity (slope of fluorescence emission per microgram DNA in the homogenate).

Neutrophil sequestration. The sequestration of neutrophils within the pancreas was evaluated by quantitating tissue myeloperoxidase (MPO) activity using a modification of the bromide-dependent chemiluminescence technique as described by Haqqani et al. (10).

Morphology. The extent of pancreatic acinar cell necrosis and interstitial edema were morphometrically quantitated by an observer who was not aware of the sample identity. For these studies, paraffin-embedded samples were sectioned (5 µm) and stained with hematoxylin and eosin. Ten randomly chosen microscopic fields (×125) were examined for each tissue sample, and the extent of acinar cell injury/necrosis was expressed as a percentage of total acinar tissue as described previously (13). Using the same slides, tissue edema was scored using a 0–3 scale in which 0 = none and 3 = severe.

In vitro studies. For these studies, dispersed rat pancreatic acini were prepared by collagenase digestion and gentle shearing as described previously (4). Acini were resuspended in HEPES-Ringer buffer (pH 7.4), which contained (in mM) 115 NaCl, 5 KCl, 1 MgSO4, 1 CaCl2, 10 HEPES, and 15 glucose and 0.1% bovine serum albumin. Viability of acini was >95% as assessed by trypan blue exclusion. After incubation of acini with various agents, the cells were washed twice with HEPES-Ringer buffer and processed for either trypsin activity or TAP measurements as described above. Amylase secretion from pancreatic acini was determined as described previously (19).

Data presentation. Results reported represent means ± SE for multiple determinations from four or more animals in each group. In the figures, vertical bars denote SE. Student’s t-test was used to evaluate the significance of changes. A value of P < 0.05 was considered statistically significant.

RESULTS

We and others (1, 6) have previously reported that both supramaximal secretagogue stimulation and ret-
Effects of CA-074me on pancreatic cathepsin B activity. As shown in Fig. 5, A and B, cathepsin B activity in the pancreas was not significantly changed during either model of pancreatitis. Pretreatment of animals with CA-074me resulted in a profound decrease in pancreatic cathepsin B activity in both the secretagogue- and the duct infusion-induced model. Under these conditions cathepsin B activity was barely detectable. A similar reduction in pancreatic cathepsin B activity was observed when control animals were given CA-074me (data not shown).

Effects of CA-074me on the severity of duct infusion-induced pancreatitis. Both models are characterized by a rise in serum amylase activity, pancreatic edema, and acinar cell necrosis (Figs. 1 and 2). Intrapancreatic TAP levels and trypsinogen activation increased in both models, indicating that intrapancreatic trypsinogen activation occurred (Figs. 3 and 4). In addition, pancreatic MPO activity in the pancreas increases in both models, indicating a sequestration of neutrophils within the pancreas occurred (Figs. 1 and 2).

Effects of CA-074me on pancreatic cathepsin B activity. As shown in Fig. 5, A and B, cathepsin B activity in the pancreas was not significantly changed during either model of pancreatitis. Pretreatment of animals with CA-074me resulted in a profound decrease in pancreatic cathepsin B activity in both the secretagogue- and the duct infusion-induced model. Under these conditions cathepsin B activity was barely detectable. A similar reduction in pancreatic cathepsin B activity was observed when control animals were given CA-074me (data not shown).
Effects of CA-074me on cerulein-induced in vitro trypsinogen activation. As shown in Fig. 6, incubation of freshly prepared acini with 1 mM CA-074me completely prevented cerulein-induced in vitro activation of trypsinogen as determined by measurement of trypsin activity and TAP levels in cellular homogenates.

Effects of CA-074me on secretagogue-induced pancreatitis. As shown in Figs. 2 and 4, the administration of CA-074me before retrograde pancreatic duct infusion with Na\(^+\)-taurocholate reduces the intrapancreatic rise in trypsin activity and MPO activity as well as the extent of acinar cell necrosis in this model of pancreatitis. Morphological changes that characterize this model, including inflammation and necrosis, are rather profound changes, CA-074me pretreatment caused only modest, but significant, reduction in the magnitude of secretagogue-induced hyperamylasemia (Fig. 1).

To rule out the possibility that the protease inhibitor protects against pancreatitis by some nonspecific effects with respect to acinar cell function or by directly inhibiting the enzymatic activity of trypsin, we studied the effects of CA-074me on cerulein-induced amylase secretion from freshly prepared acini and in vitro trypsin activity in a cell-free system. Results of these experiments indicate that the cysteine protease inhibitor by itself had no effect on either trypsin activity or on cerulein-stimulated amylase secretion from acinar cells (Fig. 8). In another in vitro study (data not shown), we found that the cysteine protease inhibitor did not directly affect the measurements of TAP in a cell-free system.

Effects of CA-074me on duct infusion-induced pancreatitis. As shown in Figs. 2 and 4, the administration of CA-074me before retrograde pancreatic duct infusion with Na\(^+\)-taurocholate reduces the intrapancreatic rise in trypsin activity and MPO activity as well as the extent of acinar cell necrosis in this model of pancreatitis. Morphological changes that characterize this model, including inflammation and necrosis, are

Fig. 5. Effect of CA-074me on pancreatic cathepsin B activity during secretagogue-induced (A) and duct infusion-induced (B) pancreatitis. Groups are as defined in Figs. 1 and 2 legends. Cathepsin B activity in the pancreas was measured, as described in the text, 6 h after the start of CER administration or duct infusion. Results are expressed relative to that observed for animals given only saline (1.6 ± 0.15 U/μg DNA; A, Control) or only subjected to sham operation (6.3 ± 0.24 U/μg DNA; B, Control). *P < 0.05 compared with value of control animals not given CA-074me.

Fig. 6. Effect of CA-074me on CER-induced in vitro trypsinogen activation. Freshly prepared rat pancreatic acinar cells were preincubated with 1 mM cathepsin B inhibitor CA-074me for 30 min. The acini were further incubated with 0.1 μM CER along with CA-074me for 30 min, and trypsin activity (A) and TAP levels (B) in homogenized acini were measured as described in the text. Values shown are expressed as means ± SE obtained from at least three different experiments.
also reduced by CA-074me pretreatment (Fig. 7). In contrast to findings with the secretagogue model, a more profound reduction in hyperamylasemia was noted after pretreatment with CA-074me in this model (Fig. 2). On the other hand, little or no change in the magnitude of pancreatic edema was observed (Fig. 7).

DISCUSSION

In the present studies, we used a pharmacological approach to inhibit acinar cell cathepsin B, a lysosomal cysteine protease belonging to the papain family of endopeptidases (15). Studies by others (2) using the agent we chose for our experiments, CA-074, have shown that it is relatively specific for cathepsin B and highly potent. It is believed to act by irreversibly binding to Cys^{29}, causing permanent inactivation of the enzyme. In addition to inhibiting cathepsin B, CA-074 can inhibit other lysosomal cysteine proteases, including cathepsins H and L, but only with a potency that is two to three orders of magnitude less than that noted for cathepsin B (2). CA-074 is highly negatively charged and, as a result, relatively impermeant to cells. To increase the likelihood that we would achieve effective intracellular levels of CA-074 in our studies, we have administered the cathepsin B inhibitor in its methyl ester form. CA-074me is readily taken up by cells, and it remains intracellularly active after deesterification by intracellular esterases (2). For in vivo studies, others used doses ranging from 4 to 80 mg/kg body wt (24, 25). In our studies, we chose to use a dose of 10 mg/kg body wt.

We have found that the administration of CA-074me markedly reduces pancreatic cathepsin B activity under resting conditions and in two dissimilar models of pancreatitis. This reduction in cathepsin B activity is associated, in both models, with 1) a reduction in intrapancreatic trypsinogen activation and 2) a reduction in the severity of pancreatitis. The observation that CA-074me pretreatment results in these changes in both models would strongly indicate that the protective effects we noted are not model specific. Rather, they suggest that our findings are applicable to the general issue of pancreatitis. Thus our observation that cathepsin B inhibition with CA-074me prevents

Fig. 7. Effect of CA-074me on morphological changes of pancreatitis. A–C: representative photomicrographs taken of samples from control mice (A), mice given CER for 6 h (B), and mice given CA-074me before CER (C). Note marked inflammation, edema, and acinar cell necrosis in the CER-alone sample that are markedly reduced in a sample taken from an animal given CA-074me before CER (compare B and C). D–F: are representative photomicrographs taken of samples from control rats (D), rats intraductally infused with Na^+TAUR (E), and rats given CA-074me before duct infusion with TAUR (F). Note inflammation and necrosis in duct infusion-alone group that are markedly reduced by the administration of CA-074me before duct infusion (compare E and F).
trypsinogen activation and reduces the severity of pancreatitis indicates that cathepsin B inhibition, with this or other agents, might reduce the severity of clinical pancreatitis as well. Interestingly, we also found that CA-074me pretreatment prevents nuclear factor (NF)-κB activation during cerulein-induced pancreatitis (data not shown). This effect on NF-κB activation is not surprising, because cysteine proteases are known to play an important role in proteasome function and proteasome function is required for NF-κB activation (9). This reduction in NF-κB activation, observed after CA-074me administration, may contribute to the reduction in pancreatitis severity we observed, because, as recently noted by Chen et al. (3), direct NF-κB activation in the pancreas can induce pancreatic inflammation. This mechanism, however, cannot explain the prevention of trypsinogen activation noted in our studies to follow CA-074me pretreatment, because direct activation of NF-κB leads to little, if any, trypsinogen activation (3). In contrast to its effects on NF-κB activation, CA-074me was not observed in our studies to alter the upregulated expression of several cytokines/chemokines (e.g., monocyte chemoattractant protein-1 and macrophage inflammatory protein-1α) in the pancreas during pancreatitis (data not shown). Presumably, this indicates that expression of those inflammatory mediators is not regulated by NF-κB activation and that at least these mediators are not, by themselves, sufficient to induce pancreatitis in the absence of trypsinogen activation.

A series of in vitro studies were performed to be certain that the cysteine protease inhibitor CA-074me does not interfere with intrapancreatic trypsinogen activation by nonspecific mechanisms. In those studies, we found that CA-074me prevents in vitro cerulein-induced trypsinogen activation (i.e., it inhibits the cerulein-induced rise in intra-acinar cell trypsin activity and TAP levels) (Fig. 6) as it does under in vivo conditions. On the other hand, CA-074me does not appear to alter acinar cell function, i.e., cerulein-stimulated acinar cell secretion of amylase is not altered in the presence of CA-074me (Fig. 8). Furthermore, CA-074me does not directly alter either trypsin activity (Fig. 8) or measurable TAP levels when evaluated in vitro in a cell-free system. From these studies, we conclude that the in vivo changes noted with CA-074me are not the result of nonspecific effects of CA-074me on either acinar cell function or the measurement of trypsinogen activation.

Mechanisms by which cathepsin B might mediate intracellular trypsinogen activation and regulate the severity of pancreatitis are poorly understood. The theory we favor suggests that, during the early stages of pancreatitis before intracellular zymogen activation, digestive enzyme zymogens and lysosomal hydrolases, including cathepsin B, become colocalized, and, within that colocalization compartment, cathepsin B catalytically activates trypsinogen (20). Although this theory remains unproven, there is a gradually enlarging body of evidence that has supported it. Work by several groups has clearly shown that, under appropriate conditions, cathepsin B can cleave the activation peptide from trypsinogen and, as a result, activate the zymogen (5, 19). Our own studies have indicated that colocalization of lysosomal hydrolases with digestive enzyme zymogens occurs before zymogen activation in experimental pancreatitis and that the colocalization of these two types of enzyme occurs within the compartment in which zymogen activation occurs (12, 21). Furthermore, in an in vitro system, we found that the cathepsin B inhibitor E64d could prevent secretagogue-induced intra-acinar cell activation of trypsinogen (19). The results of the present studies are consistent with these prior observations, and they extend our earlier work by demonstrating that, in addition to preventing trypsinogen activation, cathepsin B inhibition reduces the severity of pancreatitis when evaluated under in vivo conditions.

<Fig. 8. Effect of CA-074me on amylase secretion and trypsin activity. A: rat pancreatic acini were incubated for 30 min with different concentrations of CER in the presence and absence of cathepsin B inhibitor, CA-074me. The fraction of amylase secreted into the medium was quantitated as described in the text. Results are expressed as the percentage of maximal response. The maximal amylase secretion was 25 ± 3% of total acinar amylase content and was observed after stimulation with 0.1 nM CER for 30 min. B: different amounts of commercial trypsin were assayed in the presence and absence of 1 mM CA-074me. Trypsin activity is expressed in relative fluorescence units. Data shown are typical of three experiments.>
Halangk et al. (8) recently reported the results of studies evaluating the role of cathepsin B in pancreatitis using mice with a genetic deletion of cathepsin B. They found that deletion of cathepsin B was associated with ~50% reduction in intrapancreatic trypsinogen activation and in the severity of pancreatitis. In contrast, in the present studies, we noted a more profound reduction in both trypsinogen activation and in pancreatitis severity. These somewhat discrepant observations may indicate that other cathepsins, sensitive to CA-074me inhibition but not lost by a genetic deletion of cathepsin B, may contribute to trypsinogen activation and disease severity in pancreatitis. Further studies using agents targeting other lysosomal enzymes will be needed to address this issue. Regardless of the results of those studies, however, our present observations suggest that pharmacological interventions resulting in cathepsin B inhibition might prove useful in either preventing acute pancreatitis or reducing its severity.

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REFERENCES

3. Chen X, Ji B, Han B, Ernst SA, Simeone D, and Logsdon CD. NFkappaB activation and in the severity of pancreatitis. These somewhat discrepant observations may indicate that other cathepsins, sensitive to CA-074me inhibition but not lost by a genetic deletion of cathepsin B, may contribute to trypsinogen activation and disease severity in pancreatitis. Further studies using agents targeting other lysosomal enzymes will be needed to address this issue. Regardless of the results of those studies, however, our present observations suggest that pharmacological interventions resulting in cathepsin B inhibition might prove useful in either preventing acute pancreatitis or reducing its severity.

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