Cause-effect relationships between zymogen activation and other early events in secretagogue-induced acute pancreatitis

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Van Acker GJ, Weiss E, Steer ML, Perides G. Cause-effect relationships between zymogen activation and other early events in secretagogue-induced acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 292: G1738–G1746, 2007. First published March 1, 2007; doi:10.1152/ajpgi.00543.2006.—We have hypothesized that the colocalization of digestive zymogens with lysosomal hydrolases, which occurs during the early stages of every experimental pancreatitis model, facilitates activation of those zymogens by lysosomal hydrolases such as cathepsin B and that this activation triggers acute pancreatitis by leading to acinar cell injury. Some, however, have argued that the colocalization phenomenon may be the result, rather than the cause, of zymogen activation during pancreatitis. To resolve this controversy and explore the causal relationships between zymogen activation and other early pancreatitis events, we induced pancreatitis in mice by repeated supramaximal secretagogue stimulation with caerulein. Some animals were pretreated with the cathepsin B inhibitor CA-074me to inhibit cathepsin B, prevent intrapancreatic activation of digestive zymogens, and reduce the severity of pancreatitis. We show that inhibition of cathepsin B by pretreatment with CA-074me prevents intrapancreatic zymogen activation and reduces organellar fragility, but it does not alter the caerulein-induced colocalization phenomenon or subcellular F-actin redistribution or prevent caerulein-induced activation of NF-κB, ERK1/2, and JNK or upregulated expression of cytokemokines. We conclude 1) that the colocalization phenomenon, F-actin redistribution, activation of proinflammatory transcription factors, and upregulated expression of cytokemokines are not the results of zymogen activation, and 2) that these early events in pancreatitis are not dependent on cathepsin B activity. In contrast, zymogen activation and increased subcellular organellar fragility during caerulein-induced pancreatitis are dependent on cathepsin B activity.

tryptsinogen activation; cathepsin B; colocalization; lysosomes

STUDIES DESIGNED TO ELUCIDATE mechanisms involved in the pathophysiology of acute pancreatitis should ideally be performed using clinically derived material, but, unfortunately, that approach is not feasible because patients with acute pancreatitis are generally not identified during the earliest stages of the disease and, even if identified, access to the pancreas in patients with early acute pancreatitis is generally not possible. As an alternative, most studies dealing with the events associated with early pancreatitis have been performed using animal models of the disease, and the secretagogue (i.e., caerulein)-induced rodent model of acute pancreatitis is, perhaps, the most commonly employed and best-characterized of those models. We have used it extensively in studies designed to elucidate mechanisms responsible for the early events in pancreatitis. In those studies, we have noted that 1) lysosomal hydrolases become colocalized with digestive enzyme zymogens (15, 25) and 2) digestive zymogens become activated within acinar cells during the very early stages of pancreatitis (21). Similar events occur in each of the other pancreatitis models as well (8, 9, 17). On the basis of these as well as other observations, we hypothesized that lysosomal hydrolases catalytically activate trypsinogen during pancreatitis, and we suggested that this intra-acinar zymogen activation is the cause of the acinar cell injury/necrosis that leads to acute pancreatitis. Support for our hypothesis comes from the observation that cathepsin B, a lysosomal hydrolase, can catalytically activate trypsinogen and that trypsin can activate the other zymogens (4, 6, 10). Furthermore, both pharmacological inhibition of cathepsin B and genetic deletion of cathepsin B have been shown to diminish intrapancreatic activation of digestive enzyme zymogens during pancreatitis, and under these conditions, the severity of pancreatitis is reduced (7, 24). However, our hypothesis that a direct causal relationship exists among the colocalization phenomenon, intracellular zymogen activation, and acinar cell injury has been questioned by some investigators who have suggested that the colocalization phenomenon may be the result, rather than the cause, of zymogen activation and/or cell injury (11). Similar concerns have been raised about the potential causal relationships involving some of the other early events in pancreatitis; e.g., acinar cell cytoskeletal changes, increased fragility of lysosome-like organelles, and activation of proinflammatory events.

The currently reported studies were designed to address these mechanistic cause-effect issues and to resolve uncertainty regarding whether the colocalization phenomenon is the cause or the result of intracellular zymogen activation during pancreatitis. To this end, we took advantage of the previously reported observations 1) that effective inhibition of pancreatic cathepsin B can be achieved by intravenous administration of the cell-permeant and highly specific cathepsin B inhibitor CA-074me and 2) that under these conditions, digestive zymogen activation and the severity of caerulein-induced pancreatitis are markedly reduced (24). In the currently reported studies, we pretreated mice with CA-074me, induced pancreatitis by supramaximal stimulation with caerulein, and evaluated the effects of cathepsin B inhibition on the colocalization phenomenon and other early events that occur during secretagogue-induced pancreatitis. We show that CA-074me pretreatment does not alter the colocalization phenomenon despite the fact that the cathepsin B inhibitor prevents intrapancreatic trypsinogen activation and reduces the severity of pancreatitis. From these observations, we conclude that the colocalization phe-
nomenon is not the result of either intrapancreatic zymogen activation or cell injury during caerulein-induced pancreatitis. On the other hand, we show that pretreatment with CA-074me does prevent the increase in subcellular organellar fragility that occurs during caerulein-induced pancreatitis, and this finding leads us to suggest that this increased fragility may be the result of zymogen activation. Finally, we note that pretreatment with CA-074me does not prevent F-actin redistribution, proinflammatory transcription factor activation, or the generation of proinflammatory mediators during the early stages of this pancreatitis model, and these findings lead us to conclude 1) that these latter events are triggered by mechanisms that do not require either cathepsin B activity or intracellular cell activation of zymogens and 2) that these events may not be sufficient to induce pancreatitis in the absence of zymogen activation.

**MATERIALS AND METHODS**

All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Tufts-New England Medical Center. C57BL/6 mice, weighing 20–25 g, obtained from Taconic (Germantown, NY) were used in these studies. The animals 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. Caerulein was purchased from Sigma-Aldrich (St. Louis, MO). The trypsin substrate Boc-Glu-Ala-Arg-4-methylcumarinyl-7-amide (MCA) and the cell-permeant cathepsin B inhibitor [l-3-trans-(propylcarbamoyl)oxirane-2-carbonyl]-l-isoleucyl-l-proline methyl ester (CA-074me) were purchased from Peptides International (Louisville, KY). Anti-bodies against amylase (sheep antiserum) were purchased from The Binding Site (San Diego, CA); lysosome-associated membrane protein-1 (LAMP-1; rat monoclonal 1D4B) was obtained from The Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA); p-JNK and p-ERK1/2 were obtained from Cell Signaling (Beverly, MA); and iNOS was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 546-conjugated phalloidin was purchased from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade and purchased from Sigma-Aldrich.

**Induction of pancreatitis.** Mice were given caerulein (50 µg/kg body wt) by intraperitoneal injection at hourly intervals and killed at selected times thereafter. Some animals were pretreated with CA-074me 1 h before the start of caerulein administration. For this purpose, the zymogen granule-enriched and the lysosome-enriched pellets were resuspended in 150 µl of 0.2% (wt/vol) NaCl (4°C), and 100 µl of this suspension was added to 900 µl of prewarmed (37°C) p-nitro catechol sulfate (PNCS) buffer (111.1 mM Na-acetate, pH 5.0, 2.78 mM PNCS). The mixture was incubated at 37°C for 30 min, after which the reaction was stopped by addition of 5 ml of 1 M NaOH. Aryl sulfatase activity was then fluorometrically quantitated at 515 nm. Activity was fluorometrically measured using the specific substrate chloro-p-nitrophenyl-α-D-maltotrioside (Diagnostics Chemical, Oxford, CT) as the substrate (3). Trypsin activity was fluorometrically measured using the specific substrate MCA (Peptides International).

**Subcellular localization studies.** For immunolocalization of the digestive enzyme amylase and LAMP-1, portions of the pancreas were frozen in optimal cutting temperature (OCT) compound. Cryostat sections (8 µm) were prepared and fixed with acetone. Before antibody binding, mounted sections were first treated with sodium borohydride at a final concentration of 1 mg/ml in PBS to reduce autofluorescence. The tissue was then treated with blocking solution consisting of 1 mM L-lysine and 0.1% bovine serum albumin (BSA) in PBS, followed by incubation with primary antibodies against amylase and LAMP-1 at a dilution of 1:200 with blocking solution. Slides were washed with PBS, incubated with secondary antibodies [Texas Red-conjugated anti-sheep and fluorescein isothiocyanate (FITC)-conjugated anti-rat from Vector, Burlingame, CA] at a dilution of 1:100, and overlaid with Vectashield (Vector, Burlingame, CA). The stained samples were then examined using a Leica confocal microscope with appropriate filter sets. The extent of amylase/LAMP-1 colocalization was quantitated using CoLocalizer Pro (Co-Localization Research Software, Boise, ID) and expressed as the percentage of LAMP-1 that was colocalized with amylase. An arbitrary set threshold that allowed for reduction in background noise errors was used, and the same threshold was applied to all images.

For studies evaluating possible redistribution of subapical F-actin, 8-µm sections were treated with sodium borohydride (1 mg/ml), blocked, stained with Alexa Fluor 546-conjugated phalloidin (1 U/ml), rinsed with PBS, and covered with Vectashield. The localization of F-actin was quantitated as described earlier (12).

**Subcellular organelle fragility.** After subcellular fractionation as described above, the 1,000 g × 10 min pellet (i.e., the zymogen granule-enriched fraction to which lysosomal enzymes including cathepsin B and aryl sulfatase are redistributed during pancreatitis) was resuspended in 1 ml of MOPS buffer and incubated at room temperature (22°C) for 0–120 min. At selected times, the samples were centrifuged (10,000 g, 2 min), a 50-µl aliquot of the supernatant containing soluble material was taken for amylase assay, and the pellet was then resuspended for the remainder of the experiment.

**Activation of ERK1/2, JNK, and NF-κB.** Cytoplasmic protein extracts from pancreatic fragments were prepared as described earlier (2) in the presence of phosphatase inhibitors. ERK1/2 and JNK activation were quantitated by Western blotting using the cytoplasmic extracts and antibodies raised against the phosphorylated forms of ERK1/2 and JNK (Thr185/Tyr185; Cell Signaling, Beverly, MA). To monitor activation of NF-κB, the cytoplasmic level of inhibitor of κB (IκB) was evaluated by Western blotting. Nuclear translocation of fraction, 11% to the lysosome-enriched fraction, and 48% to the 12,000 g supernatant, which was expected to contain the endoplasmic reticulum, ribosomes, and soluble components. Thirty-two percent of cathepsin B activity was localized to the zymogen granule-rich fraction, 46% to the lysosome-enriched fraction, and 22% to the 12,000 g supernatant. Twenty-nine percent of aryl sulfatase activity was found in the zymogen granule-rich fraction, 42% in the lysosome-enriched fraction, and 29% in the 12,000 g supernatant.

**Biochemical assays.** Aryl sulfatase activity was quantitated to allow for the tracking of lysosomal hydrolases after administration of the cathepsin B inhibitor CA-074me, since under these conditions cathepsin B activity was below the measurable threshold. For this purpose, the zymogen granule-enriched and the lysosome-enriched pellets were resuspended in 150 µl of 0.2% (wt/vol) NaCl (4°C), and 100 µl of this suspension was added to 900 µl of prewarmed (37°C) p-nitro catechol sulfate (PNCS) buffer (111.1 mM Na-acetate, pH 5.0, 2.78 mM PNCS). The mixture was incubated at 37°C for 30 min, after which the reaction was stopped by addition of 5 ml of 1 M NaOH. Aryl sulfatase activity was then fluorometrically quantitated at 515 nm.

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NF-κB subunits was quantitated by ELISA using the transcription factor assay kit TransAM NF-κBp65 from Active Motif (Carlsbad, CA).

Evaluation of neutrophil chemoattractant expression by pancreas cells using quantitative RT-PCR. Quantitative RT-PCR for the housekeeping gene ARP and the neutrophil chemoattractant genes monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) was performed as previously described (18) using the following primers: ARP, (forward) 553aatgggagaacaggggga and (reverse) 1021ttgggtctcctttgctcttgtt (389 bp); IL-6, (forward) 33agttgcctctgagactgt and (reverse) 421ctctggcttctttctctctgctt (389 bp); and MCP-1, (forward) 182ctactctctgtctactctcctcc (reverse) 499gtgtaaggctctgtaa (318 bp).

Analysis of data. The results reported represent means ± SE of values obtained from multiple determinations in three or more experiments. The significance of changes was evaluated using Student’s t-test when the data consisted of only two groups or by analysis of variance (ANOVA) when three or more groups were compared. A P value ≤0.05 was considered to indicate a significant difference. In all cases, vertical error bars denote the SE, and the absence of such bars indicates that the SE is too small to illustrate.

RESULTS

Our group has previously shown that intravenous administration of CA-074me to mice markedly inhibits pancreatic cathepsin B activity and reduces the magnitude of acinar cell injury/necrosis, pancreatic edema, and pancreatic inflammation that occur during the early stages of caerulein-induced pancreatitis (24). These effects of the cell-permeant cathepsin B inhibitor on the severity of pancreatitis appear to be relatively specific, since under in vitro conditions CA-074me neither alters the pancreatic acinar cell secretory response to caerulein nor directly inhibits trypsin activity (24). Furthermore, as shown in Fig. 1, tail vein administration of CA-074me 1 h before the intraperitoneal injection of a supramaximally stimulating dose of caerulein markedly inhibits caerulein-induced intrapancreatic activation of trypsinogen. On the basis of these preliminary observations, we concluded that CA-074me might be an ideal tool for studies designed to determine whether early events in experimental pancreatitis are dependent on prior cathepsin B-induced trypsinogen activation.

Effects of CA-074me on the “colocalization phenomenon.”

Previously reported studies by our group as well as others have shown that digestive enzyme zymogens become colocalized with lysosomal hydrolases during the early stages of virtually all experimental models of pancreatitis evaluated to date (8, 9, 13–17, 25, 27). This so-called colocalization phenomenon can be detected using either techniques of subcellular fractionation or techniques of immunolocalization. In the currently reported studies, both of these complementary approaches were used to probe the effects, on this phenomenon, of inhibiting cathepsin B activity and, thus, preventing intracellular zymogen activation.

Since CA-074me is a potent cathepsin B inhibitor, our subcellular fractionation studies could not use cathepsin B activity measurements to track lysosomal enzyme localization during pancreatitis, and as an alternative to accomplish our goal, we chose to track the activity of another lysosomal enzyme (i.e., aryl sulfatase) that is not inhibited by CA-074me. As shown in Fig. 2, supramaximal stimulation with caerulein caused a time-dependent change in the relative distribution of aryl sulfatase between the lysosome-enriched and zymogen granule-enriched fractions. As expected, most of the sedimentable aryl sulfatase activity was initially detected within the lysosome-enriched fraction, but with time, aryl sulfatase became redistributed following caerulein administration. As a result, progressively more aryl sulfatase was detected within the zymogen granule-enriched fraction while less remained in the lysosome-enriched fraction, and the lysosome/zymogen granule ratio of aryl sulfatase activity fell (Fig. 2). This change in the distribution of aryl sulfatase activity mimics the change noted when cathepsin B distribution was monitored during the early stages of caerulein-induced pancreatitis, and from this observation, we concluded that aryl sulfatase activity could be used as a surrogate for cathepsin B activity in studies tracking the distribution of lysosomal hydrolases in the presence of a cathepsin B inhibitor such as CA-074me. In experiments designed to achieve that goal (Fig. 2), we found that tail vein administration of CA-074me 1 h before the intraperitoneal injection of caerulein did not alter either the magnitude or the time dependence of caerulein-induced aryl sulfatase redistribution between the lysosome-enriched and the zymogen granule-enriched subcellular fractions.

To complement subcellular fractionation studies, confocal microscopic immunolocalization studies were performed using antibodies directed against the digestive enzyme amylase and the lysosomal membrane protein LAMP-1. Representative images and quantitation of colocalized immunofluorescence are shown in Fig. 3. Supramaximal stimulation with caerulein increased the colocalization of LAMP-1 with amylase, but the prior administration of CA-074me did not alter this caerulein-induced response. Together, these subcellular fractionation and immunolocalization studies indicate that redistribution of lysosomal enzymes and their colocalization with digestive enzymes occurs despite cathepsin B inhibition and in the absence of intra-acinar cell trypsinogen activation.

Effect of CA-074me on caerulein-induced changes in subcellular organelle fragility. The subcellular fractionation studies described above, as well as many similar studies previously...
published by our group and others, indicate that the subcellular
distribution of lysosomal enzymes is altered during caerulein-
induced pancreatitis and that, as a result, those enzymes be-
come localized within digestive enzyme zymogen-containing
organelles that cosediment with the zymogen granule-enriched
fraction. To evaluate the effects of inhibiting trypsinogen
activation on the fragility of those organelles, we isolated and
incubated the zymogen granule-enriched fraction from CA-
074me-treated, caerulein-exposed animals for varying times at
22°C. Fragility was monitored by measuring the release, into
the medium, of the digestive enzyme amylase. As shown in
Fig. 4, little or no release of amylase was noted when samples
taken from saline-injected control animals were studied, but a
progressive, time-dependent release of amylase into the sus-
pending medium was observed when samples obtained from
caeulein-injected, non-CA-074me-treated animals were eval-
uated. Prior treatment with CA-074me, and thus CA-074me-
induced inhibition of cathepsin B activity and trypsinogen
activation, markedly reduced the increase in amylase release
compared with that observed in samples taken from caerulein-
injected animals that were not pretreated with CA-074me. This
finding indicates that the increase in organelle fragility ob-
served during the early stages of caerulein-induced pancreatitis
is dependent on the presence of cathepsin B activity and/or
intra-acinar cell activation of trypsinogen.

Effect of CA-074me on redistribution of subapical F-actin.
Shortly after the start of supramaximal caerulein stimulation,
disorganization of the acinar cell subapical F-actin web occurs
and F-actin becomes primarily localized at the basolateral
surface of the cells (20). Caerulein-induced F-actin redistribu-
tion can be monitored using confocal fluorescence microscopy
(Fig. 5). Within minutes of caerulein administration, the in-
tense subapical fluorescence, which gives the image the ap-
pearance of “train tracks,” disappeared, and in its place, single
phalloidin-positive lines appeared and, frequently, a “honey-
comb” pattern emerged that reflects the localization of fluores-
cence at the basolateral side of acinar cells (Fig. 5). That
redistribution of F-actin was similar in animals subjected to
supramaximal caerulein stimulation regardless of whether or
not cathepsin B had been inhibited and digestive zymogen
activation prevented by pretreatment with CA-074me. These
results indicate that subcellular redistribution of F-actin during
pancreatitis is not dependent on cathepsin B activity and/or
intracellular digestive zymogen activation.

Effect of CA-074me on caerulein-induced activation of JNK,
ERK1/2, and NF-κB. Proinflammatory cascades, including
those leading to the activation of JNK, ERK1/2, and NF-κB,
are upregulated during the early stages of caerulein-induced
pancreatitis (19). We determined the effects of inhibiting
cathepsin B and preventing digestive zymogen activation on
these processes. For this purpose, activation of JNK and
ERK1/2 was evaluated by Western blotting, using antibodies
directed against their phosphorylated (i.e., activated) forms,
whereas activation of NF-κB was examined by quantitating
both the cytoplasmic level of IκB (by Western blotting) and the
nuclear translocation of p65 (by ELISA). Samples of pancreas
were taken from animals 30 min following caerulein stimula-
tion, and the extent of activation in animals pretreated with
CA-074me was compared with that observed in pancreas
samples taken from animals that were not pretreated with
CA-074me. As shown in Fig. 6, activation of JNK, ERK1/2,
and NF-κB was observed within 30 min of caerulein admin-
istration. The magnitude of ERK 1/2 and JNK activation
decayed over the subsequent 30 min. Prior administration of
CA-074me did not alter the activation of JNK, ERK1/2, or
NF-κB, but it did prevent the decrease in ERK1/2 and JNK
activation observed over the second 30-min period (Fig. 6).

Effect of CA-074me on acinar cell generation of proinflam-
matory mediators. During the early stages of secretagogue-
induced pancreatitis, the intrapancreatic expression of several
proinflammatory mediators is increased. As shown in Fig. 7,
the increased expression of MCP-1 and IL-6 was not altered by
prior inhibition of cathepsin B despite the fact that, under these
conditions, intrapancreatic activation of trypsinogen is pre-
vented and the severity of pancreatitis is reduced (24).

DISCUSSION

Colocalization of digestive enzyme zymogens with lysosomal
hydrolases has been observed to occur during the very
early stages of virtually every experimental pancreatitis model
examined (8, 9, 13–17, 25, 27). Our group has previously
suggested that this colocalization phenomenon may play a
causal role in the intracellular activation of digestive enzymes
that occurs during pancreatitis, since the lysosomal hydrolase
cathepsin B can activate trypsinogen and trypsin can activate
the other digestive zymogens. Others, however, have argued
that rather than being the cause of zymogen activation and cell
injury, the colocalization phenomenon might be the result of
zymogen activation and/or cell injury (11). In this regard, the
colocalization phenomenon could represent a protective re-
sponse by facilitating the degradation, by lysosomal hydrol-
ases, of inappropriately activated digestive enzymes within
acinar cells.

To resolve this controversial issue, we have performed a
series of studies in which the lysosomal hydrolase cathepsin B

Fig. 2. Administration of CA-074me does not prevent caerulein-induced
lysosomal enzyme redistribution. Mice were given 10 mg/kg CA-074me or
vehicle by intravenous injection, and 1 h later they began receiving intraperi-
toneal injections of caerulein (50 μg/kg). After mice were killed at varying
times, the pancreata were subjected to subcellular fractionation. The distribu-
tion of aryl sulfatase between the lysosome-enriched and the zymogen granule-
enriched fractions was monitored by measuring aryl sulfatase activity using
p-nitroacetateh sulfate as a substrate. The ratio of aryl sulfatase activity in
these two fractions is shown in the y-axis. Results are means ± SE from 3
different mice in each group. caer, Caerulein.
and perhaps, other lysosomal hydrolases as well) was inhibited by the intravenous administration of CA-074me. In previously published work, our group has shown that pretreating mice with this cell-permeant cathepsin B inhibitor virtually eliminates measurable pancreatic cathepsin B activity, prevents the intrapancreatic activation of trypsinogen that follows supramaximal stimulation with caerulein, and markedly reduces the extent of pancreatic edema, pancreatic inflammation (i.e., increased pancreatic myeloperoxidase activity), and acinar cell injury/necrosis that occur during the early stages of caerulein-induced murine pancreatitis (24).

Our initial goal in the currently reported studies was to determine whether preventing zymogen activation would, by itself, alter or prevent the colocalization phenomenon, because if that were the case, one might reasonably conclude that the colocalization phenomenon was in some way the result of zymogen activation. On the other hand, results indicating that preventing zymogen activation does not alter the colocalization phenomenon that is noted after supramaximal caerulein stimulation would lead to the unambiguous conclusion that the colocalization phenomenon is not the result of inappropriate intracellular activation of digestive enzyme zymogens during the early stages of caerulein-induced pancreatitis.

CA-074 is a potent cysteine protease inhibitor that is relatively specific for cathepsin B. It binds irreversibly to Cys29 of cathepsin B, causing permanent inactivation of the enzyme (1).
our studies, we used a dose of 10 mg/kg. For our studies, employed CA-074me doses of 4 – 80 mg/kg body wt, and in vivo studies reported by others (22, 23) have, in general, improved by using the methyl ester form of the inhibitor. In its very cell permeant, but entry into target cells can be markedly and L. As a result of its highly negative charge, CA-074 is not also inhibit other lysosomal hydrolases, including cathepsins H and L. As a result of its highly negative charge, CA-074 is not very cell permeant, but entry into target cells can be markedly improved by using the methyl ester form of the inhibitor. In its methyl ester form, CA-074me is taken up by cells and remains in the cell after deesterification by intracellular esterases. In methyl ester form, CA-074me is taken up by cells and remains in the cell after deesterification by intracellular esterases. In vivo studies reported by others (22, 23) have, in general, employed CA-074me doses of 4 – 80 mg/kg body wt, and in our studies, we used a dose of 10 mg/kg. For our studies, CA-074me was administered by tail vein injection 1 h before the intraperitoneal injection of caerulein.

After confirming that pretreatment with CA-074me completely inhibits measurable intrapancreatic activation of trypsinogen during caerulein-induced pancreatitis in mice (Fig. 1), we embarked on a series of studies testing the effects of the cathepsin B inhibitor on the colocalization of digestivezymogens with lysosomal hydrolases. In our studies, we could not track either trypsin or cathepsin B activity for this purpose, because after pretreatment with CA-074me, the activity of both enzymes falls below the detectable threshold. As an alternative, we tracked aryl sulfatase and amylase activities in subcellular fractionation studies. To complement our studies using the technique of subcellular fractionation, we also performed studies using the technique of immunolocalization, and for those studies, we tracked the lysosomal membrane protein LAMP-1 and the digestive enzyme amylase. As shown in Figs. 2 and 3, the results of studies using these two independent but complementary techniques were similar. Supramaximal stimulation with caerulein induced subcellular redistribution of aryl sulfatase from the lysosome-enriched to the zymogen granule-enriched fraction, but administration of CA-074me before caerulein did not alter this redistribution. Supramaximal stimulation with caerulein also caused amylase and LAMP-1 to be immunolocalized to the same cytoplasmic vacuoles, but this colocalization of the digestive enzyme with the lysosomal membrane marker was not altered by administration of CA-074me. Together, these observations indicate that preventing intrapancreatic activation of trypsinogen does not alter the colocalization phenomenon that follows supramaximal secretagogue stimulation. Although these findings do not allow us to unequivocally conclude that the colocalization phenomenon is the cause of digestivezymogen activation, they are not compatible with the claim that either the process of trypsinogen activation or the presence of active trypsin triggers the colocalization phenomenon.

In addition to the colocalization phenomenon and activation of digestive enzymezymogens, the early phases of secretagogue-induced pancreatitis are characterized by several other acinar cell events, including an increase in 1) organellar fragility, 2) basolateral distribution of subapical F-actin, 3) activation of proinflammatory transcription factors, and 4) expression of certain proinflammatory mediators. Some of these events have also been noted during the early phases of other experimental pancreatitis models, but before the current stud-

![Fig. 4.](image)

Fig. 4. Inhibition of cathepsin B and prevention of trypsinogen activation prevents the caerulein-induced increase in organellar fragility. Mice were pretreated with CA-074me and then given caerulein 1 h later as described in the text. Pancreata were removed 30 min later and subjected to subcellular fractionation. The zymogen granule-enriched fractions were then incubated at room temperature for varying periods, after which samples were subjected to centrifugation (10,000 g). Rupture of amylase-containing organelles during the incubation period (i.e., organellar fragility) was quantitated by measuring amylase activity in the supernatant and expressing that activity as a percentage of the total amylase activity in the sample (y-axis). Note that caerulein treatment increased the rate of amylase release into the supernatant, but prior administration of CA-074me prevented this response to caerulein. Results are means ± SE from 3 independent zymogen granule preparations.

With two to three orders of magnitude lesser potency, it can also inhibit other lysosomal hydrolases, including cathepsins H and L. As a result of its highly negative charge, CA-074 is not very cell permeant, but entry into target cells can be markedly improved by using the methyl ester form of the inhibitor. In its methyl ester form, CA-074me is taken up by cells and remains in the cell after deesterification by intracellular esterases. In vivo studies reported by others (22, 23) have, in general, employed CA-074me doses of 4 – 80 mg/kg body wt, and in our studies, we used a dose of 10 mg/kg. For our studies, CA-074me was administered by tail vein injection 1 h before the intraperitoneal injection of caerulein.

After confirming that pretreatment with CA-074me completely inhibits measurable intrapancreatic activation of trypsinogen during caerulein-induced pancreatitis in mice (Fig. 1), we embarked on a series of studies testing the effects of the cathepsin B inhibitor on the colocalization of digestivezymogens with lysosomal hydrolases. In our studies, we could not track either trypsin or cathepsin B activity for this purpose, because after pretreatment with CA-074me, the activity of both enzymes falls below the detectable threshold. As an alternative, we tracked aryl sulfatase and amylase activities in subcellular fractionation studies. To complement our studies using the technique of subcellular fractionation, we also performed studies using the technique of immunolocalization, and for those studies, we tracked the lysosomal membrane protein LAMP-1 and the digestive enzyme amylase. As shown in Figs. 2 and 3, the results of studies using these two independent but complementary techniques were similar. Supramaximal stimulation with caerulein induced subcellular redistribution of aryl sulfatase from the lysosome-enriched to the zymogen granule-enriched fraction, but administration of CA-074me before caerulein did not alter this redistribution. Supramaximal stimulation with caerulein also caused amylase and LAMP-1 to be immunolocalized to the same cytoplasmic vacuoles, but this colocalization of the digestive enzyme with the lysosomal membrane marker was not altered by administration of CA-074me. Together, these observations indicate that preventing intrapancreatic activation of trypsinogen does not alter the colocalization phenomenon that follows supramaximal secretagogue stimulation. Although these findings do not allow us to unequivocally conclude that the colocalization phenomenon is the cause of digestivezymogen activation, they are not compatible with the claim that either the process of trypsinogen activation or the presence of active trypsin triggers the colocalization phenomenon.

In addition to the colocalization phenomenon and activation of digestive enzymezymogens, the early phases of secretagogue-induced pancreatitis are characterized by several other acinar cell events, including an increase in 1) organellar fragility, 2) basolateral distribution of subapical F-actin, 3) activation of proinflammatory transcription factors, and 4) expression of certain proinflammatory mediators. Some of these events have also been noted during the early phases of other experimental pancreatitis models, but before the current stud-

![Fig. 5.](image)

Fig. 5. CA-074me does not prevent caerulein-induced F-actin redistribution. Mice were pretreated with CA-074me or vehicle and then given caerulein 1 h later as described in the text. Pancreata were removed 30 min later, and cryostat sections were stained with phalloidin for visualization of F-actin. Note that samples taken from control, saline-injected mice showed a “train track” pattern of F-actin staining, indicating that F-actin was primarily localized to the subapical regions of opposing cells. In samples taken from caerulein-injected animals, F-actin was redistributed to the basolateral region of acinar cells and a “honeycomb” pattern of F-actin staining was seen regardless of whether the animals had been pretreated with CA-074me or vehicle.
ies, their dependence on intrapancreatic digestive enzyme activation had not been examined.

We evaluated the relationship between digestive enzyme activation and increased organellar fragility by isolating the zymogen granule-enriched subcellular fraction obtained from animals given caerulein either with or without prior administration of CA-074me. After incubating that fraction at room temperature for varying periods of time, we evaluated organelar fragility by quantitating the conversion of sedimentable amylase into nonsedimentable amylase. We found that organellar fragility was increased in samples prepared from animals given caerulein but that pretreatment of animals with CA-074me prevented that caerulein-induced increase in organellar fragility. It should be noted that this zymogen granule-

**Fig. 6.** CA-074me does not prevent caerulein-induced ERK1/2, JNK, and NF-κB activation. Mice were pretreated with CA-074me or vehicle and then given caerulein 1 h later as described in the text. Pancreata were removed 30 and 60 min after caerulein or saline administration, and total protein cytoplasmic extracts were subjected to immunoblot analysis with the antibodies raised against p-JNK (A) and p-ERK 1/2 (B). Degradation of IκB was evaluated by immunoblot analysis with antibodies against IκB (C). Nuclear translocation (i.e., activation) of NF-κB was evaluated by ELISA using anti-p65 antibodies (D). Note the increased immunoreactivity of both JNK and ERK1/2, the decrease in IκB immunoreactivity, and the increase in nuclear levels of p65 in samples obtained 30 min after caerulein administration regardless of whether the mice were pretreated with CA-074me or vehicle. *P < 0.01. NS, not significant.

**Fig. 7.** CA-074me does not prevent caerulein-induced proinflammatory cytokine expression. Mice were pretreated with CA-074me or vehicle and then given caerulein 1 h later as described in the text. Total RNA was isolated from pancreata removed 2 h after the start of caerulein administration. Quantitative RT-PCR was performed, and the number of copies of IL-6 and monocyte chemoattractant protein-1 (MCP-1) mRNA per ng of total RNA was determined. Note that caerulein caused the upregulated expression of these proinflammatory mediators and that the response to caerulein was not altered by pretreatment with CA-074me. Results are means ± SE from 3–6 mice in each group.
enriched subcellular fraction is the fraction to which lysosomal hydrolases are redistributed during the colocalization process, and it is the fraction in which digestive enzymezymogens are activated during the early phases of caerulein-induced pancreatitis. It is tempting, therefore, to speculate that in samples prepared from animals not exposed to CA-074me, the observed organellar disruption reflects the injurious effects of those activated digestive enzymes, and that the rate of organellar rupture in samples obtained from animals exposed to CA-074me is reduced because these digestive enzymes are not activated after administration of the cathepsin B inhibitor.

In contrast to marked reduction in caerulein-induced organellar fragility, which was observed following pretreatment with CA-074me, no change in caerulein-induced F-actin redistribution was observed. This finding leads us to conclude that digestive enzyme activation does not play an important role in regulating the cytoskeletal changes in acinar cells that occur during the early stages of caerulein-induced pancreatitis.

We have also examined the effects of inhibiting cathepsin B on some of the proinflammatory events that occur during the evolution of caerulein-induced pancreatitis (i.e., on the activation of JNK, ERK, and NF-κB and on the upregulated expression of IL-6 and MCP-1). In the absence of CA074-me administration, supramaximal stimulation with caerulein triggers the rapid activation of both JNK and ERK (i.e., elevated levels of p-JNK and p-ERK can be detected within 30 min of exposure to caerulein). Our finding that a similar rapid activation of both JNK and ERK was observed when caerulein was given after prior administration of CA074-me leads us to conclude that neither the inhibition of cathepsin B nor the prevention of trypsinogen activation interferes with the activation of these proinflammatory kinases. In the absence of CA074-me administration, p-JNK and p-ERK levels fell during the second 30-min period after caerulein administration, but following CA074-me administration, we found that p-JNK and p-ERK levels actually rose even higher. Together, these observation suggest that the fall in p-JNK and p-ERK levels observed during the second 30 min after caerulein administration may be the result of p-JNK and p-ERK degradation by activatedzymogens such as trypsin and that when cathepsin B inhibition preventszymogen activation, degradation does not occur and a further increase in p-JNK and p-ERK levels is observed.

Within 30 min of caerulein administration, cytosolic levels of 1κB fell and nuclear levels of the p65 NF-κB subunit rose. These changes occurred regardless of whether cathepsin B had been inhibited by pretreatment with CA-074me, a finding which leads us to suspect that NF-κB activation is not dependent on either the presence of cathepsin B activity or intrapancreatic activation ofzymogens such as trypsinogen. On the other hand, our finding that the rise in nuclear levels of p65, although still significant, was somewhat attenuated after administration of CA074-me leaves open the possibility that cathepsin B and/or activatedzymogens may play contributory roles in processes involved in NF-κB activation. Further studies are needed to resolve these uncertainties.

Finally, we have examined the effects of cathepsin B inhibition on the intrapancreatic expression of IL-6 and MCP-1 following administration of caerulein. The expression of both proinflammatory mediators was upregulated following administration of caerulein, and that upregulated expression was not altered by administration of CA074-me. These findings suggest that the expression of these two proinflammatory mediators during pancreatitis is not dependent on either cathepsin B activity or trypsinogen activation.

In previously published work (24), our group showed that administration of CA074-me interfered with neutrophil sequestration in the pancreas during caerulein-induced pancreatitis, yet in the current communication, we note that administration of CA074-me did not alter expression of at least two known neutrophil chemoattractants (i.e., IL-6 and MCP-1). At first glance, these observations might seem paradoxical, since one would expect that pancreatic sequestration of neutrophils and expression of neutrophil chemoattractants would be effected by the cathepsin B inhibitor in a similar, or at least parallel, manner. On the other hand, neutrophils are known to contain cathepsin B as well as other cathepsins potentially inhibitable by CA074-me, and neutrophil cathepsin B has been shown to play an important role in both neutrophil function and neutrophil trafficking (5, 26). Thus one explanation for our findings could be that inhibiting cathepsin B by administration of CA074-me interferes with neutrophil trafficking to the pancreas by interfering with neutrophil function and not by interfering with the generation of neutrophil chemoattractants. Alternatively, our findings may indicate that administration of CA074-me interferes with neutrophil chemoattraction to the pancreas during pancreatitis by interfering with the expression of critical neutrophil chemoattractants other than IL-6 and MCP-1. Additional studies are needed to resolve these uncertainties.

In this communication, we have reported the first studies that address the causative relationship between intra-acinar cell activation of digestive enzymezymogens and other early events in the caerulein-induced model of acute pancreatitis. We administered the cathepsin B inhibitor CA074me to completely inhibit pancreatic cathepsin B and prevent digestivezymogen activation. We found that the colocalization of digestiveenzymezymogens with lysosomal hydrolases following supramaximal stimulation with caerulein was unaltered under these conditions, and this observation leads us to conclude that the colocalization phenomenon is not dependent on either intrapancreatic activation of digestive enzymezymogens or induction of cell injury in the secretagogue-induced model. In addition, we found that the subcellular redistribution of subapical F-actin, the activation of proinflammatory transcription factors, and the upregulated expression of proinflammatory mediators following caerulein administration were unaltered, indicating that none of these responses to supramaximal secretagogue stimulation is the result of intracellular digestivezymogen activation. In contrast, we found that the increased organellefragility noted following caerulein administration was markedly reduced by CA-074me, indicating that this phenomenon is most likely caused by digestivezymogen activation, the presence of activated digestivezymogens, or the induction of cell injury. We speculate that colocalization leads tozymogen activation andzymogen activation leads to increasedorganellefragility in pancreatitis. Other events including F-actin redistribution, activation of proinflammatory transcription factors, and upregulated expression of proinflammatorymediators may be necessary, but may not be sufficient, for the evolution of pancreatitis.
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