Phosphatidylinositol 3-kinase-dependent activation of trypsinogen modulates the severity of acute pancreatitis

See related Commentary on pages 1267–1268.

Vijay P. Singh,[1] Ashok K. Saluja,[1] Lakshmi Bhagat,[1] Gijs J.D. van Acker,[1] Albert M. Song,[1] Stephen P. Soltoff,[2] Lewis C. Cantley,[2] and Michael L. Steer[1]

[1]Department of Surgery, and
[2]Division of Signal Transduction, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, USA

Correspondence to: Michael L. Steer, Department of Surgery, BIDMC, 330 Brookline Avenue, Boston, Massachusetts 02215, USA. Phone: (617) 667-4261; Fax: (617) 667-8679; E-mail: Msteer@caregroup.harvard.edu.

Ashok K. Saluja and Michael L. Steer contributed equally to this work.

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Intra-acinar cell activation of digestive enzyme zymogens including trypsinogen is generally believed to be an early and critical event in acute pancreatitis. We have found that the phosphatidylinositol 3-kinase inhibitor wortmannin can reduce the intrapancreatic activation of trypsinogen that occurs during two dissimilar experimental models of rodent acute pancreatitis, secretagogue- and duct injection-induced pancreatitis. The severity of both models was also reduced by wortmannin administration. In contrast, the NF-κB activation that occurs during the early stages of secretagogue-induced pancreatitis is not altered by administration of wortmannin. Ex vivo, caerulein-induced trypsinogen activation is inhibited by wortmannin and LY294002. However, the cytoskeletal changes induced by caerulein were not affected by wortmannin. Concentrations of caerulein that induced ex vivo trypsinogen activation do not significantly increase phosphatidylinositol-3,4-bisphosphate or phosphatidylinositol 3,4,5-trisphosphate levels or induce phosphorylation of Akt/PKB, suggesting that class I phosphatidylinositol 3-kinases are not involved. The concentration of wortmannin that inhibits trypsinogen activation causes a 75% decrease in phosphatidylinositol 3-phosphate, which is implicated in vesicle trafficking and fusion. We conclude that a wortmannin-inhibitable phosphatidylinositol 3-kinase is necessary for intrapancreatic activation of trypsinogen and regulating the severity of acute pancreatitis. Our observations suggest that phosphatidylinositol 3-kinase inhibition might be of benefit in preventing acute pancreatitis.

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Introduction

Intra-acinar cell activation of digestive enzyme zymogens such as trypsinogen is generally believed to be the triggering event that results in acinar cell injury and, subsequently, leads to acute pancreatitis. The mechanisms responsible for this activation of zymogens are not completely understood, but this issue has been the subject of intensive recent study. One hypothesis advanced to explain this phenomenon suggests that zymogen activation is the result of the colocalization of digestive enzyme zymogens with lysosomal hydrolases (1). In the normal pancreas, activation is presumably prevented by sorting events that occur during transit of newly synthesized protein through the Golgi stacks. These sorting events minimize colocalization by favoring transport of lysosome-targeted hydrolases away from the secretory pathway and into the prelysosomal compartment. Defective sorting, which is proposed to occur very early in pancreatitis, results in the subcellular redistribution of lysosomal hydrolases such that they become colocalized, along with digestive enzyme zymogens, within cytoplasmic vesicles where the lysosomal hydrolase cathepsin B can activate trypsinogen and trypsin can activate the other zymogens.

Here we report the results of studies that have further examined this issue. Using both in vivo and ex vivo systems, we have found that a phosphatidylinositol 3-kinase (PI3K) susceptible to inhibition by wortmannin and LY294002 plays a critical role in facilitating the intracellular activation of trypsinogen, that PI3K inhibition reduces the severity of two dissimilar models of acute pancreatitis, and that the relevant enzyme is a class III PI3K. Our findings suggest that interventions designed to inhibit class III PI3K may be of clinical use in either preventing pancreatitis or in reducing the severity of this frequently lethal disease.

Methods

Male CD-1 mice weighing 18–20 g were purchased from Taconic Farms, Inc. (Germantown, New York, USA), and male Wistar rats weighing 200–250 g were obtained from Charles River Laboratories (Cambridge, Massa-

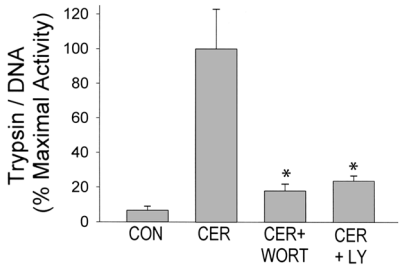

Figure 1
Trypsinogen activation in secretagogue-induced pancreatitis. Mice were given caerulein (50 μg/kg) (CER) or saline (CON) by intraperitoneal injection and sacrificed 30 minutes later. When administered wortmannin (1.4 mg/kg) (CER + WORT) or Ly294002 (100 mg/kg) (CER + LY), it was given by intraperitoneal injection 10 minutes before caerulein. Trypsin activity in homogenized pancreas samples was determined as described in the text and expressed relative to that when only caerulein was given. *$P < 0.05$ when compared with caerulein alone.

chusetts, USA). Caerulein was purchased from Research Plus (Bayonne, New Jersey, USA), wortmannin from Alexis Biochemical Corp. (San Diego, California, USA), and LY294002 from Biomol Research Laboratories (Plymouth Meeting, Pennsylvania, USA). Collagenase (type IV) was purchased from Worthington Biochemical Corp. (Lakewood, New Jersey, USA), and phospho-Akt Ab (S473) was from Cell Signaling Technology, Beverly, Massachusetts, USA. Other chemicals and reagents were purchased from sources cited previously (2) and were of the highest purity commercially available. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Beth Israel-Deaconess Medical Center.

In vivo models of pancreatitis. Secretagogue-induced pancreatitis (3) was elicited by the hourly (six times) intraperitoneal injection of caerulein (50 μg/kg; 0.2 ml/injection) to mice, whereas control animals received a comparable amount of saline. When given, wortmannin (dissolved in 4% methanol in saline, final concentration) or LY294002 (dissolved in DMSO, given 0.1 ml per animal) was administered by intraperitoneal injection in doses of 1.4 mg/kg and 100 mg/kg, respectively, 10 minutes before the first dose of either caerulein or saline. Control animals received a comparable volume of vehicle alone at the same time. Thirty minutes after the first caerulein or saline injection or 1 hour after the final caerulein or saline injection, animals were killed by CO_2 asphyxia, and tissue samples were obtained for study. Duct injection–induced pancreatitis was elicited in rats (4). After midline laparotomy and transduodenal cannulation of the pancreatic duct, 5% sodium taurocholate in saline was infused at a rate of 0.1 ml/minute. Rats received a total volume of 0.1 ml/100 g body weight. When given, wortmannin (1.4 mg/kg) or vehicle was administered by intraperitoneal injection 4 hours before (to reduce the chances of possible interaction with the anesthetic ketamine xylazine) and 12 hours after duct infusion. Twenty hours after duct infusion, animals were killed by CO_2 asphyxia, and samples were taken for study.

Evaluation of trypsinogen activation. Trypsin activity was measured fluorometrically, according to the method of Kawabata et al. (5) as described previously (6). To allow for pooling of results from several experiments, trypsin activity was calculated as the fold rise over untreated controls in each experiment. Trypsinogen activation peptide levels were quantitated by ELISA as described previously (7) using affinity-purified Ab's.

Evaluation of pancreatitis severity. 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. (8). Pancreatic edema was quantitated by measuring tissue water content and expressing it as a percentage of tissue wet weight. Serum amylase activity was quantitated as described by Kruse-Jarres (9). The extent of pancreatic acinar cell necrosis was quantitated morphometrically 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 (10).

NF-κB activation. NF-κB activation was evaluated using electromobility shift assays (EMSAs) of mouse pancreas nuclear extracts obtained 30 minutes after the start of caerulein administration. At that time, the initial peak of NF-κB activation, which occurs within acinar cells as opposed to nonacinar cell elements in the pancreas, is maximal (11).

Subcellular distribution of cathepsin B. Mouse pancreas samples obtained 30 minutes after the administration of caerulein were homogenized and subjected to differential centrifugation as described previously (12). The percentage of cathepsin B activity detected in the zymogen granule–enriched (1,300 *g* for 12 minutes) pellet and that detected in the lysosome-enriched (12,000 *g* for 10 minutes) fractions was measured and expressed as a ratio—i.e., zymogen/lysosome cathepsin

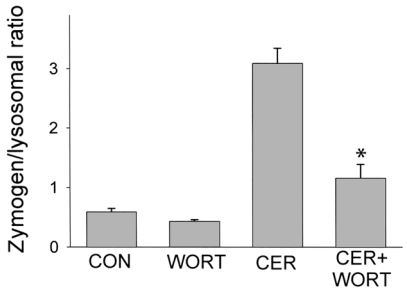

Figure 2
Lysosomal enzyme redistribution in secretagogue-induced pancreatitis. Groups are as described in Figure 1 legend. After subcellular fractionation of homogenized pancreas samples, cathepsin B in the zymogen granule–enriched fraction and lysosome-enriched fraction was measured as described in the text and expressed as a ratio. *$P < 0.05$ when CER + WORT group was compared with group given CER alone.

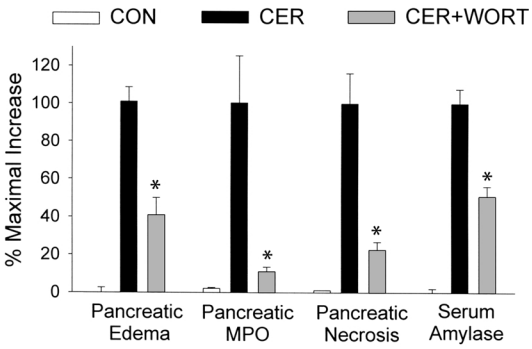

Figure 3
Severity of secretagogue-induced pancreatitis. Mice were given hourly (six times) intraperitoneal injections of caerulein (50 μg/kg) (CER) or saline (CON) and sacrificed 1 hour after the last caerulein injection. When given wortmannin (1.4 mg/kg) (CER + WORT) was administered 10 minutes before the first caerulein injection. After sacrifice, pancreatic edema, MPO activity, pancreatic necrosis, and serum amylase activity were measured as described in the text and expressed relative to that noted when only caerulein had been given. *$P < 0.05$ when compared with group given caerulein alone.

B. A rise in the zymogen/lysosome ratio for cathepsin B distribution indicates redistribution of cathepsin B from the lysosome-enriched to the heavier zymogen granule–enriched fraction (13).

Ex vivo preparation. Pancreatic acini were prepared from male Wistar rats (80–100 g) by collagenase digestion as described previously (14). The freshly prepared acini were suspended in oxygen-saturated HEPES-Ringer buffer (pH 7.4). Viability of acini, evaluated by trypan blue exclusion, was greater than 95% at the start of each experiment. In studies evaluating the effect of PI3K inhibition, acini were incubated in buffer containing wortmannin (20 nM), LY 294002 (50 μM), or vehicle for 10 minutes before addition of caerulein. Amylase secretion, when evaluated, was measured over 30 minutes at 37°C. The percentage of total amylase content that was discharged into the suspending medium over that interval was quantitated.

F-actin distribution . F-actin was localized as described by Shafer et al. (15) with minor modifications (2). Briefly, acini were fixed with formaldehyde (4%) in phosphate buffer, pH 7.0, and then allowed to attach to polylysine-coated slides, rinsed with PBS, permeabilized by exposure to PBS with Triton X-100 (0.5%) and normal goat serum (5%), and treated with sodium borohydride (1 mg/ml) to reduce background fluorescence. They were then stained with rhodamine phalloidin (5 μg/ml), rinsed with PBS, covered with a drop of Vectashield (Vector Laboratories, Burlingame, California, USA), sealed, and examined using a MRC 1024 confocal microscope, using the same settings for all images.

Assessment of PI3K activity in acini. Freshly prepared acini were preincubated with wortmannin (20 nM) or LY 294002 (50 μM) for 15 minutes. They were then stimulated with caerulein (0.1 μM) or pervanadate (100 μM) for 1, 5, or 15 minutes, washed, and lysed in lysis buffer containing protease and phosphatase inhibitors. Immunoprecipitations were carried out using 4G10 antiphosphotyrosine Ab (3 μg/ml) and lipid kinase assays were performed as described previously (16), except that exogenous PtdIns-4,5,-P2 was used as the substrate. As a positive control, purified PI3K and anti-p85 immunoprecipitates of pancreatic acinar cell lysates were included in these experiments. The lipid products were extracted, separated by thin-layer chromatography, and radiolabeled spots identified as PtdIns-3,4,5-P3 were quantified with a Bio-Rad PhosphorImager (Bio-Rad Laboratories, Hercules, California, USA).

Western blotting for Akt/PKB. Lysates of acinar cells were subjected to 10% SDS-PAGE and transferred to nitrocellulose. The immunoblots were exposed to phospho-Akt Ab (1:1,000), following the manufacturer's protocol. Proteins were visualized using enhanced chemiluminescence.

Measurement of polyphosphoinositides in intact cells. Pancreatic acini in 2 ml HEPES-Ringer buffer were labeled by incubation with 0.5 mCi/ml [^{32}P]-orthophosphate for 2 hours at 37°C. After preincubation in the presence or absence of wortmannin (20 nM, 15 minutes), they were exposed to caerulein (0.1 μM) for 8 minutes. The acini were then sedimented, washed once with PBS, and lysed in 400 μl of 1 N HCl. The acid lysate was mixed with 400 μl methanol and 400 μl chloroform. The chloroform-extracted lipids were deacylated and analyzed by HPLC, as described previously (17). For each sample, the levels of PtdIns-3-P, PtdIns-4-P, and PtdIns-4,5-P2 were normalized to the amount of phosphatidylinositol in that sample.

Analysis of data. The results reported represent mean plus or minus SE of mean values for multiple determinations from three or more separate preparations of acini (ex vivo studies) or from eight to ten animals (in vivo studies). The significance of changes was evaluated

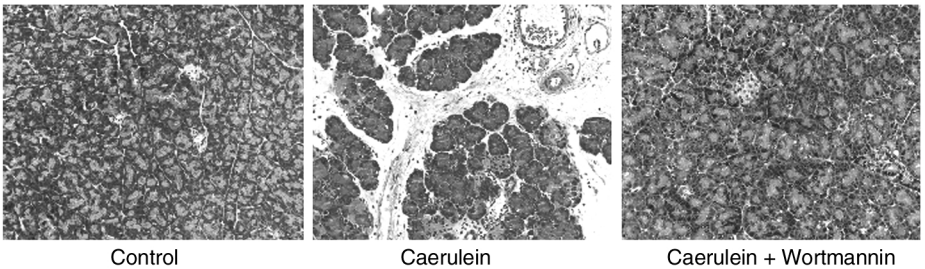

Figure 4
Histology of secretagogue-induced pancreatitis. Conditions and groups are as described in Figure 3 legend. Note marked inflammation and acinar cell necrosis in the caerulein sample, which is decreased in the caerulein + wortmannin sample.

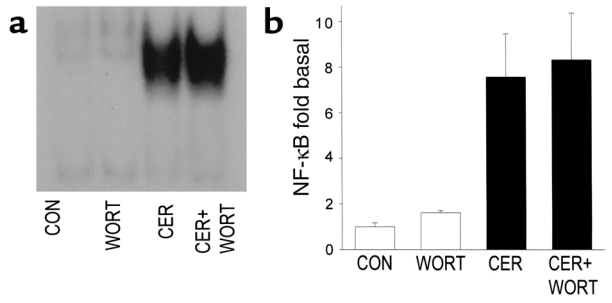

Figure 5
NF-κB activation in secretagogue-induced pancreatitis. Mice were given caerulein (50 μg/kg) by intraperitoneal injection and sacrificed 30 minutes later. Groups are as described in Figure 1 legend. (**a**) Representative EMSA for NF-κB performed as described in text. (**b**) Densitometry from three separate mice in each group.

using student's *t* test when comparing two groups and ANOVA when comparing three or more groups. *P* values of less than 0.05 were considered to be significant.

Results

Secretagogue-induced in vivo trypsinogen activation. As shown in Figure 1, supramaximal stimulation of mice with caerulein resulted in intrapancreatic trypsinogen activation that could be detected within 30 minutes after the start of caerulein administration. Trypsinogen activation was markedly reduced by prior administration of the PI3K inhibitor wortmannin (1.4 mg/kg). Trypsinogen activation after supramaximal caerulein stimulation was also markedly reduced by prior administration of another, structurally and mechanistically dissimilar PI3K inhibitor, LY294002 (100 mg/kg). Prior administration of wortmannin also markedly reduced the subcellular redistribution of cathepsin B from the lysosome-enriched to the zymogen granule–enriched fractions that was otherwise observed to occur after supramaximal stimulation with caerulein (Figure 2). This latter response to wortmannin administration was manifested by a significantly lessened magnitude in the rise of the zymogen/lysosome ratio of cathepsin B activity.

Secretagogue-induced pancreatitis. As shown in Figures 3 and 4, supramaximal stimulation with caerulein resulted in pancreatitis that was characterized 6 hours after the start of caerulein administration by the appearance of pancreatic edema, a rise in pancreatic MPO activity (reflecting neutrophil sequestration in the pancreas), pancreatic acinar cell necrosis, and a rise in serum amylase activity. Each of these features of supramaximal secretagogue stimulation were markedly reduced by the prior administration of wortmannin. In contrast, prior administration of wortmannin did not prevent the intrapancreatic activation of NF-κB, which was detected 30 minutes after the start of caerulein administration (Figure 5).

Duct injection-induced trypsinogen activation. As shown in Figure 6, injecting sodium taurocholate in a retrograde fashion into the rat pancreatic duct led to intrapancreatic activation of trypsinogen that could be detected within 20 hours of duct injection. Administration of wortmannin (1.4 mg/kg) 4 hours before and 12 hours after duct injection markedly reduced trypsinogen activation.

Duct injection–induced pancreatitis. Retrograde pancreatic duct injection with taurocholate resulted in severe hemorrhagic necrotizing pancreatitis that was characterized, 20 hours after duct injection, by a rise in pancreas MPO activity and extensive acinar cell necrosis (Figures 7 and 8). Both of these features of the duct-injection model were markedly reduced by administration of wortmannin 4 hours before and 12 hours after duct injection.

Secretagogue-induced ex vivo trypsinogen activation. Exposure of freshly prepared acini to a supramaximally stimulating concentration of caerulein (0.1 μM) for 30 minutes resulted in intra-acinar cell activation of trypsinogen that was manifested by both the appearance of intrapancreatic trypsin activity and a rise in the level of trypsinogen activation peptide (Figure 9). When the PI3K inhibitors wortmannin (20 nM) or LY294002 (50 μM) were present in the incubation medium, trypsinogen activation was prevented. Neither wortmannin nor LY294002 interfered directly with trypsin activity in broken cell preparations (data not shown).

In contrast to the effects of the PI3K inhibitors on ex vivo caerulein-induced trypsinogen activation, the biphasic dose-dependent pattern of caerulein-stimulated amylase secretion was not altered by inclusion of wortmannin in the incubation medium (Figure 10). Similarly, redistribution of F-actin from the apical pole to the basolateral areas of acinar cells, which follows exposure to a supramaximally stimulating concentration of caerulein, was not altered by wortmannin (Figure 11).

Ex vivo PI3K studies. Antiphosphotyrosine immunoprecipitates, prepared from pervanadate-treated acini, produced the class I PI3K product PtdIns-3,4,5-P3, but this did not occur when similar immunoprecipitates, prepared from acini exposed to caerulein (0.1 μM) for 1,

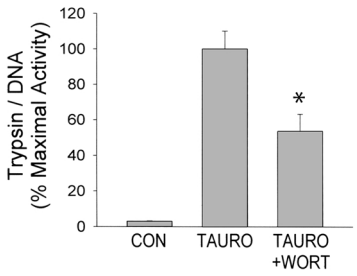

Figure 6
Trypsinogen activation in duct-injection pancreatitis. Rats underwent retrograde duct injection with sodium taurocholate (TAURO) as described in the text and were sacrificed 20 hours later. When given, wortmannin (1.4 mg/kg) was delivered by intraperitoneal injection 4 hours before and 12 hours after duct infusion. Trypsin activity in homogenates of pancreas was expressed relative to that noted for duct-infused animals not given wortmannin. *$P < 0.05$ when wortmannin groups were compared with duct-infusion group not given wortmannin.

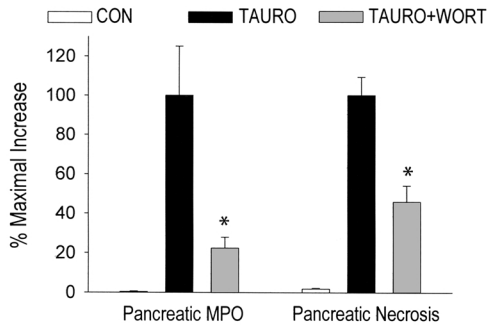

Figure 7
Severity of duct-injection pancreatitis. Groups are as described in Figure 6 legend. Values for MPO activity and pancreatic necrosis are expressed relative to animals infused but not given wortmannin. *$P < 0.05$ when compared with infused animal not given wortmannin.

5, or 15 minutes, were evaluated (not shown). Furthermore, activated Akt/PKB, the downstream signal protein activated by the class I PI3K products PtdIns-3,4-P2 and PtdIns 3,4,5-P3, was observed in lysates prepared from pervanadate-treated acini, but not in lysates prepared from caerulein-treated acini (not shown). Finally, after preloading acini with [^{32}P]-orthophosphate, measurable and similar levels of the class III PI3K product [^{32}P]-PtdIns-3-P were detected under basal and caerulein-stimulated conditions and were reduced (basal by 77%, caerulein by 75%) by exposure to 20 nM wortmannin (Figure 12). In contrast, measurable levels of the class I PI3K products [^{32}P]-PtdIns-3,4-P2 and [^{32}P]-PtdIns-3,4,5-P3 were not detected under either basal or caerulein-stimulated conditions (not shown).

Discussion

Secretagogue-induced pancreatitis, elicited by administration of a supramaximally stimulating dose of the cholecystokinin (CCK) analogue caerulein to mice or rats is the most well characterized of the pancreatitis models, and it has been extensively employed for studies probing the early events that may be critical to the evolution of the disease. It begins when caerulein binds to low-affinity CCK receptors that mediate inhibition of digestive enzyme secretion (18). The earliest changes, each noted to occur within the initial 30 minutes after the start of caerulein administration, include (a) the colocalization of lysosomal hydrolases with digestive enzyme zymogens in cytoplasmic vacuoles (a phenomenon that can be monitored by demonstrating lysosomal hydrolase redistribution from the lysosome-enriched to the zymogen granule–enriched subcellular fraction) (13); (b) intra-acinar cell activation of trypsinogen; and (c) activation of NF-κB. The mechanisms responsible for these events, as well as the relationship between each of these changes and the subsequent development of cell injury and pancreatitis, are incompletely understood.

We have found that doses of the PI3K inhibitor wortmannin that have been used by others for in vivo studies (19) markedly reduce trypsinogen activation during the early stages of secretagogue-induced pancreatitis. A similar finding was noted after administration of another agent (LY 294002, which is known to inhibit PI3K by a different mechanism) when it was given before caerulein administration in doses conventionally used for in vivo studies (20). Wortmannin, given before caerulein, markedly reduced the subcellular redistribution of cathepsin B in this model, but it did not interfere with NF-κB activation. Wortmannin administration also markedly reduced the severity of secretagogue-induced pancreatitis when evaluated 6 hours later; i.e., it reduced the extent of pancreatic edema, neutrophil sequestration within the pancreas, extent of acinar cell necrosis, and magnitude of hyperamylasemia. These observations are compatible with the conclusion that PI3K inhibition protects against pancreatitis by preventing the intracellular colocalization of lysosomal hydrolases with digestive enzyme zymogens and the intracellular activation of trypsinogen. Our findings indicate that neither enzyme colocalization nor trypsinogen activation are required for the early (i.e., 30 minutes) intrapancreatic activation of NF-κB that occurs in this model. Furthermore, our results indicate that the early activation of NF-κB does not appear to be dependent upon PI3K.

We tested the effect of wortmannin administration on another model of acute pancreatitis to determine if the effects noted with the secretagogue-induced model were specific to that model or relevant to acute pancreatitis in general. For those studies, the rat pancreatic duct was infused in a retrograde fashion with the bile salt sodium taurocholate to induce pancreatitis. This model, like the secretagogue-induced model of pancreatitis, is characterized by a rise in pancreatic trypsin activity, as well as a rise in pancreatic MPO activity and the appearance of acinar cell necro-

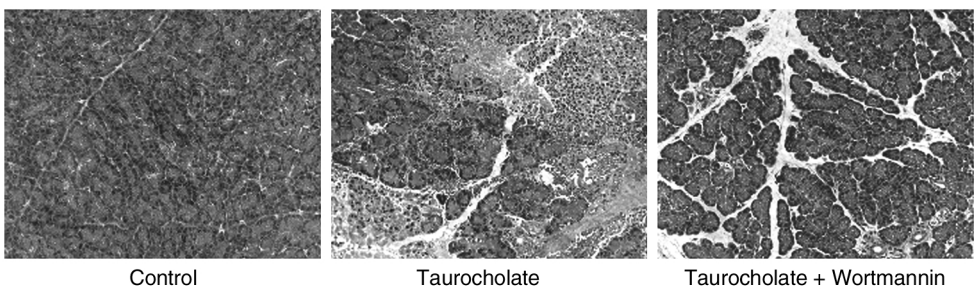

Figure 8
Duct-injection pancreatitis. Groups are as described in Figure 6 legend. Note inflammation and necrosis in animals infused with taurocholate, which is lessened by administration of wortmannin.

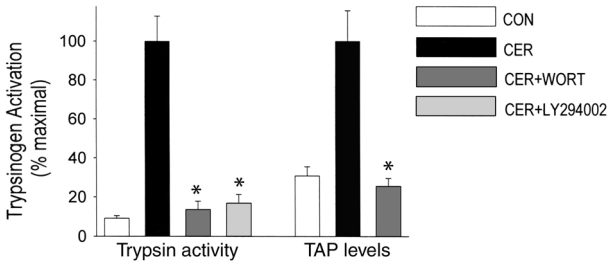

Figure 9

Ex vivo trypsinogen activation. Freshly prepared rat acini were incubated in buffer alone (CON) or buffer containing 0.1 μM caerulein (CER) for 30 minutes. Where used, 20 nM wortmannin (CER + WORT) or 50 μM LY294002 (CER + LY294002) were added to the acinar suspension for 15 minutes before adding caerulein. Acini were homogenized and used to measure trypsin and TAP levels as described in the text. Trypsin activity and TAP levels were expressed relative to that noted for samples exposed to caerulein alone. $*P < 0.05$ when compared with caerulein alone.

sis. Administration of wortmannin, at a dose conventionally used by others to inhibit PI3K activity in in vivo studies (1.4 mg/kg) 4 hours before and 12 hours after duct infusion with taurocholate markedly reduced the rise in pancreatic trypsin activity. In addition, wortmannin administration reduced the magnitude of the subsequent rise in pancreatic MPO activity and the extent of acinar cell necrosis. Even though the reduction in severity by wortmannin was significant, it is possible that the protection may have been even more marked if wortmannin had been given immediately before duct infusion. In addition, there is a component of immediate direct pancreatic injury after infusion of sodium taurocholate, attributable to the soapy action of the bile salt, such as the dissolution of cell membranes, which may not have been prevented by wortmannin.

Taken together, our observations indicate that the PI3K inhibitor wortmannin can prevent the intrapancreatic activation of trypsinogen that characterizes the early stages of two pathogenetically dissimilar models of pancreatitis and that is also believed to be an early triggering event in human acute pancreatitis. This prevention of trypsinogen activation by wortmannin is followed by a reduction in the severity of both models of the disease—an observation that suggests that agents such as wortmannin may be of use in the prevention of clinical pancreatitis.

To further explore the role of PI3K in acute pancreatitis while overcoming the limitations imposed by the in vivo condition, including potential nonspecific drug effects and difficulties in determining the concentration of drug delivered to the target cells (i.e., acinar cells), we performed a series of ex vivo studies exploring the role of PI3K in acute pancreatitis. Previous studies have shown that concentrations of caerulein in excess of those that maximally stimulate acinar cell secretion of digestive enzymes result in the inhibition of digestive enzyme secretion from pancreatic acini (14) and that those same supramaximally stimulating concentrations of caerulein (> 1 nM) cause redistribution of the subapical F-actin web to the basolateral areas of acinar cells (21). They also cause intra-acinar cell activation of trypsinogen (22), which can be detected by a rise in trypsin activity along with a rise in the trypsinogen activation peptide (TAP) level in the acini (22). We found that addition of low concentrations of wortmannin (20 nM) or LY 294002 (50 μM) prevented this ex-vivo caerulein-induced intra-acinar cell activation of trypsinogen. Since the low concentrations of wortmannin and LY294002 used in our studies are generally believed to specifically inhibit only PI3K activity, these observations support our earlier conclusion that wortmannin and LY294002 prevent in vivo trypsinogen activation by inhibiting PI3K. However, one can not completely rule out the possibility that these inhibitors may have some other nonspecific effects and are preventing trypsinogen activation and pancreatitis by mechanisms other than inhibiting PI3K. The PI3K inhibitors, however, did not alter the biphasic dose dependence or magnitude of caerulein-stimulated digestive enzyme secretion nor the redistribution of subapical F-actin to the basolateral areas of acinar cells. These latter observations exclude the possibility that the PI3K inhibitors prevent caerulein-induced trypsinogen activation by interfering with the early signal transduction events that couple cholecystokinin receptor occupancy to the stimulation and inhibition of digestive enzyme secretion. They also indicate that supramaximal stimulation of acinar cells with caerulein causes cytoskeletal changes such as F-actin redistribution by mechanisms that do not require previous intracellular activation of trypsinogen and that are not PI3K dependent.

The most well studied of the PI3K superfamily of lipid kinases are those belonging to class I and class III (23). Class I PI3Ks catalyze the 3′ phosphorylation of PtdIns-4-P, PtdIns-5-P, and PtdIns-4,5-P2, yielding PtdIns-3,4-P2, PtdIns-3,5-P2, and PtdIns-3,4,5-P3. They usually sig-

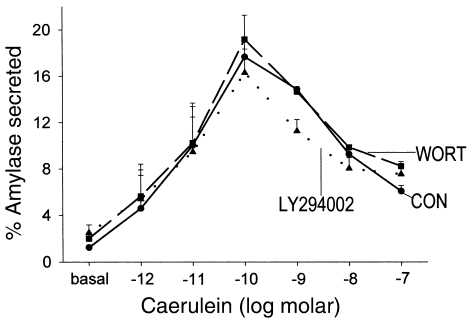

Figure 10

Caerulein-stimulated amylase secretion. Freshly prepared rat pancreatic acini were incubated in buffer alone (solid line), or buffer containing 20 nM wortmannin (dashed line), or 50 μM LY294002 (dotted line) for 15 minutes and then exposed to varying concentrations of caerulein. Amylase secretion, expressed as a percentage of total amylase content, was measured over 30 minutes as described in the text. Note unaltered amylase secretion in the presence of wortmannin.

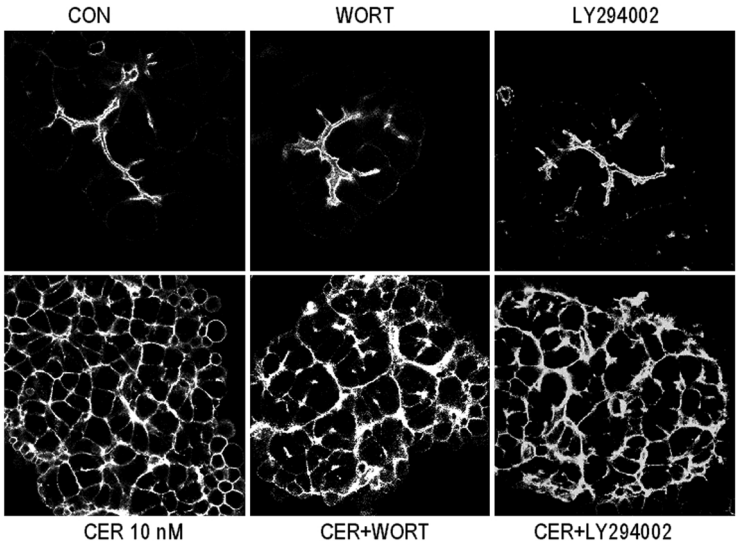

Figure 11
Effect of wortmannin on F-actin localization. Freshly prepared acini were incubated in HEPES buffer alone (CON), with wortmannin 20 nM (WORT), or LY294002 50 μM (LY294002). Some of these were incubated with caerulein 10 nM (CER) for 15 minutes or with 20 nM wortmannin (CER + WORT) or LY294002 (CER + LY294002) for 10 minutes, followed by caerulein 10 nM for 15 minutes . The samples were fixed, stained with rhodamine phalloidin, and examined by confocal microscopy as described in the text. Images are representative of those obtained from three independent experiments.

nal downstream to growth factor and G protein–coupled membrane receptors, and among the various targets of their products is the activation of the signaling protein Akt/PKB (24). Class III PI3Ks, on the other hand, catalyze the 3′ phosphorylation of PtdIns only and have only PtdIns-3-P as their product (25). Class III PI3Ks are generally considered to be constitutive enzymes that play an important role in regulating intracellular vesicle fusion events and protein trafficking (26).

Our studies designed to classify the PI3K involved in trypsinogen activation during pancreatitis were initially directed toward the class I group because of its known association with G protein–coupled receptors such as those for CCK. Acinar cells were exposed to a supramaximally stimulating concentration of caerulein, and homogenates were prepared. Antiphosphotyrosine immunoprecipitates were collected, which were then incubated with the class I PI3K substrate PtdIns-4,5-P2. As a positive control, other acini were similarly treated after exposure to pervanadate, a global PI3K activator that brings about activation by a nonreceptor mechanism. Class I PI3K product PtdIns-3,4,5-P3 was detected in samples obtained from pervanadate-treated but not caerulein-treated acini. As another test for upregulated class I PI3K activity after supramaximal stimulation with caerulein, we probed for the presence of activated (i.e., phosphorylated) Akt/PKB in acini exposed to a supramaximally stimulating concentration of caerulein. Activated Akt/PKB was detected in acini exposed to pervanadate, but not in acini supramaximally stimulated with caerulein. These negative results indicate that the PI3K, which plays a critical role in trypsinogen activation after supramaximal caerulein stimulation, is unlikely to belong to the class I PI3K group. Our results differ from those of another group (27), which has shown formation of class I PI-3K products after stimulation with caerulein. One possible explanation for the differences between our study and theirs is that we have used a supramaximal concentration whereas they have used a maximal concentration of caerulein.

Studies designed to establish a potential role for class III PI3K are complicated by the fact that the activity of class III PI3K is constitutive. Thus, changes in either activity or product levels are unlikely to occur after supramaximal stimulation with caerulein. We did note, however, that the class III PI3K product [^{32}P]-PtdIns-3-P could be detected at similar levels in control and caerulein-treated acini and that [^{32}P]-PtdIns-3-P levels were markedly reduced by wortmannin treatment of control as well as caerulein-stimulated acini. In contrast, measurable levels of the class I PI3K products [^{32}P]-PtdIns-3,4-P2 and [^{32}P]-PtdIns-3,4,5-P3 were not detected under either basal or caerulein-stimulated conditions. Taken together, these observations demonstrate the presence of a class III PI3K in acinar cells and are compatible with the conclusion that caerulein-induced intra-acinar cell activation of trypsinogen is mediated by a class III rather than a class I PI3K.

Considerable evidence has indicated that during the early stages of acute pancreatitis the intracellular activation of digestive enzyme zymogens such as trypsinogen results from a perturbation in the sorting and Golgi stack-to-lysosome trafficking of newly synthesized pro-

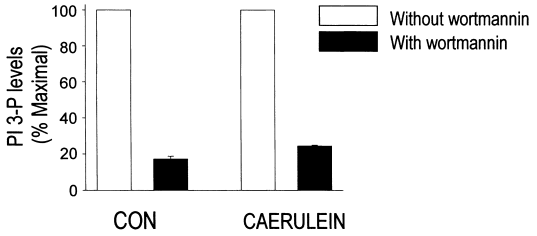

Figure 12
PI3-P levels. Rat pancreatic acini were preloaded with [^{32}P]-orthophosphate and incubated in the absence (CON) or presence (CAERULEIN) of 0.1 μM caerulein for 8 minutes with (filled bars) or without (open bars) 20 nM wortmannin. [^{32}P]-Ptdins-3-P levels were evaluated by HPLC and expressed relative to [^{32}P]-phosphatidylinositol. These values were normalized to 100% in order to permit pooling of data from three independent experiments.

teins in acinar cells (28, 29). This perturbation is believed to be responsible for the cathepsin B redistribution phenomenon that is observed after supramaximal stimulation with caerulein. It is, therefore, not surprising that a class III PI3K appears to play an important role in mediating intracellular trypsinogen activation since administration of wortmannin was observed to reduce cathepsin B redistribution and class III PI3K has been shown to play an important role in regulating Golgi stack-to-lysosome trafficking in other cell types (30–32).

Our studies lead us to propose the following working hypothesis. We suggest that caerulein-induced intrapancreatic activation of trypsinogen (and, presumably, acute pancreatitis) involves a wortmannin-sensitive (and LY 294002-sensitive) class III PI3K. With supramaximal caerulein stimulation, the class III PI3K product PtdIns-3-P levels in a critical, but unmeasurable, compartment may increase, leading to a perturbation of Golgi stack-to-lysosome trafficking. As a result, there is intra-acinar cell activation of digestive enzyme zymogens. Our findings are also compatible with an alternative hypothesis: unaltered levels of PtdIns-3-P may mediate intra-acinar cell activation of trypsinogen by acting downstream to a critical event triggered by supramaximal secretagogue stimulation (or duct injection with taurocholate). In this scenario, the class III PI3K product would act to facilitate perturbed trafficking, and PtdIns-3-P would be necessary, but not sufficient, to bring about intracellular zymogen activation. Studies testing the validity of these models and allowing us to discriminate between them are currently underway. Regardless of their outcome, and taking into account the possibility that a component of the protection afforded by wortmannin is due to its effects on the recruitment and activation of neutrophils as has been described previously (33, 34), the results reported here suggest that interventions designed to inhibit pancreatic PI3K activity, especially class III PI3K activity, may be of value in preventing acute pancreatitis.

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1. Saluja A., et al. 1989. Pancreatic duct obstruction in rabbits causes digestive zymogen and lysosomal enzyme colocalization. *J. Clin. Invest.* **84**:1260–1266.
2. Singh, V.P., et al. 2001. Serine protease inhibition causes subapical F-actin redistribution and inhibition of calcium-mediated secretion in pancreatic acini. *Gastroenterology*. **120**:1818–1827.
3. Lampel, M., and Kern, H.F. 1977. Acute interstitial pancreatitis in the rat induced by excessive doses of a pancreatic secretagogue. *Virchows Arch. A. Pathol. Anat. Histol.* **373**:97–117.
4. Aho, H.J., Koskensalo, S.M., and Nevalainen, T.J. 1980. Experimental pancreatitis in the rat. Sodium taurocholate-induced acute haemorrhagic pancreatitis. *Scand. J. Gastroenterol.* **15**:411–416.
5. Kawabata, S., et al. 1988. Highly sensitive peptide-4-methylcoumaryl-7-amide substrates for blood- clotting proteases and trypsin. *Eur. J. Biochem.* **172**:17–25.
6. Saluja, A.K., et al. 1997. Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. *Gastroenterology.* **113**:304–310.
7. Lee, H.S. 2000. Water immersion stress induces heat shock protein 60 expression and protects against pancreatitis in rats. *Gastroenterology.* **119**:220–229.
8. Haqqani, A.S., Sandhu, J.K., and Birnboim, H.C. 1999. A myeloperoxidase-specific assay based upon bromide-dependent chemiluminescence of luminol. *Anal. Biochem.* **273**:126–132.
9. Kruse-Jarres, J.D., et al. 1989. Evaluation of a new alpha-amylase assay using 4.6-ethylidene-(G7)-1-4- nitrophenyl-(G1)-alpha-D-maltoheptaoside as substrate. *J. Clin. Chem. Clin. Biochem.* **27**:103–113.
10. Hofbauer, B., et al. 1998. Effect of recombinant platelet-activating factor acetylhydrolase on two models of experimental acute pancreatitis. *Gastroenterology.* **115**:1238–1247.
11. Gukovsky, I., et al. 1998. Early NF-kappaB activation is associated with hormone-induced pancreatitis. *Am. J. Physiol.* **275**:G1402–1414.
12. Ohshio, G., Saluja, A.K., Leli, U., Sengupta, A., and Steer, M.L. 1989. Esterase inhibitors prevent lysosomal enzyme redistribution in two non-invasive models of experimental pancreatitis. *Gastroenterology.* **96**:853–859.
13. Saluja, A., et al. 1987. Subcellular redistribution of lysosomal enzymes during caerulein- induced pancreatitis. *Am. J. Physiol.* **253**:G508–G516.
14. Williams, J.A., Korc, M., and Dormer, R.L. 1978. Action of secretagogues on a new preparation of functionally intact, isolated pancreatic acini. *Am. J. Physiol.* **235**:517–524.
15. Schafer, C., et al. 1998. A role for the p38 mitogen-activated protein kinase/Hsp 27 pathway in cholecystokinin-induced changes in the actin cytoskeleton in rat pancreatic acini. *J. Biol. Chem.* **273**:24173–24180.
16. Soltoff, S.P., Carraway, K.L., Prigent, S.A., Gullick, W.G., and Cantley, L.C. 1994. ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. *Mol. Cell. Biol.* **14**:3550–3558.
17. Auger, K.R., Serunian, L.A., Soltoff ,S.P., Libby, P., and Cantley, L.C. 1989. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell.* **57**:167–175.
18. Saluja, A.K., et al. 1989. Experimental pancreatitis is mediated by low-affinity cholecystokinin receptors that inhibit digestive enzyme secretion. *Proc. Natl. Acad. Sci. USA.* **86**:8968–8971.
19. Davol, P.A., Bizuneh, A., and Frackelton., A.R., Jr. 1999. Wortmannin, a phosphoinositide 3-kinase inhibitor, selectively enhances cytotoxicity of receptor-directed-toxin chimeras in vitro and in vivo. *Anticancer. Res.* **19**:1705–1713.
20. Hu, L., Zaloudek, C., Mills, G.B., Gray, J., and Jaffe, R.B. 2000. In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002). *Clin. Cancer Res.* **6**:880–886.
21. O'Konski, M.S., and Pandol, S.J. 1993. Cholecystokinin JMV-180 and caerulein effects on the pancreatic acinar cell cytoskeleton. *Pancreas.* **8**:638-646.
22. Hofbauer, B., et al. 1998. Intra-acinar cell activation of trypsinogen during caerulein-induced pancreatitis in rats. *Am. J. Physiol.* **275**:G352–362.
23. Toker, A., and Cantley, L.C. 1997. Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature.* **387**:673–676.
24. Chan, T.O., Rittenhouse, S.E., and Tsichlis, P.N. 1999. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.* **68**:965–1014.
25. Vanhaesebroeck, B., and Waterfield, M.D. 1999. Signaling by distinct classes of phosphoinositide 3-kinases. *Exp. Cell Res.* **253**:239–254.
26. Wurmser, A.E., Gary, J.D., and Emr, S.D. 1999. Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways. *J. Biol. Chem.* **274**:9129–9132.
27. Rivard, N., Rydzewska, G., Lods, J.S., Martinez, J., and Morisset, J. 1994. Pancreas growth, tyrosine kinase, PtdIns 3-kinase, and PLD involve high-affinity CCK-receptor occupation. *Am. J. Physiol.* **266**:G62–G70.
28. Watanabe, O., Baccino, F.M., Steer, M.L., and Meldolesi, J. 1984. Supramaximal caerulein stimulation and ultrastructure of rat pancreatic acinar cell: early morphological changes during development of experimental pancreatitis. *Am. J. Physiol.* **246**:G457-G467.
29. Saito, I., Hashimoto, S., Saluja, A., Steer, M.L., and Meldolesi, J. 1987. Intracellular transport of pancreatic zymogens during caerulein supramaximal stimulation. *Am. J. Physiol.* **253**:G517–G526.
30. Peterson, M.R., Burd, C.G., and Emr, S.D. 1999. Vac1p coordinates Rab and phosphatidylinositol 3-kinase signaling in Vps45p-dependent vesicle docking/fusion at the endosome. *Curr. Biol.* **9**:159–162.
31. Lemmon, S.K., and Traub, L.M. 2000. Sorting in the endosomal system in yeast and animal cells. *Curr. Opin. Cell. Biol.* **12**:457–466.
32. Wurmser, A.E., and Emr, S.D. 1998. Phosphoinositide signaling and turnover: PtdIns(3)P, a regulator of membrane traffic, is transported to the vacuole and degraded by a process that requires lumenal vacuolar hydrolase activities. *EMBO. J.* **17**:4930–4942.
33. Naccache, P.H., et al. 2000. Stimulation of human neutrophils by chemotactic factors is associated with the activation of phosphatidylinositol 3-kinase gamma. *J. Biol. Chem.* **275**:23636–23641.
34. Young, L.H., Ikeda, Y., Scalia, R., and Lefer, A.M. 2000. Wortmannin, a potent antineutrophil agent, exerts cardioprotective effects in myocardial ischemia/reperfusion. *J. Pharmacol. Exp. Ther.* **295**:37–43.