Serine proteases mediate inflammatory pain in acute pancreatitis

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ACUTE PANCREATITIS IS A POTENTIALLY fatal inflammatory disease, which typically begins with the onset of severe abdominal pain. Although autodigestion of the pancreas by proteases such as trypsin is considered the main mechanism of the pathogenesis of acute pancreatitis, the molecular basis is still poorly understood. The activity of pancreatic digestive enzymes is strictly controlled by redundant mechanisms, including synthesis of inactive zymogens (e.g., trypsinogen, mesotrypsinogen), zymogen segregation in membrane-bound compartments that are packaged with pancreatic secretory trypsin inhibitors (PSTIs), degradation in lysosomal compartments, and restricted activation by enterokinase (ENK) located separately in the duodenum (14), where ENK cleaves trypsin-associated peptide from trypsinogen to release activated trypsins (36, 41). ENK is localized to the brush border membranes of duodenal enterocytes and is thus able to activate proteases once they have been secreted from the pancreas into the duodenum and are in direct proximity to nutrient substrates.

Trypsinogens are a functionally diverse gene family. In humans, protease serine type 1 (pssr1) encodes trypsinogen I (cationic trypsin), pssr2 encodes trypsinogen II (anionic trypsin), and pssr3 encodes mesotrypsinogen. Trypsinogen IV is a splice variant of mesotrypsinogen (47). A potential homologue of human mesotrypsinogen in rats is P23 trypsinogen, a minor isoform. Trypsin IV/mesotrypsin and P23 are resistant to polypeptide inhibitors, including the PSTIs and soybean trypsin inhibitor, and may thus remain active for prolonged period of time. However, the role of trypsin IV/mesotrypsin in disease is unknown.

The biological effects of trypsins are in part attributed to the proteolytic activation of a family of G-protein coupled receptors, the protease-activated receptors (PARs) (35). Trypsins, and other serine proteases, cleave the extracellular NH2-terminal domain, thereby unmasking a newly formed NH2-terminal that acts as a tethered ligand that binds to and activates the cleaved receptor. PAR2, which is activated by trypsins and mast cell tryptase, is strongly expressed on the luminal surface of pancreatic acinar and ductal cells, and by pancreatic sensory nerves. However, the contribution of PAR2 to pancreatitis is controversial, with reported proinflammatory and anti-inflammatory effects (14, 20, 26, 29, 31, 42).

In both experimental and human acute pancreatitis, premature cleavage of trypsinogen in pancreatic acinar cells liberates the activated serine protease trypsin, leading to cellular damage and inflammatory cell infiltration (13, 27, 29). Serine protease inhibitors block trypsinogen activation and reduce the severity of pancreatitis (8, 25, 34, 39). Genetic mutations in the cationic trypsinogen gene or in the pancreatic secretory trypsin inhibitor gene, both resulting in persistent trypsin activity (14), have been identified in patients with hereditary pancreatitis. Little is known about the role of trypsins in the pathogenesis of pancreatic inflammatory pain. Injection of a subinflammatory dose of trypsin into the pancreatic duct increased expression of c-Fos by spinal nociceptive neurons and caused mechanical hyperalgesia via PAR2 activation (16, 17). We hypothesized that inhibitor-resistant isoforms of trypsin might produce an augmented response. In the present study, we injected exogenously trypsin II, trypsin IV, and P23 into the pancreatic duct of rats pretreated or not with melagatran (MGT). MGT, originally developed as a direct thrombin inhibitor, is also a potent trypsin inhibitor (10, 11) and, as we show here, also acts as a high-affinity inhibitor of polypeptide-inhibitor-resistant trypsin isoforms. We then measured both pancreatic inflammation and
nociceptive signaling (46). To determine whether activation of endogenous trypsinogen produces pancreatic inflammation and pain via the release of trypsin isoforms, we injected ENK into the pancreatic duct following pretreatment with trypsin inhibitors with different sensitivities to the various isoforms of trypsin. Finally, to determine the contribution of inhibitor-resistant trypsins to inflammation and pain, we induced acute pancreatitis with supramaximal doses of cerulein in rats pretreated with MGT. We found that infusion of a subinflammatory dose of trypsin caused pain whereas infusion of the same dose of trypsin IV and P23 caused more robust pancreatic pain and inflammation. These effects were blocked by pretreatment with MGT. Premature activation of trypsin and its isoforms induced by intraductal injection of ENK caused pancreatic inflammation and pain. Among the trypsin inhibitors, the most pronounced reduction in ENK-induced pancreatitis pain was seen following pretreatment with MGT. Moreover, we also confirmed that MGT blocked nocifensive behavior and nociception induced by cerulein. Thus trypsin, including its minor inhibitor-resistant isoforms, contributes to pancreatic pain, and specific inhibitors of these isoforms could be novel therapies for pancreatitis pain.

METHODS

Animals. Sprague-Dawley rats (male, 225–275 g; Charles River Laboratories, Hollister, CA) were kept in a temperature-controlled environment with 12:12-h light-dark cycle with free access to food and water. All procedures performed were approved by the University of California, San Francisco Institutional Animal Care and Use Committee and in compliance with the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD).

Materials. Rabbit anti-c-Fos was from Chemicon (Temecula, CA) and biotinylated goat anti-rabbit IgG was from Vector Laboratory (Burlingame, CA). Porcine intestinal ENK and porcine pancreatic trypsin II-S were from Sigma (St. Louis, MO), and recombinant human trypsin IV and recombinant rat P23 have been described (22). Soybean trypsin inhibitor type II-S (STI) and trypsin inhibitor type I-P (TPI) were from Sigma, and MGT (10, 11), which has activity against both thrombin and trypsins including trypsin IV, was from Astrazeneca (Möldal, Sweden).

In vitro inhibitor characterization. The inhibition constants ($K_i$) of MGT and STI were studied for recombinant rat P23, recombinant human trypsin IV and pancreatic trypsin. IC$_{50}$ values were determined by using 150 µM tosyl-GPR-pNA as substrate ([$S$]) in 100 mM Tris-HCl, pH 8, 1 mM CaCl$_2$ at 25°C. For better comparisons, the IC$_{50}$ values were converted into binding affinity $K_i$ values, via the Cheng-Prusoff equation: $K_i = IC_{50}(1 + [S]/K_m)$ and the respective $K_m$ value of tosyl-GPR-pNA as described (22).

Intraductal infusion of proteases. Proteases were infused into the pancreatic duct as described elsewhere (17), with several modifications. Rats were anesthetized with sodium pentobarbital (50 mg/kg ip) and the abdomen was opened in the midline. The proximal common bile duct was temporarily occluded with a hemoclip to prevent flow of infused agents into the liver. The duodenum was punctured on the antimesenteric side using a 30-gauge needle near the papilla of Vater and a catheter (PE-10 tubing) that was inserted through the papilla into the biliopancreatic duct. A 6-0 Prolene stitch secured the catheter in the duct. ENK (100 U/kg), trypsin isoforms (trypsin II, trypsin IV, p23; 0.01 mg/ml) or vehicle (0.1 M PBS or 0.9% NaCl, control) (all 250 µl) were infused into the pancreatic duct over 10 min. The hemoclip and the catheter were removed before the abdomen was closed. Two and a half hours after intraductal infusion, rats were killed with pentobarbital sodium (200 mg/kg ip) and tissues were collected.

Cerulein-induced acute pancreatitis. Rats received hourly subcutaneous injections of supramaximal doses of cerulein (200 µg·kg$^{-1}$·h$^{-1}$) or vehicle (0.9% NaCl) for 6 h, after which tissues were collected (21, 22).

Administration of trypsin inhibitors. STI (8 mg/kg ip), TPI (7 mg/kg ip), or MGT (0.188 mg/kg ip) was administered 1 h before infusion of P23 or trypsin IV or ENK into the pancreatic duct or cerulein treatment.

Tissue collection. Blood was collected from the left ventricle, centrifuged (10,000 g, 10 min, 4°C), and the serum was collected for amylase activity. Rats were transcardially perfused with 100 ml of 0.1 M PBS. The body of the pancreas was snap frozen in liquid nitrogen for assay of myeloperoxidase (MPO) activity, and the tail of the pancreas was placed in 10% formalin (16 h, room temperature) for histological analysis. Rats were then perfused with 300 ml of 3.7% formaldehyde in PBS. Segments of thoracic spinal cord (T6, T8, T9, T10, T12) were immersion-fixed in 3.7% paraformaldehyde for 1 h at room temperature, cryoprotected by incubation in 30% sucrose (16 h, 4°C), and processed for localization of c-Fos by immunohistochemistry.

Serum amylase activity. Serum was assayed for amylase activity by use of Infinity Amylase Liquid Stable Reagent (Thermo Electron, Louisville, CO). Results are expressed as units per liter amylase.

MPO activity. Pancreatic tissue was assayed for MPO activity as an index of granulocyte infiltration as previously described (4). Tissue was thawed, homogenized in 20 mM phosphate buffer pH 7.4, and centrifuged (10,000 g, 10 min, 4°C). Pellets were resuspended in 50 mM PBS pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The suspensions underwent three cycles of freezing and thawing, sonication for 60 s, and centrifugation (10,000 g, 20 min, 4°C). MPO activity in the supernatant was measured using the substrate 3,3′,5,5′-tetramethylbenzidine dihydrochloride liquid substrate system (Sigma). Absorbance was assessed at 650 nm, and human neutrophil MPO (Calbiochem, San Diego, CA) was used to generate a standard curve. Results are expressed as units of MPO activity per milligram of proteins, measured with BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

TAP assays. Trypsin-associated peptide (TAP) is a stable NH$_2$-terminal hexapeptide (Val-Asp-Asp-Asp-Asp-Lys) that is liberated in equimolar amounts to trypsin after cleavage of trypsinogen. TAP is conserved among mammals (5) and is distinct from any other sequence within the trypsinogen or trypsin proteins, thereby allowing for in situ assessment of trypsinogen activation (6). Pancreatic tissue (~200 mg) was placed in 1 ml of 0.2 M Tris-HCl, pH 7.3, containing 20 mM EDTA. Samples were boiled for 15 min, homogenized for 30 s, and centrifuged (10,000 g, 10 min, room temperature). Complete protease inhibitor (Roche Biochemicals, Indianapolis, IN) was added to the supernatant. TAP was measured by using an ELISA (Biotrin International, Dublin, Ireland). Results are reported in nanomoles per liter of TAP.

Pancreas HSS. Pancreatic samples were paraffin embedded, sectioned (5 µm), and stained with hematoxylin and eosin. Pathological changes were evaluated by light microscopy (×20 objective) as described (18, 28, 44) by an investigator unaware of the experimental groups. The following seven categories were assessed on a scale of 0–5: macrolobular edema, microlobular edema, zymogen degranulation, polymorphonuclear leukocyte (PMN) infiltration, PMNs in peri-pancreatic fat, presence of vacuoles, and necrosis. The scores were tabulated and the mean value of each group served as the histology severity score (HSS).

Activation of spinal nociceptive neurons. To assess activation of nociceptive spinal neurons, c-Fos immunoreactivity (IR) was localized by immunohistochemistry (3, 4). Spinal sections (40–50 µm) were cut in the transverse plane by use of a sliding microtome and were placed in 100 mM PBS, pH 7.4. Samples were incubated in PBS
RESULTS

Effects of intraductal trypsins on pancreatitis and pancreatic pain. Pancreatic intraductal injections of subinflammatory doses of exogenous trypsin (0.01–1 mg/ml) have been reported to activate spinal nociceptive neurons in a dose-dependent manner, as shown by the increase in c-Fos expression, without evidence of inflammation (16). Since we anticipated augmented pain responses to inhibitor-resistant trypsin isoforms, we examined effects of the lowest dose of trypsin (0.01 mg/ml) reported to affect c-Fos expression. Trypsin II, trypsin IV, or P23 (0.01 mg/ml) was infused into the pancreatic duct, and 2.5 h later inflammation was assessed by measurement of serum amylase, pancreatic MPO, and HSS. Some rats were pretreated 1 h before with MGT (0.188 mg/kg ip), a trypsin inhibitor with activity against polypeptide inhibitor-resistant trypsin isoforms. Infusion of trypsin II at the selected dose did not cause pancreatic inflammation, as shown by the lack of effect on serum amylase, pancreatic MPO, and HSS (Fig. 1, A–C). In contrast, trypsin IV and P23 caused pancreatic inflammation, even at this low dose. In particular, P23 robustly increased serum amylase activity (Fig. 1A), whereas trypsin IV increased pancreatic MPO activity (Fig. 1B), and both these effects were reduced by MGT pretreatment. Moreover, trypsin IV and P23 significantly enhanced HSS (Fig. 1, C and D). These inhibitor-resistant isoforms of trypsin produced a pattern of pancreatic inflammation and injury characterized by edema, marked necrosis, and infiltration of neutrophils (Fig. 1D). Pretreatment with MGT prior to trypsin IV altered some of the individual end points assessed. Thus there was a decrease in interlobular edema. However, this was offset by an increase in vacuoles in pancreatic acinar cells, such that the overall HSS was un-
changed. Similarly, pretreatment with MGT prior to P23 decreased interlobular edema, but increased intralobular edema, zymogen loss, neutrophil infiltration, and necrosis were apparent. Thus the HSS obtained in this group was increased compared with that seen in animals injected with P23 alone.

To examine whether injection of inhibitor-resistant trypsin isoforms caused activation of pancreatic nociceptive pathways, we quantified c-Fos IR in the superficial laminae I/II of the spinal levels T6–T12. As expected, intraductal infusion of trypsin II increased c-Fos IR in all pancreatic spinal levels, with a significant effect in T9, compared with vehicle (Fig. 2A). The inhibitor-resistant isoforms trypsin IV and P23 had more robust nociceptive effects than those of trypsin II, as shown by the significant increase of c-Fos IR at all pancreatic spinal levels in the superficial laminae of the dorsal horn of T8–T10 compared with vehicle (Fig. 2A). These effects were dramatically blocked by MGT pretreatment, suggesting a major role of inhibitor-resistant trypsin isoforms in mediating pancreatic pain. There were no effects on c-Fos IR in the spinal neurons of T6 or T12, indicating that effects were confined to the regions of the spinal cord that receive input from pancreatic sensory neurons (Fig. 2A). Thus a “threshold dose” of trypsin II was identified, at which activation of nociceptive pathways is measurable but effects on inflammatory end points are not significant. This same dose of inhibitor-resistant isoforms trypsin IV and P23 caused pancreatitis and pain in this model, suggesting that these trypsin isoforms could be important mediators of protease-induced pancreatic inflammatory pain.

Effects of intraductal ENK on pancreatitis. To determine whether a sufficient reservoir of activatable trypsinogens exists in vivo to produce the effects we observed with intraductal infusion of exogenous trypsin, we experimentally reproduced the unregulated activation of pancreatic endogenous trypsins by injecting ENK into the pancreatic duct. We measured serum amylase and pancreatic MPO activity and assessed pancreatic histology as indexes of pancreatitis. To confirm that the observed effects were due to trypsin release, we measured pancreatic concentration of TAP, a trypsinogen cleavage by-product and index of trypsin activation. Injection of ENK into the pancreatic duct significantly increased TAP levels in the pancreas compared with vehicle controls (Fig. 3A), thereby confirming activation of pancreatic trypsins. In addition, intraductal injection of ENK caused inflammation as shown by increased serum amylase (Fig. 3B), pancreatic MPO activity (Fig. 3C), and increased HSS (Fig. 3D). Histological findings in ENK-treated tissues included edema, a paucity of zymogen granules, polymorphonuclear leukocyte infiltration, mild necrosis, and hemorrhage (Fig. 4D). The vehicle had no effect. Thus administration of ENK into the pancreatic duct induces activation of trypsins within the pancreas and also causes inflammation.

To evaluate whether the proinflammatory effects of intraductal ENK depend on activation of endogenous trypsins, we determined whether inhibition of trypsin would ameliorate ENK-induced pancreatitis. We used three different trypsin inhibitors because of their differing sensitivities to trypsin isoforms: STI, a reversible, semiselective trypsin inhibitory peptide; TPI, a specific trypsin inhibitor; and MGT, a serine protease inhibitor that also inhibits trypsin IV and P23 (see Table 1). Administration of each inhibitor into the peritoneal cavity 1 h before ENK infusion did not affect serum amylase activity (Fig. 4A) but reduced pancreatic inflammation, as shown by a marked decrease in MPO activity and HSS (Fig. 4, B–D). Both STI and TPI improved pancreatic histological

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**Fig. 2. Effects of intraductal injection of trypsin isoforms on pancreatic nociceptive activation.** Trypsin, trypsin IV, P23 (0.01 mg/ml in 250 μl of saline), or vehicle (250 μl of saline) was injected into the pancreatic duct. Some rats were pretreated with MGT (0.188 mg/kg ip) 1 h before the surgery. After 2.5 h, c-Fos immunoreactivity (IR) was localized in the superficial laminae I/II of the dorsal horn of the spinal cord (T6–T12) and the number of c-Fos IR-positive nuclei per spinal section was determined. Trypsin II produced c-Fos IR in segment T9, whereas the inhibitor-resistant trypsin IV and P23 robustly increased c-Fos IR in all pancreatic segments (T8–T10). This effect was completely reversed by MGT pretreatment. In control mice, vehicle had no effect. The c-Fos IR did not increase significantly in the internal control spinal levels T6 or T12. *P < 0.05 vs. Veh; **P < 0.001 vs. Veh; **P < 0.01 vs. same treatment without MGT pretreatment; ^^^P < 0.001 vs. same treatment without MGT pretreatment; n = 4–6.
To better understand the effect of the inhibitors used in these experiments we also looked at their efficacy to inhibit various purified trypsins in vitro. As a convenient readout, we determined their IC50 values and converted these in Kic to be able to compare the different enzymes. To mimic an immediate effect in vivo, we did not extensively preincubate enzyme and inhibitor, but started the reaction by addition of the substrate within 5 min. The results can be found in Table 1.

Effects of intraductal ENK on pancreatic pain. To investigate the effects of endogenous trypsins on spinal nociceptive activation, we collected samples from spinal cord levels (T6–T12) 2.5 h after pancreatic intraductal injection of ENK and assessed c-Fos IR in neurons of the superficial laminae I/II of the dorsal horns. ENK injection caused a robust increase in c-Fos IR, suggesting activation of pancreatic nociceptive pathways (Fig. 5A). This pronounced effect was reversed to different extents by all trypsin inhibitors (Fig. 5B). STI diminished c-Fos IR in pancreatic spinal levels (T8–T10) to a greater extent than TPI, whereas MGT was the most effective of all. Thus ENK promoted premature activation of trypsinogen and increased trypsin activity in the pancreas, thereby causing pancreatic pain. Inhibition of trypsins, especially of the inhibitor-resistant trypsin isoforms, blocked spinal nociceptive activation induced by intrapancreatic infusion of ENK.

Inhibition of inhibitor-resistant trypsin isoforms ameliorates inflammation and pain in cerulein-induced pancreatitis. Since injection of the inhibitor-resistant trypsin IV and P23 resulted in pronounced pancreatic inflammation and activation of nociceptive neurons, we evaluated their contribution to inflammatory pain during experimental pancreatitis. To do so, we assessed the effects of pretreatment with MGT, which inhibits the serine proteases thrombin and trypsin II, and also the inhibitor-resistant isoforms trypsin IV and P23, on acute pancreatitis and pancreatic pain induced by supramaximal doses of the acinar cell secretagogue cerulein. As expected, cerulein caused a marked increase in serum amylase and MPO activity (Fig. 6, A and B) and a 5-fold increase in HSS (Fig. 6, C and D). Histological characteristics of cerulein-induced pancreatitis included changes in all of the end points examined, most strikingly in intra- and interlobular edema, neutrophil infiltration, and vacuolization. MGT significantly reduced MPO activity but did not ameliorate amylase activity or HSS. MGT pretreatment did not alter vacuole formation in acinar cells. However, decreased neutrophil infiltration occurred, and this result is consistent with our finding of diminished MPO activity in the tissue. Cerulein treatment resulted in a twofold increase in c-Fos IR in positive neurons in the dorsal horn of the pancreatic spinal level T9, but not T6 or T12, indicative of activation of pain pathways originating from the inflamed pancreas and surrounding tissues (Fig. 6D). MGT robustly decreased c-Fos IR (Fig. 6D). These results were corroborated by behavioral experiments where nociceptive behavior was measured by applying different sizes of calibrated VFFs to the abdomen of rats. Once again, cerulein treatment resulted in a robust nociceptive behavior, which was partially reversed by pretreatment with MGT (Fig. 6E). Thus MGT strongly decreased c-Fos IR and nociceptive behavior, suggesting that inhibitor-resistant isoforms of trypsin have a major role in mediating pancreatitis pain.

DISCUSSION

Trypsin IR in the serum increases early in the course of acute pancreatitis in humans (30), and urinary trypsinogen-2 levels are now used to diagnose acute pancreatitis (1); however, the role of trypsins in the pathogenesis of pancreatic inflammation has been difficult to characterize because of the concomitant secretion of trypsin inhibitors that rapidly degrade trypsin II, the most ubiquitous species of trypsin. Trypsin has been reported to mediate inflammatory pain in acute pancreatitis via PAR2 activation, but its role remains controversial (14, 20, 26, 29, 31, 42). The pancreas has abundant endogenous stores of trypsin inhibitors (8), and trypsin inhibition is an important protective mechanism against pancreatitis since cohorts of patients with recurrent acute pancreatitis have been found to have mutations in the serine protease inhibitor SPINK1 (2). Our findings suggest that minor isoforms of trypsin that are resistant to degradation by endogenous inhibitors could lead to
sustained protease signaling and thereby promote pancreatic inflammation and pain.

The pancreas has abundant endogenous stores of trypsin inhibitors (8), but inhibitor-resistant isoforms of trypsin, namely trypsin IV and P23, have been identified, whose role in pancreatitis-induced inflammatory pain has not been investigated.

In this study, we investigated for the first time the contribution of trypsin IV and P23 on pancreatic nociception. We show the important role of human trypsin IV and rat P23 in causing pancreatic inflammation and nociception. First, we observed that intraductal injection of a subinflammatory dose of exogenous trypsin II caused nociception with no evidence of inflammation, in accord with a previous study (16). Second, we injected the same subinflammatory dose of the proteinaceous inhibitor-resistant trypsin IV and rat P23 and observed that they caused pancreatic inflammation and robust nociception, which were blocked by MGT pretreatment, the only serine protease inhibitor that we show binds with affinity to trypsin IV and P23, as well as trypsin (Table 1). Third, we investigated the contribution of endogenous trypsin isoforms by pancreatic intraductal injection of ENK after pretreatment with trypsin inhibitors (STI, TPI, and MGT) with differing specificities to trypsin isoforms. Finally, we showed that pretreatment with MGT significantly decreased nociception and mechanical visceral hyperalgesia induced by acute pancreatitis, suggesting the importance of trypsin IV and rat P23 during pathological conditions. The present study supports the postulate that pre-

Fig. 4. Effects of trypsin inhibitors on ENK-induced pancreatic inflammation. Trypsins were inhibited with either soybean trypsin inhibitor type II-S (STI; 8 mg/kg ip) or trypsin inhibitor type I-P (TPI; 7 mg/kg ip) or MGT (0.188 mg/kg ip) 1 h before induction of pancreatic inflammation by ENK intraductal injection (100 U/kg in 250 µl of 0.1 M PBS). After 2.5 h, serum amylase, pancreatic MPO activity (A and B) and HSS (C and D) were measured. Intraductal ENK caused neutrophil infiltration (arrows) and hemorrhage (arrowheads) as well as edema and mild tissue necrosis. All trypsin inhibitors reduced MPO (B). **P < 0.005 vs. Veh; ***P < 0.001 vs. Veh; n = 6.

Table 1. Kic of trypsin inhibitors MGT and STI against recombinant rat P23, recombinant human trypsin IV, and pancreatic trypsin

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Recombinant Rat P23 Kic, nM</th>
<th>Recombinant Human Trypsin IV Kic, nM</th>
<th>Human Pancreatic Trypsin Kic, nM</th>
</tr>
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<tr>
<td>MGT</td>
<td>17</td>
<td>7.3</td>
<td>4.5</td>
</tr>
<tr>
<td>STI</td>
<td>230</td>
<td>1,700</td>
<td>3.7</td>
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Kic, inhibition constant; MGT, melagatran; STI, soybean trypsin inhibitor.
mature trypsin activation plays a major role in the pathogenesis of inflammation and pain during acute pancreatitis. Our findings are an important extension of previous work that suggests the major contribution of proteinaceous inhibitor-resistant trypsin isoforms in mediating pancreatic nociception and inflammation (43). Given the pharmacological profile of MGT, which also inhibits thrombin and plasmin together with trypsins (10, 11), we cannot rule out the possibility that our results may be partially due to its effect on inhibiting thrombin-induced activation of PAR1 and PAR4. Further investigation is needed to clarify this possibility. However, all of the trypsin inhibitors used in this study can inhibit thrombin and other serine proteases (24, 37). Thus we took advantage of their different selectivities to assess the relative role of various proteases.

Premature intracellular activation of trypsinogen in the pancreas leads to acinar cell injury accompanied by necrosis (9, 32). This unregulated trypsin activation is believed to occur when intracellular protective mechanisms that function to prevent trypsinogen activation or reduce trypsin activity are overwhelmed (45). Available trypsin then triggers a localized inflammatory response mediated by proinflammatory cytokines, platelet-activating factor, and substance P. Some of these inflammatory mediators, including prostanooids, bradykinin, tachykinins, serotonin, and other biological factors, are considered noxious substances that can activate or sensitize nociceptors (16). Trypsin has been demonstrated to play an important role in mediating not just the inflammatory aspects of pancreatitis, but also nociception, via PAR2 activation on peripheral nociceptive sensory nerve endings leading to the central release of substance P and calcitonin gene-related peptide in the spinal cord (16, 17). The resulting excitation of second-order neurons in the dorsal horn activates ascending pathways to the brain and leads to increasedafferent signaling, central sensitization, and potential amplification and persistence of pain. The isoforms of trypsin that mediate these effects are unknown. We hypothesized that this pathway involves not only trypsin II, but also its proteinaceous inhibitor-resistant isoforms, trypsin IV and P23.

Human trypsinogen IV is a splice variant of mesotrypsinogen (47). The NH2-terminal sequences of trypsinogen IV and mesotrypsinogen derive from different exons (38, 43, 47). However, when these zymogens are cleaved by ENK, the active COOH-terminal products, trypsin IV and mesotrypsin, are identical. In the rat, P23 is the corresponding protein and represents a minor trypsinogen isoform within the pancreas (7, 40). The main characteristics of these minor isoforms are not only the resistance to endogenous polypeptide inhibitors, but also the ability to degrade and inactivate such inhibitors, endowed by the evolutionary selection of Arg198 (43). The physiological and pathological functions of inhibitor-resistant isoforms are still largely unknown. It has been suggested that inappropriate activation of human trypsinogen IV/mesot-

Fig. 5. Effects of intraductal injection of ENK, with or without pretreatment with trypsin inhibitors, on pancreatic spinal nociceptive activation. ENK (100 U/kg in 250 μl of 0.1 M PBS) or vehicle (0.1 M PBS 250 μl) was injected into the pancreatic duct of rats. In 3 experimental groups, trypsins were inhibited with either STI (8 mg/kg ip), TPI (7 mg/kg ip), or MGT (0.188 mg/kg ip) 1 h before induction of pancreatic inflammation by ENK intraductal injection (100 U/kg in 250 μl of 0.1 M PBS). After 2.5 h, c-Fos IR was localized in the dorsal horn of the spinal cord (T6–T12) and the number of c-Fos IR-positive nuclei per spinal section was determined. ENK, but not its vehicle, produced a robust increase in c-Fos IR (A) in the superficial laminae I/II of the spinal cord that was diminished by all trypsin inhibitors. MGT, which is more selective for the inhibitor-resistant trypsin isoforms, was the most effective in blocking spinal nociceptive activation. ^^^P < 0.005 vs. Veh; ^^^^P < 0.001; vs. Veh; ***P < 0.001 vs. ENK; n = 6.
Ryprsinogen in the pancreas could degrade available trypsin inhibitor, thereby reducing its protective effect and promoting pancreatitis (43). The lysosomal protease cathepsin B activates trypsinogen IV at a higher rate than human cationic or anionic trypsinogens during pancreatitis (43). Finally, in rats with acute pancreatitis induced by the secretagogue cerulein, P23 is upregulated 14-fold, which represents the largest incremental change of any protein in the rat exocrine pancreas (7).

Our findings, that intrapancreatic infusion of the proteinaceous trypsin inhibitor-resistant isoforms trypsin IV and rat

Fig. 6. Effects of MGT on cerulein-induced acute pancreatitis and spinal nociceptive activation. MGT was injected (0.188 mg/kg ip) 1 h before pancreatic inflammation and pain were induced by cerulein (200 μg·kg⁻¹·h⁻¹ sc; 6 h). After 6 h, serum amylase and pancreatic MPO activity (A and B) and HSS (C and F) were measured; c-Fos IR (D) was localized in the dorsal horn of the spinal cord (T6–T9–T12) and the number of c-Fos IR-positive nuclei per spinal section was determined. In behavioral experiments, after 6 h rats were tested for nocifensive behavior by probing their abdomen with von Frey filaments (VFFs; E). MGT did not affect amylase activity or HSS but significantly decreased MPO activity in pancreas (B). MGT also robustly diminished spinal c-Fos activation in T9 (D). There were no significant changes in c-Fos IR in T6 or T12. Finally, MGT strongly ameliorated nocifensive behavior (E). n = 4–6; **P < 0.01 vs. Veh; ***P < 0.001 vs. Veh. ^P < 0.05 vs. Cer; ^^^P < 0.001 vs. Cer.
P23 caused robust nociception and inflammation, which were blocked by pretreatment with MGT, supporting our previous work in which we found that trypsin IV and P23 induced inflammation and hyperalgesia by cleaving PAR\textsubscript{2} at lower doses than pancreatic trypsin (22). The magnitude of spinal nociceptive activation and pancreatic inflammation induced by trypsin IV and P23 in the present study was larger than that seen with trypsin II, which did not cause inflammation at the selected dose. It is possible that resistance to degradation may potentiate their effect by prolonged signaling. In addition, trypsin IV and P23 may stimulate the generation of other proteases that can activate PARs, thereby amplifying the inflammatory and nociceptive responses. Additional studies are required to investigate these possibilities.

The results of our experiments using ENK injection into the pancreatic duct suggest the importance of release of pancreatic trypsins in both inflammation and pain in the pancreas. ENK caused release of activated trypsin as reflected by pancreatic TAP levels, and the resulting inflammatory pain was due to trypsin species since both inflammation and pain were inhibited by trypsin inhibitors. MGT pretreatment, which blocks trypsin IV and P23 activity as well as that of trypsin II, had a more profound inhibitory effect on pain than did either of the trypsin II inhibitors STI or TPI, suggesting that the difference may be due to inhibition of the proteinaceous trypsin inhibitor-resistant isoforms in the pancreas.

Finally, we evaluated the effects of pretreatment with MGT on acute pancreatitis induced by supramaximal doses of the acinar cell secretagogue cerulein. This model of acute pancreatitis has largely been used for investigating the involvement of trypsin in pain and inflammation (12, 15, 19, 20, 23, 29). Cerulein treatment caused pancreatitis, as shown by the increase in serum amylase and MPO activity and HSS; pancreatic spinal nociception activation, as confirmed by c-Fos IR increase; and behavioral visceral hyperalgesia, as measured with VFFs. MGT pretreatment reduced MPO activity, but had no effect on serum amylase activity and pancreas morphology. MGT strikingly reduced spinal nociceptive activation, by decreasing c-Fos IR, and nociceptive behavior, by decreasing withdrawal responses to VFFs. Thus MGT ameliorated pancreatitis, and especially pancreatic pain, suggesting that inhibitor-resistant isoforms of trypsin play a major role in mediating inflammatory pain. These findings support other reports that suggest the importance of trypsin IV/mesotrypsin and P23 in the pathogenesis of acute pancreatitis (7, 43).

The effect of MGT on pancreas histology was similar to that reported in a rat model of endotoxemia in which MGT pretreatment, at higher doses than the one we used in our study, improved liver and kidney function but not histology (33). Additional studies are required to determine the exact mechanism by which MGT improved pancreatic inflammation and pain, but not histological damage score. It is possible that MGT-induced inhibition of thrombin could have resulted in PAR-mediated endothelial dysfunction. In conclusion, endogenous trypsins, which are prematurely activated in the early course of acute pancreatitis, play an important role in promoting pancreatic inflammatory pain. In particular, inhibitor-resistant isoforms of trypsin, human trypsin IV and rat P23, may be important in mediating prolonged pancreatic inflammatory pain in pancreatitis.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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