Pharmacological inhibition of PAR2 with the pepducin P2pal-18S protects mice against acute experimental biliary pancreatitis

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Pharmacological inhibition of PAR2 with the pepducin P2pal-18S protects mice against acute experimental biliary pancreatitis. Am J Physiol Gastrointest Liver Physiol 304: G516–G526, 2013. Published December 28, 2012; doi:10.1152/ajpgi.00296.2012.—Pancreatic acinar cells express proteinase-activated receptor-2 (PAR2) that is activated by trypsin-like serine proteases and has been shown to exert model-specific effects on the severity of experimental pancreatitis, i.e., PAR2−/− mice are protected from experimental acute biliary pancreatitis but develop more severe secretagogue-induced pancreatitis. P2pal-18S is a novel pepducin lipopeptide that targets and inhibits PAR2. In studies monitoring PAR2-stimulated intracellular Ca2+ concentration changes, we show that P2pal-18S is a full PAR2 inhibitor in acinar cells. Our in vivo studies show that P2pal-18S significantly reduces the severity of experimental biliary pancreatitis induced by retrograde intraductal bile acid infusion, which mimics injury induced by endoscopic retrograde cholangiopancreatography (ERCP). This reduction in pancreatitis severity is observed when the pepducin is given before or 2 h after bile acid infusion but not when it is given 5 h after bile acid infusion. Conversely, P2pal-18S increases the severity of secretagogue-induced pancreatitis. In vitro studies indicate that P2pal-18S protects acinar cells against bile acid-induced injury/death, but it does not alter bile acid-induced intracellular zymogen activation. These studies are the first to report the effects of an effective PAR2 pharmacological inhibitor on pancreatic acinar cells and on the severity of experimental pancreatitis. They raise the possibility that a pepducin such as P2pal-18S might prove useful in the clinical management of patients at risk for developing severe biliary pancreatitis such as occurs following ERCP.

MATERIALS AND METHODS

Animals

All experiments employed 10- to 12-wk-old C57Bl/6 mice purchased from Charles River Labs (Wilmington, MA) or PAR2−/− C57Bl/6 mice bred from founder knock-out mice originally kindly donated by Dr. P. Andrade-Gordon (Drug Discovery, Johnson & Johnson Pharmaceutical Research and Development, Spring House, PA). All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Tufts Medical Center.

Reagents

The pepducin P2pal-18S, the NH2-terminally truncated inactive pepducin P2pal-14GF, and the PAR2-activating peptide SLIGRL-NH2 were synthesized, as previously described (27), at the microchemistry facility of Tufts University (Boston, MA). Amylase substrate (2-chloro-p-nitrophenyl-α-d-maltotrioside) was purchased from Sekisui Diagnostics (Framingham, MA). The trypsin substrate Boc-Gln-Ala-Arg-MCA and the chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-MCA were purchased from Peptides International (Louisville, Kentucky).
KY). Pluronic F-127 and fura 2-AM were from Molecular Probes (Eugene, OR). The myeloperoxidase enzyme-linked immunosensor kit was purchased from Hycult Biotechnology (Uden, Netherlands). Sodium taurocholate (NaT), tauroliothocholic acid 3-sulfate disodium salt (TLCS), and all other chemicals were of analytical grade and purchased from Sigma (St. Louis, MO).

In Vivo Studies

Induction of acute pancreatitis. Secretagogue-induced pancreatitis was elicited by administering 12 hourly intraperitoneal injections of caerulein (50 μg·kg⁻¹·injection⁻¹) as previously described (12), and mice were killed, by CO₂ asphyxia, 24 h after the first caerulein injection. Bile acid infusion-induced pancreatitis was elicited by retrogradely infusing 50 μl of 10 mM TLCS or 50 μl of 37 mM (2%) NaT in the pancreatic duct of anesthetized mice as described previously by our group (13), and, unless otherwise indicated, animals were killed 18–20 h after the start of bile acid infusion. Unless otherwise stated, P2pal-18S (10 mg/kg) was administered subcutaneously 0.5 h before either the first caerulein injection or the start of pancreatic duct infusion. The severity of models of pancreatitis was evaluated at the time of death by quantitating circulating amylose activity, pancreatic water content (i.e., edema), pancreatic myeloperoxidase activity (i.e., inflammation), and morphologic extent of acinar cell injury/death as described previously (28). We evaluated in vivo intrapancreatic zymogen activation by quantitating trypsin and chymotrypsin activity in the pancreas harvested 5 min after the completion of retrograde pancreatic duct infusion with TLCS (10 mM). The pancreas was removed and homogenized in 250 mM sucrose, 1 mM MgSO₄, and 5 mM MOPS, pH 6.5. One hundred microliters of the homogenate were added to 900 μl of 150 mM NaCl, 1 mM CaCl₂, and 50 mM Tris acetate, pH 7.6 containing 10 μM fluorescent substrate Boc-Gln-Ala-Arg-MCA or Suc-Ala-Ala-Pro-Phe-MCA to detect the trypsin and chymotrypsin activity, respectively. The results were normalized to the amount of DNA measured in the reaction mixture by determining propidium iodide fluorescence.

In Vitro Studies

Preparation of pancreatic acini. Dispersed mouse pancreatic acini were freshly prepared for each experiment by collagenase digestion as previously described (29). The freshly isolated mouse pancreatic acini were resuspended in HEPES-Ringer buffer containing HEPES-Ringer (10 mM, pH 7.4), NaCl (130 mM), KCl (5 mM), glucose (11 mM), sodium pyruvate (9 mM), MgCl₂ (1 mM), CaCl₂ (1 mM), and bovine serum albumin (0.1%). Viability of acinar cells, assessed by trypan blue exclusion, routinely exceeded 95% before the start of each experiment.

Measurement of intracellular Ca²⁺ concentration in single acinar cells. The freshly prepared acini were passed through a 150-μm Nitex mesh (Sefar American, Kansas City, MO) to reduce the size of acinar cell clusters being harvested and, thus, facilitate studies monitoring intracellular Ca²⁺ concentration ([Ca²⁺]i) in single acinar cells within those clusters. They were then loaded with fura 2 by incubation with 1 μM fura 2-AM for 10 min at room temperature. After extensive washing, the freshly prepared, fura 2-loaded acini were placed in a NUNC tissue culture chamber on a Nikon Eclipse TEU 2000 inverted microscope, and single cells within acinar cell clusters were arbitrarily selected for study. Interference filters on a rotational filter wheel allowed selection of alternate excitation wavelengths (340 and 380 nm) from a xenon light source. Images of fluorescence emission at 510 nm were collected and sent, via an intensified charge-couple device, to a dedicated digital image analysis system (IPLAB 3.6 with ratio plus; Scancoalitics, Fairfax, VA). Background subtraction was carried out independently at each of the excitation wavelengths. The ratios of fluorescence emission (510 nm) at the two excitation wavelengths (340/380 nm) were determined and expressed as the ratio of 340/380 fluorescence. At “zero time,” samples were allowed to equilibrate and, after ~3 min, the stimulant was added.

Quantitation of cell injury/death using propidium iodide. Freshly isolated pancreatic acini were preincubated with either P2pal-18S (17 μM) or vehicle in HEPES-Ringer buffer for 20 min at room temperature. After this preincubation, propidium iodide (1 μg/ml final concentration) was added to each of the samples and, to randomly selected samples, TLCS (200 or 500 μM final concentration), or NaT (0.2% final concentration), was also added. Control samples and samples receiving TLCS were incubated for an additional 3 h at room temperature, whereas those receiving NaT were only incubated for an additional 20 min. Hoechst dye 33342 was then added to each of the mixtures, and the cells were placed in a NUNC tissue culture chamber where they were examined using a Nikon A1R inverted confocal microscope. Photographs were taken of cells using NIS-Elements Microscope Imaging Software following their incubation with TLCS, or NaT, and the percentage of dead or dying cells was determined by counting the number of propidium iodide-positive nuclei (both free floating and those within clusters of cells) per total number of nuclei (i.e., those stained with Hoechst 33342) in each field. The results reported indicate the net percent of total nuclei that became propidium iodide-positive after exposure to the test agents, i.e., after subtracting out the 10 ± 3% of nuclei that were propidium iodide-positive in parallel control samples that had been incubated in the absence of test agents.

Measurement of digestive enzyme secretion in response to stimulation with caerulein, TLCS, or SLIGRL. Freshly prepared pancreatic acini were incubated with caerulein, TLCS, or SLIGRL in the presence of either 17 μM P2pal-18S or vehicle, and discharge of amylase in the suspending medium was quantitated as previously described (22).

Measurement of caerulein or bile acid-induced trypsinogen or chymotrypsinogen activation. Freshly isolated pancreatic acini were incubated with either caerulein (10 and 100 mM), TLCS (200 and 500 μM), or NaT (0.2%) for 30 min at 37°C in HEPES-Ringer buffer either with or without prior exposure to P2pal-18S (17 μM). The cells were homogenized with a Teflon/glass homogenizer, and trypsin and chymotrypsin activity was measured using the fluorogenic substrate Boc-Gln-Ala-Arg-MCA or Suc-Ala-Ala-Pro-Phe-MCA, respectively, as previously described (22, 28).

Analysis of Data

Data are expressed as mean ± SD values, and results reported were obtained from three or more independent experiments. The effects of P2pal-18S on calcium transients were evaluated by ANOVA, whereas the significance of other data reported in this manuscript was evaluated using Student’s t-test. Significant differences are those associated with a P value of <0.05.

RESULTS

P2pal-18S Inhibits Generation of Pathological Calcium Transients Induced by the PAR2-Activating Peptide SLIGRL-NH₂

The PAR2-activating peptide SLIGRL-NH₂ dose dependently triggers [Ca²⁺]i transients in pancreatic acini (29), and these calcium response patterns can be divided into three types (Fig. 1A): with low SLIGRL-NH₂ concentrations (<10 μM), “no response” is noted in most cells; at higher SLIGRL-NH₂ concentrations (10–30 μM), an “oscillatory response” occurs in which [Ca²⁺]i rises and falls to the baseline level. With increasing concentrations of SLIGRL-NH₂, a transition is noted, and progressively more cells demonstrate a peak-plateau response in which there is a large single spike in [Ca²⁺]i, followed by a fall toward, but not quite reaching, the baseline level, and fewer cells show an oscillatory response. The transition between an oscillatory response and the peak-plateau response occurs in the
presence of 100–300 μM SLIGRL-NH₂, whereas, with 1 mM SLIGRL-NH₂, one observes only a peak-plateau response (29). It is generally believed that an oscillatory response is a physiological response that reflects calcium release and reuptake by acidic stores located in the apical region of the acinar cell and that an oscillatory response is coupled to secretagogue-induced digestive enzyme secretion in pancreatic acinar cells. In contrast, the “peak-plateau response” is generally believed to be a pathological response that reflects the global release of calcium from intracellular stores as well as accelerated store-operated calcium entry. A peak-plateau calcium response is associated with intracellular digestivezymogen activation and cell injury when acinar cells are stimulated with the secretagogue cholecystokinin or caerulein (24).

As shown in Fig. 1, B and C, P2pal-18S alters the calcium response patterns associated with both low and high concentrations of SLIGRL-NH₂. In the absence of P2pal-18S, 30 μM SLIGRL-NH₂ elicits mostly a purely oscillatory response (~90% of cells), whereas the remaining cells (~10%) fail to respond altogether. In the presence of P2pal-18S, however, 30 μM SLIGRL-NH₂ fails to elicit any calcium response in most (95%) of the cells, and the remaining 5% of cells show an oscillatory response. On the other hand, 100 μM SLIGRL-NH₂, which, in the absence of P2pal-18S, elicits a peak-plateau response in 32% of the cells and an oscillatory response in 68% of the cells, only elicits either an oscillatory response (in 90% of the cells) or no response (in 10% of the cells) when combined with P2pal-18S. As a negative control, we used the NH₂-terminal truncated pepducin P2pal-14GF that has been shown to have no effect on PAR2 responses (27). Exposure to P2pal-14GF does not alter the intracellular calcium transients that are generated when the acinar cells are stimulated with 30 μM SLIGRL-NH₂ (Fig. 1B).

Fig. 2. P2pal-18S inhibits SLIGRL, but not caerulein, or tauroliothocholic acid 3-sulfate disodium salt (TLCS)-induced secretion of amylase from pancreatic acini. Freshly isolated pancreatic acini were preincubated with or without 17 μM P2pal-18S for 10 min. Caerulein (0.1 nM), TLCS (200 μM), or SLIGRL-NH₂ (100 or 1,000 μM) was then added, and the incubation was continued for an additional 30 min. The amylase in the supernatant was measured and is expressed as a percent of total. Results are averages of 4 experiments, each performed in duplicate. *P < 0.05.
P2pal-18S Inhibits SLIGRL-Stimulated Amylase Secretion

SLIGRL induces secretion of amylase by pancreatic acinar cells, and, as expected, amylase secretion in response to PAR2 stimulation is significantly reduced when the cells were pre-treated with 17 µM P2pal-18S, although it had no effect on caerulein- or TLCS-induced amylase secretion (Fig. 2).

Effects of P2pal-18S on Bile Acid-Induced Acinar Cell Calcium Transients

We and others (6, 11, 23) have shown that pancreatic acinar cells, stimulated with the bile acids NaT or TLCS, exhibit either no response, an oscillatory response, a peak-plateau response superimposed on oscillations, or a pure peak-plateau response and that the frequency of these response patterns is closely correlated with the concentration of bile acid used. We combined the peak-plateau and “oscillatory plus peak-plateau” response into one group since, in the case of both of these response patterns, the result is a sustained rise in \([\text{Ca}^{2+}]_i\) intracellular trypsinogen activation, and cell injury (13, 21). In previously published studies, we have also shown that global deletion of PAR2 or inhibition of the PAR2 agonist trypsin shifts the calcium response from a peak-plateau to an oscillatory response in wild-type acini exposed to NaT (14). As shown in Fig. 3, A and B, a similar effect is noted when acini are preincubated with the PAR2 inhibitory pepducin P2pal-18S before exposure to either NaT or TLCS. Thus, preincubation of acini with P2pal-18S reduces the frequency of peak-plateau responses from 67 to 15% in acini subsequently exposed to 0.3% NaT and from 86 to 8% in acini subsequently exposed to 250 µM TLCS. In both cases, a compensatory increase in the frequency of either an oscillatory response or no response accompanies this P2pal-18S-induced decrease in the frequency of a peak-plateau response. As shown in Fig. 3C, suppression of the calcium response pattern by P2pal-18S is not observed with acini harvested from PAR2−/− mice, demonstrating that the inhibitory effects of P2pal-18S require the presence of its cognate receptor. In control studies (data not shown), we also tested the effects of P2pal-18S on caerulein-induced \([\text{Ca}^{2+}]_i\) transients in acinar cells. A: P2pal-18S reduces peak-plateau responses triggered by exposure to 200 µM TLCS. B: P2pal-18S reduces peak-plateau responses triggered by exposure to 0.3% sodium taurocholate (NaT). C: in acini harvested from PAR2−/− mice, P2pal-18S does not alter peak-plateau responses triggered by exposure to TLCS. Conditions are as described in the legend for Fig. 1B, but the indicated bile acid was used in place of [SLIGRL-NH₂]. \([\text{Ca}^{2+}]_i\) levels were monitored as described in the text. Data shown represent pooled results obtained from 3 or more independent experiments.
transients and found that the pathological responses elicited by a supramaximally stimulating concentration of caerulein (1 nM) are not prevented by the pepducin.

**P2pal-18S Increases the Severity of Secretagogue (i.e., Caerulein)-Induced Pancreatitis**

As shown in Fig. 4, repeated administration of a supramaximally stimulating dose of the secretagogue caerulein to mice elicits a severe form of acute pancreatitis that is characterized by hyperamylasemia, pancreatic edema, pancreatic inflammation, and acinar cell injury/death. Administration of P2pal-18S to the animals 0.5 h before the start of caerulein administration does not significantly alter the extent of either hyperamylasemia or pancreatic inflammation in this model of pancreatitis, but it increases caerulein-induced pancreatic edema and markedly increases the extent of acinar cell injury/death in the secretagogue-induced model.

**P2pal-18S Reduces the Severity of Bile Acid Infusion-Induced Pancreatitis**

Intraductal infusion of either NaT (37 mM) (Fig. 5) or TLCS (10 mM) (Fig. 6) also triggers severe acute pancreatitis in mice. Administration of P2pal-18S 0.5 h before bile acid infusion markedly reduces the extent of hyperamylasemia and acinar cell injury/death in both of these models. It also significantly reduces the extent of pancreatic inflammation in the TLCS model, and, in both models, pancreatic edema is moderately but not significantly reduced by P2pal-18S administration. Similarly, pancreatic inflammation is moderately, but not significantly, reduced in the NaT model following administration of P2pal-18S.

The protective effect of P2pal-18S on TLCS infusion-induced acinar cell injury/death, noted when the pepducin is administered 0.5 h before the start of bile acid infusion, is still significant when P2pal-18S administration is delayed until 2 h after bile acid infusion.

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**Fig. 4. P2pal-18S worsens caerulein-induced pancreatitis.** Mice were treated with either vehicle or P2pal-18S (10 mg/kg, sc). Later (30 min) they received the first of 12 hourly injections of caerulein. They were killed 12 h after the last caerulein injection. Photomicrographs are representative of hematoxylin and eosin (H&E)-stained samples obtained from animals in each group. Bar in photomicrograph equals 200 μm. Serum amylase activity, pancreatic edema (i.e., water content), pancreatic inflammation (i.e., myeloperoxidase activity), and acinar cell necrosis were quantitated as described in the text. Results shown represent mean ± SD values obtained from 5 mice in each group. *P < 0.05 when mice given P2pal-18S were compared with mice receiving vehicle.
infusion but loses significant protective effects when administered 5 h after the start of bile acid infusion (Fig. 7).

**P2pal-18S Prevents In Vitro TLCS-Induced Acinar Cell Injury/Death**

As shown in Fig. 8, incubation of pancreatic acinar cells with 200 or 500 µM TLCS for 3 h or 0.2% NaT for 20 min induces cell death as measured by means of propidium iodide staining. Pancreatic acinar cells pretreated with 17 µM P2pal-18S for 30 min, however, were largely protected from TLCS- or NaT-induced pancreatic cell death (Fig. 8).

**P2pal-18S Does Not Prevent Caerulein- or TLCS-Induced Digestive Zymogen Activation**

Both caerulein and TLCS dose-dependently increase trypsin activity under in vitro conditions in freshly isolated pancreatic acini (Fig. 9), and a similar rise in chymotrypsin activity is also observed (data not shown). Prior exposure to P2pal-18S does not significantly alter these upstream responses to either TLCS or caerulein. Intrapancreatic activation of trypsinogen and chymotrypsinogen is also induced, in vivo, by retrograde intraductal infusion of TLCS and, as shown in Fig. 10, these upstream responses to TLCS infusion are not altered by pre-treatment of the animals with the pepducin P2pal-18S.

**DISCUSSION**

PAR2 is a Class A, G protein-coupled, 7-transmembrane cell surface receptor that is physio/pathologically activated when an appropriate serine protease (trypsin or mast cell tryptase) cleaves the NH2-terminal extracellular domain of the receptor, exposing a new NH2-terminus containing the activation sequence SLIGRL. That activation sequence is then free to bind, intramolecularly, to the activation site on the outer surface of the receptor, thus triggering transmembrane signaling to intracellular G proteins. In addition to being proteolytically activated by cleavage of the tethered effector arm, PAR2 can also be nonproteolytically activated by direct exposure to the peptide SLIGRL. For the most part, PAR2 activation has been noted to trigger a proinflammatory response, although, under...
certain circumstances and in certain cell types, it has also been observed to play an anti-inflammatory role (3).

It has been suggested that PAR2 might play an important role in regulating the severity of pancreatitis since 1) activated trypsin can be detected within the pancreas during clinical pancreatitis and during the early stages of both the secretagogue-induced and the bile acid infusion-induced models of pancreatitis (14, 16, 28) and 2) PAR2 is widely expressed by many cell types, including pancreatic acinar and ductal cells (2, 17). Indeed, Namkung et al. concluded that PAR2 might play a protective role in pancreatitis when they found that PAR2 activation reduces the severity of rat secretagogue-induced pancreatitis (16), and their conclusion was supported by our own subsequent studies that showed that global genetic deletion of PAR2 is associated with worsening of secretagogue-induced pancreatitis (28). Importantly, however, our studies also yielded the unexpected finding that global genetic deletion of PAR2 reduces the severity of bile acid infusion-induced pancreatitis, leading us to conclude that PAR2 exerts a model-specific effect on experimental pancreatitis, i.e., its presence or activation reduces the severity of secretagogue-induced pancreatitis but increases the severity of bile acid infusion-induced pancreatitis (14).

Although the mechanisms responsible for these apparently paradoxical responses in the two experimental pancreatitis models are not known at this time, the divergent responses might, themselves, suggest that the severity of these two models is differentially regulated. However, future studies, beyond the scope of this present communication but focused on this issue, will be needed to further clarify this issue.

In the presently reported studies, we have reexamined the role of PAR2 in regulating pancreatitis severity using a different, and more manipulable, therapeutic approach to inhibit PAR2 activity. Rather than using mice with global genetic deletion of PAR2, we have used a newly developed and novel pharmacological inhibitor of PAR2, the pepducin P2pal-18S. Prior attempts at developing effective and specific low-molecular-weight PAR2 antagonists have been unsuccessful because the various inhibitors that have been developed have been unable to prevent the protease-activated tethered ligand from interacting with the body of the receptor. Pepducins, however, are a completely new class of low-molecular-weight peptidic molecules designed to target G protein-coupled receptors on the inside surface of the receptor. They are lipidated to make them cell permeable, and their receptor specificity is based on their peptide component, which is designed to mimic all or part of the protease-activated tethered ligand.
of one of the intracellular loops of the target receptor (19). The mechanism(s) by which pepducins exert either agonist or antagonist effects is not entirely clear, but the current working hypothesis is that they act allosterically, i.e., they dimerize with the intracellular loops of their target receptor, and this dimerization event is believed to stabilize the receptor in either an active (agonist) or inactive (antagonist) conformation. P2pal-18S is an N-palmitated 18 amino acid peptide consisting of 270-RSSMDENSEKRRSAIK-287 that is designed to mimic the third intracellular loop (i3) of PAR2, which, in the pepducin, has arginine-284 replaced by a serine. It has been shown to effectively antagonize PAR2-dependent activation and chemotaxis of neutrophils, SLIGRL-induced paw edema, and mast cell tryptase-induced inflammation while having no effect on PAR1, PAR3, or PAR4 activity in various systems (27). The currently reported studies are the first to explore the effect of P2pal-18S or, indeed, any pepducin antagonist on the severity of acute pancreatitis.

The earliest pancreatic acinar cell response to PAR2 activation by either trypsin or SLIGRL-NH2 is a dose-dependent rise in \([Ca^{2+}]_i\). At low concentrations of SLIGRL-NH2, the \([Ca^{2+}]_i\) rise is purely oscillatory, but, with increasing SLIGRL-NH2 concentrations, the observed pattern shifts to one consisting first of oscillations superimposed on a peak-plateau change and then to one consisting of only a peak-plateau calcium rise (29). As shown in Fig. 1, this dose-dependent acinar cell calcium response is prevented and shifted toward higher concentrations of SLIGRL-NH2 by P2pal-18S. These results and our finding that P2pal-18S inhibits SLIGRL-NH2-induced in vitro amylase secretion from acinar cells (Fig. 2) indicate that, in mouse pancreatic acinar cells, P2pal-18S is an effective PAR2 antagonist and requires the presence of its cognate receptor (Fig. 3C) to exert its effects. Moreover, P2pal-18S has no effect on TLCS- or caerulein-induced amylase secretion. These findings are compatible with the notion that the pepducin acts as an allosteric modulator of PAR2. We cannot completely exclude the possibility that some of our findings may reflect changes that are independent of P2pal-18S direct effects on PAR2 and that they directly result from effects of P2pal-18S on CCK or bile acid pathways. However, we have no evidence to date that supports that conclusion.

We have previously shown that global genetic deletion of PAR2 worsens secretagogue-induced experimental pancreatitis but protects against bile acid infusion-induced experimental pancreatitis, and, in this current report, we show that the effects of the PAR2 antagonist replicate the effects of PAR2 deletion on the severity of these two types of experimental pancreatitis. As shown in Fig. 4, acinar cell injury/death and the morphological changes of mouse pancreatitis are either unchanged or increased by administration of P2pal-18S to animals given caerulein, whereas, in contrast, mice with bile acid infusion-induced pancreatitis elicited with either NaT or TLCS have reduced pancreatitis severity if they are given P2pal-18S (Figs. 5 and 6). The magnitude of hyperamylasemia and acinar cell injury/death as well as the morphological severity of pancreatitis are reduced in NaT-infused animals by P2pal-18S administration and, in TLCS-induced pancreatitis, these effects, along with a reduction in pancreatic inflammation, are also seen.

Our finding that P2pal-18S reduces pancreatic inflammation (i.e., pancreatic myeloperoxidase activity) in the two bile acid models is not unexpected, since the pepducin is known to directly inhibit PAR2-mediated neutrophil chemotaxis toward gradients of trypsin (27). P2pal-18S also directly reduces PAR2-dependent pancreatic injury that occurs in both of these bile acid models. On the other hand, our finding that P2pal-18S does not significantly reduce inflammation in the secretagogue-induced model may suggest that inflammation, in that model, is regulated by mechanisms that do not involve PAR2.

Our previous (14) and currently reported observations (Figs. 4–6) clearly indicate that PAR2 plays a model-specific role in regulating the severity of experimental pancreatitis, but these findings beg the question of which model most accurately
reflects the role of PAR2 in clinical pancreatitis. It is widely recognized that supramaximal secretagogue stimulation is not the clinical trigger for most cases of clinical acute pancreatitis in humans, and this recognition has led to concerns that the secretagogue-induced rodent model may not be clinically relevant. This observation spurred the development of the bile acid infusion-induced model where reflux of fluid with or without bile in the pancreatic ductal system mimics the clinical trigger for gallstone and, possibly, ERCP-induced pancreatitis, a more clinically relevant model of biliary pancreatitis (31) than the secretagogue model. If correct, we would predict that pharmacological inhibition of PAR2 activation with an antagonist pepducin such as P2pal-18S might favorably affect the course of patients with either gallstone or ERCP-induced pancreatitis.

We recognize, and even wish to emphasize, that our claim that the bile acid-infusion model is a clinically relevant model, whereas the other models may not be so is, at this point, conjectural (15) and that the only way to resolve this controversial issue may involve translational studies that directly test whether P2pal-18S or other PAR2-directed agents beneficially affect the course of clinical biliary and/or ERCP-induced pancreatitis.

The in vitro correlates of bile acid infusion-induced experimental pancreatitis in mice include bile acid-induced generation of pathological calcium transients. Notably, inhibition of PAR2 signaling with P2pal-18S reduces bile acid-induced pathological calcium transients, and this phenomenon is lost when PAR2 is genetically deleted (Fig. 3). In vitro studies reported by others have shown that bile acid-induced calcium transients in acinar cells occur before zymogen activation, and prevention of pathological calcium transients with a calcium chelator prevents intracellular zymogen activation (5, 7). These findings have generally been interpreted to mean that pathological calcium transients lead to, rather than result from, intracellular zymogen activation, but the current studies challenge this conclusion by showing that the PAR2 (i.e., trypsin receptor) antagonist can inhibit bile acid-induced generation of pathological calcium transients. An explanation for our surprising observation is not immediately obvious, but it is tempting to speculate that it 1) may indicate that there exists “cross talk” or dimerization between PAR2 and the bile acid receptor Gpbar1 and/or 2) PAR2 inhibition interferes with Gpbar1 signaling in acinar cells. Such transactivation has been shown to exist between PAR2 and epidermal growth factor (4), vascular endothelial growth factor (25), or PAR1 (8, 26). Future studies will be needed to test these hypotheses and to define the mechanisms by which P2pal-18S inhibits bile acid-induced acinar cell calcium changes.

The other in vitro correlates of bile acid-induced pancreatitis in mice include bile acid-induced cell injury/death and intra-

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**Fig. 9.** P2pal-18S does not reduce in vitro intracellular trypsinogen activation triggered by either caerulein or TLCS. Freshly isolated pancreatic acini were preincubated with or without 17µM P2pal-18S for 30 min. Caerulein (10 or 100 nM), TLCS (200 or 500 µM), or buffer alone was then added, and the incubation was continued for an additional 30 min. The acini were then homogenized, and trypsin activity in the homogenate was quantitated as described in the text. Results shown are the averages of 3 experiments, each performed in duplicate. Results obtained for samples preincubated with or without P2pal-18S were compared, and no significant difference was noted.

**Fig. 10.** P2pal-18S does not reduce in vivo bile salt-induced protease activation. Mice were pretreated with the vehicle or P2pal-18S (1 µg/kg, sc). Later (30 min), pancreatitis was induced by retrograde ductal infusion of TLCS as described in the text. After the end of the ductal infusion (5 min), the pancreas was removed, and trypsin and chymotrypsin activity was determined by means of a fluorogenic substrate. Results shown are the averages of 5 animals in each group. No difference was seen in the trypsin or chymotrypsin activity between the pretreated and untreated group.
acinar cell activation of digestive zymogens such as trypsinogen. As shown in Figs. 8 and 9, the PAR2 antagonist P2pal-18S protects acinar cells against TLCS-induced acinar cell death, but it does not alter TLCS-induced intra-acinar cell activation of trypsinogen in vitro. In addition, as shown in Fig. 10, P2pal-18S does not alter in vivo intrapancreatic activation of either trypsinogen or chymotrypsinogen in response to intraductal infusion of TLCS. These findings suggest that cell injury/death but not intra-acinar cell zymogen activation during bile acid infusion-induced pancreatitis is the result of events that may be at least in part regulated by PAR2 activation.

In summary, we report the first studies that have examined the effect of a pepducin PAR2 inhibitor on pancreatic acinar cells and on the severity of experimental pancreatitis. We have used the novel pepducin PAR2 antagonist P2pal-18S to inhibit PAR2 in mice experiencing acute pancreatitis elicited by either supramaximal secretagogue stimulation or retrograde pancreatic duct infusion with the bile acids NaTa and TLCS. In accord with our prior studies employing mice with global genetic deletion of PAR2, our currently reported studies using a pharmacological PAR2 inhibitor indicate that PAR2 plays a model-specific effect on experimental pancreatitis severity, worsening the severity of secretagogue-induced pancreatitis but reducing the severity of bile acid infusion-induced pancreatitis. Our studies suggest that PAR2 inhibition may down-regulate the severity of pancreatitis by reducing bile acid-induced generation of pathological calcium transients in acinar cells and reducing acinar cell injury during biliary pancreatitis.

Clearly, a pharmacological rather than a genetic tool for reducing pancreatitis severity will be needed for the clinical management of patients at risk for severe acute pancreatitis and, in this regard, our currently reported studies may have provided an important next step by showing that an effective PAR2 inhibitor can reduce the severity of a potentially clinically relevant model of acute pancreatitis. In this regard, our studies raise the possibility that prophylactic administration of a pharmacological PAR2 inhibitor might offer protection against ERCP-induced pancreatitis, and, when given very shortly after the onset of gallstone pancreatitis, administration of an effective PAR2 inhibitor might also reduce the severity of biliary pancreatitis. Translational studies using patients at risk of developing ERC-distributed pancreatitis or severe gallstone pancreatitis will be needed to test these hypotheses.

GRANTS

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


