Protease activation during in vivo pancreatitis is dependent on calcineurin activation

Ahsan U. Shah,1 Amna Sarwar,1 Abraham I. Orabi,1 Samir Gautam,1 Wayne M. Grant,1 Alexander J. Park,1 Adnan U. Shah,1 Jun Liu,1 Pramod K. Mistry,1 Dhanpat Jain,2 and Sohail Z. Husain1

Departments of 1Pediatrics and 2Pathology, Yale University School of Medicine, New Haven, Connecticut

Submitted 14 May 2009; accepted in final form 20 August 2009

Shah AU, Sarwar A, Orabi AI, Gautam S, Grant WM, Park AJ, Shah AU, Liu J, Mistry PK, Jain D, Husain SZ. Protease activation during in vivo pancreatitis is dependent on calcineurin activation. Am J Physiol Gastrointest Liver Physiol 297: G967–G973, 2009. First published August 27, 2009; doi:10.1152/ajpgi.00181.2009.—The premature activation of digestive proenzymes, specifically proteases, within the pancreatic acinar cell is an early and critical event during acute pancreatitis. Our previous studies demonstrate that this activation requires a distinct pathological rise in cytosolic Ca2+. Furthermore, we have shown that a target of aberrant Ca2+ in acinar cells is the Ca2+/calmodulin-dependent phosphatase calcineurin (PP2B). In this study, we hypothesized that PP2B mediates in vivo protease activation and pancreatitis severity. To test this, pancreatitis was induced in mice over 8 h by administering hourly intraperitoneal injections of the cholecystokinin analog caerulein (50 ove8hb yadministering hourly intraperitoneal injections of and pancreatitis severity. To test this, pancreatitis was induced in mice to study, we hypothesized that PP2B mediates in vivo protease activation and pancreatitis severity. To test this, pancreatitis was induced in mice over 8 h by administering hourly intraperitoneal injections of the cholecystokinin analog caerulein (50 μg/kg). Treatment with the PP2B inhibitor FK506 at 1 and 8 h after pancreatitis induction reduced trypsin activities by greater than 50% (P < 0.005). Serum amylase and IL-6 was reduced by 86 and 84% relative to baseline (P < 0.0005) at 8 h, respectively. Histological severity of pancreatitis, graded on the basis of pancreatic edema, acinar cell vacuolization, inflammation, and apoptosis, was reduced early in the course of pancreatitis. Mucolipidosis activity from both pancreas and lung was reduced by 93 and 83% relative to baseline, respectively (P < 0.05). These data suggest that PP2B is an important target of the aberrant acinar cell Ca2+ rise associated with pathological protease activation and pancreatitis.

acinar cell; tacrolimus; zymogen activation; calcium signaling; protein phosphatase 2B

An aberrant rise in cytosolic Ca2+ is required for this pathological protease activation (42, 45). Ca2+ concentrations are governed by a number of membrane pumps, buffers, second messengers, and Ca2+ channels (3). Following secretagogue-induced stimulation of acinar cells, the initial rise in Ca2+ is predominantly controlled by release from intracellular Ca2+ pools. We have recently shown that Ca2+ released by the intracellular Ca2+ channel, the ryanodine receptor (RyR), mediates premature protease activation (24). The RyR was selectively distributed in the basolateral region of the acinar cell, where protease activation is first observed. Relatively high basolateral Ca2+ elevations were dependent on RyR opening. Finally, RyR inhibition reduced premature protease activation in isolated acinar cells and in vivo. Thus, although aberrant Ca2+ release is central to the pathogenesis of protease activation and acute pancreatitis, targets of the pathological Ca2+ signal have not been identified.

An important target of Ca2+ in eukaryotic cells is the Ca2+/calmodulin-dependent serine/threonine phosphatase calcineurin (PP2B) (43). It is a heterodimeric protein with a catalytic subunit A (CN-A) and a regulatory Ca2+-binding subunit B (CN-B). The primary sequence of both subunits is highly conserved. PP2B is widely distributed in mammalian tissues and cells, including the pancreas and the pancreatic acinar cell. Although PP2B is regulated by several factors, including oxidative stress (53) and unsaturated long-chain fatty acids (26), its major activators are Ca2+ and calmodulin.

We hypothesized that PP2B is a key target of the aberrant acinar cell Ca2+ signals generated after a pancreatitis insult. We have previously shown in isolated acinar cells that PP2B inhibition reduces intra-acinar protease activation (23). In the present study, we examined whether PP2B mediates in vivo protease activation and subsequent long-term effects of pancreatitis. Acute treatment of mice with the specific PP2B inhibitor FK506 reduced intrapancreatic protease activation after 1 h of caerulein hyperstimulation and mitigated the inflammatory response over an 8-h time course. These data suggest that Ca2+-dependent events target PP2B activation early in pancreatitis and may modulate disease severity.

METHODS

Reagents and animals. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Male Swiss-Webster mice (25–30 g) were purchased from Charles River Laboratories (Wilmington, MA). Mice were fed standard laboratory chow, given free access to water, and randomly assigned to control or experimental groups. All animal treatments and euthanasia protocols were approved by the Animal Care and Use Committee.

Induction of pancreatitis. After a 12-h fast, pancreatitis was induced in mice by administering hourly intraperitoneal injections of

ACUTE PANCREATITIS IS A life-threatening inflammatory disease that affects 1 to 2 in 1,000 patients each year (51). One quarter with severe disease die, and the overall mortality rate approaches 5–10%. Activation of secreted pancreatic proenzymes, or zymogens, normally occurs in the intestinal lumen. In contrast, the premature activation of zymogens, particularly proteases, within the pancreatic acinar cell plays an early and critical role in the pathogenesis of acute pancreatitis. This conclusion is based on four fundamental observations. First, severe pancreatitis presents with morphological changes that strongly resemble those that are typical of digestive necrosis (49). Second, prior to evidence of acinar cell injury, pancreatic and serum levels of activated proteases increase early in the course of disease (11). Third, trypsinogen activation and pancreatitis is blocked following pretreatment with serine protease inhibitors (40, 45). Fourth, mutations in the cationic trypsinogen gene that may lead to enhanced activation are precursors to hereditary pancreatitis (56).
FK506 reduces pancreatic PP2B activity. FK506 (1 mg/kg) was injected intraperitoneally in mice three times over an 8-h period to inhibit PP2B activity (Fig. 1) (22). We hypothesized that pancreatic PP2B activation modulates pancreatitis by inducing pathological intra-acinar protease activation, an event that occurs within minutes of a pancreatitis insult. For this reason, FK506 was administered prophylactically 1 h before caerulein hyperstimulation as well as therapeutically during the course of pancreatitis. The aim of the study was to inhibit pancreatic PP2B. FK506 administration reduced pancreatic PP2B phosphatase activity by 61% (Fig. 2). As a positive control of the FK506 dosing regimen in another tissue, PP2B activity was reduced by 60% in kidney.

FK506 reduces intrapancreatic protease activation. In C57BL/6 mice, intrapancreatic activation of trypsin with caerulein hyperstimulation peaks at 1 and 8 h (19). The trend appeared similar in our study with Swiss-Webster mice (Fig. 3). Intrapancreatic factors primarily mediate early protease activation, whereas at later time points immune cell infiltrates also play a role (16). Notably, FK506 treatment reduced trypsin activity by 82% relative to basal levels at 1 h vs. 52% at 8 h postcaerulein hyperstimulation. There was no change in trypsin activity with FK506 administration alone at 1 and 8 h. At the early time point, pancreatic PP2B might be more influential than nonpancreatic sources. The results are consistent with our previous study, which demonstrated that FK506 pretreatment of isolated acinar cells reduced intra-acinar protease activation 1 h after caerulein hyperstimulation (23). To confirm that the in vivo results were not strain specific, a reduction in protease activation was also shown in limited experiments with C57BL/6 mice (data not shown).

FK506 reduces inflammation in pancreatitis. A time-dependent linear rise in serum amylase was observed with caerulein hyperstimulation over 8 h (Fig. 4). FK506 treatment reduced serum amylase levels by 86% at 8 h, compared with basal levels. Serum IL-6 followed a similar trend; FK506 reduced

### RESULTS

**FK506 reduces pancreatic PP2B activity.** FK506 (1 mg/kg) was injected intraperitoneally in mice three times over an 8-h period to inhibit PP2B activity (Fig. 1) (22). We hypothesized that pancreatic PP2B activation modulates pancreatitis by inducing pathological intra-acinar protease activation, an event that occurs within minutes of a pancreatitis insult. For this reason, FK506 was administered prophylactically 1 h before caerulein hyperstimulation as well as therapeutically during the course of pancreatitis. The aim of the study was to inhibit pancreatic PP2B. FK506 administration reduced pancreatic PP2B phosphatase activity by 61% (Fig. 2). As a positive control of the FK506 dosing regimen in another tissue, PP2B activity was reduced by 60% in kidney.

**FK506 reduces intrapancreatic protease activation.** In C57BL/6 mice, intrapancreatic activation of trypsin with caerulein hyperstimulation peaks at 1 and 8 h (19). The trend appeared similar in our study with Swiss-Webster mice (Fig. 3). Intrapancreatic factors primarily mediate early protease activation, whereas at later time points immune cell infiltrates also play a role (16). Notably, FK506 treatment reduced trypsin activity by 82% relative to basal levels at 1 h vs. 52% at 8 h postcaerulein hyperstimulation. There was no change in trypsin activity with FK506 administration alone at 1 and 8 h. At the early time point, pancreatic PP2B might be more influential than nonpancreatic sources. The results are consistent with our previous study, which demonstrated that FK506 pretreatment of isolated acinar cells reduced intra-acinar protease activation 1 h after caerulein hyperstimulation (23). To confirm that the in vivo results were not strain specific, a reduction in protease activation was also shown in limited experiments with C57BL/6 mice (data not shown).

**FK506 reduces inflammation in pancreatitis.** A time-dependent linear rise in serum amylase was observed with caerulein hyperstimulation over 8 h (Fig. 4). FK506 treatment reduced serum amylase levels by 86% at 8 h, compared with basal levels. Serum IL-6 followed a similar trend; FK506 reduced

### Statistical analysis

Data are expressed as means ± SD unless otherwise stated. Statistical significance was determined by a Student’s t-test.
levels at 1, 4, and 8 h by 47, 63, and 84%, respectively. It has been shown in previous studies that caerulein hyperstimulation caused a mild to moderate interstitial pancreatitis with features of pancreatic edema, basolateral vacuole formation in acinar cells, inflammatory infiltration, and apoptosis (28). Consistent with these findings, we report that FK506 treatment reduced the cumulative score at 1, 4, and 8 h by 56, 67, and 58%, respectively (Fig. 5). Saline-only and FK506-alone-treated animals had no increase in any of these parameters. Edema and vacuolization appeared within the first 1 h, consistent with previous reports (21, 40, 42, 48, 55). FK506 treatment reduced those parameters at the early time point (Fig. 6). At later time points, all parameters were significantly reduced, including inflammation and apoptosis. Mice receiving FK506 alone at 1, 4, and 8 h were indistinguishable in serum levels or histological analysis from control saline-only treated animals (Figs. 4 and 5A).

Tissue MPO activity measures neutrophil sequestration and is a reliable marker of the acute inflammatory response during pancreatitis. In pancreas, caerulein treatment by 8 h caused an increase in MPO of 15-fold above the saline-only treated group, FK506 caused a 93% reduction relative to basal levels (Table 1). In lung, within 4 h, a 4.4-fold increase was observed with caerulein treatment, but FK506 reduced those parameters at the early time point (Fig. 6). At later time points, all parameters were significantly reduced, including inflammation and apoptosis. Mice receiving FK506 alone at 1, 4, and 8 h were indistinguishable in serum levels or histological analysis from control saline-only treated animals (Figs. 4 and 5A).

Fig. 5. FK506 treatment reduces serum amylase and IL-6 elevations observed during pancreatitis. Caerulein hyperstimulation caused a time-dependent increase in serum amylase and IL-6. FK506 administration reduced levels as early as 4 h (n = 5 animals per group). *P < 0.005 compared with saline-treated and caerulein-alone groups, respectively. SE shown for IL-6.

Serum MPO activity measures neutrophil sequestration and is a reliable marker of the acute inflammatory response during pancreatitis. In pancreas, caerulein treatment by 8 h caused an increase in MPO of 15-fold above the saline-only treated group, FK506 caused a 93% reduction relative to basal levels (Table 1). In lung, within 4 h, a 4.4-fold increase was observed with caerulein treatment, but FK506 reduced those parameters at the early time point (Fig. 6). At later time points, all parameters were significantly reduced, including inflammation and apoptosis. Mice receiving FK506 alone at 1, 4, and 8 h were indistinguishable in serum levels or histological analysis from control saline-only treated animals (Figs. 4 and 5A).

Fig. 3. FK506 treatment reduces pancreatic trypsin activity in vivo. Trypsin activity was assayed from pancreatic homogenates 1 (A) and 8 h (B) after the first caerulein dose (n = 5 animals per group). *, #P < 0.005 compared with saline-treated and caerulein-alone groups, respectively. Values are expressed as means ± SE and are normalized to a maximum value.

FK506 does not increase HSP70 expression. Although FK506 is a highly specific PP2B inhibitor, isolated reports in kidney suggest that it can increase the expression of HSP70 independent of PP2B (4). HSP70 has recently been shown to be protective against the development of pancreatitis (33, 44). However, Western blotting for HSP70 in pancreas from mice treated with FK506 demonstrated no effect at 1 or 8 h postadministration (Fig. 7).

DISCUSSION

In this study, we have shown that acute treatment in vivo with the PP2B inhibitor FK506 reduced early protease activation as well as later indexes of inflammation associated with pancreatitis. Physiologically, PP2B is necessary for CCK-induced acinar cell growth (17, 46, 50) and may have a modest effect on enzyme secretion (8, 13, 23, 54). Pathological roles have also been described in other tissues such as in heart, where activation of PP2B produces cardiac hypertrophy (20). The findings of our studies are an extension of our previous work in isolated acinar cells, which demonstrated that inhibition of acinar cell PP2B reduced intra-acinar protease activa-
The present study indirectly supports the postulate that a Ca\textsuperscript{2+}-dependent acinar cell cascade is primarily responsible for the pathological activation of proteases. Previous evidence for this dependence comes from studies that examine chelation of cytosolic Ca\textsuperscript{2+} by BAPTA. When BAPTA is administered either to CCK-stimulated acinar cells in isolation (42, 45) or during in vivo bile duct ligation (32), trypsinogen activation is blocked. Furthermore, we and others have shown that an aberrant pattern of acinar cell Ca\textsuperscript{2+} signaling is necessary for protease activation. This is either due to a shift of intracellular Ca\textsuperscript{2+} release from an apical to a basolateral region of the cell (24) and/or due to sustained cytosolic Ca\textsuperscript{2+} elevations throughout the cell (42). Although the cellular targets of this pathological Ca\textsuperscript{2+} signal remain unclear, here we report a role, in vivo, for the Ca\textsuperscript{2+}/calmodulin-dependent phosphatase PP2B acting as a Ca\textsuperscript{2+} sensor in the cascade, leading to protease activation. The regulatory subunit CN-B contains four high-affinity Ca\textsuperscript{2+} binding EF-hand domains, which in conjunction with calmodulin mediate the phosphatase activity of the catalytic subunit CN-A (1). Among several known PP2B phosphatase substrates, Ca\textsuperscript{2+}-regulated heat-stable protein of 24 kDa (CRHSP-24) may contribute to premature protease activation (5, 14). CRHSP-24 is confined to the basolateral region of the acinar cell, and this localization overlaps with intracellular protease activation.

We used FK506 in our study because it is a highly specific PP2B inhibitor. It forms a complex with FK506-binding protein (FKBP) that hinders protein substrates from approaching the PP2B catalytic site (9, 12). We decided to use FK506 over another PP2B inhibitor cyclosporine (CsA) because, unlike...
FK506, CsA is known to affect mitochondrial pathways by inhibiting the mitochondrial permeability transition pore in the pancreatic acinar cell (2, 38). Although FK506 is a potent PP2B inhibitor, there may be less common PP2B-independent effects contributing to the improvement in pancreatitis. For example, in the thymus, FK506 can associate with HSP90 (27, 41) and, in the kidney, it can induce expression of HSP70 (59), proteins which have recently been shown to limit acinar cell injury during pancreatitis (2, 34). It was therefore possible that the ameliorative effects of FK506 were due to induction of HSP70. We, however, saw no elevation of HSP70 expression with FK506 treatment, consistent with our hypothesis that FK506 is acting primarily through PP2B inhibition.

The results of the study further substantiate a role for FK506 in long-term effects of pancreatitis such as inflammation. They suggest that treatment with FK506, a widely used immunosuppressant, might be clinically useful in reducing the severity of pancreatitis. Previous reports of FK506 show conflicting results. A few clinical case reports suggest an association of FK506 use with pancreatitis (31, 37, 39, 47, 52). However, in the experimental literature, some reports demonstrate improvement of disease with FK506 (15, 30, 58), whereas others show worsening (10, 25) or no effect (18). An examination of both the clinical and experimental papers suggests that chronic FK506 exposure may actually predispose individuals to pancreatitis whereas acute doses improve outcome. Although we have not critically examined this post, our data would support a beneficial role of acute, prophylactic, and intercurrent FK506 administration. The results may imply that FK506 treatment might reduce the risk of pancreatitis in high-risk situations.

### Table 1. FK506 treatment reduces pancreatic and lung MPO activity

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Caer</th>
<th>Caer + FK506</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas</td>
<td>0.02±0.01</td>
<td>0.30±0.19*</td>
<td>0.04±0.01†</td>
</tr>
<tr>
<td>Lung</td>
<td>0.16±0.09</td>
<td>0.70±0.07*</td>
<td>0.25±0.12†</td>
</tr>
</tbody>
</table>

Results are expressed as RLU/µg protein. *, †P < 0.05 compared with saline- and caerulein (caer)-only treated groups, respectively.
such as during endoscopic retrograde cholangiopancreatography.

Although we hypothesize that inhibition of pancreatic acinar PP2B leads to a reduction in protease activation and pancreatitis, a limitation of the present study is its inability to determine the primary PP2B tissue source involved. Although the observed reduction in protease activation, edema, and vacuole formation with FK506 within the first 1 h of pancreatitis induction supports our hypothesis, two major sources of PP2B expression, lymphocytes and neurons, might also contribute to the inflammatory response (35, 58). Future genetic studies designed to selectively knock down PP2B in acinar cells, for example using an elastase-specific promoter, are necessary. Another approach that examines the contribution of immune cells would be to transplant wild-type bone marrow into PP2B-deficient transgenics and thereby establish a mouse chimera that produces immune cells without a PP2B defect. Yet despite the potential sources of PP2B that might contribute to the pancreatitis response, we report here that FK506 treatment, given acutely in vivo, reduces protease activation and the observed reduction in protease activation, edema, and intrapancreatic vacuole formation with FK506 within the first 1 h of pancreatitis induction supports our hypothesis, two major sources of PP2B leads to a reduction in protease activation and pancreatitis.

ACKNOWLEDGMENTS

We thank Drs. F. Gorelick and M. Nathanson for helpful discussion throughout the study.

GRANTS

This work was supported by National Institutes of Health Grants R01 DK083327, R03 DK078707, K08 DK68116, K12 HD001401, and DK34989 (Yale Liver Center), and a Children’s Digestive Health and Nutrition Young Investigator Award (to S. Z. Husain).

REFERENCES

18. Moretti AI, Rios EC, Soriano FG, de Souza HP, Abatepaulo F, Barbeiro DF, Velasco IT. Acute pancreatitis: hypertonic saline increases...


