Enteral Exclusion Increases Map Kinase Activation and Cytokine Production in a Model of Gallstone Pancreatitis

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Key Words

Acute pancreatitis • Cholecystokinin • c-Jun NH₂-terminal kinase • Extracellular signal-regulated kinase • Stress-activated protein kinase • Cytokine • Acinar cell • Donor Rat Model

Abstract

Background: We have previously demonstrated that enteral exclusion augments pancreatic p38 mitogen-activated protein (MAP) kinase activation and tumor necrosis factor- α (TNF- α) production after bile-pancreatic duct ligation in rats. **Methods:** In the present study, we evaluated c-Jun NH₂-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) activation, and cytokine production, in pancreata of duct-ligated rats with and without duodenal bile-pancreatic juice replacement from a donor rat. We hypothesized that enteral exclusion of bile-pancreatic juice activates stress kinases and induces cytokine production in ligation-induced acute pancreatitis. **Results:** Increased JNK and ERK activation after ligation are inhibited by bile-pancreatic juice replacement. Increases in pancreatic production of IL-1 β and IL-12 after ligation are significantly subdued by replacement. In additional in vitro studies, we show that cholecystokinin- or TNF- α -stimulated nuclear transcription factor kappa-B activation in AR42J cells is inhibited by dominant negative ERK2.

Conclusions: Our novel findings using our Donor Rat Model indicate that bile-pancreatic juice exclusion induces MAP kinase activation and exacerbates cell stress and inflammation in this experimental model of gallstone pancreatitis.

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Introduction

Gallstones are the commonest cause of acute pancreatitis worldwide. Using an original surgical model, *The Donor Rat Model*, we previously showed that duodenal replacement of bile-pancreatic juice achieves substantial amelioration of pancreatic morphologic changes and hypercholecystokininemia in early ligation-induced acute pancreatitis [1, 2]. Understanding the mechanism of activation of pro-inflammatory pathways is fundamental to the elucidation of early events in disease pathogenesis [3–8]. Activation of mitogen-activated protein (MAP) kinases, translocation of transcription factors, such as nuclear transcription factor kappa-B (NF κ B), and increased cytokine production are implicated in disease pathogenesis [4, 9–11]. We hypothesize that bile-pancreatic juice exclusion from gut activates stress kinases that induce production of pro-inflammatory mediators in ligation-induced acute pancreatitis [2]. In support of this hypothesis, we

have previously shown that duodenal bile-pancreatic juice replacement from a donor rat ameliorates duct ligation-induced activation of p38 MAP kinase, limits nuclear translocation of NF κ B, and attenuates overproduction of tumor necrosis factor- α (TNF- α), IL-6 and chemokines in the pancreas [12–14].

Since MAP kinases often work in a synergistic manner, we extend our observations in the present study to investigate the MAP kinases c-Jun NH₂-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) and the cytokines interleukin-1 β (IL-1 β) and IL-12 in the pancreas using the same model. Here, we study the effect of bile-pancreatic juice replacement on activation of JNK and ERK and production of IL-1 β and IL-12 in the pancreas after duct ligation. Our results show that bile-pancreatic juice replacement subdues JNK and ERK activation, and cytokine overproduction, in ligation-induced acute pancreatitis. Our findings support our central hypothesis that bile-pancreatic juice exclusion from the gut exacerbates acinar cell stress and acute inflammation in this experimental model of gallstone-induced acute pancreatitis. We also performed *in vitro* studies to evaluate the role of ERK2 in modulating NF κ B activation in the AR42J rat exocrine pancreatic tumor cell line.

Materials and Methods

Materials

Rabbit polyclonal antibodies against total JNK (Catalog No. 9252) and phospho-JNK (pThr183/pTyr185; Catalog No. 9251) were purchased from Cell Signaling (Danvers, Mass., USA). HRP-conjugated anti-rabbit IgG secondary antibody was purchased from New England Biolabs (Beverly, Mass., USA). [³²P]-ATP (3,000 Ci/mmol) was purchased from Perkin Elmer Life Sciences/NEN (Woodbridge, Ont., Canada). Recombinant human c-Jun protein (Catalog No. sc-4113) was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Enhanced chemiluminescence (ECL) immunoblot detection reagents were from GE Healthcare Lifesciences (Piscataway, N.J., USA). The commercial Biosource ELISA kits for assay of total ERK1/2, phospho-ERK1/2, IL-1 β , and IL-12 (Catalog Nos. KHO 0081, KHO-0091, KRC 0011, and KRC 2371, respectively) were purchased from Invitrogen Corporation (Carlsbad, Calif., USA). AR42J cells and the F12K cell culture medium were purchased from the American Type Culture Collection (ATCC, Manassas, Va., USA). The recombinant adenoviral vector containing the dominant negative form of human ERK2 (DN ERK2; the dual phosphorylation site T202/Y204 mutated to A202/F204) was purchased from Cell Biolabs, Inc. (San Diego, Calif., USA; Catalog No. ADV 113). Adeno-NF κ B-luciferase was purchased from the University of Iowa Vector Core Facility [15]. The Luciferase Assay Reagent was purchased from Promega Corporation (Madison, Wisc., USA; Catalog No. E1501). Cholecystokinin-8-sulphide (CCK-8S) was pur-

Fig. 1. Diagram of the Donor Rat Model. In diseased-treated rats (Recipient Rat) the distal bile-pancreatic duct was ligated and bile-pancreatic juice was instilled via a duodenal fistula beginning immediately before duct ligation. Bile-pancreatic juice was obtained fresh from a donor rat (that was prepared with a bile-pancreatic fistula) and donated to a recipient rat using a liquid-level photodetector and peristaltic pump.

chased from Sigma Chemicals (St. Louis, Mo., USA). Rat recombinant TNF- α protein (rTNF- α) was purchased from R&D Systems Inc. (Minneapolis, Minn., USA; Catalog No. 510-RT-010).

Animal Surgery and Specimen Collection

The University of Iowa Institutional Animal Care and Use Committee approved the experimental protocols described here prior to commencement of the study. We purchased male Sprague-Dawley rats (250–325 g) from Harlan Sprague-Dawley, Inc. (Indianapolis, Ind., USA). We performed midline laparotomy under general anesthesia induced with ketamine hydrochloride (87 mg/kg), xylazine hydrochloride (13 mg/kg), and acepromazine maleate (1 mg/kg) that were administered subcutaneously. Six rats were studied in each experimental group at each time point. In diseased-controls, the distal bile-pancreatic duct was ligated at its junction with the duodenum. In the diseased-treated group, duodenal replacement of bile-pancreatic juice was begun via a duodenal fistula beginning immediately before distal bile-pancreatic duct ligation (fig. 1), as described previously [2, 12]. Briefly, bile-pancreatic juice was obtained fresh from a donor rat that was prepared with a bile-pancreatic fistula, collected in a liquid-level photodetector, and instilled into the duodenum of diseased-treated rats using a peristaltic pump. In sham-operated controls, the distal bile-pancreatic duct was dissected but not ligated. The rats were euthanized after 1 or 3 h, the pancreas was excised and snap-frozen in liquid nitrogen, and then stored at -80°C until processed. Pancreatic homogenates prepared from the stored frozen specimens were analyzed as follows: (1) immunoblots using phospho-specific JNK antibody and beta-actin antibody, (2) immune-complex kinase assay for JNK using c-Jun as substrate, and (3) ELISA for total ERK, phospho-ERK, IL-1 β and IL-12.

Immunoblotting

Western blotting was performed as previously described [16]. Pancreatic tissue was collected, portions were homogenized in 10 mM Hepes buffer (10 mM HEPES, pH 7.5, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA), and the soluble fraction was collected by centrifugation at 15,000 g. Total soluble protein was measured using the Bradford method (Bio-Rad, Hercules, Calif., USA) according to the manufacturer's instructions. Sample aliquots containing 40 µg of total protein were denatured in SDS-sample buffer (62.5 mM Tris, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromophenol blue), electrophoresed with a 4–20% gradient SDS-polyacrylamide gel (BioRad, Hercules, Calif., USA), and transferred to a nitrocellulose membrane. Protein blots were probed with specific primary antibodies (1:1,000 v/v) and developed using the appropriate secondary antibody conjugated to horse-radish peroxidase (HRP) (1:2,000 v/v). The blots were developed using the ECL method according to manufacturer's instructions. Blots were also probed with anti-β-actin antibody to evaluate sample loading.

Immunoprecipitation

Immunoprecipitation was carried out as described earlier [17]. In brief, tissues were extracted with a lysis buffer consisting of 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 1 mM benzamidine, 1 mM PMSF (phenyl methane sulfonyl flouride), and 10 µg/ml each of aprotinin, leupeptin, chymostatin, pepstatin A and antipain. Protein estimations were carried out using a commercial kit for the modified Lowry's method (Pierce Biotechnology, Rockford, Ill., USA). Tissue extracts were centrifuged at 12,000 rpm for 15 min to collect the supernatants. Aliquots of clear supernatants containing 500 µg total protein in 1 ml lysis buffer were incubated with 2 µg of specific JNK or ERK antibody overnight at 4°C followed by an additional incubation with 20 µl of a 50% suspension of protein G-agarose for 1 h at room temperature. The insoluble immune complex was collected and washed three times by brief centrifugations prior to immune complex kinase assay.

Immune Complex Kinase Assay

JNK was immunoprecipitated from tissues as described above, and followed by two additional washes with a kinase assay buffer prior to immune complex kinase assay. The assays were carried out as described earlier [17], in a total reaction volume of 20 µl containing 100 mM Tris-HCl, pH 7.0, 0.4 mM sodium ortho vanadate, 40 mM magnesium acetate, 1 mM dithiothreitol, and 30 µM calmidazolium, 10 µl of JNK immune complex, 2 µg of kinase substrate (c-Jun), 100 µM [γ -³²P]-ATP (200 cpm/pmol), and incubated at 30°C for 10 min. The reaction was stopped by addition of SDS-PAGE sample buffer, boiled for 2 min, centrifuged, and the supernatant was subjected to SDS-PAGE followed by autoradiography.

ELISA

Portions of pancreas were homogenized and total protein estimation of the soluble fraction was performed as in our immunoblotting technique described above. Total ERK, phospho-ERK, IL-1β and IL-12 levels were measured in the soluble fraction of pancreatic homogenates using a commercial kit as per the manufacturer's instructions (Biosource, Inc., Camarillo, Calif., USA; Catalog No. KRC 3013).

In vitro Studies

We studied the effect of expressing the dominant negative (DN) form of ERK2 on CCK- or recombinant tumor necrosis factor-α (rTNF-α)-stimulated NFκB activation in AR42J cells. Using a promoter construct driven only by NFκB (adeno-NFκB-luciferase vector) [15], we infected AR42J cells with a replication-deficient adenoviral vector containing either an empty vector or a DN-ERK2 expression vector. AR42J cells were thawed according to the manufacturer's instructions and grown in F-12K medium containing 20% non-heat-inactivated fetal bovine serum incubated at 37°C with 5% carbon dioxide and 95% air. In addition to adeno-NFκB-luciferase vector infection (5 MOI), we infected one million AR42J cells per well for 48 h with 5 MOI of a replication-deficient adenoviral vector containing either an empty vector or a DN-ERK2 expression vector. After 48 h, the cells were stimulated with 10 µM CCK-8S for 16 h or 1 ng/ml rTNF-α for 6 h. Nonstimulated but infected cells were used as controls. After one wash with phosphate-buffered saline, the cells were harvested using the Luciferase Assay Buffer and luciferase activity was measured on a Sirius single tube luminometer (Zylux Corporation, Oak Ridge, Tenn., USA). The relative luminescent units (RLU) of luciferase activity were normalized to the protein concentration of each sample (Bradford protein assay; Bio-Rad, Hercules, Calif., USA) prior to comparison between groups (n = 3 wells/group).

Statistical Analysis

SigmaStat software (www.spss.com; SPSS Inc., Chicago, Ill., USA) was used for statistical analysis. One-way ANOVA was used for analysis of ELISA data; six rats were studied in each experimental group at each time point and results expressed as mean ± SEM. $p < 0.05$ was considered statistically significant.

Results

We assessed the degree of acinar cell stress in pancreata of rats from our experimental groups by evaluating activation of the MAP kinases JNK and ERK. Immunoblotting of phospho-JNK and immune-complex kinase assay of JNK in pancreatic homogenates (fig. 2) show that 1 or 3 h of duct ligation are associated with JNK activation compared to sham controls, confirming our previous findings in experiments performed in a different set of rats [9]. Duodenal bile-pancreatic juice replacement in the diseased-treated group shows noticeable limitation in JNK activation after duct ligation. The immune complex kinase assay, which is more sensitive than the immunoblot technique to evaluate JNK activation, shows marked and time-dependent increases in JNK activation after 1 and 3 h of duct ligation, compared to sham controls. Equal loading of the protein samples was confirmed by an immunoblot for E-actin.

The changes in ERK activation measured by ELISA of phospho-ERK in pancreatic homogenates after duct ligation with and without enteral replacement show a similar

Fig. 2. Immunoblot of pancreatic homogenates using phospho-specific JNK antibody shows increased JNK activation after 1 or 3 h of duct ligation compared to sham that is inhibited by bile-pancreatic juice replacement. Beta-actin immunoblot confirms equal loading of lanes. Autoradiogram of immune-complex kinase assay of pancreatic homogenates using JNK antibody, and c-Jun as substrate, shows a robust increase in JNK activation after duct ligation as evidenced by progressive phosphorylation of c-Jun after 1 or 3 h of ligation. Confirming immunoblot findings, immune-complex kinase assay also shows that the increase in JNK activation after duct ligation is substantially subdued by bile-pancreatic juice replacement. The position of c-Jun was verified with molecular size standards. S = Sham-operated controls had the duct dissected but not ligated; D = diseased-controls had duct ligation; T = treated group – diseased-treated rats had duct ligation with duodenal bile-pancreatic juice replacement.

pattern of changes as seen with JNK activation. Essentially, the increases in ERK activation after 1 or 3 h of duct ligation are substantially limited by enteral replacement therapy (fig. 3). In addition, increases in phospho-ERK are not associated with corresponding increases in total ERK, indicating that the increases are due to phosphorylation (activation) of pre-existing ERK rather than a mere secondary effect of total ERK induction. Furthermore, ERK activation at 3 h of duct ligation is significantly greater than at 1 h.

Pancreatic morphological changes following 1–3 h of duct ligation are generally not sufficient to compare differences between experimental groups. Therefore, we used pancreatic IL-1 β and IL-12 production as parameters of the severity of acute inflammation in the present study. Compared to sham-operated controls, 1 or 3 h of duct ligation results in increases in pancreatic IL-1 β and IL-12 concentrations, consistent with development of acute pancreatic inflammation in our model (fig. 4). Furthermore, a progressive increase in IL-1 β and IL-12 production is seen after 3 h of duct ligation compared to 1 h of ligation. In diseased-treated rats, duodenal replacement of bile-pancreatic juice is associated with

Fig. 3. ELISA of pancreatic homogenates shows increased activation of ERK after 1 or 3 h of duct ligation, compared to sham-operated controls. The phospho-ERK levels are greater after 3 h of duct ligation compared to 1 h of ligation. ERK activation is substantially ameliorated by bile-pancreatic juice replacement. ELISA of total ERK shows no significant difference between groups. Six rats were studied in each experimental group at each time point. Results are mean $\pm$ SEM; one-way ANOVA; $p < 0.05$. Asterisk (*) indicates significant difference from S at the same time point, pound sign (#) indicates significant difference from S and D, whereas the 'at' sign (@) indicates significant difference from D at the 1-hour time point; S = Sham-operated controls had the duct dissected but not ligated; D = diseased-controls had duct ligation; T = treated group – diseased-treated rats had duct ligation with duodenal bile-pancreatic juice replacement.

amelioration of acute pancreatic inflammation as evidenced by diminished pancreatic IL-1 β and IL-12 concentrations.

As MAP kinases have been shown to regulate the expression of pro-inflammatory cytokines at the level of transcription via modulating the activation of NF κ B in several cell types [11, 18], we evaluated the role of ERK2

Fig. 4. ELISA of pancreatic homogenates shows increases in IL-1 β and IL-12 production after 1 or 3 h of duct ligation, compared to sham controls. The cytokine levels are greater after 3 h of duct ligation compared to 1 h of ligation. These increases in cytokine levels are substantially ameliorated by bile-pancreatic juice replacement. Six rats were studied in each experimental group at each time point. Results are mean $\pm$ SEM; one-way ANOVA; $p < 0.05$. Asterisk (*) indicates significant difference from S at the same time point; pound sign (#) indicates significant difference from S and D, whereas the 'at' sign (@) indicates significant difference from D at the 1-hour time point. S = Sham-operated controls had the duct dissected but not ligated; D = diseased-controls had duct ligation; T = treated group – diseased-treated rats had duct ligation with duodenal bile-pancreatic juice replacement.

in modulating transcriptional activity of NF κ B in AR42J cells (fig. 5). Stimulation of native TNF- α or CCK-A receptors promotes a significant increase in NF κ B-dependent gene expression, as measured by luciferase activity, in AR42J cells infected with the adeno-NF κ B-luciferase vector as compared to those infected with the empty vector. In both unstimulated and CCK- or TNF- α -stimu-

Fig. 5. AR42J cells infected with adeno-DN-ERK2 and adeno-NF κ B-luciferase showed diminished NF κ B luciferase activity at baseline levels and even after stimulation with CCK-8S or rTNF- α . ANOVA; $p < 0.05$. Asterisk (*) indicates significant difference from the nonstimulated empty vector control group; pound sign (#) indicates significant difference from the stimulated empty vector group; $n = 3$ wells per experimental group. RLU = Relative luminescent units.

lated cells, expression of DN-ERK2 significantly abrogates NF κ B-dependent luciferase activity. In CCK- or TNF- α -stimulated cells that express DN-ERK2 the luciferase activity is either below or similar to that of unstimulated controls. These findings indicate that inhibition of ERK2 attenuates TNF- α - or CCK-stimulated transcriptional activity of NF κ B in AR42J cells. These findings support the view that ERK regulates the activation of pro-inflammatory transcription factors, such as NF κ B, in pancreatic exocrine cells [11, 19].

Fig. 6. Schematic representation of salient early events in gallstone pancreatitis pathogenesis. Bile-pancreatic juice exclusion-induced acinar hyperstimulation imposes excess stress on the acinar cell in the presence of an obstructed duct. Acinar cell stress activates MAP kinases that induce production of inflammatory mediators. $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ play a central role in the initiation, maintenance, and propagation of pancreatic and systemic inflammation during the early stages of the pathogenesis of acute pancreatitis. $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ produced by acinar cells as a result of MAP kinase activation propagate additional MAP kinase activation.

Discussion

We present new evidence that bile-pancreatic juice exclusion from gut plays a prominent role in the mechanism of activation of pro-inflammatory pathways during the early phase of ligation-induced acute pancreatitis in rats. The results of our studies show that ligation of the distal bile-pancreatic duct, which excludes bile-pancreatic juice from the gut, is associated with activation of JNK and ERK, which enhances the overproduction of the cytokines $\text{IL-1}\beta$ and IL-12 in the rat pancreas. Using our Donor Rat Model, we show that pancreatic JNK and ERK activation, and the associated $\text{IL-1}\beta$ and IL-12 overproduction, seen after duct ligation are substantially subdued by enteral replacement of bile-pancreatic juice. Our findings indicate that, during the early phase of ligation-

induced acute pancreatitis in rats, enteral exclusion of bile-pancreatic juice augments MAP kinase activation and cytokine production and, thus, exacerbates acute inflammation of the pancreas (fig. 6).

In the present study, we also show that CCK and $\text{TNF-}\alpha$ activate $\text{NF}\kappa\text{B}$ in AR42J cells. Our results indicate that ERK regulates $\text{NF}\kappa\text{B}$ -dependent gene expression in AR42J cells stimulated with CCK or $\text{TNF-}\alpha$. These findings provide corroborative evidence that ERK activation potentially promotes activation of pro-inflammatory pathways in pancreatic exocrine cells when stimulated by G protein-coupled receptor agonists such as CCK or by cytokines. Our in vitro observations corroborate our in vivo findings, as bile-pancreatic juice exclusion is associated with substantial hypercholecystokinemia and as ligation-induced acute pancreatitis is associated with increased pancreatic production of cytokines. Therefore, AR42J cells provide a suitable model to investigate $\text{NF}\kappa\text{B}$ -dependent gene expression mechanistically prior to performing detailed in vitro studies in isolated pancreatic acinar cells or in vivo studies in experimental models of pancreatitis.

Gallstone pancreatitis is common and is potentially fatal [3, 20, 21]. As the clinical investigation of early events in the pathogenesis of gallstone pancreatitis is not practical, we must rely upon experimental models to explore the earliest acinar cell events that exacerbate acute pancreatic inflammation [1, 22]. Specific treatment strategies for gallstone pancreatitis are lacking due to the fact that several salient events in disease pathogenesis are not well understood [1, 21]. Our experimental model of duct ligation in rats is a useful means to investigate early events in the pathogenesis of gallstone pancreatitis [1, 2, 23]. Ligation of the distal bile-pancreatic duct in rats obstructs the duct, excludes bile-pancreatic juice from the gut, and induces acute pancreatitis [1, 24]. Enteral exclusion of bile-pancreatic juice results in increased acinar cell stimulation via neurohormonal pathways [25]. In a series of experiments using the Donor Rat Model, we have presented evidence that the combination of acinar cell hyperstimulation and duct obstruction exacerbates acute inflammation of the pancreas. In an earlier study, we showed that duodenal replacement of bile-pancreatic juice achieves substantial amelioration of pancreatic morphologic changes, hyperamylasemia, and hypercholecystokinemia in early ligation-induced acute pancreatitis [1]. Subsequently, we showed that trypsin and Na-taurocholate are the key components of bile-pancreatic juice in rats that exacerbate ligation-induced acute pancreatitis when excluded [24]. We also showed that the CCK-A receptor

and the cholinergic receptor on acinar cells participate in the neurohormonal hyperstimulation of acinar cells that exacerbates acute pancreatitis in this experimental model [14, 24, 25]. We then explored the hypothesis that the combination of acinar cell hyperstimulation and duct obstruction activates acinar cell stress kinase pathways that are capable of inducing pro-inflammatory cytokine production in the pancreas. We have recently shown that increased activation of p38 MAP kinase and overproduction of TNF- α in the pancreas after duct ligation are markedly diminished by duodenal bile-pancreatic juice replacement from a donor rat [12]. In the present study, we show that the increased activation of JNK and ERK and the increased production of pro-inflammatory cytokines in the pancreas after duct ligation are similarly diminished by duodenal bile-pancreatic juice replacement. Taken together, the results of our studies support our central hypothesis that *enteral exclusion of bile-pancreatic juice activates signaling intermediates that exacerbate gallstone pancreatitis* [2]. Our Donor Rat Model is a useful experimental model to investigate the mechanisms by which the enteral response to bile-pancreatic juice exclusion exacerbates duct occlusion-induced acute pancreatitis [1, 2, 12, 25].

MAP kinases such as JNK, ERK, and p38 can be activated by G protein-coupled receptor stimulation (e.g. CCK-A receptor, cholinergic receptor) and are capable of inducing pro-inflammatory cytokine production at the transcriptional level by activating transcription factors such as NF κ B [26, 27]. Our working hypothesis is that in ligation-induced acute pancreatitis in rats, bile-pancreatic juice exclusion from gut stimulates acinar cell G protein-coupled receptors via neurohormonal pathways and thus activates the MAP kinase $\rightarrow$ transcription factor activation $\rightarrow$ cytokine signal transduction pathway resulting in acinar cell hyperproduction of pro-inflammatory cytokines [2, 11]. The transcription factor NF κ B is situated in the cytoplasmic compartment of quiescent cells and is complexed with its inhibitory protein I κ B [18, 28]. Transcriptional regulation via NF κ B pathway activation involves a complex series of events. The cytosolic I κ B/NF κ B complex dissociates when I κ B is phosphorylated. Phosphorylation of I κ B causes I κ B degradation [18, 29], dissociation of the cytosolic I κ B/NF κ B complex, nuclear translocation of NF κ B, and the transcriptional upregulation of several inflammatory mediators. NF κ B induces the nuclear transcription of pro-inflammatory messengers such as cytokines, chemokines, adhesion molecules, and inducible effector enzymes [18, 19].

Investigations in experimental models have shown that the overproduction of cytokines in the pancreas occurs within the first 30 min after the onset of acute pancreatitis [5]. The morbidity and mortality of acute pancreatitis is in large part related to exocrine pancreatic overproduction of cytokines. Studies in human and experimental acute pancreatitis have underlined the central role of IL-1 β and TNF- α in the initiation, maintenance, and propagation of acute pancreatic inflammation and in the systemic spread of inflammation to major organ systems [5]. The induction of IL-1 β and TNF- α is followed by a local exacerbation of pro-inflammatory mediator production. Release of these inflammatory mediators into the general circulation manifests as a systemic hyperinflammatory state characterized by multiple organ dysfunction that is potentially fatal [4, 5]. Therefore, elucidation of the mechanism of increased cytokine production in the early phase of acute pancreatitis is of crucial clinical relevance. We have elucidated certain pathogenic mechanisms involved in cytokine production within the pancreas after duct ligation. Our findings underline the importance of the enteral response to bile-pancreatic juice exclusion in contributing to increased pancreatic IL-1 β , IL-12, TNF- α , and IL-6 production during the early phase of duct occlusion-induced acute pancreatitis [12, 14]. Furthermore, IL-1 β and TNF- α produced by acinar cells stimulate their respective cell surface receptors on acinar cells in an autocrine and paracrine fashion and thus further propagate the activation of signaling pathways that augment the production of acute inflammatory mediators [5, 10, 26, 30]. This results in a self-propagating inflammatory loop with consequent amplification of the initial inflammatory response.

The MAP kinases are a family of protein kinases that play an important role in intracellular signal transduction. However, the precise part they play in the pathogenesis of acute pancreatitis remains to be elucidated [27]. The importance of defining the role of MAP kinases in gallstone pancreatitis pathogenesis is emphasized by our finding that bile-pancreatic juice exclusion from gut augments the activation of ERK, JNK, and p38 in duct ligation-induced acute pancreatitis in rats. A few recent studies have investigated the role of MAP kinase pathways in acute pancreatitis pathogenesis. These reports by other investigators were mainly in the lethal rat model of retrograde ductal infusion of bile salts, the pancreatic necrosis model of CDE diet-induced acute pancreatitis in mice, and the non-lethal model of acute edematous pancreatitis caused by supramaximal doses of CCK analog caerulein [4, 9, 10, 27, 31–36]. Whether p38 may exacerbate acute

pancreatic inflammation or protect against it remains an area of controversy in view of contradictory findings by different groups of investigators. One group reported that p38 inhibition exacerbates caerulein-induced acute pancreatitis in rats, suggesting that p38 may have a protective rather than detrimental role [33]. In contrast, most reports support the view that p38 MAP kinase activation exacerbates acute pancreatitis [4, 31, 36, 37]. Inhibitors of JNK (CEP 1347) and ERK (U0126, PD98059) signaling pathways have been reported to ameliorate caerulein-induced acute pancreatitis in rats [33–35]. In a recent in vitro study using rat pancreatic fragments, we showed that specific inhibitors of ERK, JNK, and p38 significantly subdued caerulein-stimulated activation of the corresponding MAP kinase and attenuated production of IL-1 β and TNF- α [38].

Over the past decade, several investigators have used the AR42J rat exocrine pancreatic tumor cell line to investigate signaling mechanisms that may show parallels with the pancreatic acinar cell. One study using AR42J cells showed that CCK, but not bombesin, strongly activates ERK [39]. Another study in AR42J cells showed that caerulein-induced IL-6 production may be regulated by ERK and the transcription factors NF κ B and activator protein-1 (AP-1) [40]. A study that used both isolated pancreatic acinar cells and AR42J cells showed that TNF- α -induced NF κ B activation can also be mediated by protein kinase C- δ [41]. In dispersed pancreatic acini, the same group showed that CCK-8 and TNF- α initiate NF κ B activation by different PLC pathways that converge at the level of the protein kinase Cs (PKC- δ and - ϵ) to mediate NF κ B activation [42]. A study investigating the effect of nicotine on AR42J cells showed that it activates ERK but not p38 or JNK [43]. Lysophosphatidylcholine, a phospholipid by-product generated by phospholi-

pase A2, activated stress kinases (ERK, JNK, and p38) and increased the specific DNA-binding activity of NF κ B and AP-1 [44]. Carbachol, a cholinergic receptor agonist, activated ERK in AR42J cells [45]. These previous studies are in agreement with our strategy of using AR42J cells for pilot studies prior to performing more detailed studies in isolated pancreatic acinar cells or before embarking on extensive in vivo studies.

In summary, we have shown that duct ligation is associated with substantial increases in pancreatic JNK and ERK activation and IL-1 β and IL-12 production. We have also shown that activation of JNK and ERK and increased IL-1 β and IL-12 production after duct ligation are appreciably diminished by duodenal bile-pancreatic juice replacement. These results indicate that MAP kinase activation and increased cytokine production during the early phase after duct ligation are predominantly the result of acinar hyperstimulation following the enteral response to bile-pancreatic juice exclusion rather than purely from the mechanical effects of duct obstruction. These findings support our central hypothesis that bile-pancreatic juice exclusion from gut exacerbates cellular stress and acute inflammation in this experimental model of gallstone-induced acute pancreatitis. Our in vitro studies suggest that the pathogenesis may be mediated, in part, by ERK2 modulating CCK- or TNF- α -stimulated NF κ B-dependent gene expression in acinar cells.

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References

- Samuel I, Toriumi Y, Wilcockson DP, Turkelson CM, Solomon TE, Joehl RJ: Bile and pancreatic juice replacement ameliorates early ligation-induced acute pancreatitis in rats. *Am J Surg* 1995;169:391–399.
- Samuel I: Bile and pancreatic juice exclusion activates acinar stress kinases and exacerbates gallstone pancreatitis. *Surgery* 2008; in press.
- Saluja AK, Steer MLP: Pathophysiology of pancreatitis. Role of cytokines and other mediators of inflammation. *Digestion* 1999; 60(suppl 1):27–33.
- Yang J, Murphy C, Denham W, Botchkina G, Tracey KJ, Norman J: Evidence of a central role for p38 map kinase induction of tumor necrosis factor alpha in pancreatitis-associated pulmonary injury. *Surgery* 1999;126: 216–222.
- Norman J: The role of cytokines in the pathogenesis of acute pancreatitis. *Am J Surg* 1998; 175:76–83.
- Karne S, Gorelick F: Etiopathogenesis of acute pancreatitis. *Surg Clin N Am* 1999;79: 699–710.
- Criddle DN, McLaughlin E, Murphy JA, Petersen OH, Sutton R: The pancreas misled: signals to pancreatitis. *Pancreatology* 2007; 7:436–446.
- Hofner P, Balog A, Gyulai Z, Farkas G, Rakonczay Z, Takacs T, Mandi Y: Polymorphism in the IL-8 gene, but not in the TLR4 gene, increases the severity of acute pancreatitis. *Pancreatology* 2006;6:542–548.
- Samuel I, Zaheer S, Fisher RA, Zaheer A: Cholinergic receptor induction and JNK activation in acute pancreatitis. *Am J Surg* 2003;186:569–574.

- 10 Samuel I, Zaheer S, Nelson JJ, Yorek MA, Zaheer A: CCK-A receptor induction and P38 and NF-kappaB activation in acute pancreatitis. *Pancreatol* 2004;4:49–56.
- 11 Algul H, Tando Y, Schneider G, Weidenbach H, Adler G, Schmid RM: Acute experimental pancreatitis and NF-kappaB/Rel activation. *Pancreatol* 2002;2:503–509.
- 12 Samuel I, Zaheer S, Zaheer A: Bile-pancreatic juice exclusion increases p38MAPK activation and TNF-alpha production in ligation-induced acute pancreatitis in rats. *Pancreatol* 2005;5:20–26.
- 13 Samuel I, Yorek MA, Zaheer A, Fisher RA: Bile-pancreatic juice exclusion promotes Akt/NF-kappaB activation and chemokine production in ligation-induced acute pancreatitis. *J Gastrointest Surg* 2006;10:950–959.
- 14 Samuel I, Zaheer A, Zaheer S, Fisher R: Bile-pancreatic juice exclusion increases cholinergic M3 and CCK-A receptor expression and interleukin-6 production in ligation-induced acute pancreatitis. *Am J Surg* 2004;188:511–515.
- 15 Sanlioglu S, Williams CM, Samavati L, Butler NS, Wang G, McCray PB Jr, Ritchie TC, Hunninghake GW, Zandi E, Engelhardt JF: Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor-alpha secretion through IKK regulation of NF-kappa B. *J Biol Chem* 2001;276:30188–30198.
- 16 Kaplan R, Zaheer A, Jaye M, Lim R: Molecular cloning and expression of biologically active human glia maturation factor-beta. *J Neurochem* 1991;57:483–490.
- 17 Zaheer A, Lim R: In vitro inhibition of MAP kinase (ERK1/ERK2) activity by phosphorylated glia maturation factor (GMF). *Biochemistry* 1996;35:6283–6288.
- 18 Ghosh S, Karin M: Missing pieces in the NF-kappaB puzzle. *Cell* 2002;109(suppl):S81–S96.
- 19 Chen X, Ji B, Han B, Ernst SA, Simeone D, Logsdon CD: NF-kappaB activation in pancreas induces pancreatic and systemic inflammatory response. *Gastroenterology* 2002;122:448–457.
- 20 Ranson J, Rifkind K, Roses DF, Fink S, Eng K, Spencer F: Prognostic signs and the role of operative management in acute pancreatitis. *Surgery* 1974;139:69–81.
- 21 Ranson J: Etiological and prognostic factors in human acute pancreatitis: a review. *Am J Gastroenterol* 1982;77:633–638.
- 22 Steer ML: Workshop on experimental pancreatitis. *Dig Dis Sci* 1985;30:575–581.
- 23 Ohshio G, Saluja A, Steer ML: Effects of short-term pancreatic duct obstruction in rats. *Gastroenterology* 1991;104:1768–1779.
- 24 Samuel I, Toriumi Y, Zaheer A, Joehl RJ: Mechanism of acute pancreatitis exacerbation by enteral bile-pancreatic juice exclusion. *Pancreatol* 2004;4:527–532.
- 25 Samuel I, Joehl RJ: Bile-pancreatic juice replacement, not cholinergic and cholecystokinin-receptor blockade, reverses acinar cell hyperstimulation after bile-pancreatic duct ligation. *Am J Surg* 1996;171:207–211.
- 26 Kyriakis JM, Avruch J: Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 2001;81:807–869.
- 27 Schafer C, Williams JA: Stress kinases and heat shock proteins in the pancreas: possible roles in normal function and disease. *J Gastroenterol* 2000;35:1–9.
- 28 Song G, Ouyang G, Bao S: The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med* 2005;9:59–71.
- 29 Madrid LV, Mayo MW, Reuther JY, Baldwin AS Jr: Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. *J Biol Chem* 2001;276:18934–18940.
- 30 Fink G, Yang J, Carter G, Ward K, Ulrich P, Tracey K, Norman J: A low molecular weight macrophage inhibitor decreases severity of pancreatitis through inhibition of IL-1 and TNF production. *Surg Forum* 1996;47:137–140.
- 31 Yang J, Denham W, Carter G, Tracey KJ, Norman J: Macrophage pacification reduces rodent pancreatitis-induced hepatocellular injury through down-regulation of hepatic tumor necrosis factor alpha and interleukin-1beta. *Hepatology* 1998;28:1282–1288.
- 32 Denham W, Yang J, Norman J: Evidence for an unknown component of pancreatic ascites that induces adult respiratory distress syndrome through an interleukin-1 and tumor necrosis factor-dependent mechanism. *Surgery* 1997;122:295–301.
- 33 Fleischer F, Dabew R, Goke B, Wagner AC: Stress kinase inhibition modulates acute experimental pancreatitis. *World J Gastroenterol* 2001;7:259–265.
- 34 Wagner AC, Mazzucchelli L, Miller M, Camoratto AM, Goke B: CEP-1347 inhibits caerulein-induced rat pancreatic JNK activation and ameliorates caerulein pancreatitis. *Am J Physiol* 2000;278:G165–G172.
- 35 Clemons AP, Holstein DM, Galli A, Saunders C: Cerulein-induced acute pancreatitis in the rat is significantly ameliorated by treatment with MEK1/2 inhibitors U0126 and PD98059. *Pancreas* 2002;25:251–259.
- 36 Yang J, Denham W, Tracey KJ, Wang H, Kramer AA, Salhab KF, Norman J: The physiologic consequences of macrophage pacification during severe acute pancreatitis. *Shock* 1998;10:169–175.
- 37 Murr MM, Yang J, Fier A, Gallagher SF, Carter G, Gower WR, Norman JG: Regulation of Kupffer cell TNF gene expression during experimental acute pancreatitis: the role of p38-MAPK, ERK1/2, SAPK/JNK, and NF-kappa B. *J Gastrointest Surg* 2003;7:20–25.
- 38 Samuel I, Zaheer A, Fisher RA: In vitro evidence for role of ERK, p38, and JNK in exocrine pancreatic cytokine production. *J Gastrointest Surg* 2006;10:1376–1383.
- 39 Kiehne K, Herzig KH, Folsch UR: Differential activation of p42ERK2 and p125FAK by cholecystokinin and bombesin in the secretion and proliferation of the pancreatic amphicrine cell line AR42J. *Pancreatol* 2002;2:46–53.
- 40 Lee J, Seo J, Kim H, Chung JB, Kim KH: Signal transduction of cerulein-induced cytokine expression and apoptosis in pancreatic acinar cells. *Ann NY Acad Sci* 2003;1010:104–108.
- 41 Satoh A, Gukovskaya AS, Edderkaoui M, Daghighian MS, Reeve JR Jr, Shimosegawa T, Pandolfi SJ: Tumor necrosis factor-alpha mediates pancreatitis responses in acinar cells via protein kinase C and proline-rich tyrosine kinase 2. *Gastroenterology* 2005;129:639–651.
- 42 Satoh A, Gukovskaya AS, Nieto JM, Cheng JH, Gukovsky I, Reeve JR Jr, Shimosegawa T, Pandolfi SJ: PKC-delta and -epsilon regulate NF-kappaB activation induced by cholecystokinin and TNF-alpha in pancreatic acinar cells. *Am J Physiol* 2004;287:G582–G591.
- 43 Bose C, Zhang H, Udupa KB, Chowdhury P: Activation of p-ERK1/2 by nicotine in pancreatic tumor cell line AR42J: effects on proliferation and secretion. *Am J Physiol* 2005;289:G926–G934.
- 44 Masamune A, Sakai Y, Yoshida M, Satoh A, Satoh K, Shimosegawa T: Lysophosphatidylcholine activates transcription factor NF-kappaB and AP-1 in AR42J cells. *Dig Dis Sci* 2001;46:1871–1881.
- 45 Turner DJ, Cowles RA, Segura BJ, Mulholland MW: Cholinergic stimulation of rat acinar cells increases c-fos and c-jun expression via a mitogen-activated protein kinase-dependent pathway. *J Gastrointest Surg* 2001;5:661–672.