

ORIGINAL RESEARCH

Acinar Cell Production of Leukotriene B₄ Contributes to Development of Neurogenic Pancreatitis in MiceRafiq A. Shahid,¹ Steven R. Vigna,^{1,2} Amanda C. Layne,³ Joelle M.-J. Romac,¹ and Rodger A. Liddle^{1,4}¹Department of Medicine, ²Department of Cell Biology, and ³Department of Pathology, Duke University Medical Center, and ⁴Durham VA Medical Center, Durham, North Carolina

SUMMARY

The initiation of neurogenic inflammation in pancreatitis is unknown. This work shows that pancreatic acinar cells express 5-lipoxygenase and produce leukotriene B₄ (LTB₄). 5-lipoxygenase inhibition reduces LTB₄ secretion and pancreatitis, indicating that LTB₄ mediates neurogenic pancreatic inflammation.

BACKGROUND & AIMS: In the pancreas, activation of primary sensory nerves through the transient receptor potential vanilloid-1 (TRPV1) ion channel contributes to the early stages of development of pancreatitis. Little is known about the mechanism by which this occurs. We investigated whether leukotriene B₄ (LTB₄) is an endogenous agonist of TRPV1 and mediates pancreatitis.

METHODS: Acute inflammation was induced in the pancreata of *Trpv1*^{-/-} mice and their wild-type littermates by retrograde infusion of the main pancreatic duct with 2% sodium taurocholate (NaT) or intraperitoneal injections of caerulein. Mice were also given injections of resiniferatoxin (an excitotoxin that desensitizes TRPV1) or MK886 (a drug that inhibits LTB₄ biosynthesis). Pancreatic tissues and plasma were collected and analyzed.

RESULTS: Retrograde perfusion of the main pancreatic ducts of wild-type mice with NaT caused severe acute pancreatitis; the severity was reduced by coadministration of resiniferatoxin. *Trpv1*^{-/-} mice developed a less severe pancreatitis after NaT administration compared with controls. Administration of MK886 before perfusion with NaT also significantly reduced the severity of pancreatitis in wild-type mice. Pancreatic tissues from mice given NaT had a marked increase in the level of 5-lipoxygenase immunoreactivity specifically in acinar cells. Bile acid and caerulein induced secretion of LTB₄ by cultured pancreatic acinar cells; MK886 inhibited this process.

CONCLUSIONS: Administration of caerulein or intraductal bile acids in mice causes production of LTB₄ by pancreatic acinar cells. This activates TRPV1 on primary sensory nerves to induce acute pancreatitis. (*Cell Mol Gastroenterol Hepatol* 2015;1:75–86; <http://dx.doi.org/10.1016/j.jcmgh.2014.11.002>)

Keywords: Gallstone Pancreatitis; Mouse Model; Neurogenic Inflammation; Secretagogue; Vanilloid.

Neurogenic inflammation is caused by the local release of inflammatory neuropeptides such as substance P and calcitonin gene-related peptide from afferent sensory neurons. These peptides produce vasodilation and edema and lead to neutrophil recruitment in the affected tissue. In the pancreas, we have shown that activation of the transient receptor potential vanilloid-1 (TRPV1) ion channel expressed by primary sensory nerves plays a role in the inflammatory cascade in pancreatitis.^{1–5} We have proposed that the respective inflammatory insults cause the release of an endogenous TRPV1 agonist in the pancreas resulting in TRPV1 activation in pancreatic primary sensory nerves, which in turn release proinflammatory neurotransmitters such as substance P^{6,7} both peripherally within the pancreas itself to cause inflammation and centrally within the spinal cord to cause pain.^{8,9} However, the endogenous ligand that activates TRPV1 in pancreatitis is unknown. After the cloning and molecular characterization of TRPV1, it was discovered that leukotriene B₄ (LTB₄)¹⁰ can activate this receptor, raising the possibility that LTB₄ could be the endogenous ligand for TRPV1 after pancreatic injury. Despite this possible role, there is very limited evidence that LTB₄ is found in the pancreas.

The mechanisms involved in the pathophysiology of acute pancreatitis in humans are poorly understood. There are multiple animal models of experimental acute pancreatitis, but the relevance of most of them to human pancreatitis is unclear. One apparent exception is retrograde infusion of bile acids into the main pancreatic duct in rats and mice.^{1–3} It has been proposed that this experimental model is closely related to human biliary pancreatitis, which is thought to be caused by bile reflux into the pancreatic duct secondary to gallstone disease.⁴ There is evidence that acute pancreatitis

Abbreviations used in this paper: 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase-activating protein; G_βγ-1, G protein-coupled bile acid receptor-1; LTB₄, leukotriene B₄; MK886, 1[(4-chlorophenyl)methyl]-3-[[[1,1-dimethylethyl]thio]- α,α -dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid]; MPO, myeloperoxidase; NaT, sodium taurocholate; PCR, polymerase chain reaction; PPAR α , peroxisome proliferator-activated receptor α ; rER, rough endoplasmic reticulum; RTX, resiniferatoxin; TBS, Tris-buffered saline; TLCS, tauro lithocholic acid 3-sulfate disodium salt; TRPV1, transient receptor potential vanilloid-1.

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induced by bile acid in the mouse is initiated by the binding of bile acids to a G protein-coupled receptor in the pancreatic acinar cell apical plasma membrane. In this model, the bile acid receptor G protein-coupled bile acid receptor-1 (Gpbar-1, also known as TGR5) when activated by binding bile acids generates pathologic intracellular calcium transients, resulting in intra-acinar cell zymogen activation and acinar cell injury.⁵

In the present study, we tested the hypothesis that the mechanisms involved in acute pancreatitis induced by bile acid or secretagogue hyperstimulation include generation of an endogenous TRPV1 agonist that activates primary sensory nerves to injure the pancreas. We found that bile acids and caerulein hyperstimulation cause LTB₄ synthesis and secretion from pancreatic acinar cells and that this endogenous release of LTB₄ activates TRPV1 to contribute to acute pancreatitis.

Materials and Methods

These studies were approved by the Duke University Institutional Animal Care and Use Committee.

Animals

Mice in which the *Trpv1* gene has been deleted (*Trpv1*^{-/-}) were a kind gift of Dr. D. Julius, University of California–San Francisco. *Trpv1*^{-/-} mice and wild-type littermates were backcrossed onto a C57BL/6 background and genotyped as described previously elsewhere.¹¹

Materials

Sodium taurocholate (NaT), taurochenodeoxycholic acid 3-sulfate disodium salt (TLCS), and resiniferatoxin (RTX) were purchased from Sigma-Aldrich (St. Louis, MO), MK886 (1[[4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]- α , α -dimethyl-5-(1-methylethyl)-1*H*-indole-2-propanoic acid) was purchased from Tocris Bioscience (Ellisville, MO), and leukotriene B₄ (LTB₄) enzyme immunoassay kits were purchased from Cayman Chemical (Ann Arbor, MI). Purified mouse monoclonal anti-human 5-lipoxygenase (5-LO) primary antibody, biotinylated goat anti-mouse Ig secondary antibody, and streptavidin-horseradish peroxidase were purchased from BD Biosciences (San Jose, CA).

Induction of Acute Pancreatitis

Acute inflammation of the pancreas was stimulated by retrograde infusion of the main pancreatic duct with 2% NaT as described previously elsewhere.^{3,4} Briefly, adult male wild-type or *Trpv1*^{-/-} C57BL/6 mice were anesthetized by intraperitoneal injection of a mixture of 87.5% ketamine/12.5% xylazine, and a midline laparotomy was used to expose the first portion of the duodenum. A puncture wound was made in the antimesenteric surface of the duodenum opposite the ampulla of Vater, and a 30G catheter attached with tubing to an infusion pump was passed through the puncture wound and then into the common bile duct via the ampulla of Vater. The infusion catheter was secured in the distal common bile duct distal to the entrance of the pancreatic duct with a ligature, and

the bile duct near the liver was occluded with a bulldog clamp. The inflammatory infusate consisted of 2% NaT in 0.9% NaCl pumped into the pancreatic duct at a rate of 5 μ L/min for 10 minutes with a syringe pump. Methylene blue (1%) was included in the infusate to allow identification of leakage from the duct lumen. After 10 minutes, the catheter, ligature, and bulldog clamp were removed, and the duodenotomy was closed using a purse-string suture. The laparotomy was closed in two layers, and analgesia was achieved by subcutaneous injection of buprenorphine hydrochloride at a dose of 50 μ g/kg. The animals were given free access to food and water upon recovery. The mice were killed 24 hours after surgery by CO₂ asphyxiation and then were weighed. The pancreas was removed and weighed; a portion was frozen at -80°C for later myeloperoxidase (MPO) assay, and a separate portion was fixed overnight at 4°C in 10% formalin for the histopathologic analysis. Mixed arteriovenous blood was also collected by decapitation for serum amylase measurement.

Acute pancreatitis was also induced by secretagogue hyperstimulation using 6 hourly subcutaneous injections of caerulein at a dose of 50 μ g/kg, as previously described elsewhere.^{7,12} The mice were killed 1 hour after the last caerulein injection by CO₂ asphyxiation, and the tissues collected as described previously.

The pharmacologic treatments tested for their ability to inhibit NaT-induced acute pancreatitis included RTX, an excitotoxin that desensitizes TRPV1 when used at high concentrations,¹³ and MK886, a 5-lipoxygenase-activating peptide (FLAP) inhibitor that inhibits LTB₄ biosynthesis by blocking 5-LO activity.¹⁴ RTX was dissolved in ethanol and administered in 97.5% saline/2.5% ethanol either alone (in control experiments) or together with 2% NaT in 0.9% NaCl at a concentration of 14 μ g/mL in the pancreatic ductal infusate by retrograde infusion at 5 μ L/min for 10 minutes. RTX was given by intraductal infusion because this route of delivery is effective in desensitizing TRPV1.¹⁵ The same RTX dose was demonstrated to be efficacious in inhibiting acute pancreatitis in rats caused by pancreatic ductal infusion of low pH endoscopic retrograde cholangiopancreatography contrast solution.¹⁵ MK886 was administered as a pre-treatment 1 hour before surgery by intraperitoneal injection at a dose of 10 mg/kg dissolved in 2% ethanol/2% Tween 80/96% sterile 0.9% NaCl. Control mice were treated similarly with the vehicle alone. This dose of MK886 has previously been shown to inhibit pancreatic duct ligation-induced acute pancreatitis in rats.¹⁶

Serum Amylase Activity

The serum amylase concentration was measured as previously described elsewhere¹⁷ except that Phadebas amylase test tablets (Magle Life Sciences, Cambridge, MA) were used as substrate instead of Procion Yellow starch.

Myeloperoxidase Activity

We measured the tissue activity of MPO, an enzyme produced by neutrophils and used as a marker of inflammation associated with neutrophil infiltration, as previously described elsewhere using the substrate tetramethylbenzidine.^{18,19} The

pancreatic total protein was measured using micro-BCA kits (Thermo Scientific, Rockford, IL).

Histopathology

Portions of the pancreata were fixed overnight in phosphate-buffered 10% formalin. The tissue was then embedded in paraffin, sectioned at 5 μm , stained with H&E, and coded for examination by two investigators blinded to the experimental design. The severity of pancreatitis was graded using a modified scoring criteria previously described elsewhere.²⁰

Leukotriene B₄ Assay

Pancreatic LTB₄ levels were measured by LTB₄ enzyme immunoassay kits purchased from Cayman Chemical. Briefly, samples of pancreas were collected in 5 volumes of ice-cold 0.1 M phosphate buffer, pH 7.4, containing 1 mmol/L EDTA and 10 $\mu\text{mol/L}$ indomethacin and were homogenized for 15 seconds on ice using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at a 60% power setting for 15 seconds. After homogenization, 2 volumes of ice-cold ethanol were added to each extract, and the extracts were then incubated on ice for 5 minutes to precipitate proteins. After centrifugation at 3000g_{max} to remove the precipitated proteins, the ethanol in the supernates was removed by vacuum centrifugation. The pH of the extracts was adjusted to ~ 4.0 by addition of 1 M sodium acetate (pH 4.0). The resulting precipitate was removed by centrifugation, and the supernate was loaded onto C-18 solid phase extraction cartridges (Cayman Chemical) previously washed with methanol and distilled water, washed with distilled water, and then eluted at unit gravity with 5 mL of 99% ethyl acetate/1% methanol. The samples were then evaporated to dryness under a stream of nitrogen at 45°C, reconstituted in LTB₄ enzyme immunoassay buffer, and assayed according to the instructions of the kit manufacturer.

Acinar cell LTB₄ release *in vitro* was measured using the supernate obtained after centrifuging suspensions of pancreatic acini prepared as previously described elsewhere,²¹ preincubated for 30 minutes at 37°C, and incubated with or without the bile acid TLCS (500 $\mu\text{mol/L}$) or caerulein (100 pmol/L) for 30 minutes at 37°C with constant shaking in a water bath. When the effects of MK886 were tested, the acini were preincubated with 1 $\mu\text{mol/L}$ MK886 for 30 minutes at 37°C, and then incubated with 1 $\mu\text{mol/L}$ MK886 with or without TLCS for 30 minutes at 37°C with constant shaking in a water bath.

5-Lipoxygenase Immunohistochemistry

Immunohistochemical staining for 5-LO was performed as previously described elsewhere²² with minor modifications. Portions of the head of the mouse pancreas were taken 24 hours after duct infusion, fixed overnight in 10% buffered formalin at 4°C, and embedded in paraffin; sections 5- μm thick were prepared and mounted on glass slides. After deparaffinization and rehydration, antigen retrieval was performed by incubating the sections in 10 mmol/L citric acid (pH 6.0) in a microwave oven for 12 minutes (2

minutes high power, 10 minutes low power). The slides were then cooled to room temperature and washed in Tris-buffered saline (TBS: 50 mmol/L Tris, 0.9% NaCl, pH 7.6). Nonspecific staining was blocked by incubating the sections in full-strength normal goat serum for 30 minutes at room temperature. The sections were then incubated with the monoclonal primary antibody (mouse anti-human 5-LO; BD Biosciences) at a dilution of 1:500 in TBS-5% goat serum overnight at 4°C. After washing in TBS-5% goat serum, the sections were incubated with biotinylated secondary antibody (goat anti-mouse Ig; BD Biosciences) at a dilution of 1:100 in TBS-5% goat serum for 1 hour at room temperature. Detection of the antibody complex was performed using the streptavidin-horseradish peroxidase reaction with diaminobenzidine as chromogen. Control sections were treated identically except the primary antibody incubation was omitted. All sections were counterstained with hematoxylin for 30 seconds at room temperature.

Human pancreas was obtained from patients at the time of surgery for non-pancreas-involved conditions (controls) and for complications of trauma-induced acute pancreatitis and then treated as described previously. Samples were secured through the Duke University School of Medicine Biospecimen Repository and Processing Core (DUHS IRB Pro00035974) as approved by the institutional review board.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA from fresh pancreatic tissue was purified using the Ribopure kit (Life Technologies, Grand Island, NY) following the manufacturer's instructions. RNA (2 μg) was reverse transcribed using High Capacity RNA-to-cDNA kits (Life Technologies), and polymerase chain reaction (PCR) was performed using the Taqman gene expression master mix (Life Technologies) and Taqman gene expression assays for the *Alox5* (5-LO) and *Actb* (actin) genes (Life Technologies). *Actb* was the reference gene. Assays were performed in triplicate. The level of expression was determined using the $2^{-\Delta\Delta\text{Ct}}$ method.²³

Western Blot Analysis

Tissue from the head of the pancreas obtained after control or NaT treatment as described previously was solubilized on ice in a lysis buffer consisting of NaCl (150 mmol/L), Tris, pH 7.5 (50 mmol/L), EDTA, pH 8.0 (10 mmol/L), dithiothreitol (1 mmol/L), Nonidet P-40 (1% v/v), sodium deoxycholate (0.5% w/v), sodium dodecyl sulfate (0.1% w/v), leupeptin (10 $\mu\text{g/mL}$), aprotinin (10 $\mu\text{g/mL}$), one tablet of protease inhibitors (cOmplete-Mini; Roche Diagnostics, Indianapolis, IN), and one tablet of phosphatase inhibitor (PhosSTOP; Roche Diagnostics) per 7 mL of solution. Supernates (10 μg protein) were loaded onto 4%–12% NuPage gels (Life Technologies), electrophoresed in 3-(*N*-morpholino)propanesulfonic acid buffer, and transferred onto polyvinylidene difluoride membranes (PerkinElmer, Waltham, MA). Blots were first reacted with a mouse monoclonal anti-5-LO antibody (BD Biosciences) at a dilution of 1/1000 and then with a rabbit polyclonal anti- α -tubulin antibody (GeneTex, Irvine, CA) at a dilution of

1/10,000. Bands were detected with the West Femto substrate (Thermo Scientific).

Statistical Analysis

Results are expressed as mean \pm standard error of the mean (SEM). Mean differences between two groups were examined by the Student *t* test and mean differences among several groups by one-way analysis of variance with the Dunnett's or Tukey-Kramer posttests, using GraphPad InStat version 3.05 for Windows (GraphPad Software, San Diego, CA). $P < .05$ was considered statistically significant.

Results

To determine whether infusion of NaT into the mouse pancreatic duct causes pancreatitis, we examined several features of acute pancreatitis 24 hours after instillation of

50 μ L of 2% NaT. As shown in Figure 1A, pancreatic histology was markedly altered with distortion of acini, inflammatory cell infiltration, and tissue necrosis. Changes in specific anatomic and biochemical parameters confirmed the development of pancreatitis and were consistent with the observed histologic changes. The NaT treatment produced a significant increase in the plasma concentration of amylase 24 hours after infusion, consistent with the induction of pancreatic injury (Figure 1C). An increase in pancreatic edema, a feature common to many types of acute pancreatitis and typical of neurogenic inflammation, also was observed in NaT-treated mice as indicated by the elevated ratio of the wet weight of the pancreas to total body weight (Figure 1D). Finally, consistent with the finding of inflammatory cells in the pancreas after NaT treatment, there was a marked increase in MPO concentrations (Figure 1E). MPO is highly expressed in neutrophils and is

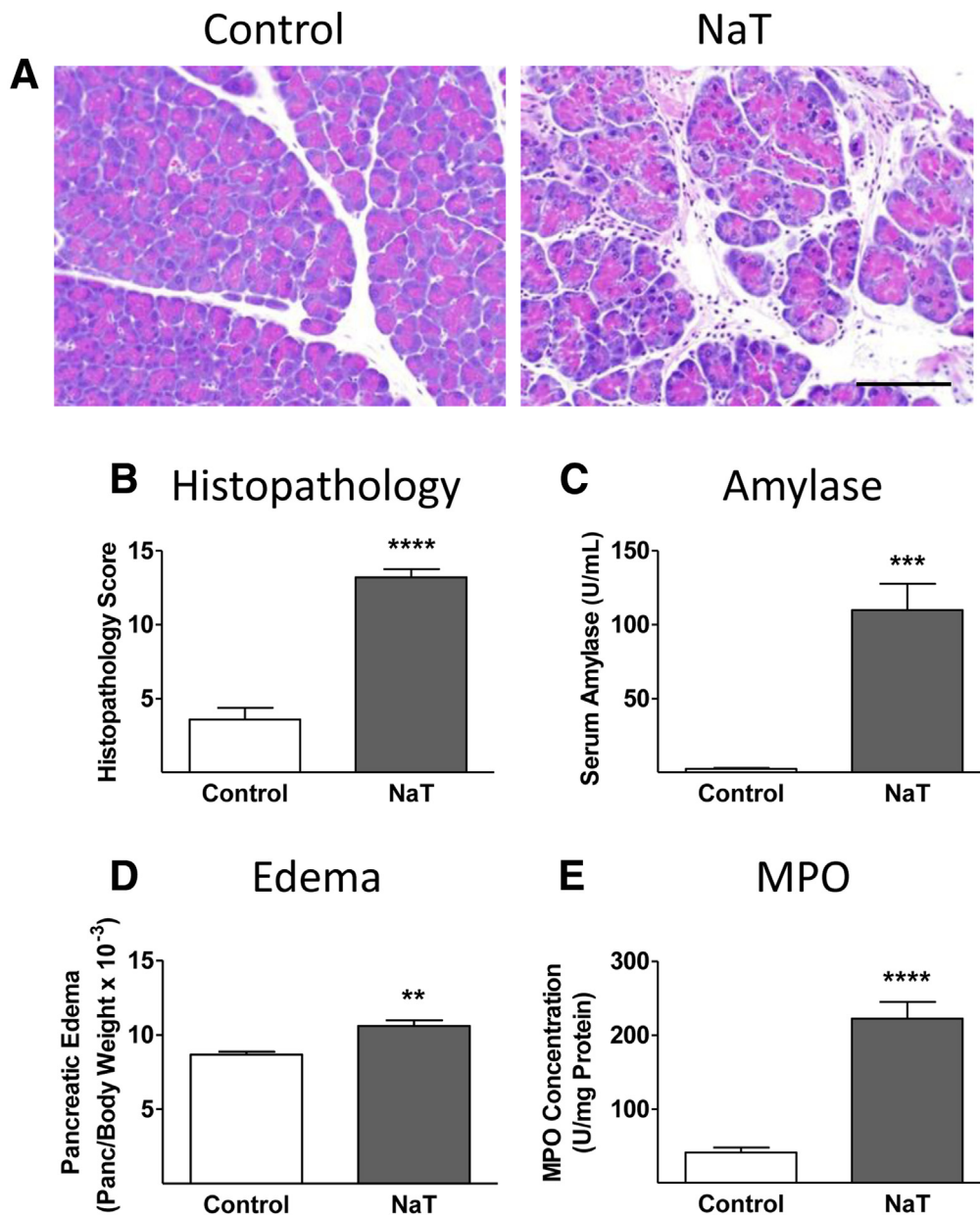


Figure 1. Intrapancreatic duct infusion of bile acids causes pancreatitis. The effects of pancreatic duct infusion of 50 μ L of 2% NaT on pancreatic histopathology, serum amylase levels, pancreatic edema, and pancreatic MPO levels are shown ($n = 6$). (A) The effects of pancreatic duct infusion with 2% NaT on pancreatic histology. (B) The effects of NaT on histopathology score (**** $P < .0001$ vs control). (C) The effects of NaT on serum amylase levels (*** $P < .001$ vs control). (D) The effects of NaT on pancreatic edema (** $P < .01$ vs control). (E) The effects of NaT on pancreatic MPO levels (**** $P < .0001$ vs control). Scale bar = 100 μ m.

commonly used as a biochemical marker of neutrophil infiltration. Together, these data indicate that bile duct infusion of NaT induces acute pancreatitis.

We previously reported that neurogenic inflammation contributes to the pathogenesis of pancreatitis produced by secretagogue hyperstimulation.^{7,12} Moreover, the manifestation of this inflammation requires the activation of the vanilloid receptor TRPV1. To determine whether bile acids induce neurogenic pancreatitis, we used two methods to examine the role of TRPV1. First, to determine whether TRPV1 mediates some or all of pancreatic edema induced by bile acid, the effect of adding a desensitizing dose of the TRPV1 excitotoxin RTX to NaT in the pancreatic duct infusion was tested. As shown in Figure 2, when RTX was coinjected with NaT, there was a marked improvement in the

histologic features of pancreatitis compared with NaT administration alone. RTX also significantly reduced blood amylase levels and pancreatic edema over control levels. Notably, duct infusion with RTX alone had no effect on pancreatic edema. Consistent with the lower numbers of inflammatory cells seen in histologic sections of the pancreas, the addition of RTX to the NaT infusion resulted in much lower MPO levels in the pancreas than seen with NaT administration alone.

The second method for evaluating the role of TRPV1 in pancreatitis induced by bile acid was to test the effects of NaT administration in mice with genetic deletion of TRPV1. *Trpv1*^{-/-} mice treated with NaT did not develop the histologic features typical of acute pancreatitis (Figure 3). The histologic scoring parameters and pancreatic MPO levels

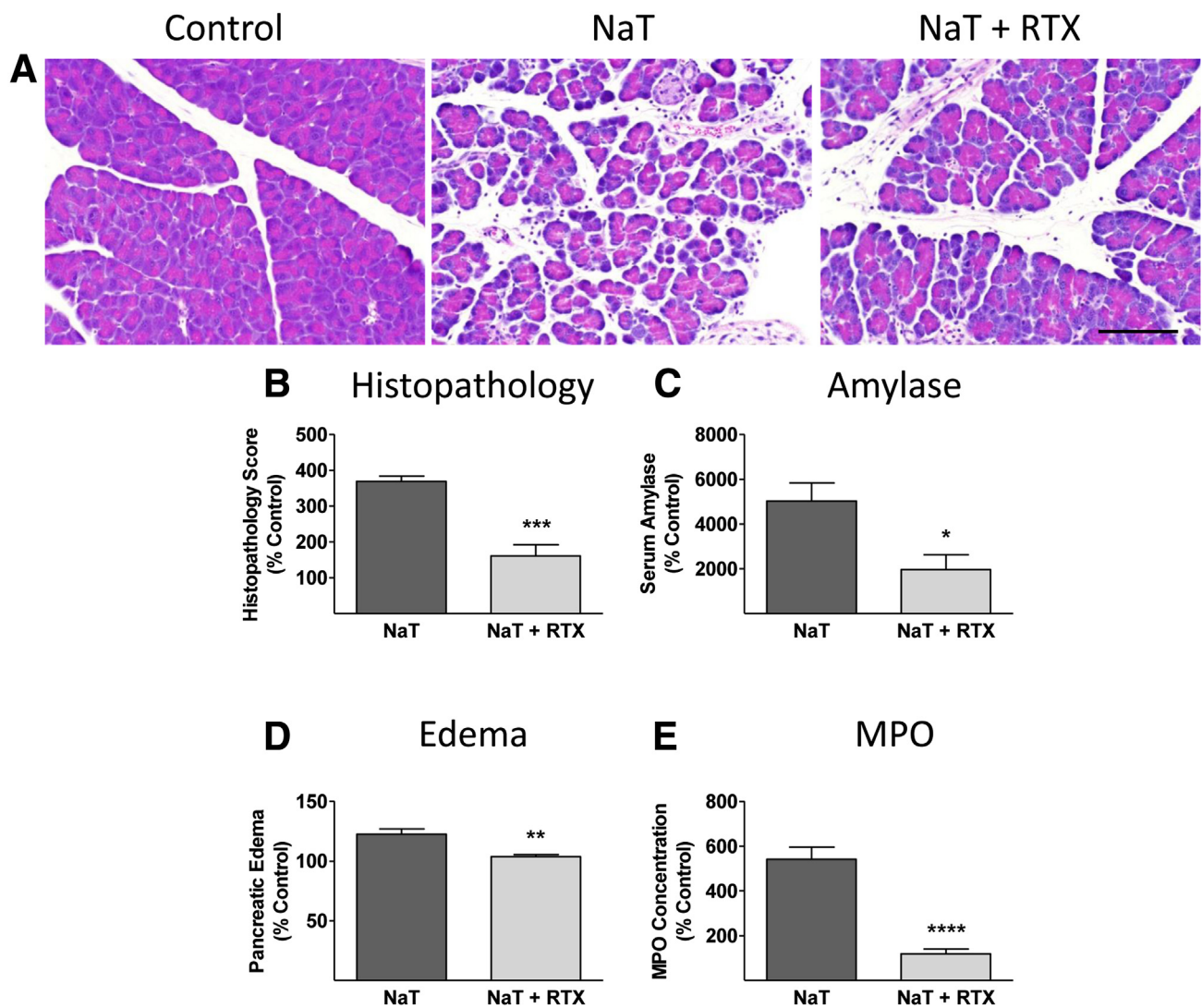


Figure 2. The effects of RTX desensitization of TRPV1 in pancreatitis induced by bile acid. NaT with or without 14 μg/mL RTX was infused into the pancreatic duct of wild-type mice (n = 6). (A) The effects of pancreatic duct infusion with 2% NaT on pancreatic histology and the protective effect of coinjection of RTX. (B) The effects of NaT and NaT + RTX on histopathology score, expressed as a percentage of the control value (***P < .001 vs NaT). (C) The effects of NaT and NaT + RTX on serum amylase, expressed as a percentage of the control value (*P < .05 vs NaT). (D) The effects of NaT and NaT + RTX on pancreatic edema, expressed as a percentage of the control value (**P < .01 vs NaT). (E) The effects of NaT and NaT + RTX on pancreatic MPO concentration, expressed as a percentage of the control value (****P < .0001 vs NaT). Scale bar = 100 μm.

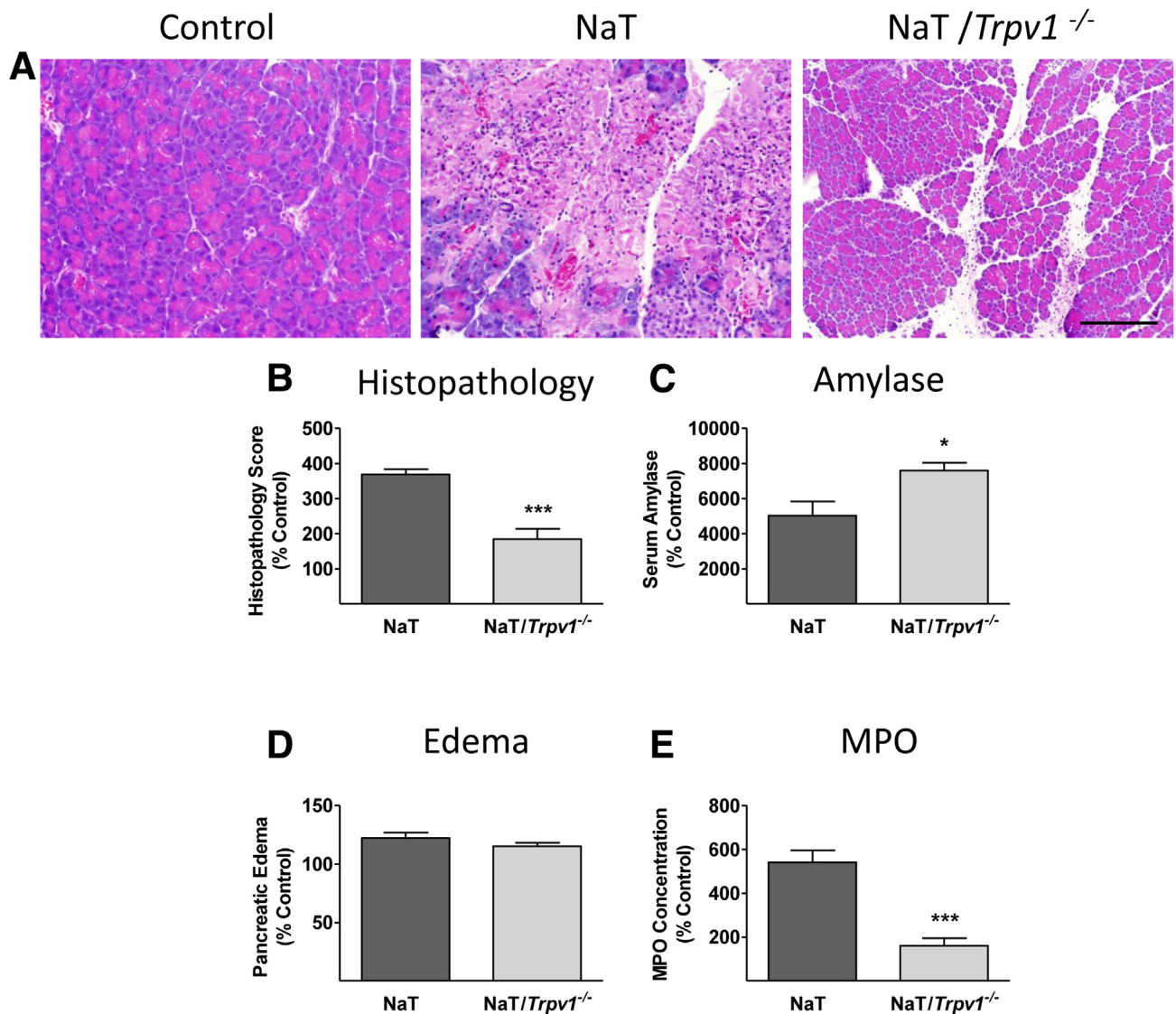


Figure 3. The effects of intraductal NaT in *Trpv1*^{-/-} mice. NaT was infused into the pancreatic duct of *Trpv1*^{-/-} mice (N = 6). (A) The effects of pancreatic duct infusion with 2% NaT on pancreatic histology in wild-type and *Trpv1*^{-/-} mice. (B) The effects of NaT on histopathology score in wild-type and *Trpv1*^{-/-} mice, expressed as a percentage of the control value (***P* < .001 vs NaT) (C) The effects of NaT on serum amylase in wild-type and *Trpv1*^{-/-} mice, expressed as a percentage of the control value (**P* < .05 vs NaT). (D) The effects of NaT on pancreatic edema in wild-type and *Trpv1*^{-/-} mice, expressed as a percentage of the control value. (E) The effects of NaT on pancreatic MPO concentration in wild-type and *Trpv1*^{-/-} mice, expressed as a percentage of the control value (***P* < .001 vs NaT). Scale bar = 100 μ m.

were significantly lower in *Trpv1*^{-/-} mice. Blood amylase levels were not lower in *Trpv1*^{-/-} mice, suggesting to us that bile acids may injure acinar cells directly or affect pancreatic release of amylase, as we had observed previously in the secretagogue hyperstimulation model.⁷ However, this process does not appear to involve TRPV1 and does not cause pancreatitis in *Trpv1*^{-/-} mice. Taken together, it appears that both pharmacologic blockade of TRPV1 with RTX and genetic deletion of TRPV1 protect against pancreatitis induced by bile acid.

Leukotriene B₄ can activate TRPV1,¹⁰ and we previously reported that LTB₄ infusion into the pancreas can induce pancreatic inflammation.¹⁶ We next sought to determine whether bile acids stimulate pancreatic LTB₄ synthesis. To

address this question, we measured the concentration of LTB₄ in pancreatic extracts 24 hours after duct infusion of NaT or 1 hour after caerulein hyperstimulation. As shown in Figure 4A, NaT and caerulein (inset) stimulated highly significant increases in pancreatic LTB₄ content compared with controls. The rate-limiting enzyme in the biosynthesis of LTB₄ is 5-LO. The cofactor FLAP is necessary for the biosynthesis to occur. Thus, inhibition of FLAP prevents LTB₄ formation. We observed that pretreatment of the NaT-treated animals with the FLAP inhibitor MK886 significantly reduced pancreatic LTB₄ levels (Figure 4A).

Next, we tested the ability of pancreatic acini to secrete LTB₄ in response to bile acid or caerulein in vitro to confirm that acinar cells are the source of LTB₄ in the pancreas and to

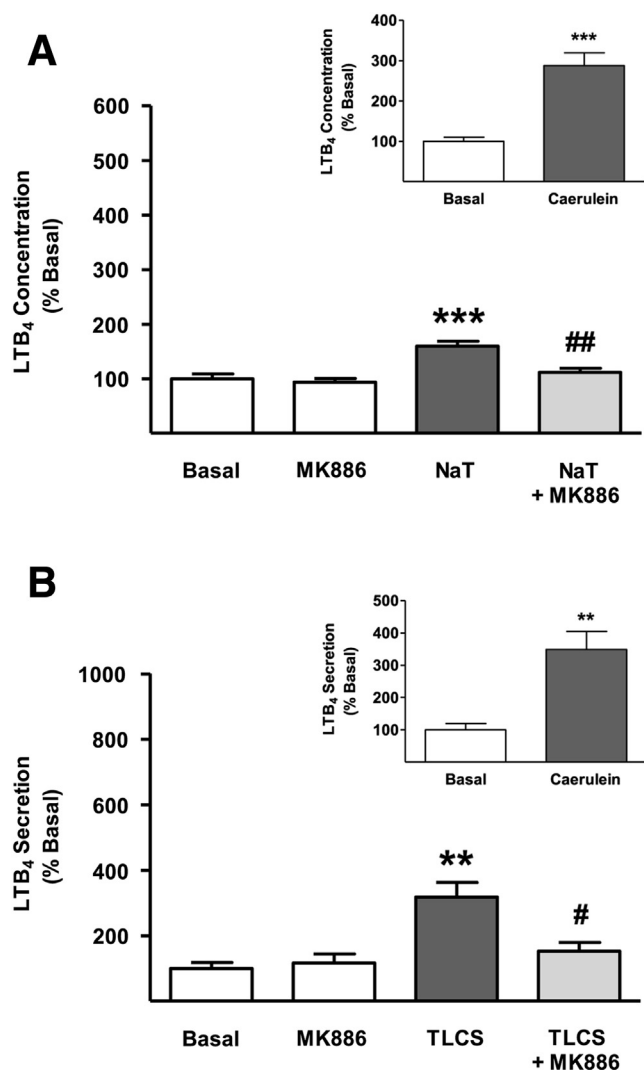


Figure 4. Bile acids stimulate LTB₄ formation in the pancreas. (A) Effect of pancreatic duct infusion of NaT on LTB₄ concentrations in the head of the pancreas in vivo and inhibition of the NaT-stimulated increase in pancreatic LTB₄ levels by pretreatment with 1 μ mol/L MK886, a FLAP inhibitor ($n = 6$; *** $P < .001$ vs control; ## $P < .01$ vs NaT). *Inset:* The effect of caerulein hyperstimulation on LTB₄ concentrations in the head of the pancreas in vivo ($n = 6$; *** $P < .001$ vs control). (B) Effect of TLCS on LTB₄ secretion from wild-type mouse pancreatic acini in vitro. TLCS (500 μ mol/L) significantly stimulated LTB₄ secretion from pancreatic acini ($n = 5-9$; ** $P < .01$ vs control) and this effect was significantly inhibited by 1 μ mol/L MK886 (# $P < .05$ vs TLCS alone). *Inset:* The effect of caerulein hyperstimulation (100 pmol/L) on LTB₄ secretion from pancreatic acini ($n = 6$; ** $P < .01$ vs control). LTB₄ levels were normalized to pancreatic protein concentrations and expressed as a percentage of the control values.

determine whether bile acids and caerulein directly stimulate LTB₄ secretion from acinar cells. We used the bile acid TLCS instead of NaT in these experiments for the following reasons: previous observations had indicated that NaT in concentrations as low as 0.2% causes nonspecific acinar cell injury in vitro,³ and TLCS, a naturally occurring bile acid in mice and humans, had been shown to cause acinar cell injury

at concentrations below detergent-like or ionophore-like effects and below pathologic relevance.^{5,24} Incubation of pancreatic acini from wild-type mice with 500 μ mol/L TLCS for 30 minutes in vitro stimulated a 3-fold increase in LTB₄ secretion over basal levels ($P < .001$; Figure 4B); caerulein hyperstimulation had the same effect (*inset*). TLCS at the nondamaging concentration of 100 μ mol/L did not cause a significant increase in LTB₄ secretion. Notably the FLAP inhibitor MK886 significantly inhibited TLCS-stimulated LTB₄ synthesis, demonstrating that pancreatic acinar cells also synthesize LTB₄ in response to bile acids.

These results raised the question of the provenance of LTB₄ in the pancreas. Leukocytes are known to be a rich source of 5-LO and LTB₄,¹⁴ and it is well known that acute pancreatitis exhibits increased neutrophil infiltration into the pancreas. However, a parenchymal origin of LTB₄ could better account for the inhibition of acute pancreatitis by TRPV1 blockade that we observed because of the close proximity of acinar cells to nerve endings. Therefore, we sought to determine the source of LTB₄ using immunohistochemistry for 5-LO, the rate-limiting enzyme in LTB₄ biosynthesis.¹⁴

Very little immunoreactive 5-LO was observed in the pancreas of control mice whose pancreatic ducts were perfused with the saline vehicle (Figure 5A). In contrast, retrograde pancreatic duct infusion with the bile acid NaT caused intense 5-LO immunostaining in acinar cells and a marked increase in the number of acinar cells showing 5-LO immunoreactivity (Figure 5A). The pancreatic ducts exhibited little immunoreactivity, but islets appeared to express small amounts of 5-LO. In bile acid-treated animals, 5-LO immunoreactivity was most prominent in the basal poles of the acinar cells, consistent with the distribution of rough endoplasmic reticulum (rER). A similar 5-LO immunostaining pattern was observed when the pancreata of NaT-treated *Trpv1*^{-/-} mice were analyzed (Figure 5A). This result indicated that bile acid-stimulated LTB₄ activation is proximal to TRPV1 stimulation in the inflammatory mechanism. Interestingly, when wild-type mice were pretreated with MK886 before the NaT perfusion, 5-LO immunostaining in the pancreas was abolished (Figure 5A), indicating that FLAP activity is required both for inflammation induced by bile acid and for increased 5-LO immunoreactivity in the pancreas.

The increased 5-LO immunoreactivity that accompanied acute pancreatitis led us to ask whether increased 5-LO expression accounted for this observation. Quantitative real-time PCR analysis revealed that there was no significant difference in *Alox5* (5-LO) mRNA expression between the pancreases of control and NaT-treated mice (Figure 5B). Similarly, Western blot analysis showed no significant difference in pancreatic 5-LO protein levels between control and bile acid-treated mice (Figure 5C and D).

To determine whether 5-LO activation was relevant to human pancreatitis, we performed 5-LO immunohistochemical staining in pancreas of patients who underwent surgery for nonpancreatic conditions (controls) and for acute pancreatitis. Similar to the findings we observed in mice, 5-LO immunoreactivity was very low in control patients (Figure 5E) but was prominently expressed in the inflamed pancreas from a patient with acute pancreatitis

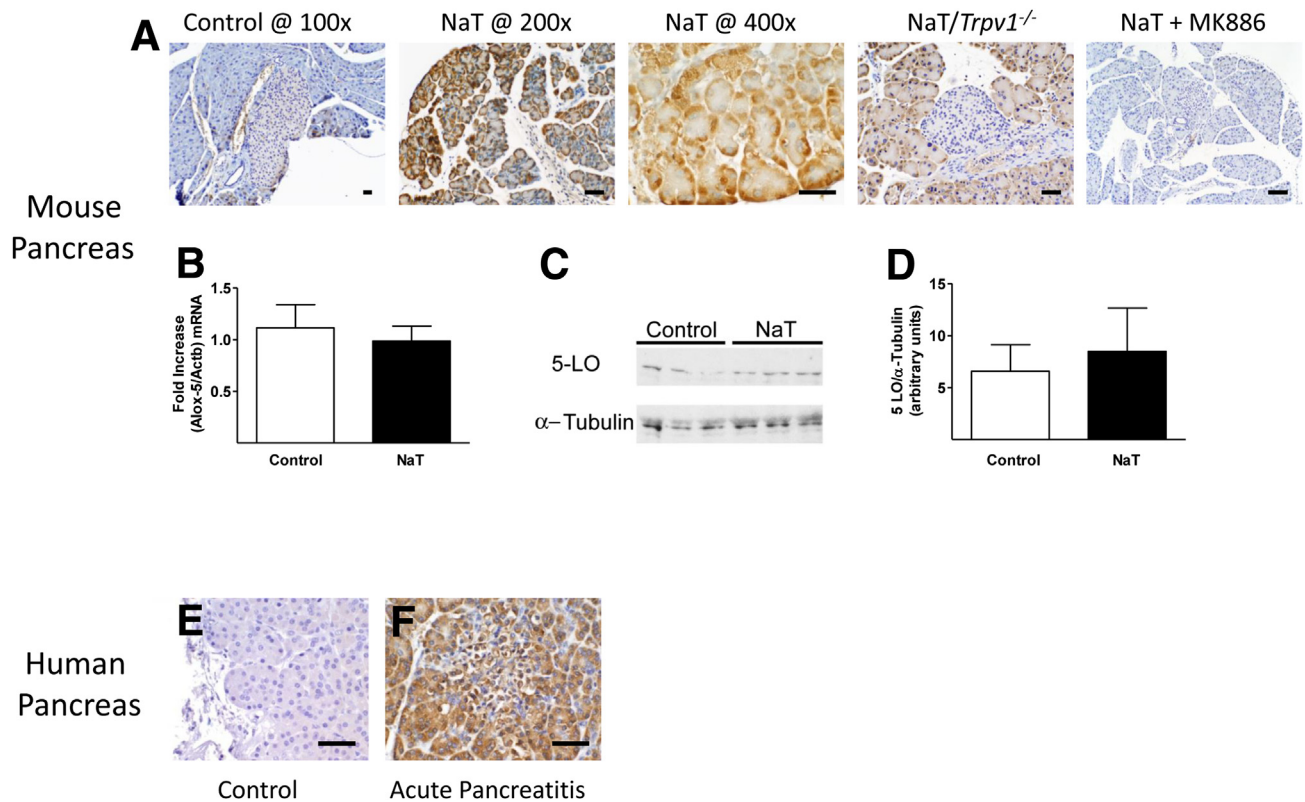


Figure 5. Bile acids regulate 5-lipoxygenase immunoreactivity in the mouse pancreas. (A) Photomicrographs of sections taken from the head of the mouse pancreas and stained immunohistochemically for 5-LO. Pancreatic duct infusion with vehicle (control) in wild-type mice resulted in little 5-LO immunoreactivity. Pancreatic duct infusion with NaT in wild-type mice (magnification 200 \times) elicited increased 5-LO immunoreactivity seen mainly in acinar cells localized mainly in the basal poles of the cells as seen at magnification 400 \times . Pancreatic duct infusion with NaT in *Trpv1*^{-/-} mice caused a similar pattern of 5-LO expression as seen in wild-type mice. Pancreatic duct infusion with NaT after pretreatment with MK886 in wild-type mice resulted in the virtual abolition of 5-LO immunoreactivity. (B) The effects of NaT on 5-LO mRNA. The pancreatic ducts of wild-type mice were retrogradely infused with vehicle (control) or NaT, and 24 hours later portions of the head of the pancreas were separately collected. RNA was purified from one portion and reverse transcribed to cDNA, and the *Alox5* gene (5-LO) was assessed by quantitative real-time PCR. There was no significant difference in the levels of 5-LO RNA in control versus NaT-treated sample. (C) Western blots showed that NaT did not affect the level of 5-LO protein expression in the head of the pancreas, and (D) densitometric analysis of the immunoblot confirmed the lack of significant differences. (E and F) The effect of acute pancreatitis on 5-LO immunoreactivity in the human pancreas: (E) control and (F) acute pancreatitis. Human acute pancreatitis patients showed a pattern of increased 5-LO immunoreactivity similar to that seen in mice. Scale bars = 50 μ m.

(Figure 5F). Moreover, 5-LO immunostaining was most intense at the basal poles of acinar cells where rER is abundant (Figure 5F).

Finally, to determine whether endogenous LTB₄ synthesis is critical for the genesis of pancreatitis, we tested the effects of the FLAP inhibitor MK886 on NaT-induced pancreatitis in vivo. As shown in Figure 6, MK886 pretreatment reduced the severity of NaT-induced histologic changes, blood amylase levels, pancreatic edema, and MPO levels. These changes of reduced pancreatitis severity are similar to those observed in RTX-treated wild-type and *Trpv1*^{-/-} mice, supporting the concept that recently synthesized LTB₄ mediates bile acid-induced pancreatitis.

Discussion

The present results demonstrate that acinar cell secretion of LTB₄ plays an important role in acute pancreatitis induced by bile acid or caerulein hyperstimulation. This is consistent

with previous studies implicating LTB₄ in experimental acute pancreatitis, such as the demonstrations that intraductal NaT in rats elicited increased pancreatic levels of LTB₄,²⁵⁻²⁷ pharmacologic inhibition of 5-LO in the same rat model protected against histologic pancreatic damage,²⁸ genetic deletion of 5-LO inhibited acute pancreatitis in the caerulein hyperstimulation model in mice,²⁹ and a dual COX-2/5-LO pharmacologic inhibitor protected rats against caerulein hyperstimulation-induced pancreatic inflammation.³⁰ However, the cellular source of LTB₄ and the mechanism by which it induces acute pancreatitis was not revealed in those studies. We show here that pancreatic acinar cells are the source of LTB₄ released in response to pancreatitis induced by bile acid or caerulein hyperstimulation, and that this LTB₄ activates TRPV1 to cause inflammation.

We first demonstrated that desensitization of TRPV1 using the TRPV1 excitotoxin RTX resulted in significant inhibition of several indices of NaT-induced pancreatitis, including pancreatic histopathology, serum amylase

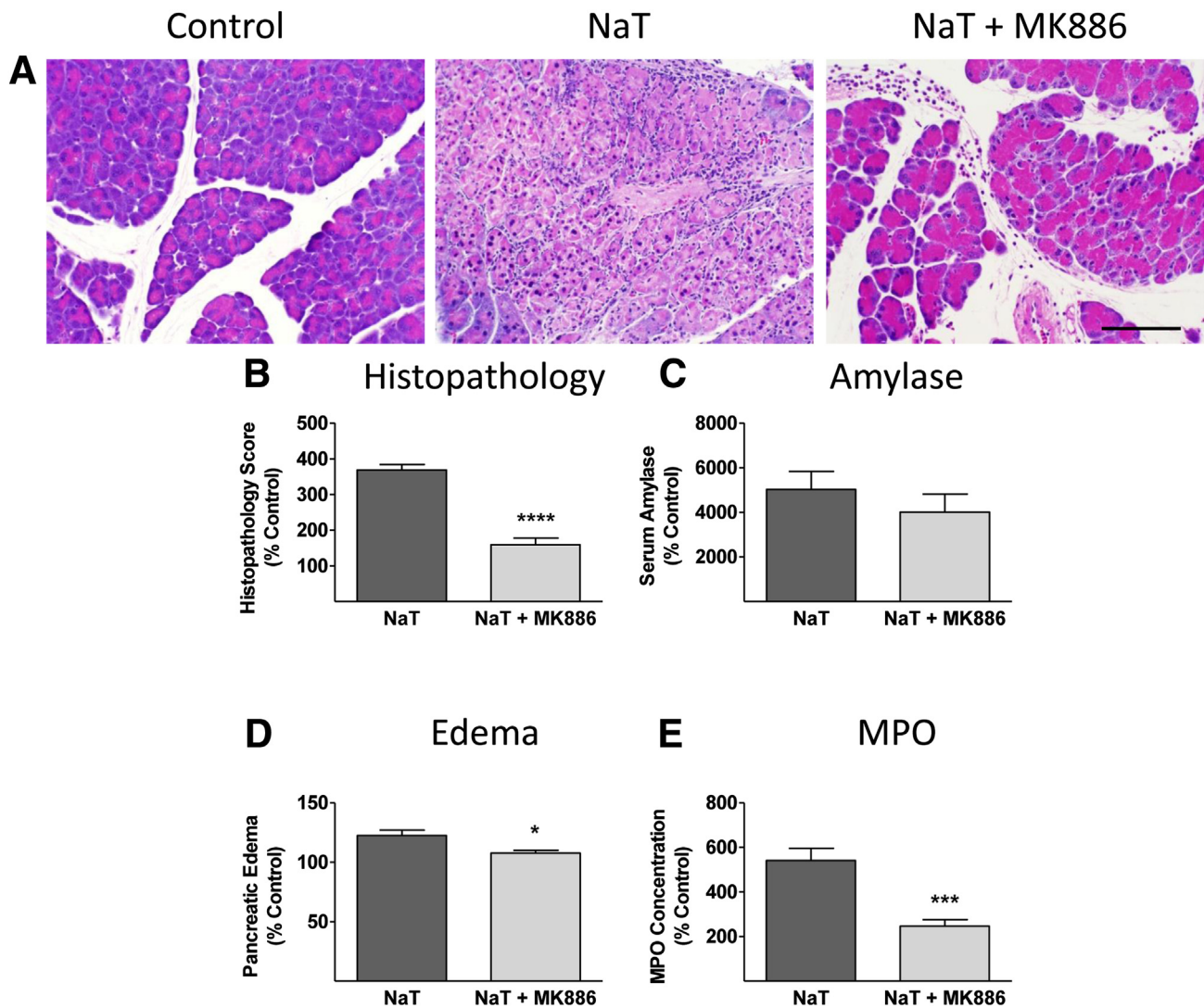


Figure 6. Bile acid-induced pancreatitis requires 5-LO activation. Wild-type mice were pretreated with the FLAP inhibitor MK886 before intrapancreatic duct infusion of NaT ($n = 6-7$). (A) The effects of pancreatic duct infusion with 2% NaT on pancreatic histology and the protective effects of pretreatment with 10 mg/kg MK886 on pancreatic histology. (B) The effects of NaT and NaT + MK886 on histopathology score, expressed as a percentage of the control value (**** $P < .0001$ vs NaT). (C) The effects of NaT and NaT + MK886 on serum amylase, expressed as a percentage of the control value. (D) The effects of NaT and NaT + MK886 on pancreatic edema, expressed as a percentage of the control value (* $P < .05$ vs NaT). (E) The effects of NaT and NaT + MK886 on pancreatic MPO concentration, expressed as a percentage of the control value (*** $P < .001$ vs NaT).

concentrations, pancreatic edema, and pancreatic MPO levels. TRPV1 gene deletion significantly inhibited pancreatic histopathology and MPO levels but had no effect on pancreatic edema. In addition, TRPV1 gene deletion did not inhibit the NaT-induced elevation of serum amylase concentrations, suggesting that this effect of NaT is a direct one on pancreatic acinar cells that does not involve proinflammatory neurotransmitter release secondary to TRPV1 activation, although some other pathway involving a TRPV1-expressing cell type may be involved because RTX treatment, which causes desensitization or loss of TRPV1-expressing cells, did significantly reduce NaT-induced edema. These results taken together indicated that NaT causes the release of an endogenous TRPV1 agonist ligand in the pancreas.

We had previously demonstrated that TRPV1 plays a role in acute experimental pancreatitis in several other

experimental models, such as secretagogue hyperstimulation,^{7,12,31} ligation of the common pancreaticobiliary duct,^{12,16} and retrograde perfusion of the pancreatic duct by endoscopic retrograde cholangiopancreatography contrast medium.¹⁵ In these models, we proposed that the respective inflammatory insults cause the release of an endogenous TRPV1 agonist in the pancreas, resulting in TRPV1 activation in pancreatic primary sensory nerves, which in turn release proinflammatory neurotransmitters such as substance P^{6,7} both peripherally within the pancreas itself to cause inflammation and centrally within the spinal cord to cause pain.^{8,9} We provided evidence in the duct ligation model of pancreatitis that the endogenous TRPV1 agonist LTB₄¹⁰ could be an endogenous TRPV1 activator generated by the inflammatory insult to the tissue.¹⁶

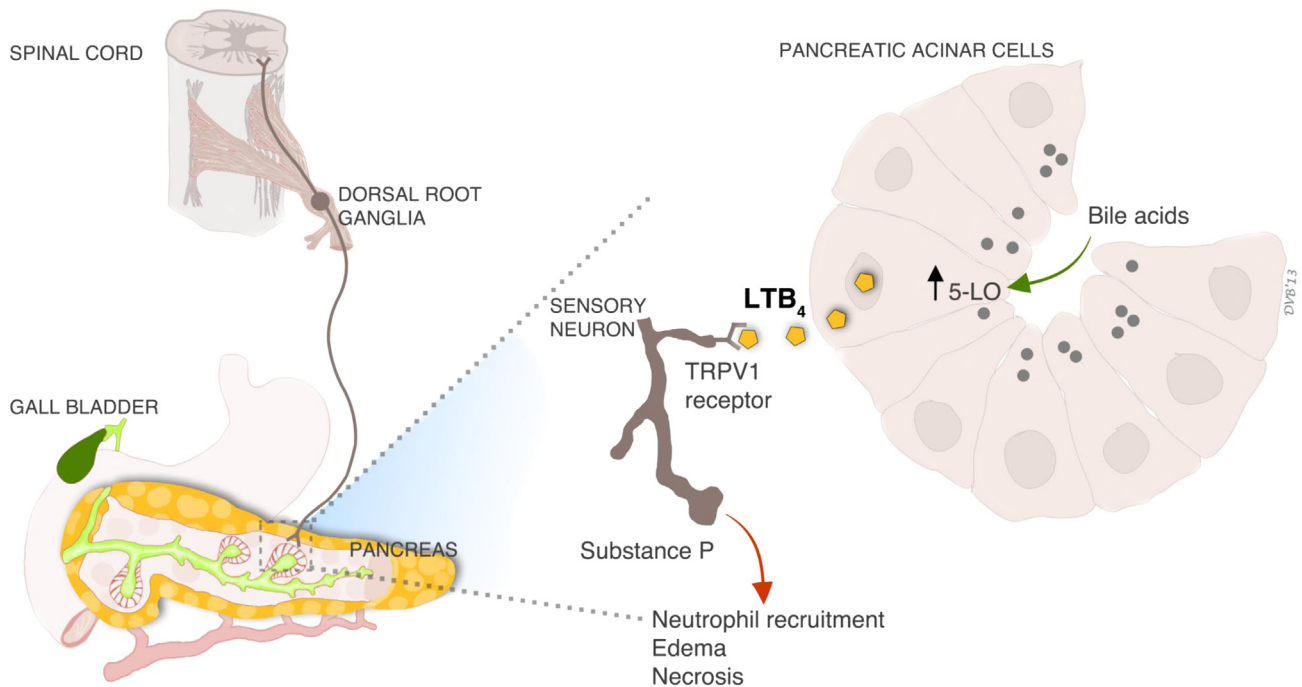


Figure 7. A model depicting the mechanisms by which intraductal bile acids cause acute pancreatitis in the mouse. Bile acids bind to the bile acid receptor Gpbar1 expressed on the apical plasma membranes of acinar cells.⁵ This interaction causes increased 5-LO activity in the acinar cell, resulting subsequently in LTB₄ synthesis and secretion from the cell. Increased pancreatic LTB₄ concentrations can then bind to TRPV1 receptors expressed by a subclass of primary sensory afferent nerves innervating the pancreas, resulting in the peripheral release of proinflammatory neurotransmitters such as substance P, which subsequently causes neutrophil recruitment, edema and necrosis, and acute pancreatitis.

Based on those previous results, we tested the hypothesis that NaT stimulates LTB₄ release in the pancreas. First, we showed that pharmacologic inhibition of FLAP by pretreatment with the drug MK886 inhibits NaT-induced pancreatic inflammation. FLAP is an essential cofactor for 5-LO activity (the rate-limiting enzyme in LTB₄ biosynthesis¹⁴). MK886 has been shown to inhibit peroxisome proliferator-activated receptor α (PPAR α) as well as FLAP,³² but PPAR α inhibition cannot explain the present findings because activation of PPAR α has been shown to reduce pancreatic injury in experimental pancreatitis.³³ Thus, a drug inhibiting PPAR α such as MK886 would be expected to enhance pancreatitis instead of reducing it, as occurred in our study. Second, we showed directly that intraductal NaT administration caused an increase in pancreatic levels of LTB₄ and that this effect was inhibited by pretreatment with MK886. These results suggest that intraductal NaT activates 5-LO in the pancreas, resulting in local production of LTB₄ and subsequent activation of TRPV1.

However, none of the previous studies had indicated the cellular source of 5-LO, so where LTB₄ was actually produced remained to be determined. Very little is known about leukotriene production in the pancreas. It was previously been shown by others that porcine³⁴ and human²² pancreatic acinar cells express 5-LO and can synthesize LTB₄. In addition, the human pancreas also expresses FLAP,³⁵ but no information was available about the distribution of 5-LO in the mouse pancreas or the effects of inflammatory agents on pancreatic 5-LO activity.

We performed immunohistochemical analysis for 5-LO in fixed sections of mouse pancreas prepared from the various experimental groups and found that very little 5-LO immunoreactivity was detectable in control animals in which the pancreatic duct was infused with the saline vehicle. When the duct was infused with NaT, however, a dramatic increase in immunoreactive 5-LO was clearly observable in acinar cells. The immunoreactive 5-LO in acinar cells was mostly seen in the basal poles of the cells, consistent with the localization of rER. Islets of Langerhans and pancreatic ducts exhibited little if any 5-LO immunoreactivity, and no differences were seen between vehicle-treated and NaT-treated animals in these parts of the pancreas. Interestingly, when 5-LO immunostaining was performed in *Trpv1*^{-/-} mice treated with NaT, the same pattern of acinar cell staining was seen, supporting the concept that LTB₄ generation precedes TRPV1 activation in this model. In addition, pretreatment of the mice with the FLAP inhibitor MK886 completely abolished 5-LO immunostaining in the acinar cells, suggesting that active FLAP is required for both 5-LO immunostaining and for 5-LO catalytic activity as demonstrated herein.

The most extensively studied cells expressing 5-LO in mammals are leukocytes.³⁶ In leukocytes, it has been suggested that translocation of 5-LO from a soluble cell compartment to an intracellular membrane compartment such as rER or nuclear membrane is required for enzymatic activity³⁶ and that the FLAP inhibitor MK886 blocks this translocation.³⁷ However, it has also been shown in human alveolar macrophages that FLAP is not involved in the

translocation of 5-LO to the nuclear envelope,³⁸ suggesting that the elimination of 5-LO immunostaining by MK886 pretreatment in our studies may be explained by some other nontranslocation mechanism. Whatever the mechanism involved, it does not appear that changes in 5-LO gene or protein expression can account for this effect on 5-LO immunoreactivity because PCR and Western blot analyses showed no changes in 5-LO expression after bile acid treatment.

Demonstrating the ability of pancreatic acinar cells to synthesize and secrete LTB₄ and showing that bile acid treatment increases pancreatic LTB₄ levels does not prove that acinar cells are the source of the increase stimulated by bile acid because there are other sources of LTB₄ in the pancreas, including, most notably, leukocytes such as neutrophils and macrophages. Therefore, we tested the response of pancreatic acinar cells in vitro, in the form of isolated pancreatic acini, to bile acid and caerulein stimulation. In this experiment, we used the natural bile acid TLCS to compare our results to those of others who have used this compound to investigate other aspects of bile acid-induced pancreatitis. In those previous studies, it was shown that TLCS at a concentration of 500 μmol/L causes unphysiologic cytoplasmic calcium release patterns and subsequent acinar cell necrosis whereas TLCS at 100 μmol/L does not.^{5,24,39} We found that 500 μmol/L TLCS and 100 pmol/L caerulein caused significant increases in LTB₄ secretion from acini in vitro whereas TLCS at a concentration of 100 μmol/L did not. This is consistent with the concept that the bile acid-induced increase in pancreatic LTB₄ secretion that results in acute inflammation via TRPV1 activation occurs at the level of acinar cells.

The effectiveness of MK886 to prevent neutrophil infiltration indicates that 5-LO activation and LTB₄ synthesis is an early event in triggering pancreatitis and precedes neutrophil recruitment. However, our data do not exclude the possibility that additional LTB₄ could be produced by neutrophils recruited to the pancreas later in the course of disease. Should this occur, additional LTB₄ could contribute to the inflammatory response in a feed-forward manner.

MK886 treatment effectively inhibited pancreatitis and appeared to improve some biochemical and histologic parameters of pancreatitis (eg, edema) to a greater extent than TRPV1 gene deletion. The reason that FLAP inhibition was more effective than TRPV1 inhibition is not known but may be explained by actions of LTB₄ on its own cognate BLT-1 or -2 receptors.

It has previously been shown that mouse pancreatic acinar cells express a G protein-coupled bile acid receptor termed Gpbar1 on their apical plasma membranes that may mediate the inflammatory effects of bile acids.⁵ These receptors are coupled to the cytoplasmic calcium changes that result in acinar cell injury in response to inflammatory concentrations of bile acids such as TLCS.^{5,40,41} Coupled with the fact that increases in intracellular calcium concentrations are known to be involved in activation of 5-LO,³⁶ this suggests that the inflammatory effects of bile acids produced in acute inflammation may be mediated by activation of Gpbar1, resulting in increased 5-LO activity and LTB₄ secretion and subsequent TRPV1 stimulation of neurogenic inflammation. However, in preliminary studies we showed that, whereas

increased intracellular Ca⁺⁺ concentrations can elicit acinar cell LTB₄ secretion, bile acid-induced LTB₄ secretion is Ca⁺⁺-independent and instead is the result of increased tyrosine kinase activity resulting in activation of extracellular signal-regulated kinases 1/2 (ERK1/2).⁴²

In summary, we have presented evidence that the inflammatory effects of NaT infused retrogradely into the pancreatic duct are mediated by generation of LTB₄ in acinar cells and subsequent LTB₄ activation of TRPV1 in primary sensory neurons (Figure 7). This pathway may be common to other causes of pancreatitis that activate intrapancreatic 5-LO. This pathophysiologic mechanism provides a conceptual basis for the potential treatment of pancreatic inflammatory diseases by inhibition of this pathway.

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Conflicts of interest

The authors disclose no conflicts.

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