Induction of chronic pancreatitis by pancreatic duct ligation activates BMP2, apelin, and PTHrP expression in mice

Cristiana Rastellini, Song Han, Vandanajay Bhatia, Yanna Cao, Ka Liu, Xuxia Gao, Tien C. Ko, George H. Greeley, Jr., and Miriam Falzon

1Department of Surgery, University of Texas Medical Branch, Galveston, Texas; and 2Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas; and 3Department of Surgery, University of Texas Health Science Center at Houston, Houston, Texas

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Rastellini C, Han S, Bhatia V, Cao Y, Liu K, Gao X, Ko TC, Greeley GH Jr, Falzon M. Induction of chronic pancreatitis by pancreatic duct ligation activates BMP2, apelin, and PTHrP expression in mice. Am J Physiol Gastrointest Liver Physiol 309: G554–G565, 2015. First published July 30, 2015; doi:10.1152/ajpgi.00076.2015.—Chronic pancreatitis (CP) is a devastating disease with no treatments. Experimental models have been developed to reproduce the parenchyma and inflammatory responses typical of human CP. For the present study, one objective was to assess and compare the effects of pancreatic duct ligation (PDL) to those of repetitive cerulein (Cer)-induced CP in mice on pancreatic production of bone morphogenetic protein-2 (BMP2), apelin, and parathyroid hormone-related protein (PTHrP). A second objective was to determine the extent of cross talk among pancreatic BMP2, apelin, and PTHrP signaling systems. We focused on BMP2, apelin, and PTHrP since these factors regulate the inflammation-fibrosis cascade during pancreatitis. Findings showed that PDL- and Cer-induced CP resulted in significant elevations in expression and peptide/protein levels of pancreatic BMP2, apelin, and PTHrP. In vivo mouse and in vitro pancreatic cell culture experiments demonstrated that BMP2 stimulated pancreatic apelin expression whereas apelin expression was inhibited by PTHrP exposure. Apelin or BMP2 exposure inhibited PTHrP expression, and PTHrP stimulated upregulation of gremlin, an endogenous inhibitor of BMP2 activity. Transforming growth factor-β (TGF-β) stimulated PTHrP expression. Together, findings demonstrated that PDL- and Cer-induced CP resulted in increased production of the pancreatic BMP2, apelin, and PTHrP signaling systems and that significant cross talk occurred among pancreatic BMP2, apelin, and PTHrP. These results together with previous findings imply that these factors interact via a pancreatic network to regulate the inflammation-fibrosis cascade during CP. More importantly, this network communicated with TGF-β, a key effector of pancreatic pathophysiology. This novel network may be amenable to pharmacologic manipulations during CP in humans.

inflammation; fibrosis; interaction; cross talk; network; signaling

The bone morphogenetic protein-2 (BMP2), apelin, and parathyroid hormone-related protein (PTHrP) signaling systems are expressed in the rodent and human pancreas (2, 3, 12, 17). BMP2 is a member of the transforming growth factor-β (TGF-β) superfamily of growth factors. Apelin (APLN) is the endogenous ligand for the APJ receptor, a G protein-coupled receptor (GPCR) [also known as apelin receptor, APLNR, or angiotensin II receptor-like 1 (Agtrl1)], a receptor related to the angiotensin receptor AT1 (30, 40, 46). PTHrP exerts multiple effects in both normal and disease states, where it modulates critical cellular functions such as proliferation, apoptosis, and differentiation (38). The activities of PTHrP are mediated by parathyroid hormone receptor 1 (PTHR1), also a GPCR. Earlier studies from our laboratories demonstrated that BMP2 and apelin suppressed the inflammatory and fibrosis responses of pancreatitis whereas PTHrP signaling promoted pancreatitis-associated inflammation and fibrosis (2, 3, 11, 12, 17). In both the repetitive cerulein (Cer-)‐induced models of acute pancreatitis (AP) and CP in the mouse, pancreatic BMP2 and apelin expression and PTHrP protein levels are upregulated robustly (3, 11, 12, 17). For the present studies, one objective was to assess and compare the effects of a second CP model, the pancreatic duct ligation (PDL) model, with those of Cer-induced CP on expression and protein/peptide levels of the BMP2, apelin, and PTHrP signaling systems in the mouse pancreas. A second objective was to identify cross-talk pathways among the pancreatic BMP2, apelin, and PTHrP signaling systems and whether TGF-β, a major effector in CP genesis and progression, interacts with these pancreatic signaling systems. A third objective was to assess the effects of BMP2 injections on pancreatic TNF-α, IL-6, ICAM, and macrophage inflammatory protein (MIP)-1α expression levels in mice with CP. The current findings showed that PDL-induced CP resulted in upregulation of pancreatic BMP2, apelin, and PTHrP signaling systems. PDL- and Cer-induced CP resulted in similar changes in the pancreatic BMP2, apelin, and PTHrP signaling systems. In terms of cross talk, BMP2 exposure upregulated apelin expression, whereas apelin and BMP2 inhibited PTHrP expression. TGF-β stimulated pancreatic PTHrP expression. Additionally, PTHrP stimulated gremelin production. Gremelin is a documented inhibitor of BMP2 activity (21, 22, 28, 37). Together, these cross-talk findings imply that PTHrP acted as an intermediate in the proinflammatory effects of TGF-β and in regulation of a novel pancreatic BMP2-apelin axis, which was protective. This pancreatic network among BMP2, apelin, and PTHrP reinforces the complex and interactive nature of endogenous protective and injurious signals evoked by CP. Based on

Address for reprint requests and other correspondence: G. H. Greeley, Jr., Dept. of Surgery, Univ. of Texas Medical Branch, 301 Univ. Blvd., Galveston, TX 77555 (e-mail: ghgjr6864@utmb.edu).
these findings and earlier work (2, 3, 11, 12, 17), the inflammation-fibrosis cascade evoked during pancreatitis may be amenable to pharmacologic interventions in the clinic.

MATERIALS AND METHODS

Animals

All mouse experiments were done in accordance with mandated standards of humane care and were approved by the Institutional Animal Care and Use Committees at the University of Texas Medical Branch in Galveston and University of Texas Health Science Center at Houston. All mice were fed a standard diet and water ad libitum. Global apelin-deficient (apelin−/−, called APKO) mice were generated by targeted homologous recombination (6, 35). APKO mice are on a mixed 129SvJ-C57Bl/6 background. Inducible pancreatic acinar cell PTHrP Δcina mice (PTHrPΔcina) were developed in our laboratories using Cre-LoxP strategy (3). Floxed PTHrPΔPPT mice were crossed with elastase-Cre transgenic mice. Cre-mediated recombination takes place in pancreatic acinar cells. In PTHrPΔcina mice, PTHrP expression is ablated in pancreatic acinar cells by acute tamoxifen treatment (3). For PDL-induced CP in adult male mice, the splenic lobe of the pancreas was selectively ligated by a ligature with aide of a surgical microscope (20). CP was also induced in mice by repetitive administration of a cholecystokinin analog, Cer (5 hour i.p. injections, 50 µg/kg, on Monday, Wednesday, and Friday mornings for 3 consecutive weeks) (17, 29, 34, 42). Mice were killed at selected intervals after PDL or start of Cer injections for harvest of the pancreas. In mice with PDL-induced CP, the ligated and nonligated lobes were collected. The nonligated gastric lobe served as control tissue for some measurements. Harvested pancreata were then processed for histological examination or extraction of total cellular RNA, total protein, or pancreatic apelin immunoreactivity. Blood was collected at death and prepared by centrifugation for measurement of serum amylase levels by means of the Phadebas Amylase Test (Pharmacia and Upjohn Diagnostics, Uppsala, Sweden) (17). To assess the influence of BMP2 administration on CP-induced upregulation of pancreatic TNF-α, IL-6, ICAM, and MIP-1α expression, CP was induced by repetitive Cer administration for 2 wk in adult male mice. Starting with CP induction, groups (n = 6–7 mice/group) of Cer-treated mice were treated for 2 wk with either vehicle or recombinant BMP2 (70 µg/kg ip, 1 time per day; gift of Medtronic Sofamor Danek). BMP2 injections continued for 2 days following cessation of Cer injections. Pancreata were harvested 16 h after the last BMP2 injection for extraction of total cellular RNA for measurement of pancreatic TNF-α, IL-6, ICAM and MIP-1α expression levels by real-time qPCR (RT-qPCR).

Pancreatic Acinar and Stellate Cells

Mouse and human acinar cells were isolated by the enzymatic dissociation method as described previously (17). Briefly, the pancreas was perfused with saline, minced with fine scissors, and digested for 15 min in 3 ml of warm isolation buffer (PBS with Ca2+ and Mg2+; 0.01% soybean trypsin inhibitor, 0.1% BSA, and 0.3 mg/ml collagenase type IV). Digestion was facilitated mechanically by continuous pipetting for 15 min. Digested tissue was then washed with 6 ml of cold isolation buffer and collected by centrifugation (1,000 rpm, 2 min). The washing step was repeated twice to remove small debris and blood cells. Acini were further purified by filtering through a sterile 860-µm mesh to remove large debris. Acini were collected by centrifugation at 1,000 rpm for 2 min.

Mouse and human pancreatic stellate cell (PSC) cultures were prepared by the outgrowth method from normal 129Sv mice or from freshly resected, nonpathological human pancreata (17). Human PSCs were cultured in high glucose DMEM containing 10% FBS, insulin-transferrin-selenium supplement X (1%), antibiotic-antimycotic (1%), gentamycin (50 mg/ml), and nonessential amino acids. Mouse PSCs were cultured in high glucose DMEM containing 10% FBS. Histological examination of PSC cultures showed a typical stellate morphology with reduced levels of vitamin A and were immunopositive for fibronectin and α-smooth muscle actin (data not shown) (12). For preparation of human pancreatic cells, diseased pancreatic tissue was obtained from CP patients undergoing surgical pancreatectomy. Discarded human pancreatic tissues that were obtained from surgical resection from cadaveric organ donors served as control tissue. Use of human tissues was approved by the Institutional Review Board at the University of Texas Medical Branch.

Chemicals

All chemicals were obtained from Sigma (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), Invitrogen (Carlsbad, CA), and MP Biomedicals (Santa Ana, CA) unless noted otherwise. Cell culture media were purchased from Mediatech (Herndon, VA) and Life Technologies (Carlsbad, CA). RNAqueous kits (AB-Ambion, Foster City, CA) were used to prepare total cellular RNA from cultured cells. Cer was purchased from Bachem California (Torrance, CA). Fibronectin and PTHrP antibodies were from Santa Cruz Biotechnology, The BMP2 antibody was purchased from R&D Systems (Minneapolis, MN) and utilized at 1:500 and 1:100 for Western blotting and immunohistochemical (IHC) analyses, respectively.

Real-Time RT-PCR Analyses

Total cellular RNA was extracted and purified from pancreatic homogenates using published procedures (2, 3, 11, 12, 17). For BMP2/BMPR2, apelin/APJ, and PTHrP/PTH1R, pancreatic expression levels are depicted in figures as fold of control, pre-Cer levels for mice with Cer-induced CP or as a ratio of ligated lobe value/unligated lobe value for mice with PDL-induced CP. For measurement of BMP2/BMPR2 mRNA levels, total RNA was reverse transcribed to cDNA using a RETROscript kit (Life Technology, Grand Island, NY). qPCR was then performed using Taqman gene expression master mix and specific gene probe sets as previously described (11). For measurement of apelin and APJ mRNA levels, real-time RT-PCR analyses were run as a two-step method using the conditions listed (17). For measurement of PTHrP and PTH1R mRNA levels, RNA (2.0 µg) was reverse transcribed into cDNA using the Applied Biosystems cDNA synthesis kit, per the manufacturer’s protocol. The first-strand cDNA was then used as a template for real-time PCR on an Applied Biosystems 7500 Real-Time PCR System using Sybr green Supermix (Applied Biosystems) and the primers described previously (2). Real-time RT-PCR analyses of pancreatic TNF-α, IL-6, ICAM, and MIP-1α mRNA levels were assessed by means of a similar method. Relative RT-qPCR assays were performed with 18S rRNA, β-actin or GAPDH as a housekeeping gene. The mouse primer sequences for TNF-α, IL-6, ICAM, MIP-1α, and 18S rRNA are given below. Primers for mouse and human gremlin were reported (24, 26).

The mouse primer sequences [forward (F); reverse (R)] for TNF-α are as follows: F- TCCAGGTTTCTTTCAAGAGGA; R- GGTGAGGAGCACACGTGATCCG; MIP-1α: F- ACGCTTCTGCTGTTTCCATTACA; R- AGGAAATATGACACGTGCGTG; ICAM: f-GCCACTTCTTGTGAAGTCTG; R- CTACGG-GCCCTGGGACG; IL-6: F- GCCCTGCGTACCCATCATG; R- CACGTTCCAGGCTTGTGCCT; R- GCAGGAATGAGATGAGTGTC; and 18S rRNA: F-GTAAACCCGTTGAACCCTCAT; R- CATCATTACCGTATGAGCCG.

Although the effects of repetitive Cer injections on pancreatic BMP2 protein levels 2, 4, and 8 wk and PTHrP protein levels 3 wk following start of Cer injections have been reported (3, 12), the effects of PDL- and Cer-induced CP on pancreatic expression levels of BMP2 and PTHrP have not been assessed in detail previously. Effects of repetitive Cer injections on pancreatic apelin and APJ expression levels, but not apelin peptide levels, have been examined (11). With the exception of pancreatic PTHrP protein levels at a single time point (2 days) following lobe ligation (3), the influence of PDL on pancre-
atic expression and peptide/protein levels of BMP2, apelin, and PTHrP has not been reported previously.

**Histological and IHC Analyses**

For histological assessment, pancreatic tissues were fixed immediately in 10% buffered formaldehyde, embedded in paraffin, and sectioned. Hematoxylin and eosin (H&E) staining was done using a standard protocol.

**Fibronectin and apelin IHC.** For assessment of pancreatic fibronectin and apelin immunostaining, entire pancreatic tissue sections were immunostained for fibronectin or apelin. Pancreatic sections were incubated overnight at 4°C with either a rabbit anti-fibronectin (1:600; Santa Cruz Biotechnology) or a rabbit anti-apelin antibody (1:1,000) followed by incubation for 0.5 h at room temperature with a biotinylated anti-rabbit antibody (1:400; Vector Laboratories, Burlingame, CA). Sections were subsequently treated for 0.5 h at room temperature with avidin/biotin complex (Vectastain Elite ABC Kit; Vector Laboratories). Slides were then stained with diaminobenzidine for 0.5–1 min, washed in distilled water, and counterstained with hematoxylin.

The polyclonal apelin antibody was generated in rabbits by G. H. Greeley using synthetic apelin-17 (modified by added tyrosine and cysteine; TYR-CYS-LYS-PHE-ARG-ARG-GLN-ARG-PRO-ARG-LEU-SER-HIS-LYS-GLY-PRO-MET-PRO-PHE) attached to keyhole limpet hemocyanin for an antigen. Images were captured with a Nikon Eclipse E600 microscope equipped with a digital camera using the SPOT imaging software (Diagnostic Instruments, Sterling Heights, MI).

**BMP2 IHC.** BMP2 staining of pancreatic sections was done using previously described methods (12). Substitution of BMP2 antibody with an isotype control antibody showed no staining (not shown).

**PTHrP IHC.** PTHrP IHC staining of pancreatic sections was performed using the peroxidase ABC kit (Vector Laboratories). Briefly, sections were incubated overnight at 4°C with polyclonal

### Table 1. Serum amylase levels in mice with PDL-induced chronic pancreatitis

<table>
<thead>
<tr>
<th>Days Post-PDL</th>
<th>Control</th>
<th>2</th>
<th>5</th>
<th>7</th>
<th>9</th>
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| Levels | 6,960 ± 476 | 45,912 ± 7,940
* | 5,754 ± 260 | 4,760 ± 266 | 6,450 ± 587 |

Values are means ± SE; n = 5–12 mice/group. Sham pancreatic duct ligation (PDL) surgery did not elevate serum amylase levels. Two days postsham PDL: 6,488 ± 612; 9 days postsham PDL: 6,295 ± 452. *P < 0.05, significantly higher vs. control serum amylase levels.

![Image](http://ajpgi.physiology.org/)

Fig. 1. Representative hematoxylin and eosin-stained sections of pancreas (A and B; ×100 magnification) and fibronectin immunostaining in the pancreas (C–F) of mice with pancreatic duct ligation (PDL). A, C, and E are from unligated lobes, and B, D, and F are from ligated lobes. The ligated lobes show a reduced acinar density, increased ductular complexes and enlarged fibrous septa (B and F; 9 days; D: 5 days post-PDL) whereas the unligated lobes show no change in gross pancreas structure (A and E: 9 days post-PDL) and marginal fibrosis (C and E: 5 and 9 days post-PDL, respectively). Acini (black arrows), ductular complexes (black arrowheads), and fibrosis (white arrowheads) are identified.

![Image](http://ajpgi.physiology.org/)

Fig. 2. Western blotting analysis of pancreatic fibronectin levels at selected intervals after PDL in mice. *P < 0.05 vs. pre-PDL value. †P < 0.05 vs. preceding value; n = 6–10 mice/group.
antibodies diluted in 2% BSA in TBS against PTHrP (H-137 or N-19; Santa Cruz Biotechnology). After three washes with TBS-Tween (TBST), sections were incubated for 30 min with biotinylated secondary antibody, washed three times with TBST, and then incubated for 30 min with ABC reagent. Lastly, the peroxidase substrate DAB was added. All sections were counterstained with hematoxylin and observed by light microscopy. For negative controls, sections were incubated with rabbit IgG (Santa Cruz Biotechnology) in place of the primary antibody. Use of the control IgG showed no staining (not shown). Images were recorded using an Olympus BX51 microscope.

Western Blotting Analyses

Pancreatic fibronectin protein levels were measured by means of the Odyssey Western blot detection system (LI-COR, Lincoln, NE) (18). In brief, membranes were blocked with LI-COR blocking buffer for 1 h at room temperature, probed with a fibronectin antibody diluted in 0.1% Tween LI-COR blocking buffer. Membranes were rinsed three times for 10 min each in 0.1% Tween phosphate buffer solution (PBST) and then probed with a goat anti-rabbit IR-Dye 680 or 800CW labeled secondary antisera in 0.1% Tween, 0.01% SDS LI-COR blocking buffer for 1 h at room temperature. Rinses were repeated after secondary labeling, and membranes were rinsed three times for 10 min in PBST and then placed in water. Membranes were imaged and quantitated by means of LI-COR Odyssey scanner and 3.0 analytical software.

Pancreatic levels of BMP2 and PTHrP proteins were measured by Western blotting analyses as described previously (12). For measurement of PTHrP protein levels, frozen pancreatic tissues were homogenized in cold 1× lysis buffer with a chemical protease/phosphatase inhibitor cocktails A and B (Santa Cruz Biotechnology). Protein concentrations were estimated using the Bio-Rad protein assay. GAPDH was used as a loading control. The signals were detected using the SuperSignal West Pico substrate kit (Pierce Biotechnology, Rockford, IL). Densitometric analysis was performed using the Alpha Innotech image analysis system (Alpha Innotech, San Leandro, CA).

Apelin ELISA

Apelin peptide levels in pancreatic extracts were measured by means of an apelin ELISA (Alpco, Salem, NH). Extracts were prepared by one of two different methods that were equally effective. In the first, extirpated pancreata were homogenized in distilled water (~1:20) containing a cocktail of protease inhibitors (protease inhibitor cocktail tablet, complete, EDTA-free, 1 tablet per 50 ml water; Roche). Extracts were then boiled for 15–20 min, cooled, and clarified by centrifugation. Supernatants were saved and pellets were resuspended in 3 ml 2 N acetic acid and allowed to sit for 1 h. Acid extracts were clarified and supernatants were combined with aqueous extracts. Extracts were lyophilized to dryness and resuspended in 250 μl of water for protein measurements. Seven-hundred and twenty microliters of apelin ELISA buffer were added to all samples for apelin determinations. For the second method, apelin immunoreactivity was extracted using a standard protein extraction method (43, 44). In brief, pancreatic samples were homogenized in lysis buffer containing 60 mM DTT, 1 mM PMSF, and Complete Mini Protease Inhibitor

Fig. 3. Top: relative BMP2 and BMPR2 expression levels in the ligated lobe at selected intervals after PDL in mice. BMP2 and BMPR2 mRNA are shown as fold of expression levels in the control, nonligated lobe (i.e., ligated/nonligated). BMP2 and BMPR2 mRNA levels are corrected for 18S rRNA levels. Bottom: relative pancreatic BMP2 and BMPR2 expression levels at selected intervals following start of cerulein (Cer) injections in mice. BMP2 and BMPR2 expression levels are shown as fold of control (Con) levels. BMP2 and BMPR2 mRNA and 18S rRNA levels were measured by real time RT-PCR. In all figures, bar graphs depict means ± SE; n = 6–8 mice/group; Con = mice without manipulations. *P < 0.05 vs. pre-PDL or control (Con) values. †P < 0.05 vs. preceding value.
Cocktail Tablet (1 tablet/10 ml). Homogenates were filtered by means of a 70-μm filter. The filtrate was clarified further by a high-speed centrifugation at 4°C, and the supernatants were harvested. Protein concentrations were determined by the method of Bradford using Bio-Rad protein assay kit (Hercules, CA).

**Statistical Analyses**

Results are expressed as the means ± SE. Data were analyzed by t-test or one-way ANOVA and subsequently with Newman-Keuls test when appropriate. For all comparisons, \( P < 0.05 \) was considered significant.

**RESULTS**

**Induction of CP by either PDL or Repetitive Cer Injections Elevates Pancreatic BMP2, Apelin, and PTHrP Signaling Axes**

The aim of this experiment was to define the influence of PDL-induced CP on pancreatic expression (mRNA) and peptide/protein levels of the BMP2, apelin, and PTHrP signaling axes.

Induction of CP by either PDL or repetitive Cer injections was verified by acute elevations in serum amylase levels and histological confirmation of CP. In mice with PDL, serum amylase levels were elevated significantly \( (P < 0.05) \) 2 days after PDL (Table 1). Five, 7, and 9 days after PDL, serum amylase levels receded and did not differ significantly compared with pre-PDL levels. Repetitive Cer administration resulted in acute elevations \( (P < 0.05) \) of serum amylase during each Cer-induced episode of pancreatitis as described previously (13). Serum amylase levels then declined after each episode to control or near-control levels. Examination of H&E-stained pancreatic sections harvested from mice with PDL showed evidence of CP (Fig. 1). H&E-stained pancreatic sections harvested from mice given Cer showed evidence of CP as reported previously (3, 12). Inflammation, parenchyma degeneration, and fibrosis were clearly evident. In the ligated lobe of mice with PDL-induced CP, abundant immunoreactive fibronectin was observed (Fig. 1).

Western blotting analysis showed that pancreatic duct ligation resulted in significant elevations of pancreatic fibronectin protein levels in mice (Fig. 2). Fibronectin protein levels were elevated maximally 2 days after PDL.

Expression (mRNA) levels of BMP2/BMPR2, apelin/APJ and PTHrP/PTH1R were assessed in pancreata harvested at numerous intervals after either PDL (4 and 19 h; 2, 5, 7, and 9 days) or start of repetitive Cer injections (1, 2, 3 wk) (Figs. 3, 4, and 5). Protein/peptide levels of BMP2, apelin, and PTHrP were measured for select times after either PDL or start of repetitive Cer injections (Fig. 6).

Four and 19 h, and 2 days post-PDL, relative expression levels of pancreatic BMP2 were unchanged in the ligated lobe compared with pre-PDL BMP2 expression levels (Fig. 3). BMP2 expression then increased ~4- and 12-fold, 5 and 7 days, respectively, after PDL. BMP2 expression levels declined significantly at 9 days post-PDL but remained elevated \( (P < 0.05) \) compared with pre-PDL expression levels. Four and 19 h and 2 days post-PDL, relative expression levels of BMPR2 in the ligated lobe were increased \( (P < 0.05) \) compared with pre-PDL BMPR2 expression levels. BMPR2 expression levels then increased gradually from approximately two- to eightfold of pre-PDL levels from 2 to 7 days after PDL. Pancreatic BMPR2 expression levels declined \( (P < 0.05) \) at 9 days post-PDL but remained elevated \( (P < 0.05) \) compared with pre-PDL levels. Pancreatic BMP2 and BMPR2 expression levels were unchanged in the control lobe following PDL (not shown). Pancreatic BMP2 protein levels increased approximately fivefold \( (P < 0.05) \) 5 days after PDL compared with levels in the control, nonligated lobe (Fig. 6A).

In mice with Cer-induced CP, relative expression levels of pancreatic BMP2 and BMPR2 were elevated maximally 1 and 2 wk after start of repetitive Cer injections (Fig. 3). BMP2 and BMPR2 elevations were ~60- to 80-, and 25- to 40-fold.
higher, respectively, of control BMP2 and BMPR2 expression levels. Three weeks after start of Cer injections, relative BMP2 and BMPR2 expression levels declined significantly but remained elevated ($P < 0.05$) compared with corresponding control levels. Pancreatic BMP2 protein levels have been shown to increase in mice with Cer-induced CP (10). IHC analysis showed that immunoreactive BMP2 levels in pancreatic acinar cells increased in mice with repetitive Cer injections or PDL (Fig. 7).

In the ligated lobe, relative expression levels of pancreatic apelin increased gradually from 19 h to 5 days post-PDL compared with pre-PDL apelin expression levels (Fig. 4). Two days after PDL, apelin expression levels were significantly higher than expression levels 19 h after PDL. Five to 9 days post-PDL, apelin expression levels were elevated maximally 10- to 15-fold of pre-PDL levels. In the ligated lobe, relative expression levels of pancreatic APJ were increased significantly from 5 to 9 days post-PDL compared with pre-PDL APJ expression levels. APJ expression levels were elevated maximally 15- to 23-fold of pre-PDL levels. Pancreatic apelin and APJ expression levels were unchanged in the control lobe following PDL (not shown). ELISA analyses showed that pancreatic apelin peptide levels increased ~13- and 7-fold, 2 and 5 days, respectively, after PDL (Fig. 6B). Pancreatic apelin peptide levels increased approximately five- and twofold, 24 h and 3 wk, respectively, after start of Cer injections (Fig. 6C).

We have reported earlier that induction of CP by repetitive Cer injections resulted in robust elevations of pancreatic apelin and APJ expression in mice (17). IHC analysis of apelin showed abundant apelin immunostaining in acinar cells of mice with either Cer- or PDL-induced CP (Fig. 7).

In the ligated lobe, relative pancreatic PTHrP expression levels were elevated significantly from 4 h to 9 days post-PDL (Fig. 5). PTHrP expression levels declined significantly at 9 days post-PDL compared with expression levels at 7 days post-PDL. Pancreatic PTHrP expression levels were increased significantly from 5 to 9 days post-PDL. Pancreatic PTH1R expression levels were increased significantly ($P < 0.05$) 1 and 2 wk after start of Cer injections. In mice with Cer-induced CP, pancreatic PTHrP protein levels were elevated approximately fourfold 3 wk after...
start of repetitive Cer injections (Fig. 6E). IHC analysis showed abundant PTHrP immunostaining in acinar cells of mice with either Cer- or PDL-induced CP (Fig. 7).

**Cross Talk Among Pancreatic BMP2, Apelin, and PTHrP**

The aim of these studies was to assess the extent of cross talk among pancreatic BMP2, apelin, and PTHrP signaling systems. Whether apelin signaling regulates pancreatic PTHrP production was assessed by measuring pancreatic PTHrP expression in mice with apelin deletion (APKO mice) and in mice with apelin treatment (Fig. 8A). In APKO mice, pancreatic PTHrP expression levels were twice those of WT levels \( (P < 0.05) \). Western blotting analysis showed that pancreatic PTHrP protein levels were approximately threefold higher in APKO mice compared with WT mice \( (W: 1.0 \pm 0.3 \text{ vs. APKO: } 2.8 \pm 0.5, P < 0.05) \). Additionally, apelin administration in WT mice \( (50 \mu g/\text{injection sc, 2 time per day, 4 days}) \) suppressed pancreatic PTHrP expression levels significantly. PTHrP expression levels were reduced by 75% \( (P < 0.05) \). Whether endogenous PTHrP signaling regulates pancreatic apelin expression was assessed by measuring pancreatic apelin mRNA levels in mice with acinar cell PTHrP deletion \( (\text{PTHrP}^{\text{Acinar}}) \) (Fig. 8B). In acinar cells harvested from \( \text{PTHrP}^{\text{Acinar}} \) mice, apelin expression levels were increased significantly \( (\sim 2.5\text{-fold}) \). The effects of BMP2 treatment on apelin and PTHrP expression were assessed in cultured pancreatic cells (Fig. 8, C and D). In cultured human PSCs, acute BMP2 treatment increased apelin expression significantly (Fig. 8C). BMP2 treatment did not affect PTHrP expression in cultured mouse acinar cells (Fig. 8D); however, BMP2 treat-
ment of cultured mouse PSCs decreased PTHrP expression levels significantly (Fig. 8D). In cultured mouse acinar cells and PSCs, TGF-β exposure increased PTHrP expression levels significantly (Fig. 8E). In cultured mouse acinar cells, PTHrP treatment increased gremlin expression levels (Fig. 8F); however, PTHrP treatment did not affect gremlin expression in cultured PSCs. TGF-β exposure increased gremlin expression levels significantly in cultured human acinar cells (Fig. 8G), which was inhibited by pretreatment with a PTHrP antagonist (PTHrP[7–34]).

BMP2 Treatment Reduced Pancreatic TNF-α, ICAM, and MIP-1α mRNA Levels in Mice with CP

Pancreatic expression levels of TNF-α, ICAM, and MIP-1α increased 32- to 125-fold (P < 0.05) in mice with Cer-induced CP (Fig. 9). TNF-α, ICAM, and MIP-1α mRNA levels were reduced by ~40, 18, and 37%, respectively, by BMP2 injections in mice given repetitive Cer injections (P < 0.05). Pancreatic IL-6 expression levels were reduced but not significantly by BMP-2 treatment in mice given Cer injections.

DISCUSSION

The present studies confirmed and extended earlier reports showing that induction of experimental CP or AP in mice resulted in robust elevations in pancreatic BMP2, apelin, and PTHrP expression (2, 3, 11, 12, 17). In the current studies, the PDL-induced experiments were done primarily to extend previous findings and to show that CP-induced changes in the BMP2, apelin, and PTHrP signaling systems were model independent. Prior data were generated in mice in which pancreatitis was induced by repetitive Cer injections (3, 11, 12, 17). Additionally, in the present report, protein/peptide levels of pancreatic BMP2, apelin, and PTHrP were measured in mice with CP. In general, the changes in pancreatic BMP2, apelin, and PTHrP protein/peptide levels paralleled the elevations in expression observed in response to either Cer- or PDL-induced CP. More importantly, showing concurrent elevations in BMP2, apelin, and PTHrP expression and protein/peptide levels in two different CP models reinforced their potential biological relevance.

PDL- and Cer-induced CP triggered significant elevations in pancreatic BMP2 and BMPR2 mRNA levels. In Cer-induced CP, elevations in pancreatic BMP2 and BMPR2 expression were much higher compared with PDL-induced elevations. In the PDL model, BMP2 and BMPR2 expression increased maximally 12- and 8-fold, respectively, whereas in the repetitive Cer model, BMP2 and BMPR2 levels increased maximally 80- and 40-fold, respectively. PDL-induced CP also triggered significant elevations in pancreatic apelin and APJ mRNA levels. These data extended prior reports that pancreatic apelin and APJ expression levels increased in the repetitive Cer-induced CP model. In the Cer model, elevations were much higher compared with PDL-induced elevations. Apelin and APJ expression levels increased maximally 30- and 50-fold, respectively, in Cer-induced CP (17), whereas pancreatic apelin and APJ expression levels increased maximally 15- and 20-fold, respectively, in the PDL model. Although speculative, the exaggerated expression elevations in the BMP2 and apelin...
signaling axes may be related to the repetitive nature of the Cer-induced pancreatic insult. Additionally, a larger portion of the pancreatic parenchyma was retained in the Cer-model, which may have supported more robust gene responses. BMP2 and PDL-induced CP increased pancreatic PTHrP and PTH1R expression levels. In general, the temporal profiles of CP-induced elevations in pancreatic BMP2, apelin, and PTHrP expression were similar. Interestingly, in contrast to the BMP2 and apelin expression changes, the magnitudes of Cer- and PDL-induced elevations in PTHrP expression were similar. The changes in protein/peptide levels confirmed the mRNA elevations in pancreatic BMP2, apelin, and PTHrP during the CP process. CP-induced elevations in expression and protein/peptide levels most likely reflect increased transcription and translation in acinar cells since these cells predominate (>90%). Invading and replicating PSCs were expected to play
significant roles in the CP-induced elevations. Furthermore, the IHC findings confirmed the CP-induced elevations in production (Western and mRNA analyses) by showing an intense immunostaining for BMP2, apelin, and PTHrP in pancreatic acinar cells of mice with Cer- or PDL-induced CP.

A second important finding of the current study was that significant cross talk took place among the pancreatic BMP2, apelin, and PTHrP signaling systems. In vitro and in vivo studies were done. Pancreatic apelin expression increased in PTHrP−/− acinar mice inferring endogenous acinar cell PTHrP inhibits pancreatic apelin production. Pancreatic PTHrP expression and protein levels increased in APKO mice inferring endogenous apelin inhibits pancreatic PTHrP production. Together, these results imply a bidirectional, negative feedback loop that targeted transcriptional regulation of apelin, and PTHrP. BMP2 exposure ramped up apelin expression in PSCs whereas BMP2 inhibited PTHrP expression in PSCs. These critical findings implied that BMP2’s anti-inflammatory and antifibrosis effects were mediated by apelin signaling and blockade of PTHrP signaling during pancreatitis. In pulmonary arterial endothelial cells, BMP2 signaling has been shown to upregulate apelin expression (36), whereas BMP4, -7, and -9 were reported to downregulate apelin expression in pulmonary endothelial vascular cells (31). Our results showed that TGF-β, a major signal behind the inflammation-fibrosis cascade in pancreatitis (10, 23, 39), upregulated PTHrP expression in acinar cells and PSCs. These findings were important since the present study showed that PTHrP upregulated pancreatic gremlin expression. Gremlin is relevant since pancreatic gremlin expression levels increased and correlated with development of pancreatic fibrosis in mice with Cer-induced CP (34), and gremlin exposure can inhibit BMP2 activity (21, 22, 28).

Because TGF-β can stimulate PTHrP expression, TGF-β’s effect on gremlin may have been mediated partly by PTHrP. In fact, treatment of cultured human acinar cells with PTHrP (7–34), a PTH1R receptor antagonist, inhibited TGF-β-induced upregulation of gremlin expression. Recent data also show that blockade of PTHrP signaling inhibited TGF-β-induced upregulation of gremlin expression in cultured mouse acinar cells (unpublished observations, Falzon M). Together, these results have significant implications for regulation of the inflammation-fibrosis cascade during CP. One implication is that TGF-β and PTHrP regulated responses to pancreatic insults by their suppression of the pancreatic BMP2-apelin axis. Additionally, because BMP2 and apelin inhibited PTHrP production, the stimulatory effects of PTHrP (and possibly TGF-β) on inflammation and fibrosis were dampened. It
should be pointed out that the “expression targeting” of the cross talk among BMP2, apelin, and PTHrP agree with the general biological effects of each ligand on the inflammatory and fibrosis responses during pancreatitis. For example, BMP2 exposure inhibits TGF-β-induced α-SMA and fibronectin production in mouse and human PSCs (11). In view of the present findings, the inhibitory effect of BMP2 signaling may be mediated directly as well as indirectly via BMP2’s regulation of pancreatic apelin, and PTHrP production. Together, these signaling relationships reinforced the complex nature of this novel pancreatic network that is proposed to play a key role in regulation of inflammation and fibrosis during pancreatitis.

As discussed earlier, results clearly showed that BMP2, apelin, and PTHrP are downstream transcriptional targets of each other. In addition, BMP2, apelin, and PTHrP target several other pathways critical for development of the inflammation-fibrosis cascade. Earlier reports from our laboratories (3, 17) showed that apelin and PTHrP signaling regulated activation of pancreatic NF-κB, a key transcription factor behind the inflammatory response of pancreatitis (1, 15, 16, 19, 33, 42, 45). PTHrP upregulated pancreatic NF-κB activity whereas apelin inhibited NF-κB activation. This upregulation of NF-κB by PTHrP may be involved in the PTHrP-induced stimulation of IL-6 and ICAM-1 expression (3). Similarly, apelin’s inhibition of NF-κB activation may play a role in the reduction of pancreatic neutrophil invasion in apelin-treated mice with pancreatitis (17). The present findings show that BMP2 treatment can reduce the inflammatory process by reducing expression levels of pancreatic TNF-α, ICAM, and MIP-1α in mice with CP. This finding extends an earlier report that leukocyte infiltration was amplified in BMPR2 knockout mice with CP (11). It should be mentioned that administration of noggin, an endogenous BMP2 antagonist, has been shown to reduce the inflammatory process in mice with Cer-induced AP implying that BMP2 signaling can be proinflammatory (5). The present findings together with these earlier data indicate that pancreatic BMP2 signaling exerts anti-inflammatory as well as proinflammatory effects. Although speculative, the influence of BMP2 signaling on the inflammatory process may be cell type and time dependent.

Other downstream targets of BMP2 are involved in its antifibrosis activity. BMP2 was shown to signal through Smad1 to reduce TGF-β-stimulated fibrosis in PSCs (11). Additionally, BMPR2/Smad1/5/8 signaling exerted a protective role against Cer-induced fibrosis by inhibition of Smad2 and p38 (MAPK) pathways (10). Based on these activities and the discovery of a pancreatic BMP2-apelin-PTHrP network, we propose that the BMP2-apelin-PTHrP network functions to regulate the inflammation-fibrosis cascade of CP.

It is important to point out that several pancreatic factors have been shown to exert either a protective or an injurious effect during pancreatitis. Examples are the fibroblast-growth factors -1 and -2 (FGF-1 and -2), IGF-1, connective tissue growth factor (CTGF), and EGF (7, 8, 10, 14, 23, 25, 39). Interestingly, the extent to which endogenous protective and injurious factors interact with each other has not been investigated. Exploiting the pancreatic BMP2-apelin-PTHrP network as well as its interaction with other endogenous protective and injurious factors represents a novel therapeutic approach to CP amelioration. We propose that these signaling axes are an integral part of the pancreas’ inherent injury response that acts to either block or amplify pancreatic deterioration in CP and that translational approaches might necessarily target numerous signals simultaneously for successful outcomes.

In summary, the current study shows that PDL-induced CP triggers elevations in the pancreatic BMP2, apelin, and PTHrP signaling systems. Our present findings corroborate and extend prior findings using the Cer-induced CP model. A more intriguing finding is that significant cross talk takes place among the pancreatic BMP2, apelin, and PTHrP signaling systems that are proposed to be part of a larger endogenous network that is recruited during pancreatitis to regulate the inflammation-fibrosis cascade during CP (Fig. 10).

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES
