Acinar cell-specific knockout of the PTHrP gene decreases the proinflammatory and profibrotic responses in pancreatitis

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1Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas; 2Department of Surgery, University of Texas Medical Branch, Galveston, Texas; 3Department of Pathology, University of Texas Medical Branch, Galveston, Texas; and 4Sealy Center for Cancer Cell Biology, University of Texas Medical Branch, Galveston, Texas

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Bhatia V, Rastellini C, Han S, Aronson JF, Greeley GH Jr, Falzon M. Acinar cell-specific knockout of the PTHrP gene decreases the proinflammatory and profibrotic responses in pancreatitis. Am J Physiol Gastrointest Liver Physiol 307: G533–G549, 2014. First published July 17, 2014; doi:10.1152/ajpgi.00428.2013.—Pancreatitis is a necroinflammatory disease with acute and chronic manifestations. Accumulated damage incurred during repeated bouts of acute pancreatitis (AP) can lead to chronic pancreatitis (CP). Pancreatic parathyroid hormone-related protein (PTHrP) levels are elevated in a mouse model of cerulein-induced AP. Here, we show elevated PTHrP levels in mouse models of pancreatitis induced by chronic cerulein administration and pancreatic duct ligation. Because acinar cells play a major role in the pathophysiology of pancreatitis, mice with acinar cell-specific targeted disruption of the Pthrp gene (PTHrP<sup>Acinar</sup>) were generated to assess the role of acinar cell-secreted PTHrP in pancreatitis. These mice were generated using Cre-LoxP technology and the acinar cell-specific elastase promoter. PTHrP<sup>Acinar</sup> exerted protective effects in cerulein and pancreatic duct ligation models, evident as decreased edema, histological damage, amylase secretion, pancreatic stellate cells (PSC) activation, and extracellular matrix deposition. Treating acinar cells in vitro with cerulein increased IL-6 expression and NF-κB activity; these effects were attenuated in PTHrP<sup>Acinar</sup> cells, as were the cerulein- and carbachol-induced elevations in amylase secretion. The cerulein-induced upregulation of procollagen I expression was lost in PSCs from PTHrP<sup>Acinar</sup> mice. PTHrP immunostaining was elevated in human CP sections. The cerulein-induced upregulation of IL-6 and ICAM-1 (human acinar cells) and procollagen I (human PSCs) was suppressed by pretreatment with the PTH1R antagonist, PTHrP (7–34). These findings establish PTHrP as a novel mediator of inflammation and fibrosis associated with CP. Acinar cell-secreted PTHrP modulates acinar cell function via its effects on proinflammatory cytokine release and functions via a paracrine pathway to activate PSCs.

parathyroid hormone-related protein; pancreatitis; acinar cells; stellate cells

ACUTE PANCREATITIS (AP) is a clinical syndrome that begins with an injury to the pancreas and results in an inflammatory response that is characterized by pancreatic edema and inflammation and is accompanied by severe epigastric abdominal pain (76). A growing body of epidemiological, etiological, and experimental data indicates that chronic pancreatitis (CP) results from the accumulated damage incurred during repeated bouts of AP (recurrent AP or RAP). The link between RAP and CP has been formalized by the sentinel acute pancreatitis event (SAPE) hypothesis model, which unifies knowledge about the molecular and cellular mechanisms underlying RAP with the ultimate development of CP (60, 76, 80). CP is associated with an increased risk of pancreatic cancer, cardiovascular disease, and severe infection (39, 78).

Multiple experimental models have been developed to simulate the pancreatic damage characteristic of human AP and CP (35). Repeated hyperstimulation with the decapeptide cerulein is a well-studied model for induction of pancreatic damage consistent with both AP and CP (35). The AP model of cerulein-induced pancreatic damage simulates that seen in human edematous pancreatitis and is evident as dysregulation of digestive enzyme production and cytoplasmic vacuolization, acinar cell death, edema formation, and infiltration of inflammatory cells into the pancreas (31). Two well-established models of pancreatic damage consistent with CP include the repetitive cerulein administration model and the pancreatic duct ligation (PDL) model. In the repetitive cerulein administration model, pancreatic damage is followed by periods of recovery and, therefore, simulates the process of RAP in humans (2, 35). Pancreatic duct obstruction due to stenosis and/or intraductal stones, resulting in blockage of pancreatic duct secretion, is an important etiologic factor in development of CP in humans (2, 35), and therefore, this model is also relevant to human disease. In both models, pancreatic damage leads to fibrotic remodeling of the pancreatic parenchyma and eventually to pancreatic insufficiency (4, 11, 32).

Parathyroid hormone-related protein (PTHrP) exerts multiple effects in both normal and disease states, where it modulates critical cellular functions, such as proliferation, apoptosis, and differentiation (65). The effects of PTHrP are mediated, in part, through paracrine and/or autocrine activation of the PTH/PTHrP receptor (PTH1R), a G protein-coupled receptor (41). In the normal pancreas, PTHrP is expressed by islet cells and regulates cell proliferation, apoptosis, and insulin release (13, 14, 71). No function for PTHrP has been reported in the exocrine pancreas. We have previously shown that PTHrP levels are elevated in the exocrine pancreas in a mouse model of cerulein-induced AP and that PTHrP plays a role in the cerulein-induced upregulation of cytokine and chemokine levels in acinar cells and extracellular matrix (ECM) proteins in pancreatic stellate cells (PSCs) (7). PTHrP also exerts proinflammatory effects in the injured kidney, in atherosclerosis, and in rheumatoid arthritis (23, 24, 42, 55, 82).

The pathophysiology of RAP involves dysfunction of acinar cells, and damage to acinar cells results in the release of inflammatory cytokines (36). Paracrine factors released by damaged acinar cells also activate PSCs, which, in turn, produce autocrine factors that maintain an activated phenotype.
exocrine cells (30), we also asked whether PTHrP is regulated have been reported between rodent and human pancreatic proinflammatory and profibrotic responses associated with pancreatic damage. Since significant interspecies differences have been reported between rodent and human pancreatic exocrine cells (30), we also asked whether PTHrP is regulated in human acinar and stellate cells in response to damage, and the consequences thereof.

MATERIALS AND METHODS

Materials

FBS was obtained from Atlanta Biologicals (Norcross, GA). Tissue culture supplies were purchased from Gibco (Carlsbad, CA). Antibodies for immunohistochemistry and immunofluorescence were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Abcam (Cambridge, MA). Alexa Fluor 488 was obtained from Invitrogen (Carlsbad, CA). Cerulein was purchased from Bachem (Torrance, CA), tamoxifen was obtained from Cayman Chemicals (San Antonio, TX), and carbamylcholine chloride (carbachol) was purchased from Sigma (St. Louis, MO). The Nova Ultra Sirius Red Stain Kit was purchased from IHCWorld (Woodstock, MD). Pthrp (1–36) and Pthrp (7–34) were purchased from Polypeptide Laboratories (Torrance, CA), tamoxifen was obtained from Cayman Chemicals (San Antonio, TX), and carbamylcholine chloride (carbachol) was purchased from Sigma (St. Louis, MO). The Nova Ultra Sirius Red Stain Kit was purchased from IHCWorld (Woodstock, MD). Pthrp (1–36) and Pthrp (7–34) were purchased from Polypeptide Laboratories (Torrance, CA) and Bachem (Torrance, CA), respectively.

Generation of mice with conditional knockout of the Pthrp gene in pancreatic acinar cells. All mice were housed in the animal facility at the University of Texas Medical Branch (UTMB) and handled according to UTMB and National Institutes of Health guidelines. All procedures were approved by the Institutional Animal Care and Use Committee at UTMB. Pthrp<sup>floxed/floxed</sup> mice were generously provided by Dr. A. Karaplis of McGill University (29, 38, 44). These mice were generated using 129/Sv-derived R1 mouse embryonic stem cells and were previously maintained on a BALB/c; 129-mixed genetic background. The generation of these mice has been described (29, 38, 44). These mice were crossed with CD-1 mice. The heterozygous offspring was crossed with inducible-Cre transgenic mice [STOCK Tg(Cre/ESR1)1Stof/SJ; Jackson Lab Stock Number 008861] (18). These mice have a tamoxifen-inducible Cre-mediated recombination system driven by the rat elastase 1 pancreatic promoter. The double heterozygous offspring were intercrossed to obtain Pthrp<sup>floxed/<sup>+/Cre</sup>+</sup> (heterozygous); Pthrp<sup>floxed/<sup>+/Cre</sup>+</sup> (homozygous); Pthrp<sup>floxed/<sup>Cre</sup>+</sup> (control) and Pthrp<sup>floxed/<sup>Cre</sup>-</sup> (control) mice. Data were generated using the Pthrp<sup>floxed/<sup>Cre</sup>+</sup> and Pthrp<sup>floxed/<sup>Cre</sup>-</sup> mice. ELA1-Cre/ERT2 transgenic mice were originally established on a B6SJL F2 background and then propagated on a CD-1 background. Thus, the genetic background of the double-homozygous mice is mixed, but predominantly CD-1. These mice were generated in collaboration with the Transgenic Mouse Facility at UTMB (director Dr. M. Wakamiya).

For genotyping, genomic DNA was isolated from tail biopsy samples and digested with BamHI. The blots were hybridized to Pthrp probe B (29) and to a Cre probe.

Induction of Cre recombinase activity. Cre recombinase activity was induced in Pthrp<sup>floxed/<sup>Cre</sup>-</sup> mice by intraperitoneal injection of tamoxifen (20 mg/ml, 100 μl/mouse), once daily for 5 days (40). Two types of controls were used: wild-type CD-1 and Pthrp<sup>floxed/<sup>Cre</sup>-</sup> mice injected with the same regimen of tamoxifen or corn oil (vehicle control), and Pthrp<sup>floxed/<sup>Cre</sup>+</sup> mice injected with corn oil. At 7 days after the end of tamoxifen treatment, mice were injected with cerulein or subjected to PDL to induce pancreatitis or were euthanized for preparation of acinar and stellate cells. Once it was established in pilot studies that similar responses were obtained from wild-type CD-1 mice and Pthrp<sup>floxed/<sup>Cre</sup>-</sup> mice injected with tamoxifen and from Pthrp<sup>floxed/<sup>Cre</sup>+</sup> injected with corn oil, then the latter mice were used as controls.

Treatment with cerulein in vivo. Pancreatitis was induced in wild-type CD-1 mice, in Pthrp<sup>floxed/<sup>Cre</sup>-</sup> mice, and Pthrp<sup>floxed/<sup>Cre</sup>+</sup> mice by repetitive intraperitoneal injection of a supramaximally stimulating dose of cerulein (50 μg/kg) at 1-h intervals (22). As a model of AP, mice (n = 6) received seven injections of cerulein and were then euthanized 1 h after the last injection. Serum amylase levels were measured 3 h after the last injection using the Phadebas amylase test kit (Lund, Sweden). Pancreatic edema was evaluated by measuring the wet-to-dry weight ratio, as described previously (26). Data are expressed as the water index (wt wet-to-dry weight ratio).

As a model for CP, mice (n = 10) received five injections of cerulein at 1-h intervals 3 days per week for 3 wk and were euthanized 4 days after the last injection (47, 70). As controls, mice that were injected with PBS used the same injection schedule. In the AP and CP models, pancreata were harvested and processed as described in the Morphological examination.

Pancreatic duct ligation. PDL was performed as described previously (62, 72, 74). Briefly, following isoflurane anesthesia, the mice (n = 10) were surgically prepared, and the pancreas was exposed by a midline abdominal incision. Using a dissecting microscope, we identified the pancreatic duct branches. The splenic duct was detected at the junction between the gastric and the splenic lobes of the pancreas on the left side of the superior mesenteric vein. The duct was ligated with a 7–0 monofilament suture at ~1 mm distal to the junction with the gastric lobe duct, avoiding any damage to vascular structures. The abdominal wall and skin were then closed with suture sutures. The unligated gastric lobe served as a control lobe.

Mice were euthanized 2 days after PDL. Previous studies have shown that, at this time point, there is significant macroscopic and microscopic pancreatic damage, as well as measurable increases in serum and mRNA cytokine levels (81).

Morphological examination. Portions of the dissected mouse pancreata were fixed immediately in 10% neutral buffered formalin for 24 h at room temperature, and then placed in 70% ethanol. Formalin-fixed tissues were embedded in paraffin, and 5-μm sections were cut from the paraffin blocks. The sections were deparaffinized in xylene, rehydrated in descending ethanol series, and prepared for hematoxylin and eosin (H&E) staining (Vector Laboratories, Burlingame, CA). Histology scores were determined on H&E-stained sections using a minimum of five high-power fields/slide (×40) for each of three slides per mouse, in a blinded manner. Histopathological changes were scored according to the criteria described by Demols et al. (17), with some minor modifications.

Procurement of human tissue. Diseased pancreatic tissue was obtained from CP patients undergoing pancreatectomy. Discarded human pancreatic tissue 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-exempt protocol at UTMB. Immediately after collection, samples were placed in liquid nitrogen and stored at −80°C. The samples were then removed from −80°C and placed into 10% neutral buffered formalin overnight at room temperature.

Immunohistochemistry analysis. Immunohistochemical staining of pancreatic sections was performed using the peroxidase ABC kit (Vector Laboratories). Briefly, sections were incubated overnight at 4°C with polyclonal antibodies (diluted in 2% BSA in TBS) against PTHrP (H-137 or N-19; Santa Cruz Biotechnology) or α-smooth muscle actin (α-SMA; Abcam). After three washes with PBS-Tween (TBST), the sections were incubated for 30 min with biotinylated secondary antibody, washed three times with TBST, 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) instead of the

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primary antibody. Images were recorded using an Olympus BX51 microscope at ×40 magnification. Images were processed using the ImageJ software (ImageJ 1.37v; National Institutes of Health, Bethesda, MD) for quantitative analysis via the color deconvolution method. Vectors for hematoxilyn (blue) and DAB (brown) were used in the analysis. The lower-limit threshold was set at 0, and the upper-limit threshold was set at 100. Measurements were taken for integrated density (calculation of area times mean gray value) for brown. Results were obtained from an average of integration density of target over nucleated cells (brown over blue) (53), using a minimum of five fields/slide for each of three slides per mouse. The number of α-SMA-positive cells was counted blindly in at least five high-power fields/slide for each of three slides per mouse.

Isolation, culture, and quality control of human and mouse acinar and stellate cells. Discarded human pancreatic tissue was obtained from surgical resection from cadaveric organ donors. Briefly, the pancreas was perfused with saline, minced, and digested for 15 min in warm isolation buffer [PBS containing Ca\(^{2+}\) and Mg\(^{2+}\), soybean trypsin inhibitor (0.01%, Sigma; cat. no. T6522), 0.1% BSA and collagenase type IV (1 mg/ml, Life Technologies-Invitrogen)]. Pancreatic tissue was rinsed and washed quickly with 3 ml of warm isolation buffer. Pancreatic tissue was finely minced and digested under the same conditions as the human tissue. The digestion was facilitated mechanically with continuous pipetting. Collagenase was inactivated by the addition of 6 ml of cold isolation buffer. The cell suspension was then washed three times in cold isolation buffer and filtered to remove large debris. Acini were collected by centrifugation, resuspended in oxygenated culture medium (DMEM + 10% FBS) containing 0.025% trypsin inhibitor, and seeded into multiwell plates coated with laminin, as described below. Acinar cell viability, assessed by Trypan blue exclusion, was routinely >98%.

Mouse PSCs were isolated using the outgrowth method (5, 34). Primary human PSCs were also isolated using the outgrowth method, with some modifications. Briefly, the pancreatic tissue was rinsed three times in DMEM with 1% penicillin/streptomycin (Life Technologies-Invitrogen) in a 100-mm dish. The tissue was cut into blocks (1–2 mm\(^3\)), placed in 75-cm\(^2\) tissue culture flasks coated with 0.002% type I collagen (Sigma), and cultured in DMEM supplemented with 10% FBS, 1% insulin-transferrinselenium-X (Life Technologies-GIBCO), 1% nonessential amino acid solution (Sigma), 50 µg/ml gentamycin solution (Life Technologies-GIBCO), and 1% penicillin/streptomycin at 37°C. The medium was changed every 3 days starting on day 3. As the colonies grew to 80–90% confluence, the tissue clumps were removed, and cells were split using 0.025% trypsin/EDTA (Life Technologies-GIBCO). The characterization of mouse and human PSCs has been described (7, 25).

For acinar cell culture, cells were plated onto laminin-coated six-well plates. The plates were coated for 2 h on ice with natural mouse laminin (50 µg/ml in PBS; Life Technologies-Invitrogen). Treatment was initiated 24 h after plating. For measurement of amylase secretion, mouse acinar cells were plated onto laminin-coated 24-well dishes. The cells were then treated with cerulein or carbachol. Cerulein (10\(^{-12}\) M to 10\(^{-7}\) M) treatment was for 2 h. In some experiments, cells were treated with PTH\(_{1\,R}\) (1–36) (10\(^{-7}\) M) during the last 15–45 min of cerulein treatment. Carbachol (10\(^{-8}\) M to 10\(^{-4}\) M) treatment was for 30 min. The supernatant was collected, and amylase levels were measured using the Phadebas amylase test kit. For PSC culture, cells were plated onto 6-well dishes. Treatments were initiated when cells reached 70–80% confluence. For measurement of mRNA and protein levels, human and mouse acinar and stellate cells were treated with 10\(^{-7}\) M cerulein for 1 to 8 h. In some experiments, the cells were pretreated for 1 h with the PTH\(_{1\,R}\) antagonist PTH\(_{1\,P}\) (7–34) (Bachem) at 10\(^{-5}\) M prior to treatment with cerulein.

**Immunofluorescence.** PSCs were plated onto eight-well chamber slides (BD Biosciences, Bedford, MA) coated with laminin as described above. When the cells had reached 70% confluence, they were fixed in 95% ethanol. Fixed cells were coinubicated with antibovine antibody (Santa Cruz Biotechnology) overnight. After washing three times with TBST, the sections were incubated in the dark for 1 h with Alexa Fluor 488. The sections were then washed three times with TBST in the dark. Nuclei were counterstained with DAPI.

**Sirius red staining.** Sirius red staining was performed on sections, which were fixed, deparaffinized, and rehydrated, as described above. After staining with hematoxilyn, sections were stained using the Picro-Red solution per the instructions of the manufacturer (IHC-World). Quantification was performed with ImageJ 1.37v, using a minimum of five fields/slide for each of three slides per mouse.

**Analysis of mRNA levels.** Total RNA was extracted from pancreatic tissue using the guanidine thiocyanate method, as previously described (27). Total RNA from isolated acinar and stellate cells was prepared using the RNeaquous isolation kit (Ambion, Austin, TX). RNA concentrations were measured by spectrophotometry. RNA quality was checked by formaldehyde agarose gel electrophoresis. RNA (2.0 µg) was then reverse transcribed into cDNA using the Applied Biosystems cDNA synthesis kit. The first-strand cDNA was used as a template for amplification, using RedTag ReadyMix PCR Master Mix (Sigma) and gene-specific forward and reverse primers. The cDNA was used for real-time PCR, performed using an Applied Biosystems 7500 real-time PCR system and the SYBR Green Supermix (Applied Biosystems, Foster City, CA). The following PTH\(_{1\,R}\) mouse primer is used: forward, CAGTTGAGATGTCCGTGTATT; reverse, GATCTCCGGCATCAGATGGT (6). The other mouse primers used are listed in Ref. 7. The human primers are listed in Table 1 (21, 33, 54, 58, 66, 67). The threshold cycle (C\(_T\)) values for each of the target genes were normalized to those of β-actin or GAPDH, and the relative expression level for each of these target genes was calculated using the formula: n-fold change = 2\(^{-\Delta C_T}\), where ΔC\(_T\) represents C\(_T\) (target sample)−C\(_T\) (control). In the case of PTH\(_{1\,R}\), individual amplicons (10 µl) were also resolved on 1% agarose gels, stained with ethidium bromide, then visualized and photographed using a Fluorchem imaging system (Alpha Innotech, San Jose, CA).

**Western blot analysis.** Frozen pancreatic tissue was homogenized in cold 1 × lysis buffer (7). Cells were washed twice with cold PBS on ice and lysed in RIPA buffer containing a protease inhibitor cocktail and phosphatase inhibitor cocktails A and B (Santa Cruz Biotechnology). Protein concentrations were estimated using the Bio-Rad protein assay. Protein levels were analyzed by Western blot analysis. GAPDH was used as a loading control. The signals were

### Table 1. List of primers used in reverse transcription/real-time PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward primer 5′-3′</th>
<th>Reverse primer 5′-3′</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH(_{1,R})</td>
<td>CTCCTTGGGCTCCACTACATTG</td>
<td>TTGAGGAACCCATGCTCTTG</td>
<td>66</td>
</tr>
<tr>
<td>PTH(_{1,P})</td>
<td>ACCCTGGAGGTCCTCCCTAAC</td>
<td>TCAAGCACCACAATGCGAGG</td>
<td>54</td>
</tr>
<tr>
<td>IL-6</td>
<td>GCCCTCGCCCATGTCCTCTT</td>
<td>GCAGATAGATGATGTTGTC</td>
<td>21</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>GCCACTTCTTCTGTAGATGTG</td>
<td>CTACGCGGCTGGAGCGG</td>
<td>33</td>
</tr>
<tr>
<td>Procollagen-I</td>
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<td>GTTACAGAGAAACAGAGG</td>
<td>58</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CAGGCAACAATCCCTGATGCTTG</td>
<td>GCCGTGTTACACCATGCTCTTG</td>
<td>67</td>
</tr>
</tbody>
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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).

Electroporation of mouse acinar cells. Acinar cells isolated from four mice were gently pelleted, washed three times with RPMI medium, resuspended in 0.5 ml of fresh medium, and placed into a cuvette for electroporation (Gene Pulser Cuvette, Bio-Rad, Hercules, CA; 0.4-cm electrode gap, 250 V, 1,500 μF capacitance and two pulses of 11 ms) (16). An NF-κB reporter sequence in the context of the −162/+44 human IL-8 promoter, cloned upstream of the luciferase reporter (hIL-8/LUC) (8, 12) was electroporated. Empty vector was used as a control. The cells were coelectroporated with a Renilla luciferase construct for standardization purposes. After electroporation, cells were kept on ice for 5–10 min before seeding onto 24-well plates in RPMI medium containing 10% FBS. After 16 h, PTHrP (1–36) or cerulein was added to the medium (final concentration 10^{-7} M). At the indicated time intervals, cell lysates were prepared, and luciferase activity was measured using the dual luciferase assay kit (Promega, Madison, WI). Empty vector control values were subtracted from the respective firefly and Renilla luciferase values. The firefly luciferase activity was normalized to Renilla luciferase activity, and the fold differences were plotted as the firefly/Renilla ratio.

Statistics. Numerical data are presented as the means ± SE. Data were analyzed by one-way ANOVA followed by the Tukey-Kramer multiple-comparisons post hoc test to determine the statistical significance of differences. Statistical analyses were performed using INSTAT Software (GraphPad Software, San Diego, CA).

RESULTS

Induction of chronic pancreatitis results in increased PTHrP levels. PTHrP levels were assessed by immunohistochemistry (IHC) and Western blot analysis in pancreatic sections from mice chronically treated with cerulein and in mice subjected to pancreatic duct ligation (PDL). A: chronic pancreatitis was induced by repetitive injection of cerulein (five injections at 1-h intervals 3 days per wk for 3 wk, with death 4 days after the last injection). Control mice received PBS. B: pancreatic damage was induced by PDL. The control lobe is the unligated gastric lobe. Mice were euthanized 2 days after PDL. Arrows point to islets, which stain positive for PTHrP, while arrowheads point to acini that stain positive for PTHrP. C: PTHrP levels, analyzed by densitometry. Cer, cerulein; PDL, pancreatic duct ligation. Each bar indicates means ± SE of data obtained from five fields/slide for three slides per mouse (six mice/treatment protocol). *Significantly different from the respective control value (P < 0.01). D: immunostaining for PTHrP in kidney (positive control). Sections from the cortex and medulla are shown. A, B, and D: H-137 anti-PTHrP antibody was used. IgG was used as a negative control. Magnification ×40. E: Western blot analysis for PTHrP in pancreata of cerulein-treated and PDL mice. Cerulein treatment and PDL were performed as described in A and B. The figure is representative of data obtained from six mice/treatment protocol.
PDL. Immunostaining for PTHrP is very low in acinar and stellate cells isolated from control mice (injected with PBS and control lobe of PDL mice) (Fig. 1, A and B). Normal kidney, which expresses high levels of PTHrP (20) was used as a positive control (Fig. 1D). In sections from control mice, PTHrP immunostaining was only evident in islets. Chronic treatment with cerulein (5 injections three times per week for 3 wk) resulted in an ~5-fold increase in PTHrP immunostaining levels in the exocrine pancreas compared with the PBS (vehicle control). In sections from control mice injected with corn oil (vehicle) controls (Fig. 1, A and C). A similar effect was observed in mice at 2 days after PDL, where PTHrP levels were ~eight-fold higher in the ligated lobe vs. the control lobe (Fig. 1, B and C). Treatment with cerulein and PDL had no significant effect on PTHrP immunostaining in islet cells (Fig. 1, A and B). These data, generated using the H-137 antibody, were reproduced with a second anti-PTHrP antibody (N-19), confirming the specificity and reproducibility of the response (data not shown). Immunohistochemical findings were confirmed by Western blot analysis, which showed that cerulein and PDL caused an increase in PTHrP levels (Fig. 1E).

Characterization of mice with Pthrp gene ablation in pancreatic acinar cells. To directly address the role of PTHrP in pancreatitis, mice with conditional knockout of the Pthrp gene in acinar cells (Pthrp<sup>Δacinar</sup>) were generated by crossing Pthrpflox/flox mice (29, 38, 44) with ELA1-Cre/ERT2 transgenic mice (Jackson Laboratories). Southern blot analysis of tail-tip genomic DNA confirmed the targeted recombination of the Pthrpflox allele (5.2-kb fragment). Southern blot analysis of Pthrpflox/+ mice showed the expected 6.2-kb fragment (wild-type allele), while 6.2-kb and 5.2-kb fragments were present in Pthrpflox/+ mice (Fig. 2A). Southern blot analysis also revealed the presence of Cre in Cre<sup>+</sup> mice (Fig. 2A).

PTHRP protein levels are very low in acinar and stellate cells of untreated wild-type mice. (Fig. 1, A and B) (7). Therefore, RT-PCR was used to confirm deletion of exon 4 in Pthrpflox/flox<sup>Cre</sup> mice treated with tamoxifen. This exon encodes most of the PTHrP protein (38). Gel electrophoresis of the PCR products obtained using primers encompassing the signal peptide (part of exon 3), and exon 4 showed the expected 220-bp fragment in acinar cells from Pthrpflox/flox<sup>Cre</sup> mice treated with tamoxifen or corn oil (vehicle control) (Fig. 2B). The 220-bp fragment was absent in acinar cells from Pthrpflox/flox<sup>Cre</sup> mice treated with tamoxifen (Fig. 2B). This 220-bp fragment was present in acinar cells from Pthrpflox/flox<sup>Cre</sup> mice injected with corn oil (Fig. 2B), confirming the validity of using corn oil-treated Pthrpflox/flox<sup>Cre</sup> mice or cells derived from these mice as controls. The 220-bp fragment was also present in Pthrpflox/+ mice treated with corn oil or tamoxifen.

Fig. 2. Characterization of mice with conditional knockout of the Pthrp gene in acinar cells (Pthrp<sup>Δacinar</sup>). A: Southern blot analysis of tail-tip genomic DNA to show the floxed Pthrp allele and the Cre transgene. The Cre transgene is regulated by a tamoxifen-inducible recombination system driven by the rat elastase 1 pancreatic promoter. Wild-type mice (+/+), and mice heterozygous (floxed/+), and homozygous (floxed/floxed) for the floxed Pthrp allele are shown. fl, flox; Cre<sup>+</sup>, Cre-positive; Cre<sup>−</sup>, Cre-negative. The wild-type PTHrP fragment is represented by the 6.2-kb band, and the floxed PTHrP fragment is represented by the 5.2-kb band. B: reverse transcription/PCR of RNA from acinar and stellate cells isolated from mice with conditional knockout of the Pthrp gene in acinar cells (Pthrpflox/flox<sup>Cre</sup>−). Cells from Pthrpflox/flox<sup>Cre</sup>− mice were used as controls. tam, tamoxifen. C: immunostaining for PTHrP in pancreatic sections from Pthrpflox/flox<sup>Cre</sup>− and Pthrpflox/+ mice. Arrows point to islets, which stain positive for PTHrP. D: PTH1R mRNA levels in acinar and stellate cells isolated from mice with conditional knockout of the Pthrp gene in acinar cells. PTH1R mRNA levels were measured by reverse transcription/real-time PCR. Values are expressed relative to the control (PTHrP<sup>+/+</sup>) acinar cell value, set arbitrarily at 1.0. Each bar is the mean ± SE of three independent experiments (three pancreata/experiment). C and D: PTHrP<sup>Δacinar</sup> = Pthrpflox/flox<sup>Cre</sup> mice injected with tamoxifen; PTHrP<sup>+/+</sup> = Pthrpflox/flox<sup>Cre</sup> mice injected with corn oil (vehicle control).
PTHrP levels in response to cerulein treatment play a role in pancreatic damage, evident by the presence of edema and necrosis (7, 22). To determine whether increased PTHrP levels in response to cerulein treatment play a role in the subsequent damage characteristic of AP, experimental pancreatitis was induced in PTHrPΔacinar and PTHrPΔ/+ mice. We first assessed the effect of cerulein on PTHrP levels in pancreata from these mice. PthrpΔacinar mice and PthrpΔ/+ mice did not alter PTH1R levels in PSCs (Fig. 2D). The PTH1R is also expressed in PSCs. Deletion of the Pthrp gene in acinar cells did not alter PTH1R levels in PSCs (Fig. 2D).

Pancreas weight in mice fed ad libitum (data not shown). Henceforth, acinar cells from PthrpΔacinar; Cre+ mice injected with tamoxifen will be referred to as PTHrPΔacinar. PthrpΔacinar; Cre+ mice injected with corn oil will be referred to as PTHrPΔ/+.

The 220-bp fragment was present in PSCs prepared from tamoxifen-treated PthrpΔacinar; Cre+ and PthrpΔacinar; Cre− mice (Fig. 2B). These data confirm that deletion of the Pthrp gene in acinar cells had no effect on PTHrP expression in PSCs. Moreover, IHC confirmed that deletion of the Pthrp gene in acinar cells did not alter PTHrP levels in islets (Fig. 2C). Pthrp gene deletion in acinar cells did not alter body weight in mice fed ad libitum (data not shown).

PTHrP signaling is mediated via interaction with the PTH1R (41). RT-real-time PCR showed that deletion of the Pthrp gene in acinar cells had no significant effect (P > 0.05) on PTH1R levels in these cells (Fig. 2D). The PTH1R is also expressed in PSCs. Deletion of the Pthrp gene in acinar cells also did not alter PTH1R levels in PSCs (Fig. 2D).

**Fig. 3.** Effect of conditional knockout of the Pthrp gene in acinar cells on pancreatic damage induced by acute cerulein treatment. A: Western blotting for PTHrP in cerulein-treated PTHrPΔacinar mice and in mice expressing PTHrP in acinar cells (PTHrPΔ/+). PTHrPΔacinar; Cre+ mice and PTHrPΔacinar; Cre− mice were injected with tamoxifen or corn oil (vehicle control) once daily for 5 days. At 7 days after the last tamoxifen injection, mice were injected with cerulein (seven injections at 1-h intervals, with euthanasia 1 h after the last injection) results in pancreatic damage, evident by the presence of edema and necrosis (7, 22). To determine whether increased PTHrP levels in response to cerulein treatment play a role in the subsequent damage characteristic of AP, experimental pancreatitis was induced in PTHrPΔacinar and PTHrPΔ/+ mice. We first assessed the effect of cerulein on PTHrP levels in pancreata from these mice. PthrpΔacinar; Cre+ mice and PthrpΔ/+ mice
PTHrP\textsuperscript{floxflox;}Cre\textsuperscript{-} mice were treated with corn oil or tamoxifen, then with cerulein or PBS. Cerulein increased PTHrP levels in the PTHrP\textsuperscript{floxflox;}Cre\textsuperscript{-} mice treated with corn oil or tamoxifen (PTHrP\textsuperscript{+/+}), and in the PTHrP\textsuperscript{floxflox;}Cre\textsuperscript{-} mice treated with corn oil (Fig. 3, A and B). The effect of cerulein on tamoxifen-treated PTHrP\textsuperscript{floxflox;}Cre\textsuperscript{-} mice was significantly attenuated (Fig. 3A); densitometric analysis showed no significant difference ($P > 0.05$) in PTHrP levels after cerulein treatment (Fig. 3B). Wild-type mice showed a similar profile as the PTHrP\textsuperscript{floxflox;}Cre\textsuperscript{-} mice (data not shown). These data ascertain the recombination efficiency of the Cre-ERT system and again confirm the validity of using PTHrP\textsuperscript{floxflox;}Cre\textsuperscript{-} mice injected with corn oil as controls.

After cerulein treatment, significant pancreatic damage was observed in sections from the PTHrP\textsuperscript{+/+} mice. This damage was evident as edema, necrotic debris, and presence of vacuoles (Fig. 3C). In contrast, PTHrP\textsuperscript{Δacinar} resulted in a significant decrease in overall pancreatic damage, as evident by a reduction in the extent of edema, presence of necrotic debris, and vacuole formation (Fig. 3C). The protective effect of PTHrP\textsuperscript{Δacinar} was confirmed by semiquantitative analysis using edema, necrotic debris, and vacuoles as parameters (Fig. 3D). Wild-type mice and PTHrP\textsuperscript{floxflox;}Cre\textsuperscript{-} mice treated with corn oil or tamoxifen showed a similar profile of pancreatic damage as PTHrP\textsuperscript{floxflox;}Cre\textsuperscript{-} mice treated with corn oil (PTHrP\textsuperscript{+/+} mice) (data not shown). Cerulein-induced pancreatic damage is associated with significant edema. Using the pancreas wet-to-dry ratio as readout, we show that cerulein induced significant edema in mice expressing PTHrP in acinar cells (Fig. 3E). The presence of edema was significantly attenuated ($P < 0.01$) in mice with deletion of the Pthrp gene in acinar cells, such that in these mice there was no significant difference ($P > 0.05$) in the wet-to-dry weight ratio of the pancreas in cerulein-treated vs. PBS-treated mice (Fig. 3E).

Deletion of the Pthrp gene in acinar cells also attenuated the effect of cerulein on serum amylase levels. Thus, while ceru-

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

Fig. 4. Effect of conditional knockout of the Pthrp gene in acinar cells on pancreatic damage in the cerulein and pancreatic duct ligation models of chronic pancreatitis. PTHrP\textsuperscript{floxflox;}Cre\textsuperscript{-} mice were injected with tamoxifen or corn oil (vehicle control) once daily for 5 days. A and B: starting 7 days after the last tamoxifen injection, mice were injected with cerulein (five injections at 1-h intervals) 3 times a week for 3 wk, then euthanized 4 days after the last cerulein injection. Control mice received the same regimen of PBS. C and D: at 7 days after the last tamoxifen injection, mice were subjected to PDL. The control lobe is the unligated gastric lobe. Mice were euthanized 2 days after PDL. A and C: H&E staining of pancreatic sections, showing morphologic changes. Magnification ×40. Representative vacuoles are indicated by arrowheads, while inflammatory cell infiltration is indicated by arrows, and edema is shown by asterisks. B and D: histology scores of pancreatic injury in the H&E-stained pancreas sections. Each bar represents the mean ± SE of five fields/slide for three slides per mouse (10 mice/treatment protocol). *Significantly different from the respective control value ($P < 0.01$). #Significantly different from the cerulein (B) or PDL (D) PTHrP\textsuperscript{+/+} value ($P < 0.01$).
lein treatment significantly increased (P < 0.01) serum amylase levels in PTHrP<sup>+/+</sup> mice, this effect was significantly attenuated in PTHrP<sup>Δacinar</sup> mice (Fig. 3F).

**PTHrP<sup>Δacinar</sup> protects against pancreatic damage in the chronic cerulein and PDL models of pancreatitis.** The chronic cerulein injection protocol and the PDL model were used to determine whether PTHrP plays a role in pancreatic damage observed in CP. Mice were euthanized 2 days after PDL because previous studies have shown that, at this time point, there is both significant macroscopic and microscopic pancreatic damage (with evidence of tissue destruction and fibrosis), as well as measurable increases in serum and mRNA cytokine levels (81). For the cerulein model, gel electrophoresis of RT-PCR products confirmed that PTHrP expression was still nondetectable at time of death (data not shown). In PTHrP<sup>+/+</sup> mice, chronic cerulein and PDL-induced pancreatic damage, evident as edema, necrosis, inflammatory infiltration, and vacuole formation (Fig. 4A). This damage was more severe in the PDL mice, with more evidence of edema and inflammatory infiltration and presence of necrotic debris and vacuoles (Fig. 4C). PTHrP<sup>Δacinar</sup> exerted a strong protective effect in both models, as seen by comparing acinar architecture and inflammatory cell infiltration (Fig. 4, A and C). The protective effect was more evident in sections from PDL mice, since the extent of damage in PTHrP<sup>+/+</sup> mice subjected to PDL was greater than that in the chronic cerulein-treated mice (Fig. 4A vs. 4C). The protective effect of PTHrP<sup>Δacinar</sup> was confirmed by semi-quantitative analysis using edema, inflammatory infiltration, necrosis, and vacuoles as parameters (Fig. 4, B and D).

**PTHrP<sup>Δacinar</sup> inhibits cerulein- and PDL-induced PSC activation and ECM deposition.** Pancreatic damage is accompanied by PSC activation, a hallmark of which is expression of α-SMA (49). To assess the role of acinar cell-secreted PTHrP in PSC activation following pancreatic damage, α-SMA levels were compared in sections from PTHrP<sup>Δacinar</sup> mice vs. PTHrP<sup>+/+</sup> mice. Chronic cerulein treatment caused PSC activation, as indicated by **α-SMA** positive cells (Fig. 5A, B, C, D, E). A significant increase in α-SMA-positive cells was observed in the PDL lobe compared to the control lobe in both PTHrP<sup>+/+</sup> and PTHrP<sup>Δacinar</sup> mice. Western blot analysis confirmed these findings, with significantly higher α-SMA levels in the PDL lobe compared to the control lobe in both PTHrP<sup>+/+</sup> and PTHrP<sup>Δacinar</sup> mice (Fig. 5E).

**Fig. 5.** Effect of conditional knockout of the **Pthrp** gene in acinar cells on stellate cell activation in the cerulein and pancreatic duct ligation models of chronic pancreatitis. **Pthrp**<sup>fl/flox;Cre</sup> mice were injected with tamoxifen or corn oil (vehicle control) once daily for 5 days. A, B, E: starting 7 days after the last tamoxifen injection, mice were injected with cerulein as described in Fig. 4. C, D, E: at 7 days after the last tamoxifen injection, mice were subjected to PDL, as described in Fig. 4. A, C: immunostaining for α-SMA. Representative positive cells are indicated by arrows. Magnification ×40. B, D: α-SMA-positive cells in the periacinar spaces of the immunostained sections. Data are obtained from six different fields/slide for three slides/mouse (10 mice/treatment protocol). *Significantly different from the respective control value (P < 0.01). #Significantly different from the cerulein (B) or PDL (D) **PTHrP**<sup>+/+</sup> value (P < 0.01). E: Western blot analysis of α-SMA levels in pancreata of cerulein-treated and PDL mice. − denotes PBS injection or control lobe.
vation in \textit{PTHrP}^{+/-} mice, evident as an increase in \(\alpha\)-SMA-positive cells localized in fibrotic areas around acini and in vascular walls (Fig. 5A). No evidence of PSC activation was seen in PBS-treated mice (Fig. 5A). After cerulein treatment, the number of \(\alpha\)-SMA-positive cells was significantly lower (\(P < 0.01\)) in sections from \textit{PTHrP}^{\Delta\text{acinar}} mice vs. \textit{PTHrP}^{+/-} mice (Fig. 5, A and B). A similar profile was observed in the PDL mice. Significant \(\alpha\)-SMA staining was seen in PDL sections from \textit{PTHrP}^{+/-} mice. The extent of PSC activation was significantly (\(P < 0.01\)) reduced in sections from \textit{PTHrP}^{\Delta\text{acinar}} mice, where \(\alpha\)-SMA staining was limited to the vascular walls (Fig. 5, C and D). These results were verified by Western blot analysis; the increase in \(\alpha\)-SMA levels after cerulein treatment and PDL was more pronounced in \textit{PTHrP}^{+/-} mice than in \textit{PTHrP}^{\Delta\text{acinar}} mice (Fig. 5E).

ECM deposition by activated PSCs plays a critical role in the development of the pathologic fibrosis characteristic of CP. Both the 3-wk cerulein treatment protocol and the PDL model resulted in an increase in collagen deposition in the periacinar and periductal regions of \textit{PTHrP}^{+/-} mice (Fig. 6, A and C). The extent of collagen deposition was significantly reduced (\(P < 0.01\)) in sections from \textit{PTHrP}^{\Delta\text{acinar}} mice (Fig. 6, A–D).

\textit{PTHrP}^{\Delta\text{acinar}} blocks the increase in IL-6 expression associated with pancreatic cell damage. The inflammatory response associated with pancreatitis is accompanied by an increase in IL-6 levels (43). Here, we assessed the effect of \textit{Pthrp} gene deletion on pancreatic IL-6 mRNA levels. Basal IL-6 levels were approximately twofold higher in pancreata of \textit{PTHrP}^{+/-} mice compared with \textit{PTHrP}^{\Delta\text{acinar}} mice (Fig. 7). In vivo chronic cerulein treatment and PDL caused an ~4–5-fold increase in IL-6 mRNA levels (Fig. 7). These effects were attenuated in pancreata of \textit{PTHrP}^{\Delta\text{acinar}} mice (Fig. 7).

\textit{PTHrP}^{\Delta\text{acinar}} suppresses the cerulein-mediated increase in IL-6 expression and blocks NF-\(\kappa\)B activity in isolated acinar cells. The effects of \textit{PTHrP} in the pancreas may be mediated via a systemic pathway and/or an autocrine/paracrine pathway at the acinar cell level. To ask whether \textit{PTHrP} regulates cytokine levels at the acinar cell level, acinar cells were isolated from \textit{PTHrP}^{\Delta\text{acinar}} and \textit{PTHrP}^{+/-} mice. These cells were then treated with cerulein in vitro. Cerulein increased IL-6 mRNA levels in \textit{PTHrP}^{+/-} acinar cells; this effect was evident after 1 h of treatment and peaked at 2 h (Fig. 8A). The same profile was obtained using cells from \textit{Pthrp}^{\text{flox/flox},\text{Cre}^-} and wild-type mice (not shown). In contrast, the effect of

![Fig. 6. Effect of conditional knockout of the \textit{Pthrp} gene in acinar cells on collagen deposition in the cerulein and pancreatic duct ligation models of chronic pancreatitis. \textit{PTHrP}^{\text{flox/flox},\text{Cre}^-} mice were injected with tamoxifen or corn oil (vehicle control) once daily for 5 days. A and B: starting 7 days after the last tamoxifen injection, mice were injected with cerulein as described in Fig. 4. C and D: at 7 days after the last tamoxifen injection, mice were subjected to PDL as described in Fig. 4. Sirius red staining in cerulein-treated (A) and PDL (C) mice. Representative periacinar Sirius red staining is indicated by arrows. Magnification \(\times 40\). B and D: Sirius red staining, analyzed by densitometry. Each bar represents the mean \pm SE of data obtained from five fields/slide for three slides per mouse (10 mice/treatment protocol). *Significantly different from the respective control value (\(P < 0.001\)). #Significantly different from the cerulein (B) or PDL (D) \textit{PTHrP}^{+/-} value (\(P < 0.01\)).](http://ajpgi.physiology.org/)
cerulein on IL-6 levels in PTHrP-h1acinar cells was significantly attenuated, such that there was no increase in IL-6 mRNA levels at any of the time points tested (Fig. 8A).

IL-6 expression is regulated by the NF-κB signaling pathway (26). To determine whether PTHrP regulates NF-κB activity, acinar cells from wild-type mice were transfected with an NF-κB-DAtargetablereporterconstruct, and then treated with PTHrP (1–36). PTHrP increased NF-κB activity; this effect was observed after 2 min of treatment and peaked at 10 min (Fig. 8B). We also asked whether PTHrP plays a role in the reported CCK-mediated activation of the NF-κB pathway (28). Treatment of PTHrP-h1acinar acinar cells with cerulein significantly increased (P < 0.001) NF-κB activity, with a peak occurring after 60 min of treatment (Fig. 8C). In contrast, cerulein had no effect on NF-κB activity in PTHrP-h1acinar cells (Fig. 8C).

**PTHrP-h1acinar suppresses the cerulein- and carbachol-induced increase in amylase release.** Both the direct and indirect effects of cerulein involve interaction with the CCK1 receptor (CCK1R) (30). Because the effects of cerulein on IL-6 levels and NF-κB activity were suppressed in PTHrP-h1acinar mice, here, we determined whether deletion of the Pthrp gene in acinar cells alters CCK1R levels. We report that the Pthrp gene deletion had no effect on CCK1R mRNA levels (data not shown).

**PTHrP-h1acinar inhibits the cerulein-induced increase in amylase secretion in vivo.** To ascertain whether the effects of cerulein on amylase secretion are specific to the CCK1R, PTHrP-h1acinar and PTHrP-h1+ acinar cells were treated with carbachol, a secretagogue which functions via the M3 muscarinic cholinergic receptors (37). Carbachol significantly increased (P < 0.001) amylase secretion from PTHrP-h1+ acinar cells, with a peak at 10−6 M (Fig. 8E). This effect of carbachol was significantly attenuated in PTHrP-h1acinar cells (Fig. 8E).

**PTHrP-h1acinar blocks the cerulein-mediated upregulation of procollagen I mRNA levels in stellate cells.** PSCs from PTHrP-h1acinar mice express PTHrP (Fig. 2B), and treatment with PTHrP (1–36) upregulates procollagen I mRNA levels in PSCs (7). Treatment with cerulein also upregulated procollagen I mRNA levels in PSCs (7). To see whether Pthrp gene knockout in acinar cells alters the response of PSCs to cerulein, PSCs isolated from PTHrP-h1acinar and PTHrP-h1+ mice were treated with cerulein in vitro. The identity of the isolated PSCs was confirmed by staining with vimentin (Fig. 9A). In PSCs derived from mice expressing PTHrP in acinar cells, cerulein significantly increased procollagen I mRNA levels. This effect was evident after 2 h of treatment and peaked at 4 h (Fig. 9B). In contrast, there was no significant effect of cerulein on procollagen I levels at any of the time-points tested in PSCs from PTHrP-h1acinar mice (Fig. 9B).

To determine whether PSCs from PTHrP-h1acinar mice lost their ability to respond to exogenous factors, PSCs were treated with PTHrP (1–36), PTHrP (10−7 M for 2 h) increased procollagen I mRNA levels in PSCs isolated from PTHrP-h1acinar mice, and there was no significant difference (P > 0.05) in the response to PTHrP in PSCs from PTHrP-h1acinar vs. PTHrP-h1+ mice (Fig. 9C). We also asked whether cerulein regulates PTHrP levels in PSCs from PTHrP-h1acinar mice. Treatment with cerulein increased PTHrP levels in PSCs from both PTHrP-h1acinar mice and PTHrP-h1+ mice. However, the pattern of induction was different, in that cerulein produced a sustained, ~1.5-fold increase in PTHrP mRNA levels in PSCs isolated from PTHrP-h1acinar mice (Fig. 9D). In PSCs isolated from PTHrP-h1+ mice, there was a gradual increase in PTHrP mRNA levels, with a peak effect at 6 h (Fig. 9D). The peak effect in PSCs from PTHrP-h1+ was significantly higher (P < 0.01) than that in PSCs from PTHrP-h1acinar mice (Fig. 9D).

**Pancreatic cell damage increases PTHrP levels in human acinar and stellate cells.** To ask whether PTHrP levels are elevated in patients with CP, and, thus, validate a role for PTHrP in human pancreatitis, PTHrP immunostaining was compared in sections from CP patients and normal pancreas. PTHrP immunostaining was elevated in CP vs. control pancreas sections (Fig. 10, A and B). These data support a role for PTHrP signaling in human pancreatic disease. The effect of pancreatic cell damage on PTHrP levels was also evaluated in isolated human acinar and stellate cells. These cells express the PTH1R, as determined by RT/real-time PCR; expression levels were comparable to those in mouse cells (data not shown). PTHrP mRNA levels were approximately three-fold higher in untreated acinar vs. stellate cells (Fig. 10C). Treatment with cerulein (10−7 M) increased PTHrP mRNA levels by approximately two- to three-fold (P < 0.01) (Fig. 10C). Cerulein also increased PTHrP protein levels (Fig. 10D).
PTHrP plays a role in the cerulein-mediated increase in proinflammatory cytokines and procollagen I levels in human cells. To determine whether PTHrP regulates IL-6 and ICAM-1 levels in human acinar cells, the cells were treated with PTHrP (1–36) (10⁻⁷ M). PTHrP increased both IL-6 and ICAM-1 levels, with a peak effect at 30 min (Fig. 11A). PTHrP (1–36) also upregulated procollagen I mRNA levels in PSCs, with a peak increase after 2 h of treatment (Fig. 11B).

To assess the role of PTHrP in the cerulein-mediated effects on proinflammatory and profibrotic mediators, autocrine/paracrine PTHrP action was blocked by pretreatment with the PTH1R antagonist PTHrP (7–34). The effects of cerulein on IL-6 and ICAM-1 levels in acinar cells and on procollagen I levels in PSCs were blocked by PTHrP (7–34) (Fig. 11, C and D). Thus, PTHrP functions as an intermediate in the cerulein effects on proinflammatory mediators and ECM proteins in human acinar andstellate cells.

DISCUSSION

AP is a clinical syndrome that begins with an injury to the pancreas and results in an inflammatory response (76). The pathophysiology of AP involves dysfunction of acinar cells, and damage to acinar cells results in the release of inflammatory cytokines (36). Paracrine factors released by damaged acinar cells also activate PSCs, which, in turn, produce autocrine factors that maintain an activated phenotype (3, 4, 49). Repeated episodes of inflammation and injury to the pancreas are then believed to lead to CP, which is characterized by destruction of acinar tissue, a sustained pancreatic inflammatory response and fibrosis (15). CP is also associated with an increased risk of pancreatic cancer, cardiovascular disease, and severe infection. PTHrP is normally expressed in pancreatic islets and regulates cell proliferation, apoptosis, and insulin release (13, 14, 71). We have previously shown that PTHrP...
levels are transiently elevated in the exocrine pancreas of mice treated with the CCK analog cerulein to induce AP (7). Here, we show that PTHrP levels are also elevated in the mouse model of CP (induced by repetitive chronic cerulein injection) and in PDL, indicating that PTHrP may play a role in sustained injury of the pancreas, leading to irreversible damage. Moreover, we provide evidence for a role of PTHrP in human pancreatic acinar and stellate cells.

Acinar cell injury results in the release of proinflammatory cytokines and chemokines, causing histological damage that is evident as cytoplasmic vacuolization, edema formation, and infiltration of inflammatory cells into the cells (31). To investigate whether the increase in PTHrP levels as a result of pancreatic damage plays a role in AP pathophysiology, we developed a mouse model with conditional knockout of the Pthrp gene in acinar cells (PTHrP\(^{\Delta\text{acinar}}\)). We provide evidence for a role for acinar cell-expressed PTHrP in AP, in that PTHrP\(^{\Delta\text{acinar}}\) exerts a protective effect. Coupled with our previous findings showing that the PTH1R antagonist PTHrP (7–34) suppresses the cerulein-induced increase in IL-6 levels in isolated acinar cells, we propose that PTHrP participates in the early stages of pancreatic injury to initiate a cascade of events that ultimately leads to proinflammatory cytokine release and pancreatic cell injury. PTHrP can function via both intracrine and autocrine/paracrine pathways (79). Future studies utilizing intraperitoneal injection of PTHrP (1–36) will help determine whether PTHrP expressed by acinar cells in response to injury functions via an intracrine pathway or whether PTHrP secreted by acinar cells exerts its effect via autocrine and paracrine pathways.

The SAPE hypothesis model links RAP with the ultimate development of CP (60, 78, 80). We used the chronic repetitive cerulein model to assess the role of acinar cell-expressed PTHrP in development of CP as a result of repeated injury followed by periods of recovery (RAP). Our data support a pivotal role for PTHrP in this model of CP, in that PTHrP\(^{\Delta\text{acinar}}\) inhibited changes in pancreatic architecture, as well as PSC activation and ECM deposition. A protective effect of PTHrP\(^{\Delta\text{acinar}}\) is also observed in the PDL model of pancreatitis, which mimics pancreatic duct obstruction due to stenosis and/or intraductal stones in humans (35). The protective effect achieved by deletion of the Pthrp gene in both models of pancreatic injury validates the specificity of the response and implies that the effects of PTHrP are not solely mediated through an effect on cerulein-induced signaling. Other models of AP, such as intraductal injection of bile salts or feeding of a choline-deficient ethionine-supplemented (CDE) diet (35), will be used in future studies to support the results obtained using the cerulein model. The effects of chronic repetitive cerulein and PDL on pancreatic IL-6 mRNA levels are also significantly attenuated in PTHrP\(^{\Delta\text{acinar}}\) mice, further strengthening our hypothesis that PTHrP exerts its effects by upregulating proinflammatory cytokine release and that suppression of PTHrP expression in acinar cells prevents pancreatic cell damage. These effects of PTHrP are likely mediated via an NF-κB-dependent pathway, as PTHrP increases NF-κB activity in acinar cells and the cerulein-mediated increase in NF-κB activity is suppressed in PTHrP\(^{\Delta\text{acinar}}\) cells. PTHrP also activates the NF-κB pathway in prostate cancer cells (8). Therefore, we conclude that the effects of PTHrP on proinflammatory cytokine release are mediated via an NF-κB-dependent pathway, and that PTHrP may function at the systemic level by enhancing release of proinflammatory cytokines and chemokines. Pthrp gene knockout also exerts a protective effect when acinar cell damage is induced in isolated cells. In combination with previous findings showing a sup-

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**Fig. 9.** A: vimentin immunofluorescence in stellate cells from PTHrP\(^{\Delta\text{Cre}}\) mice injected with tamoxifen (PTHrP\(^{\Delta\text{Cre}}\)) or corn oil (PTHrP\(^{+/+}\)). The cell nucleus was stained with DAPI (blue). B: effect of cerulein on procollagen I mRNA levels. C: effect of PTHrP (1–36) on procollagen I mRNA levels. D: effect of cerulein on PTHrP mRNA levels. B–D: PSCs isolated from PTHrP\(^{\Delta\text{acinar}}\) and PTHrP\(^{+/+}\) mice were used. Cells were treated with cerulein (10\(^{-7}\) M) or PTHrP (1–36) (10\(^{-7}\) M) for the indicated time intervals. mRNA levels were analyzed by RT/real-time PCR. Each bar or point is the mean ± SE of three independent experiments (three pancreata/experiment). B and D: values are expressed relative to the 0 time value, set arbitrarily at 1.0. *Significantly different from the corresponding PTHrP\(^{+/+}\) value (P < 0.001). #Significantly different from the 0 time value (P < 0.01). C: values are expressed relative to the control PTHrP\(^{+/+}\) value, set arbitrarily at 1.0. **Significantly different from the respective control value (P < 0.001).
pressive effect of PTHrP (7–34) on the upregulatory effects of cerulein on IL-6 levels in vitro, these data strongly support a pivotal role for PTHrP directly at the acinar cell level. One of the earliest consequences of exposure of acinar cells to supramaximal concentrations of secretagogue both in vitro and in vivo is the intra-acinar stimulation of digestive enzymes; premature intrapancreatic activation of digestive enzyme granules plays a key role in triggering AP (57). Pthrp gene knockout in acinar cells inhibited the increase in serum amylase levels in the in vivo model of cerulein-induced AP. Bothzymogen activation and stimulation of NF-κB activity have been associated with activation of specific PKC isoforms, giving rise to the pathological changes observed in AP (59, 69). Studies have implicated the novel PKC isozymes PKC-ε in these effects (59, 69). We postulate that the cerulein-induced upregulation of PTHrP expression results in PKC-ε activation, leading to amylase secretion and NF-κB activation. Pthrp gene deletion inhibits these cerulein effects, thereby limiting pancreatic damage. Future studies will address the role of PTHrP in the activation of these novel PKC isoforms. Cerulein couples through heterotrimeric G proteins of the Gq family to increase intracellular free Ca^{2+}, leading to the release of zymogen granule content (76). The effects of cerulein on NF-κB activity and amylase release also involve modulation of intracellular Ca^{2+} levels (28, 63, 68, 77). Future studies may focus on whether PTHrP_{acinar} suppresses the cerulein-induced regulation of intracellular Ca^{2+} signaling, thereby inhibiting amylase release and NF-κB activity.

The actin cytoskeleton has long been implicated in protein secretion (10). The small G proteins RhoA and Rac1 play a role in amylase secretion after stimulation with both physiological and superphysiological concentrations of cerulein (9, 10, 56). Treatment with cerulein (at 3 \times 10^{-11} M to 10^{-8} M) increases both RhoA and Rac1 activity (10). Overexpression of dominant-negative RhoA and Rac1 significantly decreases amylase secretion from isolated mouse acinar cells at this same concentration range, with RhoA and Rac1 exerting additive effects (10). RhoA and Rac1 also play a role in stimulation of amylase secretion by carbachol, a secretagogue that functions via M3 muscarinic receptors (10). PTHrP_{acinar} suppressed the effects of both cerulein and carbachol on amylase release, indicating that the PTHrP effects are not secretagogue-specific. CCK stimulation activates Rac1 and RhoA via two independent pathways, the Gα13 and PKC-α signaling pathways (56).
PTHRP activates the Rho GTPase Rac1 in colon cancer cells (45) and stimulates the formation and organization of actin stress fibers and actin expression in trophoblast outgrowth (19). On the basis of these data, we postulate that PTHrP plays a role in the secretagogue-induced amylase secretion from pancreatic acini via a mechanism involving RhoA and Rac1 signaling. Mice with Pthrp gene deletion may be defective in RhoA and Rac1 signaling, resulting in a defective actin cytoskeleton and inhibition of amylase release. Because exogenous addition of PTHrP (1–36) did not restore the cerulein-mediated increase in amylase secretion in PTHrP<sup>−/−</sup> cells, the absence of PTHrP expression in vivo may alter acinar cells in such a way as to render them unresponsive to cerulein. Alternatively, other isoforms of PTHrP may mediate the amylase response and/or PTHrP may regulate amylase secretion via an intracellular pathway. Future studies will address the role of PTHrP in the RhoA- and Rac1-mediated stimulation of amylase secretion after secretagogue stimulation.

PSCs make up ~4–7% of the total pancreatic cell content and normally exist in a quiescent phenotype (5, 49). Release of proinflammatory signals from acinar cells in response to pancreatic injury leads to PSC activation (49, 51). ECM deposition by activated PSCs plays a critical role in development of the pathologic fibrosis characteristic of CP (49). Here, we assessed the effect of PTHrP expressed by acinar cells on PSC activation. As expected, PTHrP expression in PSCs from PTHrP<sup>−/−</sup> mice was comparable to that in wild-type mice, further indicative of selective Pthrp gene knockout in acinar cells. Interestingly, PTHrP<sup>−/−</sup> mice had attenuated PSC activation and suppressed development of fibrosis in both in vivo pancreatitis models, as evident by lower α-SMA-positive PSCs and decreased collagen deposition compared with mice expressing PTHrP in acinar cells. Since PTHrP is a secreted protein (14), these data strongly indicate a role for acinar cell-secreted PTHrP functioning via a paracrine pathway in PSC activation and fibrosis associated with CP. The absence of PTHrP secretion from acinar cells protects against both the proinflammatory and profibrotic response associated with pancreatic injury. Acinar cell-secreted PTHrP may exert a direct effect in PSCs and/or may lead to the recruitment of inflammatory cells, which, in turn, activate PSCs (11, 43, 78).

The intricate relationship between acinar and stellate cells in vivo is further demonstrated by the experimental data obtained when isolated PSCs are subjected to cerulein-induced damage in vitro. PSCs express PTH1R and are responsive to PTHrP; treatment with cerulein increases PTHrP expression in these cells (7). Inhibiting PTHrP signaling suppresses the upregulatory effect of cerulein on procollagen I levels (7). In this study, we show that exogenous PTHrP increases procollagen I mRNA levels in PSCs isolated from PTHrP<sup>+/+</sup> and PTHrP<sup>−/−</sup> mice, supporting an autocrine role for PTHrP and confirming that PSCs from PTHrP<sup>−/−</sup> mice are still capable of responding to exogenous stimulants. Moreover, cerulein increases PTHrP mRNA levels in PSCs from PTHrP<sup>−/−</sup> mice, although both the magnitude and the time course profile of the effect differ. In view of the lack of effect of cerulein on procollagen I levels in PSCs from PTHrP<sup>−/−</sup> mice, we speculate that, while PSCs themselves are capable of synthesizing cytokines, such as TGF-β1, activin A, and IL-1 (3, 48, 64), which, in turn, may play a role in regulating ECM.
levels, this ability may be lost in PSCs in cell-cell contact with acinar cells lacking PTHrP expression. The in vivo interaction of PSCs with acinar cells with Pthrp gene knockout may, thus, alter PSCs in a manner that carries over and is retained by the isolated cells. The effects of cerulein on procollagen I levels in PSCs may require the presence of additional factors that are regulated by acinar cell PTHrP. In this scenario, PSCs from wild-type mice (in cell-cell contact with PTHrP-secreting acinar cells) would still respond to cerulein with an increase in procollagen I levels via a PTHrP-dependent pathway.

CCK stimulation rapidly increases pancreatic secretion in both humans and rodents (73). In rodents, this effect is mediated via both direct and indirect pathways. The direct pathway involves interaction of CCK with the CCK1R, a G protein-coupled receptor, in acinar cells (1, 50). The indirect effect involves interaction of CCK with the CCK1R expressed in afferent neurons that regulate pancreatic secretion via a vagal-vagal loop, with the final mediator being ACh (1, 50). There has been some controversy about whether the direct pathway is operational in human acinar cells. While some studies have reported that human acinar cells might lack functional CCK1R (30, 75), a more recent study has shown that physiological concentrations of CCK directly stimulate amylase release by isolated human pancreatic acinar cells (46). Our studies show that hyperstimulation of the CCK1R with cerulein increases PTHrP, IL-6, and ICAM-1 levels in human cells, and that hyperstimulation of the CCK1R with cerulein increases concentrations of CCK directly stimulate amylase release by human acinar cells in our studies were isolated as cell clusters, and PTHrP plays a role in these stimulatory effects. The human PTHrP, IL-6, and ICAM-1 levels in human cells, and that physiological concentrations of CCK directly stimulate amylase release by isolated human pancreatic acinar cells (46). Our studies show that hyperstimulation of the CCK1R with cerulein increases PTHrP, IL-6, and ICAM-1 levels in human cells, and that hyperstimulation of the CCK1R with cerulein increases concentrations of CCK directly stimulate amylase release by isolated human pancreatic acinar cells (46). We show that the response of PSCs to cerulein is accompanied by 10.220.33.3 on June 27, 2017 http://ajpgi.physiology.org/ Downloaded from

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REFERENCES


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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


