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Exposure to Radiocontrast Agents Induces Pancreatic Inflammation by Activation of Nuclear Factor- κ B, Calcium Signaling, and Calcineurin

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Abstract

Background & Aims—Radiocontrast agents are required for radiographic procedures, but these agents can injure tissues by unknown mechanisms. We investigated whether exposure of pancreatic tissues to radiocontrast agents during endoscopic retrograde cholangiopancreatography (ERCP) causes pancreatic inflammation, and studied the effects of these agents on human cell lines and in mice.

Methods—We exposed mouse and human acinar cells to the radiocontrast agent iohexol (Omnipaque) and measured intracellular release of Ca^{2+} , calcineurin activation (using a luciferase reporter), activation of nuclear factor- κ B (NF- κ B, using a luciferase reporter), and cell necrosis (via propidium iodide uptake). We infused the radiocontrast agent into the pancreatic ducts of wild type mice (C57BL/6) to create a mouse model of post-ERCP pancreatitis; some mice were given intraperitoneal injections of the calcineurin inhibitor FK506 before and after infusion of the radiocontrast agent. $\text{CnA}\beta^{-/-}$ mice were also used. This experiment was also performed in mice given infusions of AAV6-NF- κ B-luciferase, to assess activation of this transcription factor *in vivo*.

Results—Incubation of mouse and human acinar cells, but not HEK293 or COS7 cells, with iohexol led to a peak and then plateau in Ca^{2+} signaling, along with activation of the transcription factors NF- κ B and NFAT. Suppressing Ca^{2+} signaling or calcineurin with BAPTA, cyclosporine A, or FK506 prevented activation of NF- κ B and acinar cell injury. Calcineurin $\text{A}\beta$ -deficient mice

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were protected against induction of pancreatic inflammation by iohexol. The calcineurin inhibitor FK506 prevented contrast-induced activation of NF- κ B in pancreata of mice; this was observed by live imaging of mice given infusions of AAV6- NF- κ B-luciferase.

Conclusions—Radiocontrast agents cause pancreatic inflammation in mice, via activation of NF- κ B, Ca²⁺ signaling, and calcineurin. Calcineurin inhibitors might be developed to prevent post-ERCP pancreatitis in patients.

Keywords

PEP; pancreatitis pathogenesis; calcium signaling; calcineurin

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is a common gastrointestinal procedure in which an endoscope is inserted up to the duodenum and the ampulla of Vater is cannulated ¹. Radiocontrast (RC) is injected through a catheter in order to radiographically visualize the pancreaticobiliary tree. ERCPs are common procedures with an estimated annual incidence of 60 to 75 performed per 100,000 persons in the United States ². They are indispensable for the removal of impacted gall stones in the common bile duct (CBD) and various other therapeutic interventions. However, the most common iatrogenic complication of ERCP is acute pancreatitis, which is a painful, inflammatory disorder of the pancreas. The frequency of post-ERCP pancreatitis (PEP) ranges between 1% and 15% and has an overall average of 3.5% ^{3, 4}. PEP has been attributed to a combination of hydrostatic pressure in the pancreatic duct and exposure of the pancreas to RC. In high risk situations, placement of a pancreatic duct stent or rectal administration of the anti-inflammatory drug indomethacin has reduced PEP frequency ^{5–9}. The fundamental mechanism, however, by which RC induces pancreatic injury leading to pancreatitis has not been elucidated.

In the current study, we found that even brief exposure to low concentrations of RC induced aberrant cytosolic Ca²⁺ signals selectively in both mouse and human pancreatic acinar cells. RC exposure caused activation of the Ca²⁺-dependent phosphatase calcineurin, which was linked *in vivo* to pancreatic NF- κ B activation and pancreatitis and in cells to acinar NF- κ B and cell necrosis.

MATERIALS AND METHODS

Reagents and animals

RC refers primarily to iohexol (Omnipaque 300; GE Healthcare; Princeton, NJ) which is categorized as a low osmolality (672 mOsm/kg water), nonionic, iodinated (300 mg/iodine/ml) contrast medium. A second RC iopamidol (Isovue 300; Bracco Diagnostics; Monroe Township, NJ) which is in the same category as iohexol, was used to verify key findings from the study, and its use is specified in the text. NFAT-luciferase (Qiagen; Valencia, CA), NF- κ B-luciferase (Vector Biolabs; Philadelphia PA), and constitutively active calcineurin (Cn) adenoviruses were constructed as previously described ^{10–12}. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless specified otherwise. Male Swiss Webster mice (Jackson Laboratories, Bar Harbor, ME) weighing 22–

28 g were fed standard laboratory chow and given free access to water. CnA β ^{-/-} mice were of the B6129J/F1 strain and were generated by Dr. J. Molkentin¹³. Age-, sex-, and strain-matched control mice were used as wild-type (WT) controls. All animal experiments were performed using a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Intra-ductal RC infusion in mice

The procedure for retrograde infusion into the CBD and pancreatic duct has been described by Perides et al.¹⁴. Briefly, Swiss Webster mice were anesthetized with isoflurane. A midline incision was made to reveal the abdominal cavity. The duodenum was flipped to reveal its distal side and held in place by ligatures. A 30G needle was inserted through the anti-mesenteric aspect of the duodenum to cannulate the CBD. A small bull dog clamp was applied to the distal CBD (near the duodenum) to prevent back flow of the infusate into the duodenal lumen and to hold the cannula in place. A larger bull dog clamp was applied to the proximal CBD (near the liver) to prevent infusion into the liver and thus to direct flow to the pancreatic duct. Total volumes of 50–100 μ l of iohexol, iopamidol, or normal saline (NS) were infused at 10–20 μ l per min for 5 min using a P33 perfusion pump (Harvard Apparatus, Holliston, MA). Upon completion of the infusion, the bull dog clamps were released. The exterior abdominal wound was closed using 7 mm wound clips, and a single injection of buprenorphine (0.075 mg/kg) was given immediately after the surgery. Mice recovered on a heating pad for 30 min after the procedure. They were given free access to food and water after the surgery.

Detection and analysis of cellular Ca²⁺ signals from mouse and human acini

Acinar cells were loaded at room temperature with the high-affinity Ca²⁺-sensing dye Fluo-4AM (K_d =300 nM; Invitrogen). Acinar cells were plated on acid-washed glass coverslips and then mounted on a perfusion chamber. Thereupon, they were stimulated at room temperature with varying concentrations of RC (17–50%) diluted in HEPES buffer. Carbachol (1 mM) was given at the end of each experiment to confirm that the cells were intact and could mobilize intracellular Ca²⁺ stores. A Zeiss LSM710 laser scanning confocal microscope was used with a 20X, 1.4 numerical aperture objective. The dye was excited at 488 nm wavelength, and emission signals of >515 nm were collected every 2 sec. Fluorescence from individual acinar cells was recorded. Analysis of recordings was performed using ImageJ software (NIH, Bethesda, MD), and mean fluorescence over time in each region was graphed.

NFAT-luciferase activity assay

Acinar cells were infected with Ad-NFAT-luciferase following a previously described procedure^{10, 15, 16}. The construct includes a luciferase gene placed downstream of an IL-4 promoter which contains nine tandem NFAT binding sites¹⁰. Acinar cells were incubated with the NFAT-driven luciferase adenovirus for 1.5 hr prior to stimulation. All of the stated inhibitors were added for 30 min prior to stimulation with RC. NFAT-luciferase was measured using the luciferase assay system. Briefly, cells were spun at 1,000 rpm for 5 min, washed with PBS, and lysed using reporter lysis 5X buffer (Promega #E397A, Madison, WI). Samples were vortexed and spun at 12,000 g for 2 min. Supernatant was plated, and

luminescence was measured using a Synergy H1 plate reader (BioTek, Winooski, VT) and normalized to total protein.

NF- κ B-luciferase activity assay

AR42J cells were differentiated towards an acinar phenotype by administering dexamethasone (100 nM) for 48–72 hr¹⁷. AR42J cells were infected with Ad-NF- κ B-luciferase 16 h prior to stimulation using a previously described method¹². Following a wash with DMEM/F12 medium, acinar cells were evenly distributed in a 48-well plate and incubated for 30 min at 37°C. They were incubated with RC at varying concentrations and times. NF- κ B-luciferase was measured using a commercially available luciferase assay (Promega, Madison, WI). Briefly, cells were spun at 1,000 rpm for 5 min, washed with PBS, and lysed using reporter lysis 5X buffer (Promega, catalog no. E397A). Samples were vortexed and spun at 12,000 $\times$ g for 2 min. The supernatant was plated, and luminescence was measured using a Synergy H1 plate reader (BioTek, Winooski, VT) and normalized to total DNA.

Imaging pancreatic NF- κ B bioluminescence *in vivo*

An AAV6-NF- κ B-luciferase reporter was constructed, expanded in HEK293 cells, and purified as previously published^{18–20, 39}. A volume of 100 μ l of virus (10^{12} GCP/ml) was infused into the pancreaticobiliary duct at a rate of 10 μ l/min using the same technique of RC infusion described above and recently published^{21, 39}. After a 5-week recovery from the surgery, mice underwent PEP induction with or without FK506 treatment. At varying time points after the surgery, ranging from 2 to 36 hr, bioluminescence signals were obtained by first giving a subcutaneous injection of luciferin (150 μ g/g body weight) in the scruff of the neck 15 min before the start of imaging. The mice were briefly anesthetized with isoflurane and placed in the supine position in a bioluminescent imaging chamber (IVIS spectrum imager; Perkin Elmer, Waltham, MA) for 3–10 min. Average pixel intensity was measured from a pancreatic region of interest (in the upper abdomen). Raw time points from each mouse were normalized to its individual baseline intensity at time zero.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation unless otherwise stated. Statistical significance was determined using an unpaired T-test and defined as a P value $\leq$ 0.05.

RESULTS

Intraductal infusion of RC induces pancreatic injury in mice

We developed a PEP model in mice by performing a transduodenal cannulation of the CBD as detailed in the Methods (Figure 1A). Infusions were channeled into the pancreatic duct by clamping the proximal CBD and thus preventing retrograde flow into the liver.

Histological severity of pancreatitis, graded by the presence of edema, inflammatory infiltrate, and necrosis was assessed from the head of the pancreas 24 hr after the surgical procedure (Figure 1B & Supplemental Figure 1A). Serum amylase was measured 6 hr post-procedure.

Clinical reports have suggested that post-ERCP pancreatitis is due to a combination of pressure and contrast exposure to the pancreas³. Because the currently available constant pressure pumps could not infuse at low enough rates required for the biliopancreatic duct in mice (10–20 $\mu\text{l}/\text{min}$), changes in pressure were mimicked by varying the rate and volume of infusions.

We found that doubling the rate and volume of normal saline (NS) infusion increased histological severity by 33% ($P<0.05$), but it did not cause a rise in serum amylase, suggesting that pancreatitis did not reach a threshold level. Replacing the NS with RC, however, appeared to have an additive effect on histological severity, leading to a 59% increase above the intra-ductal NS sham control ($P<0.05$). Serum amylase increased by at least 50% and ranged among several batches of experiments between a 1.5- to 3.5-fold increase. Based on these findings, both increased pressure and RC exposure contribute to injury in the PEP model.

Acidic contrast has been shown to mediate severity of pancreatitis in rats²². When RC was buffered with 10 mM HEPES and the pH was clamped at 6, however, in mice there was no increased severity of pancreatitis compared to a pH of 7 (data not shown).

Most of the experiments were performed using iohexol (Omnipaque 300) which is a commonly used RC in ERCP²³. The findings were similar, however, with iopamidol (Isovue 300), which is another low osmolality, non-ionic, iodinated RC (Supplemental Figure 1B–F).

RC induces high amplitude Ca^{2+} signals in mouse pancreatic acinar cells

Since RC exposure is a major contributor to PEP, we sought to examine the mechanism by which RC initiates pancreatitis *ex vivo* in isolated pancreatic cells. Although duct cells line the pancreatic duct and ductules, over 85% of the pancreas is comprised of pancreatic acinar cells²⁴. Pancreatitis initiates within the acinar cell, and one of the earliest, critical factors in most experimental models is the induction of large-amplitude, peak-plateau (or sustained) Ca^{2+} signals^{25–27}. To test whether RC induces acinar cell Ca^{2+} signals, small clusters of 5 to 15 primary acinar cells, grouped as acini, were freshly isolated. They were loaded with the high affinity Ca^{2+} -dye Fluo-4AM and imaged in a perfusion chamber using time lapse confocal imaging (Figure 1C–F). During ERCP, even with a dedicated pancreatography that deliberately instills RC into the pancreatic duct, we estimated that the acinar lumen is exposed to RC concentrations ranging from 10% to 50% because of considerable dilution from pancreatic juice. For this reason, acini were perfused with the above dilutions of RC.

Concentrations of 17% to 20% RC elicited low-amplitude Ca^{2+} transients within 100 sec that immediately returned to baseline. As the RC was increased to 25% and then 33%, there were peak-plateau Ca^{2+} waves which propagated from the apical to basolateral region of most cells. These signals were characterized by a high amplitude (400% above baseline), followed by a sustained plateau. We observed that this Ca^{2+} signal initiated in the same way even in Ca^{2+} -free media, suggesting that RC first triggers Ca^{2+} release from intracellular stores.

All of these experiments were followed 5 min later by perfusion with the muscarinic agonist carbachol (1 mM) in order to confirm the ability of the cells to mobilize Ca^{2+} (data not shown). In limited studies, we demonstrate that RC also induces Ca^{2+} signals and calcineurin activation in live human acinar cells procured from cadaveric donors (Supplemental Figure 2A–C). The robust Ca^{2+} signals seen in pancreatic acinar cells were not observed in the HEK293 or COS7 cell lines, suggesting a certain level of selectivity of RC-induced Ca^{2+} signals for acinar cells (Supplemental Figure 2D–E).

RC induces acinar cell calcineurin activation

Since RC induces Ca^{2+} signals, we next examined whether calcineurin, a putative Ca^{2+} target in pancreatitis, is also activated. To examine calcineurin activation, the adenoviral NFAT (nuclear factor of activated T-cells)-luciferase was used, which consists of 9 tandem NFAT binding motifs from the IL-4 promoter¹⁰. Calcineurin dephosphorylation of NFAT in the cytoplasm causes translocation to the nucleus and thereby drives luciferase.

In primary acinar cells, RC caused a concentration-dependent increase in NFAT-luciferase, and the increases were due to calcineurin activation because the calcineurin inhibitors FK506 (24 μM) and cyclosporine (CsA; 16 μM) largely prevented the rise due to the RC (Figure 2). Calcineurin activation with RC was partially dependent on inositol 1,4,5-trisphosphate receptor (IP3R) activation, since 2-APB (100 μM) reduced the luciferase rise by 47% ($P < 0.05$). However, calcineurin activation was completely reduced by BAPTA-AM (64 μM). The RC iopamidol also induced NFAT activation in a concentration-dependent manner (Supplemental Figure 3). These data demonstrate that RC induces calcineurin activation in acinar cells through a rise in cytosolic Ca^{2+} .

RC causes NF- κB activation through a Ca^{2+} /calcineurin dependent pathway

A master regulator of inflammation in pancreatitis is the transcription factor NF- κB (nuclear factor of kappa light chain enhancer B), which can induce several inflammatory genes including IL-6, Spi2a, TGF- β , and IL-1 β ^{28–31}. In primary acinar cells, increasing concentrations of RC induced phosphorylation of I $\kappa\text{B}\alpha$ at Serine 32 (which is an early degradation signal³²) and nuclear translocation of p65 (Figure 3A). To determine whether RC induces NF- κB in a reporter system, we used the adenoviral NF- κB -luciferase, which requires an overnight infection followed by 6–8 hr administration of RC¹². Due to the limitations of using primary acinar cells in long term culture for this assay, we turned to the AR42J pancreatic cell line, which when primed with dexamethasone (100 nM) for 48–72 hr induces an acinar cell phenotype¹⁷. We first confirmed that RC induces Ca^{2+} signals and calcineurin activation in this cell line just as we observed in the primary acinar cells (Supplemental Figure 4). RC caused a concentration- and time-dependent increase in NF- κB -luciferase (Figure 3B). Even brief exposure of RC (i.e. 25% given for 10 min, followed by a washout) which more closely mimics exposure conditions during ERCP, led to a 5-fold increase in NF- κB luciferase above baseline. Statistically significant increases were, however, observed 60, 90, and 120 min, resulting in a 58-, 59-, and 112-fold increase above baseline, respectively ($P < 0.05$). The RC iopamidol also induced NF- κB activation in a concentration-dependent manner (Supplemental Figure 5A)

NF- κ B activation due to RC was dependent on phospholipase C (PLC), IP3Rs, and cytosolic Ca^{2+} , since luciferase levels were reduced by their respective inhibitors U73122 (5 μM), 2-APB (100 μM), and BAPTA (64 μM ; Figure 3C). A key finding was that the calcineurin inhibitors FK506 (24 μM) and CsA (16 μM) reduced NF- κ B by 73% and 79%, respectively ($P < 0.05$; Figure 3D). Taken together, RC causes NF- κ B activation through a Ca^{2+} - and calcineurin-dependent pathway.

The formulation of iohexol we used (Omnipaque 300) has an osmolality of 672 mOsm/kg at full strength, which categorizes it as a low osmolality contrast medium²³ (Supplemental Table 1). A metanalysis of a dozen randomized, controlled studies failed to demonstrate a significant difference between low or high osmolality RCs in inducing clinical PEP³³. Nonetheless, we sought to examine the contribution, if any, of osmolality on NF- κ B activation using mannitol (Supplemental Figure 5B–C). Whereas RC induced a 300-fold rise in NF- κ B at 350 mOsm/L, equimolar concentrations of mannitol failed to cause an increase. There are conflicting data about the role of oxidative stress in mediating RC nephropathy^{34, 35}. We found, however, in the acinar cell line that pretreatment with the reactive oxygen species (ROS) scavenger N-acetylcysteine (NAC; 2–12 mM) had no effect on NF- κ B activation (Supplemental Figure 5D). Another factor to consider, based on recent reports^{36, 37}, is whether the RC might contain a triglyceride lipid emulsion, that can be liberated into toxic non-esterified fatty acids by exposure to lipases in pancreatic secretions during the ERCP (Supplemental Figure 5E). The RC manufacturer, however, denied adding any emulsions. Further, pretreatment with the lipase inhibitor Orlistat did not significantly reduce the activation of NF- κ B with RC. The results suggest that RC-induced NF- κ B activation is independent of osmolality, oxidative stress, or fatty acid toxicity.

Calcineurin activation is sufficient to induce acinar cell NF- κ B activation

To determine whether calcineurin is sufficient to induce NF- κ B activation, AR42J cells were infected with an adenovirus containing a constitutively active calcineurin that is a truncated catalytic calcineurin A subunit lacking the auto-inhibitory domain¹¹(Ad- Cn; Figure 3E).

NFAT-luciferase activity was maximal after infection with Ad- Cn at greater than 1×10^5 functional viral infectious units (IFU). Even higher titers of Ad-EGFP (negative control) failed to induce NFAT-luciferase. Interestingly, there was a concentration-dependent increase in NF- κ B activation with the Ad- Cn. These data demonstrate that in the acinar cell line calcineurin activation is sufficient to induce NF- κ B.

RC causes acinar cell necrosis through a Ca^{2+} /calcineurin dependent pathway

Propidium iodide (PI) uptake in isolated primary acinar cells is a surrogate for *in vivo* acinar injury and necrosis during pancreatitis. RC exposure for 6 hr caused a concentration-dependent increase in PI uptake, which was dependent on PLC, IP3R- Ca^{2+} release, and a cytosolic Ca^{2+} rise, based on their respective inhibitors or chelator (Figure 4). Similar results were observed in the AR42J acinar cell line (Supplemental Figure 6A). In addition RC caused depletion of ATP levels in both a concentration-dependent manner (Figure 4F).

FK506, however, failed to prevent ATP depletion suggesting that *in vitro*, calcineurin is not required for the mitochondrial pathology leading to ATP depletion.

Inhibiting NF- κ B translocation with IKK-2 largely prevented PI uptake, as did the calcineurin inhibitors (Figure 4C–D). A stress responsive subunit of calcineurin is the beta isoform of calcineurin A (CnA β)¹³. Acinar cells isolated from CnA β knockout mice were protected against PI uptake due to RC (Figure 4E). Taken together, the data demonstrate that RC induces acinar cell necrosis through a Ca²⁺/calcineurin and NF- κ B-dependent pathway.

Pharmacologic and genetic inhibition of calcineurin attenuates RC-induced pancreatitis in mice

We next examined whether calcineurin mediates PEP in mice *in vivo* (Figure 5). An intraperitoneal injection of FK506 (1 mg/kg) was given 1 hr prior to intra-ductal RC infusion and then 2.5, 5.5, and 12 hr after the surgical procedure, based on previous regimens that maximally inhibited calcineurin in mice^{16, 38}. Histological severity of the pancreas following pancreatitis, measured 24 hr after the surgical procedure, was prevented and serum amylase was reduced down to the level of the NS sham condition (Figure 5A–C & Supplemental Figure 6B–C; P<0.05). This effect was as robust as therapeutic administration of the commonly administered NSAID for PEP, indomethacin (Supplemental Figure 9).

Live, dynamic imaging of pancreatic NF- κ B was achieved through a novel AAV6-mediated gene delivery of NF- κ B-luciferase into the mouse pancreas by infusing AAV6-NF- κ B-luciferase into the pancreatic duct, as detailed in the Methods³⁹. Compared to a sham-operated mouse that received intraductal normal saline, RC-infused mice had a 13-fold peak in NF- κ B luciferase 4 hr after surgery, within the region of the pancreas (Figure 5D). FK506 treatment markedly blunted the NF- κ B. In addition, the expression of IL-6, GADD45B, and IL-1 β was upregulated during RC exposure (Figure 5E). Pre-treatment with FK506 caused a significant reduction in gene expression of IL-6 and GADD45B and a trend toward reduction in IL-1 β . The results implicate calcineurin *in vivo* in inducing NF- κ B inflammatory signals. Similarly, CnA β knockout mice had reduced histological severity of PEP, although serum amylase levels were unchanged compared to wildtype mice (Figure 5F). Importantly, therapeutic administration of calcineurin inhibitors after PEP induction also protected against RC-induced pancreatitis (Supplemental Figure 7). Overall, the results demonstrate that calcineurin is a mediator of pancreatic NF- κ B and PEP in mice. Although calcineurin is a target of Ca²⁺, it can also modulate Ca²⁺ by dephosphorylating proteins that mediate Ca²⁺ homeostasis⁴⁰. However, in primary acinar cells, calcineurin inhibition did not affect RC-induced aberrant Ca²⁺ signals (Supplemental Figure 8).

DISCUSSION

The current study is to our knowledge the first demonstration, using the context of pancreatic injury, that RC exposure causes NF- κ B activation and results in epithelial and organ damage by inducing aberrant Ca²⁺ signals and activating calcineurin.

We have developed a novel model of PEP in mice. Previous models in larger animals such as dogs employed an endoscopic cannulation of the biliopancreatic ducts^{41, 42}. More recent models in smaller animals have instilled RC by performing a laparotomy and achieving transduodenal access^{22, 43}. The major benefit of the current *in vivo* work in mice was the ability to complement pharmacological inhibition of calcineurin along with the use of calcineurin knockouts.

Our current data suggest that PEP results from a combination of pancreatic ductal pressure and RC exposure, since each additively worsened PEP outcomes. The findings are consistent with clinical data which demonstrate that PEP can be mitigated by inserting a pancreatic duct stent to relieve ductal pressure, as well as by instilling only the minimum necessary amount of RC during an ERCP procedure^{5-7, 9}.

In the current work, we focused on the effect of RC exposure on the pancreas and the main parenchymal cell the pancreatic acinar cell. RC is known to cause acute kidney injury in high risk patients by inducing hemodynamic instability and it has direct toxic effects on renal cells purportedly through generation of reactive oxygen species (ROS)^{34, 44} and nuclear translocation of NF- κ B³⁵. In the acinar cell line, we found that N-acetylcysteine had no effect on NF- κ B. The results suggest that RC has differential effects, depending on the cell type. RC was shown to cause NF- κ B activation in a renal cell line but the mechanism of activation was not identified^{45, 46}. The current study is to our knowledge the first demonstration that RC causes NF- κ B activation via aberrant Ca^{2+} signals and calcineurin activation. Notably, we imaged pancreatic NF- κ B signals *in vivo* in a live, dynamic fashion by delivering NF- κ B luciferase through AAV-mediated intraductal gene delivery. This novel technique allowed us to focus primarily on pancreatic signals, without the noise from luciferase expression in adjacent organs that is inherent to transgenic mice that globally express NF- κ B luciferase reporter⁴⁷.

We found, in both mouse and human acinar cells that the Ca^{2+} transient emerged soon after RC exposure (within 1–2 min). Further, the shape of the Ca^{2+} signal in most cells mimicked a high amplitude peak plateau, which is a characteristic pathological signal that precedes acinar cell injury and pancreatitis^{25, 26, 48–53} and which would favor calcineurin activation⁵⁴. A recent report demonstrated that a long incubation (of 4 hr) with ioversol, a RC that is similar to the iohexol and iopamidol we used, caused a slow, globalized increase in baseline cytosolic Ca^{2+} levels in the NRK-52E renal cell line⁵⁵. It was suggested that the RC triggered voltage-gated Ca^{2+} channels or caused reversal of the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), so as to favor Ca^{2+} influx. The finding was supported by an earlier report that Ca^{2+} channel blockers improved RC-induced acute kidney injury in rats⁵⁶. In the context of RC-induced pancreatic injury, however, NCX does not appear to be a functional component of the pancreatic acinar cell^{25, 57}. Thus the acinar cell Ca^{2+} signals with RC were distinct from the faint Ca^{2+} signals described in the NRK-52E cell line or from the non-pancreatic cell lines we tested.

Among the putative targets of Ca^{2+} in pancreatitis, which include the MPTP and kinases, the Ca^{2+} -activated serine/threonine phosphatase calcineurin has been implicated as a critical mediator of acinar injury and pancreatic inflammation^{16, 38, 53, 58, 59}. Calcineurin is a dimer

formed by a catalytic A subunit (CnA) and a regulatory B subunit (CnB) ^{60–62}. CnA has three isoforms (alpha, beta, and gamma) and CnB has two isoforms (B1 and B2). Calcineurin is highly conserved and ubiquitous in most cells of the body, although there is differential distribution of its isoforms. The pancreatic acinar cell expresses mostly CnA β and CnB1 ⁵⁹. Calcineurin was shown to mediate similar events as aberrant Ca²⁺ including intra-acinar protease activation ⁵⁸, NF- κ B ^{53, 63}, and cell injury ⁵⁹. *In vivo*, calcineurin mediated pancreatitis due to caerulein hyperstimulation ³⁸ and bile acid infusion ¹⁶.

It is unlikely that there was a direct effect of molecular iodine on the cell or its entry (e.g. via a sodium iodine symporter ⁶⁴) because iodine dissociation in properly stored RC is minimal, and sodium iodine administration alone to renal cells does not cause cell injury ^{35, 65}. Calcineurin inhibitors are used clinically as immunosuppressants because they inhibit T-cells ⁶⁶. The current work suggests, however, that calcineurin within the acinar cell causes acinar pathology and that acinar calcineurin, by itself, might contribute to pancreatitis *in vivo*. Further work will be necessary to clarify which pool of calcineurin primarily causes pancreatitis. The study provides pre-clinical impetus to conduct clinical trials in order to test the role of calcineurin inhibitors in preventing PEP. More broadly, it is to our knowledge also the first demonstration, in any organ or cell type, that RC induces NF- κ B and cell necrosis through aberrant Ca²⁺ signals and that a central Ca²⁺ mediator is calcineurin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AAV	adeno-associated virus
CBD	common bile duct
Cn	calcineurin
Cn	constitutively active Cn
ERCP	endoscopic retrograde cholangiopancreatography
GPCR	G-protein coupled receptor
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NS	normal saline
PEP	post-ERCP pancreatitis

RC radiocontrast

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Figure 1. RC infusion causes pancreatitis *in vivo* and induces Ca²⁺ signals in mouse pancreatic acinar cells

(A) Schema for RC infusion into the distal common bile duct (arrows) using a perfusion pump (P, pancreas; D, duodenum). (B) Representative HE sections of the pancreatic head from NS sham control and RC-infused (at 20 μl/min, 100 μl volume) mice. A combination of intra-ductal RC along with an increased rate and volume induced greater histological severity and higher serum amylase levels. (n=5 animals per condition). *, P<0.05 relative to NS sham. (C) Mouse pancreatic acinar cells are loaded onto a custom-made perfusion chamber and imaged using a confocal microscope. (D) Phase contrast image and pseudo-colored images of acini loaded with the Ca²⁺ dye Fluo-4AM at baseline, during the initial rise in Ca²⁺ fluorescence, and peak fluorescence during perfusion with RC. An individual acinar cell is outlined in the dashed white line, and the red arrows indicate the progression of the Ca²⁺ signal from the apical to basal region of the cell. (E) Summation of whole cell tracings of Ca²⁺ flux with increasing concentrations of RC. (F) Amplitude and area under the curve measurements of the Ca²⁺ signal. Acinar cells were perfused with RC (25%) in the presence or absence of Ca²⁺ containing media (n=20–30 cells per condition). *, P<0.05 compared to 17% RC.

Figure 2. RC induces acinar cell calcineurin activation via Ca^{2+} mobilization and IP3Rs
 Mouse acinar cells were infected with Ad-NFAT-luciferase and stimulated with RC (A) for varying time periods (25% RC) or (B) with increasing concentrations for 5 hr. RC (9%)-induced NFAT-luciferase activity was prevented by (C) 30 min pre-treatment with the calcineurin inhibitors FK506 (24 μM) and cyclosporine (CsA; 16 μM) or (D) 30 min pre-treatment with the IP3R inhibitor 2-APB (100 μM) or the intracellular Ca^{2+} chelator BAPTA-AM (64 μM). (n=3). *, #, $P < 0.05$, relative to the control or RC alone, respectively.

Figure 3. Calcineurin is both necessary and sufficient to induce acinar cell NF-κB activation due to RC

Western blots from primary mouse acinar cells stimulated with (A) 20% RC or varying concentrations of RC for 15 min or 30 min and probed for phosphorylated IκBα or p65. Densitometry shown below for each blot. (B) Acinar-differentiated AR42J cells were infected overnight with Ad-NF-κB-luciferase and then incubated for 6 hr with increasing concentrations of RC. AR42J cells were exposed to RC (25%) for varying times (solid lines) then washed off with buffer (dashed lines) and incubated for a total of 6 hr. (C) NF-κB-luciferase activity was prevented in AR42J cells by pre-treatment with the U73122 (5 μM), 2-APB (100 μM), BAPTA (64 μM), (D) FK506 (24 μM), and CsA (16 μM). (n=3). *, #, P<0.05, relative to the control or RC alone, respectively. (E) AR42J cells were infected with increasing titers of an adenovirus carrying a constitutively active form of the catalytic calcineurin A subunit (Ad- Cn) or infected with Ad-EGFP (negative control). NFAT- and NF-κB-luciferase activity were measured. Ad-EGFP at amounts greater than 4×10^5 infectious units (IFU) failed to increase luciferase levels. Correlation between NFAT- and NF-κB-luciferase activity in AR42J cells ($R^2=0.9185$; P = 0.0002). (n=3). *, P<0.05, relative to Ad-EGFP alone.

Figure 4. RC causes acinar cell necrosis through a Ca^{2+} /calcineurin-dependent pathway (A) Acinar cells were treated with increasing concentrations of RC for 6 hr, and propidium iodide uptake was measured. RC (12%)-induced acinar cell injury (6 hr incubation) was prevented by (B) U73122 (5 μM), 2-APB (100 μM), BAPTA (64 μM), or (C) the NF- κB inhibitor IKK-2 (20 μM). (D) Inhibition of calcineurin (FK506; 24 μM , CsA; 16 μM) or (E) genetic deletion of the regulatory calcineurin A β subunit (CnA β). (F) ATP levels were measured from acinar cells treated with RC (12%) for 3.5 hr $\pm$ FK506. (n=3). *, #, $P < 0.05$, relative to the control or RC alone, respectively.

Figure 5. PEP is dependent on calcineurin

(A) Schema for the administration of FK506 (1 mg/kg) relative to the infusion of RC. (B & F) Representative H&E sections from the pancreatic head. (C & F) Overall severity score (left), and serum amylase measurements (right). (n=5 animals per group). (D) Bioluminescence from the pancreas of mice that had received intraductal infusions of AAV6- NF- κ B-luciferase. Quantification of the pancreatic NF- κ B-bioluminescent signal over 36 hr (n=3 animals per group). (F) Gene expression for IL-6, GADD45B, and IL-1 β from the pancreatic head (n=3). *, #, $P < 0.05$ compared with NS sham and RC alone, respectively.

Figure 6. Hypothesis diagram

RC exposure causes acinar cell: (1) activation of PLC; (2) generation of IP3 resulting in IP3R-induced Ca^{2+} release; (3) downstream activation of calcineurin; (4) translocation of NF- κ B to the nucleus, leading to (5) acinar cell injury and pancreatitis. The inhibitory scheme is shown in red. PIP2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol.