Mechanism of mitochondrial permeability transition pore induction and damage in the pancreas: inhibition prevents acute pancreatitis by protecting production of ATP

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ABSTRACT

Objective Acute pancreatitis is caused by toxins that induce acinar cell calcium overload, zymogen activation, cytokine release and cell death, yet is without specific drug therapy. Mitochondrial dysfunction has been implicated but the mechanism not established.

Design We investigated the mechanism of induction and consequences of the mitochondrial permeability transition pore (MPTP) in the pancreas using cell biological methods including confocal microscopy, patch clamp technology and multiple clinically representative disease models. Effects of genetic and pharmacological inhibition of the MPTP were examined in isolated murine and human pancreatic acinar cells, and in hyperstimulation, bile acid, alcoholic and choline-deficient, ethionine-supplemented acute pancreatitis.

Results MPTP opening was mediated by toxin-induced inositol trisphosphate and ryanodine receptor calcium channel release, and resulted in diminished ATP production, leading to impaired calcium clearance, defective autophagy, zymogen activation, cytokine production, phosphoglycerate mutase 5 activation and necrosis, which was prevented by intracellular ATP supplementation. When MPTP opening was inhibited genetically or pharmacologically, all biochemical, immunological and histopathological responses of acute pancreatitis in all four models were reduced or abolished.

Conclusions This work demonstrates the mechanism and consequences of MPTP opening to be fundamental to multiple forms of acute pancreatitis and validates the MPTP as a drug target for this disease.

INTRODUCTION

Pancreatic necrosis, systemic inflammatory response syndrome, multiple organ failure and sepsis are characteristic of severe acute pancreatitis (AP), which results in death of one in four patients and is without specific drug therapy.1,2 As the pancreatic acinar cell is an initial site of injury,1,3 commonly initiated by bile or ethanol excess, investigation of its behaviour in response to toxins that induce AP may identify new drug targets. This cell typifies non-exocrine exocrine cells with a high secretory turnover heavily dependent on mitochondrial production of ATP.4 While zymogen activation has
long been considered the principal mechanism of injury,\(^1\) mitochondrial dysfunction has been implicated increasingly,\(^2\) presumed consequent upon intracellular calcium overload induced by toxins that include bile acids and ethanol metabolites,\(^6\) Mitochondrial uptake of calcium drives normal cellular bioenergetics, but high calcium loads induce increasingly drastic responses culminating in necrosis.\(^13\) Mitochondrial matrix calcium overload leads to opening of the mitochondrial permeability transition pore (MPTP), a non-specific channel that forms in the inner mitochondrial membrane allowing passage of particles under 1500 Da, causing loss of mitochondrial membrane potential (Δψ\(_{\text{m}}\)) essential to ATP production; recent evidence implicates F_0F_1 ATP synthase in MPTP formation.\(^{14,15}\) MPTP opening is physiological in low conductance mode releasing calcium and reactive oxygen species (ROS) to match metabolism with workload,\(^16\) but pathological in high conductance mode compromising ATP production and inducing cell death;\(^11\) both functions are regulated by the mitochondrial matrix protein peptideyl-prolyl \textit{cis-trans} isomerase (PPI, cyclophilin) D (also known as cyclophilin F).\(^17\)

Previous limited studies found that MPTP opening can occur in pancreatitis;\(^5\) we found cyclophilin D knockout to ameliorate AP induced by ethanol and cyclosporine,\(^9\) but in a model with no clinical correlate. How the MPTP is induced in pancreatic acinar cells has not been determined, nor what role intracellular calcium might play and whether there are downstream consequences in AP. Therefore, we sought to undertake a novel, wide ranging and detailed study to determine the mechanism and significance of MPTP opening in AP.

We report that MPTP opening is critical to all forms of pancreatitis investigated, causing diminished ATP production, defective autophagy, zymogen activation, cytokine release, phosphoglycerate mutase family member 5 (PGAMS) activation\(^{18}\) and necrosis. Pharmacological or genetic MPTP inhibition in murine or human pancreatic acinar cells protected Δψ\(_{\text{m}}\), ATP production, autophagy and prevented necrosis from pancreatitis toxin-induced calcium release via inositol trisphosphate and ryanodine (IP_3R, RyR) calcium channels. This mechanism was confirmed consistently across four dissimilar, clinically relevant, in vivo models of AP. All characteristic local and systemic pathological responses were greatly reduced or abolished in cyclophilin D knockout mice (Ppif\(^{-/-}\)) and wild type (Wt) mice treated with MPTP inhibitors, confirming that MPTP opening is a fundamental pathological mechanism in AP.

**Significance of this study**

**How might it impact on clinical practice in the foreseeable future?**

- The demonstration of identical mechanisms in human as in murine pancreatic acinar cells indicates that the findings that establish MPTP opening to be of critical importance in experimental acute pancreatitis are likely to be of major importance in clinical acute pancreatitis.
- This study has shown the effectiveness in experimental acute pancreatitis of several drugs that target molecules that regulate the MPTP and that could be developed for the treatment of clinical acute pancreatitis.
- Translational drug discovery and development programmes that target the MPTP could provide specific, effective treatments for clinical acute pancreatitis.

**METHODS**

**Animals**

Cyclophilin D-deficient mice were generated by targeted disruption of the \textit{Ppif} gene\(^{20}\) and provided by Dr Derek Yellon (University College London, UK) and Dr Michael A Forte (Oregon Health and Sciences University, USA). Transgenic green fluorescent protein (GFP)-LC3 mice\(^{21}\) were a gift from Dr N Mizushima (Tokyo Medical and Dental University and RIKEN BioResource Center, Japan). All experiments comparing Wt and \textit{Ppif}\(^{-/-}\) were conducted using C57BL/6 mice; experiments using toxins on Wt cells alone used CD1 mice.

**Preparation of isolated pancreatic acinar cells and mitochondria**

Normal human pancreata samples (~1 cm\(\times\)1 cm\(\times\)1 mm, not devascularised during surgery before removal) were placed in a solution of (mM): 140 NaCl, 4.7 KCl, 1.13 MgCl_2, 1 CaCl_2, 10 D-glucose, 10 HEPES (adjusted to pH 7.35 using NaOH) at 4°C; sampling to start of cell isolation (or slicing below) was <10 min in every case. All experiments were at room temperature (23–25°C, except where stated) and cells used within 4 h of isolation. Isolation of murine and human pancreatic acinar cells was as described. Isolated murine cells were incubated at 37°C in 199 medium with or without 10 nM choleysteatin-8 (CKC-8) or 500 \mu\text{M} taurolohydrolic acid sulfate (TLCS); drug pretreatment was applied for 30 min. Mitochondria were isolated from mouse pancreas as described.\(^{23}\)

**Confocal fluorescence microscopy**

Cells and tissue were viewed using Zeiss LSM510 and LSM710 systems (Carl Zeiss Jena GmbH), typically with a 63x C-Apochromat water immersion objective (aperture at 1.2) after loading with Fluo-4 (3 \mu\text{M}; excitation 488 nm, emission >505 nm) and tetramethyl rhodamine methyl ester (50 nM; excitation 543 nm, emission >550 nm) to assess cytosolic calcium and mitochondrial membrane potential, with simultaneous measurements of NAD(P)H autofluorescence (excitation 351 nm, emission 385–470 nm) to assess mitochondrial metabolism. The protonophore carbonyl cyanide \textit{m-chlorophenyl} hydrazone (CCCP) was applied to dissipate Δψ\(_{\text{m}}\) as a positive control. ROS were assessed after loading with 5-chloromethyl-2,7'-dichlorodihydrofluorescein diacetate acetyl ester (4.5 \mu\text{M}; excitation 488 nm, emission 505–550 nm) for 10 min at 37°C.\(^{12}\) R110-aspartic acid amide (20 \mu\text{M}; excitation 488 nm, emission >505 nm) and propidium iodide (PI 1 \mu\text{M}; excitation 488 nm, emission 630–693 nm) were used to assess general caspase activation and plasma membrane rupture. Thirty random fields of view were taken of each isolate and the percentage number of cells displaying caspase activity or PI uptake counted per field, averaged across fields as mean±SEM (minimum three mice/group). PI was used in patched cells (below), as was Mg Green (4 \mu\text{M}, excitation 476 nm, emission 500–550 nm), to monitor intracellular ATP concentrations.\(^6\) Murine pancreas lobules were incubated with/without 500 \mu\text{M} TLCS and stained with Sytox Orange\(^{24}\) (500 nM, excitation 543 nm, emission >560 nm), which like PI only stains cells with ruptured cell membranes; uptake was determined every two hours by % area tissue stained.

**Patch-clamp current recording**

The whole-cell configuration was used to record I_{\text{IClCa}} from single cells while recording cytosolic calcium (Fluo-4).\(^{25}\) Patch-pipettes were pulled from borosilicate glass capillaries
Pancreas

Figure 1 Mitochondrial permeability transition pore (MPTP) inhibitors prevent mitochondrial impairment and necrosis of freshly isolated murine and human pancreatic acinar cells (confocal fluorescence; mean±SEM ratio to basal, F/F₀; n=no. of experiments). (A) Cholecystokinin-8 (CCK-8) (10 nM) induced large cytosolic calcium elevations (Fluo-4, left), falls in Δψₘ (tetramethyl rhodamine methyl ester, TMRM; positive control, protonophore carbonyl cyanide m-chloro phenyl hydrazone, CCCP, middle) and NAD(P)H autofluorescence (right), showing protection of Δψₘ and NAD(P)H by cyclosporin A (CYA, 5 μM) or bongkrekic acid (BKA, 50 μM) (pretreatment for 30 min at room temperature during loading of fluorescent dyes). (B) Taurolithocholic acid sulfate (TLCS) (500 μM) induced similar changes in calcium, Δψₘ and NAD(P)H, with similar protection by CYA and BKA. (C) Protection of Δψₘ from TLCS (500 μM) by pretreatment with DEB025 (100 nM) in murine (left) and human (middle) pancreatic acinar cells, and with TRO40303 (10 μM, right) in murine cells. (D) CYA or BKA protected cells from early plasma membrane rupture (top, % cells showing propidium iodide (PI) uptake as inset; *p<0.05 CCK-8 vs control or with inhibitor; †p<0.05 TLCS vs control or with inhibitor) but not from caspase activation (bottom, % cells showing general caspase substrate fluorescence as inset; *p<0.05, control vs all CCK-8 groups; †p<0.05 control vs all TLCS groups; white bars=5 μm). (E) Typical rise in TMRM dequench fluorescence emitted by normal fresh human pancreatic tissue slice in response to TLCS (500 μM) and protection by DEB025 (100 nM; upper panel; inset shows confocal image of human pancreatic tissue slice (mitochondrial (TMRM, red) and nuclear (Hoescht, blue) fluorescent dyes, white bar=15 μm). Lower panel shows protection from TLCS-induced PI uptake in human pancreatic acinar cells by CYA (100 nM) or DEB025 (100 nM, bottom) and (F) murine (top) and human (bottom) cells by TRO40303 (10 μM) (*p<0.05 TLCS vs control or with CYA, DEB025 or TRO40303).

Figure 2  Genetic ablation of cyclophilin D (Ppif−/−) protects pancreatic acinar cells from pancreatitis toxins (fluorescence means±SEM, F/F0).  
(A) Cholecystokinin-8 (CCK-8) (10 nM) induced cytosolic calcium elevations (Fluo-4, left) in Wt (C57BL/6) and Ppif−/− cells, with faster clearance in Ppif−/−; Δψm (TMRM, middle) and NAD(P)H (right) were preserved in Ppif−/− not wild type (Wt) cells. (B) Tauroliotholic acid sulfate (TLCS) (500 μM) induced similar calcium changes, clearing faster in Ppif−/−; whereas Δψm and NAD(P)H were preserved in Ppif−/− not Wt. (C) TLCS (500 μM) induced similar mitochondrial calcium elevations (Rhod-2, left) in Ppif−/− and Wt cells, as well as similar ROS elevations (DCFDA, middle) in Ppif−/− and Wt cells (menadione, MEN oxidant control); insets show ROS-sensitive DCFDA cell fluorescence (white bars=10 μm); ethanol (ETOH, 10 mM) and palmitoleic acid (POA, 20 μM) induced falls of Δψm (right) in Wt not Ppif−/− cells.  
(D) Significantly increased propidium iodide (PI) uptake in Wt not Ppif−/− cells after CCK-8 (10 nM) or TLCS (500 μM) (top, *p<0.05 toxin in Wt versus no toxin or toxin in Ppif−/−), but similar general caspase activation (bottom, *p<0.05 no toxin vs each toxin group). (E) Cyclophilin absence in Ppif−/− pancreas (immunoblot, left) and cytochrome c (Cyt c) cytosolic fraction immunoblots (densitometry normalised to lactate dehydrogenase (LDH), Cox IV to rule out mitochondrial contamination, right) showed Cyt c release after CCK-8 by Wt and less by Ppif−/− mitochondria.  
(F) Necrotic cell death pathway activation (Sytox Orange; SO) from TLCS (500 μM) was delayed in Ppif−/− vs Wt pancreas lobules (*p<0.05).
Pancreatitis toxin-induced acinar cell MPTP opening causes collapse of ATP production and necrotic cell death pathway activation via second messenger receptor calcium channel release

As bile acids and FAEEs induce global, prolonged acinar cytosolic calcium release via IP₃Rs and RyR calcium channels,⁶ ³³ which causes zymogen activation⁶ ³⁴ ³⁵ dependent on sustained calcium entry,⁶ we sought to determine how toxin-induced calcium release causes mitochondrial injury and pancreatic acinar cell death. Using patch clamp technology and confocal microscopy, we observed typical apical stimulus-secretion coupling calcium signals elicited by IP₃ (1–10 μM), matched by calcium-activated Cl⁻ currents⁴ ³⁵ (ICl,Ca). These signals were promptly transformed into global, prolonged (>30 s) cytosolic calcium elevations by low concentrations of TLCS (10 μM, 31 of 33 cells, figure 3A, B) or POAEE (10 μM, 34 of 37 cells, see online supplementary figure S2), followed by PI uptake in Wt cells (figure 3B and see online supplementary table). Application of the non-specific IP₃,R antagonist caffeine⁶ inhibited calcium changes and ICl,Ca from both toxins, preventing PI uptake (22 of 22 cells, figure 3A, see online supplementary figure S2 and table), demonstrating dependence of toxic transformation on IP₃Rs. Necrotic cell death pathway activation was entirely dependent on calcium influx (figure 3B and see online supplementary figure S2). Typical calcium signals and ICl,Ca elicited by the RyR ligand cyclic ADPR (cyclic ADPR, 10 μM)²⁵ were transformed by TLCS (10 μM), not POAEE; those elicited by NAADD (100 nM)²⁵ were transformed by POAEE (10 μM), not TLCS (see online supplementary figure S2). To model events in vivo, quasi-physiological concentrations of CCK-8 or acetylcholine (ACh) were tested with both toxins, again resulting in toxic transformation (24 of 24 cells, no patch pipette, figure 3C, see online supplementary figure S2 and table). Without any second messenger or secretagogue, higher toxin concentrations (TLCS, 200 μM, figure 3D; POAEE 100 μM, data not shown; both inhibited by caffeine) were required to induce global, prolonged calcium elevations. All protocols that induced such elevations sustained by external calcium entry resulted in PI uptake in Wt cells (56 of 60 cells, ≥5 cells with each protocol; figure 3B–D); patch ATP resulted in more efficient calcium clearance and prevented all PI uptake (46 of 46 Wt cells, ≥4 cells with each protocol; p<0.0001), and ATP depletion from toxic transformation without patch ATP was confirmed using Mg Green (figure 3C, see online supplementary figure S2 and table). In all Ppif⁻/⁻ cells, there was significantly more efficient calcium clearance, reduced ICl,Ca and return to baseline levels with no PI uptake, despite no patch ATP (IP₃ and TLCS, 10 μM, 17 of 17 cells; TLCS, 200 μM, 7 of 7 cells, figure 3D). These findings identify a primary role for second messenger calcium channel release in MPTP opening induced by pancreatitis toxins, resulting in declining ATP production and necrosis.

While Wt Δψm was lost after one addition of 2.5 μM CaCl₂, Ppif⁻/⁻ Δψm was lost after five successive additions (figure 4B, C). Ppif⁻/⁻ pancreatic mitochondria released only 35% less cytochrome c than Wt in 1.3 μM calcium (figure 4D), consistent with a modest contribution from MPTP opening to cytochrome c release. To further assess the significance of MPTP opening and falls in Δψm, we measured levels of PGAM5, a mitochondrial executor of necrosis.³⁷ Falls in Δψm cause PGAM5 cleavage from the inner mitochondrial membrane,³⁷ and increases in PGAM5 promote necrosis, facilitating mitochondrial fission.³⁹ After induction of CER-AP, PGAM5 was increased in Wt but significantly less in Ppif⁻/⁻ pancreata (figure 4E), indicating a mitochondrial mechanism for necrosis induced by calcium overload in AP. These changes were associated with marked ballooning of and loss of cristae in Wt but not Ppif⁻/⁻ pancreatic acinar mitochondria in CER-AP (figure 4F).

The MPTP mediates zymogen activation through impaired autophagy

Since zymogen activation is considered essential to AP and relates to disease severity,³⁸–⁴⁰ we sought to determine whether and how this is MPTP dependent. We found CCK-8-induced trypsin activity significantly inhibited in Ppif⁻/⁻ compared with Wt (figure 5A), despite no differences in the amount of trypsinogen (or amylase) between Wt and Ppif⁻/⁻ mice pancreata (figure 5B; nor cathepsin B, Bel-xL or Bel-2, data not shown). This finding indicates that MPTP opening contributes to pathological, intra-acinar zymogen activation. Zymogen activation depends on intracellular calcium overload³⁰ and accumulation of activated zymogens in AP is due to impaired autophagy.⁴¹ We therefore measured levels of microtubule-associated protein 1A/1B-light chain 3 (LC3), which in autophagy is converted from cytosolic LC3-I to lipiodated LC3-II and recruited into autophagosomal membranes, and levels of sequestosome 1 (SQSTM1, p62), which sequesters ubiquitinated protein aggregates to autophagosomes; when autophagosomes fuse with lysosomes, both LC3-II and p62 are degraded.⁴² Following induction of CER-AP that features marked falls in ATP production, acinar cell vacuolisation and zymogen activation,³⁸–⁴⁰ significant increases in LC3-II and p62 occurred in Wt pancreata, showing retarded autophagy consistent with previous data.⁴³ Increases in LC3-II and p62 were significantly attenuated in Ppif⁻/⁻ mice (figure 5C–E), indicating more efficient autophagy.⁴² We confirmed the role of MPTP opening in defective autophagy using GFP LC3 mice,²¹ crossed with Ppif⁻/⁻ mice. Analysis of LC3 puncta (autophagic vacuoles, figure 5F) as well as increases in LC3-II and p62 in GFP-LC3 versus GFP-LC3×Ppif⁻/⁻ mice (≥3 mice/group, data not shown) confirmed significant attenuation from genetic inhibition of the MPTP.

Genetic or pharmacological MPTP inhibition sustains ATP production and confers striking protection from experimental AP

To determine comprehensively the significance of these mechanisms in vivo, we compared responses of Ppif⁻/⁻ versus Wt mice in four dissimilar models of AP: CER-AP, TLCS pancreatic ductal infusions²⁷ (TLCS-AP), ethanol with POA¹¹ (FAEE-AP) and CDE-AP diet.²³ These models represent the whole spectrum of human AP, including the commonest clinical aetiologies (gallstones and ethanol) and extending from mild to lethal disease. In all models, characteristic changes occurred in serum amylase and interleukin-6 (IL-6), pancreatic trypsin and myeloperoxidase, pancreatic ATP and histopathology (figures 6 and 7, see

Figure 3  Pancreatitis toxins accelerate calcium release via second messenger receptors causing collapse of ATP production in wild type (Wt) not Ppif<sup>−/−</sup> cells (insets, representative cells, green Fluo-4 and/or red propidium iodide (PI) fluorescence, white bars=10 μm). (A) Typical calcium spikes (Fluo-4, F/F<sub>0</sub>, blue) elicited by patched IP<sub>3</sub> (1–10 μM) were transformed into global, prolonged elevations upon Taurolithocholic acid sulfate (TLCS) (10 μM) application, matched by I<sub>Cl,CA</sub> and non-specific cation currents (~30 mV, black and +10 mV, grey; inset patched cell top), inhibited by caffeine (Caf, pink); (B) top plot: toxic transformation (I<sub>Cl,CA</sub> red, no caffeine) showing PI uptake; bottom plot: without external calcium, transformed signals decreased then disappeared (I<sub>Cl,CA</sub> black, no PI uptake). (C) Top plot: toxic transformation of acetylcholine (Ach) (20 nM) signals by TLCS (10 μM), reduced by pipette ATP (4 mM) preventing PI uptake in patched (I<sub>Cl,CA</sub> and blue calcium trace) but not adjacent (purple calcium trace) cell; middle plot: toxic transformation of cholecystokinin-8 (CCK-8) (1–5 pM) signals by palmitoleic acid ethyl ester (POAEE, 10 μM) (red I<sub>Cl,CA</sub>) caused PI uptake, prevented by pipette ATP (black I<sub>Cl,CA</sub> two recordings superimposed); bottom plot: ATP decline (Mg Green; rise indicates increased ADP:ATP ratio) following toxic transformation (3 pM CCK-8 with 10 μM POAEE; carbonyl cyanide m-chlorophenyl hydrazone induced no further ATP decline). (D) Top plot: representative trace showing transformation of IP<sub>3</sub> (1–10 μM) elicited signal by TLCS (10 μM) did not induce PI uptake in Ppif<sup>−/−</sup> cells, without supplementary ATP; middle plot: TLCS (200 μM) alone induced PI uptake in Wt; bottom plot: TLCS (200 μM) did not induce PI uptake in Ppif<sup>−/−</sup> cells.
Figure 4 Genetic mitochondrial permeability transition pore (MPTP) inhibition confers resistance of pancreatic mitochondria to calcium-induced loss of $\Delta W_m$ and PGAM5 induction. (A) Representative Clark-type electrode measurement of oxygen consumption showed no difference between wild type (Wt) and $Ppif^{-/-}$ mitochondria (Mito; succinate=10 mM, ADP=200 $\mu$M, carbonyl cyanide m-chlorophenyl hydrazone (CCCP)=2 $\mu$M). (B) Typical TPP$^+$-selective electrode measurement of $\Delta W_m$ with succinate (10 mM) in free ionised calcium clamped at 1.3 $\mu$M (calcium/ethylene glycol tetraacetic acid buffers) for 10 min and (C) during pulses of calcium (25 $\mu$M), showing resistance of $Ppif^{-/-}$ mitochondria to loss of $\Delta W_m$. (D) $\Delta W_m$ (TPP$^+$-selective electrode, left) and cytochrome c (Cyt c; densitometry from Medium immunoblot, right) in the same preparations, normalised to Wt. $Ppif^{-/-}$ pancreatic mitochondria release Cyt c but less than Wt (*p<0.05, means±SEM from >3 preparations), as shown in representative Cyt c immunoblot of medium and mitochondrial pellet (Mito, Cox IV confirmed separation and equal protein loading). (E) Increase in PGAM5 in Wt caerulein acute pancreatitis (CER-AP) pancreata was significantly reduced in $Ppif^{-/-}$ with representative immunoblot (re-probed for ERK1/2 to confirm equal loading; each lane from an individual animal; 4–6 mice per group; densitometry of PGAM5 as ratio of band intensities to ERK in each sample normalised to saline-treated Wt controls, means±SEM; *p<0.01 CER-AP in Wt vs Wt controls, †p<0.05 CER-AP in $Ppif^{-/-}$ vs CER-AP in Wt). (F) Electron micrographs of pancreata showing Wt and $Ppif^{-/-}$ pancreatic acinar cells after induction of CER-AP compared with saline (SAL) controls. Wt pancreatic acinar mitochondria are markedly swollen with loss of cristae in CER-AP compared with normal morphology of $Ppif^{-/-}$ pancreatic acinar mitochondria in CER-AP and in both Wt and $Ppif^{-/-}$ saline controls (M, mitochondrion; N, nucleus; V, vacuole; ZG, zymogen granule; black bars 1 $\mu$m except top right, 2.5 $\mu$m).
In contrast, all pathological responses were greatly inhibited in Ppif−/− animals, including lung myeloperoxidase and IL-6, which mediates lung injury and lethality. In Wt, DEB025 (10 mg/kg) or TRO40303 (3 mg/kg) administered 2 h after the start of hyperstimulation in CER-AP (Figure 6C, D, see online supplementary figure S3) or 1 h after induction of TLCS-AP (Figure 7) markedly reduced or abolished all pathological changes. Protection in TLCS-AP was close to complete: all changes in Ppif−/− mice or Wt (C57BL/6) mice treated with DEB025 or TRO40303 were no or minimally
Figure 6  Genetic and pharmacological mitochondrial permeability transition pore (MPTP) inhibition markedly reduces the severity of caerulein acute pancreatitis (CER-AP). (A) CER-AP resulted in substantial elevations of serum amylase (U/L) and pancreatic trypsin (normalised to wild type (Wt) saline controls) with substantial reduction in pancreatic ATP content in Wt (*p<0.05) but not Ppif<sup>−/−</sup> mice (*p<0.05 vs CER-AP in Wt). (B) CER-AP resulted in substantial elevations of pancreatic (P) and lung (L) myeloperoxidase activity (normalised to CER-AP in Wt at 100) in Wt (*p<0.05) but not Ppif<sup>−/−</sup> mice (*p<0.05 vs CER-AP in Wt), while apoptosis scores were significantly increased in CER-AP in both Wt (*p<0.05 vs either control) and Ppif<sup>−/−</sup> (*p<0.05 vs either control). (C) Necrosis scores in CER-AP were substantially reduced in Ppif<sup>−/−</sup> and Wt with DEB025 (10 mg/kg intraperitoneal with third injection of caerulein) or TRO40303 (3 mg/kg intraperitoneal at same time points) compared to Wt with no treatment (all values means±SEM from ≥6 mice per group in all experiments; *p<0.01 CER-AP in Wt vs Wt controls; *p<0.05 CER-AP in Ppif<sup>−/−</sup> or Wt with DEB025 or TRO40303 vs CER-AP in Wt). (D) Normal pancreatic histology (Wt no treatment) contrasted with CER-AP in Wt, Ppif<sup>−/−</sup> or Wt treated with DEB025, showing extensive oedema, necrosis and inflammatory cell infiltration in Wt but not Ppif<sup>−/−</sup> and not in Wt with DEB025 (H&E, black bars=50 μm).  

Figure 7  Genetic or pharmacological mitochondrial permeability transition pore (MPTP) inhibition abolishes or markedly attenuates biochemical and histological responses of tauroliothocholic acid sulfate acute pancreatitis (TLCS-AP), fatty acid ethyl ester (FAEE)-AP and choline-deficient ethionine-supplemented (CDE)-AP. (A) Characteristic elevations in TLCS-AP of serum amylase (U/L), interleukin-6 (pg/mL), pancreatic (P) myeloperoxidase activity (normalised to TLCS-AP in wild type (Wt) at 100) and histology scores (*p<0.05 for all elevations vs sham controls) were all significantly reduced in $Ppif^{-/-}$ or in Wt treated with DEB025 or TRO40303 ($^\dagger$p<0.05 vs TLCS-AP in Wt without treatment). (B) Characteristic elevations in lung (L) myeloperoxidase activity (normalised to TLCS-AP in Wt at 100; *p<0.05 vs sham controls) were significantly reduced in Wt treated with TRO40303 (†p<0.05 vs TLCS-AP in Wt without treatment). (E) Representative histology showing protective effects of $Ppif^{-/-}$ in TLCS-AP, of DEB025 on TLCS-AP in Wt and of $Ppif^{-/-}$ in FAEE-AP and CDE-AP.

For more than a century following an original postulate by Chiari,48 pancreatitis has been viewed as an autodigestive disease consequent on pathological zymogen activation.3 34 35 39 45 In experimental AP, zymogens are activated inside acinar cells within minutes of toxin exposure,1 3 30 41 which this work has shown to result from induction of the MPTP, caused by and contributing to calcium overload. Sustained calcium overload may activate degradative calpains, phospholipases or other enzymes44 and damage zymogen granules, inducing autophagic45 and/or endolysosomal49 responses that activate digestive enzymes. Such activation was not completely prevented by MPTP inhibition, however, likely from global cytosolic calcium overload that was seen to be more effectively cleared in *Ppif*−/− cells, without which overload no enzyme activation occurs.30 Nevertheless, intracellular expression of trypsin per se without mitochondrial injury leads to apoptotic not necrotic pathway activation45 and trypsinogen activation does not appear necessary for either local or systemic inflammation;32 knockout of cathepsin B greatly reduces trypsinogen activation with little effect on serum IL-6 or lung injury.19 Hereditary pancreatitis caused by cationic trypsinogen gene mutations rarely features clinically significant pancreatic necrosis;31 52 further, systemic protease inhibition has had little success as a clinical strategy,53 suggesting that while zymogen activation contributes, it is not the critical driver of AP. This study, however, shows that MPTP opening triggers defective autophagy, while inhibition of MPTP opening preserved ATP supply, increased the efficiency of autophagy and decreased zymogen activation. Together with major effects of MPTP opening on PGAM5 activation that implements necrosis,51 52 and on local and systemic inflammatory responses, these findings now place mitochondrial injury centrally in AP.

Our new data show that in pancreatic acinar cells IP3Rs and RyRs that loads pancreatic acinar mitochondria, which are markedly sensitive to calcium signals.25 The mitochondrial calcium overload induces high conductance MPTP opening and dissipates Δψm, initiating collapse of ATP production, diminished calcium clearance, PGAM5 activation and subsequent necrosis. Importantly for a disease without specific treatment, pharmacological MPTP inhibition29 47 administered after AP induction came close to preventing all injury, notably in the clinically relevant TLCS-AP.

**DISCUSSION**

This study demonstrates that MPTP opening is critical to experimental AP, mediating impaired ATP production, defective autophagy, zymogen activation, inflammatory responses and necrosis (figure 8), features of AP at molecular, cellular and whole organism levels.1 Our previous work identified metabolic effects of MPTP opening specific to ethanol. Here we have established the general significance of MPTP opening as a central mechanism in the pathogenesis of AP and the primary role of calcium overload in this. The patch clamp data show how tight control of cytosolic calcium elevations essential to normal stimulus–load in this. The mitochondrial permeability transition pore (MPTP) plays a critical role in the development of acute pancreatitis. Exposure to pancreatic toxins leads to a sustained rise in cytoplasmic calcium that crosses the inner mitochondrial membrane (IMM) to enter the mitochondrial matrix. Consequent cyclophilin D (CypD) activation promotes MPTP opening across the IMM, causing mitochondrial depolarisation and impaired ATP production. These induce PGAM5 activation and retarded autophagy, downstream mechanisms in acute pancreatitis (upper panel). When MPTP opening is inhibited by genetic (Ppif−/−) or pharmacological means (DEB025 or TR040303), mitochondrial membrane potential is preserved and ATP production sustained. This maintains the integrity of pancreatic acinar cells that clear calcium more effectively and prevents the development of acute pancreatitis (lower panel) (MPTP drawn after reference 14).

**Figure 8** Summary diagram: the mitochondrial permeability transition pore (MPTP) plays a critical role in the development of acute pancreatitis. Exposure to pancreatic toxins leads to a sustained rise in cytoplasmic calcium that crosses the inner mitochondrial membrane (IMM) to enter the mitochondrial matrix. Consequent cyclophilin D (CypD) activation promotes MPTP opening across the IMM, causing mitochondrial depolarisation and impaired ATP production. These induce PGAM5 activation and retarded autophagy, downstream mechanisms in acute pancreatitis (upper panel). When MPTP opening is inhibited by genetic (Ppif−/−) or pharmacological means (DEB025 or TR040303), mitochondrial membrane potential is preserved and ATP production sustained. This maintains the integrity of pancreatic acinar cells that clear calcium more effectively and prevents the development of acute pancreatitis (lower panel) (MPTP drawn after reference 14).
transformation by different toxins was specific to different second messengers, identifying potential for a variety of deleterious effects. ATP deficiency may be further exacerbated by fatty acids released on hydrolysis of FAEEs or triglycerides, which may inhibit beta oxidation. Without sufficient ATP, cytosolic calcium overload produces a vicious circle in which high-affinity, low-capacity sarcoplasmic reticulum calcium transport ATPase (SERCA) and plasma membrane calcium ATPase (PMCA) pump clearance of cytosolic calcium is impaired, further mitochondrial injury sustained and necrotic cell death accelerated. Although the toxicity of cytosolic calcium overload depends on calcium store recharging a generalised mechanism of pancreatic injury and necrosis, confirmed in murine and human pancreatic acinar cells, pancreas lobules and tissue slices. Pancreatic necrosis drives the inflammatory response, which may be further exacerbated by deficiencies in mitochondrial permeability transition pore.

Whereas the vast majority of previous studies undertaken to determine mechanisms and/or new targets in AP have used only one model, our four models are broadly representative of a consistent calcium overload to be due completely to release from the cell. Specific second messenger receptor blockade demonstrated calcium overload to be due completely to release from their calcium channels, not direct effects of toxins on calcium entry or extrusion.

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Acknowledgements The authors thank Dr George Perides and Professor Michael Steer who taught R.M. techniques for TLCS-AP.

Collaborators The authors are indebted to members of the NIH Pancreas BRU: Diane Latawiec for technical assistance, Dayani Rajamohanar for pancreatic lobule assays, Euan McLaughlin for whole cell assays with caffeine and Paula Ghane, Christopher Halloran, John P Neoptolemos and Michael GT Raraty for provision of human pancreas tissue samples. The authors wish to thank Dr Samuel W. French, Christopher Halloran, John P Neoptolemos and Michael GT Raraty for provision of human pancreas tissue samples. The authors wish to thank Dr Samuel W. French, Christopher Halloran, John P Neoptolemos and Michael GT Raraty for provision of human pancreas tissue samples. The authors wish to thank Dr Samuel W. French, Christopher Halloran, John P Neoptolemos and Michael GT Raraty for provision of human pancreas tissue samples. The authors wish to thank Dr Samuel W. French, Christopher Halloran, John P Neoptolemos and Michael GT Raraty for provision of human pancreas tissue samples.

Contributors RM and OAM are co-first authors. ASG and RS are co-senior authors. ASG and RS designed and supervised the study. RM, OAM, IVO, WH, JM, MC, MAJ, LW, DBM, MCC and MA performed the experiments. BG, RMP, SS, AVT, OHP, SJ, IG, JDM, DNC and the NIH Pancreas BRU made technical and scientific contributions. RS, ASG, IG, RM and OAM wrote the paper.

Funding This work was supported by US Veterans Administration Merit Review (ASG), NIH grants ROI DK59936 (ASG) and R01AA19730 (IG and OAM), Southern California Research Center for Alcoholic Liver and Pancreatic Diseases (SJG and ASG), American Gastroenterological Association Foundation Designated Research Scholar Award in Pancreatitis (OAM), Russian Federation BR grant 09-04-00739 (IVO), UK/China Postgraduate Research Scholarship for Excellence (WH), Liverpool China Scholarship Council Award (IW), CORE, UK (RM, JM, MAJ), UK Medical Research Council (DMB, MC, MCC, OHP, AVT, DNC and RS), Royal College of Surgeons of England (MAJ) and UK NIHR Biomedical Research Unit Funding Scheme (MA, AVT, DNC and RS).

Competing interests BG is an employee of Debiopharm Research and Manufacturing S.A., who supplied DEB025, RMP and SS are employees of Trophos SA, who supplied TRO40303; RS has received research funding from Debiopharm Research and Manufacturing S.A. and has acted as a consultant for Novartis International A.G.

Ethics approval Human pancreatic samples were donated by patients undergoing surgery for left-sided or unobstructing pancreatic tumours as approved by Liverpool Adult Local Research Ethics Committee (Ref: 03/12/242/2). Animal protocols were approved by UK Home Office (PPL 40/3320) and animal research committee of Veterans Affairs Greater Los Angeles Healthcare System, as per NIH guidelines.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement Further unpublished data identified in the text of this article can be made available to bona fide researchers after communication with the corresponding author.

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Pancreas


Mechanism of mitochondrial permeability transition pore induction and damage in the pancreas: inhibition prevents acute pancreatitis by protecting production of ATP

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Gut published online June 12, 2015

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