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Multimodal Transgastric Local Pancreatic Hypothermia Reduces Severity of Acute Pancreatitis in Rats and Increases Survival

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Abstract

Background & Aims: Acute pancreatitis (AP) of different etiologies is associated with activation of different signaling pathways in pancreatic cells, posing challenges to development of targeted therapies. We investigated whether local pancreatic hypothermia, without systemic hypothermia, can reduce severity of AP, induced by different methods in rats.

Methods: A urethane balloon, with two polyurethane tubes was placed inside the stomach of the rats. AP was induced in Wistar rats by administration of caerulein or glyceryl-trilinoleate (GTL). Cold water was then infused into the balloon to cool the pancreas. Pancreatic temperatures were selected based on those found to reduce acinar cell injury. An unperfused balloon was used as a control. Pancreatic and rectal temperatures were monitored, and an infrared lamp and/or heating pad were used to avoid generalized hypothermia. We collected blood, pancreas, kidney, and lung tissues and analyzed them by histology, immunofluorescence, immunoblot, cytokine and chemokine magnetic bead, and DNA damage assays. The effect of hypothermia on signaling pathways initiated by caerulein and GTL was studied in acinar cells.

Results: Rats with pancreatic cooling developed less severe GTL-induced AP compared with rats that received the control balloon. In acinar cells, cooling reduced the lipolysis induced by GTL, increased the micellar form of its fatty acid, reduced the increase in cytosolic calcium, prevented loss of mitochondrial membrane potential (by 70%–80%), and resulted in a 40%–50% reduction in uptake of a fatty acid tracer. In rats with AP, cooling reduced pancreatic necrosis by 48%, reduced serum levels of cytokines and markers of cell damage, decreased markers of lung and renal damage. The pancreatic cooling increased the proportions of rats surviving 6 hrs after

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induction of AP (to 90%, from less than 10% of rats that received the control balloon). In rats with caerulein-induced AP, pancreatic cooling reduced pancreatic markers of apoptosis and inflammation.

Conclusions: In rats with AP, transgastric local pancreatic hypothermia reduces pancreatic necrosis, apoptosis, inflammation, and markers of pancreatitis severity, and increases survival.

Keywords

animal model; DAMP; exocrine pancreas; glucose

INTRODUCTION:

AP has multiple etiologies, each of which can initiate multiple parallel signaling pathways^{1,2} which eventually cause cell injury or inflammation. So targeted pharmacotherapy, as noted historically³⁻¹³, may be ineffective since parallel pathways may bypass the inhibited one, causing AP to progress. For e.g. caerulein at concentrations relevant to AP activates protein kinase C isoforms^{14,15}, inhibits secretion¹⁶, reorganizes actin¹⁷, causes blebbing^{18,19} and increases cytosolic calcium from multiple pools^{20,21}. There is associated calcineurin signaling²², co-localization of cathepsin B^{23, 24} with trypsinogen, generating trypsin²⁵⁻²⁷, derangement of autophagy²⁸, and parallel activation of pro-inflammatory transcription factors^{29,30}, causing cytokine, chemokine generation³¹, which worsen pancreatic inflammation. Concurrent reactive oxygen species generation³², glutathione depletion^{33,34}, cell death pathways³⁵, mitochondrial depolarization³⁶, may all worsen AP. These multiple signaling pathways, like those of bile acids³⁷ and fatty acid^{13,38,39} (which cause local and systemic injury), pose a formidable challenge, and may explain why mechanistically sound and targeted monotherapies for AP did not provide clear benefits in clinical trials³⁻¹³.

We therefore planned an approach which could slow down multiple signaling steps, and therefore be broadly relevant irrespective of the etiology of pancreatitis. Additionally, it would need to be rapidly inducible, such as in the emergency room, and be therapeutically relevant- by being efficacious after the onset of disease. Hypothermia is one such modality. However, due to the visceral location of the pancreas, external cooling is not a viable option, without causing generalized hypothermia, and its depressant effects on multiple systems such as the cardiovascular system and lungs. We thus planned local pancreatic hypothermia. Since the stomach lies anterior to the pancreas and covers most of its surface, we hypothesized that cooling the pancreas via a cooling gastric balloon would be feasible, while minimizing systemic hypothermia. This local hypothermia could thus have the potential advantages of being multi-modal, rapidly inducible, reversible, and being relevant to the diverse etiologies of acute pancreatitis with minimal toxicity. Since AP severity cannot be predicted early on, we chose to test our hypotheses in one of the most severe AP models (intraductal glyceryl tri-linoleate; GTL^{40,41}) along with the mildest (caerulein in rats) for safety and efficacy. The following study presents our findings using transgastric local pancreatic hypothermia as related to these two pancreatitis models.

METHODS:

These are described in detail in the supplementary section. In summary, all *in vitro* or *ex-vivo* studies are from 3 or more replicate experiments. All *in vivo* studies represent 8–10 animals per group. Data shown as bar graphs are mean $\pm$ SD. The box plots show the mean (dotted line), median (solid line), 25th and 75th percentiles and 10th and 90th percentiles (whiskers). Unless otherwise indicated, “*” depicts significant ($p < 0.05$) difference from control and “†” a significant difference in the cooling group vs. without cooling.

RESULTS:

Pancreatic necrosis and systemic injury occurs in severe pancreatitis via lipolytically generated fatty acids.

Before testing pancreatic hypothermia, we chose our models by directly comparing pancreatic injury and organ failure induced by a common unsaturated fatty acid (UFA), to mild AP induced by caerulein. UFAs are elevated in human severe pancreatitis^{38, 39, 42, 43}, and linoleic acid (LA)-comprises 15–25% of fat in both rodents, humans^{44,45} and in human pancreatic necrosis collections^{38, 39}. Since visceral fat comprises 2–10% of body weight⁴⁶ we used LA at 0.1% body weight, which is in the range noted in patients with necrotizing pancreatitis^{38, 39}, and has been previously used by others⁴⁷. Intraperitoneal LA caused pancreatic necrosis ($4.3 \pm 1.2\%$ vs. $0.1 \pm 0.1\%$ in controls, $p < 0.01$) similar to palmito-oleic acid with ethanol^{48, 49}, along with shock (drop in carotid pulse distension) and renal failure (Fig. 1A), and a 100% mortality by 48 hours. None of these were noted with caerulein (necrosis $0.6 \pm 0.9\%$, $p = 0.27$ vs. con, Fig. 1B) when followed daily to 72 hours with Q12 hour dosing (data not shown), as shown previously³⁸. Thus LA causes both local and systemic injury in AP in contrast to caerulein which is mild. Therefore testing hypothermia in these models is relevant, since severity cannot be reliably predicted early on in clinical AP.

We next studied if LA and caerulein are mechanistically distinct using *in vitro* experiments. Since LA would be generated lipolytically during AP, we studied if the hydrolysis its triglyceride (glyceryl trilinoleate; GTL) induced acinar cell necrosis *in vitro*. As seen in figure 1C, GTL (30 μ M) caused 80% necrosis within 4 hours. Lipase inhibition with orlistat completely prevented GTL, but not LA (300 μ M) induced injury and reduced fatty acid (FFA) generation, proving that lipolysis of GTL to LA induces acinar injury. In contrast caerulein did not generate FFAs and caused less injury ($21 \pm 2.8\%$) which was unaffected by orlistat- thus proving that the two agents are mechanistically distinct. Other differences included caerulein increasing active caspase-3 (Fig 1E)¹³, but not drastically reducing ATP concentrations (Fig 1F), unlike LA or GTL. LA and GTL increased the binding of near infra-red 2-deoxyglucose (2-DG) to acinar cells. Orlistat reduced the GTL induced increase, while that due to LA was unaffected (Fig 1G). These findings are consistent with fatty acid mitochondrial toxicity³⁹ reducing ATP synthesis, which increases glucose demand and 2-DG binding, similar to human pancreatitis^{50–55}, and reinforce the mechanistically distinct nature of the caerulein and GTL models.

Severe AP but not mild AP increases the metabolic demand of the pancreas.

We went on to verify the significance of our findings *in vivo* using infra-red 2-DG (NIR 2-DG), Caerulein AP did not increase NIR 2-DG uptake (metabolic demand), whether studied over the whole animal (Fig 3A), the pancreas (Fig 2B,C) or the kidneys (Fig 2B, D), the tubules of which would have been damaged during severe AP^{38–40, 56}. Conversely GTL pancreatitis, which causes 60–70% pancreatic necrosis, respiratory and renal failure⁴⁰, and 100% mortality, increased NIR 2-DG retention (Fig 2A), over both the pancreas and kidneys *in-vivo* and when examined *ex-vivo* (Fig 2B–D). These findings concur well with our *in vitro* findings (Fig 1) and clinical pancreatitis^{50–55}, and thus provide an intermediary target to determine severity and study the efficacy of hypothermia while treating AP.

Cooling reduces lipotoxicity via synergistically slowing multiple mechanisms.

We first determined the temperatures required to significantly reduce the GTL induced injury in acinar cells, with a goal of understanding the underlying mechanisms and applying these *in vivo*. Since Fig 1C–G indicated that acinar necrosis requires GTL's lipolysis to LA, we studied the effect of hypothermia on these phenomena. On incubating acinar cells with GTL (300 μ M, 4 hours) at different temperatures, we noted cooling progressively reduced cell injury, providing 45–90% protection at 25°C vs. 37°C (Fig 3A?–C). Terminal end points (Fig 3B, C) were the most protected. We then studied hypothermia's effect on upstream steps. Lipolysis of the GTL by recombinant human pancreatic triglyceride lipase (PNLIP; 1 μ g/ml, in the presence of 2 fold molar excess of colipase) was reduced by 40–50% at 25°C vs. 37°C (Fig 3D). Cooling reduced the Ca_i increase and prevented the loss of mitochondrial membrane potential (ψ_m) by 70–80% at 25°C (Fig 3E, F). The lipase inhibitor orlistat completely inhibited the increase in Ca_i , ψ_m (Fig 3E', F'). In comparison to GTL, LA induced Ca_i increase and ψ_m were reduced by 30–40% by cooling to 25°C (Fig 4A, B). The above findings support the hypothesis that cooling synergistically reduces LA formation, as well as LA induced Ca_i , thus having a greater effect on GTL mediated Ca_i , ψ_m changes. Since fatty acid toxicity involves inhibition of mitochondrial ATP production, we studied the effect of cooling on uptake of the fatty acids tracer BODIPY FL C16, and noted it to be reduced by 40%–50% by cooling to 25°C vs. 37°C (Fig 4C). Thus reducing fatty acid membrane incorporation contributes cooling's protective effects.

We have previously shown that fatty acid toxicity is not due to detergent like membrane damage³⁹, and therefore LA would need to be monomeric to integrate into membranes. We thus studied micellar breakdown of LA using isothermal titration calorimetry, by injecting sub-micellar concentrations (10 μ M/injection every 200 seconds) from a 1mM LA stock ($\approx 10\times$ critical micellar concentration⁵⁷) into PBS (Figure 4D, E show representative raw tracings, please see supplementary data for details in technique). The Y-axis in Fig 4D, E represent the heat released (if negative) or absorbed (if positive) with each 10 μ M injection. As can be seen, micellar breakdown is endothermic (Lower red line in Figure 4D) at 37°C. At 25°C, this micellar breakdown was much weaker (lower Blue line Figure 4E, which shows smaller magnitude of heat rate/injection vs. 37°C) and its magnitude decreased much sooner, and therefore ceased at a lower concentration. When plotted as a function of concentration (Fig 4F) the magnitude of enthalpy (K Cal/mol) at 25°C started reducing before 50M, but at 37°C this was noted after 100 μ M. When plotted as change in enthalpy (ΔH)/

change in concentration (C), the peak or nadir of which represents the critical micellar concentration (CMC)⁵⁸; the CMC of LA changed from 150 μ M at 37°C to 50–60 μ M at 25°C (Fig 4G). Therefore cooling reduces the concentration of LA available for signaling in the monomeric form (Fig 6H). Overall, a 12°C reduction in temperature from 37 to 25°C, by slowing consecutive steps involved in lipotoxic injury by a small extent (i.e. lipolytic generation of LA, its micellar breakdown, membrane incorporation and ψ m increase), had a large effect on the major terminal end point of acinar necrosis (>80%, Fig 3B,C). Thus cooling, by slowing multiple steps by a small magnitude, synergistically provides a large degree of protection over multiple steps. This is summarized in Fig 6H. We next went on to study the feasibility and relevance of this *in vivo*.

Transgastric local pancreatic hypothermia is rapidly inducible and reversible

Based on *in vitro* data, we targeted a pancreatic temperature of 25°C *in vivo*. As most of the pancreas is covered by the stomach (Supp. Fig 1A), we designed a cooling balloon perfused with a dual catheter system and placed it in the stomach (Supp. Fig 1B, C) orogastrically, and externalized the tubes via laparotomy. While using external heating (infrared lamp, heating pad), and rectal temperature monitoring, we perfused the balloon with cold water (Supp. Fig 1C, D). Two thermocouple probes (orange, Fig 5B) placed in the pancreatic head and tail showed similar pancreatic temperature averaging between 25 and 27°C to be achievable within 1–2 hours and sustainable for the duration of the planned study without a drop in rectal temperatures. Cooling did not cause pancreatic, gastric or liver injury (supplemental figure 2, 3). Cessation of cooling normalized pancreatic temperatures within 1 hour (Supp. Fig 1D). Based on this we designed studies to test the efficacy of therapeutic transgastric pancreas cooling for treating pancreatitis, in which cooling was initiated after AP induction (Figure 5A).

Transgastric local pancreatic hypothermia reduces local and systemic injury during severe pancreatitis.

In vivo, 25.8 $\pm$ 0.6°C was achieved in the pancreas 1–2 hours after initiating AP (Figure 5A, B). AP increased NIR 2-DG uptake (4.4 $\pm$ 3.3 fold control, Fig 5C, D) in the exocrine pancreas (Fig 5E) while sparing the islets. Cooling reduced this (1.9 $\pm$ 0.8 fold) unlike balloon placement alone (4.1 $\pm$ 1.5 fold). Within 1 hour of GTL infusion, there was 21 $\pm$ 4% necrosis (dashed line, Fig 5F) which increased to 70 $\pm$ 4% over 6 hours. Cooling reduced this (43 $\pm$ 6%) unlike balloon placement alone (64 $\pm$ 4%, $p=0.38$, Fig 5F, F1).

Mechanistically, cooling reduced serum LA levels (Fig 6A), systemic injury markers, i.e. damage associated molecular patterns [DAMPs; circulating dsDNA, and histone complexed DNA fragments (Fig 6B, B1)], and cytokines (Fig 6C–C2) associated with severe pancreatitis^{59–63} unlike balloon placement alone. Pancreatic cooling without pancreatitis did not affect serum cytokines (supplementary figure 4). Systemic injury measured as apoptotic cells in the lung (Fig 6D) and kidneys, increase in BUN (Fig 6E, E1) and shock (Fig 6F) [noted as a drop in carotid pulse distention (pulse Dist. from 637 $\pm$ 171 μ m to 199 $\pm$ 18 μ m)] were prevented by cooling unlike balloon placement alone at the mean mortality time (3.5 hours) in the GTL group. The significant decrease in oxygen saturation was also improved in the cooling group (supplementary Fig 5).

Most significantly cooling improved survival from <10% at 6 hours (mean 3.5 hours) to 90% (Fig 6G). Therefore as shown in Fig 6H, therapeutic local pancreatic hypothermia, by slowing multiple steps including the lipolysis of intrapancreatic triglyceride and downstream signaling, not only reduced the local pancreatic injury, but also the increase in DAMPs, hypercytokinemia, shock, renal, lung injury and mortality that followed.

Transgastric local pancreatic hypothermia reduces cell injury and inflammation during mild pancreatitis.

Since AP is mostly mild, we aimed at testing hypothermia in caerulein AP (Fig 1B). We first determined the target temperature *in vitro* (Fig 7A–C). In rat pancreatic acini, caerulein (100nM) induced upstream events, i.e. peak and plateau cytosolic calcium levels (Cai), and I κ -B α degradation (Fig 7A, B) were reduced significantly at 25°C, similar to cooling's effect on trypsinogen activation and chemokine upregulation described previously⁶⁴. Interestingly, terminal end points including apoptosis (active caspase-3; Figure 7B, middle panel) and LDH leakage at 4 hours, were reduced at between 31 and 34°C (Fig 7C). We thus targeted an *in vivo* temperature of 33±1°C after inducing pancreatitis (Figure 7D).

AP was induced after a single injection of caerulein (50µg/kg), given about 30 minutes before the induction of cooling as in the time line of figure 5A. Over all it took about 1 hour for target pancreatic temperatures to be achieved (Fig 7D). Cooling significantly reduced inflammatory cell infiltration (Myeloperoxidase) and apoptosis (TUNEL positive cells) normally noted in this model after 6 hours (Figure 7E, F). The increase in serum amylase by 1 hour of caerulein pancreatitis (2636±211 U/L vs. 403±27 U/L in controls) was not further reduced by cooling (2799±1002U/L), since the disease was already established by the time cooling was induced. Thus multimodal *in vivo* benefits during caerulein induced AP (Fig 7G) and the GTL model (Fig 5, 6) support that local pancreatic hypothermia is beneficial in mechanistically different AP models, which also had different severity.

DISCUSSION:

In this study we note the benefits of therapeutic localized transgastric pancreatic hypothermia on the severity of mechanistically distinct mild and severe models of pancreatitis. First we note lipolytically generated or directly administered linoleic acid (LA; which is abundant in pancreatic necrosis^{38, 39, 65}) worsens local and systemic injury (Fig 1) and increases metabolic demand (Fig 2). In contrast caerulein induces pancreatitis without inducing organ failure or increasing metabolic demand. Thereafter, we note cooling slows multiple sequential and distinct parallel steps induced by caerulein or GTL (Fig 3, 4, 6H, 7F). This culminates in large and significant reductions in the terminal end points of cell injury between 25°C (for GTL) and 34°C (for caerulein), while individual upstream steps are slowed by a smaller magnitude at these temperatures. Lastly we note that therapeutic pancreatic cooling without generalized hypothermia reduces local injury in both models, and systemic injury, mortality in the severe GTL model (Fig 6).

Our studies provide evidence that localized pancreatic hypothermia avoids complications of generalized hypothermia including hypotension⁶⁶, vascular leak, hypovolemia⁶⁷,

hemoconcentration⁶⁸ worsening renal failure and respiratory failure⁶⁹, a worse cytokine response⁷⁰ or pancreatic edema⁷¹ (Supplementary figs 2–4).

The multimodal nature of hypothermia reduces LA generation (Fig 3D, 6A) from GTL lipolysis and reduces the ability of LA to signal (Fig 3E, 3F, 4), culminating in reduced pancreatic acinar necrosis (Figure 3B, 3C 5F, F1). The increase in pancreatic 2-DG uptake is consistent with LA inhibiting mitochondrial complexes I and V³⁹, and is not seen in caerulein pancreatitis (Figure 2B). The reduction in caerulein induced Cai, I κ -Ba degradation, apoptosis and previously shown reduced chemokine increase with cooling⁶⁴, reduce inflammation and TUNEL positive cells (Figure 7E,F) during caerulein AP and support this multimodality.

Therapeutic pancreatic hypothermia was achieved 1–2 hours after AP induction and improved markers of local and systemic injury (Fig 5–7). Similar to GTL pancreatitis, serum LA⁴³, nucleosomes and histones (DAMPs) are increased in patients with severe AP^{72, 73}, and predict mortality. DAMPs can induce sterile inflammation⁷⁴, disrupt the plasmalemma⁷⁵, increase IL-1 β via the inflammasome^{76, 77} and further increase DAMP release (e.g. HMGB1⁷⁸). Serum IL-1 β ⁶³, TNF- α ⁶⁰ and IL-6^{59, 60} can be high in clinical severe AP, like in GTL induced AP (Fig 6C–C2), but unlike caerulein AP (Supplementary figure 4). Cooling, by reducing LA generation, necrosis (Fig 1, 3, 5F, F1), reduced DAMPs, IL-1 β , TNF- α and IL-6 levels (Fig 6B–C2). While mechanistically unexplored, the reduced cytokine levels by cooling could be multifactorial. I.e. due to reduced cytokine production downstream of DAMPs^{79,80} which were reduced (Fig 6H), or reduced signaling by transcription factors such as the NF- κ B/I κ B (Fig 7B). Reduced pancreatic MPO (Fig 7E) provides a functional significance to reduced NF- κ B/I κ B signaling, and chemokine mRNA levels⁶⁴ induced by cooling. Cooling's impact on pancreatitis associated lung injury was noted as improved oxygen saturations (Supplementary figure 5) and reduced TUNEL positive cells (Fig 6D), which are a hallmark of lipotoxic ARDS^{39, 81–84}. Similarly, cooling reduced the severity of shock, noted as improved pulse distention (Fig 6F), and reduced renal tubular injury and renal failure (Fig 6E, E1)⁴⁰. This improvement in multiple parameters of systemic injury culminated in dramatically improved survival in this otherwise lethal model (Fig 6G).

Fat necrosis has been associated with severe human AP for over a 100 years^{39, 85–87}. LA is present in high amounts in human pancreatic fat and necrosis^{39, 45} and sera of patients with severe AP^{43, 88}. Therefore LA induced signaling is highly relevant to AP. GTL or LA, likely increase 2-DG uptake (Fig 1G, Fig 5C–E) due to mitochondrial injury and reducing ATP^{39, 41} (Fig 1F) in the exocrine pancreas, which gets necrosed (Fig 5E) unlike islets. Interestingly, this concurs with insulin, glucose supplementation protecting from lipotoxicity⁸⁹. Increased 2-Deoxy-2-[18F] fluoroglucose uptake also occurs in human AP^{50–55}. Caerulein, does not increase 2-DG uptake (Fig 2) or cause necrosis. The notion that non-invasive studies of 2-DG uptake may predict severe AP is further supported by previous studies showing a lower uptake during mild biliary vs. severe biliary AP⁴¹.

Over all this study may provide a clinical tool to predict AP severity using 2-DG mimetics, along with a rapidly inducible, minimally invasive therapy for AP irrespective of its etiology.

While our study is limited by the need to keep rats with a gastric balloon sedated; pilot studies in pigs, on the feasibility of placing a gastric balloon and keeping them unsedated and comfortable are favorable (data not shown). Therefore we would like to discuss the relevance of our work from a clinical perspective. While clinical AP is diagnosed within a few hours, typically in the emergency room, its severity is hard to predict, and often the etiology remains unknown. The current, and previous studies⁴¹ suggest that early stratification into mild vs. severe AP based on 2-DG uptake (in combination with to other methods like BISAP/ SIRS criteria) may allow for choosing the target pancreatic temperatures. Lower temperatures (e.g. 25°C) would be relevant to treating predicted severe AP vs. higher temperatures (e.g. 31–34°C) to mild AP, which may translate to reduced hospital stay. We hypothesize that the rapid, multimodal effect of cooling could prevent clinical worsening over the first several hours or days, till a definite approach (e.g. plasmapheresis or stone extraction) is undertaken, or the toxin (e.g. alcohol) is metabolized or excreted. This modality would likely be inexpensive, since similar technologies (balloon with catheters, e.g. Minnesota tube for bleeding varices) do already exist, albeit for a totally different purpose.

In summary, herein we provide the basis for novel, translatable, scientifically sound methods of predicting severe AP and reducing its severity irrespective of its etiology. These harness the potential of 2-DG mimetics to predict severe AP early on in the disease, based on which patients can be stratified into the lower pancreatic temperature vs. mild cooling groups. The ability to rapidly induce therapeutic pancreatic hypothermia, with its synergistic, multimodal benefits, and rapid reversibility along with avoidance of generalized hypothermia, support its use as an early intervention for AP irrespective of its etiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper:

2-DG	Near infrared 2-deoxyglucose
ALT	alanine aminotransferase
AP	acute pancreatitis
BUN	blood urea nitrogen
CER	caerulein

DNA	deoxyribonucleic acid
DAMPS	death associated molecular patterns
ELISA	enzyme-linked immunosorbent assay
FA	fatty acid
GTL	glyceryl tri-linoleate
LA	Linoleic acid
H&E	hematoxylin and eosin
IL-1β	interleukin-1 beta
IL-6	interleukin-6
linoleic	acid
NF-κB/I	κ B, nuclear factor kappa-light- chain-enhancer of activated B cells
LDH	lactate dehydrogenase
MPO	myeloperoxidase
MSOF	multi-system organ failure
NEFA	non-esterified fatty acid
UFA	unsaturated fatty acid
PI	propidium iodide
SD	standard deviation
TNF-α tumor	necrosis factor-alpha
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
CMC	critical micellar concentration

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Figure 1: Comparison of direct injury induced by linoleic acid (LA) and caerulein (CER). *In vivo* studies showing effect of LA (A) and caerulein pancreatitis (B) on pancreatic histology, induction of shock as measured by carotid pulse distension (pulse Dist.), and renal failure using serum BUN 24 hours after AP induction. Arrows in the images point to loss of cell outlines in necrotic areas. C-G are *In vitro* studies showing the effect of inhibiting lipolysis with orlistat (orli; 50M final concentration) on acinar cell injury. LDH leakage (C) and free fatty acid release (FFA; D) into the medium over 3 hours induced by GTL; glyceryl tri-linoleate (300 μM), LA (300μM), caerulein (CER; 10⁻⁷M). Formation of cleaved caspase-3 as measured by western blotting (E), ATP concentrations (F), and uptake of near infra-red 2-Deoxyglucose (2-DG; G) induced by the same agents. * indicates a significant increase induced by the agent over controls. † indicates a significant reduction in the parameter induced by orlistat.

Figure 2: Comparison of pancreatic and systemic increase in metabolic demand during caerulein (CER) and GTL induced pancreatitis.

A: Near infra-red 2-deoxyglucose (NIR 2-DG) imaging of rats, comparing the time course of NIR 2-DG uptake over the pancreas (dashed polygon) during CER and GTL induced pancreatitis. The NIR 2-DG was given 10 minutes before inducing pancreatitis. Note the higher uptake in the GTL group over time. **B:** *Ex-vivo* visualization of NIR 2-DG uptake in the pancreas and kidneys compared to gross morphology (first and fourth column of images). Quantification of radiant efficiency of 2-DG uptake in the pancreas (**C**) and Kidney (**D**) *ex-vivo* as induced by the different AP models.

Figure 3: Effect of cooling on parameters on acinar injury and signaling induced by GTL. Effects of incubation with GTL (300 μ M) at different temperatures on ATP levels (A), PI uptake (B) and LDH leakage (C) at the end of 4 hours of incubation. Dashed lines indicate threshold for 80% reduction vs. 37°C. D: Effect of cooling on fatty acid generation by lipolysis of GTL (300 μ M) by recombinant human pancreatic triglyceride lipase (1 μ g/ml, with 2 μ g/ml colipase). Effect of GTL (300 μ M) on cytosolic calcium increase (E) and mitochondrial depolarization (F) in pancreatic acini at 37°C and 25°C. Alternatively to determine the effect of inhibition of lipolysis, these were measured in the presence of orlistat (E', F'). * depict a significant difference compared to 37°C.

Figure 4: Mechanisms of the protective effect of cooling on lipotoxic fatty acid signaling. Effect of cooling to 25°C vs. 37°C on the increase in cytosolic calcium (**A**), and mitochondrial depolarization (**B**) induced by linoleic acid (LA; 300µM), and uptake of the fatty acid tracer BODIPY-C16 over 15 minutes (**C**). The quantification of fluorescence intensity is shown in the bar graphs. Representative isotherms from isothermal titration calorimetry at 37°C (red, **D**) and 25°C (Blue, **E**) showing injection of PBS (top tracing) or LA (10µM/ injection every 200 seconds from a 1mM stock in PBS, lower tracing) into PBS. Upward deflection indicates endothermic reaction. Note the reduced magnitude at 25°C. **F**:

Change in enthalpy of micellar breakdown at 37°C (red) and 25°C (blue) as a function of concentration of LA injected. Note that micellar breakdown is sustained for higher concentrations of LA at 37°C than at 25°C. The points in the graphs show the mean $\pm$ SD of 3–4 experiments. **G:** Effect of cooling on critical micellar concentration (CMC) shown as an arrow and calculated as the change in enthalpy (ΔH) over the corresponding change in concentration (ΔC). The lines shown depict an average of 3–4 experiments. Note that CMC is lower at 25°C vs. 37°C.

Figure 5: Effect of cooling (C) or balloon placement (B) on pancreatic parameters during GTL (G or GTL) induced pancreatitis compared to controls (Con).

A: Experimental set up and time line. Note that pancreatitis was induced before initiating cooling **B:** Time line of pancreatic and rectal temperatures after induction of cooling. **C:** Images of NIR-2DG (2-DG) measured at 800nm (upper panel) or nonspecific fluorescence at 700nm (second panel from top) in blocks from pancreatic head (PH) and tail (PT), these are quantified in **D**. **E:** Fluorescent photomicrographs show 2-DG uptake (green) to be specifically seen in the exocrine pancreas, while sparing the islets (red). **F:** Pancreatic necrosis quantified as % acinar area. Dashed line denoted the amount of necrosis already present at 1 hour, i.e. before a temperature of 25°C was reached. **F1:** Photomicrographs show representative H&E stained sections. Note large areas of diffuse light pink staining consistent with necrosis

Figure 6: Effect of cooling (C) or balloon placement (B) on systemic parameters during GTL (G or GTL) induced pancreatitis compared to controls (Con).

Serum levels of linoleic acid (C18:2) (A), dsDNA (B), Histone complexed DNA fragments concentration (B1) and cytokines (C, C1, C2) in rats from different groups. Lung (D) and kidney (E) sections stained for TUNEL using Cy5 probe. Co-localization with nuclei (DAPI) is seen as magenta. Box plot in D shows quantification in lung. **E1:** Serum BUN. **F:** pulse distention (Pulse Dist.) in different groups. **G:** Survival curve comparing groups; GTL alone (solid grey), GTL+ balloon (G+B, dotted grey) and GTL+ cooling (G+C, black dashed), controls (solid black). **H:** Schematic summarizing the effects of cooling (blue) on deleterious mediators and signaling (red) during GTL induced AP.

Figure 7: Effect of hypothermia on caerulein induced cell death signaling in acinar cells and pancreatic injury *in vivo*:

A-C: *in vitro* studies. Acinar cell intracellular calcium (Ca_i ; **A**) in response to 100nM caerulein (arrow) at 37°C (black circle) and 25°C (hollow circle). **B**) Western blots of I- κ B, and active caspase-3 using amylase as loading control on lysates from acinar cells stimulated for 60 minutes with 100nM caerulein (CER) at 25°C, 31°C, 37°C. **(C)** Effect of cooling on cell injury at 4 hours as measured by LDH leakage. **D:** *In vivo*, Pancreatic and rectal temperatures after induction of cooling. Pancreatic myeloperoxidase (MPO; **E**) and apoptotic cells (**F**) in rats given caerulein (C) alone, with a cooled balloon (C+Co) or with a balloon that was not cooled (C+B). **G:** Schematic summarizing the effects of different temperatures (mentioned in white text of background) on mediators or signaling induced by 100nM caerulein or during caerulein AP. Note milder cooling reduces more terminal events vs. upstream events. The “64” indicates the reference number showing the evidence.