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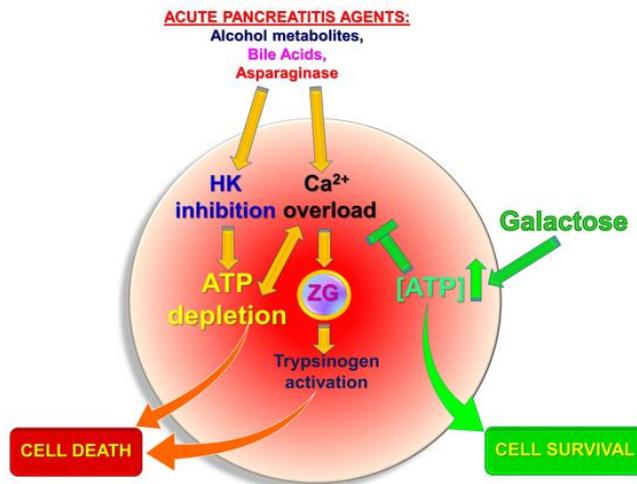
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Galactose protects against cell damage in mouse models of acute pancreatitis

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Summary

Acute pancreatitis (AP), a human disease in which the pancreas digests itself, has substantial mortality with no specific therapy. The major causes of AP are alcohol abuse and gallstone complications, but it also occurs as an important side effect of the standard Asparaginase-based therapy for childhood acute lymphoblastic leukaemia. Previous investigations into the mechanisms underlying pancreatic acinar cell death induced by alcohol metabolites, bile acids or Asparaginase indicated that loss of intracellular ATP generation is a significant factor. In isolated mouse pancreatic acinar cells or cell clusters, we now report that removal of extracellular glucose had little effect on this ATP loss, suggesting that glucose metabolism was severely inhibited under these conditions. Surprisingly, we show that replacing glucose with galactose prevented or markedly reduced the loss of ATP and any subsequent necrosis. Addition of pyruvate had a similar protective effect. We also studied the effect of galactose *in vivo* in mouse models of AP induced either by a combination of fatty acids and ethanol or Asparaginase. In both cases, galactose markedly reduced acinar necrosis and inflammation. Based on these data we suggest that galactose feeding may be used to protect against AP.

authors equally contributed to this study

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Author contributions: SP, JVG, TMT, OG, SS, OHP and OVG designed the study. SP, JVG, TMT, OG and OVG conducted and analysed experiments. SP, JVG, OHP and OVG wrote the manuscript. All authors read and approved the final draft of the manuscript.

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Introduction

Acute pancreatitis (AP) is an inflammatory disease that originates in the exocrine pancreas, where inactive pancreatic pro-enzymes become prematurely activated inside the pancreatic acinar cells (PACs), digesting the pancreas and its surroundings (1,2). The main causes of AP are excessive alcohol and fatty food intake and gallstone disease, comprising about 80% of all cases (3). Stimulation of PACs with alcohol metabolites or bile acids leads to aberrant calcium signalling due to excessive release from intracellular stores followed by activation of massive Ca^{2+} entry through store operated Ca^{2+} release activated Ca^{2+} (CRAC) channels causing intracellular Ca^{2+} overload (2,4,5).

Another cause of AP is the L-Asparaginase treatment of acute lymphoblastic leukaemia (ALL) (6,7). According to Cancer Research UK, there were 760 new cases of ALL diagnosed in the UK in 2014. The incidence rates for ALL are highest in children aged 0-4 (2012-2014). Anti-leukemic drugs based on L-Asparaginase are currently used in the clinic as an effective treatment of childhood ALL (8-12). However, in up to 10% of the cases the Asparaginase treatment has to be truncated due to development of AP, a serious and incurable illness (6,7,13-17). Although Asparaginase-based drugs have been used in the clinic for many years (8), the mechanism of this side effect has not been well explored and understood.

We have recently made progress in understanding the mechanism of Asparaginase-induced acute pancreatitis (AAP) (18). Our key findings include the activation of protease activated receptor 2 (PAR2) as well as calcium overload and loss of ATP in PACs. These findings provide the first mechanistic insight into the process by which Asparaginase treatment of ALL may cause AAP. The Asparaginase effect on cancer cells relies on the depletion of asparagine, which the malignant cells cannot produce by themselves, as opposed to normal cells (19,20). However, the AP inducing side effects of Asparaginase do not depend on presence or absence of asparagine (18). In contrast, the AP-inducing side effect of Asparaginase is caused by the activation of a signal transduction mechanism involving PAR2 which, via a number of steps, causes cytosolic Ca^{2+} overloading and reduction in the intracellular ATP level. The reduction of energy supply inhibits both the plasma membrane Ca^{2+} ATPase (PMCA) and the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) (21-23). We have recently shown that restoration of energy supply, by the addition of pyruvate, provides an astonishingly high degree of protection against pancreatic necrosis (18).

We have now analysed the role of glycolysis in AP in more detail, *in vivo* and *in vitro*, and specifically compared the effects of pyruvate, galactose (24) and glucose on the functional and morphological features of AP and AAP. Based on these data, we propose a simple and promising way to rescue intracellular ATP levels in AP and AAP patients.

Results

ATP loss is the common hallmark of AP

It has been established previously that ATP loss in AP is a critical part of the pathological mechanism in PACs, irrespective of whether it has been initiated by alcohol metabolites or bile acids (BA) (1,22,25). As previously described (18) we have assessed intracellular changes in ATP concentration by using Magnesium Green (MgGreen) fluorescence measurements. As most of the intracellular ATP will be in the form of Mg-ATP, a reduction of the ATP concentration will increase the fluorescence intensity of MgGreen due to the increase in free Mg^{2+} concentration. We have studied the effect of Asparaginase in PACs and found that 30 min of exposure to this agent caused a $45.8 \pm 4.8\%$ loss of ATP (Fig.1A). The ATP reduction induced by Asparaginase was very similar to that elicited by exposure to the non-oxidative alcohol metabolite palmitoleic acid ethyl ester (POAEE, $40.9 \pm 4.9\%$), and palmitoleic acid (POA, $66.9 \pm 4.9\%$) (26) or a bile acid mixture (BA, $51.6 \pm 3.3\%$) (Fig.1A), while removal of glucose for 30 min led to a substantially smaller reduction ($15.5 \pm 0.95\%$). Interestingly, removal of glucose did not significantly increase ATP depletion induced by POA or BA, but partially increased ATP depletion induced by BA (Fig.1A).

Since the majority of cellular ATP is produced by glucose metabolism, we have compared the effect of a glucose free-medium on necrosis to that induced by Asparaginase, POAEE, POA, or BA (Fig.1B). In these experiments, lasting 2 hours we found that removal of glucose produced a comparable level of necrosis to all other pathological agents ($14.8 \pm 0.5\%$, $p < 0.0001$), but did not significantly exacerbate the effects of Asparaginase ($p > 0.059$), marginally increased POA-elicited necrosis (from $20.0 \pm 0.3\%$ to $22.2 \pm 0.7\%$, $p < 0.01$) (Fig.1B), and somewhat increased BA-induced necrosis (from $18.3 \pm 1.1\%$ to $29.4 \pm 2.5\%$, $p < 0.008$). The fact that removal of glucose did not further increase the extent of necrosis induced by Asparaginase or POA may suggest that glucose metabolism is already so strongly inhibited by these two agents that removal of external glucose has practically no additional effect.

Pyruvate and galactose rescue bile- and alcohol metabolite- induced pathology

In our previous study into the mechanism by which Asparaginase evokes pathological changes in isolated PACs (18), we have shown that inclusion of pyruvate in the bathing solution provided remarkable protection against necrosis. We furthermore demonstrated that the reduction in the intracellular ATP level caused by Asparaginase was significantly diminished when pyruvate was present (18). In addition to pyruvate we decided to test galactose for its effectiveness in protection against alcohol- and bile-induced pancreatic pathologies. Galactose very significantly reduced the ATP loss caused by the alcohol metabolites POAEE (Fig.2A,B) and POA (Fig.2D,E) and also essentially prevented the necrosis induced by these agents (Fig.2C,F). Pyruvate had a very similar effect (Fig.2F). A

similar protective effect of pyruvate was also found in the case of bile-related pathology. Pyruvate substantially reduced the ATP loss elicited by BA (Fig.2G,H) and both pyruvate and galactose almost entirely eliminated the BA-induced necrosis (Fig.2I).

Pyruvate and galactose protect against Asparaginase-induced necrosis

The ability of galactose to protect against necrosis induced by POAEE, POA or BA (Fig.2C,F,I) has prompted us to also test the effect of galactose on Asparaginase-induced pathology (18). Both, pyruvate and galactose, at either 1 mM (Fig.3A,B) or 10 mM (Fig 3C) had similar protective effects against Asparaginase-induced necrosis in PACs. Interestingly, the presence or absence of glucose made no difference to the extent of the necrosis (Fig.1B). These data suggest that glucose metabolism is severely affected by Asparaginase, but that energy supply can be rescued by galactose or pyruvate joining the glycolysis cycle.

Galactose and pyruvate, but not glucose, rescue the Asparaginase-induced pathology

With regards to the primary action of Asparaginase on PACs, we have previously shown that this agent evokes a sustained elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) due to interaction with PAR2 (18). Fig.4A-B show that both pyruvate and galactose very markedly reduced the Asparaginase-elicited $[Ca^{2+}]_i$ rise. In control experiments pyruvate and galactose did not change the frequency of Ca^{2+} oscillations induced by either CCK ($p>0.3$, $n=11$ and $p>0.9$, $n=11$ respectively) or Asparaginase ($p>0.1$, $n=33$ and $p>0.7$, $n=17$, respectively).

There was also no significant difference with regard to the amplitude of spikes induced by CCK ($p>0.8$, $n=157$ and $p>0.8$, $n=46$ respectively). The amplitude of Asparaginase-induced oscillations was reduced by 20% ($p<0.0001$, $n=39$) in the presence of pyruvate and by 15% ($p<0.02$, $n=26$) in the presence of galactose. These relatively minor effects are probably due to the increase in the cytoplasmic ATP level and, therefore, Ca^{2+} uptake after release.

Previously we have shown that Asparaginase inhibits Ca^{2+} extrusion from PACs, most likely due to the reduced availability of ATP (18).

Although the Asparaginase-elicited sustained rise in $[Ca^{2+}]_i$ depends on increased Ca^{2+} entry (18), this could be compensated by an increase in the rate of active Ca^{2+} extrusion, if an adequate supply of ATP is available. It would seem possible that ATP supply is enhanced in the presence of pyruvate or galactose and that this could be the mechanism by which the toxic $[Ca^{2+}]_i$ rise is inhibited. We therefore tested this hypothesis by assessing changes in the intracellular ATP concentration (Fig.4C,D) as well as changes in NADH and FAD (Supplemental SFig.1A,B).

Asparaginase induced a substantial intracellular ATP loss (Fig.4C-E), in line with reduction of NADH (SFig.1A). Replacement of glucose with pyruvate or galactose (both 10 mM, Fig.4C,D)

or adding 1mM pyruvate or galactose (Fig.4E) markedly reduced the Asparaginase-induced ATP loss. Replacing glucose with pyruvate or galactose was very effective in protecting against ATP loss and we therefore compared our results in the presence and absence of glucose and pyruvate (Fig.4F-H). The ATP loss was substantially higher in the absence of pyruvate (red and orange traces) regardless of the presence or absence of glucose. Comparison of the 'areas under the curve' shows that 1mM pyruvate (blue and green traces) significantly reduced the ATP loss (Fig.4G), whereas the presence of 10 mM glucose did not ($p>0.05$). Comparison of the amplitudes (Fig.4H) showed very similar results, namely that the glucose-independent ATP loss was markedly reduced by 1 mM pyruvate. Asparaginase also affected the mitochondrial potential (SFig.2A-F) and the mitochondrial Ca^{2+} levels (SFig.3A-F), but, pyruvate and galactose restored these parameters to near control levels.

Pyruvate and galactose increase intracellular ATP levels

All the three AP-inducing factors that we tested substantially inhibited ATP production and both Asparaginase and POA severely inhibited glucose metabolism. Galactose can enter the glycolysis cycle, skipping its first step and does not depend on hexokinase activity. Our data may therefore indicate that glucokinase/hexokinase activity is inhibited during the induction of AP. Both galactose and pyruvate provide an additional source of ATP and increase intracellular ATP levels (Fig.5A,B).

The glucose analogue 2-Deoxy-D-glucose (2-DG) (27), which inhibits glycolysis via its indirect actions on hexokinase, induced a substantial ATP loss (Fig.5C), necrosis (Fig.5D) and $[Ca^{2+}]_i$ elevations (Fig.5E,F), very similar effects to those induced by Asparaginase (Fig.4A-D and Fig.3B and Fig.5D). Pyruvate significantly reduced the 2-DG-induced sustained $[Ca^{2+}]_i$ elevation (Fig 5E,F). The rescue effects of galactose were completely blocked by the glucose transport inhibitor phloretin (SFig.1C), suggesting that only hexokinase inhibition could explain the ATP depletion observed in AP.

Hexokinase activity is inhibited *in vitro* by POA and BA

To test our hypothesis that AP-inducing agents cause intracellular ATP loss by reducing hexokinase activity, we measured *in vitro* the activities of the three major human hexokinases present in the pancreas (28,29). We found that POA markedly reduced the activity of hexokinase 1 and partially reduced hexokinase 2 activity (Fig.6AB). Whereas POA had no effect on glucokinase (HK4), BA markedly reduced HK4 activity (Fig.6C), but had no effect on HK1 and 2 (Fig.6A-C). In control experiments we found that the only other enzyme present in the cuvette (glucose-6-phosphate dehydrogenase) was not affected by either POA or BA ($p>0.6$ and $p>0.4$ respectively, as compared with control $n=5$). We also

measured the activity of hexokinase 3, that has relatively low abundance in most tissues except myeloid cells, but we did not find any significant inhibition by either POA or BA as compared to control (n=5). POAEE partially, but significantly, inhibited hexokinase 1 ($p < 0.004$, n=3) but did not affect other hexokinases. The Western blot (Fig.6D) shows that HK1, HK2 and HK4 are all present in mouse PACs. We conclude that the pathological hexokinase inhibition, particularly of HK1 by POA and HK4 by BA, play the key role in the ATP depletion that is such a significant feature of AP. In line with these data, a relatively high concentration of insulin (100 nM), stimulated hexokinases and rescued Asparaginase-, POA- and BA-induced necrosis (SFig.1D). An increased glucose concentration (30 mM) only stimulated glucokinase and, therefore, rescued Asparaginase- and POA- but not BA-induced necrosis (SFig.1D).

Galactose administration protects from alcohol-induced AP *in vivo*

To test whether our findings could lead to a rational treatment of AP, we focussed our attention specifically on the possibility that galactose might be helpful, as this sugar has already been included as part of human trials for the treatment of glycogen storage disease type 1b (Fabry's disease), nephrotic syndrome, congenital disorders of glycosylation and has not been shown to have any negative effects (30-33). Galactose, an essential component of human breast milk (up to 70 mM during the first month (34)), is quite stable in solution, relatively slowly metabolised as compared to pyruvate and has been administered both by intraperitoneal injections and feeding (drink) protocols (35-37). We tested the protective effect of galactose *in vivo*, in a realistic mouse model in which AP was induced by a mixture of POA and alcohol (FAEE-AP (38)). As shown in Fig.7A-E, galactose significantly improved the histology score (Fig.7E) and reduced the degrees of edema (Fig.7B), inflammation (Fig.7C) and necrosis (Fig.7D). Galactose also rescued the alcohol-induced increase in amylase activity (SFig.4A), interleukin 6 (IL6), SFig.4B) and intracellular trypsin (SFig.5A-H). Control glucose feeding did not affect amylase activity (SFig.4A) but was able to partially restore IL6 levels. The weight loss, typically seen in AP was partially prevented by galactose but not glucose (SFig.4C,D). Overall, galactose had a remarkable protective effect against experimental alcohol-related AP.

Galactose administration inhibits Asparaginase-induced AP *in vivo*

The experiments shown in Fig.3B,C indicate that it might be possible to use galactose to boost energy production *in vivo* to counteract the toxic effects of Asparaginase. We have therefore developed a mouse model of Asparaginase-induced AP, using an approach similar to that developed for studying AP induced by alcohol metabolites, bile and caerulein (38).

Asparaginase injections resulted in significantly increased histology scores, high degrees of edema, inflammation and necrosis (Fig.8A-E) that were similar to those reported for other AP models (38). As seen in Fig.8A-E, galactose significantly reduced the histology score and the degrees of edema, inflammation and necrosis towards much lower values in both feeding, and combination of injection and feeding, protocols with similar efficacy. The weight loss typical for AP was also partially reduced (SFig.4D). Therefore, we conclude that galactose could become a new effective supplemental treatment for AAP.

Discussion

It is well established that the initial stages of AP are characterised by intracellular Ca^{2+} overload, causing inadequate function of the mitochondria leading to reduction of ATP production, premature intracellular activation of digestive enzymes and cell death mainly by necrosis (1,2).

Our new data reveal that AP-inducing agents such as alcohol and fatty acids, bile and Asparaginase markedly reduce glucose metabolism in PACs leading to reduced ATP synthesis and therefore substantial ATP loss. The combination of cytosolic Ca^{2+} overload and ATP depletion leads to profound cellular necrosis that could be avoided by ATP supplementation (22).

We have now shown that the addition of pyruvate or galactose rescues cells from injury induced by all the principal agents inducing AP. Removal of glucose from the medium does not significantly affect the ATP loss and necrosis induced by these agents, indicating that glucose metabolism is severely inhibited. Phloretin, the glucose transport inhibitor (39) also completely blocked the galactose rescue effect (SFig.1C). Glucose and galactose are known to enter the cells by the same transporters (40), but galactose is converted to glucose-6-phosphate by several enzymes without involving hexokinases (41,42). We therefore conclude that hexokinase inhibition is likely to play an important role in the ATP depletion that is a significant element in the development of AP.

Our *in vitro* experiments (Fig.6) suggest that both alcohol metabolites and bile acids directly affect hexokinase enzymes, hexokinase 1 and 4 respectively, whereas the Asparaginase effect is indirect (18). The direct inhibition of hexokinases reduces but does not abolish ATP production (SFig.6A-C), as there can still be some production by a number of metabolic pathways. However, cellular ATP is severely depleted and at the same time cells are overstimulated by pathological substances, making recovery virtually impossible. Galactose addition *in vivo* (as well as pyruvate *in vitro*) rescues the cells from ATP depletion and hence necrosis.

A relatively high dose (100 nM) of insulin reduced all POA- (26), BA- and Asparaginase-induced necrosis (SFig.1D), most likely by potentiating hexokinases (28,29). An increased

glucose concentration (30 mM) potentiates glucokinase which has a low affinity for glucose (29) and also reduced both POA- and Asparaginase-induced necrosis (SFig.1D). However, such an increased glucose level failed to rescue BA-induced necrosis (SFig.1D). This is in line with our data regarding the inhibition of glucokinase by BA (Fig.6C), whereas both POA and Asparaginase have striking similarities in their pathological mechanisms, likely inhibiting hexokinase 1 (Fig.6A). Although both insulin and high glucose levels were effective *in vitro*, none of them could of course be employed *in vivo*. In contrast, galactose feeding, which appears to have no negative side-effects would be a potentially valuable therapy against AP. Galactose could also be used preventively, which could be of particular importance in cases where there is a significantly enhanced risk of AP (43), for example when treating ALL with Asparaginase. Our results indicate that galactose would be a valuable addition to the current Asparaginase treatment protocol. Substitution of drinking water in mouse models with a 10 mM galactose solution significantly reduced all pathological scores in both Asparaginase- and alcohol metabolites-induced AP. Since this approach has been successful in treating experimental AP in the mouse, induced by several different agents, and relies on increasing the intracellular ATP/prevention of depletion of ATP, it might become useful also for treating other diseases with ATP loss and subsequent necrosis as well as counteracting similar side effects of other drugs.

With regards to the clinical treatment of patients with AP, there is currently a debate about high versus low energy administration in the early phase of acute pancreatitis (44). The protocol for a current multi-centre randomised double-blind clinical trial only deals with the question of the potential merit of high-energy enteral tube feed versus zero-energy enteral tube feed (44). Our new results now suggest a need for clinical trials potentially using galactose instead of glucose in enteral tube feeds for patients in the early phase of AP.

Materials and methods

Chemicals and reagents

Fluorescent dyes – Fluo-4-AM, MgGreen-AM and propidium iodide (PI) were purchased from Thermo Fisher Scientific (UK). Collagenase was obtained from Worthington (Lorne, UK), Asparaginase was purchased from Abcam (UK), palmitoleic acid ethyl ester (POAEE) was from Cayman Chemical (UK). All other chemicals were purchased from Sigma (UK). C57BL/6 J mice were obtained from Jackson Laboratory (Charles River, UK).

Antibodies

Primary antibodies: Anti-HK1 (ThermoFisher Scientific) mouse monoclonal antibody (7A7) (MA5-15675) 1/500; Anti-HK2 (Abcam) mouse monoclonal antibody [1E8-H3-F11] ab131196 1/500; Anti-HK4 (GCK) (ThermoFisher Scientific) rabbit polyclonal antibody (PA5-15072) 1/500; Anti-b-actin (Santa Cruz Biotechnology) mouse polyclonal antibody (sc-47778) 1/500. Secondary antibodies: Pierce Goat anti-rabbit IgG, (H+L) peroxidase conjugated antibody (ThermoFisher Scientific) 31460 1/5,000; Goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) sc-2005 1/1000.

Isolation of PACs

Cells were isolated as previously described (18). Briefly, animals were sacrificed according to the Animal Scientific Procedures Act, 1986 and approved by the Ethical Review Committee of Cardiff University. After dissection, the pancreas was digested using collagenase-containing solution (200 IU/ml, Worthington, UK) and incubated in a 37°C water bath for 14–15 min. The extracellular solution contained: 140 mM NaCl, 4.7 mM KCl, 10 mM Hepes, 1 mM MgCl₂, 10 mM glucose, pH 7.3, and CaCl₂ 1 mM and osmolality checked by Osmomat 030. All *in vitro* experiments have been conducted using this solution, unless otherwise stated.

Fluorescence measurements

For measurements of [Ca²⁺]_i, isolated PACs were loaded with Fluo-4-AM (5 μM; excitation 488 nm; emission 510-560 nm) following the manufacturer's instruction. Measurement of intracellular ATP was performed with MgGreen, which senses changes in [Mg²⁺]_i at concentrations around the resting [Mg²⁺]_i (18). PACs were incubated with 4 μM MgGreen AM for 30 min at room temperature (excitation 488 nm; emission 510-560 nm). ATP depletion mixture (4 μM CCCP, 10 μM oligomycin and 2 mM iodoacetate) was applied for a final 10 min of each experiment to induce maximum ATP depletion (21). Asparaginase was used in concentration of 200 IU/ml, POAEE 500 μM (from the stock solution in ethanol, Cayman Chemicals UK), POA 30 μM (from 30 mM stock in ethanol) and Sodium Choleate (BA) 0.06% unless stated otherwise.

Necrotic cell death was assessed with propidium iodide (PI) uptake as previously described (excitation 535 nm; emission 617 nm) (4). The total number of cells showing PI uptake was counted in ≥3 series of experiments for each treated group (>100 cells per each sample) to provide a percentage as the mean ± SEM.

All experiments were performed at room temperature using freshly isolated cells attached to coverslips of the perfusion chamber. Fluorescence was imaged over time using Leica SP5 two-photon, Leica TCS SPE and Zeiss spin-disk confocal microscopes.

***In vivo* models of Asparaginase- and fatty acid ethyl ester-induced AP**

All animal studies were ethically reviewed and conducted according to UK Animals (Scientific Procedures) Act of 1986, approved by the UK Home Office. The animal procedures and experimental protocols were approved by Animal Care and Ethics Committees at Cardiff School of Biosciences, Cardiff University. Before and throughout the experiment unless otherwise denoted, mice were maintained in plastic cages with corn cob bedding; tap water and commercial pelleted diet were freely provided. To establish Asparaginase-induced AP, C57BL6/J mice received four daily (24 hours apart) intraperitoneal (IP) injections of Asparaginase in phosphate-buffered saline (PBS) at 20 IU/g. Control mice only received PBS IP injection. Treatment groups were defined as follows: galactose (180 mg/kg/day) fed (in drinking water 24 hours before the first Asparaginase IP and all the following days during injections) followed by Asparaginase injection (20 IU/g) or galactose (180 mg/kg/day) fed with galactose (180 mg/kg/day) and Asparaginase (20 IU/g) IP (n = 5-8 mice/group). Mice were sacrificed 96 hours after first injection and pancreas was extracted for histology or isolation of PACs. Blood was also collected for amylase and IL6 measurements.

In FAEE-induced AP (FAEE-AP) group, mice received two intraperitoneal injections of ethanol (1.35 g/kg) and POA (150 mg/kg) at 1 h intervals as previously described (37). The treatment group animals were fed with galactose (180 mg/kg/day) as described previously. Animals were sacrificed at 24 hours after the final injection.

Histology

Pancreatic tissue was fixed in 4% formaldehyde, embedded in paraffin, and histological assessment was performed after H&E staining of fixed pancreatic slices (4 μ m thickness). Evaluation was performed on ≥ 10 random fields (magnification: x200) by 2 blinded independent investigators grading (scale, 0–3) edema, inflammatory cell infiltration, and acinar necrosis as previously described (38), calculating the means \pm SEM (n = 3-5 mice/group).

Hexokinase activity

To assay inhibitory effects of POA and BA on activity of hexokinase 1 (HK1), hexokinase 2 (HK2) (Novus Biological) and hexokinase 4 (HK4) (Enzo Life Sciences), NADH generated by glucose-6-phosphate dehydrogenase was detected at 340nm as described in manufacturers' protocols for the Hexokinase assay kit (MAK091, Sigma, UK).

Western Blotting

Equal amounts of proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4-12% SDS Bis Tris gels, ThermoFisher Scientific) and blotted; membranes were probed with primary and then secondary antibodies.

Measurements of mitochondrial membrane potential ($\Delta\Psi_m$)

For measurements of mitochondrial membrane potential ($\Delta\psi_m$) in PACs, we have used the dequench mode as previously described (25). Freshly isolated pancreatic cells were loaded with 20 μ M TMRM for 25 min at room temperature. Cells were then washed and re-suspended in extracellular solution. Fluorescence was excited by a 535 nm argon laser line, and emission was collected above 560 nm. All experiments were conducted by using a Leica TCS SPE confocal microscope with a 63x oil immersion objective. The region of interest for analysing the change of mitochondrial membrane potential was the whole cell.

Measurements of mitochondrial Ca^{2+}

For mitochondrial calcium [Ca^{2+}]_m measurements (45), freshly isolated PACs were loaded with 10 μ M Rhod-2-AM for 48 min at 30°C. After incubation the cells were centrifuged for 1 min and re-suspended in extracellular solution. The fluorescence of Rhod-2 was excited using 535 nm laser line and the emitted light was collected above 560nm.

Enzyme activity and Interleukin 6 measurements.

Serum amylase was determined by spectrophotometer measurements at 405 nm (Jenway, Geneflow, UK) using Amylase Activity Assay Kit (MAK009, Sigma UK) according to manufacturer's instructions.

For visualization of trypsin activity, pancreatic acinar cells were incubated in extracellular solution containing 10 μ M rhodamine 110, bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride (BZiPAR) (Molecular Probes) (4), according to manufacturer's instructions. BZiPAR were excited with a 488-nm laser line; emission was collected at 508-530-nm.

Interleukin 6 (IL6) was determined by enzyme-linked immunosorbent assay (Abcam, UK).

ATP measurements.

Isolated PACs have been incubated during 2 hours with either POA, BA or Asparaginase with appropriate controls. Cellular ATP was determined in the homogenised cell preparation using ATP Essay kit (Sigma UK), according to manufacturer's instructions.

Statistical analysis

Data were presented as mean \pm SEM. Statistical significance and p-values were calculated using Student's 2-tailed *t*-test or ANOVA, with **p* < 0.05 and ***p* < 0.01 considered statistically significant and ****p* < 0.001 was considered as highly significant.

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Footnotes

Abbreviations used:

AP	acute pancreatitis
ALL	acute lymphoblastic leukaemia
AAP	L-Asparaginase-induced acute pancreatitis
ASNase	L-Asparaginase
CRAC	calcium release activated calcium channels
PAR2	protease activated receptor
PAC	pancreatic acinar cell
PMCA	plasma membrane Ca^{2+} ATPase
SERCA	sarco-endoplasmic reticulum Ca^{2+} ATPase
POA	palmitoleic acid
POAEE	palmitoleic acid ethyl ester
FAEE	fatty acid ethyl ester
2-DG	2-Deoxy-D-glucose
PI	propidium iodide

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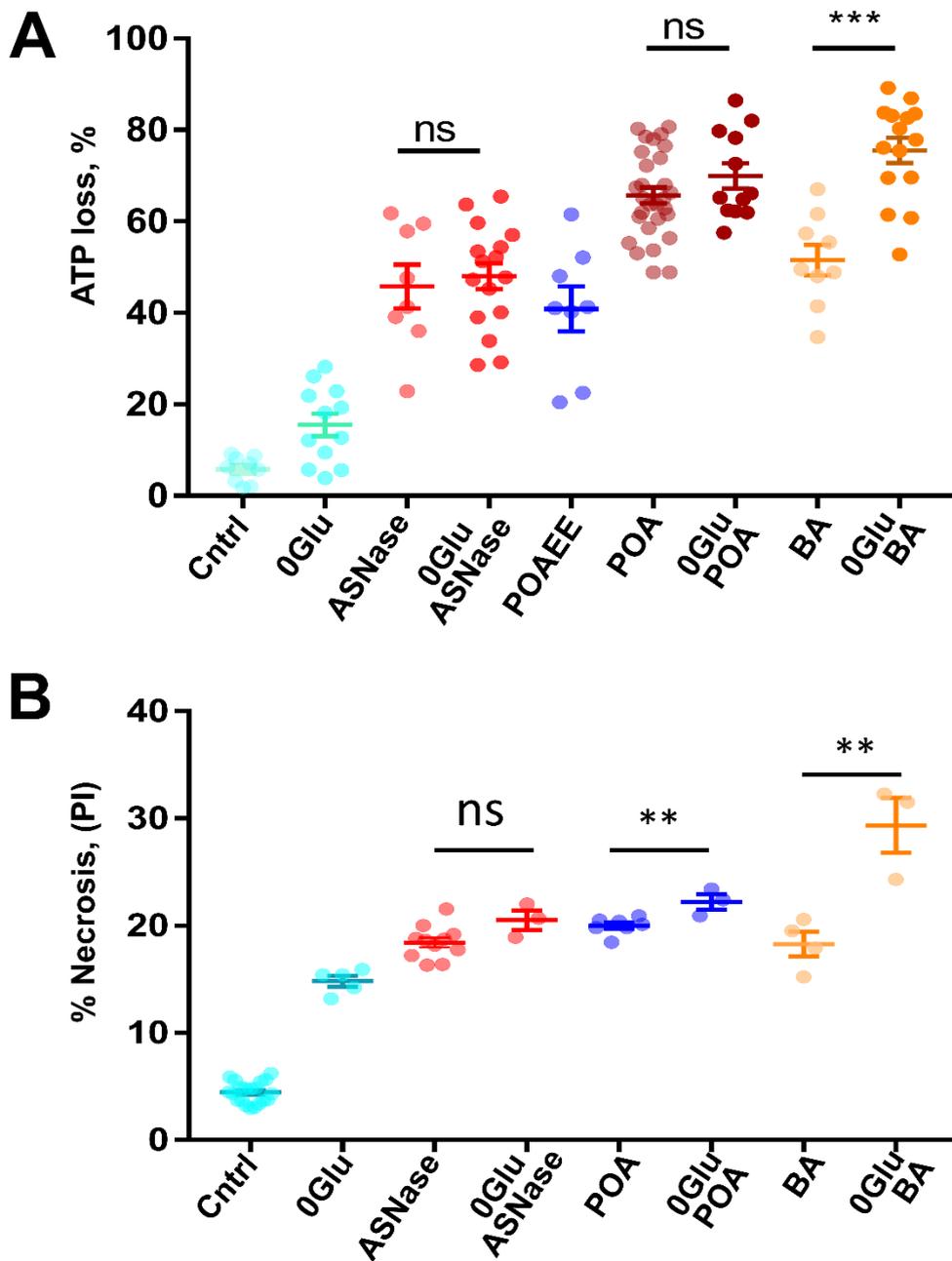


Figure. 1. Asparaginase (ASNase), non-oxidative alcohol metabolites (POAEE and POA) and bile acids (BA) all induce substantial ATP loss and cell necrosis.

- A. Comparison of cellular ATP depletion in PACs after treatments of cells for 30 min with or without glucose (0 G), or application of ASNase, POAEE, POA or BA. Level of ATP loss (measured by MgGreen) is shown as a percentage of full depletion by a mixture of CCCP, oligomycin and iodoacetate. Dots represent ATP loss (%) in each cell. Bars present mean \pm SEM.
- B. Summary of cell necrosis measurements in PACs treated with ASNase, POA or BA for 2 hours in the presence or absence (0G) of 10 mM glucose as compared with control. Removal of glucose had little effect on ASNase and POA but increased BA-induced necrosis. Cells were stained with propidium iodide (PI). Dots represent series of experiments with $n > 100$ cells in each sample. Bars present mean \pm SEM.

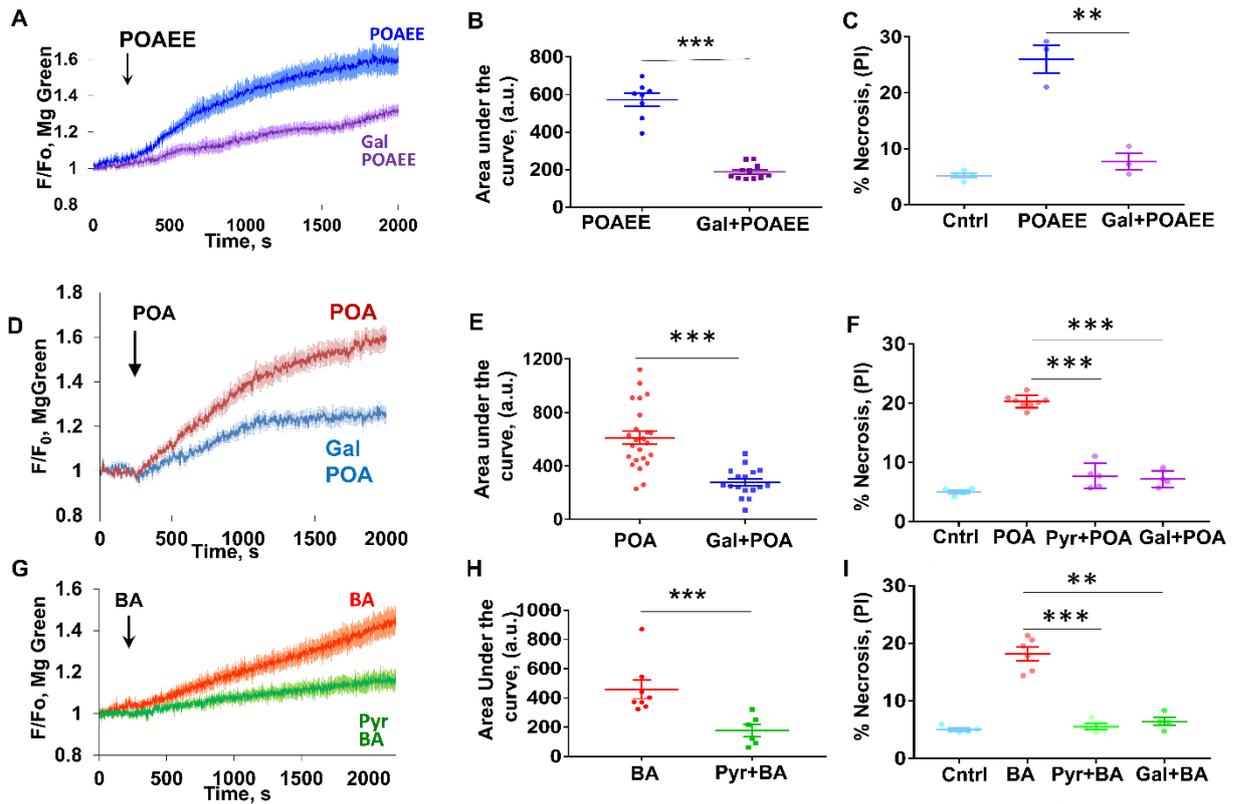


Figure 2. Pyruvate and galactose provide substantial protection against alcohol- and bile- induced ATP loss and necrosis in PACs.

- A.** POAEE (500 μ M) – induced ATP depletion is markedly reduced by adding 1 mM galactose. Averaged traces with error bars (POAEE, n=8; Galactose + POAEE, n=11).
- B.** ‘Area under the curve’ comparison of traces shown in **A**. Galactose reduced POAEE-induced ATP depletion ($p < 0.0001$).
- C.** POAEE-induced necrosis was significantly reduced by adding 1 mM galactose ($p < 0.003$; 3 series of experiments with more than 100 cells in each sample).
- D.** POA (50 μ M) – induced ATP depletion is reduced by replacing glucose with 10 mM galactose. Averaged traces with error bars (POA, n=24; Galactose + POA, n=17).
- E.** ‘Area under the curve’ comparison of traces shown in **D**. Galactose significantly reduced POA-induced ATP depletion ($p < 0.0001$).
- F.** POA-induced necrosis was reduced significantly by replacing glucose with either 10 mM pyruvate or 10 mM galactose ($p < 0.0001$ in both series; dots represents a series of experiments with >100 cells in each sample).
- G.** Bile acid mixture (BA)-induced ATP depletion is reduced by adding 1mM pyruvate. Averaged traces with error bars (BA, n=8, BA+ pyruvate n=6).
- H.** Area under the curve comparison of traces shown in **G**. Pyruvate significantly reduces BA-induced ATP depletion ($p < 0.002$).
- I.** BA-induced necrosis is reduced to nearly control level by replacing glucose with 10 mM pyruvate ($p < 0.00015$; 5 series of experiments with more than 100 cells in each sample) or 10 mM galactose ($p < 0.008$; 4 series of experiments with more than 100 cells in each sample).

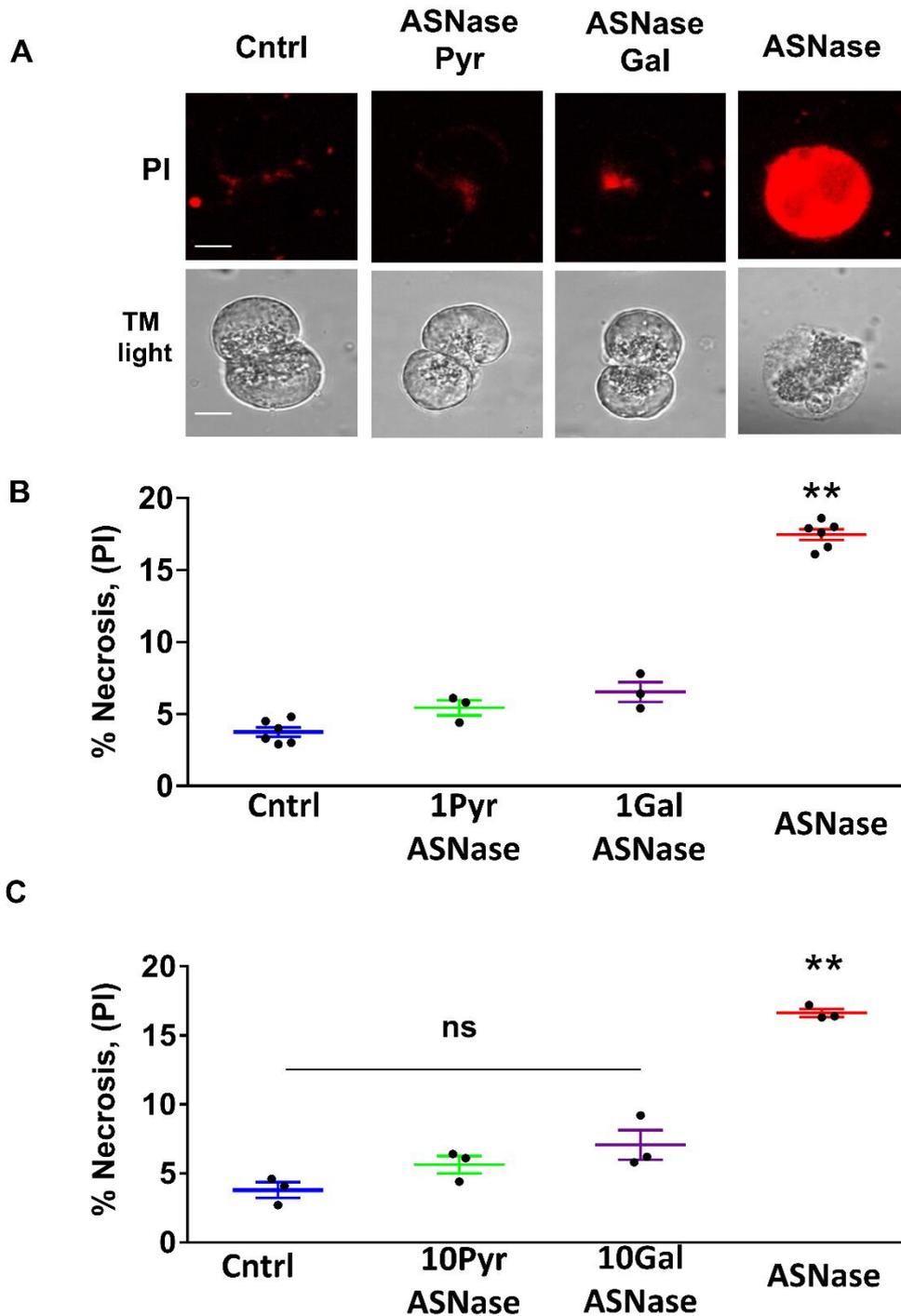


Figure 3. Pyruvate and galactose significantly reduce the level of Asparaginase-induced necrosis.

A. Representative images of cells from experiments shown in B and C.

B. In PACs, 1 mM pyruvate ($5.4 \pm 0.5\%$, three series with $n > 300$, $p < 0.0002$) or 1 mM galactose ($6.5 \pm 0.7\%$, three series with $n > 300$, $p < 0.0004$) reduce the ASNase -induced necrosis level as compared to ASNase alone ($17.5 \pm 0.4\%$, six series with $n > 300$).

C. Complete replacement of extracellular glucose (10 mM) with pyruvate (10 mM) ($5.6 \pm 0.6\%$, three series with $n > 300$, $p < 0.0001$) or 10 mM galactose ($7.1 \pm 1.1\%$, three series with $n > 300$, $p < 0.001$) reduces significantly the ASNase-induced necrosis level as compared to the control level ($3.8 \pm 0.6\%$, three series with $n > 300$).

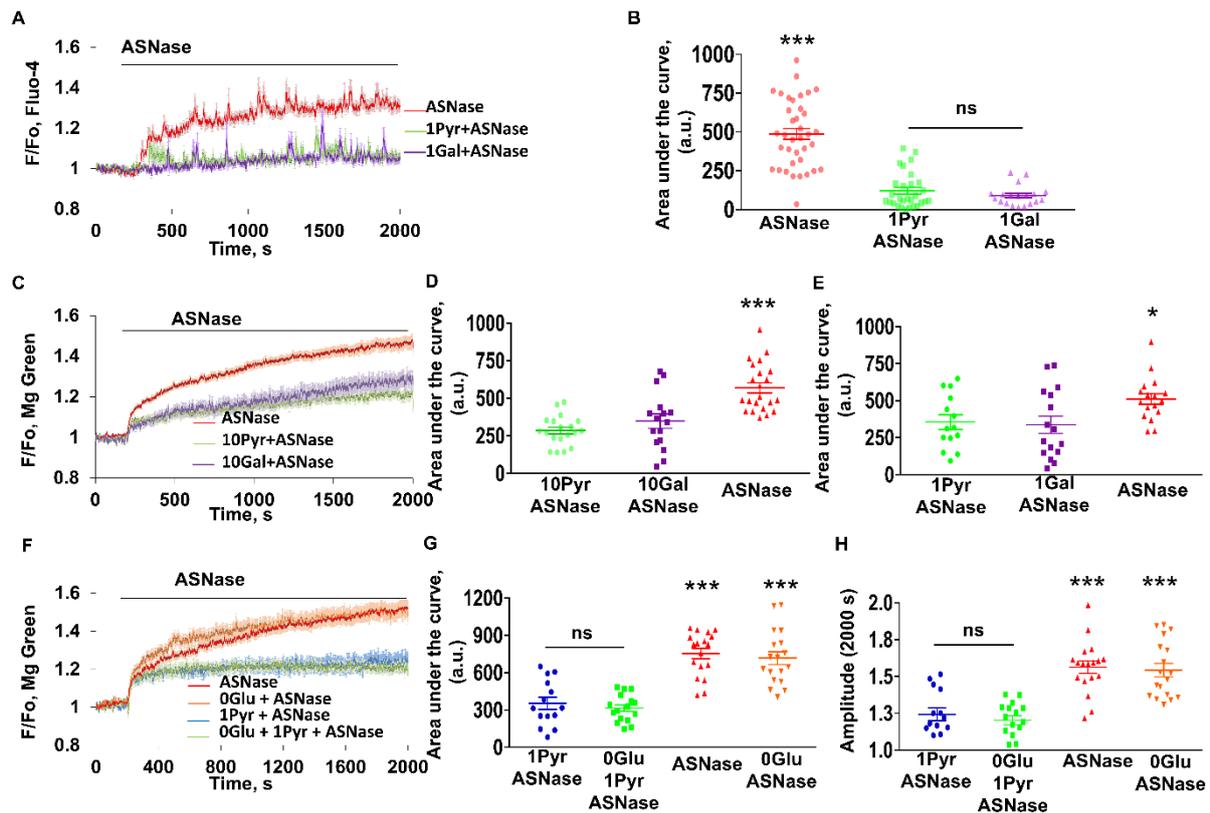


Figure 4. Asparaginase-induced Ca^{2+} overload and ATP loss were substantially reduced by galactose or pyruvate, whereas glucose removal did not affect the ATP loss.

A. ASNase elicits an elevated $[\text{Ca}^{2+}]_i$ plateau with repetitive Ca^{2+} transients on top, averaged traces with error bars shown (red, $n = 35$). Green trace shows reduced response to ASNase in the presence of 1 mM pyruvate (after 5 min of preincubation, $n = 33$). Purple trace shows reduced response to ASNase in the presence of 1 mM galactose (after 15min of preincubation, $n=17$).

B. Comparison of the 'areas under the curve' shown in A during first 30min of the $[\text{Ca}^{2+}]_i$ change in response to ASNase in the presence of pyruvate (green) and galactose (purple) or asparaginase alone (red) ($p < 0.0001$). Both pyruvate and galactose reduced significantly the $[\text{Ca}^{2+}]_i$ rise induced by ASNase.

C. ATP loss was evaluated using MgGreen. Average traces show that complete replacement of extracellular glucose (10 mM) with pyruvate (10 mM) (green, $n = 19$) or galactose (10 mM) (purple, $n = 16$) for 30 min markedly reduces $[\text{Mg}^{2+}]_i$ change induced by ASNase (red, $n = 38$).

D. Quantitative analysis of experiments of the type shown in C (area under the curve during the 30 min of ASNase application). Pyruvate (green) and galactose (purple) markedly reduced the ATP loss evoked by ASNase (red, $p < 0.0005$).

E. Quantitative analysis of experiments like in C but with 1mM of either pyruvate (green, $n=14$) or galactose (purple, $n=16$) added before the application of ASNase. Bars show the 'area under the curve' recorded during 30 min of ASNase application. Both treatments reduced significantly the ATP loss ('area under the curve') ($p < 0.015$).

F. ASNase induces substantial ATP loss in the absence of pyruvate irrespective of whether extracellular glucose was present (red, $n = 21$) or absent (orange, $n = 17$). In contrast, adding pyruvate (1 mM) dramatically decreased ATP depletion induced by ASNase irrespective of the absence (green trace, $n = 16$) or presence of 10 mM glucose (blue trace, $n = 14$).

G. Quantitative analysis of experiments shown in F by comparing 'areas under the curve' during 30 min of ASNase application. Pyruvate (blue and green) was highly protective against ATP depletion induced by ASNase, ($p < 0.0001$) regardless of presence or absence of glucose ($p > 0.05$).

H. Comparison of amplitudes of traces at 2000 sec shown in F. Pyruvate (blue and green) is highly effective against cellular ATP depletion induced by ASNase, $p < 0.0001$ regardless of presence (red) or absence of glucose (orange).

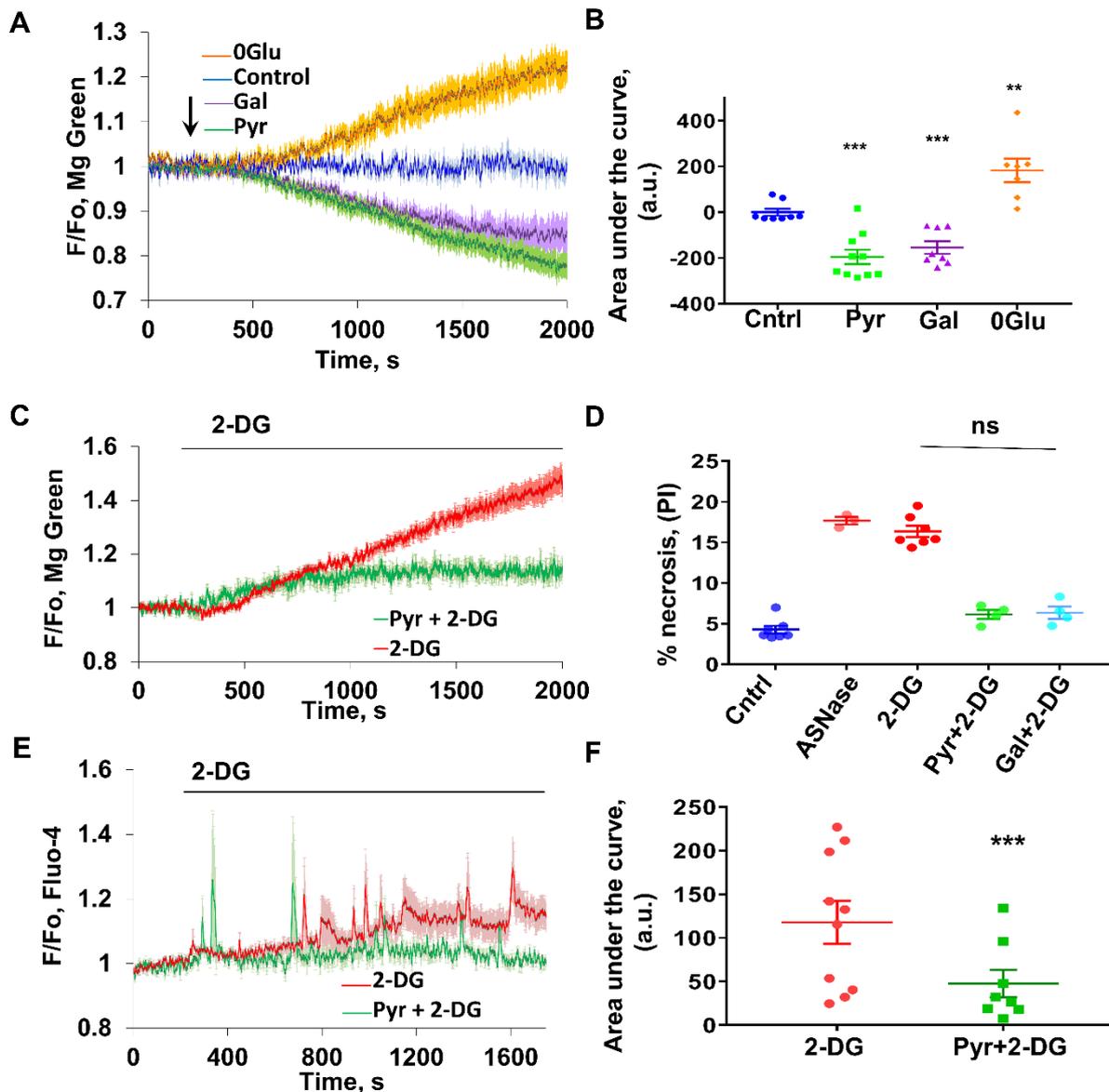


Figure 5. Pyruvate and galactose increase intracellular ATP levels and protect cells against ATP depletion induced by 2-deoxyglucose (2-DG).

- Glucose removal induces substantial ATP depletion, whereas pyruvate or galactose boosts ATP production. Average traces show normalized changes of MgGreen fluorescence in PACs in the presence (blue trace, $n = 8$) or absence (orange trace, $n = 7$) of 10 mM glucose or in the presence of pyruvate (1 mM; green trace, $n = 10$) or galactose (1 mM; purple trace, $n = 8$).
- Comparison of 'areas under the curve' for experiments shown in A.
- Pyruvate markedly reduces ATP depletion induced by 10 mM 2-Deoxy-D-glucose (2-DG). Averaged trace (shown with error bars) represents the result of application of 2-DG in the absence (red trace, $n = 9$) or presence of 1 mM pyruvate (after 5 min pre-incubation, green trace, $n=7$).
- Comparison of necrotic cell death levels induced by 2 hours incubation of PACs with 10 mM 2-DG or ASNase with control (non-treated cells) (PI stained cells, $p = 0.36$, three series of experiments with $n > 100$ cells in each sample).
- Average traces show normalized changes of Fluo-4 fluorescence in PACs induced by 10 mM 2-DG

alone (red trace, n=10) or after 5 min pre-incubation of cells and continuous presence of 1mM pyruvate (green trace, n = 8) for 25 min.

F. Quantitative analysis of experiments of the type shown in E by comparing 'areas under the curve' for 25 min of the recording after application of 10 mM 2-DG ($p < 0.001$).

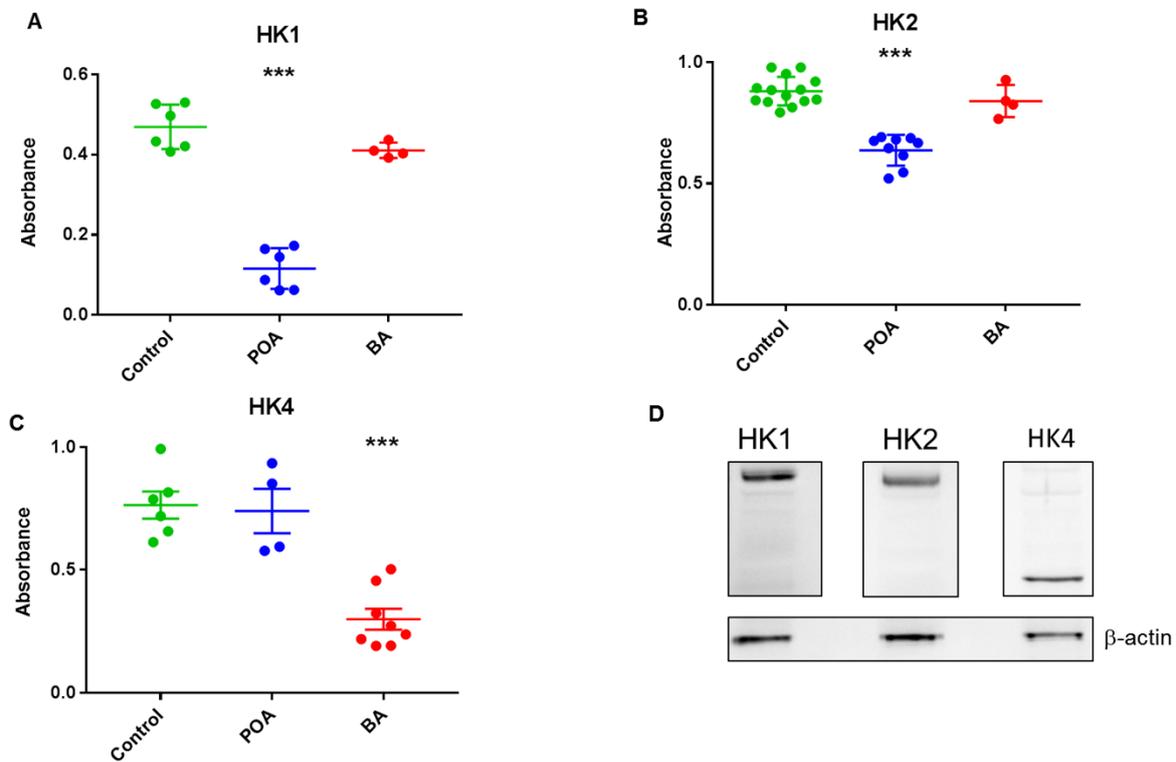


Figure 6. Hexokinase activity is significantly inhibited *in vitro* by POA and BA.

- Hexokinase 1 activity (HK1) is reduced significantly by 0.1mM POA (n=6, $P < 0.0001$,) but not changed significantly by 0.05% BA (n=4, $p > 0.13$) as compared with control (n=6).
- Hexokinase 2 activity (HK2) is reduced significantly by 0.1mM POA (n=9, $p < 0.0001$,) but not affected by 0.05% BA (n=4, $p > 0.3$) as compared to control (n=13).
- Hexokinase 4 activity (HK4) is reduced significantly by 0.05% BA (n=8, $p < 0.0001$,) but not affected by 0.1mM POA (n=4, $p > 0.8$) as compared to control (n=6).
- Western blot analysis of the expression levels of HK1, HK2 and HK4 in PACs (representative case, repeated 3 times with similar results).

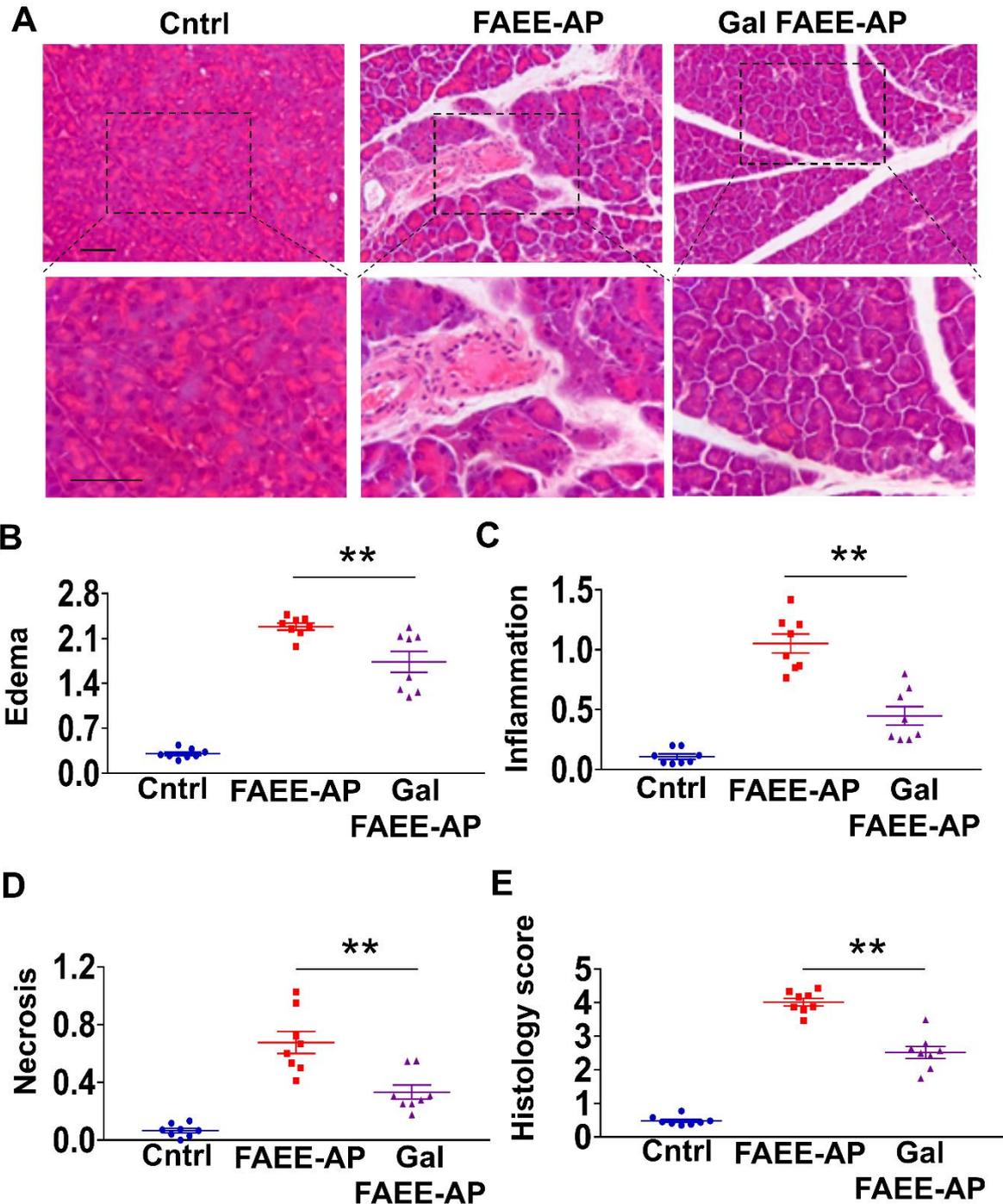


Figure 7. Galactose protects against alcohol-induced acute pancreatitis *in vivo*.

A. Galactose improved significantly the pathological scores in FAEE-AP. Representative H&E images of pancreas histology slides showing normal pancreatic histology (saline injection), typical histopathology from FAEE-AP without or with galactose (Gal) feeding (10 mM). Lower row of images shows zoomed parts of the images above (H&E; scale bar: 50 μ m).

B-E. Overall histopathological score (E) and components: edema (B), inflammation (C) and necrosis (D). All detrimental changes induced by POA and ethanol were significantly ameliorated by galactose ($p < 0.007$). Values are means \pm SE of 3-5 mice per group).

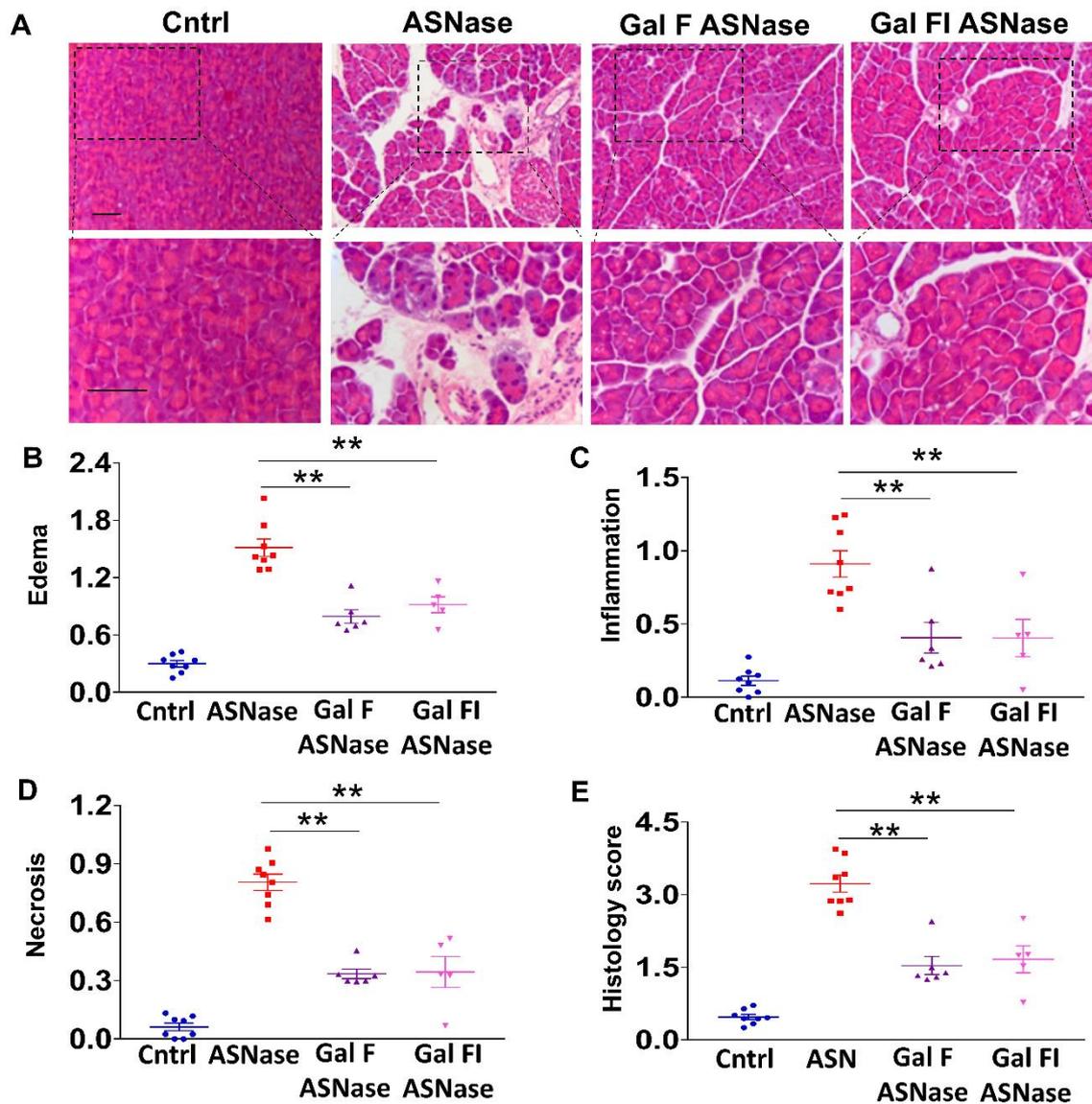


Figure 8. Asparaginase-induced acute pancreatitis is substantially reduced by galactose *in vivo*.

A. Representative H&E images of pancreas from slides showing normal pancreatic histology (saline), typical histopathology from AAP model (ASNase 20 IU/g), and typical histopathology from treatment groups: galactose feeding (Gal F) and combination of galactose feeding and galactose injection (Gal FI). Lower row of images shows zoomed parts of the images above (H&E; scale bar: 50 μ m).

B-E. Edema (B), inflammation (C), necrosis (D) and overall histopathological score (E) in ASNase-induced AP and the effects of the two different galactose treatment protocols (180mg/kg/day of galactose in each protocol). All detrimental changes induced by ASNase were significantly ameliorated by galactose ($p < 0.004$, values are means \pm SE of 3-5 mice per group).