CRUCIAL ROLE OF PANCREATIC DUCTS IN THE INITIATION AND PROGRESSION OF PANCREATITIS

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Ph.D. Thesis

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Number of full publications:	16 (5 first author)
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ABBREVIATIONS

$(ATP)_i$	intracellular ATP level
BAPTA-AM	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BCECF-AM	2'7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester
$[Ca^{2+}]_i$	intracellular Ca2+ concentration
cAMP	cyclic AMP
CBE	Cl^-/HCO_3^- exchanger
CFTR	cystic fibrosis transmembrane conductance regulator Cl^- channel
CFTRinh-172	CFTR inhibitor-172
FA	fatty acid
FAEE	fatty acid ethyl ester
FURA-2-AM	5-Oxazolecarboxylic acid, 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acetoxymethyl ester
H2DIDS	dihydro-4,4'-diisothiocyanostilbene-2,2'-disulfonic acid
J(*B*-)	transmembrane base flux
KO	knockout
NBCe1-B	Na^+/HCO_3^- contransporter
NHE	Na^+/H^+ exchanger
NBD	nucleotide binding domain
PDEC	pancreatic ductal epithelial cells
PA	palmitic acid
POA	palmitoleic acid
POAEE	palmitoleic acid ethyl ester
pH_i	intracellular pH
pH_L	intraluminal pH
PKA	protein kinase A
RT-PCR	real-time reverse transcription polymerase chain reaction
SLC26	solute carrier family 26
WT	wild-type

1. INTRODUCTION

The exocrine pancreas consists of two different cell types. The pancreatic acinar cells produce and secrete the digestive enzymes, whereas the pancreatic ductal epithelial cells (PDEC) secrete high quantity of HCO_3^--rich low viscosity fluid. The alkaline pancreatic fluid secretion, in response to meal washes the digestive enzymes out of the pancreatic ductal tree and neutralises the acidic chyme entering the duodenum. The function of the pancreatic ductal fluid and HCO_3^- secretion used to be underestimated; however recent findings suggest that it plays a central role in the physiology and pathophysiology of the pancreas. Importantly, HCO_3^- neutralises protons secreted by the acinar cells and keeps trypsinogen and most probably other proteases in an inactive form We also have to highlight that one of the most common pathogenic factors for acute pancreatitis (bile acids) impair ductal HCO_3^- secretion which likely contributes in a major manner to the pancreatic damage. However the exact mechanism of the inhibitory effects of bile acids has not been revealed yet, moreover we have no information about the effects of the other most common pathogenetic factor (ethanol) on the pancreatic ductal secretion.

1.1. The physiology of the pancreatic ductal HCO_3^- secretion

Pancreatic ductal HCO_3^- secretion can be divided to two separate steps, first the accumulation of the HCO_3^- ions in the cells via the basolateral membrane and second the secretion into the ductal lumen across the apical membrane. The basolateral accumulation is carried out by a Na^+/HCO_3^- cotransporter (NBCe1-B), which transports 1 Na^+ and 2 HCO_3^- into the cells, driven by the high intracellular Na^+ gradient. Another possible mechanism for the HCO_3^- accumulation is the passive diffusion of CO_2 trough the cell membrane, followed by the carbonic anydrase mediated conversion of CO_2 to HCO_3^-. The electroneutral Na^+/H^+ exchanger (NHE1) might also contribute to the HCO_3^- accumulation, although its role differs among species, it is essential for intracellular pH (pH_i) homeostasis. On the luminal membrane PDEC express electrogenic Cl^-/HCO_3^- exchangers (SLC26A6, which operates with a 1 Cl^- : 2 HCO_3^- stoichiometry and possibly SLC26A3, which transports 2 Cl^- : 1 HCO_3^-) and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. The electrogenic Cl^-/HCO_3^- exchange allows the pancreatic ductal cells to transport HCO_3^- into the ductal lumen and establish the very high (140mM) maximal intraluminal HCO_3^- concentration during stimulated secretion, resulting in an intraluminal HCO_3^- level which is ~5-6 fold higher compared to the cell interior. It is important to note that CFTR mutations, which are associated with exocrine pancreatic insufficiency, also establish a major deficiency in the apical CFTR-dependent Cl^-/HCO_3^- exchange activity.

Pancreatic ductal HCO_3^- secretion is a strongly ATP dependent processes. CFTR, also called ABCC7, a member of the ATP-binding cassette transporter superfamily, has two nucleotide binding domain (NBD1 and NBD2). During the activation of CFTR, protein kinase A (PKA) uses ATP to phosphorylate and activate the R domain of CFTR. This phosphorylation step is followed by the

binding of two Mg-ATP molecules on the inter-NBD interface of the NBD domains, leading to the channel gating. PKA-dependent phosphorylation of the CFTR R domain is also required for the interaction of the R domain with the STAS domain of the SLC26 Cl^-/HCO_3^- exchangers, which increases the overall open probability and therefore the activity of CFTR. Moreover, evidence suggests that NHE1 acts as an ATP-binding transporter; thus, ATP may directly activate NHE1, however its activity does not require ATP hydrolysis.

1.2. Pathophysiological role of pancreatic HCO_3^- secretion

The physiological role of pancreatic ductal HCO_3^- secretion has been investigated in details, however recent evidences suggest that this process is playing a curtail role in the pathophysiology of the pancreas as well. Earlier Freedman et al. showed that impaired ductal electrolyte and fluid secretion in CFTR knockout mice leads to acinar cell damage and to primary defect in membrane trafficking at the apical plasma membrane of acinar cells. In an elegant study Ooi et al. demonstrated that the risk of developing pancreatitis was much higher in CF patients, who had milder CFTR mutations (type IV and V) and were pancreatic sufficient compared to those who had severe mutations and were pancreatic insufficient. The importance of the intraluminal pH was further confirmed by showing that protons co-realsed during exocytosis cause significant acidosis in the lumen of the acini. Physiological stimulation of exocytosis causes a decrease in the extracellular pH of up to 1 pH unit. Pathophysiological stimuli using supramaximal concentration of cerulein evokes more enhanced and prolonged acidification of the lumen. Bhoomagoud et al. showed that that an acute acid load given in vivo enhanced cerulein-induced trypsinogen activation and pancreatic oedema. These findings also suggest that low pH environments might play an important role in the pathogenesis of acute pancreatitis.

1.3. The effects of etiological factors of pancreatitis on the exocrine pancreas

1.3.1. Ethanol

One of the most common causes of acute pancreatitis is excessive ethanol consumption, although the pathogenesis of alcohol-induced acute pancreatitis remained elusive. Ethanol alone had no detectable effect on pancreatic acinar cells, however non-oxidative ethanol metabolites (fatty acid ethyl esters; FAEE and fatty acids; FA) induced a sustained $[Ca^{2+}]_i$ elevation leading to necrosis and depolarized the mitochondria, which was abolished by BAPTA-AM preincubation. Importantly, ATP supplementation via a patch pipette prevented the formation of sustained $[Ca^{2+}]_c$ elevation during the administration of palmitoleic acid (POA). There are less information available about the effects of ethanol, or ethanol metabolites on pancreatic ductal cells. Earlier Yamamoto et al. showed that a low concentration (1mM) of ethanol induced a $[Ca^{2+}]_i$ elevation and augmented secretin-stimulated fluid secretion in guinea pig pancreatic ducts. They also observed a weak inhibition of the stimulated fluid secretion during the administration of 100mM ethanol. The stimulatory effect of 1mM ethanol was

abolished by BAPTA-AM preincubation, suggesting that it was mediated by the $[Ca^{2+}]_i$ elevation. Besides these results we have no further information regarding the effects of ethanol, or ethanol metabolites on the pancreatic ductal HCO_3^- secretion.

1.3.2. Bile acids

Similarly to non-oxidative ethanol metabolites, bile acids induced Ca^{2+} release from both the ER and acidic intracellular Ca^{2+} stores through activation of IP_3R and ryanodine receptors in isolated pancreatic acinar cells. Moreover, Voronina *et al*. showed that taurolithocholicacid 3-sulfate (TLC-S) decreased $(ATP)_i$ in pancreatic acinar cells and caused the loss of $(\Delta\Psi)_m$, which was not influenced by BAPTA-AM pretreatment. Using isolated guinea pig pancreatic ducts Venglovecz et al. demonstrated that the non-conjugated bile acid chenodeoxycholate (CDC) a has dose-dependent dual effects on pancreatic HCO_3^- secretion, which might be explained by the type of Ca^{2+} signals evoked by CDC. Low concentrations (100µM) of CDC induced repetitive, short-lasting Ca^{2+} oscillations, which stimulated HCO_3^- secretion from the luminal membrane of PDEC. The oscillations were abolished by the IP_3R inhibitor caffeine, or xestospongin C and the phospholipase C (PLC) inhibitor U73122. Preincubation of the PDEC with the intracellular Ca^{2+} chelator BAPTA-AM prevented the Ca^{2+} signals and also abolished the stimulatory effect of 100µM CDC on HCO_3^- secretion. In contrast, high concentrations (1mM) of CDC induced a toxic sustained Ca^{2+} elevation, which inhibited the acid/base transporters including the basolateral NHE, NBCe1-B and the luminal CBE. Notably, BAPTA-AM preincubation failed to prevent the inhibitory effect of CDC on the HCO_3^- secretion, suggesting a Ca^{2+}-independent cellular toxicity which has not been clarified yet.

2. AIMS OF THE STUDY

I. Excessive ethanol consumption is one of the most common cause of acute pancreatitis, but it is not know in details how ethanol, or ethanol metabolites influence the pancreatic ductal secretion. Therefore the aim of this study was to characterize the effects of ethanol and ethanol metabolites on the pancreatic ductal epithelial cells.

II. Earlier we showed that the non-conjugated bile acids can inhibit the pancreatic ductal HCO_3^- secretion in high concentration, but the mechanism of inhibition remained elusive, therefore we aimed to assess the inhibitory mechanisms of bile acids.

3. MATERIALS AND METHODS

3.1. Culturing of Capan-1 pancreatic ductal adenocarcinoma cell line

Capan-1 cells were used for experiments between 20-60 passages. Cells were cultured according to the distributors' instruction. For the intracellular pH (pH_i) measurements cells were seeded onto polyester permeable supports. For intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) or intracellular ATP level ($(ATP)_i$) measurements cells were seeded onto 24mm-diameter cover glasses and for mitochondrial membrane potential ($(\Delta\Psi)_m$) cells were seeded onto glass bottom dishes.

3.2. Isolation and culture of guinea pig pancreatic ducts

4-8 week-old guinea pigs were sacrificed by cervical dislocation and intra/interlobular ducts were isolated by enzymatic digestion and microdissection from the pancreas and cultured overnight as previously described. Single pancreatic ductal cells were isolated as described previously.

3.3. Maintenance of CFTR knockout mice

CFTR knockout mice were originally generated by Ratcliff et al. The mice were congenic on the FVB/N background. The animals were kept at a constant room temperature of 24°C with a 12 h light–dark cycle. The mice received electrolyte drinking solution containing polyethylene glycol (PEG) and high HCO_3^- and a fibre-free diet. The mice used in this study were 6-8 weeks old and weighted 20-25 grams, the gender ratio was 1:1 for all groups.

3.4. *In vitro* measurement of pH_i, $[Ca^{2+}]_i$, $(ATP)_i$ and $(\Delta\Psi)_m$

Isolated guinea pig pancreatic ducts, or Capan-1 cells were incubated in standard HEPES solution and loaded with BCECF-AM (1.5μmol/L), Fura2-AM (2.5μmol/L), MgGreen-AM (5μmol/L), or TMRM (100nmol/L) respectively for 30 min at 37°C. The HCO_3^- efflux across the luminal membrane was determined by NH_4Cl pulse and luminal Cl^- withdrawal as described previously. For $(\Delta\Psi)_m$ measurements glass bottom petri dishes were perfused continuously with solutions containing 100nmol/L TMRM at 37°C at a rate of 2-2.5ml/min.

3.5. *In vitro* measurement of pancreatic fluid secretion

Fluid secretion into the closed luminal space of the cultured guinea pig pancreatic ducts was analysed using a swelling method. The ducts were transferred to a perfusion chamber. Bright-field images were acquired at 1 min intervals using a CCD camera. Digital images of the ducts were analysed using Scion Image software to obtain values for the area corresponding to the luminal space in each image.

3.6. Magnetic resonance imaging of the exocrine pancreatic fluid secretion

Magnetic resonance imaging (MRI) was performed to measure the pancreatic exocrine function as described previously before and 24 hours after intraperitonal injection with the mixture of

1.75 g/kg ethanol and 750 mg/kg palmitic acid. Strong T2-weighted series of the complete abdomen were acquired before and after retroorbital injection of secretin. The volume of intestinal fluid was assessed before and after secretin stimulation and total extracted volume (TEV) was calculated.

3.7. Electrophysiology

Single PDEC and Capan-1 cells were prepared as described above. Few drops of cell suspension were placed into a perfusion chamber. Membrane currents were recorded with an Axopatch1D amplifier using whole cell at 37°C. Current-voltage (I/V) relationships were obtained by holding Vm at 0mV and clamping to ±100mV in 20mV increments. Analyses were performed by using pClamp6 software after low-pass filtering at 1kHz.

3.8. Quantitative real-time reverse transcription polymerase chain reaction (qPCR)

Total RNA was purified from individual cell culture samples using the RNA isolation kit. All the preparation steps were carried out according the manufacturer's instructions. Gene expression was performed using the TaqMan probe sets of CFTR gene. The final relative gene expression ratios were calculated as ΔCt values.

3.9. Immunofluorescence

Cultured cells. For CFTR immunostaining Capan-1 cells were fixed in 4% paraformaldehyde (PFA). For CFTR detection cells were incubated with anti-NBD2 monoclonal primary CFTR antibody obtained from CF Foundation.

Guinea pig pancreatic tissue. Guinea pigs were treated with a mixture of 0.8g/kg ethanol and 300mg/kg palmitic acid *i.p.*. The animals were sacrificed 3, 6, 12 and 24 h after the injection. Primary antibody „Mr Pink" (rabbit polyclonal antibody against human CFTR) was applied.

3.10. Statistical Analysis

All data are expressed as means±SEM. Significant differences between groups were determined by analysis of variance.

3.11. Ethical Approvals

All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Academies Press, Eight Edition, 2011), and were approved by Committees on investigations involving animals at the University of Szeged and also by independent committees assembled by local authorities.

4. RESULTS

4.1. Low concentration of ethanol stimulates, whereas high concentration of ethanol and fatty acids inhibit the HCO_3^- secretion in pancreatic ductal epithelial cells

To investigate the effects of ethanol and ethanol metabolites on the pancreatic ductal epithelial HCO_3^- secretion we used Capan-1 human polarized pancreatic cell line. During the experiments the apical and the basolateral side of the Capan-1 PDEC were perfused separately, which allowed us to selectively change the composition of the apical or basolateral solutions. Using two independent method (apical Cl^- removal and NH_4Cl pulse technique) we showed that low concentration of ethanol (10mM) stimulated, high concentration of ethanol (100mM), or POA (100, 200µM) significantly decreased apical Cl^-/HCO_3^- exchange activity.

4.2. High concentration of ethanol and fatty acids inhibit the CFTR Cl^- current in pancreatic ductal epithelial cells

Next we directly detected the effects of ethanol and ethanol metabolites on the CFTR Cl^- current. Exposure of Capan-1 cells to 10µM forskolin increased basal whole cell currents, which was stimulated by 10mM ethanol the and inhibited by 100mM ethanol or 200µM POA. In both cases, the inhibition was voltage-independent and irreversible.

4.3. High concentration of ethanol and fatty acids inhibit the HCO_3^- secretion and the CFTR Cl^- current in guinea pig pancreatic ductal epithelial cells

To confirm our observations we used isolated guinea pig pancreatic ducts, since the guinea pig pancreas secretes a juice containing ~140mM $NaHCO_3$ as does the human gland. Using isolated ducts we showed that administration of 100mM ethanol, or 200µM POA for 30 min markedly reduced the pancreatic HCO_3^- secretion confirming our observations on Capan-1 cells. We detected the effects of ethanol and ethanol metabolites on the CFTR Cl^- current in primary epithelial cells as well. Exposure of isolated guinea pig PDEC to 100mM ethanol or 200µM POA significantly decreased the forskolin-stimulated CFTR currents.

4.4. Ethanol and fatty acids inhibit the pancreatic ductal ductal fluid secretion

To detect the pancreatic ductal fluid secretion *in vitro* we used isolated guinea pig pancreatic ducts. Administration of 100mM ethanol, or the non-oxidative ethanol metabolite POA (200µM) for 30 min markedly reduced the pancreatic fluid secretion. To assess the effects of ethanol and ethanol metabolites to the *in vivo* exocrine pancreatic secretion, we used MRI cholangiopancreatography to measure the total excreted volume (TEV) in anesthetised mice. We compared the TEV of wild type (WT) animals to CFTR knockout mice, which was significantly lower. We reassessed the secretion 24

h after the *i.p.* injection of 1.75g/kg ethanol and 750mg/kg palmitic acid (PA), which markedly impaired TEV in WT and almost completely abolished in CFTR KO mice.

4.5. Low concentration of ethanol stimulates both the apical Cl^-/HCO_3^- exchanger and CFTR via intracellular Ca^{2+} signalling in PDEC

We showed, that administration of 10μM CFTR Cl^- channel inhibitor CFTR(inh)-172 or 500μM SLC26A6 inhibitor H_2DIDS for 15 min could not prevent the stimulatory effect of 10mM ethanol alone, however, administration of both inhibitors at the same time totally prevented the stimulatory effect of low concentration of ethanol. We went further and found that administration of 10mM ethanol induced short lasting, repetitive Ca^{2+} spikes in 43% of the Capan-1 cells. Administration of the inositol-triphosphate receptor (IP_3R) antagonist caffeine, or the phospholipase C (PLC) inhibitor U73122 completely abolished the Ca^{2+} response and stimulatory effect of 10mM ethanol.

4.6. High concentration of ethanol and fatty acids inhibit both the apical Cl^-/HCO_3^- exchanger and CFTR in PDEC

The above mentioned two inhibitors (CFTR(inh)-172; H_2DIDS) were used to evaluate the involvement of CFTR Cl^- channel and SLC26A6 in the inhibitory mechanisms of ethanol and POA as well. Both methods showed that pre-treatment of the cells for 15 min with either 10μM CFTR(inh)-172 or 500μM H_2DIDS strongly decreased the inhibitory effects of ethanol and POA suggesting that both transport mechanisms are inhibited.

4.7. High concentration of ethanol and fatty acids induce sustained Ca^{2+} release in PDEC

High concentration of ethanol (100mM) induced moderate, but sustained $[Ca^{2+}]_i$ increase in PDEC, whereas POAEE had no effect. POA in low concentration (50μM) evoked small $[Ca^{2+}]_i$ elevation, but in high concentrations (100 and 200μM) induced sustained $[Ca^{2+}]_i$ rise, which was inhibited by the ryanodin receptor (RyR) inhibitor Ruthenium Red, whereas, by the IP_3R inhibitor caffeine and the PLC inhibitor U73122. Co-administration of the inhibitors almost totally blocked the initial $[Ca^{2+}]_i$ increase, confirming that the initial $[Ca^{2+}]_i$ elevation is due to release from the ER via the activation of IP_3R and RyR.

4.8. High concentration of ethanol and fatty acids induce $(ATP)_i$ depletion and decrease mitochondrial membrane potential in PDEC

Measurement of $(ATP)_i$ using MgGreen-AM fluorescent Mg^{2+} indictator revealed 100mM ethanol and 100 or 200μM POA markedly and irreversibly decreased $(ATP)_i$. To further characterize the effects of ethanol and ethanol metabolites on the mitochondrial function, we showed that 100mM ethanol and 100-200μM POA markedly and irreversibly irreversible decreased $(\Delta\Psi)_m$.

4.9. The inhibitory effects of ethanol and fatty acids on HCO_3^- secretion are mediated by sustained $[Ca^{2+}]_i$ elevation and $(ATP)_i$ depletion

We tested the effects of intracellular Ca^{2+} chelation on the inhibitory effects of ethanol and POA using 40μM BAPTA-AM preincubation for 30 min. We detected that the preincubation completely abolished the inhibitory effect of 100mM ethanol and 200μM POA on pancreatic ductal HCO_3^- secretion suggesting that their inhibitory effect was mediated by the sustained elevation of $[Ca^{2+}]_i$. Moreover we showed that intracellular ATP depletion induced by Deoxyglucose/Iodoacetamide-CCCP significantly decreased HCO_3^- secretion, very similar to the effects of 200μM POA, or 100mM ethanol (not shown).

4.10. Ethanol and non-oxidative ethanol metabolites cause translocation and expression defect of CFTR in PDEC

To investigate the effects of ethanol and ethanol metabolites on the protein expression levels, Capan-1 cells were incubated with ethanol, POAEE or POA and changes in CFTR expression were measured. We showed that high concentrations of ethanol, POAEE and POA time-dependently decreased both the mRNA and protein expression of CFTR. To test whether these effects could be observed *in vivo* as well, guinea pigs were injected *i.p.* with 0.8g/kg ethanol and 300mg/kg palmitic acid (PA). Apical CFTR expression was significantly decreased 12 and 24 h after the treatment.

4.11. The effects of bile acids on the mitochondrial morphology and $(ATP)_i$ level in pancreatic ductal epithelial cells

To investigate the effects of high concentration of bile acids on the mitochondrial morphology isolated pancreatic ducts were exposed to 1mM CDC, which strongly damaged all of the mitochondria and decreased the intracellular ATP level.

4.12. The effect of $(ATP)_i$ depletion on the bicarbonate secretion of pancreatic ductal epithelial cells

To characterize the effects of $(ATP)_i$ depletion on the activities of NHE, NBC and CBE, we used the NH_4Cl pulse technique in HCO_3^--buffered solution. Combined administration of CCCP and DOG/IAA significantly inhibited the activity of the acid/base transporters. These observations suggest that depletion of $(ATP)_i$ is the key element which inhibits NBC, NHE and CBE.

5. DISCUSSION

In this present work we have demonstrated that ethanol, as well as its non-oxidative metabolites cause impairment of pancreatic ductal fluid and HCO_3^- secretion via toxic cellular Ca^{2+} signaling and break down of the mitochondrial ATP production. The same mitochondrial damage was found in these cells upon the administration of non-conjugated bile acid. These results highlight the central role of mitochondrial damage in the pathogenesis of acute pancreatitis.

5.1. The effects of ethanol and non-oxidative ethanol metabolites on the pancreatic fluid and HCO_3^- secretion

Pancreatic tissue metabolizes ethanol mainly via the non-oxidative pathway mediated by FAEE synthases (FAEES), which combine ethanol and FA and produce FAEE. A clinical study showed that blood FAEE concentration was elevated in parallel with ethanol concentration during alcohol consumption; but FAEE remained increased longer in the serum compared to ethanol. Moreover, compared with the liver, pancreatic FAEES activity is higher, which creates the possibility for the local accumulation of non-oxidative ethanol metabolites. Werner et al. showed that FAEE infusion induced pancreatic edema, intrapancreatic trypsinogen activation, and vacuolization of acinar cells. Recently Huang et al. developed a novel model of alcohol-induced pancreatitis using a combined *i.p.* injection of ethanol and FA, where the pharmacological inhibition of non-oxidative ethanol metabolism decreased the pancreatic damage.

We demonstrated that pancreatic ductal HCO_3^- secretion plays central role in the physiology of the exocrine pancreas, maintaining the intraductal pH , therefore in our experiments we used different *in vivo* and *in vitro* techniques to clarify the acute effects of ethanol and ethanol metabolites on fluid and HCO_3^- secretion and CFTR Cl^- current in PDEC. Importantly, our MRI cholangiopancreatography experiments showed that in CFTR KO mice the ductal secretion is remarkably diminished compared to WT, moreover, ethanol and PA strongly impaired ductal secretion in both groups. Besides the fluid transport we characterized the effects of ethanol and it metabolites on HCO_3^- secretion. Our results showed that ethanol in low concentration stimulates, whereas in high concentrations inhibits HCO_3^- secretion and decreases CFTR activity. Similar dual effects of ethanol on fluid secretion were highlighted earlier. In our study, the stimulatory effect of 10mM ethanol on HCO_3^- secretion was mediated by IP_3R-dependent Ca^{2+} release from the ER. In contrast, high concentrations of ethanol and POA induced sustained $[Ca^{2+}]_i$ elevation mediated by both the IP_3R and RyR as well as extracellular Ca^{2+} influx. Notably, similar toxic Ca^{2+} elevation was found in pancreatic acinar cells, and in other cell types leading to premature protease activation and cell death. It is well documented that sustained $[Ca^{2+}]_i$ elevation causes mitochondrial Ca^{2+} overload , which impairs $(\Delta\Psi)_m$ and ATP production . Very recently ethanol was shown to sensitize pancreatic mitochondria to activate the mitochondrial permeability transition pore, leading to mitochondrial failure. In this study, high concentrations of

ethanol and POA also induced $(ATP)_i$ depletion and decreased $(\Delta\Psi)_m$. Although the toxic effects of ethanol and POA were similar to those of high concentration of bile acids, in this study chelation of $[Ca^{2+}]_i$ abolished the inhibitory effect of ethanol and POA on HCO_3^- secretion. This observation indicates, that ethanol and POA, in a similar manner to trypsin, induce a sustained rise in $[Ca^{2+}]_i$, which damage the mitochondria and inhibit the pancreatic fluid and HCO_3^- secretion.

One of the important observations of this study is that upon alcohol and fatty acid administration the expression of CFTR is decreased on the luminal membrane of PDEC. The *in vitro* experiments in human PDEC and the *in vivo* experiments in guinea pig clearly demonstrated that alcohol and its non-oxidative metabolites indeed strongly decrease CFTR expression without damaging PDEC. This effect can be attributed at least partially to the chronic cytoplasmic ATP-depletion, considering that CFTR conformation maturation is an ATP-sensitive process at the ER. These results indicate that chronic exposure of PDEC to ethanol or ethanol metabolites compromise both the biosynthetic processing and peripheral stability of the channel.

Association between CFTR gene mutations and the risk for the development of acute recurrent or chronic pancreatitis provides strong evidence that mutations in CFTR and/or insufficiency of electrolyte and fluid secretion by pancreatic ductal cells lead to increased risk for pancreatitis. Heterozygous carriers of *CFTR* mutations are at an increased risk of chronic pancreatitis , moreover Ooi et al. demonstrated that the risk of developing pancreatitis was much higher in CF patients, who had milder CFTR mutations (type IV and V) and were pancreatic sufficient compared to those who had severe mutations and were pancreatic insufficient . In the pathogenetic model proposed in this study, the risk of developing pancreatitis inversely correlates with CFTR function. However, in other studies the association between *CFTR* gene mutation and alcoholic pancreatitis was inconsistent. Very recently LaRusch et al. elegantly demonstrated that *CFTR* gene mutations that don't cause typical cystic fibrosis, but disrupt the WNK1-SPAK-mediated HCO_3^- permeability of the channel, are associated with pancreatic disorders. In animal model of pancreatitis Dimagno earlier showed that CFTR KO mice developed more severe acute pancreatitis after cerulein hyperstimulation than WT mice and recently Pallagi et al. had the same observation in mice with genetic deletion of Na^+/H^+ exchanger regulatory factor, which regulates CFTR expression.

5.2. The effects of high concentration of chenodeoxycholate on the pancreatic fluid and HCO_3^- secretion

Besides heavy ethanol consumption bile acids are the other most common etiological factors of pancreatitis. Small bile stones can enter the common bile duct and get stuck at the papilla of Vater, blocking both the outflow of bile and pancreatic juice. Although the exact pathogenesis of biliary pancreatitis is still a matter of debate, according to the common channel theory bile acids can reach the pancreatic tissue via the pancreatic ductal tree. During biliary reflux the PDEC are the first cell type

reach by bile acids, but the earlier studies focused mainly on acinar cells. Taurolithocholic acid 3-sulphate (TLC-S) was shown to induce Ca^{2+} release from the ER and acidic Ca^{2+} stores in pancreatic acinar cells and to inhibit SERCA pump activity. Voronina *et al*. showed that TLC-S decreases the $(ATP)_i$ in pancreatic acinar cells and induces loss of $\Delta\Psi$, which was also not influenced by BAPTA-AM pretreatment.

In PDE cells the non-conjugated bile acid CDC induced toxic sustained Ca^{2+} signalling and inhibited acid/base transporters including the basolateral Na^+/H^+ exchanger, Na^+/HCO_3^- cotransporter and the luminal Cl^-/HCO_3^- exchanger . In these series of experiments, BAPTA-AM did not prevent the inhibitory effect of CDC on HCO_3^- secretion, suggesting the presence of a Ca^{2+} independent inhibitory mechanism. In this study we provided evidence, that high concentration of CDC induced severe morphological damage of the mitochondria (mitochondrial swelling and disruption of the inner mitochondrial membrane) and consequent $(ATP)_i$ depletion. Notably, the preincubation of the pancreatic ducts with BAPTA-AM failed to prevent the mitochondrial damage induced by CDC, suggesting a Ca^{2+}-independent mechanism underlying the observed mitochondrial damage in response to CDC in guinea pig PDE. Pancreatic ductal HCO_3^- secretion is a strongly ATP dependent processes. According to our findings, the ATP depletion directly inhibited pancreatic ductal HCO_3^- secretion. Our finding is in accordance with other authors results, where they show that depletion of $(ATP)_i$ inhibits many of the ion transporters such as the NHEs in different cell types . Mitochondrial damage was found in *in vivo* models of acute pancreatitis as well, where L-lysine was used to induce acute pancreatitis in rats. L-lysine induced mitochondrial damage, which preceded the activation of trypsinogen or NF-κB activation. These results suggest that L-lysine may directly damage mitochondria and that mitochondrial injury is the initiating factor in this pancreatitis model. Halangk *et al*. showed that, in the rat, during acute pancreatitis induced by supramaximal cerulein stimulation the mitochondrial oxidative phosphorylation and ATP production are drastically decreased.

6. ACKNOWLEDGEMENTS

Background: Alcohol-induced and biliary pancreatitis are the two most common forms of acute pancreatitis. Notably, there is no specific treatment against this inflammatory disease suggesting the lack of knowledge of pathophysiological mechanisms involved in the initiation and progression of the diseases. Although recent evidence suggest that pancreatic ductal epithelial cells (PDEC) play important role in the pathogenesis of pancreatitis the effects of ethanol, fatty acids and their metabolites on PDEC has not been investigated yet. On the other hand bile acid in millimolar concentration has been shown to inhibit the pancreatic ductal HCO_3^- secretion, however the exact mechanism of inhibition remained elusive.

The **aim** of this study was to characterize the effects of ethanol and ethanol metabolites on the pancreatic ductal epithelial HCO_3^- secretion and to dissect the inhibitory effect of non-conjugated bile acids on the pancreatic HCO_3^- secretion.

Methods: We studied the effects of ethanol, fatty acids, and fatty acid ethyl esters on secretion of pancreatic fluid and HCO_3^-, levels and function of CFTR, and exchange of Cl^- for HCO_3^-, in pancreatic cell lines, as well as in tissues from guinea pigs and CFTR-knockout mice following administration of alcohol and fatty acids. We detected the changes of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), ATP level ($(ATP)_i$), and mitochondrial membrane potential ($(\Delta\Psi)_m$) upon the administration of ethanol, ethanol metabolites, or chendoxycholate using fluorescent indicators. The mitochondrial morphology was assessed by electron microscopy.

Results: Low concentration of ethanol stimulated the pancreatic HCO_3^- secretion via IP_3 mediated Ca^{2+} release from the endoplasmic reticulum (ER). High concentration of ethanol and fatty acids inhibited secretion of fluid and HCO_3^-, as well as CFTR activity, in pancreatic ductal epithelial cells. These effects were mediated by sustained increases in $[Ca^{2+}]_i$, depletion of $(ATP)_i$, and depolarization of mitochondrial membranes. Moreover in pancreatic cell line and guinea pig pancreatic tissue administration of ethanol, or non-oxidative ethanol metabolites reduced the expression of CFTR. Similarly, high concentration of the non-conjugated bile acid chenodeoxycholate disrupted the mitochondrial structure and consequently depleted $(ATP)_i$ in guinea pig pancreatic ductal cells. The $(ATP)_i$ depletion induced by the inhibition of the cellular ATP production significantly impaired the HCO_3^- secretion.

Conclusions: In this present work we have demonstrated that ethanol, as well as its non-oxidative metabolites cause impairment of pancreatic ductal fluid and HCO_3^- secretion via toxic cellular Ca^{2+} signaling and break down of the mitochondrial ATP production. Very similar mitochondrial damage was found in these cells upon the administration of non-conjugated bile acid. These results highlight the central role of mitochondrial damage in the pathogenesis of acute pancreatitis.

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