The role of Ca^{2+} signaling in the physiology and pathophysiology of the pancreatic ductal cells

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Ph.D. Thesis

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LIST OF FULL PAPERS RELATED TO THE THESIS

Articles closely related to the subject of the thesis and cited in the thesis:

I. Pallagi P*, **Görög M***, Papp N, Madácsy T, Varga Á, Tim Crul, Szabó V, Molnár M, Dudás K, Grassalkovich A, Szederkényi E, Lázár Gy, Venglovecz V, Hegyi P, Maléth J. Bile acid- and ethanol-mediated activation of Orai1 damages pancreatic ductal secretion in acute pancreatitis.
J Physiol. 2022 Jan 26. doi: 10.1113/JP282203. Online ahead of print.
[IF_{2021}: 5.182]

*** Megosztott első szerzők**

II. Fanczal J, Pallagi P, **Görög M**, Diszházi G, Almássy J, Madácsy T, Varga Á, Csernay-Biró P, Katona X, Tóth E, Molnár R, Rakonczay Z Jr, Hegyi P, Maléth J. TRPM2-mediated extracellular Ca^{2+} entry promotes acinar cell necrosis in biliary acute pancreatitis.

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[IF_{2020}: 5.182]

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Lab Invest. 2020 Jan; doi: 10.1038/s41374-019-0300-3

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1. Introduction

1.1. The exocrine pancreas

The pancreas is a complex organ with exocrine and endocrine parts and located in the retroperitoneal region of the upper abdominal cavity. The exocrine pancreas consists of two main types of cells: acinar and ductal cells. Acinar cells secrete an isotonic, NaCl-rich fluid containing a multitude of enzymes and precursor enzymes. It has long been implied that the main function of pancreatic ductal epithelial cell (PDEC) is to provide a mechanical framework for acinar cells. Although the duct cells create 5% of the pancreas, a large proportion of the secreted pancreatic fluid is due to the ductal cells. The amount of daily secreted pancreatic fluid in humans is between 1 liter and 2,5 liter. The physiological function of alkaline secretion is in one hand to neutralise protons secreted by acinar cells, to flush digestive enzymes to the duodenum, and to neutralise the gastric acid entering the duodenum.

1.2. Physiology of pancreatic ductal HCO_3^- secretion

The secreted Cl^- by acinar cells is exchanged for HCO_3^- by the ductal cells to produce an alkaline fluid, which is essential for normal digestion. The accumulation of HCO_3^- inside the ductal cell across basolateral membrane thought to be mediated by direct mechanism through Na^+/ HCO_3^- cotransporters (pNBC1) or indirectly via the passive diffusion of carbon dioxide (CO_2) across the cell membrane. CO_2 is converted to carbonic acid by the carbonic anhydrase enzyme and after hydrolyzed to HCO_3^- and H^+. Backward transport of protons is mediated by Na^+/H^+ exchangers (NHEs) and an H^+-ATPase. HCO_3^- secretion can occur through the apical membrane of PDECs via solute carrier family 26 (SLC26) anion exchangers and the cystic fibrosis transmembrane conductance regulator (CFTR). Although CFTR is predominantly a Cl^- channel, it can also handling HCO_3^- and other anions. Gray et al. have reported that CFTR is 3-5 times more selective for Cl^- than HCO_3^-.

1.3. Regulation of pancreatic ductal secretion by Ca^{2+} signaling

Complex signaling system is responsible for the physiological regulation of pancreatic ductal HCO_3^- secretion, in which intracellular Ca^{2+} play crucial roles, however uncontrolled Ca^{2+} release can lead to intracellular Ca^{2+} overload and toxicity, including mitochondrial damage and impaired ATP production. There are two mechanisms which can maintain the balance of the intracellular Ca^{2+} concentration: the Ca^{2+} entry into the cytosol via the plasma membrane ion channels and the Ca^{2+} clearance from the cytosol via the plasma membrane Ca^{2+}

pump. If we would be able to block the entry or maintain the clearance, we could reduce the toxicity of sustained Ca^{2+} signals and thus cell damage.

1.3.1. Calcium entry

The release of endoplasmic reticulum (ER) Ca^{2+} stores by biologically active compounds (such as bile acids, or fatty acid ethyl esters) activates the influx of the extracellular Ca^{2+} via the Orai1 Ca^{2+} channel. This process is referred as store operated Ca^{2+} entry (SOCE). During SOCE an agonist binds to and activates IP_3 receptors (IP_3R) and causes the release of Ca^{2+} from ER, which leads to oligomerization and conformational switch of Stim1. Stim1 binds to Orai1 and trigger Ca^{2+} influx. Although SOCE is part of the physiological Ca^{2+} signaling events in non-excitable cells, under pathological conditions it significantly contributes to the sustained intracellular Ca^{2+} overload. The other player of Ca^{2+} entry is transient receptor potential melastatin-2, which is sensitive to oxidative stress and can trigger the elevation of intracellular Ca^{2+} concentration. Two different publications confirmed the role of selective Orai1 inhibition by GSK-7975A and CM-128 in the reduction of the severity of AP via decrease of bile acid-mediated extracellular Ca^{2+} influx and sustained Ca^{2+} overload in pancreatic acinar cells. Although the beneficial effect of selective Orai1 inhibition in AP is well-established, precisely how Orai1 inhibition affects pancreatic ductal secretion is unknown.

1.4. Pathophysiological Ca^{2+} signal generation in ductal cells

Recent publications confirmed the benefical role of physiological Ca^{2+} signaling in the regulation of PDEC. Howerver, several study showed that sustained $[Ca^{2+}]_i$ elevation was induced by the most frequent pathogenic factors for acute pancreatitis, such as bile acids, non-oxidative ethanol metabolites and trypsin in pancreatic ductal cells, which inhibits the cellular functions. These results suggest, that uncontrolled Ca^{2+} release can lead to sustained intracellular Ca^{2+} elevation which caused cellular damage during acute pancreatitis.

To confirm the role of Ca^{2+} signaling in the regulation of pancreatic HCO_3^- secretion, dose-dependent dual effects of non-conjugated bile acid chenodeoxycholate (CDC) were demonstrated by Venglovecz et al, which can depend on the type of Ca^{2+} signal. Repetitive, short-lasting Ca^{2+} oscillations evoked by low concentrations (100 μM) of CDC stimulate HCO_3^- secretion from the luminal membrane of PDEC. Different type of inhibitors, such as IP_3R inhibitor caffeine, xestospongin C or the PLC inhibitor U73122 abolished the oscillations. If the cells were preincubated with intracellular Ca^{2+} chelator BAPTA-AM, the Ca^{2+} signals and the stimulatory effect of 100 μM CDC on HCO_3^- secretion were also abolished. In contrast,

administration of high concentrations (1 mM) CDC induced a toxic sustained Ca^{2+} elevation and caused mitochondrial damage resulting the depletion of ATP production which changes might explain the inhibition acid/base transporters including the basolateral NHE, NBCe1-B and the luminal CBE. In contrast to the effect of low concentration of CDC, BAPTA-AM preincubation could not prevent the formation of Ca^{2+} signal, mitochondrial damage and the inhibitory effect of CDC on the HCO_3^- secretion. This finding suggest that bile acid has a direct, Ca^{2+}-independent effect to mitochondria. This toxic effect might be explained by the protonophoric-like effect of the high concentration of CDC that can affect the mitochondrial ATP production.

Excessive ethanol consumption is the other leading cause of AP. Similarly to bile acids, ethanol and non-oxidative ethanol metabolites can evoke similar intracellular changes in PDEC and also has a dual effects on pancreatic ductal HCO_3^- secretion. Administration of low concentration (1 mM) of ethanol increase the elevation of $[Ca^{2+}]_i$, and secretin-stimulated fluid secretion in guinea pig pancreatic. If 100 mM ethanol was used by Yamamoto et al weak inhibition of stimulated fluid secretion was observed. BAPTA-AM preincubation abolished the stimulatory effect of low concentration of ethanol which suggest that it was mediated by the $[Ca^{2+}]_i$ elevation. In our experiments administration of low concentration ethanol increase HCO_3^- secretion via stimulation of phospholipase C and IP_3-mediated Ca^{2+} release from the endoplasmic reticulum (ER) in CAPAN-1 cells. On the other hand, high concentration of ethanol and non-oxidative ethanol metabolites (POA) evoke sustained intracellular Ca^{2+} elevation by releasing Ca^{2+} from the ER via IP3 and ryanodine receptor activation. Additionally, depleted ATP level was observed, which cause cell necrosis and decrease HCO_3^- secretion in PDEC via inhibition of the apical Cl^-/ HCO_3^- exchanger and CFTR. BAPTA-AM preincubation completely abolished the inhibitory effects of ethanol and POA, suggesting that the inhibition was mediated by the sustained $[Ca^{2+}]_i$ elevation.

Early protease activation in the pancreas is a hallmark of the pathogenesis of acute pancreatitis. In our previous work, the effects of trypsin on the pancreatic ductal epithelia was investigated and showed that luminal administration of trypsin or PAR2 antagonist peptide inhibit the luminal Cl^-/ HCO_3^- exchanger and CFTR via elevation of intracellular Ca^{2+} concentration. Moreover, the inhibitory effect of trypsin was abolished by BAPTA-AM preincubation, similarly to the inhibitory effects of ethanol and POA.

1.5. Transient Receptor Potential Melastatin 2

Transient receptor potential melastatin-2 (TRPM2) is a member of Melastatin subfamily of TRP proteins and has been identified to act as a cellular redox-sensor. TRP channels are nonselective cation channels that are activated by different chemical and physical stimuli. TRPM2 is also permeable for Ca^{2+} and play crucial role in different physiological and pathophysiological processes associated with redox signaling and oxidative stress. TRPM2 is expressed in numeous different cell types and organs including pancreatic β cells, spleen, neurons, bone marrow cells and immune cells such as T lymphocytes, macrophages and neutrophils. Oxidative stress induced by ROS activate the channel and it can be also became active by free ADP-ribose in synergy with free intracellular Ca^{2+}. TRPM2 has a crucial role in the development of inflammatory disorders. Chemokine production were increased by Ca^{2+} influx via TRPM2 in monocytes, resulting to elevation of neutrophil infiltration in inflammatory bowel disease. Furthermore, the role of TRPM2 has been established in the pathogenesis of irradiation-induced xerostomia. Liu et al. demonstrated that the elevated ROS production induced by irradiation activated TRPM2 and lead to extracellular Ca^{2+} overload in salivary glands, which damaged acinar cells and resulted loss of acinar cell function (saliva production) in the salivary glands. It has been also suggested, that TRPM2 is involved in diabetic stress-induced mitochondrial fragmentation in pancreatic β cells. Abuarab *et al.* demonstrated that ROS production is enhanced and TRPM2 is activated by high extracellular glucose concentrations, which caused permeabilization of lysosomal membranes and induced Zn^{2+}-mediated mitochondrial fission. Several publications proved that TRPM2 could play key role in the regulation of insulin secretion and could represent a new target for diabetes therapy, however, the role of TRPM2 in other secretory function of the pancreas, such as fluid and HCO_3^- secretion of PDEC, has not yet been investigated.

2. Aims

It is evident that Ca^{2+} signaling has important role in the regulation of pancreatic ductal physiology and pathophysiology, however several crucial questions are not answered yet. Thereofre during my Ph.D. studies I investigated the expression and function of two plasma membrane Ca^{2+} channels in pancreatic ductal cells.

Aim 1.

Inhibition of the Orai1 Ca^{2+} channel prevented the sustained elevation of the intracellular Ca^{2+} in pancreatic acinar cells and significantly decreased the severity of the experimental acute pancreatitis. However, the role of Orai1 channel in the physiological and pathophysiological function of the pancreatic ductal epithelial cells is currently unknown.

Specific aims:

1. To characterize the expression and function of Orai1 in pancreatic ductal cells.
2. To examine the role of Orai1 in bile acid and ethanol+PA induced ductal cell damage.
3. To investigate the role of Orai1 inhibition on pancreatic ductal function during AP.

Aim 2.

TRPM2 is a non-selective cation channel that mediates extracellular Ca^{2+} influx during oxidative stress in several cell types, however the presence and role of TRPM2 in pancreatic ductal cells is unknown.

Specific aims:

1. To examine the expression and functional activity of the TRPM2 channel in isolated pancreatic ductal fragments.
2. To characterize the role of TRPM2 in bile acid induced pancreatic ductal cell damage.

3. Materials and methods

3.1. Animals

2 months male FVB/N mice (20-25g) were used. TRPM2 knockout mice were generously provided by Yasuo Mori. The knockout mice were generated from a C57BL/6 background as described previously. Mice were genotyped using a standard polymerase chain reaction (PCR) assay. 2 mounths male mice (20-25g) were used. The animals were kept at 22-24°C with a 12 h light–dark cycle. Mice were used with adherence to the NIH guidelines and the EU directive 2010/63/EU for the protection of animals used for scientific purposes. The studies was approved by the National Scientific Ethical Committee on Animal Experimentation under licence number XXI. /2523/2018 and XXI. /1541 /2020.

3.2. Isolation of mouse pancreatic ductal fragments

Pancreatic ductal fragments were isolated following pentobarbital-induced terminal anesthesia by microdissection after enzymatic digestion.

3.3. Isolation of mouse pancreatic acinar cells

WT and TRPM2 KO mice were sacrificed using terminal anesthesia with 250 mg/bwkg sodium pentobarbital. Acinar cells were isolated from the pancreas with enzymatic digestion

3.4. Fluorescent microscopy

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), intracellular Cl^- concentration ($[Cl^-]_i$), or intracellular pH (pH_i) were measured by using FURA2-AM, MQAE, BCECF-AM flurescent dye, respectively. Isolated mouse pancreatic ductal fragments were attached to a poly-L-lysine-coated coverslip as the base of a perfusion chamber. The ductal fragments were imaged using Olympus IX71 fluorescent microscope equipped with an MT-20 illumination system The signal was captured by a Hamamatsu ORCA Flash 4.0 V3 CMOS camera through a 20X oil immersion objective with a temporal resolution of 1 sec. Ratiometric image analysis was performed by Olympus excellence software.

3.5. Immunofluorescence staining

Upon freezing in Shandon Cryomatrix, isolated pancreatic ducts were sectioned and stained. Isolated mouse pancreatic ducts were labelled with Trypan Blue (Trypan Blue solution, Sigma, Cat.No. 93595-50ML) for 10 min then frozen -20°C in Shandon Cryomatrix (ThermoFisher Scientific, Cat. No.: 6769006) and sectioned 7µm thicks with cryostat (Leica CM 1860 UV) at -20°C. After the fixation and blocking the sections they were incubated with anti-mouse ORAI1 antibody or an ATTO-594 conjugated rabbit polyclonal primary antibody against TRPM2 in 1:200 dilution overnight on 4°C. Nuclear labelling was performed with Hoechst33342 for 15 minutes and visualized with a ZEISS LSM 880 confocal 7 microscope equipped with at 40x water based immersion objective.

3.6. Gene expression analysis and gene knockdown

Total messenger ribonucleic acid (mRNA) from acini and ductal fragments was purified with NucleoSpin RNA XS kit according to the manufacturer's instructions. One µg mRNA was used to synthetize complementary deoxyribonucleic acid (cDNA) with iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA; Cat. No.: 1708890). Conventional PCR amplification was performed with DreamTaq Hot Start DNA Polymerase and cDNA-specific Orai1 primers (forward: 5' CTTCGCCATGGTAGCGAT 3'; reverse: 5' TGTGGTGCAGGCACTAAAGA 3') for 35 cycles. For gene knockdown studies, isolated mouse ductal fragments were transfected with 50 nM pre-designed siRNA for Stim1 or siGLOGreen transfection indicator with Lipofectamine 2000 in feeding media for 24h.

3.7. In vivo acute pancreatitis models

In the cerulein-induced AP model, AP mice received 7 hourly injections of cerulein (50 µg/kg, i.p.) whereas control animals received injections containing physiological saline (i.p.) solution.

One hour after the first cerulein injection, CM-5840 (20 mg/kg, i.p.) was administered. Twelve hours after the first cerulein injection, mice were sacrificed with pentobarbital (85 mg/kg, i.p.).

Biliary AP was induced by intraductal administration of 4% Na-taurocholate. Briefly, mice were anesthetized with a ketamine and xylazine (respectively 125 mg/kg and 12.5 mg/kg, i.p.) cocktail followed by median laparotomy, the common biliopancreatic duct was cannulated across the duodenum. One hour after the 4% Na-TC infusion, the animals received CM-5480 (20 mg/kg, i.p.). Twenty-four hours after the operation, the mice were anesthetized with pentobarbital (85 mg/kg, i.p.) and sacrificed through exsanguination through the heart.

In the acute alcohol-induced pancreatitis model, mice received 2 hourly injections of ethanol (1.35 g/kg, i.p) mixed with palmitoleic acid (150 mg/kg). One hour after the first and directly before the second ethanol /POA injection, CM-5480 (20 mg/kg, i.p.) was administered. Control mice received 200 µl physiological saline (i.p.) instead of ethanol/POA. Twenty-four hours after the first ethanol/POA treatment, the mice were sacrificed under pentobarbital (85 mg/kg, i.p.) anaesthesia.

For all experimental models histological parameters (edema, inflammatory cell infiltration, and necrosis of the samples) were monitored to estimate the severity of induced pancreatitis.

3.8. In vivo measurement of pancreatic fluid secretion

In all experimental pancreatitis models, pancreatic fluid was collected *in vivo* directly before sacrifice. Mice were anesthetized with ketamine/xylazine cocktail (respectively 125 mg/kg and 12.5 mg/kg, i.p.) and placed on a heated pad to maintain body temperature. The operation was performed as described in case of 4% Na-TC-induced AP. Following stimulation with secretin (0.75 Clinical Unit/kg, i.p.) for 30 min, the pancreatic juice was collected and the secretory rate was calculated as µl/body weight g for 1 h.

3.9. Statistics

Statistical analysis was performed with Graphpad Prism software. All data are expressed as means ± SEM. Both parametric (one-way analysis of variance) and nonparametric (Mann Whitney test and Kruskal-Wallis test – used for analysis of the acinar cell survival assay) tests were used based on the normality of data distribution. A p value below 0.05 was considered statistically significant.

4. Results

4.1. Orai1 is expressed on the apical plasma membrane of pancreatic ductal epithelia

First, we analyzed the expression of Orai1 in mouse primary pancreatic ductal epithelial cells. End-point PCR analysis of acini and ductal fragments showed that the Orai1 gene is expressed both in pancreatic acinar and ductal cells. When immunofluorescent labelling of Orai1 was performed on ductal cells, the confocal images showed that Orai1 channels were expressed on the apical membrane of the pancreatic ductal cells and in the surrounding cells also. Next, to demonstrate Orai1 functionality in isolated mouse pancreatic ductal cells, SERCA pump was inhibited with 25μM cyclopiazonic acid (CPA) in Ca^{2+} -free medium to deplete ER Ca^{2+} stores. Under these conditions, Ca^{2+} influx into the cytosol was induced by re-addition of extracellular Ca^{2+}, which was significant inhibited by perfusing Orai1 inhibitors at a concentration of 10μM. Although the maximal inhibition did not increase when using higher CM-5480 concentrations resulting in a similar decrease of the plateau phase (45.15±3.41% at 10 μM CM-5480 vs. 52.38±2.45% at 30 μM CM-5480, respectively), it was achieved significantly faster at 30 μM CM-5480.

As the plateau phase of the Ca^{2+} signal under the applied conditions is a mixture of Ca^{2+} influx and efflux, which may affect the characterization of the Orai1 mediated Ca^{2+} entry, we also applied another protocol. Addition of CM-5480 before the re-addition of extracellular Ca^{2+} significantly decreased the extracellular Ca^{2+} influx. Of note, despite the inhibition of the Orai1 channels, a significant proportion of the extracellular Ca^{2+} influx remained active in every case suggesting that other Ca^{2+} influx channels may contribute to the extracellular Ca^{2+} influx in ductal cells.

4.2. The inhibition of Orai1 abolishes toxin-induced extracellular Ca^{2+} influx in the pancreatic ductal epithelia

Next, we examined the effect of Orai1 channel inhibition on bile acid- or ethanol+ palmitic acid (PA)-induced intracellular Ca^{2+} elevation. 250 μM CDC induced sustained elevation of intracellular Ca^{2+} concentration in isolated mouse pancreatic ductal fragments. Reaching a stable plateau, the ductal fragments were challenged with 10 μM CM-5480, which reduced the extracellular CDC -induced Ca^{2+} influx significantly. 100 mM ethanol and 200 μM PA also induced intracellular Ca^{2+} elevation in ductal fragments. However, as the plateau phase of the evoked Ca^{2+} signal was not clearly separated from the peak in case of ethanol-, and PA treatment, CM-5480 was applied simultaneously with the ethanol and PA treatment. Similar to

CDC, the extracellular ethanol-, and PA-induced Ca^{2+} influx significantly decreased for CM-5480 administration. These results suggest the potential of CM-5480 to prevent Ca^{2+}-overload-mediated functional and morphological damage of ductal cells associated with biliary- or ethanol-induced AP.

4.3. Inhibition of Orai1 prevents bile acid- and ethanol-induced decrease of HCO_3^- secretion and CFTR function in pancreatic ductal epithelia

HCO_3^- secretion –the primary function of pancreatic ductal epithelia– is significantly impaired by bile acid- and ethanol-mediated sustained Ca^{2+} elevation and mitochondrial damage. To assess the potential protective effect of Orai1 inhibition on ductal HCO_3^- secretion, we treated isolated mouse ductal fragments with CDC or ethanol/PA in the presence or absence of CM5480 and compared HCO_3^- efflux across the apical membrane. The isolated mouse pancreatic ducts were perfused with 20 mM NH_4Cl in HCO_3^-/CO_2-buffered solution to measured HCO_3^- secretion across the apical membrane of pancreatic ductal cells. NH_4Cl containing solution triggered a rapid alkalization caused by the passive NH_3 uptake of the cells, which was followed by a slow recovery of the alkaline pH - due to the SLC26 Cl^-/HCO_3^- exchanger - and CFTR-mediated HCO_3^- [88] efflux (i.e. secretion) from the ductal epithelia to resting pH_i. We found that 250 μM bile acid and 100 mM ethanol+200 μM PA significantly inhibited bicarbonate secretion, which was significantly increased by 10 μM CM-5480. To calculate the base flux [$J(B^-)$] values (calculated as $\Delta pH/\Delta t$ [88]), the initial recovery rates were measured over the first 30 s.

Intracellular Cl^- concentration was measured also by microfluorimetry and an intracellular Cl^- concentration sensitive fluorescent indicator MQAE was used. The fluorescent signal emitted by MQAE is inversely correlated with intracellular Cl^-. When we removed extracellular Cl^- from the HCO_3^-/CO_2- buffered solution, intracellular Cl^- decreased due to Cl^- efflux through the CFTR channel. 250 μM CDC and 100 mM ethanol + 200 μM PA significantly reduced Cl^- efflux. Treatment of these ductal cells with CM-5480 the Cl^- efflux significantly increased. These results suggest that CM-5480 treatment has the potential to prevent the AP-induced inhibition of the pancreatic ductal HCO_3^- secretion and CFTR activity.

4.4. Bile acid- and alcohol-induced Orai1-mediated extracellular Ca^{2+} entry depends on Stim1 activation

Exposure of ductal cells to bile acids and ethanol release Ca^{2+} from intracellular stores –most prominently from the ER [89,90]– which induces a conformational change of Stim1 triggering

Orai1-mediated Ca^{2+} influx. Isolated ductal cells were treated with specific siRNA to knock down Stim1 expression and in order to assess the involvement of Stim1 in bile acid- and alcohol-induced ductal cell functional impairment. Treatment of pancreatic ductal cells with siStim1 did not alter CFTR-mediated HCO_3^-- or Cl^- secretion. Whereas CDC or ethanol/PA significantly impaired HCO_3^- secretion or CFTR activity in siGLO-Green-treated pancreatic ductal cells, this was prevented in siStim1-treated cells. These results suggest that bile acids and ethanol induce Orai1-mediated Ca^{2+} influx in a Stim1-dependent manner.

4.5. Inhibition of Orai1 preserves pancreatic ductal secretion in vivo during acute pancreatitis

We wanted to analyze AP-mediated changes of fluid secretion in vivo. Upon establishment of experimental AP, secretin-induced fluid secretion was measured in vivo in anesthetized mice. In all three AP groups the in vivo fluid secretion was significantly decreased (i.e. cerulein-, Na-TC-, and ethanol/POA-treated animals). CM-5480 treatment alone did not affect secretin-stimulated pancreatic secretion. In fact, in both cerulein- and ethanol/POA-treated animals, CM5480 significantly improved in vivo fluid secretion to levels comparable to the untreated – healthy– control group. Moreover, in the Na-TC-treated group, CM5480 resulted in an almost twofold increased fluid secretion compared to the Na-TC group; however, the difference failed to reach statistical significance. To confirm these results, we studied HCO_3^- secretion through fluorescence microscopy, using the alkali load technique on isolated pancreatic ductal fragments from CM-5480- and cerulein-treated mice. Cerulein significantly decreased HCO_3^- secretion in the ductal fragments, this secretory activity was preserved in ductal fragments derived of cerulein-induced AP mice receiving CM-5480. Importantly, these results confirmed that inhibition of Orai1 preserves the ductal ion and fluid secretion both in vitro and in vivo in different forms of AP.

4.6. Functional TRPM2 channels are present in pancreatic ductal cells

After the characterization the role of Orai1 in the pancreatic ductal secretion, we wanted to investigate the expression and function of an other Ca^{2+} channel, TRPM2, which play role in the Ca^{2+} entry process. Immunofluorescent labelling of TRPM2 showed an atypical expression on isolated ducts. We have found that H_2O_2-induced oxidative stress activated TRPM2. 1 mM H_2O_2 induced an increased $[Ca^{2+}]_i$ in TRPM2 WT pancreatic ductal cells, than in TRPM2 KO ductal cells (0.30 ± 0.06 vs 0.10 ± 0.013, respectively). In these cells, Ca^{2+} elevation was significantly lower in Ca^{2+} free conditions. This suggests that the sustained elevation of $[Ca^{2+}]_i$

in response to H_2O_2 was largely due to TRPM2-channel-mediated influx of extracellular Ca^{2+}. Because HCO_3^- secretion is the primary function of the ductal epithelia, the HCO_3^- efflux across the apical membrane was compared between WT and TRPM2 KO ducts using fluorescence pH_i measurements. With this assay, no difference in the activities of the apical and basolateral proteins was found between WT and TRPM2 KO ducts. CDC markedly inhibited the HCO_3^- secretion both in TRPM2 KO and WT ductal cells, the genetic knockout of TRPM2 had no protective effect, suggesting that bile acids affect ductal cells via a TRPM2-independent mechanism.

5. Discussion

Sustained elevation of intracellular Ca^{2+} is a hallmark in the development of AP-mediated cellular injury. Although selective Orai1 inhibitors –limiting the uncontrolled extracellular Ca^{2+} influx– prevented acinar cell damage and decreased the severity of AP in multiple animal models, precisely how Orai1 inhibition affects the pancreatic ductal cell functions is currently unknown. During my work we first demonstrated that Orai1 resides in the apical membrane of the pancreatic ductal cells where it mediates extracellular Ca^{2+} influx upon ER Ca^{2+} store depletion. Next, we provided evidence that bile acid- and ethanol-mediated SOCE-activation contribute to sustained intracellular Ca^{2+} elevations leading to damaged ductal secretion and cell death. Finally, prevention of intracellular Ca^{2+} overload with selective Orai1 inhibitors preserved pancreatic ductal ion and fluid secretion and maintained exocrine pancreatic secretion during AP.

Expression of Orai1 in exocrine pancreas acinar cells was previously described by two independent groups. Lur et al. demonstrated Stim1-translocation and Orai1-activation in the lateral and basal plasma membrane, whereas Hong et al. reported a more pronounced Orai1 expression in the apical membrane. In our experiments, Orai1 expression was observed on the apical membrane of ductal cells. Although the significance of this polarized expression pattern is currently unknown, it may be of importance in reuptake of intraluminal Ca^{2+} secreted by the acinar cells during digestive enzyme secretion. Interestingly, CM-5480-mediated functional inhibition of Orai1 did not completely abolish the ER store depletion-induced extracellular Ca^{2+} influx. In fact, in the current study, we achieved a maximal inhibition of around 50% –both in case of 10 μM and 30 μM CM-5480– suggesting that additional PM-residing Ca^{2+} channels contribute to SOCE in ductal cells. Interestingly, genetic deletion of the TRPC3 Ca^{2+} channel resulted in a 50% reduction of receptor-stimulated SOCE in pancreatic acinar cells and prevented bile acid- and ethanol metabolite-induced sustained Ca^{2+} elevation and intracellular

trypsin activation. These beneficial effects ultimately resulted in reduced cerulein-induced AP severity *in vivo*. Similar results were achieved with the specific TRPC3 inhibitor Pyr3. However, the contribution of TRPC3 to SOCE in pancreatic ductal cells is currently unknown.

On the other hand, the most common biologically active molecules inducing AP –including bile acids, non-oxidative ethanol metabolites, and trypsin– induce toxic, sustained intracellular Ca^{2+} elevation in the exocrine pancreas. Previous data indicated that the non-conjugated bile acid CDC dose-dependently impairs pancreatic HCO_3^- secretion via sustained Ca^{2+} elevation and subsequent mitochondrial damage in pancreatic ductal cells and isolated pancreatic acinar cells.

Considering the detrimental effect of heavy ethanol consumption - in combination with non-oxidative ethanol metabolites (such as fatty acid ethyl esters (FAEE)) - on acinar and ductal cells, our group previously demonstrated ethanol / POA-mediated impaired activity of the apical SLC26 Cl^-/HCO_3^- exchanger and CFTR Cl^- channel together with decreased HCO_3^- secretion in ductal cells. Mechanistically, ethanol and POA induced a sustained Ca^{2+} elevation through IP_3- and ryanodine receptor-mediated Ca^{2+} release from the ER combined with extracellular Ca^{2+} influx; a mechanism which was also described in pancreatic acinar cells. The involvement of Orai1 in the development of AP was first highlighted by Gerasimenko et al. by demonstrating that Orai1 inhibition decreased acinar cell necrosis in vitro. In fact, selective GSK-7975A-mediated inhibition of Orai1 inhibited SOCE in a concentration-dependent manner and reduced the sustained Ca^{2+} elevation, trypsin activation, and acinar necrosis upon FAEE exposure. Others found that GSK-7975A and CM_128 –developed by CalciMedica– markedly impaired bile acid-induced extracellular Ca^{2+} influx and sustained Ca^{2+} overload in pancreatic acinar cells and significantly decreased pancreatic edema, inflammation, and necrosis in experimental models of AP. By using CM-5480 – another selective Orai1 channel blocker by CalciMedica currently in clinical trials– Waldron et al. showed that inhibition of SOCE prevented trypsinogen activation, acinar cell death, NF-κB and NFAT activation, and inflammatory responses in multiple *in vitro* and *in vivo* models. In our study, inhibition of Orai1 in pancreatic ductal cells significantly decreased the bile acids and ethanol / PA-mediated extracellular Ca^{2+} influx in pancreatic ductal fragments and organoids. The CM-5480-mediated inhibition of Orai1 was sufficient to significantly improve the in vitro HCO_3^- secretion and CFTR activity in pancreatic ductal cells. As specific siRNA knockdown of the ER Ca^{2+} sensor protein Stim1 reproduced the effect of selective pharmacologic Orai1 inhibition, CDC- and ethanol/PA-induced activation of Orai1 seems to be Stim1 dependent.

The importance of pancreatic ductal fluid and HCO_3^- secretion in the physiological function of the exocrine pancreas is supported by several independent studies. Di Magno et al. demonstrated that CFTR knockout mice –in which the exocrine pancreatic secretion was impaired– develop more severe cerulean-induced AP which is accompanied with increased pancreatic edema, neutrophil infiltration, and expression of inflammatory mediators. In addition, our group showed that genetic deletion of Na^+/H^+ exchanger regulatory factor-1 (NHERF-1) –a scaffolding protein that anchors CFTR to the apical PM– reduces pancreatic fluid and HCO_3^- secretion in mice. Compared to wild type littermates, NHERF-1 KO mice developed more severe experimental AP upon cerulein hyperstimulation or bile acid infusion to the main pancreatic duct. In addition, the alcohol-induced impairment of CFTR function and expression resulted in increased severity of experimental AP. In the current study, we confirmed previous reports by indicating that in vivo CM-5480-mediated inhibition of Orai1 in mice markedly decreases the severity of AP in three different model systems with independent pathogenic triggers. Importantly, in all three AP models, we demonstrated improved secretin-stimulated in vivo pancreatic fluid secretion in CM-5480-treated animals. Notably, at the tested dose, CM-5480 had no effects on the secretin-stimulated in vivo pancreatic fluid secretion itself in the control groups.

Restoration of pancreatic fluid secretion could have significant beneficial impact on the disease outcome. In a healthy pancreas, the digestive enzymes produced by the acini are washed out by HCO_3^--rich fluid into the duodenum where it neutralizes the local pH. Previously, our group demonstrated pH-dependent autoactivation of trypsinogen together with elevated trypsinogen activity in acidic environment indicating the primordial role of HCO_3^- to prevent early autoactivation of trypsinogen. In addition, Zeng et al. reported that pharmacological correction of CFTR expression and activity rescues pancreatic acinar cell function and reduces autoimmune pancreatitis-induced inflammation, further highlighting the importance of proper ductal function in the disease outcome. Recently, the role of Saraf –an Orai1 channel regulator protein– was reported in AP. In contrast to constant expression levels of Stim1 and Orai1, expression levels of Saraf decreased during AP in both mice and human. In addition, whereas Saraf knockout mice developed more severe AP accompanied by increased Ca^{2+} influx in acinar cells, its overexpression reduced acinar Ca^{2+} influx and decreased AP severity.

Very recently, a phase 2, open-label, dose-response clinical study evaluated the safety of Auxora –a selective Orai1 inhibitor drug developed by CalciMedica– in patients with AP, SIRS, and hypoxemia. In this clinical study, the patients received low- or high-dose Auxora plus

standard of care (SOC). Overall, no differences in the number of serious adverse events with Auxora compared to SOC alone were reported. Of patients with moderate AP receiving low-dose Auxora, 36.5% improved to mild AP. Very interestingly, patients receiving Auxora better tolerated solid foods, had less persistent SIRS, and had a reduced hospitalization rate compared to SOC. It is tempting to speculate that the increased tolerance towards solid food may be explained by improved exocrine pancreatic secretion as observed in our current study. Based on these results, further clinical studies are needed to clarify the utility of Orai1 inhibition in AP patients.

As mentioned in the introduction, the Ca^{2+} entry is also mediated by the TRPM2 channel, a ROS-sensitive channel. To study this we characterized of TRPM2 expression in mouse pancreatic ducts, which was on the luminal membrane. The expression of TRPM2 has been demonstrated previously in different cell types, including inflammatory cells, myocytes and epithelial cells, to our knowledge, we demonstrated first the expression of TRPM2 in the exocrine pancreas. When isolated TRPM2 WT ductal cells were challenged with H_2O_2 to increase ROS significantly increased the $[Ca^{2+}]_i$, which was due to the TRPM2-channel-mediated influx. Redox signals have been demonstrated to sensitise TRPM2 in other cell types. Those increased intracellular Ca^{2+} concentration at physiological body temperature, which plays an important role in the regulation of macrophage functions. Perides et al. demonstrated that activation of the G-protein-coupled cell surface bile acid receptor (Gpbar1 or TGR5) at the pancreatic acinar cells leads to sustained Ca^{2+} elevation and intracellular activation of digestive enzymes. On the other hand, CDC dose-dependently increased the intracellular Ca^{2+} level and inhibited HCO_3^- secretion in pancreatic ductal cells. In our experiments, CDC also decreased the HCO_3^- secretion both in TRPM2 KO and TRPM2 WT ductal cells, but genetic deletion of TRPM2 had no protective effect, suggesting that bile acids affect ductal cells via a TRPM2-independent mechanism.

Taken together, we show that genetic deletion of TRPM2 protects pancreatic ductal cells from sustained intracellular Ca^{2+} overload triggered by H_2O_2. We also report that inhibition of Orai1 protects pancreatic ductal cells from sustained intracellular Ca^{2+} overload triggered by bile acids and ethanol in combination with non-oxidative ethanol metabolites. Importantly, this protection seems to be sufficient to maintain crucial ductal functions –such as fluid and HCO_3^- secretion– both in vitro and in vivo during AP. Taking into account that Auxora is currently in Phase 2b clinical trials to treat severe AP, our current results can further contribute to the development of specific pharmacological treatments for AP.

6. Summary of new observations

- This is the first study demonstrating the expression of Orai1 in the apical membrane of pancreatic ductal epithelial cells
- We described the functional activity of Orai1 in isolated mouse pancreatic ductal cells
- The inhibition of Orai1 abolishes bile acid or ethanol+palmitic acid-induced extracellular Ca^{2+} influx in the pancreatic ductal epithelia thereby prevent the Ca^{2+}-overload-mediated functional and morphological damage of ductal cells
- Inhibition of Orai1 prevents bile acid- and ethanol-induced decrease of HCO_3^- secretion and CFTR function in pancreatic ductal epithelia
- Our results suggest that bile acids and ethanol induce Orai1-mediated Ca^{2+} influx in a Stim1-dependent manner.
- Inhibition of Orai1 preserves the ductal ion and fluid secretion both *in vitro* and *in vivo* in different forms of AP
- This is the first study demonstrating the expression and function of TRPM2 in pancreatic ductal epithelial cells
- Sustained elevation of $[Ca^{2+}]_i$ in response to H_2O_2 was largely due to TRPM2-channel-mediated influx of extracellular Ca^{2+}
- CDC inhibited the HCO_3^- secretion both in TRPM2 KO and TRPM2 WT ductal cells, the genetic knockout of TRPM2 had no protective effect, suggesting that bile acids affect ductal cells via a TRPM2-independent mechanism.

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