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The effects of Orai1 channel inhibition in chronic pancreatitis

Ph.D. Thesis

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List of publications

List of publications related to this thesis:

I. **Viktória Szabó**, Noémi Csákány-Papp, Marietta Görög, Tamara Madácsy, Árpád Varga, Aletta Kiss, Bálint Tél, Boldizsár Jójárt, Tim Crul, Krisztina Dudás, Mária Bagyánszki, Nikolett Bódi, Ferhan Ayaydin, Shyam Jee, László Tiszlavicz, Kenneth Stauderman, Sudarshan Hebbar, Petra Pallagi, József Maléth; Orai1 calcium channel inhibition prevents progression of chronic pancreatitis. **THE JOURNAL OF CLINICAL INVESTIGATION INSIGHT** (2023) D1 **IF: 9.484**

List of publications not related to this thesis:

I. Bálint Tél, Noémi Papp, Árpád Varga, Viktória Szabó, Marietta Görög, Petra Susánszki, Tim Crul, Aletta Kis, Ingrid H Sendstad, Mária Bagyánszki, Nikolett Bódi, Péter Hegyi, József Maléth, Petra Pallagi; Thiopurines impair the apical plasma membrane expression of CFTR in pancreatic ductal cells via RAC1 inhibition. **CELLULAR AND MOLECULAR LIFE SCIENCES** (2023) D1 **9.234**

II. Tamara Madácsy, Árpád Varga, Noémi Papp, Bálint Tél, Petra Pallagi, Viktória Szabó, Aletta Kiss, Júlia Fanczal, Zoltán Rakonczay Jr., László Tiszlavicz, Zsolt Rázga, Meike Hohwieler, Alexander Kleger, Mike Gray, Péter Hegyi, József Maléth; Impaired regulation of PMCA activity by defective CFTR expression promotes epithelial cell damage in alcoholic pancreatitis and hepatitis. **CELLULAR AND MOLECULAR LIFE SCIENCES** (2023) D1 **IF: 9.234**

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IV. Petra Pallagi, Marietta Görög*, Noémi Papp, Tamara Madácsy, Árpád Varga, Tim Crul, Viktória Szabó, Melinda Molnár, Krisztina Dudás, Anna Grassalkovich, Edit Szederkényi, György Lázár, Viktória Venglovecz, Péter Hegyi, József Maléth; Bile

acid- and ethanol-mediated activation of Orai1 damages pancreatic ductal secretion in acute pancreatitis. **THE JOURNAL OF PHYSIOLOGY** (2022) D1 **IF: 6.228**

V. Surya Henry, Viktória Szabó, Enikő Sutus, Melinda K. Pirity; RYBP is important for cardiac progenitor cell development and sarcomere formation. **PLOS ONE** (2020) Q1 **IF: 3.240**

VI. Gergő Kovács, Viktória Szabó, Melinda K. Pirity; Absence of Rybp compromises neural differentiation of embryonic stem cells. **STEM CELLS INTERNATIONAL** (2016) Q2 **IF: 3.540**

VII. Olga Ujhelly, Viktória Szabó, Gergő Kovács, Flóra Vajda, Szilvia Mallok, János Prorok, Károly Acsai, Zoltán Hegedűs, Stefan Krebs, András Dinnyés, Melinda K. Pirity; Lack of Rybp in Mouse Embryonic Stem Cells Impairs Cardiac Differentiation. **STEM CELLS AND DEVELOPMENT** (2015) Q2 **IF: 3.777**

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Introduction

Chronic pancreatitis (CP) is a progressively inflammatory disease characterized by both functional and structural harm to the pancreas. CP sufferers encounter malabsorption, post-pancreatitis diabetes, severe pain, and a notable decline in quality of life. Roughly 20-30% of acute pancreatitis (AP) patients experience recurrences, with approximately 10% progressing to CP. This is linked to a 50% mortality rate within 20-25 years post-diagnosis due to infections, malnutrition, and recurrent pancreatitis (RAP) complications. CP is also the most potent risk factor for pancreatic cancer, elevating the risk by 13.3-fold, with a lifetime risk of around 50% for those with idiopathic and hereditary CP. In advanced CP stages, pancreatic tissue damage is irreversible, rendering pharmacological restoration of pancreatic functions impossible. Current clinical approaches rely on surgical, endoscopic interventions, pancreatic enzyme replacement, pain management, and nutritional support, lacking FDA-approved CP therapies. The disease progression model proposes sentinel AP events triggering RAP, leading to early CP, which evolves into established and end-stage CP. While pancreas changes might resolve in RAP and early CP, they become permanent in advanced stages. Thus, intervening during RAP or early CP could potentially impede progression and address unmet needs.

CP's pathogenesis involves intricate interplay of genetic and environmental factors damaging acinar and ductal cells, activating pancreatic stellate cells (PSCs), and immune cells. Identifying shared pathogenic events in these cells for potential drug targets is crucial. Intracellular Ca^{2+} signaling, a universal pathway in eukaryotic cells including pancreatic ones, stands out as an example. Sustained Ca^{2+} elevation is a hallmark of acute and chronic inflammatory diseases like pancreatitis. This involves agonist-stimulated release of Ca^{2+} from endoplasmic reticulum (ER) stores, activating STIM1 and facilitating extracellular Ca^{2+} influx through plasma membrane Ca^{2+} channels like Orai and TRPC. This process, store-operated Ca^{2+} entry (SOCE), is regulated by ER proteins (e.g. SARAF) and Orai1. Selective Orai1 inhibition has shown promise in diminishing Ca^{2+} overload and reducing severity of pancreatitis. However, Orai1's potential benefits in RAP or early CP remain unexplored. Moreover, it's unclear if selective disruption of Orai1-mediated extracellular Ca^{2+} influx can hinder functional damage and fibrosis in CP. This study aims to extensively investigate SOCE's role in RAP and early CP pathogenesis, evaluating Orai1 inhibition's effect on disease progression with focus on various exocrine pancreas cell types.

Methods

Human samples

Human pancreatic tissue samples (from control and CP patients) were obtained from formalin-fixed paraffin-embedded (FFPE) tissue samples collected at the Department of Pathology, University of Szeged. The diagnosis of CP was established based on the clinical history and histological findings. Control pancreas samples were obtained from cadaver organ donors, who had no documented pancreatic disease.

Animals

8–12-week-old FVB/N mice weighing 20-25 g were utilized in our experiments. The mice were kept at a constant room temperature of 22-24°C with a 12-hour light–dark cycle and free access to food and drink. The gender ratio was 1:1. VRF1(P) standard rodent food and standard bedding were used. The treatments were carried out during the light cycle.

Induction and evaluation of recurrent acute and chronic pancreatitis

RAP and CP was induced by repetitive intraperitoneal (i.p.) cerulein injections. The mice received 3 or 5 series of 8 hourly physiological saline (p.s., control group) or 50 µg/bwkg cerulein injections every third day. The selective Orai1 inhibitor CM5480 (i.p.; 20 mg/bwkg; provided by CalciMedica) or vehicle was administered daily on the last 5 consecutive days (after 3 episodes of RAP). 24 hours after the last cerulein injections, the mice were anesthetized and *in vivo* pancreatic fluid secretion was measured as described previously. The severity of CP was assessed by determining the pancreas weight/body weight ratio, histological parameters and by biochemical assays.

Isolation of pancreatic ductal fragments, acinar and stellate cells from mouse pancreas

After terminal anesthesia the pancreas was removed and digested with collagenase, then intra/interlobular ducts were isolated under stereomicroscope. For pancreatic acinar and stellate cell isolation, the pancreas was enzymatically digested, minced into 1-3 mm^3 pieces and placed into shaking water bath at 37°C for 20 min (acinar) or 45 min (PSC) as described previously by *Fanczal et al.* and *Meng et al.* with modifications. Acinar cells were collected and resuspended in supplemented Media 199 and used within 4 hours. After centrifugation and filtration of PSCs, the pellet was plated with feeding media. The medium was changed every other day. PSC cultures were maintained for one week until subsequent analysis.

Measurement of intracellular Ca^{2+}, Cl^- and pH by fluorescence microscopy

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) and Cl^- ($[Cl]_i$) concentration, or intracellular pH (pH_i) of isolated cells were evaluated by microfluorometry as described earlier by using Fura-2-AM (2 μmol/l), MQAE (2 μmol/l), or BCECF-AM (1 μmol/l) fluorescent dye, respectively.

Immunohistochemistry and immunofluorescence labelling

For histopathology or IHC pancreata were fixed in formalin, paraffin embedded and 4 μm thick sections were cut. For IHC, specific antibodies were used for immune cell and other examined markers. For immunofluorescence staining whole samples were cryosectioned or PSCs seeded onto cover glasses were fixed and labelled with primary antibodies overnight then secondary antibodies for 2 hours.

Gene expression analysis

mRNA from human FFPE and mouse pancreas samples was isolated by NucleoSpin totalRNA FFPE XS kit or NucleoSpin® RNA Plus kit. mRNA from PSCs was isolated by NucleoZOL based on the manufacturer's instructions. 1 μg mRNA was reverse transcribed and 50 ng cDNA was used for quantitative real-time PCR analysis in a LightCycler® 96 System. The fold change in transcript levels was calculated by normalizing the threshold values to *Psmb6* (to *Cftr* and in case of *in vitro* experiments), *GAPDH* (to *SARAF*) and *Rpl13a*.

Western blot analysis

Pancreatic tissues were sonicated in RIPA lysis buffer then protein concentrations were determined by the BCA assay. 20 μg of protein was loaded per sample to the gel then transferred onto PVDF membrane. Proteins were detected by probing with anti–TMEM66 (SARAF) and anti-beta-actin followed by anti-rabbit–HRP and developed with Clarity Western ECL Substrate before being visualized on the ChemiDoc Imaging System.

Oil Red O staining, in vitro proliferation, migration and cell death measurements

PSCs were plated into cell culture dishes and stained with Oil Red O solution. The cell densities were determined by Crystal Violet staining. Alternatively, Crystal Violet dye was solubilized and optical density was measured with a CLARIOstar® Plus plate reader. To analyze the cell cycle phase ratio PSCs were incubated with Bromodeoxyuridine (BrdU) for 1 hour, labeled with anti-BrdU antibody and DAPI, then flow cytometry analysis was performed. The living/apoptotic/necrotic cell ratio was determined by Apoptosis/Necrosis Assay Kit in CLARIOstar® Plus plate reader. For the wound healing assay PSCs were grown to confluency for 1 week and a cell-free region was made by scratching the cell monolayer and monitor cell

migration. Images were taken at 0-hour, 10-hours, and 20-hours after scratch by Zeiss Primovert microscope and analyzed with Fiji software.

Statistics

Statistical analysis and strength of significance were performed with GraphPad Prism software. All data are expressed as means ± SEM. Shapiro-Wilk normality test was applied. Both parametric (Unpaired t-test or one-way analysis of variance with Tukey's multiple comparisons test) and nonparametric (Mann-Whitney test and Kruskal-Wallis test) tests were used based on the normality of data distribution. P value below 0.05 was considered statistically significant.

Study approval

The NIH rules and the EU directive 2010/63/EU were followed while using animals. The National Scientific Ethical Committee on Animal Experimentation granted the study license number XXI./1541/2020. The collection and use of human samples, including cadaver donor pancreas, was carried out in accordance with EU norms and was approved by the Hungarian Medical Research Council's Regional Committee of Research Ethics under license number 37/2017-SZTE.

Results

SARAF expression is impaired in CP contributing to excess extracellular Ca^{2+} entry in pancreatic acinar cells

The amount of SARAF is decreased in AP, whereas STIM1 and Orai1 remained unchanged suggesting that SARAF might be more sensitive to pathological stimuli compared to other components of SOCE. In tissue samples from CP patients', IHC revealed a remarkable decrease in the amount of SARAF in pancreatic acinar cells compared to the control pancreas. Moreover, the mRNA expression of *SARAF* was decreased in the CP tissue samples as well. For further investigations, we induced RAP in mice by repetitive cerulein injections to trigger the development of early and established CP. In this model the IHC showed that the amount of SARAF in pancreatic acinar cells was significantly lower in the cerulein-treated mice compared to the control. Whereas the mRNA expression of *Saraf* was not changed significantly upon cerulein-treatment. The subcellular distribution of SARAF in pancreatic acinar cells revealed a reticular intracellular expression pattern, presumably in the ER, while Orai1 localized mainly in the basolateral membrane of the control pancreatic acinar cells. Moreover, the immunofluorescence staining confirmed that the amount of pancreatic SARAF remarkably

decreased in the cerulein-treated mice, whereas the amount of Orai1 showed no detectable changes. Notably, Orai1 was present in the peri-acinar PSCs as well. Control and cerulein-treated mice were treated with CM5480 during the last five days of CP induction then pancreatic acini were isolated. To assess whether the decreased presence of SARAF has any impact on the intracellular Ca^{2+} homeostasis we evaluated SOCE in isolated acinar clusters from control and cerulein-treated mice. SOCE was activated by the re-addition of 5 mM extracellular Ca^{2+} after depletion of ER Ca^{2+} stores with 25 μM cyclopiazonic acid (CPA) in Ca^{2+}-free solution. Under these conditions, SOCE was significantly higher in the CP group. In contrast, when the mice were treated with 20 mg/bwkg CM5480, SOCE was significantly reduced in the control and cerulein-treated animals as well. Based on these data, we conclude that the expression of SARAF significantly decreases in pancreatic acinar cells during CP, which could lead to excess extracellular Ca^{2+} influx by disturbed regulation of Orai1. Moreover, the increased Orai1-mediated Ca^{2+} entry can be prevented by Orai1 inhibition.

Inhibition of Orai1 decreases the severity of CP in mice

The augmentation of Orai1 function due to impaired SARAF protein expression may contribute to the cell damage in early CP; therefore, we hypothesized that the inhibition of Orai1 would decrease the severity of CP. Three episodes of RAP (3x8 cerulein injections) resulted in clear signs of the early CP including acinar cell atrophy, fibrosis and the presence of acinar-to-ductal metaplasia. Whereas after 5x8 cerulein injections the histology corresponded to established CP with severe atrophy and fibrosis. This was further confirmed by Crossmon's trichrome staining, which also revealed that 3x8 cerulein decreased the parenchyma percent and moderately increased tissue fibrosis. In addition, the pancreas weight/body weight ratio also reduced upon 3x8 cerulein-treatment. These parameters were significantly worsened in the mice treated with 5x8 cerulein injections accompanied by the weight loss of these animals suggesting the progression of the chronic inflammation. Importantly, *in vivo* administration of CM5480 significantly improved all parameters. No significant difference was detected between the mice receiving 3x8 cerulein injections (early CP) and the mice treated with 5x8 cerulein injections+CM5480 suggesting that the inhibition of Orai1 efficiently prevents the progression of early CP to established CP. The extent of fibrosis was also determined by the measurement of the pancreatic hydroxyproline (HyP) concentration, which is a major component of collagen. In line with the histology parameters, 5x8 cerulein injections significantly increased the pancreatic HyP concentration, which was lowered by CM5480. Next, we wanted to assess whether the inhibition of Orai1 restores the impaired SARAF protein expression observed in

CP. Our results showed that the *in vivo* treatment with CM5480 restored SARAF protein expression in pancreatic acinar cells of cerulein-treated mice, whereas it had no relevant effect on the *Saraf* mRNA expression. In contrast, gene expression of *Orai1* was significantly elevated in CP, which was decreased by the CM5480 treatment. Markedly, the elevated *Orai1* gene expression was not translated to elevated protein expression suggesting that it may be compensated by other mechanisms. Notably, we observed an altered intracellular localization of SARAF, which translocated to the apical part of the acinar cells from the reticular localization pattern upon Orai1 inhibition. This could be explained by the redistribution of ER to the apical pole of the acinar cells. More importantly, the amount of detectable SARAF was significantly increased in the cerulein-treated mice upon Orai1 inhibition These observations were also supported by western blot analysis, which confirmed that the pancreatic expression of the SARAF is remarkably impaired by cerulein treatment, while CM5480 administration partially restored the protein expression. Beta-actin was used as an internal control. It was previously described that the increased number of inflammatory cells result in the elevated expression of beta-actin protein, which was also detected in this case. Thus, inhibition of Orai1 with CM5480 restores the impaired expression of SARAF in pancreatic acinar cells and remarkably reduced the severity of CP by diminishing or even abolishing disease progression.

Orai1 inhibition impairs the tissue inflammation in CP

Chronic inflammation is the hallmark of CP that leads to permanent tissue damage and loss of pancreatic functions; therefore, we assessed the effect of Orai1 inhibition on the pro-inflammatory cytokine expression and immune cell infiltration in the pancreas. As expected, mRNA levels of proinflammatory cytokines – tumor necrosis factor alpha (*Tnfa*), interleukin-1β (*Il1b*) - and transforming growth factor β1 (*Tgfb1*) were significantly elevated in cerulein-treated mice and were markedly lowered by the administration of CM5480 in the CP group. Notably, the inhibition of Orai1 decreased the expression of *Il1b* and *Tgfb1* in the control animals as well, which could be explained by the role of Orai1 in the immune system. To decipher the immune cell infiltration during CP the percentage of CD3-positive cytotoxic and helper T-cells; CD8-positive cytotoxic T-cells; CD19-positive B-lymphocytes; F4/80-positive macrophages and myeloperoxidase-positive (MPO) neutrophil granulocytes were determined and normalized to the total cell numbers in a field of view. Similar to the mRNA expression results, we detected a remarkable elevation of immune cell infiltration of the pancreas in CP, which was significantly impaired by Orai1 inhibition, whereas CM5480 had no significant effect in the control animals. These results indicate that inhibition of Orai1 reduces pro-

inflammatory immune cell infiltration and cytokine expression, thereby alleviating the inflammatory processes in CP.

CM5480 significantly improves the diminished acinar and ductal cell functions in CP

Next, we wanted to clarify whether the impaired inflammation and tissue fibrosis is sufficient to maintain crucial exocrine pancreatic functions, such as acinar cell enzyme production, or ductal ion and fluid secretion. IHC and enzyme activity measurements revealed that the tissue alpha-amylase and elastase activities were significantly diminished in mice treated with cerulein, which was significantly improved by Orai1 inhibition in the CP animals, but not affected in the controls. This indicates that the number of functional acini is highly decreased in CP, but significantly improved by CM5480 treatment. Additionally, secretin-stimulated *in vivo* ductal fluid secretion was significantly impaired by cerulein-treatment. In contrast, this was completely restored to the control level in mice treated with cerulein and CM5480. To measure ductal HCO_3^- secretion, pancreatic ductal fragments were isolated from mice in each experimental group and intracellular alkalization was triggered with the administration of 20 mM NH_4Cl in HCO_3^-/CO_2 buffered extracellular solution. Under these conditions, the inhibition of Orai1 had no effect on the HCO_3^- extrusion in the control group, whereas cerulein-treatment significantly diminished the ductal HCO_3^- secretion, which was prevented by CM5480 treatment. The mislocalization and impaired function of CFTR in pancreatic ductal cells are known characteristics of CP; therefore, we also investigated the effect of Orai1 inhibition on these features. The intracellular Cl^- levels were investigated with MQAE, which reflects the CFTR-mediated Cl^- extrusion. Removal of extracellular Cl^- increased the fluorescent intensity, which was significantly reduced by cerulein. *In vivo* CM5480 treatment improved the cerulein-induced decrease significantly suggesting a restoration of CFTR function. Immunolocalization revealed that in control ducts, CFTR and Occludin (used as an apical membrane and barrier integrity marker) localized to the apical surface; however, in the cerulein-treated mice a translocation of CFTR was observed into the cytoplasm of ductal epithelial cells. Importantly, inhibition of Orai1 was able to restore the apical plasma membrane localization of CFTR. Finally, analysis of *Cftr* mRNA expression showed a statistically measurable decrease in the cerulein and cerulein+CM5480 treated groups, but these are unlikely biologically meaningful alterations. In summary, our data demonstrated that the inhibition of Orai1 can prevent the damage of pancreatic acinar and ductal cells and restores the physiologically essential exocrine pancreatic functions.

CM5480 inhibits the activation of PSCs

To understand how Orai1 inhibition decreased pancreatic fibrosis in CP, we investigated the effect of CM5480 on PSCs. Immunofluorescence staining with the stellate cell markers alpha smooth muscle actin (α-SMA) and vimentin (VIM) showed relatively low levels in the control pancreas, which was significantly increased by cerulein administration and double positive cells (activated PSCs) clearly showed periacinar localization. Orai1 inhibition significantly reduced the relative intensities of α-SMA and VIM, indicating a decrease of activated PSCs. On the other hand, *Acta2* (the gene encoding α-SMA) mRNA expression, which was elevated in the cerulein-treated group, was not decreased by CM5480. The decrease of α-SMA protein expression by Orai1 inhibition was also confirmed by IHC. Additionally, immunostaining of glial fibrillary acidic protein (GFAP), a panPSC marker (stains the quiescent and activated PSCs) revealed that PSCs were present in the control groups in relatively low percentage but were increased after cerulein-treatment. Notably, the increase in GFAP was not prevented by Orai1 inhibition. In contrast, the ratio of GFAP and α-SMA-positive PSCs indicated that activated PSCs were dominantly present in CP and were remarkably diminished by CM5480. PSCs are known to activate rapidly in *in vitro* culture (within 48 h), which was utilized to study the effect of Orai1 inhibition on the activation process. Therefore, PSCs were isolated from control mice and maintained *in vitro* up to one week. PSCs were treated with 10 μM CM5480 or vehicle every other day for 1 week and were immunostained for VIM, GFAP, α-SMA and Orai1. The relative intensities of VIM and GFAP were lower, while the intensity of α-SMA was higher in the control group indicating the activation of PSCs in the control sample. In contrast, CM5480 treatment increased VIM and GFAP but decreased the α-SMA expression suggesting inhibition of the PSC activation. Additionally, Oil Red O staining revealed that the lipid droplets within the PSCs were relatively rare in the myofibroblast-like cells in the control culture, while CM5480 treatment significantly increased the percentage of the round-shaped lipid droplet containing quiescent PSCs. Gene expression analysis showed no change of *Orai1*, *Saraf* and *Tgfb1* mRNA levels, while *Acta2* and Fibronectin-1 (*Fn1*) expression, which are markers of activated PSCs, significantly reduced upon Orai1 inhibition. Notably, activated myofibroblast-like PSCs in the control culture displayed spontaneous Orai1 puncta formation (8.51±1.373% of the cells), which is a well-described feature of Orai1 activation, which was accompanied by extracellular Ca^{2+} influx. Such cells were not detected in the CM5480-treated cultures. In addition, the intensity of Orai1 was significantly higher in the control, compared to the CM5480-treated cells. These results demonstrated that inhibition of Orai1 prevented the

transformation of quiescent-to-activated PSCs, which can explain the significantly impaired progression of fibrosis in CP.

Orai1 inhibition decreases the proliferation and impairs the migration of activated PSCs

Activation of PSCs greatly increases their proliferation and migration. To investigate the effect of Orai1 inhibition to these parameters mouse pancreatic tissue was immunostained for the mitotic marker phospho-Histone H3 (Ser10) (pHH3). While pHH3-positive cells were only sporadically detected in the control groups, cerulein-treatment significantly increased the number of proliferating cells within the acinar clusters (intra-acinar) and in the inter- or periacinar space. Notably, significantly more proliferating cells were observed in the inter-acinar areas in the cerulein+CM5480 treated group. Since pHH3 also marks apoptotic cells, cerulein+vehicle and cerulein+CM5480 pancreatic tissues were costained for pHH3 and cleaved-PARP (a marker of apoptosis). Double-positive cells were absent in cerulein treated samples but were present in the inter-/periacinar area of CM5480-treated group (35.25±0.83%) beside the pHH3 single-positive cells. For further analysis PSCs were isolated and cultured in vitro. First, the number of PSCs in culture was assessed by staining with Crystal Violet, which showed a significantly lower cell number in the CM5480-treated samples based on image analysis and colorimetric measurement as well. *In vitro* pHH3 staining was performed to determine whether the decreased cell number was due to increased apoptosis or decreased proliferation, but no significant difference was found between the control and CM5480-treated cultures. Similarly, no difference was observed in the gene expression of the proliferation marker *Ki-67* and H3 clustered histone 4 (*H3c4*), a core component of the nucleosome. Next, we quantified the percentage of bromodeoxyuridine (BrdU) positive cells (marking the newly synthetized DNA) with flow cytometry, which showed that the number of cells in the S phase was significantly higher in the CM5480-treated sample as compared to control. Concomitant with this increase, we have also observed a decrease of average number of cells in G1 and G2/M phases in the treated cultures. These results indicate that the S to G2/M transition was slowed down or blocked by the inhibition of Orai1, pointing to the importance of SOCE in this cell cycle phase in PSCs. Another plausible explanation for the lower cell number in the Orai1 inhibited sample could be an increased rate of cell death. This was further supported by the increased number of PARP-positive PSCs *in vivo*, indicative of enhanced apoptosis. To test this, the late apoptosis/necrosis and living cell ratio was determined, which showed a moderate decrease of living cells and an increase of apoptotic cells in the CM5480-treated group; however, the difference may not be biologically relevant. The gene expression of the late

apoptotic marker *Casp3* was moderately lower in the CM5480-treated cell cultures, whereas PARP-staining showed no difference. Altogether, these data suggest that the lower cell number in the CM5480 treated samples was caused by the impaired proliferation and not by increased cell death. Finally, the migration of PSCs was compared by determining the wound healing at 0, 10 and 20 h. The images showed that the inhibition of Orai1 significantly impaired the speed of PSC migration as the percentage of wound closure was significantly lower after 10 and 20 hours of recovery. In summary, we can conclude that inhibition of Orai1 significantly impairs the S to G2/M transition, cell proliferation and migration of PSCs.

Discussion

Our study investigated the role of Orai1 inhibition in preventing the progression of early chronic pancreatitis (CP) using a combination of *in vitro* and *in vivo* experiments. Chronic pancreatitis is a debilitating disease characterized by pancreatic tissue damage, inflammation, fibrosis, and impaired exocrine functions. Our study focused on the molecular mechanisms involving the Orai1 protein, which is responsible for regulating intracellular calcium (Ca^{2+}) levels. Dysregulation of Ca^{2+} signaling has been implicated in the pathogenesis of AP and CP. First, we identified impaired expression of the SARAF protein in pancreatic acinar cells of CP patients. This reduction in SARAF expression was associated with excessive extracellular Ca^{2+} entry into the cells. We used an Orai1 inhibitor, which effectively mitigated this excessive Ca^{2+} influx, restored SARAF expression, and halted the progression of early CP. These positive effects of Orai1 inhibition included reduced tissue damage, decreased fibrosis, lower inflammatory cell infiltration, preserved production of digestive enzymes by acinar cells, and maintenance of ductal secretory function. Consequently, this study suggests that Orai1 inhibition holds promise as a targeted therapy for preventing the development of end-stage CP.

We also examined the impact of Orai1 inhibition on various cell types within the exocrine pancreas. Inflammatory cells, particularly mononuclear cells like macrophages, play a critical role in the onset and progression of CP. Orai1 has a central role in the activation of T lymphocytes, and the study demonstrated that inhibiting Orai1 reduced the expression of key cytokines involved in CP pathogenesis. Orai1 inhibition also diminished immune cell infiltration into pancreatic tissue, further supporting its potential therapeutic role. The effects of Orai1 inhibition extended to pancreatic acinar and ductal cells. In CP, dysfunction of these cells leads to impaired secretion of digestive enzymes and bicarbonate-rich fluid. We found that Orai1 inhibition allowed pancreatic acinar cells to continue producing enzymes, prevented

impairment of ductal cell function, and restored proper cellular localization of a key chloride channel (CFTR) responsible for regulating fluid secretion.

The role of PSCs in fibrosis development was also explored. In CP, PSCs transition from a quiescent state to an activated, fibrosis-promoting state. The study demonstrated that Orai1 inhibition prevented PSC activation, reduced proliferation and migration of fibrosis-promoting PSCs, and contributed to decreased fibrosis. We noted that our findings were relevant to multiple proposed mechanisms of CP pathogenesis and highlighted that Orai1 inhibition addressed factors such as tissue damage, inflammation, oxidative stress, and ductal dysfunction, all of which contribute to CP development. Despite the promising results, there are still open questions; the suitability of the mouse model for modelling human CP and the possibility of potential tissue regeneration following CP induction need to be further investigated. Furthermore, our research has highlighted the potential impact of Orai1 inhibition on immune function, especially in long-term use.

Finally, we found that blocking Orai1 could effectively prevent the progression of early CP. Our findings suggested that inhibiting Orai1 had broad effects on various cellular processes involved in CP pathogenesis, making it a promising therapeutic strategy to pursue further. We suggest that further research into the benefits of Orai1 inhibition in patients with RAP and early CP is worthwhile.

Novel observations

- Orai1 inhibition mitigates Ca^{2+} influx and restores SARAF expression: Inhibiting Orai1 effectively reduced unregulated Ca^{2+} influx and restored SARAF expression.
- Preservation of tissue and cellular functions: Orai1 inhibition was associated with reduced tissue damage, showing less edema and fibrosis with increased parenchyma amount.
- Reduced the inflammatory parameters: CM5480 significantly decreased inflammatory cell infiltration and inflammatory marker gene expression.
- Helped to preserve the function of the acini, such as acinar enzyme production, evidenced by the increased activity of tissue amylase and elastase.
- CM5480 maintained ductal secretory function, increased HCO_3^- and Cl^- secretion and restored apical localization of the CFTR protein.
- Orai1 inhibition and PSCs: The study revealed that inhibiting Orai1 prevented the activation, proliferation, and migration of PSCs, which are involved in excessive ECM

protein secretion and fibrosis, suggesting a potential role of Orai1 inhibition in controlling fibrosis development in CP.

- Addressing key mechanisms in CP pathogenesis: The study demonstrated that inhibiting Orai1 targeted several key mechanisms underlying CP, highlighting the potential of Orai1 inhibitors as a comprehensive treatment option for this disease.

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