THE ROLE OF AQUAPORINS IN ACUTE PANCREATITIS

In this 3 years' project, we attempted to characterize the role of aquaporin (AQP) water channels in the physiology and pathophysiology of the pancreas and our goal was to identify novel therapeutic targets in the treatment of acute pancreatitis (AP). Recently, 13 AQPs (AQP 0-12) have been identified in mammals, from which several isoforms have been shown to be expressed in the pancreas; however, it is still unknown how these multiple AQPs are regulated and how they contribute to the development of AP. Our hypothesis was that impaired expression of AQPs may have a significant role in the decreased pancreatic fluid secretion and therefore, in the pathomechanism of AP.

1) Expression of AQPs on pancreatic ductal cells

Pancreatic ductal epithelial cells (PDECs) play a fundamental role in the maintenance of normal pancreatic function by providing a HCO_3^-- rich isotonic solution which washes out the digestive enzymes from the pancreas and provide an optimal pH environment. In the first step we investigated the presence of AQPs on PDECs. We studied three, different ductal cell lines, namely the Capan-1, Panc-1 and Miapaca-2. Using real-time PCR technique, mRNA expression of AQP1-12 was found in the Capan-1 and Panc-1 cells, whereas in Miapaca-2 only AQP-1, -3, -5, -6, -7 and -11was detected. The presence of AQP1, -3 and -6 was also confirmed at protein level in the Capan-1 cells. Since among these cell lines, only Capan-1 expresses the CFTR Cl^- channel, we followed our examinations using this cell line.

2) Effect of bile acids, ethanol and its non-oxidative metabolite on the expression of AQPs

In the next step, we wanted to study the effect of pancreatitis-inducing factors on the expression of AQPs. Gallstone obstruction and heavy alcohol consumption are one of the major causes of AP. Capan-1 cells were treated with bile acids (chenodeoxycholic acid (CDC) and glycochenodeoxycholic acid (GCDC)), ethanol (EtOH) and its metabolites (palmitoleic acid (POA) and palmitoleic acid ethyl esther (POAEE)) in different concentrations for different time points (6, 12, 24 and 48 h) and the mRNA expression of AQPs was investigated by real-time PCR. Among these agents, CDC induced the highest decrease, which was dose- and time-dependent. The inhibitory effect of EtOH, POA and POAEE appeared after 12 h, mainly at higher concentrations and largely recovered after 48 h in the continued presence of these noxas. The decreased expression of AQPs have been also confirmed at protein level, using immunohistochemistry. Interestingly, incubation of the cells in normal culture media after the treatment completely restored the expression of AQPs in all treated groups, except the CDC-treated group.

3) Effect of bile acids on the water permeability of Capan-1

In the following, we tried to characterize the effect of bile acids on the function of AQPs. We did several attempts in order to set a reliable and repeatable technique for the measurement of transepithelial water permeability (P_f) of Capan-1 cells. Measurement of P_f is a fairly difficult technique therefore we tried different methods (microplate reader and confocal microscopy) to find out which is the most suitable technique. Both techniques use the fluorescens dye, calcein-AM, which fluorescence intensity changes with the cell volume. In hypoosmotic solution, the

free cytoplasmic concentration of the dye increases due to the swelling of the cells, whereas in hyperosmotic solution, just the opposite happens and the fluorescence intensity of the dye decreases.

During the experiments with microplate reader we faced with the fact that this technique is not suitable to measure P_f. The biggest disadvantage of this technique was that the visualization of the cells is not possible during the measurement; therefore, the changes in cell volume cannot be following. Other problem with this technique is that the pancreatitis-inducing factors inhibit the proliferation of the cells, therefore the intensity of the basal fluorescence.

We have also tried to set up P_f measurement using confocal microscopy. After a several failed attempts we have finally managed to set up a method which allows the reproducible measurement of P_f through Capan-1 cells. Using this method, we found that CDC dose-dependly decreased the P_f of Capan-1 cells.

4) Investigation of AQP expression in human pancreatic duct

Using the laser microdissection technique our aim was to isolate intra-interlobular pancreatic ducts from paraffin-embedded, human pancreatic tissue sections (obtained from normal, acute and chronic pancreatitis patients), and compare the mRNA expresssion of the 12 AQP isoforms using RT-PCR. We microdissected pancreatic ducts from tissue section (10 sections in each group) and then isolated mRNA for the RT-PCR reaction. Unfortunately, the quality and quantitiy of the isolated mRNA was not good enough to run a PCR.

In addition, we also investigated the protein expression of AQPs in human tissue sections using immunohistochemistry. Strong AQP1 staining has been detected both at the luminal and basolateral membrane of intralobular and intercalar pancreatic ducts and on the plasma membrane of centroacinar cells, whereas antibodies against AQP3, -5 and -6 has not shown any staining. We also showed that expression of AQP1 was significantly higher in chronic pancreatitis patients compare to control, which indicate that in chronic pancreatititis upregulation of AQPs may try to compensate the decreased fluid secretion.

5) Examination of HCO_3^- and fluid secretion in AQP knock out mice

We used both *in vitro* and *in vivo* approaches in order to investigate pancreatic secretion in AQP-1 and -4 knock out (KO) mice.

A) Measurement of HCO_3^- secretion in isolated pancreatic ducts
We have isolated intra-interlobular pancreatic ducts both from wild-type (WT) and AQP-1 and -4 KO animals. Pancreatic HCO_3^- secretion was measured using the Cl^- withdrawal technique. Our results showed that intracellular pH (pH_i) alkalinisation induced by removal of luminal Cl^- was significantly reduced in AQP-1 (42±3,2%) and AQP-4 (52±4,5%) KO vs. WT mice indicating that HCO_3^- secretion was impaired in AQP-1 and AQP-4 KO ducts.

B) Measurement of fluid secretion in isolated pancreatic ducts
To investigate if ductal fluid secretion was also compromised in KO mice, the rate of fluid secretion was measured over 30-40 min using sealed ducts and the swelling

technique (Pallagi et al., Crit Care Med, 2014). Initially, ducts were perfused with the standard HEPES-buffered solution and then perfusion was switched to the standard HCO_3^-/CO_2-buffered solution to initiate HCO_3^--dependent secretion. In the absence of secretagogue, we could not detect any significant changes in the volume of either the WT or the AQP KO ducts. Stimulation of the WT ducts with 5 μM forskolin caused dynamic swelling of the ducts as a result of fluid secretion into the closed luminal space. In contrast, ducts from AQP-1 and AQP-4 KO mice showed no or only a slight response to forskolin.

C) Measurement of fluid secretion using magnetic resonance imaging
We also examined the rate of pancreatic juice secretion *in vivo* in anesthetized mice. We used magnetic resonance imaging cholangiopancreatography to measure total excreted volume in WT and AQP KO mice in collaboration with the Greisfald University. On retro-orbital injection of 10 U/kg body weight secretin, the increase in total excreted volume (TEV) in WT animals (0.023 TEV/cm^3) was significantly higher than in AQP-1 (0.0041 TEV/ cm^3) and AQP-4 KO animals (0.0068 TEV/ cm^3).

Taken together, these results demonstrate that pancreatic fluid and HCO_3^- secretion was significantly reduced in AQP-1 and AQP-4 KO compared to WT animals. Since previous studies have demonstrated that decreased fluid secretion of ductal cells may contribute to the development of AP (Hegyi et al., Am J Gastroenterol 2010), our data suggest, that the absence of AQPs increases the risk of AP development.

6) Cerulein-induced pancreatitis in AQP-1 and AQP-4 knock-out mice

In order to investigate the role of AQPs in the pathomechanism of acute pancreatitis WT and AQP-1 and -4 KO mice were given 10 hourly injections of either physiological saline (control) or supramaximal doses of cerulein (50 μg/kg per injection) i.p. to induce acute pancreatitis. After saline injection the pancreas had normal histology for both WT and KO animals. In contrast, i.p. injections of cerulein caused extensive cell damage. We have found that the rates of necrosis were markedly higher in the AQP-1 and -4 KO compared to WT mice. There were, significant differences in the amount of interstitial edema (2.0 ± 0.11, for WT vs. 2.6 ± 0.2 for AQP-1 KO and 3.4 ± 0.3 for AQP-4 KO) and leukocyte infiltration (1.72 ± 0.08 for WT vs. 1.85 ± 0.13 for AQP-1 KO and 2.25 ± 0.23 for AQP-4 KO) between cerulein-treated WT and AQP KO animals

7) Connection between CFTR and AQPs

We have also investigated the relationship of AQPs with ion channels. It has been demonstrated that water secretion is driven by cystic fibrosis transmembrane conductance regulator (CFTR)-dependent Cl^- transport in a variety of epithelia. Moreover, studies in *Xenopus* oocytes showed enhanced osmotic cell swelling after expression and cAMP-dependent activation of CFTR. Burghardt and co-workers showed colocalization of AQP1 and AQP5 with CFTR at the apical membrane of intercalated duct cells. These studies suggest coupled functional work between Cl^- transport driven by CFTR and water transport. It has been also shown that CFTR-dependent stimulation of osmotic water permeability is absent in airway

cells derived from cystic fibrosis (CF) patients indicating that interaction between CFTR and AQPs has a pathophysiological impact on the lung disease in CF. However, no information is available whether a similar interaction could be observed in the pancreas. Therefore, in this study we also planned to investigate the relation between AQPs and CFTR in the PDECs.

A) Expression of AQPs in CFTR KO mice
   Immunohistochemical staining of the pancreas obtained from CFTR KO mice showed decreased expression of AQP-1 compared to WT mice

B) Expression of CFTR in AQP KO mice
   In order to investigate whether the absence of AQP-1 also affect the expression of CFTR, pancreas was removed from AQP-1 KO mice and the expression of CFTR was investigated by IHC. Unfortunately, despite several attempts, we were unable to set an IHC protocol for CFTR staining, therefore this part of the study is still under examination.

C) Activity of CFTR in AQP KO mice
   In order to investigate CFTR activity intra-interlobular ducts were isolated from the pancreas of AQP-1 KO and WT mice and then single pancreatic ductal cells were obtained from the ducts for patch clamp experiments. Activity of CFTR was measured after forskolin administration using the whole cell configuration of the patch clamp technique. We showed that CFTR activity significantly decreased in ductal cells obtained from AQP-1 KO animals compared to WT animals.

Taken together these data suggest that there is a strong interaction between CFTR and AQPs which may explain the decreased fluid secretion in AQP KO animals.

In conclusion, we demonstrated that AQPs play an important role both in the physiology and pathophysiology of the pancreas which was confirmed by numerous methods, including *in vivo* and *in vitro* examinations on different experimental models (cell lines and primer tissue) and also on AQP gene-modified animals. Therefore, we believe that after the completion of the remaining experiments we will be able to publish our result in a prestigious gastroenterological journal.