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Investigation of the stimulatory effect of type 1 diabetes mellitus on the ion transport mechanisms of pancreatic ductal epithelial cells and a novel method for their study

Ph.D. thesis

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1. INTRODUCTION

1.1. The pancreas

The pancreas is dual gland composed of an exocrine part, which secretes digestive enzymes to break down nutrients in the intestinal lumen, and a dispersed endocrine part – the islets of Langerhans – which secretes hormones into the blood stream to regulate metabolism. The endocrine part of the pancreas is unique in the way that it is dispersed and embedded into the exocrine tissue, rather than forming an anatomically separate gland.

The functional unit of the endocrine pancreas is the islet of Langerhans. Islets are mainly composed of five secretory cell types: α- (~35%), β- (~55%), δ- (~5%), PP- (<1%), and ε-cells (~0.15%). β-cells are usually most abundant, and their main role is insulin secretion, which is induced by post-prandial increase of blood glucose concentrations. Insulin lowers blood glucose level by inducing the uptake and storage of nutrients by other organs and reduces their *de novo* synthesis. α-cells principally secrete glucagon, which is induced by hypoglycaemia. In the liver, glucagon induces glycogenolysis and gluconeogenesis to increase and maintain blood glucose level inside a physiological range. δ-cells secrete somatostatin, which in the islet is a potent paracrine inhibitor of insulin and glucagon release. PP-cells produce pancreatic polypeptide (PP), which is released into the circulation after a meal, stimulated mainly by the *vagus* nerve. PP has effects on gastrointestinal motility, gastric acid production, glucose metabolism, and satiety. ε-cells secrete ghrelin, which stimulates growth hormone release, appetite, energy storage, and gastrointestinal motility.

The functional unit of the exocrine pancreas is the acinus, which consists of pyramidal acinar cells forming a central cavity, to where they secrete a fluid, rich in Cl^-, proteins, zymogens, and enzymes. These enzymes are drained from the central cavity into an intercalated duct. Multiple acini and intercalated ducts form a larger lobule with a main intralobular duct. Intralubolar ducts form even larger interlobular ducts, which combine to form the main pancreatic duct, which drains the enzymes into the duodenum. This tree-like ductal system is composed of pancreatic ductal epithelial cells (PDECs), which play a key role in the production of pancreatic juice by adding water to dilute, and replacing Cl^- to HCO_3^- to alkalinize the secretions of acinar cells. The key transporters of HCO_3^- secretion are most highly expressed in the intercalated ducts, which are suggested to be the main sites of fluid and HCO_3^- secretion in the human pancreas. As the juice flows along the ductal tree, it is sequentially modified to obtain increasingly higher concentrations of HCO_3^-. The alkaline juice functions to prevent

premature activation of the digestive enzymes and to neutralize acid chyme entering the duodenum from the stomach.

1.1.1. Ion transport mechanisms of pancreatic ductal epithelial cells

The transporters involved in fluid and HCO_3^- secretion are differentially expressed on the apical and basolateral membrane. Furthermore, ion transport mechanisms differ in cells located in the proximal versus the distal regions of the ductal tree. HCO_3^- can enter the cell via multiple ways through the basolateral membrane. One way is passive diffusion of CO_2 into the cell, where it is hydrated by intracellular carbonic anhydrase (CA), and the H^+ ions produced are exported through the basolateral membrane by the Na^+/H^+ exchanger (NHE-1). The electrochemical driving force of Na^+ is generated by the functioning of the Na^+/K^+ ATPase pump and K^+ channels. However, the overwhelming majority of HCO_3^- is actively transported into the cell by the $1Na^+/2HCO_3^-$ cotransporter, NBCe1-B. Secretion of HCO_3^- through the apical membrane and into the lumen occurs via the solute carrier family 26 member 6 (SLC26A6) $2HCO_3^-/1Cl^-$ exchanger, with the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) Cl^- channel providing the recycle of Cl^- ions. The transcellular movement of HCO_3^- generates a negative electrical potential in the lumen that drives the paracellular movement of Na^+ ions. Water follows Na^+ and HCO_3^- osmotically via both paracellular and transcellular routs, facilitated by aquaporin 1 and 5 (AQP1, -5). Because the poor basolateral and high apical permeability of duct cells to Cl^-, most of the luminal Cl^- is reabsorbed in proximal ductal segments due to Cl^-/HCO_3^- exchange activity. In the distal duct, the decreased luminal Cl^- concentration results in rapid reduction of $[Cl^-]_i$. When $[Cl^-]_i$ drops under 10 mM, the Cl^--regulated kinase, with-no-lysine kinase 1 (WNK1) becomes activated and changes the pore size of CFTR, making it more permeable to HCO_3^-. At the same time, WNK1 inhibits apical SLC26 anion exchangers through the STE20/SPS1-related proline/alanine-rich and oxidative stress responsive kinase 1 (SPAK/OSR1) pathways, preventing their activity in reverse mode, and thus, the reabsorption of luminal HCO_3^-. This mechanism enables 140 mM HCO_3^- concentration or higher in the final pancreatic juice.

1.1.2. Regulation of HCO_3^- secretion in pancreatic ductal epithelial cells

HCO_3^- secretion in PDECs is triggered via the cAMP/PKA pathway and potentiated by the Ca^{2+} signalling pathway. Upon entry of acidic chyme from the stomach into the proximal duodenum, neuroendocrine S-cells release SCT, which is the primary activator secretagogue of PDECs. SCT binds to its G-protein coupled receptor, localized in the basolateral membrane of

PDECs, which activates adenylate cyclase (AC). The generation of cAMP results in the activation of PKA, which phosphorylates and activates key mediators of HCO_3^- secretion, like CFTR. Activation of the cAMP/PKA pathway may also occur via the VIP receptor, VPAC1, and beta-adrenergic receptor activation. Other important agonists of PDECs are CCK and acetylcholine, which function to potentiate SCT-induced HCO_3^- secretion. CCK-B and muscarinic M2, M3 receptors, localized in the basolateral membrane of PDECs, activate phospholipase C (PLC) to generate inositol 1,4,5-trisphosphate (IP_3), which triggers Ca^{2+} release from intracellular storages. Intracellular Ca^{2+} levels are further amplified by Ca^{2+}-activated Ca^{2+} channels in the plasma membrane. The Ca^{2+} signalisation pathway may stimulate HCO_3^- secretion by activating the Ca^{2+}-dependent AC and Ca^{2+}-activated Cl^- channels.

The synergism of the two molecular pathways in PDECs is mediated by the IP_3 receptor binding protein released with IP_3 (IRBIT). In the unstimulated state, IRBIT is bound to the IP_3 receptor (IP_3R) and prevents its association with IP_3. Phosphorylation of IP_3R serine residues by PKA increases the affinity of the receptor to IP_3 and reduces its affinity to IRBIT. When stimulation of Ca^{2+} signalisation produces IP_3, IRBIT becomes dissociated from the IP_3R and recruits protein phosphatase 1 (PP1) and calcineurin to dephosphorylate vesicular NBCe1-B, CFTR, and SLC26A6, promoting their translocation to the plasma membrane. Furthermore, in the plasma membrane, IRBIT mediates the removal of specific inhibitory phosphate groups of CFTR and NBCe1-B, enhancing their activity, while also reducing the inhibitory effect of intracellular Cl^- on NBCe1-B.

1.1.3. The cystic fibrosis transmembrane conductance regulator Cl^- channel

CFTR belongs to the ATP-binding cassette (ABC) transporter superfamily and conducts Cl^- and HCO_3^- along the electrochemical gradient. It is an essential component of epithelial HCO_3^- secretion in the pancreas, lung, lacrimal and salivary glands, and other organs. Mutations in the CFTR gene can impair functions of the channel, leading to dysregulation of epithelial secretion and the disease, CF. Importantly, at low $[Cl^-]_i$, CFTR activity is modulated by WNK1 to increase its HCO_3^- permeability by potentially binding to elbow helix 1 of CFTR TDM1 and changing the pore size of the channel from 4.8 Å to 5.7 Å. The increased pore size favours the passage of HCO_3^- versus the smaller Cl^- ion. Additionally, the lasso motif of TMD1 is proposed to affect channel gating by interaction with the R domain. Through binding to Elbow helix 1, which is closely connected to – and may able WNK1 interaction with – the lasso motif, WNK1 may also affect the gating of CFTR. CFTR affects a multitude of other transporters through direct interaction. CFTR has a PDZ (PSD95/Discs-large/ZO-1) ligand at

the C-terminus end of TMD2 that can bind to PDZ domains of other proteins. Such proteins include the Na^+/H^+ exchanger regulatory factor (NHERF) family of scaffold proteins and SH3 and multiple ankyrin repeat domains 2 (Shank2). Through these scaffolds, CFTR forms functional complexes with other transporters and regulates them through direct interaction. The R domain of CFTR interacts with the sulphate transporter and anti-sigma factor antagonist (STAS) domain of SLC26 anion exchangers, hence, the two transporters are mutually activated by PKA. At the same time, PKA inhibits transporters involved in HCO_3^- salvation in the apical membrane of epithelial cells, i.e. NHE-3 and NBCn1. This process is also mediated by interaction with CFTR through NHERFs. Therefore, it has been proposed that HCO_3^- secretion and reabsorption is controlled by complex interactions between CFTR, SLC26 anion exchangers, NHE-3, and NBCn1, connected through their PDZ domains. Interestingly, direct interaction with CFTR also might be necessary for the membrane deployment of CA IV. Mutations in CFTR can affect its functions in diverse ways. CFTR mutants that lack Cl^- channel activity, but are able to activate SLC26 anion exchangers retain most of epithelial Cl^-/HCO_3^- exchange. In contrast, the activity of SLC26A6 is markedly reduced in mice expressing the aforementioned ΔF508 CFTR. Other mutants that reach the plasma membrane and are able to function as Cl^- channels may still be associated with pancreatic insufficiency.

1.2. Type 1 diabetes mellitus and pancreatic exocrine insufficiency

T1DM is a chronic disease characterized by insulin deficiency due to autoimmune-induced pancreatic β-cell loss that leads to hyperglycaemia and related complications. Stage 1 of T1DM is marked by the presence of more than one autoantibody against insulin, glutamic acid decarboxylase (GAD), protein tyrosine phosphatase IA-2 or IA-2β, or zinc transporter 8, while stage 2 is characterized by dysglycaemia or glucose intolerance. Since both stages are asymptomatic, patients usually present to the clinic at stage 3 with symptoms of hyperglycaemia, i.e. polyuria, polydipsia, enuresis, weight loss, and blurred vision. Long-term complications developed due to hyperglycaemia include microvascular (i.e. retinopathy, nephropathy, and neuropathy) and macrovascular (i.e. cardiovascular disease, cerebrovascular accidents, and peripheral vascular disease) defects, as well as pancreatic exocrine insufficiency (PEI). PEI is a complication affecting about 40% of T1DM patients. Several factors are involved in the development of PEI in T1DM. (1) Enteropancreatic reflexes may become impaired due to autonomic diabetic neuropathy. (2) Microvascular damage in the pancreas may lead to insufficient perfusion and ischemia, which contributes to the development of pancreatic

atrophy and fibrosis. (3) Hyperglycaemia was shown to inhibit SCT-induced pancreatic exocrine secretion. (4) Oxidative stress, hyperglycaemia, and cytokines released from infiltrating immune cells activate pancreatic stellate cells, which play a central role in pancreatic fibrosis. Lastly and most importantly, (5) the impaired insulin output from islets has a reduced trophic effect on the pancreatic parenchyma, which is physiologically accustomed to exceptionally high insulin levels. Accordingly, reduced pancreatic volume and acinar atrophy has been shown in patients with T1DM, and it has been demonstrated that the lack of insulin negatively affects acinar enzyme secretion. In contrast to acinar cells, there is considerably less information regarding the effect of diabetes on PDECs. Therefore, our aim in this thesis was to investigate the effect of T1DM on the ion transport mechanisms of PDECs.

1.3. Experimental systems for the functional study of PDECs

1.3.1. Experimental models widely used in pancreas research

2D cell cultures, 3D organoid cell cultures, and isolated ductal segments are the most widely used experimental model systems for the research of PDECs. However, these models are prone to phenotypical changes due to enzymatic digestion during sample isolation, culturing time, and the lack of original intercellular interactions of cells.

1.3.2. Pancreatic tissue slices

The acute tissue slice technique is an *in situ* experimental system that is widely used in research investigating the structure and function of the brain, as well as the liver, adrenal gland, and retina. The technique was first optimized by Speier and Rupnik to study β-cells and it was thereafter successfully used in the structural and functional study of both acinar and β-cells. In contrast to the experimental systems mentioned before, the preparation of tissue slices (TSs) does not require enzymatic digestion. Direct intercellular junctions and interactions are involved in maintaining pancreatic development, cellular differentiation and functional regulation. Since tissue architecture is well preserved in the slice, this technique is suitable for both morphological and functional imaging, without the risk of phenotypic changes. Electrophysiological studies using patch-clamp or microfluorimetric measurements can be conveniently implemented to TSs. Additionally, the TS technique may allow investigation of the interactions between cells of the exocrine and endocrine component of the pancreas, a field of increased focus in our workgroup.

2. AIMS OF THE STUDY

T1DM is associated with reduced quality of life, severe long-term complications, and substantial costs for the health-care system and the individual. The prevalence of T1DM is steadily increasing world-wide, however, our knowledge on the mechanisms of the disease and our ability to reduce disease-associated complications like PEI are still incomplete. Some of the few available studies on pancreatic ductal ion transport mechanisms in T1DM are outdated, or have yielded conflicting results. Because PDECs play an essential role in the maintenance of pancreas integrity, identification of the mechanism through which ductal fluid secretion is altered in diabetes may bring us closer to understanding the pathogenesis of exocrine insufficiency. Furthermore, the experimental systems widely used to study ductal secretion do not enable examination of PDECs in their physiological environment.

Therefore, our aims in the **first** part of this thesis were

I. to determine the effect of T1DM on the activity and expression of the main ductal ion transporters,
II. to define the role of CFTR in the changes of ductal ion transport in T1DM,
III. to study the underlying mechanisms in the changes of ductal ion transport in T1DM,

and our aim in the **second** part of this thesis was

IV. to investigate whether TSs are suitable for the functional examination of the pancreatic ducts.

3. MATERIALS AND METHODS

3.1. Induction of diabetes

Type 1 diabetes was induced using the low-dose STZ protocol of the Diabetic Complications Consortium. 4 weeks after the first injection, the mice were then sacrificed and exsanguinated through cardiac puncture. The pancreas was removed and a portion was processed for hematoxylin/eosin staining. Small pieces of the pancreas were fixed in 3% glutaraldehyde for transmission electron microscopy. The other portion of the pancreas was used for the isolation of ducts.

3.2. Isolation of pancreatic ducts and measurement of intracellular pH

The pancreas of WT and CFTR KO mice was used for the isolation of intra/interlobular ducts by enzymatic digestion and microdissection. Changes in intracellular pH (pH_i) were measured using the pH-sensitive fluorescence dye, 2,7-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF), and the alkaline load technique.

3.3. Measurement of HCO_3^- secretion

To estimate HCO_3^- efflux, the activity of the Cl^-/HCO_3^- exchanger was measured by the NH_4Cl pre-pulse technique and the Cl^- withdrawal technique. For the NH_4Cl pre-pulse technique, ducts were exposed to 20 mM NH_4Cl in HCO_3^-/CO_2-buffered solution, causing influx of NH_3 across the membrane and resulting fast pH_i increase. After maximal alkalization, pH_i begins to recover, reflecting the rate of HCO_3^- secretion. After the removal of NH_4Cl, pH_i drops due to the dissociation of intracellular NH_4^+ to H^+ and NH_3. The initial rate of recovery from the acid load reflects the activity of NHEs and NBC.

In the Cl^- withdrawal technique, Cl^- is removed from the external solution that causes alkalization of pH_i, which results from the reverse operation of the Cl^-/HCO_3^- exchanger. The initial rate of alkalization and the rate of recovery from alkalosis directly reflects the activity of the exchanger in reverse and normal mode respectively.

3.4. Measurement of CFTR activity

CFTR activity was analysed by measuring intracellular Cl^- concentration using N-(Ethoxycarbonylmethyl-6-Methoxyquinolinium) Bromide (MQAE) fluorescent dye and forskolin. Because of the quenching effect of Cl^- on the dye, the fluorescence intensity of MQAE is inversely proportional to Cl^- concentration. Pancreatic ducts were perfused with

HCO_3^-/CO_2-buffered solution containing 20 μM forskolin. The readings were displayed as fluorescence ratio (F/F0) and a linear trend line was plotted on the curve for the first two min (120 sec) of forskolin stimulation. The area under the plotted curve was calculated using the definite integral of the equation.

3.5. Measurement of pancreatic fluid secretion

To estimate the rate of ductal fluid secretion *in vitro*, we measured the forskolin-stimulated swelling of isolated intra-interlobular ducts using video microscopy. Pancreatic fluid secretion was assessed *in vivo* in as well, by cannulation of the main pancreatic duct and collection of juice in anesthetised mice.

3.6. Immunolabelling of frozen tissue sections

Cryomatrix-embedded frozen ducts were cut into 10 μm-thick sections. Immuno-fluorescence labelling was done based on the protocol provided at www.novusbio.com. The images were evaluated using Image J software. The measured area was restricted to the layer of epithelial cells and to a constant threshold value of intensity. Red intensity values marking the antigens of interest were normalized to the green intensity values representing β-actin.

3.7. Quantitative reverse transcription PCR (RT-qPCR)

Intra-interlobular pancreatic ducts were isolated from 6 normal and 6 diabetic mice and pooled for total RNA extraction. Total RNA was reverse-transcribed and qPCR was performed using TaqMan probe sets. The results were calculated by the $2^{-\Delta\Delta CT}$ method.

3.8. ELISA assays

Plasma samples obtained by cardiac puncture were transferred to fluoride/heparin-coated capillaries and centrifuged. Pancreas homogenates were prepared by grinding 100 mg tissue in liquid nitrogen using a mortar and pestle and dissolving it in 1 mL ice-cold protease inhibitor cocktail. SCT and CCK assays were done as per the manufacturer's instructions.

3.9. Preparation of serial tissue sections from Giemsa-injected whole mouse pancreas

10% Giemsa dissolved in 1.5% low-melting-point agarose was injected into the common bile duct of the mice. The pancreas was removed, cleaned, and the divisions of head, body, and tail were cut into three separate pieces. Each pieces were subsequently embedded in cryomatrix and cut into 15 μm sections.

4. RESULTS

4.1. Morphology of the exocrine pancreas in diabetes

The number and size of islets decreased by the destruction of insulin-producing beta-cells due to STZ treatment. Additionally, low levels of fibrosis, acinar atrophy, and leukocyte infiltration, were developed. In contrast, the structure of the ducts was preserved. Electron microscopic examination of the pancreas showed enlarged mitochondria and a fragmented mitochondrial internal membrane structure in the acini.

4.2. Pancreatic ductal fluid secretion increases in diabetes

For the *in vitro* measurements, the rate of fluid secretion was estimated from the change of luminal volume of isolated ductal segments. Following stimulation with forskolin, the amount of fluid secreted by the diabetic ducts was significantly higher than that of non-diabetic ducts. *In vivo* fluid secretion studies revealed similar results. Both basal and secretin-stimulated (1 CU/kg) fluid secretion were significantly increased in diabetic mice, compared to normal mice.

4.3. The activity and expression of ductal acid/base transporters is increased in diabetes

The alkaline load method was used to measure the activity of acid–base transporters in isolated ducts of normal and diabetic mice. Both alkali and acid regeneration were increased in diabetic mice compared with control mice, indicating that diabetes increases the activity of these transporters. To confirm this result, the Cl^- withdrawal method was used. Similarly to the alkaline load method, the exchanger showed increased activity in diabetes.

Next, we examined mRNA and protein expression of the major acid–base transporters in isolated intra-interlobular pancreatic ducts of normal and diabetic mice. RT-qPCR and immunofluorescence labelling showed increased expression of CFTR, ANO-1, NHE-1, and AQP-1 in diabetic mice.

4.4. The central role of CFTR in diabetes-induced HCO_3^- secretion

The CFTR Cl^- channel plays an essential role in ductal HCO_3^- secretion and showed increased expression in diabetes. Administration of 20 μM forskolin increased Cl^- efflux in MQAE fluorescence readings of isolated perfused ductal segments. This increase was significantly higher in diabetes.

To confirm the role of CFTR in diabetes-induced HCO_3^- secretion, we examined the activity of the Cl^-/HCO_3^- exchanger in the absence of CFTR, using CFTR KO mice using the alkaline load technique. In the absence of CFTR, the rate of HCO_3^- secretion was significantly reduced. Diabetic KO mice exhibited increased HCO_3^- secretion rates compared with their non-diabetic counterparts; however, this increment fell short compared with that observed between diabetic and non-diabetic WT mice and did not reach statistical significance. In the absence of CFTR, the diabetes-induced activity of NHE and/or NBC was significantly decreased. Additionally, in CFTR KO mice, the rate of fluid secretion was far below that of WT. The presence of diabetes slightly increased the amount of fluid secretion in CFTR KO mice, but this did not reach statistical significance. Taken together, these results indicate that CFTR plays an important role in the increased fluid and HCO_3^- secretion induced by diabetes.

4.5. Effects of high glucose on the activity of acid/base transporters

T1DM is often associated with hyperglycaemia; therefore, we determined the effect of acute and chronic glucose treatment on the activity of the transporters using the alkaline load technique. Neither acute, nor chronic glucose administration showed difference in the rate of regeneration from alkalosis compared to control ducts. However, chronic treatment increased the rate of recovery from acidosis, indicating that the activity of NHE-1 and/or NBCe1-B increases in response to chronic high glucose. The activity of the Cl^-/HCO_3^- exchanger was also examined using the Cl^- withdrawal technique; however, neither the acute nor the chronic high glucose treatment caused any significant change in activity.

4.6. Plasma levels of secretin and cholecystokinin in diabetic mice

Neither plasma SCT nor CCK levels differed between the control and diabetic mice. Secretin was also examined in pancreatic homogenates, which showed a slight, although not significant increase in diabetic animals. Finally, RT-qPCR revealed that the expression of SCTR was significantly increased in the ducts of diabetic, however, we could not confirm this finding on the protein level using fluorescence immunolabelling.

4.7. Structural integrity of ducts in pancreatic TSs

Intra- and inter-lobular ducts of the head and the body showed strong nuclear staining by Giemsa, whereas weak or no staining was found in the tail, and no staining of the surrounding tissue was found. The luminal membrane showed strong CFTR labelling and appeared structurally intact, suggesting normal functioning of PDECs.

5. DISCUSSION

The negative long-term effects of diabetes on the exocrine pancreas are numerous; yet, our understanding of the underlying mechanisms is still incomplete, particularly regarding to PDECs. In the present study, we showed that the fluid and HCO_3^- secretion of ductal cells increases as a result of diabetes. This process likely involves activation and overexpression of CFTR, NHE-1, and possibly ANO-1.

First, we examined how T1DM affects the morphology of the exocrine and endocrine pancreas using electron microscopy. The most characteristic difference between the normal and diabetic pancreas was the morphological change of mitochondria in acinar cells, which had a rounder, less elongated shape in diabetes. Additionally, enlargement of the mitochondria and disruption of the inner membrane structure were also observed. Similar results were observed in the intermediate cells (characterised by containing both zymogen and hormone storage granules) of T1DM patients and in the beta cells of T2DM patients. In contrast to acini, ductal mitochondria remained mostly intact, which suggests that ductal functions are preserved in T1DM. Mitochondria play an important role in maintaining the normal energy production and balance of the cells, therefore, damage of acinar mitochondria probably contributes to the development of PEI.

Using *in vivo* and *in vitro* approaches, we demonstrated that the amount of pancreatic fluid was significantly increased in streptozotocin-induced diabetic mice. This was observed under both basal conditions and under stimulation of the cAMP/PKA pathway by forskolin or SCT, suggesting an increased activation of CFTR, and suggesting that the sensitivity of stimulated ductal fluid secretion to SCT is also increased.

The rate of fluid secretion primarily depends on the rate of HCO_3^- secretion, and therefore, on the activity of ductal ion transporters. The major route for HCO_3^- secretion in the ductal cells is the apically localized Cl^-/HCO_3^- exchanger, SLC26A6, which mediates the electrogenic exchange of 1 Cl^- and 2 HCO_3^-. Using the alkaline load method, we found that the activity of the Cl^-/HCO_3^- exchanger is increased in diabetes. The Cl^- withdrawal technique allows for the direct measurement of exchanger activity. Similarly to the alkaline load method, the exchanger showed increased activity in diabetes, at least in normal mode. Interestingly, there was no difference in the degree of alkalosis, suggesting that the activity of the exchanger in reverse mode is not affected by diabetes. However, it is also possible that the increased HCO_3^- efflux, (but not influx) is mediated by CFTR, which becomes activated by WNK1 if $[Cl^-]_i$ drops under 10 mM.

Because the CFTR Cl^- channel and the Cl^-/HCO_3^- exchanger interact with each other through NHERs and are mutually regulated, we measured the activity of CFTR using the Cl^- sensitive fluorescent dye, MQAE, and forskolin, which induces CFTR by activating AC. We detected increased CFTR activity in diabetic ducts, which most probably contributes to the increased rate of HCO_3^- secretion.

The alkaline load technique also enables the investigation of alkalizing transporters by measuring the rate of regeneration from acidosis. We observed a significant increase in acidic recovery similarly to the alkaline recovery, indicating that the activity of alkalizing transporters was also increased as a result of diabetes. In ductal cells, the major alkalizing transporters are the basolateral NHE-1 and NBCe1-B. NBCe1-B transports HCO_3^- into the cell, whereas NHE removes H^+, a by-product of CA activity. Consequently, if the activity of the anion exchanger increases, it must be supported by the increased activity of NHE-1 and NBCe1-B, as confirmed by our experiments.

To identify the mechanism responsible for increased transporter activity in diabetes, we first tested the expression of the major acid–base transporters. We found that both the mRNA and protein expression of CFTR was significantly increased in diabetic ducts, partly explaining increased CFTR activity. We also found that other acid–base transporters involved in ductal HCO_3^- secretion are overexpressed as well. Increased NHE-1 expression was also observed in diabetic ducts, however, we did not find alterations in the expression of NBCe1-B. This may indicate that during increased secretion in diabetes, a substantial proportion of intracellular HCO_3^- is accumulated via the activity of CA and NHE-1, rather than NBCe1-B.

Among the SLC26 anion exchangers, SLC26A3 and SLC26A6 isoforms are present in the apical membrane of PDECs and play major role in ductal HCO_3^- secretion. Although our functional experiments revealed increased Cl^-/HCO_3^- exchange activity in diabetes, we found no difference in the expression of SLC26A3 and SLC26A6 compared to the control. This means that increased anion exchange activity probably occurs due to increased activation of SLC26A3 and SLC26A6 by CFTR, rather than overexpression of the exchangers.

The expression of AQP-1 was also increased in diabetes. AQP-1 facilitates transcellular water movement and, as the major water channel on ductal cells, it is responsible for most of the volume of the pancreatic juice. The overexpression of AQP-1 in diabetes is probably a compensatory mechanism for increased anion secretion because transcellular ion movements are osmotically followed by water.

An exciting finding of ours is that the Ca^{2+}-activated Cl^- channel, ANO-1, is also expressed in PDECs. Although, the presence of a Ca^{2+}-activated Cl^- current has been described

in pancreatic ductal cells, the molecular identity of this channel remained undetermined. Our study demonstrates the presence of ANO-1 on the luminal membrane of murine PDECs. Activation of ANO-1 is triggered by divalent cations – mainly Ca^{2+} – which can be antagonized by Mg^{2+}. Several signals have been described to activate the channel, such as extracellular ATP, decrease of pH_i, and heat stress. Since increase in $[Ca^{2+}]_i$ leads to the activation of the channel in acinar cells and salivary ducts, it is conceivable that the channel becomes activated in pancreatic ductal cells through the Ca^{2+}-signalisation pathway. Both murine and human PDECs show relatively large Ca^{2+}-activated Cl^- current density, and some of the major secretagogues of PDECs (e.g. acetylcholine and CCK) act through the Ca^{2+}-signalisation pathway. Furthermore, the ion selectivity ANO-1 can shift from Cl^- to HCO_3^- by modulation of its pore size, similarly to CFTR. Therefore, we hypothesize that in addition to CFTR, ANO-1 also functions in close co-ordination with apical transporters and acts as an alternative pathway for HCO_3^-, as has been shown in acinar cells. We also found that diabetes increases the expression of ANO-1, therefore, it may also contribute to increased HCO_3^- secretion. In the end however, further functional studies are needed to clarify the role of ductal ANO-1 in pancreatic fluid secretion. Our results indicate that the increased expression of CFTR, ANO-1, and NHE-1 stimulates Cl^-/HCO_3^- exchange. This suggests that finding methods which induce upregulation of these transporters may prompt the development of new therapies in the treatment of CF or acute and chronic pancreatitis.

The central role of CFTR in the increased ductal secretory activity was further confirmed by our results produced in CFTR KO mice. As expected, both fluid and HCO_3^- secretion were significantly decreased in the ducts of CFTR KO mice. When diabetes was induced in KO mice, there was a small increase in both fluid and HCO_3^- secretion, but was not statistically significant. This indicates that the presence of functionally active CFTR is essential for the stimulatory effect of diabetes on ductal secretion. Yet, the small increase observed in diabetic CFTR KO animals may indicate that the activity of other apical transporters, like ANO-1 and the Cl^-/HCO_3^- exchangers, are also affected by diabetes.

In our subsequent experiments we sought to identify the stimuli responsible for the increased activity/expression of transporters observed in diabetes. A notable factor affecting ion transport mechanisms – in the renal proximal tubules and also in pancreatic ducts – in T1DM is hyperglycaemia. Using the alkaline load and Cl^--withdrawal techniques, we found that neither acute, nor chronic glucose treatment affected anion exchange activity. However, chronic glucose treatment did increase the rate of regeneration from acidosis, which suggests that hyperglycaemia directly increases the activity of NHE-1 and/or NBCe1-B. Previous studies

showed that high extracellular glucose increases NHE-1 activity in distal nephron cells, vascular myocytes, and lymphoblasts of patients with diabetic nephropathy, possibly through the protein kinase C or the Mek/Erk1/2/p90(RSK) and p38MAPK pathways.

Another way by which ductal fluid and HCO_3^- secretion may increase in diabetes is the altered response to major secretagogues, like SCT and CCK. SCT is the primary hormone influencing the rate of HCO_3^- secretion in PDECs. Activation of basolateral SCTRs increases intracellular cAMP levels, which activate PKA. PKA subsequently activates CFTR and SLC26 anion exchangers and inhibits NHE-3 and NBCn1 within NHER-linked functional complexes, thereby inducing apical HCO_3^- transport in PDECs. CCK, produced mainly by enteroendocrine I-cells, acts on CCKBR and induces elevation of intracellular Ca^{2+} levels in PDECs. Through the Ca^{2+} signalisation pathway, CCK can potentiate SCT-induced HCO_3^- secretion mainly by activating IRBIT, but also by potentially activating the Ca^{2+}-dependent AC and Ca^{2+}-activated Cl^- channels, like ANO-1. We found no changes in plasma SCT and CCK levels, which suggest that T1DM probably does not affect the secretory activity of duodenal S-cells and enteroendocrine I-cells. However, we found that mRNA expression of SCTR is significantly increased in the ducts of diabetic mice. An increase was also observed on the protein level, but was not significant. We speculate that secretin exerts a greater stimulatory effect on ductal cells in mice with induced T1DM, however, further studies are needed to confirm this hypothesis.

In the second part of this thesis, we examined whether the acute pancreas slice technique is suitable for the functional examination of ductal cells. Giemsa staining of nuclei of PDECs, but not other structures within the pancreatic TS shows that the injected agarose gel is retained inside the ducts. Furthermore, based on the examination of pancreatic sections, the gel travels as far as the interlobular ducts of the body of the murine pancreas. The rupture of ducts and gel leakage would result agarose lumps in the tissue and would prevent appropriate gel distribution inside the ductal tree, ultimately resulting in poor tissue support during microtomy.

In a physiological state, CFTR is transferred to the apical membrane, indicated by well-defined apical staining of PDECs. The folding of CFTR is relatively complicated compared to other proteins and the maturation efficiency of even WT CFTR is only ~30%. Due to toxic substances or pathological conditions like chronic pancreatitis, CFTR accumulates in the endoplasmic reticulum and shows cytoplasmic localization. Therefore, the apical localization of CFTR on PDECs within the pancreatic TS is probably reflective of undisturbed cellular physiology, which was confirmed also by functional experiments. Finally, we conclude that the structure and function of PDECs is preserved in the pancreatic TS and that this model is suitable for the study of PDECs and their interactions.

6. CONCLUSION AND NOVEL FINDINGS

In conclusion, our results demonstrate that the stimulatory effect of T1DM on ductal HCO_3^- secretion is a complex and multifactorial process. The activity of the SLC26 anion exchangers is enhanced by the upregulated expression of ductal acid–base transporters, particularly CFTR and NHE-1. Additionally, high extracellular glucose stimulates alkalizing transporters, such as NHE-1, which may also contribute to increased secretion. Furthermore, because ANO-1 is present in PDECs and its expression is upregulated in T1DM, it may also be involved in increased secretion. The role of increased ductal secretion in T1DM, and whether it is a temporary or permanent condition, is not known. Based on clinical studies, the most accepted view is that exocrine functions are mostly impaired by long-standing diabetes. An explanation for this is that the stimulatory effect of insulin is absent in T1DM. On the other hand, our study showed that ductal secretion increases as a result of diabetes, at least in the initial stage. This would not be unprecedented, since patients with diabetic kidney disease initially exhibit increased glomerular filtration rate to compensate for decreased Na^+ delivery due to hyperglycaemia. As the disease progresses over time, glomerular filtration rates slowly decline. Therefore, we propose that increased ductal HCO_3^- secretion serves as a protective mechanism in T1DM and therefore represents a potential therapeutic target for the prevention or treatment of diabetes. Although this hypothesis is promising, further studies are needed in this area.

Our main novel findings are that

I. STZ-induced T1DM has a stimulatory effect on ductal fluid and HCO_3^- secretion,

II. the activity of the SLC26 anion exchangers is enhanced in T1DM due to upregulated expression of ductal acid–base transporters, mainly CFTR and NHE-1,

III. high extracellular glucose stimulates alkalizing transporters, such as NHE-1,

IV. ANO-1 is present in PDECs and its expression is upregulated in T1DM,

V. the acute pancreas tissue slice is a suitable model for the study of PDECs.