Physiological and pathophysiological investigation of lacrimal and pancreatic ductal epithelial 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. **Katona M**, Vizvári E, Németh L, Facskó A, Venglovecz V, Rakonczay Z Jr, Hegyi P, Tóth-Molnár E. Experimental evidence of fluid secretion of rabbit lacrimal gland duct epithelium. *Invest Ophthalmol Vis Sci.* 2014;55(7):4360-7.
[IF_{2014}: 3.661]

II. **Katona M**, Hegyi P, Kui B, Balla Zs, Rakonczay Z Jr, Rázga Zs, Tiszlavicz L, Maléth J, Venglovecz V. A novel, protective role of ursodeoxycholate in bile-induced pancreatic ductal injury.
Am J Physiol Gastrointest Liver Physiol. Accepted for publication
[IF_{2014}: 3.737]

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III. Maléth J, Balázs A, Pallagi P, Balla Z, Kui B, **Katona M**, Judák L, Németh I, Kemény LV, Rakonczay Z Jr, Venglovecz V, Földesi I, Pető Z, Somorácz Á, Borka K, Perdomo D, Lukacs GL, Gray MA, Monterisi S, Zaccolo M, Sendler M, Mayerle J, Kühn JP, Lerch MM, Sahin-Tóth M, Hegyi P. Alcohol disrupts levels and function of the cystic fibrosis transmembrane conductance regulator to promote development of pancreatitis.
Gastroenterology. 2015;148(2):427-39.e16.
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Number of full publications: **3 (2 first author)**
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LIST OF ABBREVIATIONS

$(ATP)_i$	intracellular ATP level
BCECF-AM	2'7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
cAMP	cyclic adenosine monophosphate
AE	anion exchanger (Cl^-/HCO_3^- exchanger)
CA	carbonic anhydrase
CaCC	calcium-activated Cl^- channel
CFTR	cystic fibrosis transmembrane conductance regulator Cl^- channel
EIPA	5-(N-Ethyl-N-isopropyl)amiloride
FURA-2-AM	5-Oxazolecarboxylic acid, 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acetoxymethyl ester
DIDS	diisothiocyanostilbene-2,2'-disulfonic acid
LG	lacrimal gland
LGDEC	lacrimal gland ductal epithelial cells
NBC	Na^+/HCO_3^- contransporter
NHE	Na^+/H^+ exchanger
PDEC	pancreatic ductal epithelial cells
pH_i	intracellular pH
pH_L	intraluminal pH
PKA	protein kinase A
SLC26	solute carrier family 26

1. INTRODUCTION

Vectorial transport of water and electrolytes is a fundamental physiological function of epithelial cells which ensures the appropriate volume and electrolyte composition of the various fluids. In exocrine glands, fluid and electrolyte secretion is carried out by both acinar and ductal cells. The composition of fluid, secreted by these cell types varying among glands. Acinar cells mainly secrete isotonic, plasma-like fluid which is mostly modified by the ductal cells. The key transporters mediating acinar fluid and electrolyte secretion are the $Na^+/K^+/2Cl^-$ cotransporter type 1 (NKCC1), the Ca^{2+}-activated Cl^- channels (CaCC) and the Ca^{2+}-activated K^+ channels. Whereas, ductal fluid and electrolyte secretion are mainly mediated by the Na^+/HCO_3^- cotransporter (NBC), the Cl^-/HCO_3^- anion exchanger (AEs), the cystic fibrosis transmembrane conductance regulator Cl^- channel (CFTR) and the CaCC. Decrease or damage of ductal fluid secretion can contribute to the development of numerous diseases such as pancreatitis, cystic fibrosis (CF) or dry eye disease.

1.1. Lacrimal gland

Preocular tear film is an essential protector of the ocular surface. Tear is produced predominantly in the exocrine tubuloacinar LGs. Appropriate amount of balanced electrolyte, protein and mucin composition of fluid secreted by the LG is fundamental for maintaining preocular tear film integrity. Such as other exocrine tubuloacinar glands, LG is composed mainly of three types of cells: acinar, ductal and myoepithelial cells. Acinar cell functions are widely studied, resulting in broad spectrum of information, however much less is known about the possible secretory function of duct cells. It has been proposed that primary acinar fluid is modified by ductal secretory processes: an elevated K^+ and Cl^- content of the final product evolves during passage of fluid through the ductal tree. Although acini are the determining structures of tear production, the secretory role of ducts beside their piping function was also suspected for a long while. Modifications of K^+ and Cl^- content of primary acinar fluid by ductal secretory processes were also proposed earlier. However, the role of LG ductal epithelium on fluid, electrolyte and protein secretion is not well understood. The first experimental model for the investigation of lacrimal duct function was adapted and applied by our group. Using short-term cultured duct segments allowed studying the role and regulation of various ion transporters in the lacrimal duct. These results showed the functional presence of a Na^+ dependent proton efflux mechanism (NHE) and a Cl^- dependent HCO_3^- efflux mechanism (AE) using pH-sensitive fluorescent dye in LG ductal cells. Ca^{2+} and cAMP signaling pathways in activation of epithelial fluid and electrolyte secretion are well studied in different gland types such as pancreas and salivary gland, however we are lacking of information about LG secretory functions. Dry eye disease is one of the most frequently diagnosed ocular surface pathology. Presently substitution of tear fluid with arteficial tear drops is the main, mostly insufficient, intervention to manage dry eye without treating the underlying pathologic alterations since the causative factors of the disease is mostly unknown. Our knowledge about LG function is far from complete and therefore the more detailed understanding of the physiology and pathophysiology of the lacrimal gland is essential.

1.2. Pancreas

There are two major cell types in the exocrine pancreas, the acinar and ductal cells. Both cell types play role in the secretion and the release of the pancreatic juice. Acinar cells secrete pancreatic juice rich in NaCl and digestive enzymes which travels through the pancreatic ductal system into the duodenum. Ductal cells secrete a HCO_3^- rich fluid and modify the electrolyte composition of the juice by the secretory and absorptive processes. Recent evidences suggest that pancreatic HCO_3^- secretion is playing a crucial role in the pathophysiology of the pancreas. Impaired ductal electrolyte and fluid secretion leads to acinar cell damage and to primary defect in membrane trafficking at the apical plasma membrane of acinar cells. Intraluminal pH is also play a central role in normal pancreatic functions, since luminal acidosis may elevate the risk of AP. Different etiological factors also can contribute to the development of AP such as excessive ethanol consumption, smoking, viral infections and also gallstones. The obstruction of the common biliopancreatic duct by a gallstone is a frequent cause of AP. Although, the exact mechanism is not completely understood. One of the most accepted theories is that bile reflux into the pancreatic ductal system leads to AP. We know from animal studies, that retrograde infusion of bile acids into the pancreatic duct triggers pancreatitis. Therefore the cytotoxic effects of bile acids have been widely investigated in the pancreas. Initial studies, in which the main pancreatic duct was perfused with various bile acids, demonstrated that in the mM range hydrophobic bile acids cause mucosal damage and increase the permeability of the pancreatic ducts to different ions. Bile acids also induced Ca^{2+} release from the intracellular Ca^{2+} stores. Previous results from our workgroup demonstrated that the nonconjugated bile acid chenodeoxycholic acid (CDCA) has dose-dependent dual effects on pancreatic HCO_3^- secretion, which might be explained by the intracellular Ca^{2+} signals, decrease in the ATP_i, mitochondrial damage and the inhibition of acid/base transporter activity caused by CDCA. The cytotoxic effect of CDCA is basically attributed to its detergent characteristic, which is responsible for the disruption of the membrane integrity and consequently the release of intracellular constituents. However, several studies suggest that non-detergent effects of bile acids are also involved in the bile-induced cellular injury. It has been recently demonstrated that CDCA induces ATP release from both ductal and acinar cells, which probably play a role in the CDCA-induced $[Ca^{2+}]_i$ elevation. In the absence of ATP_i, the acid/base transporters fail to function properly, which finally causes decreased fluid and HCO_3^- secretion. Impaired fluid secretion can lead to pancreatic injury and likely contributes to the development of pancreatitis. Nevertheless not every bile acid has cell damaging effect. Ursodeoxycholic acid (UDCA) is a secondary, hydrophilic bile acid, which is currently used for gallstone dissolution and considered as first-choice therapy in cholestatic diseases. The mechanism by which UDCA increases liver function is not completely understood. Basically, there are three concepts for the action of UDCA: (i) stimulation of hepatobiliary secretion (ii) displacement of the hydrophobic, toxic bile acids from the liver and (iii) direct cytoprotection against toxic bile acids. The cytoprotective effects of UDCA or its taurin-conjugated form, tauroursodeoxycholic acid (TUDCA) have been widely investigated in the liver. Studies on hepatocytes have shown that UDCA pretreatment significantly reduces bile acid-induced opening of the mitochondrial

permeability transition pore (mPTP) and consequently apoptosis, indicating that stabilization of the mitochondrial membrane, at least in part, plays an important role in the cytoprotective action of UDCA. This remarcable effect of UDCA on bile acid-induced hepatocellular injury raises up the question, whether UDCA has a similar effect on the pathophysiology of other bile acid-related diseases.

2. AIMS OF THE STUDY

2.1. Investigation of lacrimal gland ductal secretion

Our understanding of lacrimal gland function is far from complete and below that can be experienced in other areas of exocrine glands. Until recently there were limited experimental methods available for the investigation of LG duct function. Therefore our aims were to determine the osmotic water permeability of ductal epithelium by means of calculation of filtration permeability (P_f) and to investigate lacrimal gland ductal fluid secretion by means of measurement of fluid secretion evoked by potential agonists (forskolin and carbachol) seeking for evidence of secretory function of lacrimal ductal system.

2.2. The effect of bile acids on pancreatic ductal cells

Previous studies shown that bile acids such as CDCA strongly inhibits ion transporters through the destruction of mitochondrial function, resulting in impaired pancreatic ductal fluid and HCO_3^- secretion. This effect of bile acids may have significance in the pathomechanism of AP. UDCA is known to protect the mitochondria against hydrophobic bile acids and has ameliorating effect on cell death. There the aim of the study was to investigate the effect of UDCA pretreatment on CDCA-induced pancreatic ductal injury on isolated *in vitro* and to study the effect of UDCA pretreatment in CDCA-induced AP *in vivo*.

3. MATERIALS AND METHODS

3.1. Animals

Adult male New Zealand white rabbits weighing 2-2.5 kg were used (Devai Farm Kondoros Hungary). The animals were narcotised with a mixture of ketamine (40 mg/ml) and xylazine (10 mg/ml) and were euthanized with pentobarbital overdose (80 mg/kg). LGs were carefully dissected as described earlier.

Guinea pigs weighing 150-250 g were used in order to examine the effect of bile acids on pancreatic ductal HCO_3^- secretion. Animals were killed humanly by cervical dislocation, and then the pancreas was removed.

Male SPRD weighing 200-250 g rats were used for experiments as described below in the section of the „Induction of pancreatitis”.

All animals were kept at a constant room temperature of 22±2 ^oC, under 12-h light and dark cycles, and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary).

3.2. Ethics

All animal experiments were conducted in compliance with the Guide for the Care and Use of

Laboratory Animals (National Academies Press, Eight Edition, 2011), with the 2010/63/EU guideline and the Hungarian 40/2013 (II.14.) government decree and were approved by the Institutional Animal Care and Use Committee of the University of Szeged (I-74-3/2012 MÁB) and also by an independent committee assembled by national authorities (XII./3773/2012.). LG experiments were also conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

3.3. Isolation and culture of lacrimal and pancreatic duct segments

Rabbit LG and guinea pig pancreatic intra/interlobular ducts were isolated and then cultured overnight in +37^{O}C as described above. In case of the isolation of pancreatic ducts all used mediums were supplemented with 0.01% trypsin inhibitor.

3.4. Measurement of osmotic water permeability and ductal fluid secretion of lacrimal gland interlobular duct epithelium

Ends of LG ducts sealed during overnight incubation forming a closed luminal space. Fluid secretion (or osmotically determined fluid movement) into the closed intraluminal space of the ducts resulted in ductal swelling which could be analyzed using the videomicroscopic method. The initial lumen length (L_0) and the lumen area (A_0) were measured directly from the pixel intensities on the first image. To study water permeability, ducts were perfused with isotonic HEPES-buffered solution (290 mosM) for 5 minutes after equilibration, than the perfusate was changed to hypotonic HEPES-buffered solution (145 mosM). The osmotic water permeability constant ([P_f]=µm/s) was calculated using the initial volume ($V_0=\pi R_0^2 l_0$), the initial slope of the relative volume increase ($d(V/V_0)/dt$), the initial luminal surface area ($2\pi R_0 l_0$), and the molar volume of water ($V_w=18x10^{12}$ µm/mol). In the case of fluid secretion measurement, carbachol or forskolin were added to the perfusate after 10 minutes superfusion with HEPES-buffered or HCO_3^-/CO_2-buffered solution. At the end of each experiment, perfusion was changed to hypotonic solution for 5 minutes in order to confirm epithelial integrity.

3.5. Bile acid treatments

Pancreatic ducts were treated with bile acids as follows: no treatment (control group), 5 minutes CDCA (1 mM) treatment (CDCA group), 24 h UDCA (0.5 mM) treatment (UDCA group) and 24 h preincubation with 0.5 mM UDCA and then parallel incubation for further 5 minutes with 1 mM CDCA (UDCA+CDCA group).

3.6. Measurement of intracellular Ca2+ concentration, pH and ATP level

Isolated guinea pig pancreatic ducts were incubated in HEPEs-buffered solution and loaded with BCECF-AM (1.5 µM), Fura-2-AM (2.5 µM), MgGreen-AM (5 µM) or TMRM (1 µM) respectively for 30-45 min at 37^{O}C. Acid/base transporter activity was investigated by NH_4Cl pulse technique in the presence and the absence of extracellular HCO_3^-.

3.7. Measurement of mitochondrial permeability transition pore opening

To measure mitochondrial inner membrane permeabilization and/or the opening of the mitochondrial permeability transition pore (mPTP), we used the calcein-cobalt dequenching technique. Pancreatic ducts were loaded with calcein-AM (1 μM) for 30 min then with $CoCl_2$ (1 mM) for further 10 min. Fluorescence signals (495/515 nm) were normalised to initial fluorescence intensity (F/F_0) and expressed as relative fluorescence.

3.8. TUNEL cell death assay

For detection of cell death we used the terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labelling (TUNEL) assay (Roche Diagnostics, Mannheim, Germany). Intra-interlobular pancreatic ducts were treated with bile acids as previously described. Control and bile acid-treated isolated duct segments were fixed with 4% paraformaldehyde overnight and then cryosection and staining of the samples were performed according to the manufacturer's protocol. The CDCA-treated groups were incubated for 3 h in culture media before fixation.

3.9. Transmission electron microscopy

For electron microscopic studies, ducts were fixed in 2.5% glutaraldehyde immediately after isolation("Keratoconjunctivitis Sicca and Corneal Ulcers.," n.d.). Samples were then postfixed in 1% osmium tetroxide, dehydrated in a series of graded ethanols, and subsequently embedded in epoxy resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate. Tissue sections were analyzed under a transmission electron microscope (CM10; Philips, Eindhoven, The Netherlands).

3.10. Induction of acute pancreatitis

Male SPRD rats were used for AP experiments. Animals were fasted 12 h before the surgical procedure. Ursodeoxycholic acid (Ursofalk) was purchased from Dr Falk Pharma Ltd. UDCA was dissolved in 2 mL of tap water and 250 mg/kg of body weight was administered orally (gavage) for two weeks. Control animal were only treated with same amount of tap water. Last UDCA treatment was performed one day before the pancreatitis induction. Anestethised rats were shaven and the abdominal cavity was opened with median laparotomy. The common bile duct was temporary occluded with a vessel clip. Duodenum was punctured with a 0.4-mm diameter needle connected to polyethylene tubing. The polyethylene tube was placed into the major pancreatic duct and 1 ml/kg 1 % sodium-CDCA (dissolved in physiologic saline) was administered. Control animals received intraductal physiological saline instead of bile acid. After bile acid or physiological saline infusion, vessel clip and the polyethylene tube were removed and the abdominal wall and skin were closed. Rats were sacrificed 24 h later of the surgical procedure.

3.11. Statistical Analysis

Data are expressed as means±SEM. Significant difference between groups was determined by analysis of variance. Probability values of $p<0.05$ were accepted as being significant in every cases.

4. RESULTS

4.1. Investigation of lacrimal gland ductal secretion

4.1.1. Osmotic water permeability of lacrimal gland interlobular duct epithelium

The perfusion of the ducts with hypotonic solution the luminal area resulted in rapid increase in the luminal area, indicating that the NaCl gradient caused rapid water flux into the closed luminal space. P_f proved to be 60.53 µm/s (± 19,76).

4.1.2. Forskolin-stimulated fluid secretion of lacrimal gland interlobular ducts in the presence and in the absence of HCO3-

LG duct secretion to forskolin stimulation were investigated both in HCO_3^- free (HEPES-buffered) and in HCO_3^-/CO_2-buffered solutions. At first step ducts were superfused with HEPES-buffered solution for 10 minutes, and then 10 µmol/l forskolin was added to the bath. Ducts remained unchanged in HEPES-buffered solution, but during forskolin stimulation, rapid, sustained luminal volume increase was observed which remained relatively unchanged over a 30-minutes period. To evaluate the possible role of HCO_3^- in forskolin-evoked fluid secretion, ducts were superfused with HCO_3^-/CO_2-buffered solutions for 10 minutes, and then forskolin was added to the bath. There was no detectable secretion in HCO_3^-/CO_2-buffered solution, while rapid secretion could be observed as an effect of forskolin stimulation without decline in the swelling response over a 30 minutes period. The secretory effect of forskolin did not differ in HEPES-buffered and in HCO_3^-/CO_2-buffered solution.

4.1.3. Effects of different inhibitors on fluid secretion

The above described results indicate that LG interlobular ducts are able to secrete fluid in response to forskolin stimulation in HEPES-buffered solution in the absence of extracellular HCO_3^-, another anion than HCO_3^-, most probably Cl^- can be the driving force of fluid secretion. To study the source of basolateral Cl^- uptake, we tested the role of NKCC1 by means of examination of the effect of cotransport inhibitor bumetanide. Bumetanide (100 µmol/l) completely blocked forskolin-evoked fluid secretion in HEPES-buffered, and also in HCO_3^--buffered solutions. Bumetanide was applied after 10 minutes of forskolin stimulation. Pretreatment with bumetanide completely inhibited forskolin evoked fluid secretion in HCO_3^--buffered solution. The complete inhibition of fluid secretion by bumetanide in HCO_3^--buffered solution suggests the central role of Cl^- transport and only the marginal role of HCO_3^- transport in this process.

4.1.4. The role of basolateral HCO_3^- transporters in fluid secretion

To study the contribution of basolateral HCO_3^- and Cl^- transporters, we tested the role of inhibition of basolateral transporters potentially contributing to the HCO_3^- transport. NHE was inhibited by 3 µmol/l EIPA, and Na^+-HCO_3^- cotransporter was inhibited by 500 µmol/l DIDS. In HCO_3^- -buffered solution, administration of EIPA and DIDS had no effect on forskolin-evoked fluid secretion while simultaneous administration of 30

µmol/l bumetanide, 3 µmol/l EIPA and 500 µmol/l DIDS completely inhibited fluid secretion evoked by forskolin. These results suggest the key role of Cl^- transport in fluid secretion in rabbit lacrimal gland interlobular ducts.

4.1.5. Carbachol-stimulated fluid secretion of lacrimal gland interlobular ducts in the presence and in the absence of HCO_3^-

Carbachol in 100 µmol/l was used as the lowest concentration producing the maximal obtainable effect in the experiments described thereinafter. Effect of carbachol treatment on secretion of lacrimal gland ducts was investigated both in HEPES-buffered and in HCO_3^-/CO_2-buffered solutions. In the first series of experiments ducts were perfused with HEPES-buffered solutions for 10 min, followed by administration of 100 µmol/l carbachol to the bath solution. Fluid secretion proved to be biphasic consisting of a continuous swelling in the first 5 minutes followed by a plateau phase. To investigate the role of HCO_3^- in carbachol evoked fluid secretion, ducts were perfused with standard HEPES-buffered solution for 5 min followed by 10 min perfusion with HCO_3^-/CO_2-buffered solution. 100 µmol/l carbachol was then administered to the bath and swelling response was detected. The kinetics of carbachol stimulated ductal fluid secretion was very similar to those that observed in HEPES-buffered solution. The stimulatory effect of carbachol could be detected in the first 5 minutes, followed by a plateau phase. Administration of parasympatholytic atropine resulted in a complete abolishment of carbachol-evoked fluid secretion in both solutions. Calculated secretory rates did not show a substantial difference measured in HEPES-buffered and HCO_3^-/CO_2 -buffered solutions .

4.2. Effect of bile acids on pancreatic ductal cells

4.2.1. Effect of UDCA and CDCA on pH_i

Several studies have shown that bile acids induce intracellular acidification in various cell types. At first step, we have investigated the effect of CDCA and UDCA on basal pH_i of pancreatic ducts. Administration of CDCA induced a dose-dependent intracellular acidification in Hepes-buffered solution. The effect of CDCA on pH_i was reversible, following the removal of bile acid from the external solution, the pH_i completely returned to the basal level. UDCA also induced a dose-dependent decrease in pH_i, however the effect of UDCA was much smaller compared to CDCA. We repeated these experiments in HCO_3^-/CO_2-buffered solution, where the two bile acids induced similar degrees of pH_i decrease.

4.2.2. Effect of UDCA pretreatment on the inhibitory effect of CDCA on the acid/base transporters

We used the ammonium pre-pulse technique to investigate the acid-base transporters of the pancreatic ducts. Acute administration of UDCA did not affect the rate of recovery from the acid or alkali load neither in Hepes-buffered nor in HCO_3^-/CO_2-buffered solutions. In contrast, 1 mM CDCA strongly inhibited the activity of the acid/base transporters. Next we tested whether UDCA administration can influence the inhibitory effect of CDCA. When UDCA and CDCA were added simultaneously, UDCA was unable to prevent the inhibitory

effect of CDCA on the ion transporters in all of the investigated concentration. Several studies have shown that prolonged incubation of the cells with UDCA is needed to exert its protective effect. Therefore, in the next step, we pretreated the ducts with UDCA for various time periods (5h and 24 h) and the effect of CDCA on the ion transporters was examined. A 5 h preincubation of the ducts with UDCA (0.1, 0.25, 0.5 and 1 mM) did not affect the response to CDCA. However, 24 h pretreatment with UDCA significantly decreased the toxic effect of CDCA both in Hepes and in HCO_3^-/CO_2-buffered solutions. Pretreatment of the ducts with 0.5 mM UDCA prevented the inhibitory effect of CDCA on NHE, CBE and NBC.

4.2.3. Effect of UDCA pretreatment on the CDCA-induced calcium signaling

Numerous studies have indicated that bile-induced toxic calcium signalling is an initial step in the development of AP. We have previously shown that high concentration of CDCA induces (Ca^{2+}) elevation in ductal cells. So in the next step we have examined whether UDCA pretreatment has any effect on the CDCA-induced Ca^{2+} signalling. Administration of 0.5 mM UDCA alone had no significant effect on $[Ca^{2+}]_i$ (data not shown). In contrast, 1 mM CDCA induced high and partially reversible Ca^{2+} signalling in pancreatic ducts. Preincubation of the ducts with 0.5 mM UDCA for 24 h did not affect the CDCA-induced increase in $[Ca^{2+}]_i$, indicating that the protective effect of UDCA is unlikely to be caused be the prevention of the elevated $[Ca^{2+}]_i$.

4.2.4. Effect of UDCA pretreatment on the CDCA-induced mitochondrial injury

To further analyse the protective effect of UDCA on ductal mitochondria, we also investigated $\Delta\Psi_m$ and mPTP upon administration of bile acids. Administration of CDCA resulted in a huge increase in TMRM fluorescence intensity, indicating that this bile acid induced marked mitochondrial depolarization. Preincubation of the ducts with 0.5 mM UDCA reduced the CDCA-induced depolarization by 69.4±4.6%. Since ATP depletion and mitochondrial depolarization are caused by mPTP induction, next we investigated the effect of CDCA on the opening of mPTP using the calcein-cobalt technique. Treatment of the calcein-loaded pancreatic ducts with 1 mM CDCA decreased the fluorescence excitation of calcein, approximately 1 min after the addition of the bile acid. Similarly to the $\Delta\Psi_m$ experiments, 24 h pretreatment of the ducts with 0.5 mM UDCA had a preventive role on mitochondria and decreased the CDCA-induced mPTP opening by 72.1±4%. Administration of UDCA alone did not affect mPTP. Mitochondrial morphology of the ducts was also examined by electron microscopy. No morphological alterations were observed in the control and UDCA-treated groups. In contrast, incubation of the ducts with 1 mM CDCA for 5 min resulted in mitochondrial swelling and the loss of the mitochondrial inner membrane. This swelling could be prevented by UDCA pretreatment. Moreover, the integrity of the mitochondria was also maintained in the CDCA+UDCA-treated ducts compared to the ducts only treated with CDCA.

4.2.5. Effect of UDCA pretreatment on the CDCA-induced cell death

Ducts were treated with 1 mM CDCA for 5 min, then were incubated in culture media for further 3 h in order to leave time for development of cell death. Cell death was assessed by TUNEL staining. Incubation

of the pancreatic ducts with CDCA resulted in a significant increase in cell death compared to control, non-treated ducts. Although, both apoptosis and necrosis can be characterised by DNA fragmentation, the presence of intact cell organelles, cellular shrinkage and the lack of cellular content release indicate that CDCA rather induces apoptotic cell death than necrosis. 24 h preincubation with UDCA (0.5 mM) alone caused only a small degree of DNA fragmentation in the ductal cells, but significantly reduced the CDCA-induced apoptotic cell death by 63.3±5.7 %.

4.2.6. *In vivo* investigation of the effect of UDCA pretreatment in CDC-induced AP model

In order to investigate the protective effect of UDCA under *in vivo* conditions, we utilized a CDCA-induced pancreatitis model. We used rats as the model animals because we were unable to administer CDCA intraductally in guinea pigs due to the anatomical topography of the main duct. Serum amylase activities were significantly elevated after retrograde infusion of CDCA (CDCA group, 983±100 U/l) compared to intraductally administered physiological saline (control group, 396±50 U/l). Pretreatment of UDCA for two weeks did not influence the serum amylase activities (UDCA group, 424.7 ± 20) in control animals; however, it was significantly decreased (582 ± 50 U/l) in the UDCA+CDCA group versus the CDCA group. Pancreatic water content was significantly elevated after retrograde infusion of CDCA (80±1%) compared to intraductally administered physiological saline (60±1%). A two-week pretreatment with UDCA showed no influence on the pancreatic water content (61±2%) in the UDCA group. However in the UDCA+CDCA group, it was significantly decreased (65±2%) versus to the CDCA group.

5. DISCUSSION

5.1. Investigation of lacrimal gland ductal secretion

In contrast to many secretory epithelia, we are lacking of published calculations of the water permeability of the ductal epithelium, even through this parameter is essential in determining the ability of the fluid transport. It has been estimated that ductal cells may produce as much as 30% of the total volume of the lacrimal gland fluid, thus the relative contribution of ductal epithelial cells to water secretion cannot be neglected. The summarized actions of basolaterally and apically located ion transporters, co-transporters and exchangers can produce an osmotic gradient which may determine the direction of water flow. Water passively follows secreted ions depending on the osmotic gradient and on the permeability of the epithelial structure. Similarly to pancreatic ducts, the isolated lacrimal gland duct segments seal spontaneously during overnight culturing, forming closed sac-like structures. Secretory processes of ductal epithelium into the closed intraluminal space result in swelling of the ducts as the luminal space fills with the fluid secreted as seen in the case of isolated pancreatic ducts. The ductal volume changes can be analyzed by videomicroscopy.

We measured the initial rate of swelling of LG ducts after a sudden drop in bath osmolarity caused by a Na^+-gradient in order to characterize the water permeability properties of the structure. There was only minimal connective tissue surrounding the basolateral surface of the epithelium thus the unstirred layer effect could be neglected. According to our observations, the sealing of the ends of the ducts results in complete

restoration of epithelial integrity and swelling of the ducts happens without leakage in the vast majority of cases. The value of P_f proved to be 60,53 μm/s (± 19,76). This P_f value is lower, than the highly water permeable pancreatic ductal epithelium (160-170 μm/s, measured in rat pancreatic ducts) or the kidney proximal tubule (100-500 μm/s, different species) and very similar to the distal airways of the guinea pig (60 μm/s), to the rat cholangiocytes (50 μm/s) or to the cultured corneal endothelium in mice (74 μm/s). The P_f value showed by our experiments is clearly sufficient to support fluid secretion, and proves that rabbit lacrimal gland ductal epithelium is be able to secrete fluid in response to secretagogues.

Forskolin stimulation was initiated a brisk and continuous swelling response in rabbit lacrimal gland interlobular ducts. Fluid secretion was unaffected by inhibition of HCO_3^- transport mechanisms, but completely abolished when basolateral Cl^- uptake was blocked by bumetanide suggesting the predominant role of Cl^- transport mechanisms over HCO_3^- secreting processes in lacrimal duct fluid secretion in rabbit. As bumetanide is a well known inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter located on the basolateral membrane of the duct cells, this transport process can be the main route of cellular cloride uptake.

Cholinergic stimulation with carbachol resulted in a biphasic secretory response with a faster initial and a plateau second phase in our experiments. The kinetics of swelling response was in accordance with the carbachol-evoked changes of intracellular calcium levels of lacrimal ductal cells reported earlier by our laboratory. Carbachol-evoked secretory pattern of lacrimal ducts was similar to those found in pancreatic ducts. Parasympatholytic atropine abolished the stimulatory effect of carbachol, suggesting the involvement of muscarinic cholinoceptors.

In conclusion, besides water permeability, forskolin and carbachol induced fluid secretory capability of lacrimal gland ductal cells were demonstrated in the present work. Our results strongly support the hypothesis, that lacrimal gland ductal system is actively involved in lacrimal fluid secretion. Further studies are needed to clarify the effects of various stimulatory agents and the role of ion transport mechanisms in this process. Future results may contribute to the development of targeted pharmacological interventions in order to improve deteriorated lacrimal gland function in dry eye disease.

5.2. Effect of bile acids on pancreatic ductal cells

The pathomechanism of biliary AP is still not fully understood. Numerous studies indicated that bile reflux into the pancreas may lead to pancreatic injury and as a result can induce AP. Therefore, a better understanding of the mechanisms underlying bile-induced pancreatic injury and its prevention may provide novel therapeutic tools for the treatment of AP.

To study the effect of CDCA and UDCA we used isolated intra-interlobular pancreatic ducts, which is a reliable *in vitro* model for investigating pancreatic ductal damage. The CDCA concentration used in this study was previously shown to induce intracellular Ca^{2+} signalling, mitochondrial injury and inhibition of the acid-base transporters, whereas the concentrations of UDCA were chosen on the basis of the literature data.

Our results indicaded that 24 h pretreatment of pancreatic ducts with 0.5 mM UDCA significantly reduced the inhibitory effect of CDCA on the acid/base transporters. Studies on pancreatic acinar cells have

indicated that the toxic effect of hydrophobic bile acids is mediated by a sustained Ca^{2+} signalling. We have previously shown that high concentration of CDCA induces a huge and long-lasting elevation of $[Ca^{2+}]_i$ in pancreatic ducts. When pancreatic ducts were exposed to UDCA for 24 h and CDCA was then added, the extent of calcium elevation did not change, indicating that the protective effect of UDCA is unlikely to be caused by the reduction of Ca^{2+} signalling. This finding is in accordance with our previous observation on pancreatic ducts, where preincubation of the cells with a specific calcium chelator, BAPTA-AM was unable to prevent the inhibitory effect of CDCA on acid-base transporters.

Numerous studies support the concept that mitochondrial damage plays a central role in the bile acid-induced cellular injury and that UDCA pretreatment is able to attenuate the toxic effect of hydrophobic bile acids on mitochondria. Therefore, in the next step we investigated the protective effect of UDCA on the function and morphology of pancreatic ductal mitochondria. Administration of CDCA alone induced mPTP opening in the ductal cells. In contrast to the effects of CDCA, UDCA alone caused no significant changes in mitochondrial function. However, UDCA pretreatment was able to prevent the CDCA-induced mPTP, mitochondrial membrane perturbation and the consequently formed decrease in membrane potential. Moreover, UDCA prevented the CDCA-induced ATP_i loss, which provides further evidence that UDCA pretreatment is beneficial to avoid mitochondrial injury. This conclusion was confirmed by electron microscopic studies which showed normal appearance of mitochondria in the UDCA+CDCA group compared to the CDCA group, where mitochondrial swelling and disruption of the inner mitochondrial membrane were observed. The mechanism underlying the mitochondrial protective effect of UDCA is not clear. One of the main inducers of mPTP is Ca^{2+} overload and oxidative stress. Although, we have shown that UDCA pretreatment had no effect on the extent of CDCA-induced Ca^{2+} elevation, we did not investigate the effect of UDCA on the total Ca^{2+} load. It is possible that the protective effect of UDCA is due to the reduction of Ca^{2+} overload or by the inhibition of reactive oxygen species (ROS) production, however further studies are needed to confirm these hypotheses.

Mitochondrial dysfunction is often associated with cell death either by the reduction of ATP_i levels or by irreversible alterations in the mitochondrial membrane permeability which induces the release of apoptotic signalling molecules from the mitochondria. Administration of CDCA induced marked DNA fragmentation in intact pancreatic ducts. We speculate that impairment of mitochondrial function plays a central role in this mechanism, but other signalling pathways may be involved. The apoptotic effect of CDCA and other hydrophobic bile acids have been examined in more detail in hepatocytes. ROS generation, mPTP induction, cytochrom c release and activation of downstream caspases have been shown to be associated with apoptosis. It has been also demonstrated that the glycin-conjugated form of CDCA, directly stimulate Fas-dependent cell death due to the activation of the Fas receptor which is independent from the mitochondrial pathway. 24 h pretreatment with UDCA effectively reduced the CDCA-induced apoptosis as indicated by significantly decreased DNA fragmentation which further confirms the cytoprotective effect of UDCA. The exact mechanism by which UDCA exerts its protective effect was not investigated by our group; however, our results and previous studies on hepatocytes strongly indicate that mPTP inhibition by UDCA is one of the key

mechanisms in the reduction of CDCA-induced cell death in PDECs.

In vivo experimental models are essential to confirm *in vitro* results. In order to extend our study, we also tested the protective effect of UDCA in animal models. There is no accepted bile acid-induced pancreatitis model in guinea pigs; therefore we induced AP in rats by intraductal injection of low concentration of CDCA. Under these experimental conditions, CDCA induced acinar cell damage and also increased serum amylase activity. The CDCA-induced acinar cell injury, hyperamylasemia and pancreatic edema was markedly reduced in the UDCA-treated group. The protective effect of UDCA can be attributed to its ability to reduce mitochondrial injury both in acinar and ductal cells. However, further *in vitro* experiments are needed to prove that UDCA pretreatment may exert a protective effect against the CDCA-induced acinar cell damage.

Understanding the early injury mechanisms induced by hydrophobic bile acids is extremely important to find a therapeutic target to reduce pancreatic injury. In this study, we confirm and extend our previous observations that mitochondria is a key target in the CDCA-induced cellular injury in PDECs. The hydrophilic bile acid, UDCA inhibits CDCA-induced apoptosis probably by the stabilization of mitochondrial membrane via the block of membrane depolarization and mPTP and also by prevention of mitochondrial swelling. Several studies have focused on the inhibition of cellular injury during AP in order to stop or delay the progression of the disease. UDCA may represent a novel option against the bile-induced ductal injury, however issues for the therapeutic application of this bile acid in AP need further investigation.

6. SUMMARY

6.1. Investigation of lacrimal gland ductal secretion

In the first part of this study our **AIM** was to investigate the osmotic water permeability of LG duct epithelium by means of calculation of filtration permeability and to investigate LG ductal fluid secretion with the following **METHODS**: Experiments were performed on isolated rabbit LG duct segments maintained in short-term culture. Osmotically determined fluid movement or fluid secretion into the closed intraluminal space of cultured LG interlobular ducts was analyzed using video microscopic technique.

Therefore our **RESULTS** were:

- The end of the LG ducts sealed after overnight incubation forming a closed luminal space.
- For the calculation of osmotic water permeability, ducts were initially perfused with isotonic HEPES buffered solution, and then with hypotonic HEPES buffered solution. Filtration permeability was calculated from the initial slope of the relative volume increase.
- Secretory responses to carbachol or to forskolin stimulation were also investigated. Forskolin stimulation resulted in a rapid and sustained secretory response in both solutions. Forskolin- stimulated fluid secretion was completely inhibited by bumetanide both in HEPES buffered and in HCO_3^-/CO_2 buffered solutions, suggesting the central role NKCC1.
- Administration of carbachol initiated a rapid but short secretory response in both HEPES buffered and in HCO_3^-/CO_2 buffered solutions. Atropine completely abolished the carbachol-evoked fluid secretion.

CONCLUSIONS from the results:

(i) A new method was introduced to investigate LG duct function.

(ii) Water permeability of rabbit LG duct epithelium was measured by calculating filtration permeability.

(iii) Fluid secretion of LG duct cells induced by carbachol or forskolin was also demonstrated. These results provide calculated values of lacrimal duct osmotic permeability and direct experimental evidence of LG duct fluid secretion.

6.2. Effect of acids on pancreatic ductal cells

Earlier studies showed that CDCA strongly inhibits pancreatic ductal HCO_3^- secretion through the destruction of mitochondrial function, which may have significance in the pathomechanism of AP. UDCA is known to protect the mitochondria against hydrophobic bile acids and has ameliorating effect on cell death in heptocytes. Therefore, our **AIM** was to investigate whether UDCA pretreatment has any effect on CDCA-induced pancreatic ductal injury.

To study ductal functions under phatophysiological conditions we used the following **METHODS:**

- Guinea pig intra-interlobular pancreatic ducts were isolated by collagenase digestion. Ducts were treated with UDCA for 5 and 24 h and the effect of CDCA on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), pH (pH_i), morphological and functional changes of mitochondria, and the rate of apoptosis were investigated.
- AP was induced in rat by retrograde intraductal injection of CDCA (0.5%) and the disease severity of pancreatitis was assessed by measuring standard laboratory and histological parameters.

Using *in vitro* and *in vivo* approaches our **RESULST** showed that UDCA pretreatment:

- completely prevented the inhibitory effect of CDCA on ductal acid-base transporters,
- decreased the rate of CDCA-induced mitochondrial injury and cell death
- reduced the severity of experimental AP induced by CDCA.

Therefore our **CONCLUSION** is that these results clearly demonstrate that UDCA:

(i) suppresses the CDCA-induced pancreatic ductal injury by reducing apoptosis and mitochondrial damage

(ii) reduces the severity of CDCA-induced AP.

The protective effect of UDCA against hydrophobic bile acids may represent a novel therapeutical target in the treatment of biliary AP.

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