Role of transmembrane chloride transporters in the fluid secretion of lacrimal gland duct cells

Eszter Vizvári, M.D.

Ph.D. Thesis

Doctoral School of Clinical Medicine

Supervisor: Edit Tóth-Molnár, M.D., Ph.D.,

Department of Ophthalmology,

University of Szeged,

Szeged, Hungary

2017

LIST OF RELATED FULL PAPERS

The thesis is based on the following publications:

I. Vizvári E, Katona M, Orvos P, Berczeli O, Facskó A, Rárosi F, Venglovecz V, Rakonczay Z Jr., Hegyi P, Ding C, Tóth-Molnár E. Characterization of Na+-K+-2Cl- cotransporter activity in rabbit lacrimal gland duct cells. Invest Ophthalmol Vis Sci. 2016; 57(8): 3828-3856.

IF: 3.303

II. 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): 4360-4367.

IF: 3.404

Other related publication:

III. Kalász H, Tekes K, Pösténvi Z, **Vizvári E**, Sótonyi P, Szabó D, Tóth-Molnár E. Pharmacokinetics of selegiline in a rabbit model. *Letters in Drug Design and Discovery* 2016; 13(8): 752-756.

IF: 1.1

1. INTRODUCTION

Preocular tear film is an essential protector of the ocular surface. It is secreted by the lacrimal functional unit which is composed of the ocular surface tissues, lacrimal glands (LG), and their interconnecting sensory and autonomic innervation. This functional unit ensures the stability of the tear film which is essential for the health of ocular surface. Dysfunction of this unit may result in dry eye which is the most common ophthalmic medical problem. Our knowledge about the function of LG is far from complete and therefore more detailed understanding of the physiology and pathophysiology of the LG is essential. Several reports have suggested that LG ducts may play a decisive role in tear production, albeit no definite, especially functional evidence has ever been shown. Recently two new methods have been published to study LG duct epithelium during the past decade. Ubels and colleagues introduced the laser capture microdissection technique in LG research to perform gene expression studies on collected duct cells from frozen rat LG sections. Another experimental technique - which is the first method allowing functional studying of LG ducts - was described by our laboratory. The new isolation technique results in viable duct segments suitable for functional studies. Role and regulation of various ion transporters in lacrimal duct can be studied with the use of these isolated, short-term cultured duct segments. With the use of isolated duct segments, we started to investigate LG duct functions in the last few years. In a recently published paper by our laboratory, video-microscopic technique was adapted and was used for the first time in lacrimal duct research to investigate fluid secretion of isolated LG duct segments. This technique was originally developed by Fernandez-Salazar et al. for the measurement of fluid secretion of pancreatic ducts. In our video-microscopic fluid secretion experiments, fluid secretion was almost unaffected by inhibition of basolateral HCO_3^- transporters whereas was completely abolished, when basolateral Cl^- uptake was blocked with 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 Na-K-2Cl-cotransporter 1 (NKCC1), which is located on the basolateral membrane of LG duct cells, this transport mechanism appears to be the main route of cellular Cl^- uptake. Our findings - based on indirect evidences - strongly suggested the importance of cystic fibrosis transmembrane conductance regulator (CFTR) in ductal fluid secretion in rabbit, as forskolin - a well-known activator of CFTR via the elevation of cytosolic cAMP levels – resulted in a significant swelling response in our experimental set-up. Our earlier results suggested the pivotal role of NKCC1 in basolateral Cl^- uptake and the key function of

CFTR on the apical membrane in intraluminal Cl^- secretion. Since our knowledge about the role of NKCC1 and CFTR in ductal secretion is limited, further investigations have been initiated to study the role of these transporters in the secretory processes of LG ducts. Although experimental evidence was found for expression of NKCC1 on the basolateral membranes of LG duct cells of rat, rabbit and mouse there is no available data about the role and function of NKCC1 in LG duct epithelium apart from our secretory results. Important role of CFTR has been widely demonstrated in the maintenance of homeostasis in different secretory epithelia including pancreas, salivary glands, sweat glands and airways epithelium. In contrast, role of CFTR in LG function is only partially investigated and its involvement in tear secretion is unknown. Accumulating evidences from rat and rabbit LG gene expression studies demonstrated the predominant expression of CFTR in LG duct cells. These studies were mostly descriptive without exploring the functional aspect of CFTR in LG secretion. In our earlier work with rabbit lacrimal duct segments, we were unable to carry out direct investigation of the apically located CFTR since the luminal space cannot be reached in the isolated, fragile, small sealed ducts. Availability of transgenic mouse models carrying genetic defects in CFTR allows the direct examination of the role of CFTR. Presently we conducted the first study in lacrimal gland research which focuses on the functional role of CFTR in LG secretion with the use of CFTR transgenic mouse model.

2. AIMS OF THE STUDY

Previous studies of our laboratory suggested important role of NKCC1 in LG duct function. Role of CFTR was also suggested, since elevation of cytosolic cAMP is tend to activate CFTR on the apical membrane. Therefore aims of our experiments were:

1) to investigate the functional role and the activity profile of NKCC1 in isolated LG ducts.

2) to determine the role of CFTR in tear secretion and in the maintenance of ocular surface integrity as well as the presence and function of CFTR in LG ductal secretion using LG ducts isolated from wild type (WT) and CFTR knock-out (KO) animals.

3. METHODS

Animals

LG ducts isolated from adult male New Zealand white rabbits were used in all NKCC1 experiments while wild type and CFTR knock-out mice were used in our CFTR investigations. All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols has been approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary and conformed to the Directive 2010/63/EU of the European Parliament.

Measurement of tear secretion and corneal fluorescein staining

Tear production was measured in anesthetized mice using phenol red impregnated cotton threads. Ocular surface integrity was evaluated by applying fluorescein sodium into the conjunctival sac, followed by slit-lamp biomicroscopy through cobalt-blue filter. Staining was assessed by using the NEI grading system.

H&E staining and immunofluorescence

Dissected LG tissues, from both WT and KO mice, were fixed, cut and stained with H&E. Cryostat sections were rehydrated, then fixed. Following overnight incubation with primary antibodies the samples were incubated with secondary antibody for 1 hour.

Isolation and culture of lacrimal duct segments

LGs were dissected and following incubation steps the tissue pieces were transferred to a glass microscope slide and viewed under stereo microscope. Inter-, and intralobular ducts were micro-dissected and after microdissection, intact LG ducts were transferred to the culture solution in a Petri dish. Ducts were cultured overnight.

Intracellular pH measurement with fluorophotometry

Ducts were loaded with the pH-sensitive fluorescent dye BCECF-AM. Thereafter, the ducts were continuously superfused and intracellular pH was measured using an imaging system. ROIs of 5 to 10 cells were excited at 490 nm and 440 nm and the 490/440 fluorescence emission ratio was then measured at 535 nm.

Measurement of NKCC1 activity

Ammonium pulse technique was used to measure the activity rate of NKCC1 by determining the rate of intracellular acidification caused by NH_4^+ entry into the cells via this transport

mechanism. An increase in fluorescence ratio corresponds to the elevation of intracellular pH.

Measurement of fluid secretion of LG ducts

Videomicroscopic technique was used for measurement of ductal fluid secretion. Fluid secretion into closed intraluminal space of cultured lacrimal gland interlobular ducts was analyzed with the swelling method using an inverted microscope.

Statistical analysis

A mixed ANOVA model was used for the calculation of NKCC1 activity, and also for the calculation of ductal fluid secretion. Kruskal-Wallis test with Dunn method was used for the analysis of tear secretion. SPSS 22 statistical software (IBM, New York, USA) was used to analyze the data, which were presented as means ± SEM. A p value of less, than 0.05 was regarded as significant.

4. RESULTS

4.1 BASOLATERAL CHLORIDE TRANSPORT OF LG DUCT CELLS: ROLE OF NKCC1

Localization of NKCC1 in rabbit LG

Immunofluorescence demonstrated presence of NKCC1 on the basolateral membranes of both acinar and ductal cells in rabbit LG, with the intensity being more pronounced in acinar cells.

Characterization of NKCC1 activity in rabbit LG ducts

We tested the hypothesis that NKCC1 can transport NH_4^+ instead of K^+ with a resultant change in intracellular pH. Ammonium induced acidification was reduced in the presence of high K^+ concentration in the superfusate, indicating competition between K^+ and NH_4^+. To determine whether NH_4^+ transport in duct cells occurs via a Na^+-dependent pathway, the NH_4^+ pulse was repeated with tetramethylammonium-chloride instead of NaCl in the superfusate. Ammonium-induced cell acidification was reduced significantly in the absence of Na^+. To test the existence of a bumetanide-sensitive basolateral transport system (indicative for the presence of NKCC1), standard NH_4^+ pulse was administered in the presence and absence of bumetanide. In the presence of bumetanide NH_4^+- induced cell acidification was significantly reduced in duct cells. These results confirm the existence of a Na^+ dependent, bumetanid-

sensitive pathway in the duct cells where K^+ transport is in competition with NH_4^+ suggesting the functional involvement of NKCC1.

Influence of repeated $NH4^+$ pulse on the slope of second phase acidification

To determine whether repeated ammonium pulse administration itself could influence the slope of the second phase, three consecutive pulses were added to the same duct segment. The slope of the second phase was stable during repeated pulses; thus, the ‘‘fatigue’’ effect of the repeated base administration should not be taken into consideration during calculation of NKCC1 activity.

Basal activity of NKCC1

To determine whether basal activity of NKCC1 affects the measurements, the slope of second phase acidification was determined and compared during $NH4^+$ pulse in the presence and absence of bumetanide. Basal activity of NKCC1 was negligible and statistically not significant.

Activation of NKCC1 by low cytosolic Cl^-

Isolated LG duct segments were preincubated in Cl^--free solution for 20 minutes, then $NH4^+$ pulse was performed in the presence and absence of bumetanide in these experiments. Low cytosolic Cl^- increased the activity of NKCC1 in a statistically significant manner.

Activation of NKCC1 by hyperosmolarity

To investigate the role of hyperosmotic environment in the activation of NKCC1, ducts were preincubated with bath solution of 390 mOsm/l in the absence and presence of bumetanide. Hyperosmotic challenge, that is elevation of bath osmolarity from 290 mOsm to 390 mOsm increased the activity of NKCC1 significantly indicating that hyperosmolarity has an important role in the activation of NKCC1.

Effects of carbachol, PMA, and Ca^{2+} ionophore A23187 in the activation of NKCC1

Acetylcholine analogue carbachol was used to investigate the effect of cholinergic agonists in the activation of NKCC1. $NH4^+$ pulse was administered in the absence and presence of bumetanide after preincubation with carbachol. The slope of second-phase acidification was increased compared to the control as a result of cholinergic stimulation. Bumetanide treatment did not change the slope of second phase acidification during NH^{4+} pulse,

indicating that carbachol had no significant effect on NKCC1 activity. For further elucidation of the role of cholinergic cellular signaling pathways in the activation of NKCC1 in rabbit LG ducts, the effects of Ca^{2+} ionophore A23187 and protein kinase C (PKC) activator PMA was investigated. Preincubation with Ca^{2+} ionophore A23187 was followed by $NH4^{+}$ pulse administration with and without bumetanide in the superfusate. Calcium ionophore A23187 did not result in activation of the cotransporter. In the next series of experiments, effect of PMA was tested. Ammonium pulse was administered to the ducts in the absence and presence of bumetanide after preincubation with PMA. Bumetanide treatment slightly but significantly reduced the slope of second phase acidification.

Activation of NKCC1 by VIP

We also investigated the effect of VIP in the activation of NKCC1. $NH4^{+}$ pulse was administered in the absence and presence of bumetanide after preincubation with VIP. VIP treatment increased the rate of second phase acidification, which was reduced by bumetanide indicating the VIP- induced marked increase in NKCC1 activity.

Activation of NKCC1 by forskolin and cell permeable cAMP analogue 8-bromo cAMP

$NH4^{+}$ pulse was administered after a 5-minute incubation with forskolin in the absence and presence of bumetanide. Forskolin increased the rate of second phase acidification, which was reduced by bumetanide in a significant manner, indicating the forskolin-induced increase in NKCC1 activity. To further verify the effect of elevated cytosolic cAMP level in the activation of NKCC1, cell permeable cAMP analogue was used. Ammonium pulse was administered in the absence and presence of bumetanide after preincubation with 8-bromo cAMP. A statistically significant increase in NKCC1 activity caused by cell-permeable cAMP analogue 8-bromo cAMP was found.

4.2. ROLE OF CFTR-MEDIATED CHLORIDE TRANSPORT IN LACRIMAL SECRETION

Role of CFTR in tear secretion and corneal fluorescein staining using CFTR KO mice

Tear secretion was measured in three age groups in both WT and KO mice, at 8-10 weeks of age, at 14-16 weeks of age and at 20-24 weeks of age. Data from both eyes were averaged and evaluated. Tear secretion of KO animals in all age groups were significantly lower as

compared to their WT littermates. To study the effect of age results were calculated not only vertically (i.e. separately in different age groups, comparing WT and KO measurements, see above) but also horizontally, with the comparison of tear secretion in different age groups separately in WT and in KO animals. Tear secretion of WT mice measured at the age of 14-16 weeks was significantly higher compared to the 8-10 weeks group. However, no significant differences were observed between secretion data at the age of 14-16 weeks and 20-24 weeks. We could not find statistically significant differences in tear secretion of KO animals measured in different age groups. Fluorescein staining was evaluated in both WT and KO mice at 8-10 weeks of age and at 20-24 weeks of age. Data from both eyes were pooled together. Corneal staining score was significantly lower in WT mice in both age groups compared to KO animals.

H&E staining of LGs from WT and CFTR KO mice

No obvious structural changes have been observed in KO LG tissues as compared to their age-matched WT counterparts.

Immunofluorescence staining for CFTR of WT and CFTR KO LGs

CFTR protein could be found in the apical membrane of LG duct cells deriving from WT animals. Strong staining was detected in the ducts whereas acinar cell membranes showed no signs of staining. As anticipated, CFTR protein could not be identified in CTFR KO LGs.

Forskolin-induced fluid secretion of LG duct segments isolated from WT and CFTR KO mice

Effect of forskolin stimulation on WT and CFTR KO LG interlobular and intralobar duct segments isolated from 14-24 weeks old animals were investigated both in HEPES- buffered and in HCO_3^- buffered solutions. Forskolin stimulation resulted in a continuous, sustained swelling response in WT ducts. In contrast, no forskolin-evoked fluid secretion could be measured in CFTR KO ducts. Using HCO_3^-/CO_2-buffered solution, forskolin stimulation resulted in a rapid secretion in WT ducts. CFTR KO ducts remained unchanged with no secretory response observed during forskolin stimulation. The secretory effect of forskolin in WT ducts did not differ significantly in HEPES-buffered and in HCO_3^-/CO_2- buffered solution.

5. DISCUSSION

Dry eye is the most common ocular surface disease affecting millions of people worldwide. Despite recent developments in the past few years, our treatment options are still limited, rendering the management of this debilitating disease very challenging. Tear secretion is a complex process with the involvement of the main and accessory LGs, corneal and conjunctival epithelial cells and the Meibomian glands, etc. LG is the main source of fluid, electrolyte and proteins in the tear, and deficiency in its secretion results in aqueous deficient dry eye. Our understanding of the physiological and pathological mechanisms of LG secretion is limited, despite its critical importance in developing new treatment strategies. Unfortunately, duct cells have been understudied for many years as compared to acinar cells, although recent advances clearly indicated that these duct cells play critical and indispensable roles in LG production. In the scope of our laboratory is the clarification of the role of LG ducts in the secretory process of the gland. In the present work we aimed to investigate the main chloride transport systems of the ducts cells ie. the NKCC1 on the basolateral (interstitial) membrane and the CFTR on the apical (luminal) side.

LG duct secretion is mediated by an array of ion transporters and channels including NKCC1, even though its role in LG duct secretion is not well understood. Our present work evidenced the expression of NKCC1 in the basolateral membranes of duct cells from rabbit LG by immunofluorescence. Investigations of various factors that may influence the activity of NKCC1 in LG duct cells were also performed. Hyperosmotic environment led to a marked increase of NKCC1 activity in rabbit LG duct cells in our experiments. On the other hand, we could not demonstrate notable effect of cholinergic agonist carbachol in the activation of NKCC1. This finding is in agreement with our previous results ie. cholinergic stimulation of isolated rabbit LG duct segments resulted in a very weak fluid secretory response. To further clarify the role of the cholinergic cellular signaling pathways, effects of Ca^{2+}-ionophore (A23187) and PKC activator PMA were measured. Elevation of cytosolic Ca^{2+} level with Ca^{2+} ionophore did not cause activation of NKCC1 in our experiments. In contrast, direct stimulation of PKC with its potent activator PMA resulted in a significant increase of NKCC1 activity, even though the rate of activation of the cotransporter was very weak. This contradiction between carbachol effect (no activation of NKCC1) and PMA effect (activation of NKCC1) might be explained by the weaker extent of activation of PKC during cholinergic effect compared to the direct and robust activation of the enzyme by PMA. Lack of effect of carbachol on NKCC1 might be explained also by the rapid cholinergic-evoked internalization

of the cotransporter. Besides acetylcholine, parasympathetic nerves also release VIP. This transmitter acts predominantly through elevation of cytosolic cAMP level, the minority of its action thought to be mediated by Ca^{2+} signaling. We could demonstrate a considerable increase of NKCC1 activity evoked by VIP stimulation.

Involvement of CFTR in the secretory process of various epithelial cells is widely investigated. Defective Cl^- and consequent fluid secretion can be observed in the pancreatic ducts, in the airways epithelium or in the salivary glands when CFTR protein is missing or aberrant. Unfortunately, little is known about the role CFTR may play in LG function. In the present study, we demonstrated decreased tear secretion and increased corneal fluorescein staining in KO mice in all three age groups studied, as compared to age-matched WT mice, suggesting CFTR plays a critical and essential role in tear secretion and in the maintenance of ocular surface integrity. We also found significant increase in tear secretion in 14-16 weeks old WT mice, as compared to the 8-10 weeks old WT animals. This increase could not be observed in KO mice which may indicate the impaired reserve capacity of tear secretion during the examined life period in KO mice. Our results show that KO mice is a useful model to investigate the role of CFTR may play in LG secretion.

Histological examinations did not reveal any significant morphological differences between the WT and KO LG tissues either in the 8-10 weeks or in the 20-24 weeks old group. These histological results demonstrated that functional deterioration may precede morphological alterations during the course of disease progression. Our immunohistochemical examinations proved the strong predominance of CFTR protein in duct cells compared to the acini. Therefore to further elucidate the role of CFTR in LG function, we used LG duct segments isolated form WT and CFTR KO mice. Complete absence of forskolin-stimulated fluid secretion on video-microscopy observed in CFTR KO ducts represents the important role of CFTR may play in LG duct secretion in mice. Lack of cAMP-mediated fluid secretion in CFTR KO ducts demonstrates that CFTR can be the only cAMP-dependent transporter on the luminal surface of duct cells in mouse LG. Considering the strong predominance of CFTR protein in LG ducts, CFTR could influence LG secretion through the modification of ductal secretion. Cl^- secretion through CFTR may be a major contributor to the transmembrane electrochemical gradient and subsequent electrolyte and water movements and therefore defects in CFTR may significantly compromise Cl^- and water secretion from LG ducts. Our present results provide evidence, that CFTR affects not only corneal and conjunctival epithelial cell function, but also LG secretion.

6. SUMMARY

In conclusion, our results presented here demonstrate the functional presence of NKCC1 in rabbit LG duct cells, providing further support that this transporter can be the main route of basolateral Cl^- uptake. Our data also demonstrated decreased tear secretion and impaired ocular surface integrity in CFTR KO mice, suggesting the important role of CFTR may play in LG function and in the maintenance of ocular surface integrity. Our functional studies by employing our isolated duct segment model, suggest that CFTR plays a pivotal role in the fluid secretion of LG duct system. Further studies are needed to clarify whether modification of CFTR function may serve as a potential target to stimulate LG secretion and therefore can be an option in treating aqueous deficient dry eye.

Conclusions of the studies on LG ducts presented in the thesis are:

1) **NKCC1 is expressed on the basolateral membrane of the LG duct cells in rabbit**
2) **The transporter can be activated by low cytosolic Cl^- level, hyperosmolarity of bath media, elevated intracellular cAMP level, and in a smaller extent, by direct activation of PKC.**
3) **For the first time in LG research, CFTR KO mice were used to study the role of CFTR in ocular surface integrity and in the function of the LG.**
4) **CFTR KO mice exhibited considerably decreased tear secretion and increased corneal fluorescein staining compared to WTs, suggesting the pivotal role of CFTR in physiological tear secretion and in the maintenance of ocular surface health.**
5) **Strong predominance of CFTR protein was demonstrated on the basolateral membranes of duct cells compared to acinar cells in the LGs of WT mice.**
6) **Using our isolated duct segment model developed earlier, fluid secretion of LG ducts from WT and CFTR KO mice was investigated. Forskolin-induced elevation of cytosolic cAMP level resulted in strong secretory response in WT ducts, while no secretion was observed in KO ducts.**

ACKNOWLEDGEMENT

First of all I express my gratitude and appreciation to **Dr. Edit Tóth-Molnár** who introduced me to the experimental work and always helped and guided me during my PhD studies. Her attitude, inspiration and advices helped me to complete this work.

I am very grateful to **Professor András Varró** for giving me the opportunity to work in a highly inspiring scientific environment in the Department of Pharmacology and Pharmacotherapy.

I am indebted to **Professor Péter Hegyi** and **Professor Zoltán Rakonczay** for providing me all tools and instruments of the Pancreas Research Group needed in our experiments.

I wish to thank **Professor Andrea Facskó** for granting the necessary time for my experimental work.

I especially grateful to **Orsolya Berczeli, Dr. Máté Katona** and **all members of the Pancreas Research Group** for their continuous support and help during my entire work.

Last but not least I would like to express my sincere gratitude to **my parents** for their endless love and support in my all efforts.