Adrenergic regulation of lacrimal gland ductal fluid secretion: role and intracellular mechanisms of adrenergic stimulation

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PhD Thesis

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2021

LIST OF FULL PAPERS RELATED TO THE THESIS

Szarka D, Elekes G, Berczeli O, Vizvári E, Szalay L, Ding C, Tálosi L, Tóth-Molnár E.
Alpha-Adrenergic Agonists Stimulate Fluid Secretion in Lacrimal Gland Ducts.
Invest Ophthalmol Vis Sci 2020 Dec 1;61(14):3. doi: 10.1167/iovs.61.14.3.
Scimago: Q1/D1 Impact factor: 3.46 (2019)

Berczeli O, **Szarka D**, Elekes G, Vizvári E, Szalay L, Almássy J, Tálosi L, Ding C, Tóth-Molnár E.
The regulatory role of vasoactive intestinal peptide in lacrimal gland ductal fluid secretion: A new piece of the puzzle in tear production.
Mol Vis. 2020 Dec 6;26:780-788. eCollection
Scimago: Q2 Impact factor: 2.21 (2019)

1. INTRODUCTION

Tear film is a complex mixture of substances secreted from multiple sources on the ocular surface, including the main and accessory lacrimal glands, the Meibomian glands, and the goblet cells. The appropriate amount and composition of the tear film is critical for a healthy, intact ocular surface. The lacrimal gland is a tubuloacinar exocrine gland what secretes electrolytes, water, proteins, and mucins - known as lacrimal gland fluid - into the tear film.

The lacrimal gland is composed of three major cell types: acinar, ductal, and myoepithelial cells. The main secretory cells are the pyramidal shaped polarized acinar cells that comprises about 80% of the gland. The ductal cells are also linked by tight junctions resulting in polarization of the cells and contribute to one-way secretion of lacrimal fluids. Most researches have been focusing on the function of acinar epithelial cells and much less efforts have been paid to the research of the ductal system even though its important role in lacrimal gland function has been assumed for a long time. Lack of functional experimental methods hindered the research of physiology and pharmacology of lacrimal gland ducts. An isolated duct model was developed in our laboratory with the modified adaptation of the method used in pancreas duct research. Using this model and a video-microscopy method, experimental evidence of fluid secretion of rabbit lacrimal gland ducts was given, confirming the important role of ducts in tear secretion. The isolated duct segment model is also suitable for investigation of intracellular mechanisms of the duct system. Using this model our research group identified and studied the functional activity of several ion transporters in the polarized duct cells. These experiments confirmed the functional presence of Na^+/H^+ exchanger, Cl^-/HCO_3^- exchanger and NKCC1 on the basolateral side of rabbit lacrimal gland ducts. CFTR KO mice model were used to study the role of parasympathetic regulation in lacrimal gland duct function and to study the role of CFTR in the secretory mechanism of ducts. Carbachol- and VIP-induced fluid secretory experiments provided direct functional evidence of the importance of parasympathetic regulation in lacrimal gland ducts.

Regardless of the results mentioned above, autonomic regulation of the ductal function is far not fully explored. Parasympathetic pathways are rated as the main regulatory system of lacrimal gland function while sympathetic effects have been supposed to play an indirect role through blood flow regulation. There are increasing evidence, however, that sympathetic stimulation - apart from the hemodynamic effects - plays a direct and important role in the protein secretion of the lacrimal gland. Although earlier reports suggested that both α and β

adrenergic agonists could result in protein secretory response in whole lacrimal gland pieces of mouse, the role of α-adrenergic receptors is suggested to be more relevant. Furthermore, intracellular mechanisms mediating α-adrenergic stimulation in lacrimal gland involve additional pathways beside the conventional route through activation of phospholipase C. This conception is supported by the well documented fact, that the dominant α-adrenergic receptor subtype present in the lacrimal gland is the α_{1D} and not the most common α_{1A} or α_{1B} subtypes.

The intracellular mechanisms of α_{1D} adrenergic receptor activation are not clearly understood. Additionally, involvement of the NO/cGMP pathway was suggested in the phenylephrine-induced protein secretion of lacrimal gland in rat. However, all these results were obtained from studies investigating the effect of adrenergic stimulation on acinar cells or on whole lacrimal gland pieces. Therefore, the effect of adrenergic stimulation as well as the intracellular mechanisms underlying this process in ducts of lacrimal glands are completely unknown.

2. AIMS OF THE STUDY

Although there is a growing amount of information about the function of adrenergic innervation in lacrimal gland, the available studies provide data particularly on protein secretion of acinar cells. The role of adrenergic effect in the regulation of lacrimal gland ductal fluid secretion is unknown. Therefore, aims of the present study were:

1) to investigate the effect of adrenergic stimulation on fluid secretion of isolated lacrimal gland duct segments

2) to assess the type and subtype of the involved adrenergic receptors

3) to study the underlying intracellular mechanisms.

3. MATERIALS AND METHODS

3.1. Animals

FVB/N mice were used in our study. The animals were 12-16 weeks old and weigh of 18-22 g. Both gender were used in a 1:1 ratio for all experiments. Mice were narcotized intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg) and euthanized with pentobarbital overdose (100 mg/kg) then the exorbital lacrimal glands were dissected. All

experiments were conducted in compliance with the statement of the Association for Research in Vision and Ophthalmology for the Use of Animals in Ophthalmic and Vision Research. The protocol 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.

3.2. Isolation and culture of lacrimal duct segments

Lacrimal glands 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 lacrimal gland ducts were transferred to the culture solution in a Petri dish. Ducts were cultured overnight.

3.3. Measurement of fluid secretion of lacrimal gland 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.

3.4. Measurement of intracellular Ca^{2+} level with microfluorophotometry

Intracellular Ca^{2+} level was measured using Ca^{2+}-sensitive fluorescent dye FURA 2AM. Changes in intracellular Ca^{2+} level were measured using an imaging system (Xcellence; Olympus). Small areas (region of interests: ROIs) of 5 to 10 cells were excited in each intact duct with light at 340 and 380 nm, and the 380/340 fluorescence emission ratio was measured at 510 nm. Results were expressed as maximum value of the 380/340 fluorescence emission ratio [F_{max} ($_{380/340}$)]. One measurement was obtained per second.

3.5. Statistics

For the analysis of ductal fluid secretion, effects of the stimulatory agents (phenylephrine, isoproterenol, noradrenaline) were taken into account as “fixed effects”. The effect of the individual “duct" and the “duct and effects of phenylephrine / isoproterenol / noradrenaline interaction” (we assumed that the value of the effect of the stimulatory compounds depend on the individual duct) were taken into account as random effects in the model. For the investigation of the inhibitory effect of L-NAME, ODQ and BMY-7378, data were expressed as the percent change of the luminal volume (LV) above baseline LV (baseline LV was considered 1.0). A mixed ANOVA model was used for statistics, by using SigmaPlot version

12.5 (Systat Software Inc., San Jose, CA, USA), results were presented as means ± SEM. A 'p' value of less, than 0.05 was regarded as significant.

4. RESULTS

4.1. Effect of adrenergic agonists on fluid secretion of lacrimal gland ducts

4.1.1. Noradrenaline

Isolated mouse lacrimal gland ducts were stimulated with various concentrations (5, 10 or 20 μM) of the natural adrenergic agonist noradrenaline to determine the secretory response and dose-response relationship. Noradrenaline stimulates both α- and β-adrenergic receptors causing a complete adrenergic upset. Application of noradrenaline initiated a dose-dependent, rapid fluid secretory response. The most effective concentration of noradrenaline proved to be 10 μM, higher concentration (20 μM) did not result in further increase in the secretory response of the investigated ducts.

4.1.2. Phenylephrine

To analyze the role of various adrenergic receptors in the observed adrenergic secretory response, effects of selective α_1 and β_1 adrenergic stimulations were investigated. In the α_1-adrenergic studies, ducts were stimulated with phenylephrine. Various concentrations (5, 10 or 20 μM) were used to determine the secretory response and dose-response relationship. To ensure the blockade of β-adrenergic receptors, phenylephrine was administered in the presence of β-adrenergic antagonist propranolol (1 μM). Secretory response of ducts given to phenylephrine was similar to those caused by noradrenaline: application of phenylephrine evoked a rapid fluid secretory response. The optimal concentration of phenylephrine found to be 10 μM: higher concentration (20 μM) did not result in any further increase in the fluid secretion of the ducts. Therefore, this concentration (10 μM) was used throughout the additional phenylephrine experiments. No statistically significant difference was detected between the extent of the fluid secretory rates evoked by phenylephrine in the presence of propranolol vs. noradrenaline ($p=0.42$) and the kinetics of these stimulated secretions were also similar.

Earlier studies demonstrated, that α-adrenergic receptor subtype present in the acinar epithelial cells of lacrimal gland is the $\alpha_{1D.}$ Therefore, we investigated whether this receptor subtype is involved in the α-adrenergic secretory response of mouse lacrimal gland ducts. Isolated lacrimal gland duct segments were pre-incubated with different doses of selective α_{1D} receptor antagonist BMY-7378 (1, 10, 100 and 200 μM) for 30 minutes and then phenylephrine (10 μM) was added to the superfusate containing α_{1D} receptor antagonist. BMY-7378

completely abolished phenylephrine-induced ductal fluid secretion proving the role of α_{1D} -adrenergic receptors in the observed secretory response.

4.1.3. Isoproterenol

Effect of β-adrenergic stimulation on ductal fluid secretion was also investigated. β-adrenergic agonist isoproterenol was administered in the presence of α-adrenergic antagonist phentolamine (10 μM) to ensure the selective β-adrenergic stimulation. Isoproterenol failed to elicit any detectable secretory effect in all applied concentrations (50, 100 or 200 μM).

4.2. Investigation of intracellular mechanisms underlying phenylephrine-induced ductal fluid secretion

4.2.1. Effect of endothelial nitric oxide synthase (eNOS) inhibitor L-NAME on phenylephrine-induced ductal fluid secretion

Since the mechanisms underlying α_{1D}-adrenergic receptor stimulation involve NO/cGMP pathway in acinar cells, the role of this intracellular pathway was investigated in the next series of experiments. Lacrimal gland ducts were pre-incubated with different doses of eNOS inhibitor L-NAME (1, 10, 100 or 200μM) for 30 minutes and then 10 μM of phenylephrine was added to the bath containing eNOS inhibitor. Phenylephrine-evoked ductal fluid secretion was reduced by L-NAME in a dose-dependent manner. However, even at the maximal inhibition effect of L-NAME, a significant difference ($p=0.023$) was found between baseline LV and LV measured following phenylephrine stimulation in the presence of L-NAME. These results suggest that although administration of L-NAME reduced, but not completely abolished the phenylephrine-induced fluid secretion of isolated lacrimal gland ducts.

4.2.2. Effect of guanylyl cyclase inhibitor ODQ on phenylephrine-induced ductal fluid secretion

To investigate the role of cGMP-pathway in phenylephrine-induced ductal fluid secretion, lacrimal gland ducts were pre-incubated with different doses of guanylyl cyclase inhibitor ODQ (0.1, 1, 10 or 100 μM) for 30 minutes and then 10 μM of phenylephrine was added to the bath containing guanylyl cyclase inhibitor.

Inhibition of guanylyl cyclase with ODQ decreased phenylephrine-induced LV increase in a dose dependent manner. Maximal inhibition occurred at 10 μM ODQ concentration. Although the inhibitory effect of ODQ was visible, a significant difference ($p=0.0008$) was

proved between baseline LV and LV measured following phenylephrine stimulation in the presence of ODQ. Effect of ODQ administration was similar to the one that L-NAME produced in the previous experiments: it reduced, but not completely inhibited phenylephrine-induced ductal fluid secretion.

4.2.3. Phenylephrine-evoked Ca^{2+} signaling in isolated lacrimal gland duct segments

While α_{1D} receptor blockage with BMY-7378 completely abolished phenylephrine-induced ductal fluid secretion, inhibition of eNOS or guanylyl cyclase considerably reduced but couldn't completely blocked it. We hypothesized in the background of this phenomenon that the elevation of intracellular Ca^{2+} level as a consequence of α_{1D} adrenergic receptor activation may contribute to the fluid secretion of the ducts.

To investigate this theory, in the next series of experiments changes of intracellular Ca^{2+} level were measured in response to phenylephrine stimulation. In these experiments, applied concentration of phenylephrine was 10 μM similarly to the fluid secretion experiments. Stimulation of α_1-adrenergic receptors by phenylephrine resulted in a small, but statistically significant increase in intracellular Ca^{2+} level of ducts ($p=0.012$). The extent of this increase was much smaller, compared to the response we observed previously during carbachol stimulation in epithelial cells of isolated mouse lacrimal gland ducts.

4.2.4. Effect of co-administration of L-NAME with Ca^{2+} -chelator BAPTA-AM on phenylephrine-induced ductal fluid secretion

In contrast to BMY-7378, eNOS inhibitor L-NAME considerably reduced but not completely abolished phenylephrine-induced ductal fluid secretion. To investigate the potential role of phenylephrine-evoked elevation of intracellular Ca^{2+} level, effect of L-NAME inhibition on phenylephrine-induced secretion was investigated in the presence of intracellular Ca^{2+}-chelator BAPTA-AM. Co-administration of L-NAME and BAPTA-AM completely blocked phenylephrine-induced ductal fluid secretion.

4.2.5. Effect of co-administration of ODQ with Ca^{2+} -chelator BAPTA-AM on phenylephrine-induced ductal fluid secretion

Phenylephrine-induced secretion was also studied in the combined presence of ODQ and intracellular Ca^{2+}-chelator BAPTA-AM. Isolated ducts were pre-incubated with ODQ (100 μM) and BAPTA-AM (10 μM) in these experiments. A complete inhibition of phenylephrine-induced ductal fluid secretion was observed following co-administration of ODQ and BAPTA-AM: change of LV was negligible and non-significant compared to baseline value.

5. DISCUSSION

Tear secretion is regulated by the autonomic nervous system. Fibers from both the parasympathetic as well as the sympathetic ganglion innervate the gland and exert positive and negative control over the secretion. Parasympathetic pathways are rated as the main regulatory system of lacrimal gland function. Role of parasympathetic stimulation on ductal fluid secretion was revealed by our laboratory: secretory effects of carbachol and VIP in isolated lacrimal gland ducts were proved and analyzed in our previous studies.

Beside the generally accepted decisive role of parasympathetic innervation, there are accumulating experimental evidence about the direct effect of the sympathetic system on lacrimal gland function. However, all results, suggesting the role of sympathetic regulation were obtained from studies performed on acinar cells, on whole lacrimal gland or gland pieces.

In my thesis the experimental evidence of the role of adrenergic effect in the regulation of lacrimal gland ductal fluid secretion is presented. Application of the natural adrenergic transmitter noradrenaline induced a rapid and robust fluid secretion in the isolated ducts. Considering the intense response observed, sympathetic stimulation may have more functional significance than previously believed. As noradrenaline stimulates both α and β adrenergic receptors, we investigated the pharmacological background of the observed secretory response. Stimulation of α-adrenergic receptors with phenylephrine in the presence of β-adrenergic blocker propranolol resulted in a pronounced ductal fluid secretion similar to those observed during application of noradrenaline. In contrast, no detectable fluid secretion was observed by the activation of β-adrenergic receptors with isoproterenol in the presence of α-adrenergic antagonist phentolamine.

These results are in agreement with a previously published study, where high density of α-adrenergic receptors and very weak presence of β-adrenergic receptors were found in lacrimal gland ducts by immunostaining. Our results solidly suggest the involvement of sympathetic nervous system in the regulation of ductal fluid secretion and the decisive role of α-adrenergic receptors in the sympathetic neurotransmission of this regulatory process.

Generally, three subtypes of α1-adrenergic receptors, α_{1A}, α_{1B} and α_{1D}-subtypes have been identified using pharmacological, biochemical and molecular biology methods. The lacrimal gland tissue contains both α_{1B}- and α_{1D}-adrenergic receptors. The α_{1D} is the dominant between these two subtypes, while no α_{1A}-adrenergic receptors were detected by either binding or RT-PCR studies. Despite widespread distribution, there is limited knowledge regarding the role of α_{1D}-adrenergic receptors in cellular functions. The role and function of α_{1D}-adrenergic receptors in lacrimal gland ducts is not known. In our experiments selective α_{1D} receptor blocker BMY-7378 could completely abolish the phenylephrine-induced ductal fluid secretion proving the involvement of this receptor subtype in the sympathetic innervation of lacrimal gland ducts.

In exocrine tissues α1-adrenergic agonists - in general - activate phospholipase C. However, increasing body of evidence shows that the α_{1D}-adrenergic receptor subtype may activate different signaling pathways and any or some of them may play role in the lacrimal gland.

To elucidate the intracellular mechanisms underlying α_{1D}-adrenergic stimulated ductal fluid secretion, the role of NO/cGMP pathway was investigated in our experiments. Both eNOS inhibitor L-NAME and guanylyl cyclase inhibitor ODQ reduced but couldn't completely block phenylephrine-evoked ductal fluid secretion. This finding partially differed from results obtained by Hodges et al in rat lacrimal gland acinar cells where application of either L-NAME or ODQ resulted in a complete blockade of phenylephrine-induced protein secretion. Although our results proved the involvement of NO/cGMP pathway an additional and obviously NO/cGMP pathway-independent mechanism was supposed in the background of the observed partial blockade. Since α-adrenergic stimulation is generally linked to Ca^{2+} signaling, the effect of phenylephrine on intracellular Ca^{2+} level was investigated. Phenylephrine stimulation resulted in a small but significant elevation of intracellular Ca^{2+} level. To specify the role of observed Ca^{2+} signaling in the α-adrenergic stimulation-enhanced fluid secretion, a further series of experiments were performed. In these experiments, participation of Ca^{2+} signaling was excluded by intracellular Ca^{2+} chelator BAPTA-AM. Under these circumstances, blockade of NO/cGMP either by L-NAME or ODQ completely abolished the phenylephrine-induced ductal

fluid secretion, showing the apparent role of a NO/cGMP pathway-independent Ca^{2+} signaling mechanism.

6. SUMMARY

In conclusion, our data strongly suggest the direct role of α adrenergic stimulation in lacrimal gland ductal fluid secretion. Both the lack of isoproterenol-induced fluid secretory response and the similar secretory effects of noradrenaline and phenylephrine suggest that the determining adrenergic pathway is via α-adrenergic receptors in mouse lacrimal gland ducts.

Inhibition of phenylephrine-induced ductal fluid secretion by α_{1D} adrenergic receptor antagonist or by reduction of fluid secretion by either eNOS or guanylyl cyclase inhibitors suggest that α-adrenergic agonists use the NO/cGMP pathway through α_{1D} receptor stimulation to increase fluid secretion, but involvement of a NO/cGMP pathway-independent Ca^{2+} signaling mechanism is also assumed.

Conclusions of the studies on adrenergic stimulation of isolated mouse lacrimal gland ducts presented in the thesis are:

1. **Our results prove the involvement of sympathetic nervous system in the regulation of ductal fluid secretion**
2. **α-adrenergic stimulation caused a rapid fluid secretory response in the isolated mouse lacrimal gland duct segments, therefore the α-adrenergic effect may play a significant direct role in the regulation of ductal fluid secretion**
3. **No detectable secretory effect was observed by the activation of β-adrenergic receptors**
4. **Selective α_{1D} receptor blocker BMY-7378 (100 μM) completely abolished phenylephrine-induced ductal fluid secretion proving the role of α_{1D} -adrenergic receptors in the observed secretory response**
5. **E-NOS inhibitor L-NAME (100 μM) and guanylyl cyclase inhibitor ODQ (10 μM) reduced, but not completely abolished the phenylephrine-induced fluid secretion of isolated lacrimal gland ducts showing the important but not exclusive role of a NO/cGMP pathway**
6. **Phenylephrine-evoked elevation of intracellular Ca^{2+} level has a minor but apparent role via Ca^{2+} signaling in the α-adrenergic stimulation-enhanced ductal fluid secretion.**

ACKNOWLEDGEMENTS

First and foremost I would like to express my gratitude to my mentor and guide **Dr. Edit Tóth-Molnár** who has been a constant source of ideas, knowledge, support and encouragement. With her passion about her work and her moral values, she taught me not just how to be a better professional but also a better human being.

I am grateful to **Prof. Dr. András Varró**, the former head of Department of Pharmacology and Pharmacotherapy who gave me opportunity to work at his DepartmentI also would like to say thank you for **Prof. Dr. Péter Hegyi** and **Dr. Viktória Venglovecz** for the opportunity to do my research in their laboratory.

I would like to thank my colleagues from the **Lacrimal Gland Research Group**, **Gréta Elekes, Dr. Orsolya Berczeli, Dr. Eszter Vizvári** and **all members of the Pancreas Research Group** for their support and help during my work and also the for the days that put a smile on my face.

I am mentioning these names at the end of the section, yet these were the people who have encouraged me **my parents, my brother dr. Ádám Szarka, Renáta Bordás, Noémi Nikoletta Smaniotta**. Their constant support, not just emotionally, but actually taking care of my things, has kept me going on.