The effect of alcohol and cigatette smoking on the iontransport processes of esophageal epithelial cells

Cigarette smoking and/or heavy alcohol consuption are among the few, potentially risk factors, which may contribute to the development of esophageal diseases. Previous studies demonstrated that smoking increase the risk of GERD, esophagitis and Barrett's esophagus (BE) development and also enhance the rate of BE transformation to cancer. Regarding to alcohol, opinions are contradictory but agree that long-term alcohol consumption sensitizes the esophagus to certain esophageal diseases. In contrast, very few experimental studies have been conducted, therefore the mechanism by which smoking or ethanol make the esophagus more sensitive to reflux-induced diseases, is not completely known. The esophageal epithelial cells provides the structural integrity of the esophagus. These cells are exposed to duodenogastric reflux even multiple times a day, therefore several protective mechanisms, such as esohageal tissue resistance, developed in order to avoid reflux-induced esophageal injury. The esohageal tissue resistance is highly determined by the **ion transport processes** across the esophageal epithelial cells. These transport mechanisms ensure a HCO_3^--rich mucus layer on the luminal surface of the epithelial cells and also contribute to the establishment of the intracellular acid buffering systems. In this project our hypothesis was that ethanol and cigarette smoke extract alter the activity and/or expression of ion transporters which damage the esophageal tissue resistance and make the esophagus more susceptible to acidic reflux.
Since esophageal ion transport processes are a less studied area, there is no comprehensive study describing what ion transporters are found on esophageal epithelial cells (EECs). Therefore, we conducted a literature search as a first step. As a result, we described the currently identified functionally active ion transporters in the EECs and discussed the pathological significance of these transporters in different esophageal lesions (Becskeházi et al. 2020).

Establishment of experimental system for functional investigation of esophageal epithelial ion transport processes

In order to study esophageal ion transport processes cell lines are available which are derived from human esophagus with metaplasia (CP-A), dysplasia (CP-D), and adenocarcinoma (OE-33). Although these cell lines are suitable for the study of pathological processes, they are not the most suitable for the study of physiological processes. To examine the effects of tobacco smoke and ethanol on normal cells, we obtained a normal esophageal cell line (HET-1A) in addition to the existing ones. Unfortunately, maintaining normal cells is very expensive and cumbersome, and in our case, despite several attempts, HET-1A cells regularly detached and died during the culturing. So this normal cell line could not be maintained and therefore we were unable to perform the experiments we planned on it. However, in the research plan, we also designed experiments on another type of normal cell, namely on primary EECs isolated from guinea pig. The methodology was successfully adapted and optimized for our own experiments. EECs were obtained after enzymatic digestion of the esophageal mucosa. The primary cells proved viable, could be maintained in culture media for 6-12h and were suitable for functional measurements.

In addition, another experimental model was also used to characterize normal esophageal epithelial iontransport processes. We generated 3D esophageal organoids (EOs) from two different mouse strains (CD-1 and C57BL/6) and we proved that this model is suitable to study esophageal ion transport mechanisms (Korsós et al. 2021). We showed that EOs form a cell-filled structure with a diameter of 250–300 µm and generated from epithelial stem cells as shown by flow cytometry analysis. Using conventional PCR and immunostaining, the presence of Slc26a6 Cl^-/HCO_3^- anion exchanger (AE), Na^+/H^+ exchanger (NHE), Na^+/HCO_3^- cotransporter (NBC), cystic fibrosis transmembrane conductance regulator (CFTR) and anoctamin 1 Cl^- channels were detected in EOs. One of the interesting finding of our study is the presence of CFTR Cl^- channel on EOs. Immunolocalization illustrated that both peripheral and central cells highly express CFTR. Costaining of CFTR and Slc26a6 revealed some colocalization, mainly in cells on the periphery, indicating that the two transporters interact with each other. In order to investigate the activity of most of these transporters, microfluorimetric technique was used. We initially determined the resting pH and total buffering capacity of the cells. We found that the starting pH of the organoids was nearly 7.6 which is unusually high in epithelial cells and is presumably explained by the excessive activity of the alkalizing transporters that act against acidosis. Microfluorimetric techniques revealed high NHE, AE, and NBC activities. The CFTR activator forskolin dose-dependently increased the activity of CFTR, although the response to forskolin was relatively low even in the presence of supramaximal concentrations, indicating that CFTR channel activity is lower than usually observed for secretory epithelia. The presence of the CFTR inhibitor CFTRinh-172 decreased the activity of AE indicating that CFTR regulates the activity of AE. This type of interaction was not previously described in the esophagus. In this study, we uncovered for the first time the presence of the major epithelial ion transporters in EOs and showed that EOs provide a relevant and suitable model system for studying the ion transport mechanisms of esophageal epithelial cells.

Effect of smoking on iontransporters of esophageal epithelial cells

After physiological examination of the ion transport processes of EECs, we examined how the cells behave under pathological conditions and whether this plays a role in the development of esophageal diseases. In the first step, we examined the effect of cigarette smoke extract (CSE) on the activity and expression of ion transporters (Becskeházi et al. 2021). Among the acid–base transporters, we focused on NHE-1, since this exchanger has a multiple role in the cells, including the regulation of pH_i, cell volume, proliferation, migration, and invasion. First, we have investigated how CSE affects NHE activity of the normal EECs isolated from the guinea pig. Pretreatment with different concentration of CSE significantly reduced the activity of NHE. In order to investigate the chronic effects of smoking, guinea pigs were exposed to cigarette smoke for one, two and four months, respectively, and then NHE activity was examined. Similarly to acute CSE treatment, chronic treatment decreased NHE activity. The decreased expression of NHE-1 associates with cellular acidosis, which may increase the risk of cancer development, as a greater number of DNA damage and thus mutations develop in an acidic environment. In contrast, CSE increased NHE-1 activity in the metaplastic cells and decreased the activity in the dysplatic cells. Although, CSE treatment did not cause significant differences in mRNA expression of NHE-1 in any of the cell lines, increased the protein

expression of metaplastic cells. The increased activity and expression of NHE-1 in the metaplastic CP-A cells presumably a defense mechanism by which the cells try to maintain the normal pH homeostasis. In contrast, decreased activity of NHE-1 in the dysplastic, CP-D cells indicates damaged compensatory pH regulatory mechanisms. The results obtained for the metaplastic cell line were also confirmed with human surgical specimens. Protein expression of NHE-1 was investigated in normal squamous epithelium and in BE samples obtained from patients with smoking and non-smoking history. Patients who had never smoked or not smoked for more than a year were classified as non-smokers, while patients who had been smokers for at least 20 years were classified as smokers. Only patients with known smoking status were included in the analysis. As controls, normal esophageal biopsy samples and the intact tumor-free margin of surgically resected esophageal cancer were used. Weak NHE-1 expression was detected in the normal esophageal epithelium, and it was further reduced by smoking. In BE, strong NHE-1 expression was observed, mainly at the basolateral membrane of the columnar cells. In smokers, NHE-1 expression increased, and staining was detected not only in the plasma membrane but in the cytoplasm as well. Interestingly, strong NHE-1 staining was also observed in the glands. There was no significant difference between the intestinal and non-intestinal metaplasia, neither in the smoker nor in the non-smoker group.

An interesting observation of our study is that CSE treatment slightly reduced the proliferation of metaplastic cells, while it increased the proliferation of dysplastic cells. In order to clarify the role of NHE-1 in the CSE-induced proliferation, we downregulated NHE-1 by specific siRNA transfection. In the absence of NHE-1, the CSE-induced proliferation increased in the metaplastic cell line, suggesting that NHE-1, in addition to being essential in maintaining the normal pH of cells, also performs an important protective function and regulates cell proliferation against toxic agents. In the more advanced dysplastic state, inhibition of NHE-1 had no effect on the CSE-induced proliferation, indicating that, in dysplasia, the proliferative effect of CSE is independent from NHE-1.

Taken together we have shown that smoking affects NHE-1 function in normal, metaplastic and dysplastic cells differently. Under normal conditions, smoking reduces the activity and expression of NHE-1, resulting in the acidosis of pHi. Disturbance of the pH homeostasis can lead to cell deaths or to the malignant transformation of the cells. In the metaplastic state, smoking increases the function of NHE-1, which is presumably a compensatory mechanism that prevents the onset of cancerous processes by keeping the intracellular pH in the normal range. As the expression of NHE-1 decreases, this protective mechanism disappears and the proliferative potential of the cells increases. In contrast to BE, decreased activity or expression of NHE-1 had no effect on smoking-induced proliferation in the dysplastic state indicating the involvement of other mechanisms. We propose that upregulation of NHE-1 is the part of a protective mechanism against the harmful effects of smoking; however, further investigation would be needed to support this hypothesis. Direct increase in NHE-1 expression by using NHE-1 agonists or the use of transgenic mice models in which the SLC9A1 gene is modified would give a more complete picture of the role of NHE-1. Nevertheless, the present results indicate that direct augmentation of NHE-1 function may provide new avenues for decreasing the damaging effect of smoking.

The above experiments were also performed on the OE-33, adenocarcinoma cell line. Very low NHE-1 expression was detected in this cell line, which did not change significantly with CSE

treatment. Therefore, further studies are needed to characterize the effect of CSE on ion transporters.

Effect of alcohol on iontransporters of esophageal epithelial cells

In the second half of the project, we investigated the effect of alcohol on ion transport processes in EECs. Experiments are in progress, but our recent results indicate that treatment with ethanol, similarly to smoke extract, increases NHE-1 activity and mRNA expression in metaplastic CP-A cells. EtOH did not significantly alter CP-A cell proliferation, however, upon silencing of the NHE-1 gene, ethanol increased cell proliferation, suggesting that NHE-1 activity and expression increase with ethanol treatment, which is able to prevent the proliferative effect of ethanol on the cells. In dysplastic CP-D cells, ethanol treatment did not significantly alter NHE-1 expression, but strongly inhibited the activity of the exchanger. Ethanol treatment increased proliferation of CP-D cells, which was not significantly affected by NHE-1 knock down. In adenocarcinoma OE-33 cells, no change in NHE-1 expression was detected with ethanol treatment, but a small decrease in NHE-1 activity was observed. Ethanol treatment increased the proliferative potential of OE-33, although, further studies are needed to determine whether NHE-1 has any role in ethanol-induced proliferation.

In summary our results indicate that both smoking and alcohol consumption alter the function of NHE-1. Because NHE-1 plays an essential role in the pH homeostasis of epithelial cells, changes in the function of NHE-1 disrupts normal intracellular pH regulation, which may play a role in the progression of certain esophageal diseases. Our results suggest that increased function of NHE-1 in metaplastic cells plays a protective role against cellular acidosis and try to keep the intracellular pH in the normal range. In contrast, decreased activity of this exchanger in the dysplastic CP-D cells indicates damaged compensatory pH regulatory mechanisms. Based on our studies to date, NHE-1 does not play a role in the effect of smoking on the adenocarcinoma cell line, but further studies are needed to support this hypothesis.

Since the esophageal epithelium is not a typical secretory epithelia, ion transport processes are less studied. Nevertheless, our results show that ion transporters play an important pathological role in the esophagus; therefore, targeted modulation of transport processes may open up new possibilities in the therapy of esophageal diseases.

Papers related to the topic of the project:

1. Becskeházi E, Korsós MM, Erőss B, Hegyi P, Venglovecz V. OEsophageal Ion Transport Mechanisms and Significance Under Pathological Conditions. *Frontiers in physiology*;855,15 p., 2020 **IF: 4.566 Q2**
2. Korsós MM, Bellák T, Becskeházi E, Gál E, Veréb Z, Hegyi P, Venglovecz V: Mouse organoid culture is a suitable model to study esophageal ion transport mechanisms. *Am. J. Physiol-Cell Physiol*; 321(5):C798-C811, 2021 **IF: 4.249 Q1**
3. Becskeházi E, Korsós MM, Gál E, Tiszlavicz L, Hoyk Zs, Deli MA, Köhler Z.M, Keller-Pintér A, Horváth A, Csekő K, Helyes Zs, Hegyi P, Venglovecz V. Inhibition of NHE-1 Increases Smoke-Induced Proliferative Activity of Barrett's Esophageal Cell Line. *Int. J. Mol. Sci*;22(19), 2021 **IF: 5.923 Q1**

Papers not related to the topic of the project, in which NKFIH support was indicated:

1. Gál E, Dolensek J, Stozer A, Pohorec V, Ébert A, Venglovecz V. A novel in situ approach to study to studying pancreatic ducts in mice. *Frontiers in Physiology;* 10:938, 2019 **IF: 3.45 Q2**
2. Barreto SG, Habtezion A, Gukovskaya A, Lugea A, Jeon C, Yadav D, Hegyi P, Venglovecz V, Sutton R, Pandol SJ. Critical thresholds : key to unlocking the door to the prevention and specific treatments for acute pancreatitis. *Gut*;70(1):194-203, 2020 **IF:23.059 Q1**
3. Gál E, Veréb Z, Kemény L, Rakk D, Szekeres A, Becskeházi E, Tiszlavicz L, Takács T, Czakó L, Hegyi P, Venglovecz V. Bile accelerates carcinogenic processes in pancreatic ductal adenocarcinoma cells through the overexpression of MUC4. *Scientific Reports*;10, article number: 22088, 2020 **IF: 4.13 Q1**

Conference presentations related to the topic of the project:

1. Becskeházi E, Vér K, Rábóczki B, Venglovecz V. Alcohol dose-dependently impairs the function of Na+/H+ exchanger in guinea pig esophageal epithelial cells. *Central European Journal of Gastroenterology and Hepatology/Gasztroenterológiai és hepatológiai szemle*, Suppl.1, p88, 2018
2. Becskeházi E, Gál E, Székács I, Rábóczki B, Venglovecz V. Alcohol and smoking alter ion transport mechanisms of esophageal epithelial cells. *Central European Journal of Gastroenterology and Hepatology/Gasztroenterológiai és hepatológiai szemle*, Suppl 1 p87, 2019
3. Korsós M, Venglovecz V. Setting up the oesophageal organoid culture and investigation of oesophageal iontransport mechanism. *Central European Journal of Gastroenterology and Hepatology/Gasztroenterológiai és hepatológiai szemle*, Suppl 1 p121, 2019
4. Becskeházi E, Gál E, Korsós M, Venglovecz V. Smoking changes iontransport mechanisms of guinea pig esophageal epithelial cells and human esophageal cell lines. *Central European Journal of Gastroenterology and Hepatology/Gasztroenterológiai és hepatológiai szemle*, Vol. 6, Suppl 2 p35, 2020
5. Korsós MM, Bellák T, Becskeházi E, Venglovecz V. Esophageal organoid culture is a novel model to study epithelial ion transport mechanisms. *Central European Journal of Gastroenterology and Hepatology/Gasztroenterológiai és hepatológiai szemle*, Vol. 6, Suppl 2 p62, 2020

Theses related to the topic of the project:

1. Szekács István (2018) The effect of alcohol and cigarette smoke extract on the expression of ion transporters in OE33 esophageal adenocarcinoma cell line. SZTE-ÁOK, Farmakológiai intézet, témavezető: Dr. Venglovecz Viktória
2. Virág Melinda (2018) Az etanol hatása a nyelőcső epitél-sejtek sav/bázis transzportereire. SZTE-ÁOK, Farmakológiai intézet, témavezető: Dr. Venglovecz Viktória
3. Rábóczki Bettina (2019) The effect of alcohol and cigarette smoke extract on the iontransport mechanisms of guinea pig esophageal epithelial cells and OE-33 adenocarcinoma cell line. SZTE-ÁOK, Farmakológiai intézet, témavezető: Dr. Venglovecz Viktória