Role of epithelial cells in gastrointestinal diseases of the oesophagus and the pancreas

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Summary of Ph.D. thesis

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Publications

List of publications included in the thesis

I. **Becskeházi, E.**, Korsós, M. M., Gál, E., Tiszlavicz, L., Hoyk, Z., Deli, M. A., Köhler, Z. M., Keller-Pintér, A., Horváth, A., Csekő, K., Helyes, Z., Hegyi, P., & Venglovecz, V. (2021). Inhibition of NHE-1 Increases Smoke-Induced Proliferative Activity of Barrett's Esophageal Cell Line. *International journal of molecular sciences*, *22*(19), 10581. https://doi.org/10.3390/ijms221910581

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II. Gál, E., Veréb, Z., Kemény, L., Rakk, D., Szekeres, A., **Becskeházi, E.**, Tiszlavicz, L., Takács, T., Czakó, L., Hegyi, P., & Venglovecz, V. (2020). Bile accelerates carcinogenic processes in pancreatic ductal adenocarcinoma cells through the overexpression of MUC4. *Scientific reports*, *10*(1), 22088. https://doi.org/10.1038/s41598-020-79181-6

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List of publications related to the subject of the thesis

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IV. **Becskeházi, E.***, Korsós, M. M.*, Erőss, B., Hegyi, P., & Venglovecz, V. (2020). OEsophageal Ion Transport Mechanisms and Significance Under Pathological Conditions. *Frontiers in physiology*, *11*, 855. https://doi.org/10.3389/fphys.2020.00855

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List of other publications

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1. Introduction

1.1 Epithelial cells in the gastrointestinal tract

The epithelium is comprised of closely related epithelial cells, which are connected with each other via special structures. Important transport processes are proceeded through them, which are essential for the proper functioning of the human body. Polarization is the most characteristic feature of the epithelial cells, which means, that basal and apical surfaces are differentiated on these cells that vary in functional and biochemical properties, thereby ensuring unidirectional flow of substances. Gastrointestinal (GI) epithelial cells perform diverse functions in the body. During my research, my investigations focused at the function of GI epithelial cells, specifically the function of oesophageal and pancreatic epithelial cells. Although these two organs fulfil different functions in the body, notably, the oesophagus forwards food, whereas the pancreas takes part in the digestion, the common feature in them is, that epithelial cells play important role in both organs, and the disturbances of these epithelial cells can lead to the development of different inflammatory and cancerous diseases. In this thesis, I would like to present my research of these two organs, to the greater extent the oesophagus, and to the lesser extent the pancreas.

1.2 The oesophagus

1.2.1 Anatomical and histological overview of the oesophagus

The oesophagus is a tube-shaped organ that connects the pharynx and the stomach; it commences at the level of cricoid cartilage, continues in the thorax in the superior and posterior mediastinum, then crosses the diaphragm, where it ends in the cardia of the stomach. The oesophagus has two sphincters at its two ends, the upper oesophageal sphincter (UOS) and the lower oesophageal sphincter (LOS). The wall of the oesophagus is comprised of four layers, namely (from the inside) mucosa, submucosa, muscularis externa and adventitia.

1.2.2 Oesophageal defence mechanisms

The oesophagus possess defence mechanisms that prevents oesophageal damage. This is comprised of antireflux mechanisms, luminal clearance mechanisms, and tissue resistance. Antireflux mechanisms consists of the function of LOS and diaphragm. Luminal clearance mechanisms include gravity, saliva, swallowing, that together shortens the contact time with luminal content. Tissue resistance comprises three components, namely pre-epithelial, epithelial and post-epithelial defence mechanisms. The pre-epithelial defence includes mucus

layer, unstirred water and bicarbonate ions. Epithelial defence has structural and also functional components. Structural components prevent the diffusion of certain ions and molecules from the luminal space to the intra- and intercellular space. Beside cell restitution and proliferation, epithelial ion transporters, intra- and extracellular buffers have a great importance among functional mechanisms. They have a crucial role in compensation of acidification caused by transcellular or paracellular entry of H^+. The main post-epithelial defence mechanism of the oesophageal mucosa is blood flow.

1.2.3 Ion transporters of oesophageal epithelial cells (OECs)

Ion transporters are located on both the basolateral and the apical side of the squamous epithelial cells (SECs), and the distribution of different ion transporters develops an individual pattern that ensures unidirectional ion diffusion. Coordinated function of the ion transporters maintains not only a pH homeostasis, which is essential for cell functions, but it is also important in cell growth, migration, differentiation, restitution and cell volume. As a part of the epithelial defence, ion transporters have a considerable function in the physiology of the GI tract. Numerous articles have identified the presence of ion transporters in the GI cells, and their role in some diseases was also described, but only a few focused on the ion transport mechanisms in the oesophagus. To date, anion exchangers (AEs), Na^+/HCO_3^- co-transporter (NBC), Na^+/H^+ exchanger (NHE), Na^+/K^+/ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), cystic fibrosis transmembrane conductance regulator (CFTR), Ca^{2+} sensitive Cl^- channel, basolateral and apical K^+ channels, K^+/Cl^- co-transporter (KCC), three subunits of epithelial Na^+ channel (ENaC) have been identified in the oesophagus.

1.2.4 Physiological function of Na^+/H^+-exchanger-1 in oesophageal epithelial cells

Na^+/H^+-exchangers (NHEs) are integral membrane proteins, that mediate Na^+ influx and H^+ efflux in 1:1 stoichiometry in cells, using the energy of electrochemical Na^+ gradient provided by Na^+/K^+/ATPase. NHE-1 is located on the basolateral plasma membrane of the OECs. NHE-1 is identified as a housekeeping gene, and it is also involved in e.g. cell proliferation, migration and differentiation, indicating it complex role. However, in the oesophageal epithelium, the main function of NHE-1 is to prevent intracellular acidification by extruding H^+, therefore maintain the pH_i homeostasis of the cell in case of acidic luminal content.

1.2.5 Pathophysiological relevance of Na^+/H^+-exchanger-1 in the oesophagus

1.2.5.1 Na^+/H^+-exchanger-1 in gastro-oesophageal reflux disease and Barrett's oesophagus

On a long term of gastro-oesophageal reflux disease (GORD), the oesophageal epithelium adapts to the acidic environment, and Barrett's oesophagus (BO) develops, in which the stratified squamous epithelium is replaced by columnar metaplasia in the lower section of the oesophagus. The underlying mechanisms are not completely clear yet. Many studies have raised the possible involvement of NHE-1 and some articles also showed elevated NHE-1 expression in GORD and BO. In contrast, in normal tissue samples and cell lines only weak NHE-1 expression could be detected.

1.2.5.2 Na^+/H^+-exchanger-1 in oesophageal cancer

Oesophageal cancer is the 8th most common cancer around the world, that has a poor 5-year survival rate. It has two main histological type, oesophageal squamous cell carcinoma (OSCC) and oesophageal adenocarcinoma (OAC), from which OAC is strongly associated with BO. In oesophageal malignancies, there are less experimental data is available about the molecular mechanisms in association with NHE-1 and the results are contradictory.

1.2.6 The effect of smoking on the oesophagus

Smoking is one of the most important etiological factors in the development of oesophageal diseases. Clinical studies presented clear results that smoking elevates the risk of BO and OAC. In BO patients, smoking promotes progression and doubles the risk to high-grade dysplasia or cancer. Surprisingly, the effect of smoking on OECs has been investigated in only one basic research study, which suggested the role of Na^+ transporters in the effects of tobacco smoke; however, it did not identify the transporters involved, so the exact mechanism remains unknown.

1.3 The pancreas

1.3.1 Anatomy and function of the pancreas

The pancreas is an elongated, retroperitoneal, accessory digestive gland, which is situated transversely between duodenum and spleen. This organ has both exocrine and endocrine functions. The exocrine part of the pancreas is responsible for the secretion of digestive enzymes and HCO_3^--rich fluid that together give pancreatic juice. Langerhans-islets

create the lesser, endocrine part of the pancreas, that produce essential hormones for glucose homeostasis, namely insulin, glucagon, somatostatin.

1.3.2 Role of gastrointestinal epithelial cells in the pancreas

The human pancreas secretes 2,5 L of alkaline, isotonic pancreatic juice each day, which has a main role in the normal function of the organ. Acinar cells are polarized epithelial cells and create an acinar segment, where they produce isotonic fluid containing NaCl. Pancreatic duct can absorb Cl^- and secrete a relatively high concentration of HCO_3^- and water, with which it neutralizes the acidic acinar fluid. Intercalated ducts collect acinar fluid, then flow in to intralobular, interlobular and interlobar ducts that finally reach the main duct.

1.3.3 Duct-related pancreatic diseases

Pancreatic cancer (PC) is an aggressive, highly malignant cancer type in the GI tract, which has a very low 5-year survival rate. The diagnosis is difficult and often late, since there is lack of symptoms and due to retroperitoneal location of the pancreas, it can be problematic to examine this organ. More than 80% of PC are ductal adenocarcinoma (PDAC) and in 60% located in the head of the pancreas. As they grow, they cause obstructive jaundice, which means a poor prognostic sign. Pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasm (IPMN) are considered the precursor lesions of PDAC.

1.3.4 Bile acids as etiological factor in pancreatic cancer

BAs play a significant role in lipid digestion, absorption of fat-soluble vitamins and they are also key molecules in signalling pathways related to metabolism, gene expression or cell proliferation. The effect of BAs on pancreatic cells is a highly important topic in PC research. Although numerous studies described, that BAs, especially hydrophobic BAs, may contribute to the development and progression of GI cancers, there is no such a study that investigated the effect of the most common human BAs in PC.

2. Aims of the study

There is a lack of information about the effect of smoking on the pathophysiology of BO and its progression. As we have seen in the introduction, ion transporters have a highlighted role in the prevention of oesophageal injury. Based on this, we hypothesized, that expression and function of the main epithelial ion transporter, namely NHE-1, alters by a harmful action such as smoking and it has a significance in the pathophysiology of smoke-induced oesophageal injury. Aims in the first part of the thesis were:

I. to investigate the effect of cigarette smoke extract (CSE) on the activity and expression of NHE-1 isoform in primary OECs originated from guinea pig, in human metaplastic and dysplastic BO cell lines *in vitro*
II. to examine the impact of smoking on NHE-1 function in guinea pig model *in vivo*
III. to analyse the effect of smoking on NHE-1 expression in healthy and BO patients
IV. to investigate the role of NHE-1 in the effect of CSE

Numerous scientific articles have explained the carcinogenic feature of BAs. The majority of PC is located in the head of the pancreas, the growing tumour causes an obstruction of the common channel and provoke an obstructive jaundice. The exact effects of certain BAs are not clearly understood on the progression of PC. Aim in the second part of thesis was:

V. to estimate proliferative activity of pancreatic ductal adenocarcinoma cells after treatment with various BAs.

3. Materials and Methods

3.1 Human samples

Retrospective data collection of patients was performed by the approval of the Ethics Committee of the University of Szeged (No. 4658), according to Helsinki Declaration and GDPR. Patient information was retrieved concerning the histopathological samples collected between January 2018 and May 2021 from a medical database used in Albert Szent-Györgyi Medical School, University of Szeged, eMedSolution. BO patients were included based on age, diagnosis, state of the disease, smoking history, histopathological description of oesophageal lesion. Formalin-fixed, paraffin-embedded oesophageal tissue samples of patients were retrieved from the Archive of Department of Pathology, University of Szeged. Patients were

basically classified into two groups: smoking group with a smoking history of more than 20 years and non-smoking group who had never smoked or had not smoked for at least a year. As a control, tumour-free resection margins and normal oesophageal biopsy samples were used. The average age of BO patients was 55.1 ± 4.6 years in the smoking group (n = 7), and 57.3 ± 3.8 years in the non-smoking group (n = 20). The average age in the control group was 38.5 ± 11.5 years in the smoking group (n = 3), and 59.7 ± 3.8 years in the non-smoking group (n = 7).

3.2 Animals

4-12 week-old, male guinea pigs were used for our experiments. Animals were kept at room temperature (23 ±1°C), at 12:12 h light-dark cycle in plastic cages and had free access to laboratory chow and water completed with Vitamin C. All animal experiments were performed in accordance the Guide for the Care and Use of Laboratory Animals (United States, Department of Health and Human Services) and by the approval of the local Ethical Board of the University of Szeged, Hungary.

3.3 Cell lines

CP-A (human metaplastic oesophageal epithelial cell line) and CP-D (human dysplastic oesophageal epithelial cell line) and Capan-1 (human pancreatic adenocarcinoma) cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were seeded when being 90-100% confluent and CP-A and CP-D cells were used between 3-19 passage numbers, and Capan-1 cells between 30-35 passage numbers.

3.4 Cigarette smoke exposure

The effect of chronic cigarette smoke exposure was studied using smoking chamber at the Department of Pharmacology and Pharmacotherapy, University of Pécs. Male guinea pigs were divided into three groups according to the period of cigarette smoke exposure (1-, 2- and 4-months exposure, n=3/group). All animals were 5 months old at the time of sacrifice. Animals were exposed to whole body cigarette smoke exposure 5 times a day, 10 minutes each time using a TE2 closed-chamber manual smoking system. During the experiment, 3R4F Kentucky Research Cigarettes were smoked and ventilated inside the chamber. 24-48 hours after the last CSE exposure, animals were sacrificed, and OECs were isolated. Age-matched control animals were exposed to air. All experiments were performed in accordance with the institutional guidelines under approved protocols (No.: XII./2222/2018, University of Pécs).

3.5 Cigarette smoke extract and treatment

Cigarette smoke extract (CSE) was prepared at the Department of Pharmacognosy, University of Szeged. Briefly, mainstream smoke from 15 filterless 3R4F Kentucky Research Cigarettes (12 mg tar and 1.0 mg nicotine/cigarette), was continuously bubbled through 20 mL of distilled water. CSE was then sterile-filtered through a 0.22-μm filter and the dry weight was measured. CSE solution was than diluted to the desired concentration using HEPES or culture media. Cells were treated with cigarette smoke extract for 6, 24 and 72 hours in concentrations of 1, 10 and 100 μg/mL.

3.6 Isolation of guinea pig oesophageal epithelial cells

Our workgroup optimized a protocol for guinea pig OECs based on the work Kalabis et al. Briefly, animals were sacrificed by cervical dislocation, the oesophagus was removed, cut longitudinally, rinsed in Hank's Balanced Salt Solution (HBSS) and digested in dispase I solution (2 U/mL) for 40 minutes. After digestion, the inner, epithelial layer of the oesophagus was detached from the submucosa and rinsed in fresh HBSS. Then the epithelial layer was incubated in 0,25% Trypsin-EDTA solution supplemented with 1% (v/v) antimycoticum/antibioticum for 2 ×15 minutes, and the Eppendorf tube was vortexed several times during the incubation period to promote tissue disintegration. Filtered Soybean trypsin inhibitor solution was added to the tube and the whole lysate was centrifuged for 5 minutes at 1000 rpm. The cell pellet was diluted in keratinocyte serum free media supplemented with 1% (v/v) antimycoticum/antibioticum and seeded onto cover glasses and incubated until use.

3.7 Immunohistochemistry

Immunohistochemical analysis of NHE-1 expressions was performed on 4% buffered formalin-fixed sections of human oesophageal samples embedded in paraffin. The 5 μm–thick sections were stained in an automated system. Briefly, the slides were deparaffinised, and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 (10 min). Antigenic sites were disclosed by applying citrate buffer in a pressure cooker (120 °C, 3 min). To minimize non-specific background staining, the sections were then pre-incubated with milk (30 min). Subsequently, the sections were incubated with a human anti-NHE-1 (1:100 dilution, 30 min, Alomone Laboratories) primary polyclonal antibody and exposed to LSAB2 labeling for 2 × 10 min. The immunoreactivity was visualized with 3,3′-diaminobenzidine (10 min); then the sections were dehydrated, mounted and examined. NHE-1 expressing cells were identified

by the presence of a dark red/brown chromogen. A semi-quantitative scoring system was used to evaluate the expression of NHE-1. The intensity of staining (0 = negative, 1 = weak, 2 = moderate and 3 = strong) and the percentage of positive cells(1 = 0–25% of the cells are positive, 2= 25–50% of the cells are positive, 3 = 50–75% of the cells are positive and 4 = 75–100% of the cells are positive) were scored and then the composite score was obtained by multiplying the intensity of staining and the percentage of immunoreactive cells.

3.8 Western blot

Cells were lysed in Cell Lysis Buffer supplemented with complete EDTA-free protease inhibitor. Then samples were centrifuged at 2500 rpm for 20 minutes at 4 °C, and the supernatants were used. Protein concentration in the samples was determined by using a BCA assay or Bradford reagent, and equal amounts of proteins (20 or 30 μg) were resolved on polyacrylamide gel and transferred onto Protran or PVDF membranes. Membranes were incubated overnight with rabbit polyclonal anti-NHE-1, mouse monoclonal anti-GAPDH or mouse monoclonal anti-α-Tubulin antibody followed by the incubation with the appropriate HRP-conjugated secondary antibody. The peroxidase activity was visualized by using the enhanced chemiluminescence assay or with Clarity Chemiluminescence Substrate. Signal intensities were quantified by using the QuantityOne software or Image Lab Software, version 5.2. The results from each membrane were normalized to the GAPDH or α-Tubulin values and compared to the 6-hour control.

3.9 Measurement of intracellular pH

Cells were seeded onto 24 mm cover glasses which was placed on the stage of an inverted microscope (IX-71) connected with an Xcellence imaging system (Olympus, Budapest, Hungary). Cells were incubated with a pH-sensitive fluorescence dye, BCECF-AM for 30-60 minutes according to cell type. Cells were perfused with solutions at 37°C at a 5-6 ml/min perfusion rate. Average 5-12 regions of interest (ROIs) were marked in each measurement and one image was taken per second using a CCD camera. The cells were excited with 440 and 495 nm wavelengths, and the 440/495 ratio was detected at 535 nm. One pH_i measurement was obtained per second. *In situ* calibration of the fluorescence signal was performed using the high K^+-nigericin technique.

3.10 Determination of buffering capacity

The total buffering capacity (β_{total}) of cells was estimated according to the NH_4Cl pre-pulse technique, as previously described. Briefly, OECs were exposed to various concentrations of NH_4Cl in a Na^+- and HCO_3^--free solutions. The total buffering capacity of the cells was calculated using the following equation: $\beta_{total} = \beta_i + \beta_{HCO3-} = \beta_i + 2.3 \times [HCO_3^-]_i$, where β_i refers to the ability of intrinsic cellular components to buffer changes of pH_i and was estimated by the Henderson–Hasselbach equation.

3.11 Measurement of Na^+/H^+ exchanger activity

For evaluating the activity of NHE-1, NH_4Cl pre-pulse technique was used. Cells were exposed to NH_4Cl (20 mM) for 3 minutes, which caused a sudden pH_i elevation due to the diffusion of NH_3 into the cells. After the removal of NH_4Cl, the pH_i decreased rapidly followed by a pH_i regeneration. The rate of the acid regeneration (the first 60 s) reflects the activity of NHEs in standard HEPES-buffered solutions. In order to quantify NHE-1 activity, the measured rates of pH_i change ($\Delta pH/\Delta t$) were converted to transmembrane base flux $J(B^-)$ using the equation: $J(B^-)=\Delta pH/\Delta t \times \beta_i$, where β_i is the intrinsic buffering capacity. For the calculation of $J(B^-)$, the β_i value at the start point pH_i was used.

3.12 RT-qPCR

Total mRNA was isolated using RNA isolation kit of Macherey-Nagel according to manufacturer's instructions. The concentration of RNA was determined by spectrophotometry. Two micrograms of total RNA were reverse transcribed using High-Capacity cDNA Archive Kit according to manufacturer's instructions. Quantitative real-time PCR was carried out on a Roche LightCycler 96 SW. TaqMan probe set of *SLC9A1* was used to check gene expression. Target gene expression levels were normalised to β-actin (*ACTB*), and then using the $\Delta\Delta C_T$ method, relative gene expression was calculated. Fold changes were represented ($2^{-\Delta\Delta CT}$). Values below 0.5 and above 2.0 were considered significant.

3.13 *SLC9A1* gene silencing

SLC9A1 gene silencing was performed on the cell lines as follows. 3×10^5 cells were seeded on a 6-well plate in antibiotic free growth medium, incubated overnight and then medium was changed to 800 µL/well fresh, complete growth medium without antibiotics. *SLC9A1* gene silencing was performed at 40-50% confluency. 100 pmol SLC9A1 siRNA was dissolved in 250 µL Opti-MEM serum free media. Depending on the transfection length and cell line, 5-7.5 µL Lipofectamine 2000 was added to 250 µL Opti-MEM and incubated for 5

minutes at room temperature. Then prepared siRNA solution and Lipofectamine 2000 were mixed and incubated at room temperature for 20 minutes to form complexes. Complexes were added to the wells, mixed gently by rocking the plate back and forth, and incubated for 72 hours at 37°C. After transfection, RT-qPCR and immunocytochemistry was performed to estimate mRNA and protein levels.

3.14 Evaluation of cytotoxicity

100 µL of cell suspension was seeded into a 96-well cell culture plate at a 2×10^4 cells/well density and incubated overnight. Then cells were treated with CSE (1, 10 or 100 µg/mL) for 6, 24 and 72 hours, then Cytotoxicity Detection Kit Plus was used, according to the kit instructions. Lactate dehydrogenase (LDH) activity was measured at 490 nm. Control groups were determined according to kit instructions. The LDH release induced by Triton-X 100 was assigned as 100%. Percentage of cytotoxicity was calculated using the following formula: Cytotoxicity (%) = (exp. value –low control / high control-low control) × 100.

3.15 Proliferation

10^3 CP-A or CP-D cells per well were seeded into a 96-well plate (100 µL/well) in complete growth medium and incubated overnight. Cells were then treated with CSE (1 and 10 µg/mL) for 6, 24 and 72 hours. After the treatments, CCK8 assay was used according to the manufacturer's instructions. For determining the proliferation of Capan-1 cells, 5×10^3 cells per well were seeded into a 96-well plate (100 µL/well) in complete growth medium and incubated overnight. Cells were then treated with BAs (glycocholic acid (GCA); taurocholic acid (TCA); glycodeoxycholic acid (GDCA); taurodeoxycholic acid (TDCA); glycochenodeoxycholic acid (GCDCA); taurochenodeoxycholic acid (TCDCA)) in two different concentrations (100 and 500 µM), for 24, 48 and 72 hours. After treatments, we have performed CCK8 assay.

3.16 Statistical analysis

Results were described as means ± SEM. For statistical analysis, one-way ANOVA, and Student's t-test were used. $p \leq 0,05$ were considered significant.

4. Results

4.1 Effect of smoking on the proliferation and viability of oesophageal cells

CP-A and CP-D cells were seeded in 96-well plates, allowed to adhere overnight and treated with 1, 10 and 100 μg/mL CSE for 6, 24 and 72 hours. At the end of the incubation period, cell viability was investigated using Cytotoxicity Detection Kit Plus from Roche. We have found that in case of both cell lines, lower concentrations of CSE (1 and 10 μg/mL) caused moderate cell death, around 20% in all three incubation periods. In contrast, 100 μg/mL CSE induced a time-dependent, high cell death rate. 72 hours after the CSE treatment, cell death was above 80%. As 100 μg/mL concentration of CSE was toxic for the cells, we continued investigating the proliferative activity only in lower concentrations (1 and 10 μg/mL) of CSE. After treating the cells with CSE for 6, 24, and 72 hours, proliferative activity of the cells was estimated using CCK-8 assay according to manufacturer's instructions. Proliferation of CP-A cells dose-dependently decreased after 24 and 72 hours CSE treatment compare to control. In CP-D cells proliferative rate increased in the 72-hour treatment groups, at both concentrations.

4.2 Na^+/H^+-exchanger-1 activity and expression in metaplastic and dysplastic Barrett cell lines

For investigating the function of NHE, NH_4Cl pre-pulse technique was performed. In the absence of HCO_3^-, the rate of recovery from acidosis reflects the activity of NHEs. Our workgroup has previously shown that the primary NHE isoform is NHE-1 in CP-A and CP-D cell lines, and NHE-1 has the greatest role in pH_i regeneration. Recovery rate from acidosis, consequently functional activity of NHE-1, was significantly higher in CP-A cells (BF: 5.47 ±0.52) than in CP-D cells (BF: 3.36 ±0.24). We have also examined the mRNA and protein expression of NHE-1 in these cell lines at certain time points (6, 24 and 72h). RT-qPCR was performed for investigating mRNA expression, and data were normalized to β-actin (*ACTB*). We have found that there was no significant difference between mRNA expression of NHE-1 in CP-A and CP-D cells at either incubation time. Similarly, to RT-qPCR results, Western blot analysis showed that protein levels were entirely the same at all-time points, in both cell line.

4.3 Effect of cigarette smoke extract on Na^+/H^+-exchanger-1 activity and expression in metaplastic and dysplastic Barrett cell lines

We have studied the acute effects of CSE on NHE-1 activity by performing the above mentioned NH_4Cl pre-pulse technique. Cells were incubated with CSE (1, 10 and 100 μg/mL) for one hour before the measurements. In CP-A cells we have observed that 1 μg/mL CSE reduced the activity of NHE-1 compared to the control (5.47±0.52 to 3.08 ± 0.55). However, treatment with higher concentrations of CSE provoked an elevated function of this exchanger,

and 100 μg/mL CSE pre-treatment caused a more than 2-fold elevation (8.18 ± 1.3 at 10 μg/mL CSE and 12.28 ± 0.73 at 100 μg/mL CSE). In case of CP-D cells, CSE decreased the activity of NHE-1 in all three groups. For expressional studies we have treated the cells with 1 μg/mL and 10 μg/mL of CSE for 6, 24 and 72 hours and implemented qPCR and Western blot analysis. We did not detect any significant changes in mRNA expression of NHE-1 after CSE treatment in any of the cell lines. In spite of these results, in CP-A cells protein levels were elevated in almost all groups. Surprisingly, in CP-D cells there was an elevated protein level only in the 6-hour 1 μg/mL CSE treatment group, and no significant differences could be seen in the other groups.

4.4 Effect of smoking on Na^+/H^+-exchanger-1 activity in normal oesophageal cells

In order to investigate how CSE affects NHE activity under physiological conditions, we studied the effect of CSE on normal OECs isolated from guinea pigs. The same concentrations were used as for the cell lines, and the cells were pre-treated with CSE in the same manner. NHE-1 activity was significantly reduced by CSE treatment (from 12.19 ± 0.46 to 4.64 ± 0.94 at 1 μg/mL CSE, to 3.96 ± 0.43 at 10 μg/mL CSE and to 4.49 ± 0.4 at 100 μg/mL CSE, respectively). In chronic studies, we have investigated the long-term effects of smoking on OECs using a guinea pig model. Male guinea pigs were selected for three groups depending on the period of cigarette smoke exposure. OECs were isolated according to the isolation protocol mentioned earlier and NH_4Cl pre-pulse technique was performed in order to estimate changes in NHE-1 function. Results were similar to the acute experiment results; after long-term cigarette smoke exposure the recovery rate was reduced in all three groups, compared to their own control groups, hence cigarette smoke exposure decreased NHE-1 activity.

4.5 Effect of long-term smoking on Na^+/H^+-exchanger-1 expression in human oesophageal samples

NHE-1 immunostaining of biopsy samples from normal squamous epithelia and BO was performed. Patients were classified into four groups based on their diagnosis and smoking history. Protein expression was determined with a semi-quantitative scoring system, and DAB intensities were calculated for quantification. In normal, non-smoker group, low NHE-1 expression could be detected and it was even lower in the smoker group. On the contrary, in BO patients' samples there was a strong NHE-1 expression, which was further increased by smoking.

4.6 Effect of knockdown of *SLC9A1* gene on the proliferation of metaplastic and dysplastic oesophageal epithelial cell lines

In order to investigate whether the altered expression or activity of NHE-1 has any role in the effect of CSE on proliferation, we silenced the *SLC9A1* gene, using specific siRNA. The efficiency of silencing was investigated at both mRNA and protein levels. In CP-A cells, NHE-1 knockdown reduced the rate of proliferation at each incubation time, suggesting that NHE-1 is essential for the normal function of the cells. In the CP-D cells, the lack of NHE-1 protein initially increased the rate of proliferation, whereas no significant difference was observed with additional incubation times. In the absence of NHE-1, CSE treatment increased the rate of proliferation in the CP-A cells in almost all treated groups. For CP-D cells, proliferation increased alone in the 72-hour treatment group.

4.7 Effect of bile acids on the proliferation of Capan-1 cells

Cells were incubated with GCA, TCA, GDCA, TDCA, GCDCA and TCDCA for 24, 48 and 72 hours in two different concentrations, then we estimated proliferative activity of the cells using CCK-8 assay. BAs increased proliferation of Capan-1 cells almost in all treated groups. TDCA decreased proliferative activity of the cells at 100 μM, especially after 24 h (0.83±0.06), and increased it (1.64±0.02) at 500 μM at each incubation time.

5. Discussion and new findings

In the present work we examined the cellular alterations of epithelial cells in the pathophysiology of gastrointestinal diseases. In the greater part of the thesis, we studied the effect of CSE on the NHE-1 of OECs, and the role of smoking in the pathophysiology of BO. In the lesser part we investigated the effect of different BAs, that are commonly found in the human bile, on the proliferative activity of PDAC cells.

New findings of our study are the followings:

- Activity of NHE-1 varies in normal, metaplastic and dysplastic oesophageal cells and smoking influences NHE-1 differently in each cell type.
- **Under physiological circumstances**, both the activity and expression of NHE-1 is reduced by smoking, resulting in an intracellular acidosis. According to scientific literature, alterations in pH optimum can induce apoptosis, or via trans-differentiation, the appearance of metaplasia, in which the cells are more prone to malignant transformation.

- **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 BO, decreased activity or expression of NHE-1 had no effect on smoking-induced proliferation **in the dysplastic state** indicating the involvement of other mechanisms.

Our results strengthen the observation, that smoking has a significant role in the pathomechanism of BO. NHE-1 has an essential function in pH homeostasis of the cells. In normal OECs, smoking reduces the activity of NHE-1, and on a long-term NHE-1 expression is also reduced. The worsening alkalising function can no more maintain normal pH range, and this state leads to metaplastic transformation of the oesophageal epithelium. In metaplastic cells, NHE-1 expression and activity is also higher in order to compensate the pH alterations, that smoking or other toxic agents caused. NHE-1 also participates in proliferation, and in absence of NHE-1, metaplastic cells react with high proliferative activity for smoking. All these results confirm that metaplastic cells attempt to compensate the effect of toxic agent, such as smoking. These cells have a high potential to malignant transformation and dysplastic state can easily occur. In dysplastic cells, the activity of NHE-1 is significantly lower than in metaplastic cells, and smoking does not influence expression or the activity of this ion transporter. In NHE-1 knockdown dysplastic cells proliferation does not change, but a long-term treatment enhances it. Based on these findings, NHE-1 has a protective role in metaplastic state, but dysplastic cells do not respond to CSE treatment indicating, that these changes would be not alterable in this cell type. It would be an interesting question, if NHE-1 expression or activity would change in case of cessation of smoking, or whether these alterations are reversible. Nevertheless, it is highly recommended for every smoker patient to quit smoking as soon as possible to reduce the chances of oesophageal malignancies.

Taken together, 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. Nevertheless, the present results indicate that direct augmentation of NHE-1 function may provide new avenues for decreasing the damaging effect of smoking.

The carcinogenic effect of BAs was strengthened by our results, as they elevated proliferative activity, an important character of cancerous cells. This feature may make the PC more aggressive. This additional observation might be a relevant information in terms of therapy.

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