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ORIGINAL ARTICLE

Esophageal barrier function and tight junction expression in healthy subjects and patients with gastroesophageal reflux disease: functionality of esophageal mucosa exposed to bile salt and trypsin *in vitro*

ELEONORA VICTORIA CHARLOTTA BJÖRKMAN[1], ANDERS EDEBO[1,2], MIHAI OLTEAN[3] & ANNA CASSELBRANT[2]

[1]*Department of Gastrosurgical Research and Education, Institute of Clinical Sciences, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden,* [2]*Department of Gastrosurgical Research and Education, Institute of Clinical Sciences, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden, and* [3]*The Transplant Institute, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden*

Abstract

Background and aims. Gastroesophageal reflux disease (GERD) is associated with impaired epithelial barrier function. However, the influence of acid and/or bile acids on human esophageal epithelial barrier function and the tight junction (TJ) proteins has not been fully elucidated. The aim of the study is to investigate the esophageal barrier function and TJ expression in healthy subjects and patients with GERD. The functionality of esophageal mucosa exposed to bile salt deoxycholic acid (DCA) and trypsin has been studied *in vitro.* ***Material and methods.*** Endoscopic biopsies from healthy controls and patients with GERD-related symptom with endoscopic erosive signs, as well as esophageal mucosa taken from patients undergoing esophagectomy were evaluated in Ussing chambers and by western blot and immunohistochemistry. ***Results.*** The esophageal epithelium from GERD patients had lower electrical resistance and higher epithelial currents than controls. Claudin-1 and -4 were significantly decreased in GERD patients. The bile salt DCA in the low concentration of 1.5 mM and trypsin increased the resistance and claudin-1 expression, while the higher concentration of 2.5 mM DCA and trypsin decreased the resistance and the claudin-3, -4 and E-cadherin expressions. ***Conclusion.*** In addition to acidic reflux, duodenal reflux components, such as bile salts and trypsin, have the potential to disrupt the esophageal barrier function, partly by modulating the TJ proteins. However, the expression of TJ is dependent on both the refluxed material as well as the concentration of the bile salt.

Key Words: *barrier function, esophageal epithelium, gastroesophageal reflux disease tight junctions protein*

Introduction

An intact gastrointestinal barrier is essential for fundamental cell and tissue functions as well as for preventing harmful substances from entering into the tissue and circulation. Epithelial paracellular passage of potentially noxious agents plays an important pathophysiological role in the initiation of a number of common gastrointestinal diseases, such as gastroesophageal reflux disease (GERD) [1]. The esophageal mucosa faces an environment that can be quite aggressive, with mechanical wear and tear, as well as heating (or even burning) or cooling after swallowing food boluses. Moreover, gastric acidic contents or gastroduodenal contents including bile and pancreatic enzymes exert a chemical challenge during reflux episodes. The ability of the epithelium to tolerate such stress depends on the balance between the intensity of the noxious stimulation and the protective mechanisms. By unknown reasons, GERD patients have an impaired ability to maintain this barrier property of the epithelium [2].

The integrity of the epithelial surface is dependent on the mechanical cohesion between epithelial cells

Correspondence: Anna Casselbrant, PhD, Department of Gastrosurgical Research and Education, Sahlgrenska Academy, Bruna stråket 20, SE413 45 Göteborg, Sweden. Tel: +46 31 342 6858. Fax: +46 31 41 18 82. E-mail: anna.casselbrant@gastro.gu.se

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that are constituted by structures called apical junction complexes which consist of adherence junctions, desmosomes and tight junctions (TJs). The TJ complex holds adjacent cells together, making the epithelium act as a barrier, although still allowing some passage, which in turn is charge- and size-selective and sensitive to various signaling entities [3,4].

Recently, studies have shown that an early event in the pathogenesis of GERD is an acid-induced increase in paracellular permeability in the esophageal epithelium [5,6]. Jovov et al. showed that E-cadherin is an important factor in order to improve the esophageal barrier [5], while others have shown different expression profiles for claudins in patients suffering of acidic reflux [7]. However, the role of duodenal reflux, containing bile salts and pancreatic enzymes (with special attention to trypsin), on the epithelial permeability is almost lacking in humans. This is odd because studies have shown that the duodenal refluxate is especially important in the development of Barrett's esophagus [8–10].

Therefore, the first aim of the present study was to elucidate the electrical epithelial resistance in healthy controls and in GERD patients, as well as in esophageal mucosa treated with the bile salt deoxycholic acid (DCA) and trypsin *in vitro*. The second aim was to elucidate the expression profile of TJ proteins in the respective above-mentioned human esophageal mucosa.

Material and methods

Subjects and tissue specimens

Ethics. The study was approved by the Ethical Committee of Gothenburg University as well as by the Regional Ethical Review Board in Gothenburg and was performed in accordance with the Declaration of Helsinki. All individuals were informed verbally and in writing and signed a consent form.

Esophageal biopsies were obtained from healthy volunteers (n = 26, 16 males, mean age: 26 years, range: 21–37 years) and from individuals with GERD (n = 17, 11 males, mean age: 39 years, range: 20–60 years). The enrolled GERD patients had been referred to the outpatient endoscopy unit due to reflux symptoms and the patients were requested to abstain from proton pump inhibitors and H2 receptor antagonist medication for at least 2 weeks before the endoscopic procedure. All individuals were subjected to an upper endoscopy with a high-resolution magnification instrument and the biopsies were collected using biopsy forceps (Endotherapy disposable biopsy forceps FB-234U Olympus, Hamburg, Germany) from the squamous epithelium within ~2 cm above the gastroesophageal junction in the 3 O'clock position with the patient in left lateral position [11]. An additional biopsy from the erosive area (red streak/esophagitis) was collected from the GERD patients, where all individuals were scored to be grade A according to the Los-Angeles classification system for reflux esophagitis.

Esophageal mucosal specimens were also obtained from patients undergoing esophagectomy for malignancy of the lower esophagus (n = 10, 9 males, mean age: 67 years, range: 57–78 years). The esophageal specimens were obtained from squamous mucosa ~4–8 cm above the lower esophageal sphincter. Care was taken so that the resected tissue used in the experiment was collected as far as possible from any pathological area, that is, neoplastic changes, as assessed macroscopically.

All specimens were immediately placed in Krebs solution for Ussing chamber experiments or snap-frozen in liquid nitrogen for western blot analysis or fixed in 4% formaldehyde for immunohistochemistry analysis.

Ussing chamber experiments

The esophageal biopsies for the Ussing chamber experiments were transported to the laboratory in ice-cold oxygenated (95% O_2 and 5% CO_2) Krebs solution with the following composition (in mM): 118.1 NaCl; 4.7 KCl; 2.5 $CaCl_2$; 1.2 $MgSO_4$; 1 NaH_2PO_4; 25 $NaHCO_3$ and 11.1 glucose. The endoscopic biopsies were mounted in vertical mini-Ussing Chambers (Warner instruments, Hamden, CT, USA), containing biopsy inserts with a diameter of 1.5 mm resulting in the square area of 0.018 cm^2. The esophageal surgical specimens were mounted in conventional Ussing chambers with the square area of 0.29 cm^2 (Warner instruments, Hamden, CT, USA). After mounting, each half chamber was filled with 5 ml Krebs solution. The Krebs solution was maintained at 37°C and continuously oxygenated with 95% O_2 and 5% CO_2 and was stirred by a gas flow in the chambers. Three to six preparations could be retrieved from a single individual. The potential difference (PD) was measured with a pair of matched calomel electrodes (REF401, Radiometer analytical, Denmark). The Ussing pulse method (UPM) was used in determining the tissue's epithelial electrical resistance (Rep) and the epithelial ion current (Iep) was received by using Ohm's law, where I = U/R (current = voltage/resistance, i.e., Iep = PD/Rep). The UPM has previously been described [6], but briefly the method is based on the concept that the epithelium consists of a capacitor and resistor coupled in parallel. Separate trains of short current pulses induce a voltage response in the tissue and charge the

epithelial capacitor, which gradually is discharged when the current ends. The epithelial voltage response, specifically, is received from the discharge curve, and by knowing the magnitude of the applied current the Rep can be calculated. The data were collected using an amplifier and specially constructed software developed in LabView (National Instruments, Austin, TX, USA). The surgical esophageal epithelium had to exhibit a negative PD of ≥1 mV, whereas the endoscopic biopsies had to have a lumen negative PD of ≥0.25 mV to become included in the experiment based on previous experimental experiences [6].

Experimental procedures. Endoscopically acquired mucosal specimens: after mounted in the Ussing chambers the biopsies were left for 30 min to allow basal conditions to be established. Baseline parameters were then measured over 20 min.

Surgically acquired mucosal specimens: after an equilibration period of 30 min, basal parameters were measured over 20 min. After baseline, the epithelial integrity was challenged by adding a combination of 1.5 or 2.5 mM of the bile salt DCA (Sigma-Aldrich, Stockholm, Sweden) and 0.04 mM trypsin (bovine pancreas Type 1; Sigma-Aldrich) to the luminal side, or vehicle as control, and the electrical parameters were measured after 20, 40 and 60 min. After the experiment, the specimens were snap-frozen in liquid nitrogen for later western blot analysis.

Western blot analysis

The frozen specimens were sonicated in a PE buffer (10 mM potassium phosphate buffer, pH 6.8 and 1 mM Ethylenediaminetetraacetic acid (EDTA)) containing 10 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS: Boehringer Mannheim, Mannheim, Germany) and protease inhibitor cocktail tablet Complete™ (Roche Diagnostics AB, Stockholm, Sweden). The homogenate was then centrifuged (10,000 *g* for 10 min at 4°C) and the supernatant was analyzed for protein content by the Bradford method [12] and was stored at −70°C. The samples were diluted in sodium dodecyl sulfate buffer and heated at 70°C for 10 min and then loaded on a NuPage 10% Bis-Tris gel and the electrophoresis was run using a MOPS buffer (Invitrogen AB, Lidingö, Sweden). The gel was loaded with a prestained molecular weight standard (SeeBlue, NOVEX, San Diego, CA, USA). After the electrophoresis, the proteins were transferred to a polyvinyl difluoride transfer membrane (Hybond, 0.45 μm, RPN303F, Amersham, Buckinghamshire, UK). Membranes were then incubated with specific antibodies directed at the claudin-1, -2, -3, -4, -5, E-cadherin and occludin, respectively (see Table I). An alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit IgG antibody (Santa Cruz) and CDP-Star (Tropix, Bedford, MA, USA) were used as a substrate to identify immunoreactive proteins by means of chemiluminescence. Images were captured by a Chemidox XRS cooled CCD camera and analyzed with Quantity One software (Bio-Rad laboratories, Hercules, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, IMG-5143A, Imgenex, BioSite, San Diego, CA, USA) was used as control for equal loading, and for each tested sample the optical density of primary antibody/GAPDH represented the result. The membrane was stripped for reprobing with other primary antibodies using a stripping buffer (Re-blot Plus Mild Solution (10×), Millipore, Temecula, CA, USA).

Table I. Antibodies used in western blot and immunohistochemistry.

Target protein	Primary antibody	Protein size (kDa)
Claudin-1	Rabbit anti-claudin-1, 51–9000 (Invitrogen AB, Lidingö, Sweden)	22
Claudin-2	Rabbit anti-claudin-2, 51–6100 (Invitrogen)	22
Claudin-3	Rabbit anti-claudin-3, 34–1700 (Invitrogen)	22
Claudin-4	Mouse anti-claudin-4, 32–9400 (Invitrogen)	22
Claudin-5	Mouse anti-claudin-5, 35–2500 (Invitrogen)	22–24
E-Cadherin	Mouse anti-E-cadherin, 33–4000 (Invitrogen)	120
Occludin	Mouse anti-occludin, 33–1500 (Invitrogen)	65

Immunohistochemistry

The intraepithelial distributions of the TJ proteins were assessed by immunohistochemistry. The fixed esophageal mucosal specimens were dehydrated and embedded in paraffin. Sections were deparaffinized and antigen retrieval was performed by boiling the slides in 50 mM citrate buffer (pH 6) for 15 min followed by 20 min cooling time. After inhibition of endogenous peroxidase activity and species-specific protein blocking, the slides were incubated overnight at 4°C with the primary antibody (see Table I). Negative control sections were incubated with normal rabbit/mouse IgG instead of primary antibody. The slides were then incubated with a biotinylated secondary antibody and the protein–antibody complex was detected using horseradish peroxidase–streptavidin and developed with the color 3,3′-diaminobenzidine (CSA II kit K1497 staining systems, Dako, Stockholm, Sweden). The sections

Table II. Electrical characteristics of endoscopically acquired esophageal mucosa.

	PD (-mV)	Rep ($\Omega^{\star}cm^2$)	Iep ($\mu A/cm^2$)
Healthy controls (n = 18, N = 98)	1.22 ± 0.1	70.8 ± 5	23.4 ± 1.8
GERD (n = 10, N = 46)	1.47 ± 0.2	44 ± 4 ⋆	46.9 ± 6.8 ⋆

PD = Potential difference; Rep = Epithelial electrical resistance; Iep = Epithelial electrical current, GERD = Gastroesophageal reflux disease.
Values are given as mean ± SEM; number of individuals are indicated as n and preparations are indicated as N; ⋆ denotes differences between groups by $p \leq 0.001$.

were counterstained with hematoxylin (Santa Cruz), mounted and examined through a Nikon Microphot FXA microscope (Nikon Corporation, Tokyo, Japan).

Statistical analysis

Comparisons of relative changes in Ussing parameters were performed by Student's t-test for paired or unpaired values when appropriate. Kruskal-Wallis and Mann–Whitney U-test for independent variables and Wilcoxon signed-rank test for related variables were used for analyzing the differences in protein expression. Data were presented as means ± SEM. Individuals are denoted n and preparations/observations N. The statistical software program SPSS version 19.0 was used (SPSS, Chicago, IL, USA) and a p-Value of ≤ 0.05 was considered significant.

Table III. Baseline electrical characteristics of surgically acquired esophageal mucosa.

	PD (-mV)	Rep (Ω cm^2)	Iep ($\mu A/cm^2$)
Esophageal mucosa (n = 10, N = 29)	3.9 ± 0.4	151.9 ± 35.48	66.1 ± 10.4

PD = Potential difference; Rep = Epithelial electrical resistance; Iep = Epithelial electrical current.
Number of individuals are indicated as n and preparations are indicated as N.

Results

Electrical characteristics in human esophageal epithelium

Endoscopically acquired mucosal specimens: The electrical parameters are displayed in Table II. The epithelial electrical resistance was lower (p-Value ≤0.001), while the ion currents were higher (p-Value ≤0.001) in the normal epithelium taken from GERD patients compared to healthy controls (some preliminary results of the parameters have been presented in previous publications [6,13]).

Surgical acquired mucosal specimens: The baseline epithelial electrical parameters in tissues acquired at surgery are shown in Table III. In presence of 1.5 mM DCA and 0.04 mM trypsin, the epithelial electrical resistance increased by 100% (Figure 1, p-Value = 0.029), while the PD decreased by 20%. In contrast, exposure of the epithelium with 2.5 mM DCA and 0.04 mM trypsin rapidly decreased the epithelial resistance by 80% (Figure 1, p-Value ≤ 0.001), while the PD decreased by 55% from baseline.

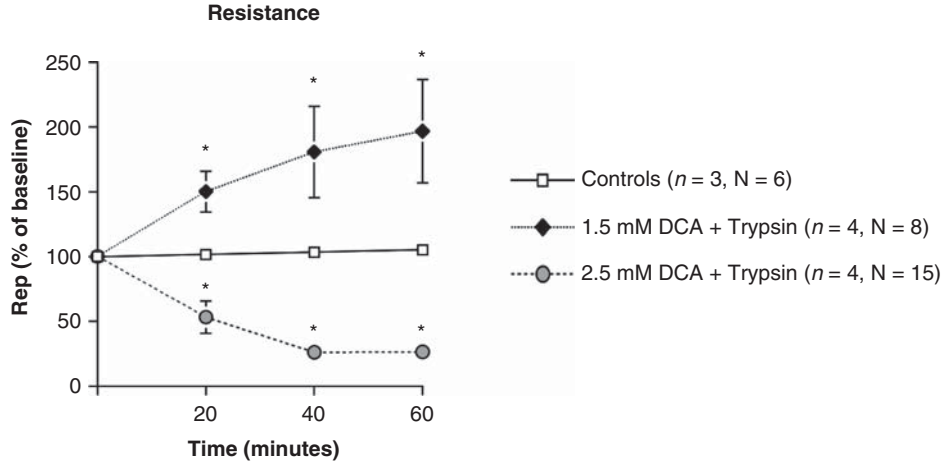

Figure 1. Schematic representation of Ussing chamber experiments on human esophageal epithelium. The epithelial electrical resistance increased in presence of the bile salt DCA in concentration of 1.5 mM and 0.04 mM trypsin, whereas it decreased in presence of 2.5 mM DCA and 0.04 mM trypsin. Significant differences compared to controls are indicated with asterisks ⋆ (Student's t-test). Abbreviations: n: number of individuals, N: number of preparations.

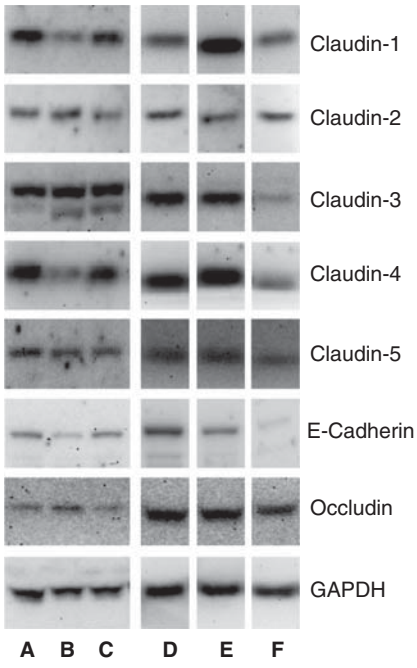

Figure 2. Protein bands on the western blot membrane are shown. Representative samples of protein bands of claudin-1, -5, E-cadherin and occludin and the loading control GAPDH in healthy controls (A), red streak (B), GERD mucosa (C), control sample after Ussing experiment (D), sample treated with 1.5 mM DCA + 0.04 mM trypsin in Ussing chamber (E) and sample treated with 2.5 mM DCA + 0.04 mM trypsin in Ussing chamber (F) are shown.

Expression of TJs proteins in healthy controls and GERD patients

All proteins investigated by western blot were detected in both healthy individuals and GERD patients (Figure 2). The protein expressions of claudin-1 (p-Value = 0.021) and claudin-4 (p-Value = 0.049) were significantly decreased in esophageal endoscopic biopsies taken in the red streak area from GERD patients compared with healthy subjects (Figure 3A and B). The claudin-1 and -4 expressions (p-Value = 0.028) were also decreased in the red streak area compared to the gastroendoscopically unchanged mucosa taken from GERD patients (Figure 3A, B). Claudin-3, claudin-5, E-cadherin and occludin were unchanged. However, both the E-cadherin and occludin expressions had a tendency to decrease in the red streak area compared with the unchanged mucosa from GERD, but the difference did not attain statistical significance (p-Value = 0.063 for both) (data not shown). Immunohistochemistry was used to localize the TJ proteins (no comparison between healthy individuals and GERD were made). Claudins-1 and -4 were detected throughout the epithelium with particularly strong junctional staining in the stratum spinosum (brown color, Figure 4A and D). Claudin-5 was also observed in the stratum spinosum but not so intense (Figure 4E). Claudins-2 and -3 were localized preferably to the basal and suprabasal zone of the epithelium (Figure 4B and C). The E-cadherin showed a distinct junctional staining from the basal cell layers up to stratum spinosum (Figure 4F), while occludin was found mainly in the entire epithelium (Figure 4G).

Expression of TJ proteins after DCA and trypsin

After the esophageal mucosa was exposed to 1.5 mM DCA and 0.04 mM trypsin in the Ussing chambers, the protein expression of claudin-1 was significantly increased (p-Value = 0.032), whereas the other junction proteins remained unchanged (Figure 5). In contrast, after exposure to the higher DCA concentration of 2.5 mM and trypsin in the Ussing chambers, the epithelial expressions of claudin-3 (p-Value = 0.016),

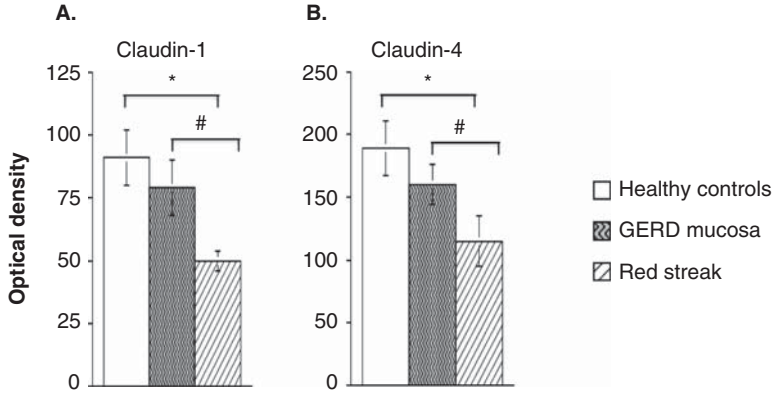

Figure 3. Schematic representation of results from the western blot showing the protein expression of claudin-1 (A) and claudin-4 (B) in esophageal epithelial biopsies taken from healthy subjects ($n = 8$) and in the normal epithelium and red streak area from GERD patients ($n = 7$). Significant differences are indicated with asterisks (*p-Value between groups using Mann–Whitney test) (#p-Value for related variables using Wilcoxon signed-rank test).

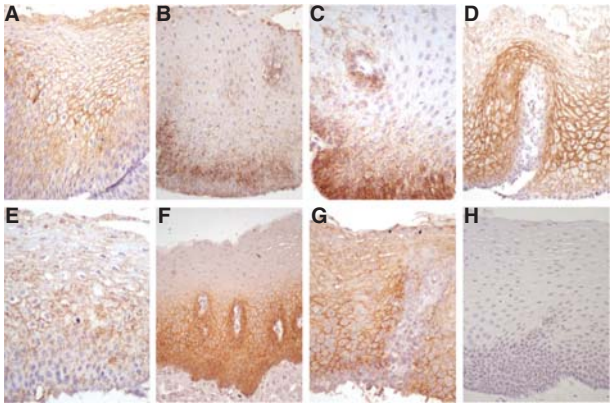

Figure 4. Illustration of localization of TJs protein in the human esophageal epithelium. The panels show immunohistochemical staining of (A) claudin-1, (B) claudin-2, (C) claudin-3, (D) claudin-4, (E) claudin-5, (F) E-cadherin, (G) occludin and (H) negative control. No comparisons between groups were made.

claudin-4 (p-Value = 0.032) and E-cadherin (p-Value = 0.008) were significantly decreased, while the expressions of Claudins-1, -2, -5 and Occludin were unchanged (Figure 6).

Discussion

The results of this investigation demonstrated that GERD patients have an impaired ability to maintain the barrier function of the esophageal epithelium. Acids as well as bile salts in the refluxate influence the paracellular permeability in the esophageal epithelium. GERD patients in this study had increased permeability compared with healthy subjects that can partly depend on the decreased claudin-1 and -4 protein expressions. The bile salt concentration is also of importance; in low concentration, the epithelial resistance increased probably due to modulation and increase of claudin-1 protein expression. In contrast, high concentration of DCA and trypsin immediately decreased the resistance probably as a consequence of decreased claudin-3, -4 and E-cadherin protein expressions.

In the present study, we used the UPM for assessment of the epithelial electrical resistance and PD. This method has recently been validated concerning if the transepithelial permeability, using different probes, is considered to reflect the assessed epithelial resistance [6]. The authors conclude that the Ussing pulse methodology is very useful in investigating the epithelial integrity and suitable for investigating esophageal endoscopic biopsies [6,13].

Ussing chamber preparations using biopsies obtained during endoscopy found differences that discriminated between healthy individuals and individuals with reflux disease. As shown in Table I, the GERD epithelium outside the red streak had almost half the resistance value and double the epithelial current value than biopsies from the mucosa in healthy controls. These results showed that GERD patients had alterations in their epithelial properties indicating disease-related events. The lower tissue resistance of GERD patients is in line with previous

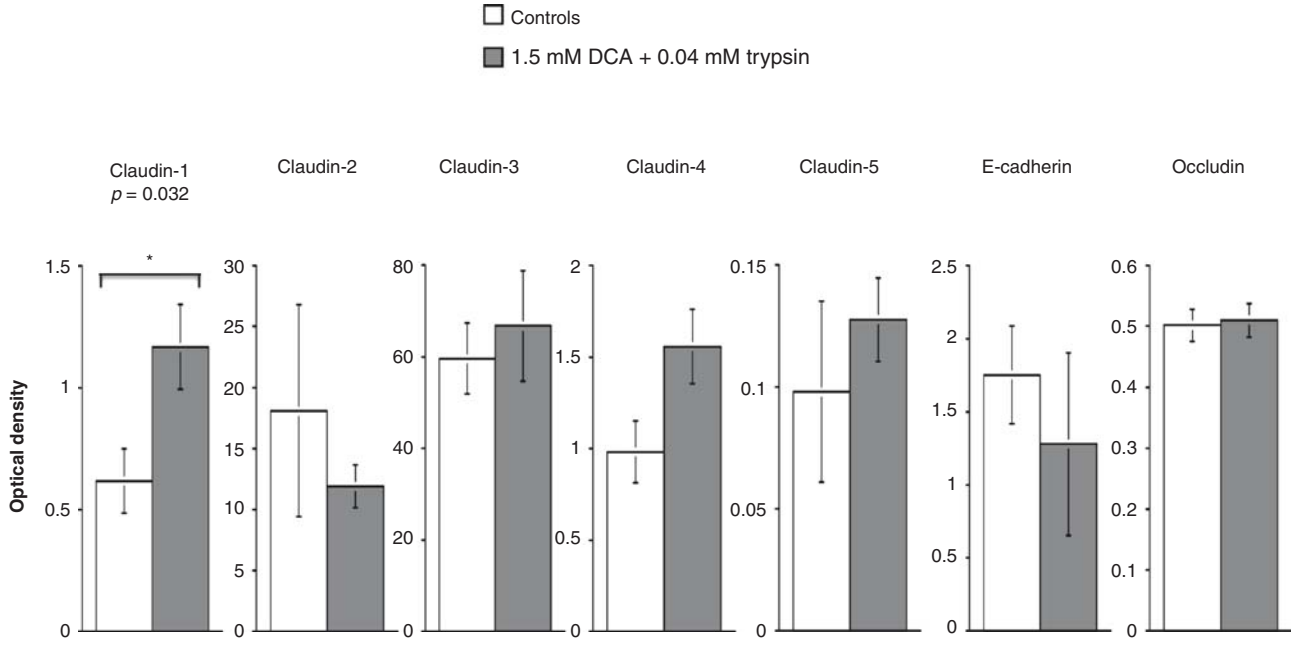

Figure 5. Schematic representation of western blot results of the TJ protein in human esophageal epithelium after treatment of the bile salt DCA in concentration of 1.5 mM and 0.04 mM trypsin *in vitro*. The expression of claudin-1 was significantly increased after treatment.

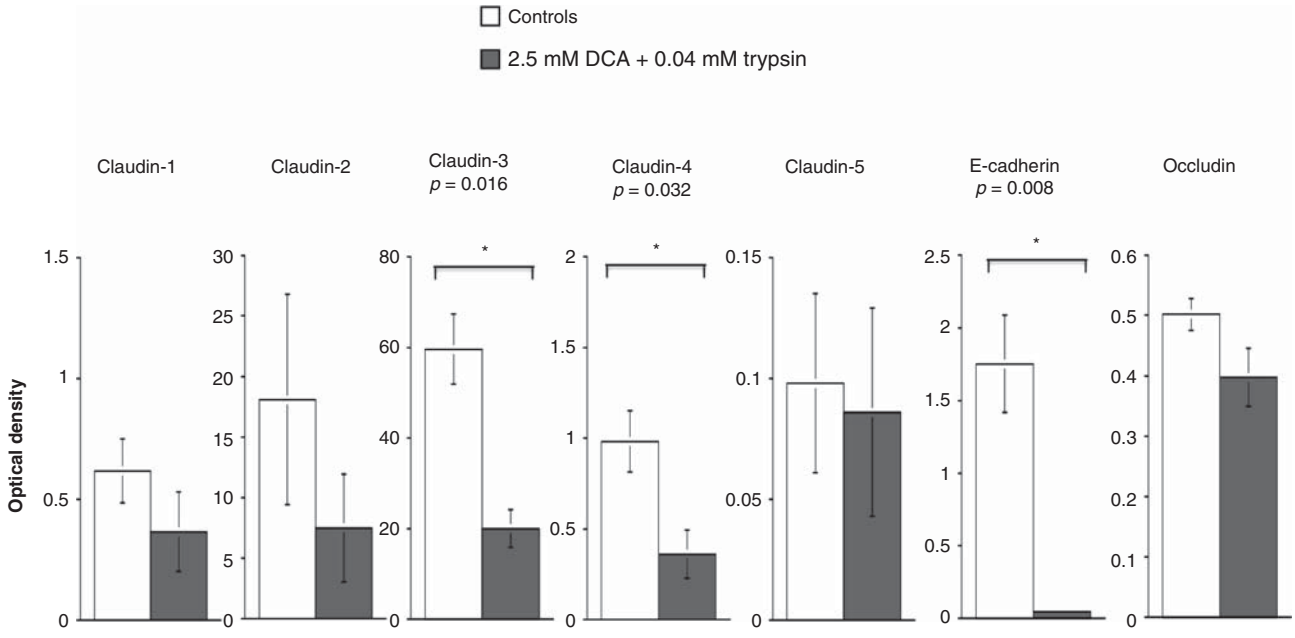

Figure 6. Schematic representation of western blot results of the TJ protein in human esophageal epithelium after treatment of the bile salt DCA in concentration of 2.5 mM and 0.04 mM trypsin *in vitro*. The expressions of claudin-3, -4 and E-cadherin were significantly decreased after treatment.

studies [5,13,14], where tissue alterations, such as dilated intercellular spaces (DIS), probably were the cause of the observed lower epithelial resistance. In the present study, the protein expressions of claudin-1 and -4 were significantly decreased in the GERD group compared to the healthy subjects. A previous study by Oshima et al. [15], using a stratified squamous epithelial cell-like culture system, also showed that claudin-4 is susceptible to acid stimulation and may be related to disruptions of the barrier function.

Gastric acid is a major factor in the development of esophagitis. The patient groups used in this study were requested to abstain from proton pump inhibitors and H2 receptor antagonist medications for at least 2 weeks before the endoscopic procedure. However, it cannot be excluded that the refluxate includes not only gastric acid but also bile acid and pancreatic enzymes. Recently, it has become widely accepted that mixed gastric and bile acid reflux is the dominant pattern of reflux in patients with severe GERD [14,16,17]. Gastric acid combined with bile salts seems to be more harmful to the esophageal epithelial layers than gastric acid alone [18]. There are studies showing that patients with Barrett's esophagus and adenocarcinoma have increased duodenal reflux compared to GERD patients without metaplasia, indicating that duodenal refluxate might be a risk factor for developing specialized intestinal metaplasia and adenocarcinoma [9]. Different concentrations of bile salts have been reported in humans [14,19,20]. In healthy volunteers, bile acids in *esophageal aspirates* are almost undetectable, whereas the concentrations in GERD patients range from 0 to 1 mM [19]. Gastric bile acid concentrations in healthy controls is between 0.3 and 2 mM and in GERD it can increase to >5 mM [21,22].

The composition of the refluxate that regurgitates into the esophagus in patients with ongoing acid suppression therapy is different from that in patients not undergoing therapy. The main bile acids present in the patient's refluxate are taurocholic acid and glycocholic acid, but when using acid suppression therapy, higher levels of secondary bile acids, such as DCA, are detected [23]. Therefore, we used DCA in the present study. Here, we showed that DCA in the lower concentration of 1.5 mM and 0.04 mM trypsin did not affect the esophageal barrier as expected. Surprisingly, the epithelial electrical resistance increased. At the same time, the claudin-1 protein expression significantly increased, which could be related to this increased resistance. Although the cellular viability clearly decreased, as displayed by the reduction in PD, the increased resistance could be due to cell swelling and diminished intercellular spaces that would have counteracted a possible resistance decrease. In contrast, when using the higher DCA concentration of 2.5 mM and 0.04 mM trypsin, the epithelial resistance rapidly decreased to the very low value of 80%, whereas PD gradually decreased, reaching 55% of baseline after 60 min. The TJ expressions in the corresponding tissue after challenge showed several diminished significances, where claudins-3, -4 as well as E-cadherin decreased.

Several previous reports have indicated that the disruption of barrier function is related to delocalization and loss of TJ complexes between epithelial cells [5,15,24–26]. However, except Jovov et al.'s study [5], this is the first study on human esophagus. Although TJ complexes are composed of several different classes of proteins, which interact in a coordinated manner to form epithelial barriers, the claudins are clearly essential TJ proteins for the paracellular barrier properties. Claudins fall into two functional categories: pore-forming claudins that increase permeability through formation of paracellular channels and barrier-building claudins that have been associated with a more general barrier tightening function [27]. Claudin-2 is one of the best characterized pore-forming claudin, and an increase in claudin-2 expression has been found in inflammatory bowel disease and ulcerative colitis, where it weakens the intestinal barrier [28]. In contrast, claudins-1, -3 and -4 are characterized as key contributors to the barrier function and decreased expressions of these claudins are described to increase the paracellular permeability [28]. The present immunohistochemistry showed localization of all investigated claudins, where particularly claudin-1 and -4 were intense in more or less all cell layers throughout the epithelium, compared to claudins-2, -3 and -5. There are, however, additional proteins that are required to form functional junctional complexes. Most notable are the proteins zonula occludens-1 and -2 and occludin that bind directly to claudins and link them to the actin cytoskeletal network [27]. Also E-cadherin, a component of the adherens junction, is known to be important in junctional barrier function in most epithelia, including that of the esophagus [5]. Immunoreactivity of occludin was found in almost all cell layers and also staining of E-cadherin was observed mainly in the lower compartments of the epithelium in the esophageal biopsies. Previously, the study by Jovov et al. showed that the deletion of E-cadherin results in DIS and a marked increase in paracellular permeability in GERD patients [5]. This was not supported in the present GERD-patient groups, even if a tendency of decreased E-cadherin as well as occludin expressions were observed. However, the present observed permeability increase in GERD epithelia could very well be due to the decreased expressions of claudins-1 and -4.

In summary, using esophageal biopsies obtained during endoscopy, we found that GERD patients had increased epithelial permeability compared to healthy controls and that this is in association with decreased claudin-1 and -4 protein expressions. Moreover, on challenging esophageal mucosa tissue with bile salt DCA and trypsin *in vitro*, low concentration were reflected by increased epithelial resistance and claudin-1 protein expression, while a higher concentration immediately disrupted the barrier, partly by modulating, that is, decreasing, the amounts of claudin-3, -4 and E-cadherin.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Barlow WJ, Orlando RC. The pathogenesis of heartburn in nonerosive reflux disease: a unifying hypothesis. Gastroenterology 2005;128:771–8.

[2] Orlando RC. The integrity of the esophageal mucosa. Balance between offensive and defensive mechanisms. Best Pract Res Clin Gastroenterol 2010;24:873–82.

[3] Farquhar MG, Palade GE. Junctional complexes in various epithelia. J Cell Biol 1963;17:375–412.

[4] Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2001;2:285–93.

[5] Jovov B, Que J, Tobey NA, Djukic Z, Hogan BL, Orlando RC. Role of E-cadherin in the pathogenesis of gastroesophageal reflux disease. Am J Gastroenterol 2011; 106:1039–47.

[6] Björkman E, Casselbrant A, Lundberg S, Fändriks L. In vitro assessment of epithelial resistance in human esophageal and jejunal mucosae and in Caco-2 cell layers. Scand J Gastroenterol 2012;47:1321–33.

[7] Mönkemuller K, Wex T, Kuester D, Fry LC, Kandulski A, Kropf S, et al. Role of tight junction proteins in gastroesophageal refux disease. BMC Gastroenterol 2012;12:128.

[8] Richter JE. Gastroesophageal reflux disease. Best Pract Res Clin Gastroenterol 2007;21:609–31.

[9] Jankowski JA, Harrison RF, Perry I, Balkwill F, Tselepis C. Barrett́s metaplasia. Lancet 2000;356:2079–85.

[10] Wild CP, Hardie LJ. Reflux, Barrett́s esophagus and adenocarcinoma: burning questions. Nat Rev Cancer 2003;3: 676–84.

[11] Edebo A, Vieth M, Tam W, Bruno M, van Berkel AM, Stolte M, et al. Circumferential and axial distribution of esophageal mucosal damage in reflux disease. Dis Esophagus 2007;20:232–8.

[12] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[13] Björkman E, Edebo A, Casselbrant A, Helander HF, Bratlie SO, Vieth M, et al. The renin angiotensin system in the esophageal mucosa of healthy subjects and patients with reflux disease. Scand J Gastroenterol 2013;48:147–59.

[14] Farre R, van Malenstein H, De Vos R, Geboes K, Depoortere I, Vanden Berghe P, et al. Short exposure of oesophageal mucosa to bile acids, both in acidic and weakly acidic conditions, can impair mucosal integrity and provoke dilated intercellular spaces. Gut 2008;57:1366–74.

[15] Oshima T, Koseki J, Chen X, Matsumoto T, Miwa H. Acid modulates the squamous epithelial barrier function by modulating the localization of claudins in the superficial layers. Lab Invest 2012;92:22–31.

[16] Farre R, Fornari F, Blondeau K, Vieth M, De Vos R, Bisschops R, et al. Acid and weakly acidic solutions impair

mucosal integrity of distal exposed and proximal non-exposed human oesophageal. Gut 2010;59:164–9.
[17] Souza RF. The role of acid and bile reflux in oesophagitis and Barrettś metaplasia. Biochem Soc Trans 2010;38:348–52.
[18] Nakos A, Zezos P, Liratzopoulos N, Efraimidou E, Manolas K, Moschos J, et al. The significance of histological evidence of bile reflux gastropathy in patients with gastro-esophageal reflux disease. Med Sci Monit 2009;15:313–18.
[19] Nehra D, Howell P, Williams CP, Pye JK, Beynon J. Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity. Gut 1999;44:98–602.
[20] Vaezi MF, Singh S, Richter JE. Role of acid and duodeno-gastric reflux in esophageal mucosal injury: A review of animal and human studies. Gastroenterology 1995;108: 1897–907.
[21] Schindlbeck NE, Heinrich C, Stellaard F, Paumgartner G, Muller-Lissner SA. Healthy controls have as much bile reflux as gastric ulcer patients. Gut 1987;28:1577–83.
[22] Fiorucci S, Distrutti E, Di Matteo F, Brunori P, Santucci L, Mallozzi E, et al. Circadian variations in gastric acid and pepsin secretion and intragastric bile acid in patients with reflux esophagitis and in healthy controls. Am J Gastroenterol 1995;90:270–6.
[23] Theisen J, Nehra D, Citron D, Johansson J, Hagen JA, Crookes PF, et al. Suppression of gastric acid secretion in patients with gastroesophageal reflux disease results in gastric bacterial overgrowth and deconjugation of bile acids. J Gastrointest Surg 2000;4:50–4.
[24] Oltean M, Joshi M, Björkman E, Oltean S, Casselbrant A, Herlenius G, et al. Intraluminal Polyethylene Glycol Stabilizes Tight Junctions and Improves Intestinal Preservation in Rat. Am J Transplant 2012;12:2044–51.
[25] Chen X, Oshima T, Tomita T, Fukui H, Watari J, Matsumoto T, et al. Acidic bile salts modulate the squamous epithelial barrier function by modulating tight junction proteins. Am J Physiol 2011;301:203–9.
[26] Chen X, Oshima T, San J, Fukui H, Watari J, Miwa H. Bile salts disrupt human esophageal squamous epithelial barrier function by modulating tight junction proteins. Am J Phys 2012;303:199–208.
[27] Overgaard CE, Daugherty BL, Mitchell LA, Koval M. Claudins: Control of barrier function and regulation in response to oxidant stress. Antioxid Redox Signal 2011;15:1179–93.
[28] Amasheh S, Milatz S, Krug SM, Markov AG, Gunzel D, Amasheh M, et al. Tight junction proteins as channel formers and barrier builders. Ann NY Acad Sci 2009;1165:211–19.