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ORIGINAL ARTICLE

The renin–angiotensin system in the esophageal mucosa of healthy subjects and patients with reflux disease

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Abstract

Objective. Components of the renin–angiotensin system (RAS) were recently discovered in the esophagus, which could be of interest in relation to gastroesophageal reflux disease (GERD). The present study was undertaken to confirm and further investigate the expression of RAS in healthy and refluxed exposed human esophageal mucosae. ***Methods.*** Esophageal biopsies were obtained from healthy subjects ($n = 34$) and individuals with erosive reflux disease (ERD, $n = 28$). Evaluation of general morphology and histological signs of reflux as well as investigation of gene transcript, protein expression and localization of various RAS components using RT-PCR, ELISA, western blot and immunohistochemistry were performed. Physiological effects of the AT2R were investigated in Ussing chamber experiments. ***Results.*** The study confirmed histological signs of reflux in ERD and expression of ACE, AT1R, AT2R and CatD in all examined specimens. In addition, the main effector peptide AngII, the pro-hormone AGT, the Mas receptor and the angiotensin-forming enzymes renin, CMA, CatG and NEP were present. Individuals with reflux disease had higher transcription activity of ACE and AT1R, increased protein levels of AT2R and lower levels of MasR. AT2R stimulation increased the ion currents in healthy epithelium, whereas epithelium from individuals with reflux disease exhibited no significant response. ***Conclusions.*** The study demonstrated that a local RAS is present in the human esophageal epithelium. Some RAS components were significantly altered in individuals diagnosed with ERD suggesting involvement in the pathophysiology of GERD.

Key Words: *angiotensin II type 2 receptor, gastroesophageal reflux disease, human esophageal mucosa, renin–angiotensin system, Ussing chamber*

Introduction

Increased incidence and prolonged exposure of gastroesophageal reflux can damage the esophageal epithelium and lead to gastroesophageal reflux disease (GERD). The diagnosis of the disease is based on symptom perception and on endoscopic evaluation. If the distal esophageal mucosa contains mucosal breaks, the condition is termed erosive reflux disease (ERD). The ability of the epithelium to tolerate reflux episodes depends on the balance between the intensity of the noxious stimulation and the protective mechanisms of the tissue. By unknown reasons the ability to maintain barrier properties is impaired in GERD [1,2], and the disease is for example associated with dilated intercellular spaces (DIS) and signs of increased proliferation such as elongation of papillae length (PL) [3,4].

Previous investigations conducted by our group have demonstrated the presence of renin–angiotensin system (RAS) components in the human esophagus [5,6]. RAS is involved not only in body fluid balance and blood circulation, but also in growth, proliferation, inflammation and carcinogenesis [7,8], making

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it of particular interest in relation to GERD. The classical view of RAS constitutes the conversion of angiotensinogen (AGT; hepatic origin) to angiotensin I (AngI) by the circulating enzyme renin (renal origin). By actions of angiotensin-converting enzyme (ACE), AngI is converted to angiotensin II (AngII), which is regarded as the main effector of the system exerting its effects via the AngII type 1 receptor (AT1R) and type 2 receptor (AT2R). However, AngII formation can also occur through other pathways, i.e. by other locally expressed enzymes such as chymase and cathepsin D and G [7,9]. In addition, several other angiotensin peptides exist that could have physiological relevance, e.g. angiotensin 1-7 (Ang1-7) mediating its effects via the Mas oncogene receptor (MasR) [10]. Originally, RAS was ascribed only a systemic mode of action, by AngII acting as a circulating hormone, but lately several local tissue RAS have also been discovered, for example throughout the gastrointestinal tract [11,12]. The aim of the present study was to confirm the presence of RAS in the human esophageal mucosa and to investigate alternative AngII-forming enzymatic pathways. In addition to expression and localization of RAS in healthy squamous epithelium, a second aim was to investigate potential aberrations related to ERD. The results showed that the mucosal protein expressions of the AT2R and MasR were altered in individuals diagnosed with ERD. A third aim of the present study was therefore to compare functionality of the AT2R in esophageal epithelium of healthy individuals with that of patients with ERD.

Methods

Human subjects

Esophageal biopsies were obtained from healthy volunteers ($n = 34$, male 18, mean age 30 years, range 21–61 years) and from individuals with ERD ($n = 28$, male 16, mean age 42, range 20–67; of Caucasian origin). The enrolled ERD patients had been referred to the outpatient endoscopy unit due to reflux symptoms. The ERD patients were requested to abstain medication with proton pump inhibitors or H_2 antagonists for 14–15 days before the endoscopic examination in order to re-establish clinical ERD. Other kinds of antacids were offered as escape medication if necessary due to severe reflux symptoms. All individuals were subjected to an upper endoscopy with a high-resolution magnification video endoscope and the biopsies were collected using biopsy forceps (Endotherapy disposable biopsy forceps FB-234U Olympus, Hamburg, Germany). Biopsies were taken within 1 cm from the gastroesophageal junction in the 3 o'clock position [13]. In the ERD patients the biopsies were collected both directly in the mucosal break area (ERD m.b.) and from the endoscopically normal epithelium (ERD n.e.) 1 cm lateral to the mucosal break but from the same height. The ERD patients were scored according to the Los Angeles classification for reflux esophagitis [14] and 25 individuals were classified as Grade A and 3 as Grade B.

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

Tissue histology

Biopsies were fixed in neutral buffered 4% formaldehyde, dehydrated, embedded in paraffin and cut into 3-μm thick sections that were mounted on glass slides. For general histomorphology, the sections were deparaffinized and stained with hematoxylin–eosin and evaluated concerning DIS, PL and thickness of the basal cell layer (BCL). BCL and PL were measured in μm, whereas DIS was estimated on an arbitrary five-step scale being either absent (0), slight focal (1), slight diffuse (2), moderate (3) or marked (4). The histopathological investigator was blinded to the source of the biopsy, the subject's group affiliation, etc.

Transcription activity

Reverse transcriptase polymerase chain reaction (RT-PCR) was used to investigate *m*RNA presence and levels of the RAS components: ACE, AGT, AT1R, AT2R and renin in healthy and ERD epithelia. The primer sequences and PCR products sizes are listed in Table I [15,16].

The biopsies were put in RNA STAT-60 (Nordic BioSite AB, Stockholm, Sweden) and snap frozen in liquid nitrogen immediately after collection. Upon analysis, the frozen tissue was homogenized and total RNA extracted according to the manufacturer's instructions using phenol-chloroform extraction and ethanol precipitation. Reverse transcription from 2.5 μg of total RNA was carried out using the SUPERSCRIPT™ First-Strand Synthesis System (Invitrogen, Lidingö, Sweden) with Oligo (dT) Primers (Life Technologies, Täby, Sweden). Resulting cDNA was stored at -20°C until use. Lightcycler Q-PCR (Roche Diagnostics AB, Stockholm, Sweden) was performed using the FastStart DNA Master SYBR Green I (Roche Diagnostics AB, Stockholm,

Table I. Reverse transcriptase PCR primer sequences and product sizes.

Target *m*RNA	Primer sequences	Size (bp)
ACE	F:5′-CCGATCTGGCAGAACTTC-3′ R:5′-GTGTTCCAGATCGTCCTC-3′	408
AGT	F:5′-TCCACCTCGTCATCCACA-3′ R:5′-GGCTCCCAGATAGAGAGA-3′	329
AT1R	F:5′-GGCCAGTGTTTTTCTTTTGAATTTAGCAC-3′ R:5′-TGAACAATAGCCAGGTATCGATCAATGC-3′	210
AT2R	F:5′-GTTCCCCTTGTTTGGTGTAT-3′ R:5′-CATCTTCAGGACTTGGTCAC-3	253
Renin	F:5′-GGTGTCTGGCAGATTCAA-3′ R:5′-CGGTTGTTACGCCGATCA-3	404

Abbreviations: PCR = polymerase chain reaction; bp = base pair; ACE: angiotensin-converting enzyme, AGT = angiotensinogen; AT1R and AT2R = angiotensin II type 1 and type 2 receptor, respectively.

Sweden). RT-PCR was performed, containing 2 μL of each RT-sample, using the hot-start technique. $MgCl_2$ concentration was optimized to 4 mM to obtain the highest signal intensity and lowest background. For each pair of primers, a standard curve was produced, using 3–6 dilutions of a positive sample. The copy numbers of the genes were interpolated using the standard curves and each tested target gene was expressed per μg total RNA by dividing the copy numbers of the PCR products with the total RNA concentration measured by GENios spectrophotometer (TECAN, Salzburg, Austria). Quantification analyses were performed using software supplied by Roche Diagnostics (Mannheim, Germany).

Protein expression and localization

AngII-ELISA. AngII levels in healthy individuals and ERD patients were investigated by ELISA analysis. The frozen biopsies were defrosted and proteins solubilized by sonication in PE-buffer (10 mM potassium phosphate buffer, pH 6.8, and 1 mM EDTA) containing a protease inhibitor cocktail (Complete-tablet, Riche Diagnostics, Stockholm, Sweden) and CHAPS detergent (10 mM 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate; Boehringer Mannheim, Mannheim, Germany). The resulting homogenate was centrifuged (10,000 *g* for 10 min at 4°C) and the supernatant's protein content analyzed using the Bradford method [17]. The ELISA plate and reagents were prepared and the analysis performed in accordance to the manufacturer's instructions (angiotensin II enzyme immunoassay kit A05880-96 wells, Spi Bio Bertin Pharma, Montigny le Bretonneux, France). The principle of the assay is based on that immobilized anti-AngII antibodies in the bottom of each well bind to potential AngII peptides in the solubilized biopsy samples. The antibody–AngII complex forms a yellow compound together with a tracer (acetylcholinesterase) and a chromogen (Ellman's reagent) and each sample's concentration is colorimetrically determined (absorbance at 405 nm, TECAN, Salzburg, Austria) when compared with a prepared standard curve on the same ELISA plate.

Western blot analysis. Western blot was used for semi-quantitative analyses of protein expression levels of the investigated RAS components, listed in Table II, in healthy subjects and ERD patients. The proteins were solubilized in the same way as for the ELISA samples described above. Then, the samples, as well as positive controls, were diluted in sodium dodecyl sulfate buffer and heated to 70°C, before the proteins in each sample were separated by gel electrophoresis using NuPage 10% Bis-Tris gel and MOPS-buffer (Invitrogen, Lidingö, Sweden). One lane on the gel was loaded with a prestained molecular weight standard (SeeBlue, Novex, San Diego, CA, USA) to which the molecular weight of the samples could be compared. The separated proteins on the gel were transferred onto a membrane (polyvinylidene difluoride membrane; Amersham, Buckinghamshire, UK) and incubated, at 4°C over night, with respective primary RAS antibody or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), working as a loading control. To enhance detection signal an alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit/goat anti-mouse/donkey anti-goat) was linked to the primary. Signals were detected by adding the reagent CDP-Star (Tropix, Bedford, MA, USA) that produces a chemoluminescent light when reacting with the alkaline phosphatase on the secondary antibody.

Images were captured (Chemidox XRS cooled charge-coupled device camera) and analyzed using the software program Quantity One (camera and software from Bio-Rad Laboratories, Hercules, CA, USA). After imaging, the membrane was put in a re-blot mild antibody stripping solution (Millipore, Solna, Sweden), resulting in removal of the antibody,

Table II. Antibodies used in western blot and immunohistochemistry.

Primary antibody	Catalog#	Supplier	Positive control
ACE	ab85955	Abcam, Cambridge, UK	Kidney extract, Sc-2255, Santa Cruz Kidney extract 1345-N, Nordic BioSite AB
AGT	AF3156	R&D Systems, Abingdon, UK	Kidney extract 1345-N, Nordic BioSite AB
AT1R	Sc-1173	Santa Cruz Biotechnology, Heidelberg, Germany	PC-12 Sc-2250, Santa Cruz
AT2R	Sc-9040	Santa Cruz Biotechnology, Heidelberg, Germany	HepG2 Sc-2227, Santa Cruz
CatD	Sc-10725	Santa Cruz Biotechnology, Heidelberg, Germany	HepG2 Sc-2227, Santa Cruz
CatG	Sc-33206	Santa Cruz Biotechnology, Heidelberg, Germany	
CMA	LS-B2147	LifeSpan Biosciences, Inc., Seattle, WA, USA	Achalasial tissue containing mast cells
MasR	AAR-013	Alomone Labs Ltd., Jerusalem, Israel	
NEP	AB5458	Millipore AB, Solna, Sweden	Kidney extract 1345-N, Nordic BioSite AB
Renin	Sc-22752	Santa Cruz Biotechnology, Heidelberg, Germany	Kidney extract Sc-2255, Santa Cruz
GAPDH	IMG-5143A	Nordic BioSite AB, Täby, Sweden	Loading control

Abbreviations: ACE = angiotensin-converting enzyme; AGT = angiotensinogen; AT1R and AT2R = angiotensin II type 1 and type 2 receptor, respectively; CatD and CatG = cathepsin D and G, respectively; CMA = chymase; MasR = Mas oncogene receptor; NEP = neprilysin; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

after which the membrane could be incubated with another RAS antibody and the procedure repeated.

Immunohistochemistry. Immunohistochemistry (IHC) was used to demonstrate the intraepithelial location of the various RAS components in the esophageal tissue (no comparisons were done between healthy and ERD epithelia). Antigen retrieval was performed on deparaffinized slides by boiling them in 10 mM citrate buffer (pH 6.0) in a microwave oven for 10 min. After blockade of peroxidase activity, slides were preincubated with serum block followed by incubation with respective primary antibody (see Table II; minimal staining of at least three slides per antibody in separate individuals) for 15 min to 2 h in room temperature according to the suppliers' recommendations. Negative control sections were incubated with normal goat/mouse/rabbit IgG instead of primary antibody. To enhance detection signal, the slides were incubated with a conjugated (biotin/horseradish peroxidase, HRP) secondary antibody (anti-rabbit/anti-mouse/anti-goat). The protein-antibody complex was detected using an amplification system (fluorescyltyramide hydrogen peroxidase, i.e. in the Dako kit) and a reagent (streptavidin-HRP/anti-fluorescein-HRP), where after development was performed with the substrate 3,3′-diaminobenzidine (Santa Cruz sc-2053 and Dako K1497/K1501 staining systems).

Functional assessment

Ussing chamber experiments were performed to elucidate actions of the AT2R in the normal epithelia of healthy subjects and patients with ERD (i.e. outside the mucosal break). The biopsies were put in oxygenated Krebs solution (mM: 118.1 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.0 NaH_2PO_4, 25 $NaHCO_3$ and 11.1 glucose) and mounted in vertical mini-Ussing Chambers (Warner instruments, Hamden, CT, USA) containing a biopsy insert with a diameter of 1.5 mm resulting in the square area of 0.018 cm^2. Biopsies were positioned in the middle of each chamber, creating a luminal and serosal side, and the resevoirs were filled with 5 mL Krebs solution that was kept at 37°C and continuously oxygenated and stirred with a gas flow of 95% O_2 and 5% CO_2.

Transepithelial PD was continuously measured with a pair of Ag/AgCl voltage electrodes (Warner instruments, Hamden, CT, USA). The Ussing pulse method (UPM) was used to determine the epithelial electrical resistance (R_{ep}). The epithelial ion current (I_{ep}) was calculated using Ohm's law by I = U/R (current = voltage/resistance, i.e. I_{ep} = PD/R_{ep}). The UPM is based on the concept that the epithelium acts as a capacitor and resistor coupled in parallel [18]. 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 voltage response of the epithelium, specifically, is received from the discharge curve and by knowing the magnitude of the applied current the R_{ep} can be calculated. The data were collected using an amplifier and specially constructed software developed in LabView (National Instruments, Austin, TX, USA).

After being mounted in the Ussing chamber, each biopsy was let to equilibrate for 30 min before baseline measurements were performed over a period of 20 min. To be eligible for experimentation, each preparation had to exhibit a lumen-negative potential difference (PD) of at least 0.3 mV at baseline. This criterion was based on previous pilot experimental experience where an initial PD < 0.3 mV indicated a later specimen failure. In the present study, 8%

of the mounted biopsies were discarded due to this reason.

The selective AT2R agonist, C21 [19], was added stepwise to the serosal reservoir in two concentrations (1×10^{-7} M and 1×10^{-6} M in chamber) with 5 min interval and the effects recorded 10 min subsequent to the first addition. Untreated specimens were run in parallel in each setting and served as time controls. Viability of these preparations at the end of the study period was confirmed by addition of amiloride (0.1 mM in chamber) resulting in an expected 40–60% reduction of I_{ep} and PD. The selectivity of C21 was tested in healthy specimens only: C21 was then added in presence of the AT2R antagonist PD123319 (1×10^{-6}M in chamber; added 10 min before C21; Sigma-Aldrich, Stockholm, Sweden). PD123319 *per se* was also investigated in this group.

Data analysis and statistics

Comparisons of BCL thickness, PL and relative changes of Ussing parameters were performed by Student's t-test for paired or unpaired values, when appropriate, using the statistical software program SPSS 18.0 (SPSS, Chicago, IL, USA). Kruskal–Wallis and Mann–Whitney U-test for independent variables and Wilcoxon's signed rank test for related variables were used analyzing the differences in DIS, transcription activity and protein expression. A p-Value of ≤ 0.05 was considered significant; the number of individuals was denoted n and preparations/observations N.

Results

Histological appearance of the squamous mucosa

The epithelium from the area of a mucosal break in the ERD patients was morphological altered with significantly thicker basal cell layer ($p = 0.029$), longer papillae ($p = 0.001$) and wider intercellular spaces ($p = 0.018$) compared with epithelium from healthy controls (Figure 1A–C). Furthermore, ERD patients had significantly ($p = 0.038$) longer papillae in their mucosal break area than in their normal epithelium (Figure 1B).

Gene activity of RAS

The transcription activity is shown in Table III. Renin, particularly, showed low levels independent on background condition. The amount of ACE ($p = 0.019$) and the AT1R ($p = 0.003$) were significantly higher in the mucosal break area of ERD patients compared with control subjects whereas the AT2R was not ($p = 0.061$). Interestingly, ACE gene expression was increased also in normal mucosa of ERD patients compared with controls ($p = 0.034$).

Protein expression and localization of RAS

AngII was detected in all groups, without any significant difference (Figure 2). AGT, renin (precursor and mature form), ACE, CatD and NEP were detected by western blot in all groups (no significant difference; Figure 3A–F), but not CMA and CatG (not shown in figure). The AT2R levels were significantly higher ($p = 0.05$) in the normal epithelium of ERD patients compared with in their mucosal break area as well as to healthy subjects (Figures 3H and 4), while the AT1R expression was on a reasonably similar level in all three groups (Figure 3G). Lower expression of the MasR was displayed in the mucosal break area of ERD patients compared with healthy volunteers ($p = 0.03$, Figures 3I and 4).

The intraepithelial localizations of the components, shown in Table IV, were typically membranous or cytosolic and often localized around the papillae. Some of the components were also found in the blood vessel walls and sometimes around/in the nuclei. AGT and its proteolytic enzyme renin were observed in the intermediate and basal epithelium, along the papillae and blood vessel structures (Figure 5A and B). Also the AGT-converting enzymes CatD and CatG were observed in the cytosole and CatG and renin were also present seemingly in the nuclei, particularly in stratum basale (Figure 5B and C). The enzymes ACE and chymase, that both form AngII, were observed in the entire epithelium and particularly chymase showed a pronounced staining (Figure 5D). ACE was also localized to the blood vessel walls in the epithelium and lamina propria (Figure 5G). The AT1R and AT2R were observed in the entire squamous epithelium (Figure 5E and F), even in the superficial cell layers. The AT1R showed a distinct staining in the basal cell layers, peripapillary and occasionally in the stratum spinosum. Ang1-7's formation enzyme, NEP, and receptor, MasR, were localized to the cell cytosole of the entire epithelium. NEP was also found in the intermediate epithelium around/in cell membranes and MasR on/in the nuclei (Figure 5H and I). Control sections incubated with normal goat/mouse/rabbit IgG instead of primary antibody did not display any immunoreactivity (not shown in figure).

AT2R stimulation

Basal electrical parameters are displayed in Table V. The epithelial electrical resistance at baseline was lower, while the potential difference and ion currents

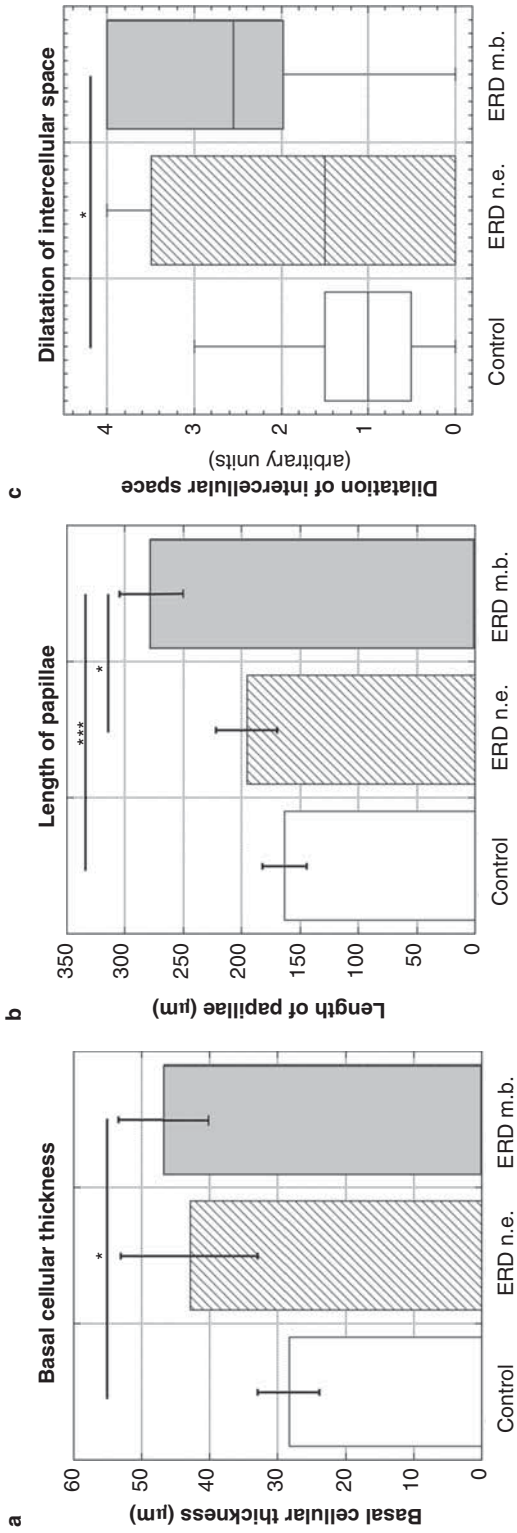

Figure 1. Histological alterations in relation to ERD. (A) Thickness of stratum basale, (B) length of intraepithelial papillae and (C) dilatation of epithelial intercellular spaces from macroscopically normal squamous mucosa of control subjects ($n = 11$) and of ERD outside ($n = 10$; n.e.: normal epithelium) and in the mucosal break area ($n = 11$; m.b.: mucosal break). Panel (A) and (B) are presented as mean ± SEM and (C) as median with interquartile and total ranges. Significant differences are indicated with asterisks: $*p \leq 0.05$ and $***p \leq 0.001$.

Table III. Gene transcripts in squamous mucosae.

	Control		ERD Normal epithelium			ERD Mucosal break area		
	Median (range)	*n*	Median (range)	*p*	*n*	Median (range)	*p*	*n*
ACE	0 (0–6.65)	11	8.24 (0–279)	0.034*	10	10.0(0–56.0)	0.019*	11
AGT	4.41 (0–62.9)	11	119.74 (0.05–2888)	0.121	10	30.67 (0.65–450)	0.250	11
AT1R	0.93 (0.21–3.56)	11	17.09 (0.02–94.3)	0.260	10	6.67 (0.35–49.0)	0.003**	11
AT2R	0.47 (0–5.17)	11	8.35 (0.04–66.1)	0.105	10	4.74 (0.04–21.7)	0.061	11
Renin	0.24 (0–2.74)	11	0.95 (0–40.0)	0.193	10	0.07 (0–7.17)	0.921	11

Abbreviations: ACE = angiotensin-converting enzyme; AGT: angiotensinogen; AT1R and AT2R = angiotensin II type 1 and type 2 receptor, respectively.
Data are normalized to total RNA and are given as median values and range; *n* = number of individuals. Significant differences from control are indicated with asterisks (* $p \leq 0.05$ and ** $p \leq 0.01$).

were higher in the normal epithelium of ERD patients compared with healthy subjects (some preliminary results of the baseline parameters, specifically, have been presented in a previous publication [18]). The results of AT2R stimulation are shown in Figure 6, where AT2R stimulation with C21 increased the PD and I_{ep} by 10% and 9%, respectively (both with $p < 0.01$) in healthy individuals ($n = 10$, $N = 14$) compared with time controls ($n = 11$, $N = 16$; no addition of C21). A similar effect was seen in ERD patients ($n = 10$, $N = 18$) but the difference upon AT2R stimulation was not statistically significant from their untreated time controls ($n = 10$, $N = 15$; Figure 6). No changes in the resistance occurred upon AT2R stimulation in neither healthy subjects or ERD patients (data not shown). The antagonist PD123319 blocked the C21 effect when the two compounds were added together (potential difference -1%±6 and I_{ep} -3%±6; $n = 9$, $N = 14$) and addition of only the antagonist itself ($n = 10$, $N = 13$) had no significant effect compared with time control (data not shown in figure).

Discussion

The results confirmed the presence of RAS components in the epithelium of the distal human esophagus [5,20]. Moreover, in addition to the previously described components ACE, AT1R, AT2R and CatD, the present investigation also identified the peptide AGT, the additional AngII-forming enzymes renin, CatG and chymase, as well as the main effector, AngII, itself in the epithelium. The data also suggested activity of the angiotensin peptide Ang1-7, since its generating enzyme NEP and receptor MasR were demonstrated. Interestingly, several of the detected components appeared to be altered in the mucosa of ERD patients, suggesting an involvement of RAS in the pathogenesis of this disease. When investigating functionality in Ussing chamber experiments the AT2R appeared to be primarily involved in regulation of epithelial ion currents, and this effect differed between healthy and ERD individuals.

From being looked upon as a fairly uncomplicated blood borne system RAS is today regarded as a complex mesh of potential tissue specific enzymatic pathways generating bioactive fragments from the pro-hormone AGT, and particularly the system's

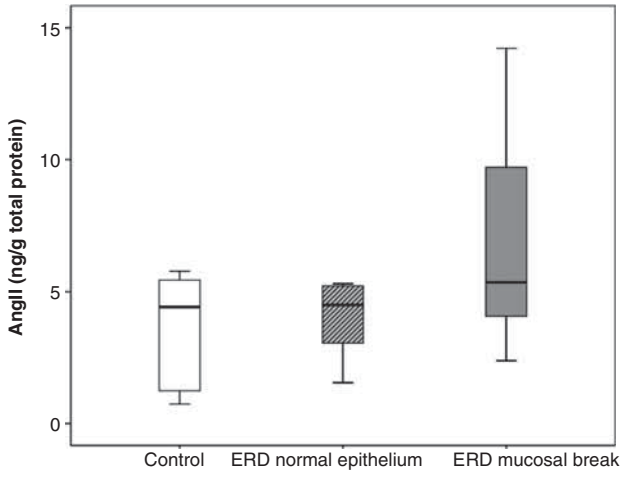

Figure 2. Angiotensin II levels. AngII levels in relation to total protein amount in healthy control subjects ($n = 8$), in the ERD normal epithelium ($n = 7$), and in the mucosal break area ($n = 7$). Data are expressed as box-and-whiskers plots showing median values, interquartile, and total ranges.

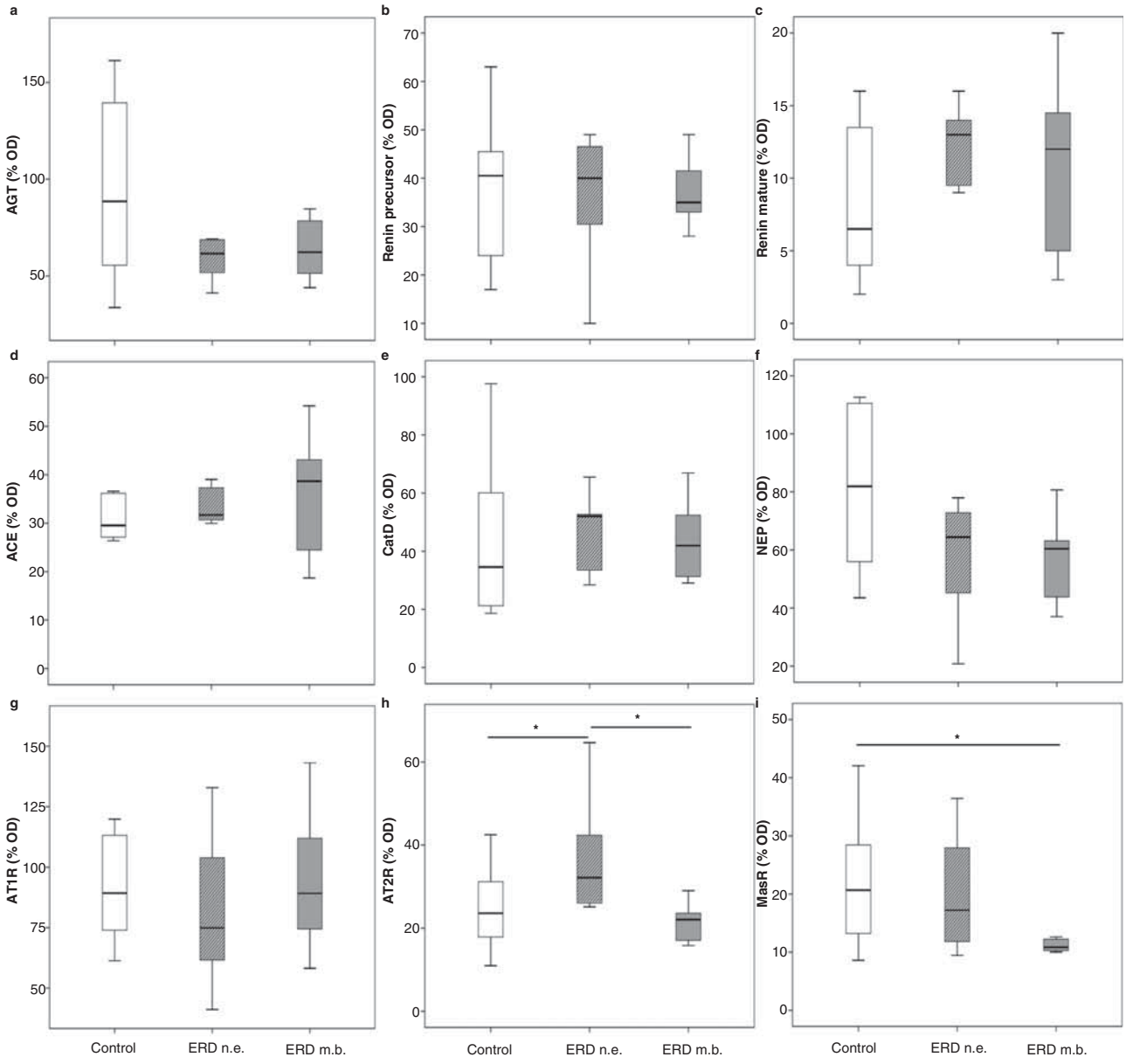

Figure 3. RAS proteins analyzed by western blot. (A) Angiotensinogen, (B) precursor renin, (C) mature renin, (D) angiotensin-converting enzyme, (E) cathepsin D, (F) neprilysin, (G) angiotensin II type 1 and (H) type 2 receptor and (I) Mas oncogene receptor in healthy control subjects ($n = 8$) and in the normal epithelium (n.e.) and mucosal break area (m.b.) of ERD patients ($n = 7$). The optical density (OD) of protein amounts is semi-quantitative and in relation to the housekeeping protein GAPDH of respective sample. Data are expressed as box-and-whiskers plots with median values, interquartile, and total ranges. Significant differences are indicated with asterisks ($*p \leq 0.05$).

main effector peptide AngII. The role of RAS in the human esophageal mucosa is not established, but the present investigation suggests involvement in epithelial ion transport. Previous data from our laboratory have shown that the two subtypes of AngII receptors are located close to the subepithelial blood vessels suggesting vasoregulatory actions. Several angiotensin-forming enzymes were localized peripapillary close to or in the blood vessel walls (e.g. AGT, renin, ACE) indicating perivascular formation of angiotensins, very probably supported by RAS components in the blood circulation. Furthermore, as confirmed in the present study both two AngII subtype receptors, AT1R and AT2R, were shown to be located in the esophageal epithelium [5]. The signal transmission by AngII in the esophageal stratified epithelium appears more enigmatic than only composed of potential vascular regulation. Many angiotensin-forming enzymes were found throughout the mucosa, suggesting local production of AngII with

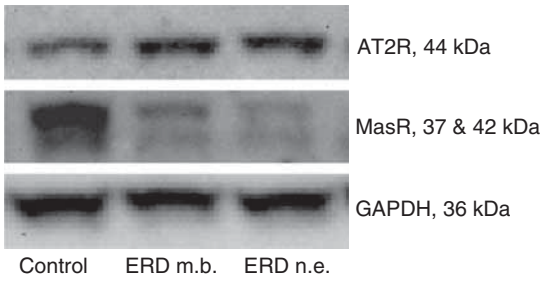

Figure 4. Protein bands as displayed on the western blot membrane. The figure shows samples from the AT2R, the MasR and the loading control GAPDH in healthy controls (Control) and in the mucosal break (ERD m.b.) and the normal epithelium (ERD n.e.) of ERD patients.

the ability to exert its effects even in the superficial epithelium close to the esophageal lumen.

The elevated gene-transcript activity of ACE and the AT1R in ERD mucosa can probably be related to the well-established pro-inflammatory actions of the AT1R, including tissue proliferation and angiogenesis [8,21,22]. The signs of gene transcription of RAS components strongly support that the system is locally active. However, from a functional perspective the presence of a certain protein is more relevant than the presence of gene transcripts. Gene transcription and protein expression are processes with very different time resolution and a gene transcript signal can occur and disappear before the corresponding protein is detectable. This is probably the explanation of the present contrasting results for the AT1R and AT2R gene and protein expressions, respectively. Renin is also known to be involved in inflammation and proliferation [23] and the present observation of this enzyme in the nuclei suggests that renin might governs transcription of such mediators in the esophageal epithelium. Although no difference in CatD levels between groups were observed in the present study, a previous study has shown that the enzyme is elevated in relation to esophagitis, Barrett's esophagus and esophageal adenocarcinoma [20]. This could be due to that the enzymatic activity of CatD is enhanced in the acidic environment occurring at reflux. Moreover, CatD has been shown to restrain apoptosis following bile-induced cell damage that in turn could promote carcinogenesis [20]. Consequently, CatD could be of particular interest at both acidic and bile containing reflux events. Taken together, many actions of RAS may be connected and even promote processes leading to esophagitis and increased proliferation.

In contrast to the pro-inflammatory character of the AT1R, both the AT2R and the MasR are described to have anti-inflammatory properties, be tissue protective and prevent pathological processes [8,10,22,24]. Thus, it can be speculated that the observed decrease of Mas receptors in the mucosal break area may indicate a reduced anti-inflammatory signal. Furthermore, the AT2R expression was increased in the normal ERD epithelium, suggesting an adaptive up-regulation of this receptor as part of a mucosal protective response to refluxate attacks. In line with this hypothesis is a recently published retrospective analysis indicating that AT1R antagonists support the healing of reflux esophagitis induced by proton pump inhibition [25]. These healing-promoting effects are likely not only as a result of blockade of the AT1R-mediated pro-inflammatory response, but also due to cross-talk [8] and AngII-mediated stimulation of the unopposed AT2R.

The finding of higher AT2R protein expression in ERD compared with mucosae from healthy

Table IV. Localizations of various RAS proteins in the esophageal epithelium.

Protein	Stratum superficiale	Stratum spinosum	Stratum basale	Peripapillary
ACE	+	+	+	+ vessels
AGT	–	+	+	+ vessels
AT1R	+	+ membranous, cytosolic	+ membranous, possibly cytosolic	+
AT2R	+	+ membranous, cytosolic	+ membranous, cytosolic	+
CatD	+ cytosolic	+ cytosolic	+ cytosolic	+ cytosolic
CatG	+	+ nuclear, cytosolic	+ nuclear	+
CMA	+	+	+ cytosolic, around the nucleus	+
MasR	+	+ cytosolic, around the nucleus	+	+
NEP	+	+ membranous, cytosolic	+	+
Renin	+	+ membranous	+ nuclear	+ vessels

Abbreviations: ACE = angiotensin-converting enzyme; AGT = angiotensinogen; AT1R and AT2R = angiotensin II type 1 and type 2 receptor, respectively; CatD and CatG = cathepsin D and G, respectively; CMA = chymase; MasR = Mas oncogene receptor; NEP = neprilysin; GAPDH = glyceraldehyde 3-phosphate dehydrogenase. No comparisons between groups were made.

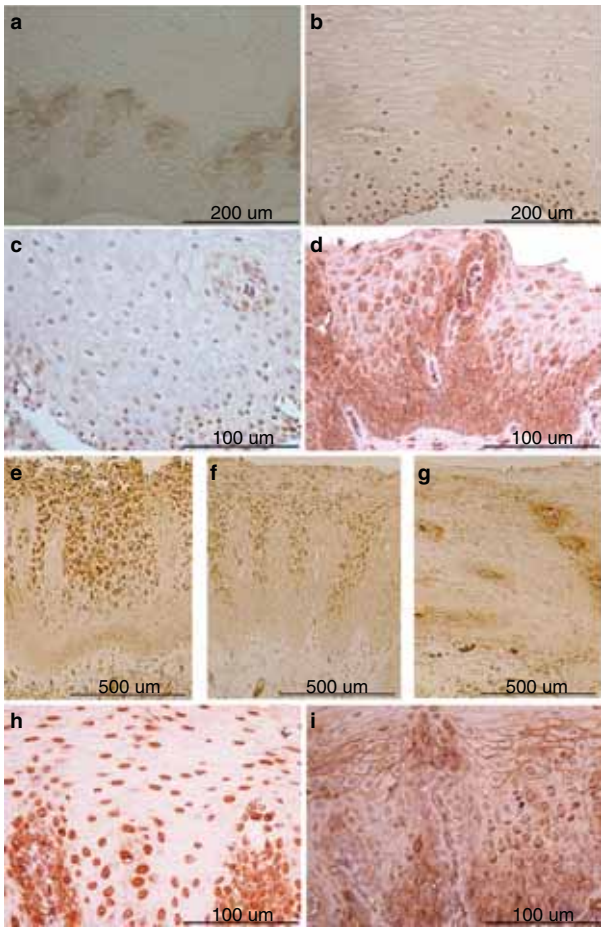

Figure 5. Localization of RAS components in the esophageal epithelium. The panels show immunohistochemical staining of: angiotensinogen (A), renin (B), cathepsin G (C), chymase (D), angiotensin II type 1 receptor (E), angiotensin II type 2 receptor (F), angiotensin-converting enzyme (G), Mas oncogene receptor (h) and neprilysin (I) (hematoxylin–eosin staining in sections C, D, H, and I). No comparisons between groups were made.

individuals motivated a functional analysis by use of Ussing chambers. The present Ussing chamber experiments showed that the electrical parameters at baseline differed between healthy subjects and ERD patients. A lower electrical resistance was recorded in the normal epithelia from ERD patients confirming data from another Ussing chamber study [2]. Low epithelial resistance indicates increased paracellular conductivity (i.e. the permeability to charged molecular species = ions) and can be explained as due to the dilated intercellular spaces observed in the ERD patients of the present study. The small-sized endoscopic biopsies necessitated the use of small aperture Ussing chambers. The assessed absolute values of PD and epithelial resistance of "mini-Ussing devices" are generally lower as compared with conventional Ussing chambers. This is ascribed to the increased ratio between circumferential edge-damage and total area of the mucosa under study in the small aperture Ussing setup [26,27]. Moreover, in the present study the magnitude of the electrical current generated by the epithelium (I_{ep}) was markedly higher than corresponding values published by others using short-circuit current conditions (I_{sc}) [26]. The reason for this is that the UPM technique first assesses the epithelial resistance after which I_{ep} is calculated according to Ohm's law using the instant PD [18]. The I_{ep} value obtained by UPM therefore represents the situation where the actual transepithelial charge-gradient determines the epithelial ion transport. By definition such an electrochemical gradient does not exist during short-circuited conditions (PD = 0) and the epithelial transport may thus be influenced by this procedure. Hence, the UPM approach appears advantageous from a physiological perspective, but the quantitative precision of the two methods remains to be fully elucidated.

It was a bit surprising that the PD and ion currents were elevated in the normal ERD epithelium compared with healthy epithelium. This picture could be compatible with an increased secretory state in the ERD mucosa, probably as part of a mucosa protective response to the acidic reflux load. However, it was considered beyond the scope of the present investigation to elucidate these findings in detail. Focus was instead put on the up-regulated AT2R expression observed in the normal epithelium of ERD patients. When the AT2R was stimulated with the selective agonist C21, both the electrical transepithelial potential difference and epithelial current increased in the mucosa from healthy subjects. The present results confirm observations from a previous Ussing chamber study performed in our laboratory, where AT2R stimulation also increased I_{ep} [5]. In those experiments, AT1R stimulation reduced I_{ep} and increased the resistance [5]. It should be noted that that study investigated esophageal mucosae from patients undergoing esophagectomy due to malignant disease, whereas the present study used endoscopic biopsies

Table V. Baseline characteristics of electrical parameters.

	PD (-mV)	R_{ep} (Ω*cm^2)	I_{ep} (μA/cm^2)
Healthy controls (n = 15, N = 57)	1.1 ± 0.1	66 ± 7	24 ± 3
ERD normal epithelium (n = 10, N = 33)	1.7 ± 0.3*	38 ± 4 *	57 ± 9*

Abbreviations: PD = potential difference; R_{ep} = epithelial electrical resistance; I_{ep} = epithelial electrical current.
Values are given as means ± SEM, number of individuals are indicated n and preparations N and * denote differences between groups by $p \leq 0.05$.

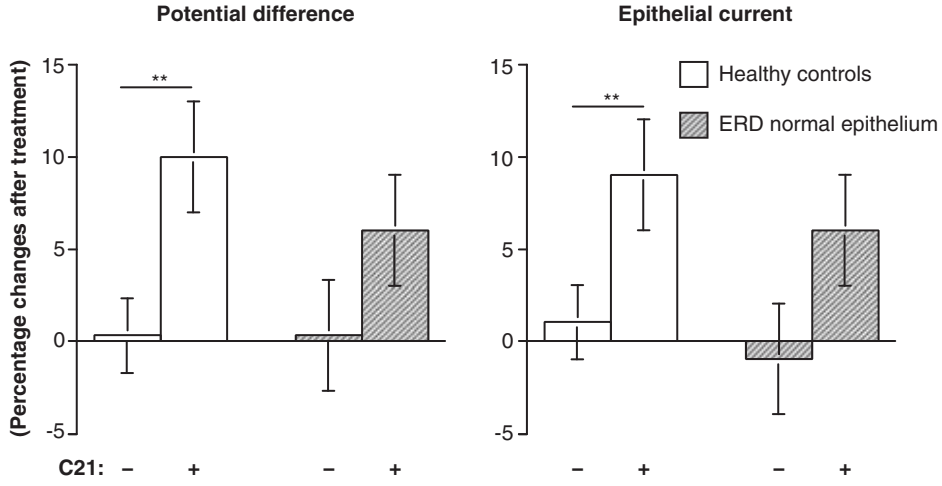

Figure 6. Effects of AT2R stimulation. The esophageal potential difference and epithelial electrical current in healthy subjects increased significantly upon AT2R stimulation with C21 (+ denotes presence of C21, 1*10-7 and 1*10-6 M) compared with time controls (– denotes absence of C21). No statistical difference was observed in the normal epithelium of ERD individuals upon AT2R stimulation compared with time controls. Data are expressed as mean ± SEM and in percentage change from baseline. Significant differences are indicated with asterisks: **p $\leq$ 0.01.

from healthy volunteers and individuals with ERD. Moreover, the present results confirm that the changes in electrical parameters following addition of C21 were truly AT2R-mediated since the selective antagonist PD123319 successfully blocked them.

Because the PD became more lumen-negative upon AT2R stimulation, the changed ion currents were composed of either increased net absorption of cations and/or secretion of anions. The involved cellular mechanisms remain to be identified but it is known that AngII can influence various transporters [28–30] and that the AT2R specifically, is described to be involved in Na^+, H^+ and HCO_3^- transport. In the intestinal tract of rats, the AT2R stimulated bicarbonate secretion [31] and in the vascular smooth muscle cells of mice the receptor blocked an amilorid sensitive Na^+/H^+ exchanger [32]. Interestingly the basolateral Na^+/H^+ exchanger, NHE-1, as well as the sodium pump activity have shown to be increased in GERD [33], and NHE-1 also intensely expressed in the columnar cells of Barrett's esophagus [34]. Influencing basal ion exchangers can lead to subsequent changes of other ion transporters, e.g. apical Na^+/H^+ exchangers and HCO_3^- transport [35]. Na^+/H^+ and Cl^-/HCO_3^- transporters are of particular interest for the esophageal epithelium because of their essential role in regulating intracellular acidity [26] and hence protecting the mucosa from refluxed gastric acid. Of note in this regard is the present finding that despite the higher AT2R levels in the epithelium of ERD patients, stimulation with the agonist C21 did not cause significant effects in the Ussing chamber experiments. This implies that the AT2R is less active in ERD patients, which possibly can be due to a functional defect or to receptor internalization. An alternative explanation is that the already elevated ion currents at baseline reduced the AT2R response capability. The baseline shift can be due to endogenous AngII stimulating the numerous AT2Rs in the ERD condition, or to other so far unknown stimulatory mechanisms. Future studies are warranted to elucidate which transporters the AT2R might influence and why it seems to be less effective in mucosae from ERD patients. The present study was not dimensioned for gender analysis, but this is also a relevant issue for future research.

RAS is a regulatory super-system and some of its components might be of interest as targets for novel pharmaceuticals or have the potential of becoming valuable biomarkers of GERD. Although the presently investigated compounds were considered as being good representatives of RAS, several additional bioactive components exist downstream AngII, e.g. AngIII and AngIV. Moreover, contributing to the complexity of RAS is that angiotensin peptides have different affinities to various receptors, that effects are concentration-dependent and sometimes inverse at certain thresholds and that certain receptor subtypes counterbalance each other's actions [7,8,12].

In summary, the present study confirms that a local RAS is present in the human esophageal mucosa. The study also revealed that several alternative angiotensin-forming pathways were in place.

Furthermore, the results showed that the epithelial AT2R and MasR proteins were altered in GERD patients. Ussing chamber recordings demonstrated a lower epithelial electrical resistance and higher epithelial current in ERD mucosa compared with normal mucosa. AT2R stimulation with a selective receptor agonist increased the ion currents in healthy epithelium but not in normal epithelium of ERD patients despite that the AT2R protein expression was higher in ERD. Involved cellular mechanisms could be connected to regulation of intracellular acidity but are so far unknown and should be elucidated in detail in future studies.

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