The Renin Angiotensin System in the Human Esophageal Mucosa

– expression, actions and potential involvement in reflux disease

Doctoral Thesis

Eleonora Björkman

Department of Gastrosurgical Research and Education
Institute of Clinical Sciences
at Sahlgrenska Academy
University of Gothenburg

Gothenburg, Sweden
2012

UNIVERSITY OF GOTHENBURG

Correspondence:
Eleonora Björkman
Department of Gastrosurgical Research and Education
Institute of Clinical Sciences at Sahlgrenska Academy, University of Gothenburg
Bruna stråket 20, S-413 45, Göteborg, Sweden
Phone: +4631-342 93 09 and +4631-342 68 59
Fax: +4631-41 18 82
e-mail: eleonora.bjorkman@gastro.gu.se and ellen_bjorkman@hotmail.com

http://hdl.handle.net/2077/30563
ISBN 978-91-628-8586-1

Printed by Kompendiet, Aidla Trading AB, Gothenburg, Sweden

ABSTRACT

The renin angiotensin system (RAS) is a classical endocrine system, regulating body fluid balance and blood circulation. Recent research has shown that the system is being also locally expressed and active in several organs and tissues. Components of RAS have been discovered throughout the gastrointestinal tract and have, in addition, been found in the human esophagus. It was hypothesised that RAS could be of interest in relation to gastroesophageal reflux disease (GERD), which is a prevalent clinical condition, where gastric content backflows into the esophagus and causes troublesome symptoms. The general aim of the present thesis was to confirm the presence and further investigate RAS in healthy and reflux exposed human esophageal mucosae.

Esophageal biopsies were collected from healthy volunteers and GERD patients. The gene activity and protein expression of various RAS components were investigated using RT-PCR, western blot, ELISA and immunohistochemistry. The square wave current pulse analysis was investigated for its applicability in Ussing chambers for assessing mucosal *epithelial* resistance (R_{ep}), which in turn permits calculation of the epithelial ion current (I_{ep}).

All investigated RAS components were detected and several of these were significantly altered in relation to reflux disease. Particular attention was paid to the induced expression of the angiotensin II type 2 receptor (AT2R), and to the reduced expression of the angiotensin IV (AngIV) receptor (AT4R) in certain areas in the mucosae from patients with erosive reflux disease (ERD). Using the validated Ussing chamber method, it was found that biopsies from reflux exposed mucosa exhibited lower R_{ep} and higher I_{ep} at baseline. Upon AT2R stimulation the healthy individuals responded with increased I_{ep}, while no significant change was observed in relation to ERD, despite the higher AT2R expression. The peptide AngIV also stimulated the net epithelial current, although the response was small in the mucosae from ERD patients.

The thesis demonstrates that a substantial local RAS is present in the human esophageal mucosa, and it is likely that also angiotensins other than Angiotensin II are produced. Particularly, the AT2R, seems to have reduced response capability in individuals with reflux disease. The expressional and functional alterations suggest that RAS might be involved in the pathophysiology of GERD.

LIST OF PAPERS

The thesis is based on two accepted papers and on one manuscript that in the text will be referred to their Roman numerals.

I. Björkman E, Casselbrant A, Lundberg S, Fändriks L.
In vitro assessment of epithelial electrical resistance in human esophageal and jejunal mucosae and in Caco-2 cell layers. *Scand J Gastroenterol.* 2012 Nov;47(11):1321-33.

II. Björkman E, Edebo A, Casselbrant A, Helander HF, Bratlie SO, Vieth M, Fändriks L.
The renin angiotensin system in the esophageal mucosa of healthy subjects and patients with reflux disease. *Accepted Scand J Gastroenterol.* 2012.

III. Björkman E, Edebo A, Fändriks L, Casselbrant A. In vitro actions by angiotensin IV on the esophageal epithelium in healthy subjects and GERD patients. *In manuscript*

SAMMANFATTNING PÅ SVENSKA

Matstrupsslemhinnans flerskiktade skivepitel förhindrar att skaldiga ämnen tar sig in i vävnaden. Dock kan ett återflöde av magsaft (reflux) reta slemhinnan och orsaka gastroesofageal reflux sjukdom (GERD) med symptom så som halsbränna, sura uppstötningar och bröstsmärtor. Av okänd anledning verkar GERD-patienter ha en nedsatt förmåga att upprätthålla en skyddande epitelial barriär, vilket kan innebära att reflux lättare tränger in i vävnaden och orsaka skada (1, 2). Epitelets permeabilitet (genomsläpplighet) kan studeras i Ussingkammarförsök genom att mäta vävnadens resistans och jonströmmar. Metoden kan vara svårutförd eller inkludera mätvärden från underliggande epiteliala strukturer. Därför är behovet av en enkel Ussingkammarmetod med klinisk användbarhet önskvärd. Orsaken till nedsatt barriärfunktion i samband med refluxsjukdom är fortfarande okänd, men en studie som utförts i vårt laboratorium har indikerat att renin angiotensinsystemet (RAS) påverkar epitelets permeabilitet (3) och därmed barriäregenskaper. RAS är ett regulatoriskt hormonsystem med väl kända effekter på blodtryck och vätskebalans. Systemet har relativt nyligen visat sig finnas lokalt i mag-tarmkanalen (4, 5) och kan vara involverat i processer så som inflammation, tillväxt och cellspecialisering (6, 7). RAS är ej väl utforskat i matstrupen och har aldrig undersökts i relation till refluxsjukdom, vilket är relevant med tanke på systemets kraftfulla regulatoriska egenskaper, dess inblandning i inflammation och dess eventuella påverkan på vävnadens genomsläpplighet. Det övergripande syftet med avhandlingen är att utreda om RAS är närvarande och aktivt i human matstrupsslemhinna och om någon förändring föreligger vid refluxsjukdom.

Hypoteser:

- att ett lokalt RAS med fler olika angiotensiner finns närvarande och är aktivt i den humana matstrupsslemhinnan
- att RAS är förändrat vid refluxsjukdom
- att Ussingpuls metoden (UPM) är en användbar metod för att studera epitelets genomsläpplighet och att metoden kan särskilja på frisk respektive refluxutsatt slemhinna

UPM utvärderades och användes för att studera vävnadsresistans och jontransporter i epitel från matstrupen och jejunum, samt i Caco-2-celler. RAS-komponenters genaktivitet, proteinuttryck och proteinlokaliseringen studerades med hjälp av RT-PCR, western blot, ELISA och immunohistokemi i både frisk och refluxutsatt matstrupsslemhinna. Effekterna av

angiotensin II typ 2 receptorn (AT2R) och angiotensin IV (AngIV) undersöktes med UPM i Ussingkammarförsök.

Resultaten visade att Ussingpulsmetoden var en enkel och användbar metod för att studera epitelets permeabilitet. De elektriska UPM-parametrarna visade att refluxutsatt matstrupsslemhinna hade en lägre vävnadsresistans och förhöjda jonströmmar, vilket i sin tur visar på förändringar i epitelets barriäregenskaper. Resultaten demonstrerade även att ett omfattande lokalt RAS existerade och var aktivt i den humana matstrupsslemhinnan. Flera av systemets komponenter hade ett förändrat uttryck i refluxutsatt slemhinna, vilket tyder på att systemet är influerat av eller involverat i GERD. Det förhöjda AT2R-uttrycket i refluxslemhinnan skulle kunna innebära en förhöjning av anti-inflammatoriska processer och fungera vävnadsskyddande. AT2R och AngIV påverkade epitelets jonströmmar, men vilka jonkanaler som påverkades återstår att utreda, men skulle förslagsvis kunna involvera jontransporter som inverkar på den intracellulära syraregleringen. Den funktionella betydelsen av refluxslemhinnans förändrade AT2R-nivå och aktivitet bör således utredas vidare. Sammanfattningsvis visar avhandlingen att ett omfattande renin angiotensinsystem är på plats och verksamt i human matstrupsslemhinna och att systemet är förändrat i relation till refluxsjukdom. Det är möjligt att stimulering eller inhibering av någon RAS beståndsdel, till exempel AT2R, skulle kunna stärka matstrupsslemhinnan att stå emot reflux. Renin angiotensinsystemet kan möjligtvis vara ett potentiellt mål för utvecklingen av nya och spännande behandlingar mot halsbränna.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACE	angiotensin-converting enzyme
ACE2	angiotensin-converting enzyme 2
AGT	angiotensinogen
AngI	angiotensin I
Ang1-7	angiotensin 1-7
Ang1-9	angiotensin 1-9
AngII	angiotensin II
AngIII	angiotensin III
AngIV	angiotensin IV (IRAP, OTase)
AP-A	aminopeptidase A
AP-B	aminopeptidase B
AP-M	aminopeptidase M
AT1R	angiotensin II, type 1 receptor
AT2R	angiotensin II, type 2 receptor
AT4R	angiotensin IV receptor
BCL	basal cell layer
BE	Barrett's esophagus
CatA	cathepsin A
CatD	cathepsin D
CatG	cathepsin G
CC	current clamping
cDNA	complementary DNA
C_{ep}	epithelial electrical capacitance
CMA	mast cells chymase
DAP	dipeptidyl aminopeptidases
DCA	deoxycholic acid
DIS	dilated intercellular spaces
EAC	esophageal adenocarcinoma
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ERD	erosive reflux disease
ERD n.e.	ERD normal epithelium
ERD m.b.	ERD mucosal break
FD4	fluorescein isothiocyanate-dextran 4000-Mw
FH	functional heartburn
FSS	fluorescein sodium salt 376-Mw
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GEJ	gastroesophageal junction
GERD	gastroesophageal reflux disease
GI	gastrointestinal tract
H_2RA	histamine H_2-receptor antagonists
IBD	inflammatory bowel disease
IBS	irritable bowels syndrome
I_{ep}	epithelial electrical current
IHC	immunohistochemistry
I_{sc}	short circuit current
LES	lower esophageal sphincter
MasR	mas oncogene receptor
MC	mast cells
*m*RNA	messenger RNA
Mw	molecular weight
NEP	neprilysin
NERD	nonerosive reflux disease
P_{app}	apparent permeability coefficient
PCP	prolyl carboxypeptidase
PD	potential difference
PGE_2	prostaglandin E2
PL	papillae length
PO	prolyl oligopeptidase
PPIs	proton pump inhibitors
RAS	renin angiotensin system
R_{ep}	epithelial electrical resistance
RPR	renin-prorenin receptor
R_{sub}	subepithelial resistance
RT-PCR	reverse transcriptase polymerase chain reaction
R_{trans}	transmural resistance
Scc	short circuit current technique
TLESR	transient lower esophageal sphincter relaxation
TO	thimet oligopeptidase
UES	upper esophageal sphincter
UPM	Ussing pulse method
WB	western blot

I. INTRODUCTION

This thesis explores the renin angiotensin system (RAS) in the human esophageal mucosa and its potential role in influencing important epithelial features. RAS is an endocrine system that primarily is known for its role in regulating body fluid balance and blood circulation. The peptide Angiotensin II (AngII) is regarded as the system's main effector, responsible for exerting "the classical effects". However, the system contains several angiotensins with biological activity that are formed and broken down by various enzymes. Moreover, expression of RAS has been discovered locally in different tissues, where it can influence various processes.

The system, has for example, been detected throughout the gastrointestinal tract (GI), and it has been suggested to participate in regulation of mucosal absorption, secretion, blood flow, motor activity and inflammatory signalling. Components of RAS were recently found in the human esophagus, where it was shown to modulate muscle contractions and epithelial transport processes, which in turn could influence the mucosal barrier properties. The latter is of great interest in relation to gastroesophageal reflux disease (GERD), when reflux (backflow into the esophagus) of gastric contents causes troublesome symptoms such as heartburn and chest pain.

GERD symptoms are very common and affect the daily life of many individuals. The pathophysiology behind GERD is complex, involving not only the reflux as such, but also the mucosal ability to withstand the refluxate, as well as the central modulation of sensory information. Despite that proton pump inhibitors (PPIs), reducing the gastric acid production, improve the quality of life for many patients, there are still individuals not benefiting from this therapeutic principle and for which no good alternatives exist.

The present thesis project was undertaken in an attempt to connect these unmet clinical needs regarding GERD, with the bioscientific possibilities of the presence of RAS in the esophageal mucosa. The scientific background to the project and the results from new research are summarised and discussed below.

II. THE ESOPHAGUS

Gross anatomy and function

The esophagus constitutes the first part of the gastrointestinal tract and is a muscular tube with the main function of transporting fluids and food boluses from the pharynx into the stomach. The organ is approximately 25 cm long and extends through the thoracic cavity and enters the abdomen via the diaphragm. Alternating contractions and relaxations of the muscles in the esophageal wall create peristaltic waves transporting the food onward. The peristalsis is induced upon swallowing, which also results in relaxation of the upper esophageal sphincter (UES). When the bolus reaches the distal part of the esophagus the lower esophageal sphincter (LES) relaxes (8). LES also plays an important role in preventing the backflow of gastric content from the stomach into the esophagus. The primary peristaltic wave can be accompanied by secondary contractile waves until the passage is sustained. Both the peripheral and the local gastrointestinal enteric nervous system regulate peristalsis and sphincter activity. The innervation of the esophagus is primarily through the vagus nerve. The proximal part of the esophagus is composed of striated muscles that are regulated by somatic nerve fibres arising from the nucleus ambiguous in the brainstem. The mid part of the esophagus is composed of both striated and smooth muscles, while the distal part is composed of only smooth muscles that are controlled by sympathetic spinal nerves and by parasympathetic innervation arising from the dorsal motor nucleus of the vagus (8).

Tissue structure

The esophagus is subdivided into various tissue layers: *mucosa, submucosa, muscularis externa* and *adventitia*. The outermost layer is the *adventitia* that consists of connective tissue that covers and attaches the organ. Next to the adventitia is the *muscularis externa*, which is composed of the *longitudinal and circular muscles layers* that are separated by a ganglion of nerve cells, the *myenteric plexus*. On the inside of the muscular layers is the *submucosa*, being composed of connective tissue, blood vessels, lymph vessels, mucus secreting glands and a submucosal nerve plexus (8).

The mucosa is the innermost layer and faces the lumen. The mucosa is divided into *muscularis mucosae*, *lamina propria* and the *stratified epithelium* (Figure 1). The muscularis mucosae decrease the tension and retail movements to the mucosal surface. The lamina

propria extends as papillae in the epithelium and contributes mechanical support with its connective tissue and nourishment with its blood vessels. The layer also contains various inflammatory cells, lymphatics and nerve fibres (9).

The human esophageal epithelium is squamous and non-keratinized, and can be divided into three parts: the germinative *stratum basale* (basal cell layer), the metabolic active *stratum spinosum* (intermediate or prickle cell layer) and the *stratum superficiale* (corneum). The epithelium is constantly renewed, and as the cylindrical cells in the basal cell layer are disconnected from the basal lamina and start to migrate towards lumen they subsequently become mature and more flattened. As the cells move from the basal layers they get further away from the blood supply, i.e. oxygen and nourishment, and finally degeneration and extrusion of dead cells occur at the epithelial surface (9, 10).

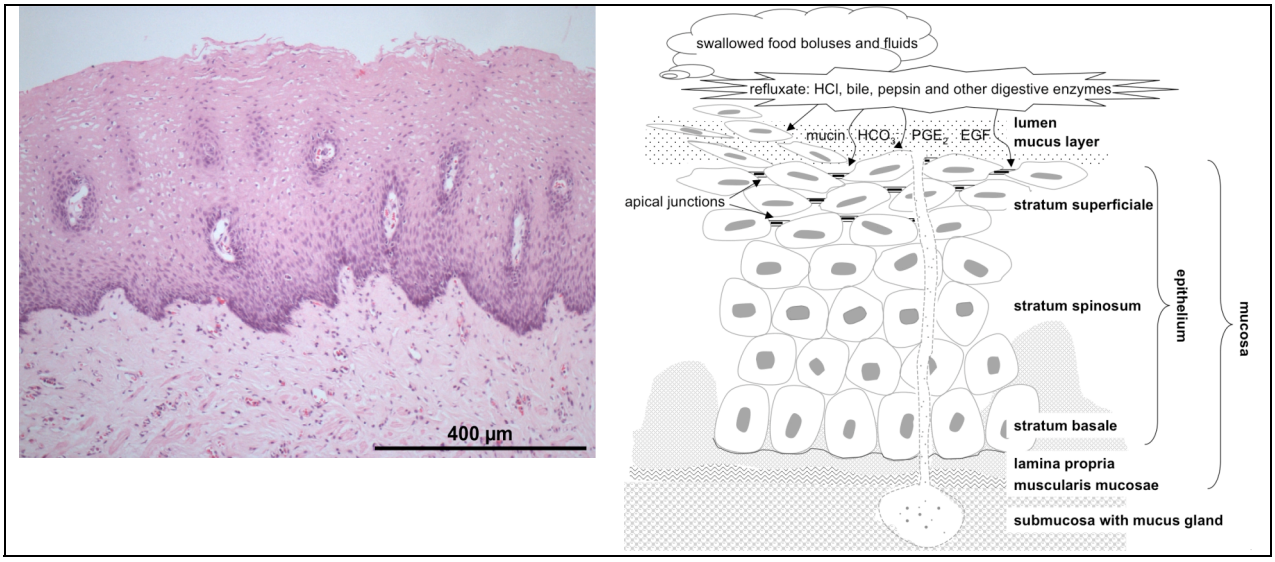

Figure 1. The esophageal mucosa
The left panel shows the esophageal epithelium as seen in light microscopy and the right panel is a schematic representation of components contributing to the mucosal barrier.

The mucosal barrier

The esophagus is regularly subjected to mechanic, thermal and chemical stimuli from ingested foods and fluids, as well as to backflow of gastric contents. The mucosa is protected against potential noxious actions by several features collectively called *the mucosal barrier*, involving the tissue structure, muscle activity, anatomical factors, neural sensory and reflexes, blood supply and a mucus layer. The epithelium itself comprises a major part of the mucosal protection, but pre- and sub-epithelial factors also contribute.

Pre-epithelial barrier

The lower esophageal sphincter together with the diaphragm creates a pressure barrier between the esophagus and stomach that plays an important role for minimizing the reflux (backflow) of gastric contents (11), while peristalsis-induced luminal clearance is crucial for limiting the duration of contact between noxious substances and the mucosa. A boundary is also created by the un-stirred water layer that resides in the lumen in close connection to the epithelial cells. The layer originates from esophageal submucosal and salivary glands, and the secretion influences moistening, digestion and the mucosal clearance. The submucosal glands are located in the connective tissue, with ducts protruding through the squamous epithelium and distributing the secretion onto the mucosal surface (10). The secretion contains water, mucins, bicarbonate, epidermal growth factors (EGF) and prostaglandins (i.e. PGE_2) (12). The bicarbonate neutralizes acid in the gastric refluxate and EGF protects and restitutes tissue integrity (13). The salivary secretion can increase at intraluminal acidification, probably through mediation of pH-sensitive chemoreceptors in the mucosa (12, 13). However, the esophageal bicarbonate-mucus layer is not as extensive as the gastric or duodenal layers and the esophageal epithelial cells *per se* do not secrete any bicarbonate (14).

Epithelial barrier

The stratified esophageal epithelium constitutes an effective barrier, where transepithelial transport can occur transcellularly (through cells) or paracellularly (between cells) (Figure 2). The hydrophobic epithelial cell membranes are selectively permeable and normally resistant to undesirable substances such as acidic gastric contents (14). Although small water-soluble molecules and electrolytes may pass paracellularly, the passage is markedly restrained. The paracellular permeability is dependent on apical structures between the cells that are formed by the tight junctions, adherens junctions and desmosomes, which normally create a rather tight barrier in the esophagus (15). The adherens junctional protein E-cadherin has, for example, been shown to be important for tight junction assembly and hence for the establishment of junctional resistance (1).

The paracellular passage is charge and size selective and sensitive to various factors such as digestive contents, cytokines, microorganisms and drugs (16). Refluxed gastric acid, i.e. the hydrogen ions, can diffuse into the intercellular spaces and the cells, but the acid is normally buffered by bicarbonate, phosphates and proteins or is taken care of by various membranous

ion transporters such as the basolateral sodium dependent chloride-bicarbonate exchanger and the basolateral sodium-hydrogen ion exchanger (14). Bicarbonate is either produced intracellularly by the enzymatic activity of carbonic anhydrases or supplied by the blood.

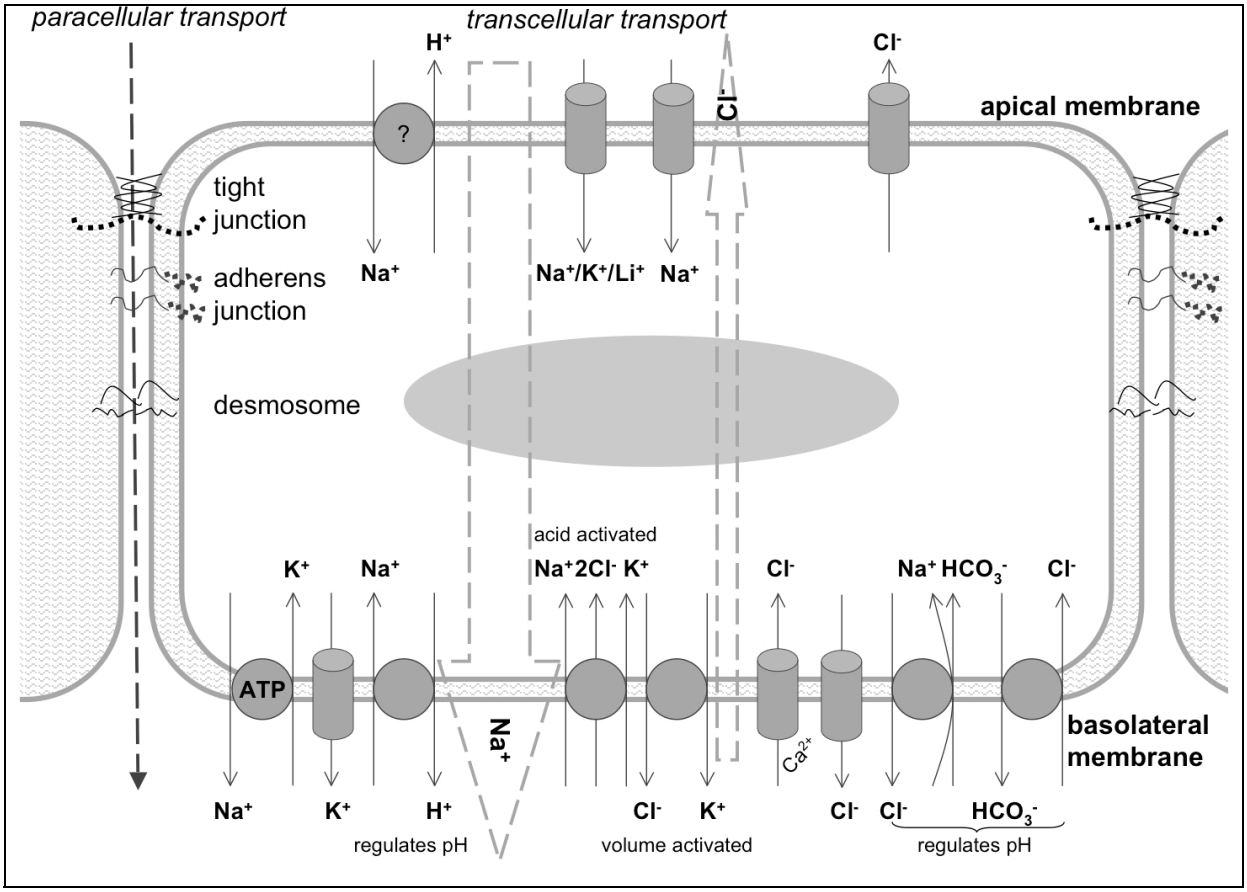

Figure 2. Transcellular and paracellular transport
Esophageal transepithelial transport processes occurring both transcellularly and paracellularly, although the latter is markedly restricted, (17-19).

Sub-epithelial barrier

The major part of the sub-epithelial defence is constituted by the basement membrane and the blood perfusion that delivers various substances such as energy substrates, oxygen and bicarbonate and removes carbon dioxide and hydrogen ions. Interestingly, the rate of the blood flow can be increased upon acid exposure and injury of the tissue (14). This is important to prevent further damage to the underlying tissue layers and also to allow for infiltration of phagocytes that can remove injured cells (20).

Causes of barrier dysfunction

Impaired integrity can occur when some of the protective mechanisms are lost or the amount and frequencies of reflux episodes are abnormal. Anatomical reasons for loss of barrier function can be a dysfunctional diaphragm, defective basal LES pressure and/or transient lower esophageal sphincter relaxations (TLESR). Sometimes a hiatus hernia is present where the stomach protrudes into the thorax (Figure 3). Hiatus hernia can be induced by a dysfunctional diaphragm (11) or by prolonged longitudinal muscle contractions that shorten the esophagus and lead to relaxation of the LES (8). The flap valve created by the angle between the esophagus and the upper part of the stomach, the so-called angle of His (Figure 3), is also of great importance since the function of the valve diminishes upon widening of the angle (11). Interestingly, inflammation as a consequence of acidic reflux has been suggested to lower the LES pressure, increase the longitudinal muscle contractions and impair the peristalsis (14, 21).

A weakened mucosal barrier can also be due to an insufficient mucus layer or to low content of EGF that will increase the risk of epithelial damage. It has been shown that the EGF amount is diminished at acid exposure or in inflamed mucosa (13). Other reasons can be that the barrier properties of the epithelium are weakened, making it easier for noxious substances to diffuse into the tissue (2, 14).

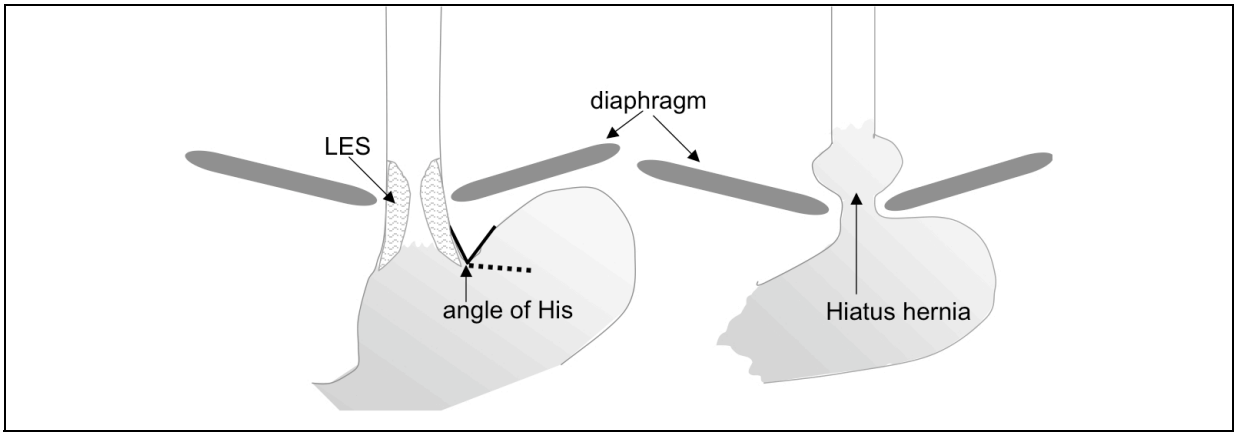

Figure 3. The gastroesophageal junction
A schematic representation of the lower esophageal sphincter (LES), diaphragm and angle of His (dotted line depict a wider angle). The right panel shows a Hiatus hernia where the stomach protrudes into the thorax.

III. GASTROESOPHAGEAL REFLUX DISEASE – GERD

An imbalance between the noxious stimuli and the mucosal barrier protection can lead to gastroesophageal reflux disease (GERD). GERD is defined in the Montreal definition as "a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications" (22). The pathophysiology behind GERD is complex, involving the degree of reflux, the state of the mucosal barrier and the modulation of sensory information in the central nervous system. 'Physiological' gastroesophageal reflux episodes occur regularly in all individuals, especially after a meal when the LES pressure is low (11) and the stomach is satiated or during sleep when the regular luminal acid clearance is reduced due to the absence of gravity and salivary secretions (12), although the LES pressure is at its highest at night (11).

In general, the mucosal barrier should be able to withstand these 'physiological' reflux episodes, but during conditions with impaired barrier properties the epithelium could be challenged and troublesome symptoms may occur. Excessive reflux is another causative factor behind GERD. Various pathological conditions, particularly valvular dysfunction of the LES, can increase the reflux volume and frequency of the reflux episodes. Obesity is an example of a common condition that is strongly associated with GERD. The abdominal fat accumulation increases the intra-abdominal pressure and thereby also the risk of backflow of gastric contents into the esophagus. Regardless of the reason, an excessive amount of refluxate can overcome the esophageal epithelial barrier properties and cause mucosal stress. The refluxate contains various substances that have the potential to damage tissue and elicit symptoms. These substances include gas, bile salts, pepsin and other digestive enzymes as well as gastric acid of which the latter is regarded as the most noxious (14).

GERD symptoms

Studies indicate that the prevalence of weekly symptoms of GERD is around 20% (23) in the Western world and that the disease is more common in Caucasian and Hispanic than in Asian and African populations (24, 25). The most common symptoms of GERD are heartburn, regurgitation, trouble swallowing and hoarseness. Another indication of reflux can be immense chest pain, sometimes mimicking symptoms of coronary heart disease. This is due to sensory nerve fibres from the esophagus converging with nerves from the heart (8).

Diagnostics

The diagnosis of GERD is primarily based on symptoms combined with endoscopic evaluation of the mucosal appearance and usually also on the response to acid suppression by proton pump inhibitors (PPIs). To identify acidic reflux events a 24-48 hour pH-metry can be performed immediately above the LES, measuring time with acidic exposure. The exposure episodes may or may not be related to symptoms. More sophisticated diagnostic methods can be used in unclear cases. The direction, transit time and nature of the refluxed content, e.g. liquid, gas, acidity and alkalinity, can be assessed using an impedance-monitoring catheter. Refluxed bile can be assessed by the Bilitec® method, where an intra-esophageal catheter monitors the light absorption of luminal bile. The intraluminal pressure and hence the LES pressure and peristalsis can be measured by manometry, while radiology is useful for investigating the appearance of a large hiatus hernia.

Clinical subdivision

GERD can be differentiated into subgroups based on symptom perception, endoscopic evaluation and often some functional examination. The Montreal classification of GERD is patient and symptom centered, where patients with typical GERD symptoms are classified into *esophageal symptoms* (with or without esophageal injury) and patients with associated symptoms (e.g. cough, laryngitis and asthma) into *extra-esophageal* symptoms (22). Today, however, both the symptoms and endoscopic appearance are often used to classify GERD (Figure 4 and 5). Even though the mucosa is endoscopically normal, changes may be observed microscopically, such as dilated intercellular spaces (DIS), enlarged papillae length (PL) and thicker basal cell layer (BCL) (26, 27).

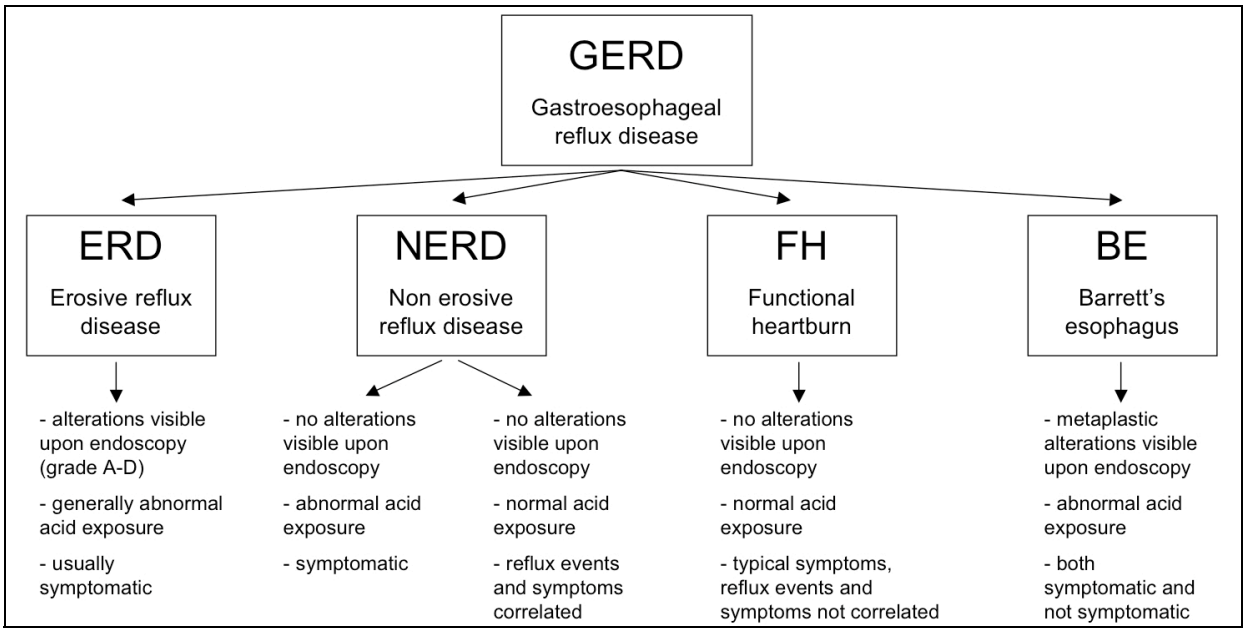

Figure 4. GERD subgroups
The different GERD subgroups and their common characteristics.

Erosive reflux disease – ERD

As a result of reflux, the esophageal mucosa can be inflamed and contain mucosal breaks (Figure 5). The condition is then referred to as erosive reflux disease (ERD). The diagnosis is made endoscopically and the mucosal alterations graded upon appearance according to the Los Angeles classification for reflux esophagitis, where the definition of a mucosal break is "an area of slough or erythema with a discrete line of demarcation from the adjacent, more normal looking mucosa" (28). Symptoms and acidic reflux events are almost always closely associated in individuals with ERD (29) and hence symptoms are often improved following acid suppressive therapy with PPIs.

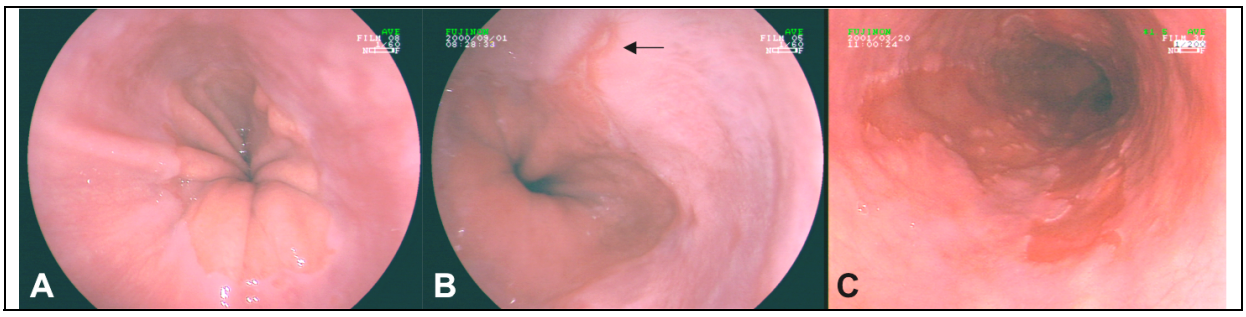

Figure 5. Endoscopic esophageal mucosal appearance
The junction between the squamous mucosa and the glandular columnar mucosa in a healthy individual (A), a patient with erosive reflux disease (B) and a patient with Barrett's esophagus (C). The arrow in B shows erosion. (The pictures are published in agreement with the owner Anders Edebo)

Nonerosive reflux disease – NERD

Individuals can perceive reflux symptoms (sometimes very severe) even though they do not have any mucosal alterations upon endoscopic examination. The condition is then termed nonerosive reflux disease (NERD) and may include as many as 70% of GERD patients (30). The acid exposure time is abnormal in some individuals, while it is normal in others. According to the Rome III criteria, NERD should only be diagnosed if the symptoms are associated with gastroesophageal reflux and the mucosa appears normal endoscopically. The frequency of successful responses to acid suppressive therapy is lower in patients with NERD than ERD (29). This could be due to weakly acidic reflux also being able to elicit symptoms that may be a consequence of mucosal hypersensitiveness or to lowered tissue resistance (2, 14).

Functional heartburn – FH

Sometimes reflux symptoms are not associated with acidic reflux events and the individuals respond poorly to acid suppressive therapy (30). That subgroup is often referred to as functional heartburn (FH). The reasons for symptoms unrelated to intra-esophageal acidity are not completely known, but may include anatomical and muscular dysfunctions, sensitization of neurons, impairment of digestion and reflux of non-acidic substances (29, 30).

Barrett's esophagus – BE

If the reflux is severe and chronic the stratified squamous esophageal epithelium can be replaced and converted (metaplasia) into columnar lined epithelium of fundic, cardiac or specialized intestinal cell types. This condition is termed Barrett's esophagus and constitutes around 2-15% of the GERD patients (31, 32). The condition is considered to be an adaptation of the tissue to acid exposure. However, Barrett's esophagus is associated with a diminutive but significant increased risk of 0.1-0.5% per year of developing into esophageal adenocarcinoma (EAC), which corresponds to a 10-125 fold higher lifetime risk than in the general population (32, 33).

Treatment

Reflux symptoms can be improved by omitting certain foods and liquids such as fat, spices,

coffee, alcohol and tobacco, as well as by weight loss in obese individuals. PPIs are generally the first treatment of choice for GERD. The PPIs reduce gastric acid production by inhibiting the H^+/K^+ ATPase pump (proton pump) of the hydrochloric acid producing parietal cells of the stomach. Other acid suppressive therapies include histamine receptor antagonists (H_2RA) that decrease histamine's stimulating effect on the parietal cells and hence inhibit gastric acid secretion. It is also possible to reponate a hiatus hernia and/or to perform a fundoplication, which is a surgical procedure where the upper part of the stomach is wrapped around the lower part of the esophagus, resulting in strengthening of the LES function and in re-creation of the angle of His. Barrett's esophagus can, in cases with signs of mucosal malignant transformation, be treated with esophagectomy, endoscopic resection and/or ablation techniques.

The problem

The prevalence of GERD is high and seems to increase in the Western population. Hence, the disease affects the daily life of many people and is an important background factor to the growing incidence of EAC (23). The introduction of PPIs some thirty years ago improved the quality of life for many GERD patients. Initially, PPI treatment was considered to be *the solution* for all patients with GERD, but it is now evident that as many as 20-50% of patients with reflux symptoms are 'PPI resistant' (34). For these individuals no good treatment alternatives exist, especially not for NERD patients without mucosal breaks or in FH where the symptoms are not associated with acidic reflux. Moreover, in cases with confirmed acidic reflux, it is not completely understood why the acid causes heartburn and pain.

So, there are still a significant number of individuals for which no potent treatment exists. To resolve this therapeutic gap more research is needed to elucidate; the underlying mechanisms of GERD; how symptoms are elicited; and why such large differences exist between various individuals. It is intuitive that the refluxate as such is important for the occurrence of symptoms and/or mucosal disease. Much research has been devoted to the composition of the refluxate (acidic, weakly acidic, non-acidic, bile containing, etc.) and to the basis for its occurrence (hiatus hernias and TLESR, etc.). This thesis focuses more on the esophageal mucosal ability to withstand refluxate and more specifically on the role of the mucosal barrier properties.

The mucosal barrier and GERD

The relationship between the barrier properties and the reflux load will determine whether symptoms or even mucosal injury will develop. An impaired mucosal barrier may allow influx of various refluxed factors (acidic as well as non-acidic), activating sensory nerves that reside in the mucosa and underlying tissue layers. A seemingly macroscopically normal mucosa may also provoke symptoms (20), particularly in the presence of sensitized spinal sensory neurons, thus with lowered activation thresholds (29). Interestingly, it has been reported that the epithelium of NERD patients appears to have dilated intercellular spaces due to alterations in the apical junction structures. This in turn increases the permeation of noxious substances with the potential of eliciting reflux symptoms and influencing proton-gated ion channels, which might lead to sensitization and neurogenic inflammation (14, 29). If considerable amounts of acid reach the intercellular spaces, the normal buffering and neutralization capacity can be disturbed due to the activation of basolateral acid-absorbing transporters (14). Hence, the regulation of the transepithelial permeability represents an area of considerable interest, both with regard to the understanding of GERD pathophysiology and also as a potential therapeutic target. Recent research from our laboratory has revealed the presence of components of the renin angiotensin system (RAS) in the human esophagus (3, 35). As RAS is a well-known and potent regulatory system, it may hypothetically influence the esophageal barrier dynamics. The present research project was started as an attempt to elucidate this possibility.

IV. THE RENIN ANGIOTENSIN SYSTEM - RAS

The renin angiotensin system is an endocrine system that regulates body fluid balance and blood circulation. The system can also influence other functions such as inflammation, cell growth, proliferation and carcinogenesis and, in addition, have a local and intracellular mode of action (7). The system contains various angiotensins of various peptide lengths that are formed and broken down by several different enzymes and where the octapeptide Angiotensin II (AngII) is regarded as the main effector peptide of the entire system (Figure 6).

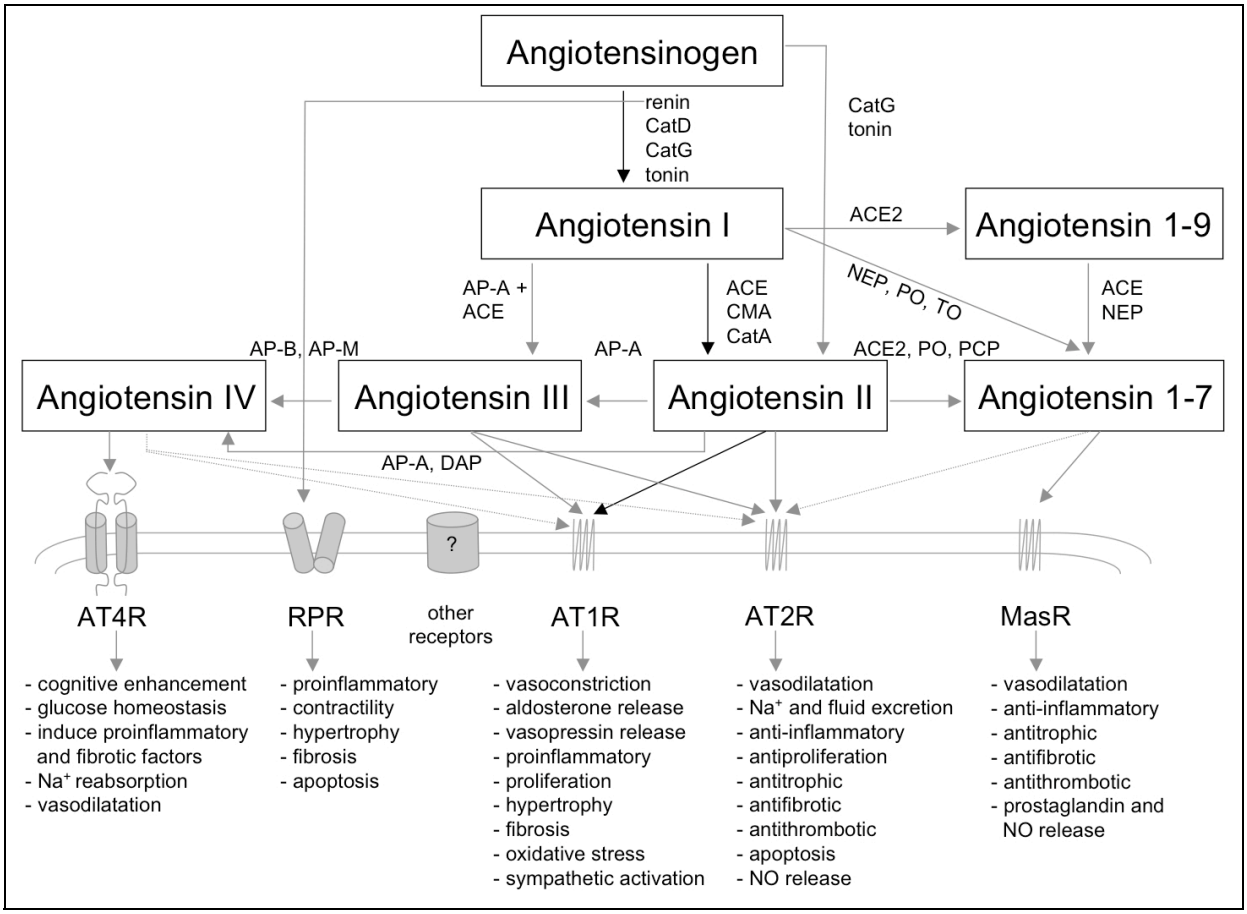

Figure 6. The renin angiotensin system
An enlarged view of the renin angiotensin system (RAS) with numerous pathways and effects. Abbreviations; ACE: angiotensin-converting enzyme, AP-A, AP-B and AP-M: aminopeptidase A, -B and -M, AT1R and AT2R: angiotensin II type 1 and type 2 receptor, AT4R: angiotensin IV receptor, CatA, CatD and CatG: Cathepsin A, -D and -G, CMA; mast cells chymase, DAP: dipeptidyl aminopeptidases, MasR: mas oncogene receptor, NEP: neprilysin, PCP: prolyl carboxypeptidase, PO: prolyl oligopeptidase (an endopeptidase), RPR: renin-prorenin receptor, TO: thimet oligopeptidase (an endopeptidase), (4, 5, 7, 36, 37).

The classical RAS – an endocrine system

Angiotensinogen (AGT), angiotensin I (AngI) and angiotensin II (AngII), together with their

formation enzymes renin and angiotensin-converting enzyme (ACE), have been regarded as the main components of the system and responsible for the main functions, i.e. regulating fluid balance and blood pressure (7). The classical RAS pathway starts by AGT from the liver being converted into AngI by the actions of the circulating enzyme renin. Renin is released from the juxtaglomerular cells in the kidney in response to low hydrostatic pressure, low sodium concentration in the afferent arteriole or increased sympathetic nerve activity. AngI is mainly considered to be a pro-hormone, but has some direct vasoconstrictor actions. AngI is converted into AngII by ACE, which is found in the vasculature. AngII activates the AngII type 1 receptor (AT1R), which has vasoconstrictive effects leading to higher blood pressure, and the receptor also promotes aldosterone secretion which in turn causes the kidneys to retain water and sodium, resulting in increased blood volume. In addition to the AT1R, AngII can bind to the AngII type 2 receptor (AT2R), which often counterbalances the actions of the AT1R.

The tissue-located RAS

A novel view of RAS has emerged from the discovery of numerous peptide-degrading enzymes in various tissues. Some of these can constitute alternative AngII-generating pathways as well as catalyzing the formation of other 'angiotensins' with biological activity. Importantly, data are accumulating to suggest that many components of this complex system can be expressed locally and thereby regulate the functional state of the tissue in a paracrine and intracrine mode of action (7). A brief review of the literature is given below:

AngII generating pathways

Local AngII generation can occur through various enzymatic processes, e.g. by renin and ACE but also through alternative pathways by chymase (CMA), tonin, Cathepsin- A, D (CatD) and G (CatG) (Figure 6).

Renin was previously considered to be purely proteolytic and involved in the degradation of AGT. Novel data have demonstrated that renin and its proenzyme, prorenin, also exert independent proinflammatory and profibrotic signalling via renin-prorenin receptors (RPR). The receptors are abundant in the heart, the brain and the placenta, but have also been found in the kidney and the liver. RPR seems to recruit prorenin and angiotensinogen, enhancing the

catalytic activity of receptor bound renin (7).

CatD can generate AngI from AGT. CatD and renin share an overall structural homology and topology with similar active sites. CatD seems to be released after a myocardial infarction and is likely to contribute to AngII formation, which in turn has been connected to maladaptive remodelling of the myocardium (38, 39).

Chymase (CMA) can, in addition to ACE, enzymatically generate AngII from AngI. CMA is released from secretory granules of mast cells (MC) (40). When stored in MC, chymase is enzymatically inactive and produces AngII only in pathological situations associated with MC degranulation (7). CatG is also expressed by MC (40) as well as by myelomonocytic cells (particularly the neutrophils), and can generate AngII directly from AGT (41). Although CatG has broader peptidase specificity, it is generally less potent compared with chymase but could still be responsible for a considerable part of the AngII production and influence the inflammatory processes in certain tissues and species (41, 42).

Other angiotensins

AngI or AngII can be broken down into smaller peptides: angiotensin 1-9 (Ang1-9), 1-7 (Ang1-7), 2-8 (AngIII) and 3-8 (AngIV). These peptides were initially thought to have no or little biological activity, but have now been shown to bind to specific receptors and exert various functions.

Ang1-7 binds to the Mas oncogene receptor (MasR) and is proposed to mediate vasodilatation and antithrophic effects, counterbalancing many actions of the AT1R (7). The peptide is e.g. generated by the enzymatic activity of ACE, angiotensin-converting enzyme 2 (ACE2) or neprilysin/neutral endopeptidase (NEP). ACE2 has high affinity for AngII and is an exopeptidase that is structurally similar to ACE but resistant to pharmacological ACE inhibitors.

The AngII fragments AngIII and AngIV are also biologically active (37). AngIII is generated from AngII by the enzymatic activity of aminopeptidase A (AP-A) or alternatively from AngI by the combined actions of AP-A and ACE (43). AngIV is then generated by the degradation of AngIII by the actions of aminopeptidase B (AP-B), aminopeptidase M (AP-M; also known

as aminopeptidase N and CD13) or dipeptidyl aminopeptidases (DAP). AngIII acts through the AT1R and AT2R, and AngIV through the angiotensin IV receptor (AT4R). The AT4R is also called insulin-regulated aminopeptidase (IRAP) and oxytocinase (OTase), depending on its detection in various tissues and species (37). The best-known AT4R effects are related to the central nervous system where it influences e.g. memory retrieval, but it is also known to influence vasodilatation, sodium reabsorption, intracellular signalling and insulin-regulated glucose uptake (37, 43).

Gastrointestinal RAS

Numerous RAS components have been discovered throughout the gastrointestinal (GI) tract. AGT, renin, ACE, AT1R and AT2R have all been found in the stomach, the small intestine and the colon (5). The AngII receptors and ACE2 are greatly expressed at the luminal brush border membrane in both the colon and the small intestine (4) and expression of NEP has been shown in the human gastric mucosa (44).

Various functions of RAS in the GI tract have been proposed such as absorption and secretion of fluid and electrolytes, as well as glucose and amino acid transportation. RAS also seems to influence GI blood flow, motility and inflammation, and could be involved in the pathological processes of esophageal motility disorders, stomach ulcerations, irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and carcinogenesis (4, 5).

Esophageal RAS – a link to GERD?

Research in our laboratory has shown that AGT, renin, ACE, AT1R and AT2R are present in the human esophageal musculature, where they influence contractions and the high-pressure zone at the gastroesophageal junction (GEJ) (35). The RAS components ACE, AT1R and AT2R were observed in the vasculature and the two AngII receptors also in the epithelium, where they modulated the epithelial ion transport and electrical resistance (3). Because these functional modalities have been proposed to reflect mucosal barrier properties, it was speculated that the local RAS in the esophageal mucosa might be involved in the epithelial barrier impairment observed in GERD. Hence, this hypothetical involvement of RAS in the pathophysiology of GERD became the starting point for the research project of this thesis.

V. AIMS OF THE THESIS

There is a lack of knowledge about RAS in the esophagus, especially regarding alternative AngII generating pathways and the presence and functions of additional angiotensins, e.g. Ang1-7, AngIII and AngIV. The general aim of this thesis was, therefore, to confirm the presence and gain new knowledge about the distribution and function of RAS components in the healthy esophageal mucosa and to look for aberrations associated with GERD (i.e. ERD). The specific aims were:

1. To confirm and further map the presence of RAS in the distal human esophageal mucosa of healthy subjects
2. To investigate the distribution of RAS components in the mucosae from patients diagnosed with ERD
3. To establish an *in vitro* method for assessment of the epithelial electrical resistance in human esophageal mucosa
4. To investigate *in vitro* mucosal effects of some RAS components of particular interest

VI. METHODOLOGICAL CONSIDERATIONS

Subjects, tissue and cell culture

All studies included in the thesis were performed in accordance with the ethical principles regarding human experimentations stated in the Declaration of Helsinki. All subjects participated voluntarily and were informed verbally and in writing before they signed a consent form. The Ethics Committee at the University of Gothenburg and the Regional Ethical Review Board in Gothenburg approved the studies.

Tissue handling

Tissue collection, handling and preparation were performed in a standardized manner, e.g. refrigeration, transportation to the laboratory, buffer preparations and tissue stripping. On collection, the tissue specimens were immediately placed in fixation medium, Krebs solution or liquid nitrogen depending on the subsequent analysis method.

Esophageal tissue

In paper I, esophageal mucosa was obtained from patients (n=14) undergoing esophagectomy for malignancy at the esophagogastric junction. The esophageal specimen was considered to be normal for two reasons: it was collected as far as possible from the pathological area and normal histological appearance was confirmed. However, it cannot be entirely precluded that the tissue, and hence the results, could be influenced by the surgical procedure, the patient's disease history, preoperative medication and radiation.

Endoscopic esophageal biopsies from healthy volunteers and reflux patients were used in papers I (healthy n=7 and ERD n=8), II (healthy n=34 and ERD n=28) and III (healthy n=19 and ERD n=14). The normal biopsies were collected at the distal esophagus, within 1 cm from the GEJ, in the 3´o clock circumferential position, where mucosal breaks and histo-pathological changes are most prevalent (45). The ERD biopsies were collected in and just outside the mucosal break area, at the same height. The mucosae of all ERD patients were graded according to the Los Angeles classification system for reflux esophagitis. To avoid disturbances in the analysis, patients using anti-hypertensives directed towards any RAS

component were not included. For two weeks prior to endoscopy, the enrolled patients had to abstain from anti-acid medication to resume a symptomatic ERD picture.

One advantage of taking endoscopic biopsies is that human mucosal samples become more available both from healthy individuals and from certain patient cohorts. The biopsies were taken from the same tissue location (in contrast to the esophagectomy specimens) with the same equipment and for the most part also by the same endoscopist, making the results more comparative. One factor that could potentially influence the present results was that the mean age was higher in the individuals with reflux compared with the healthy controls.

Jejunal tissue

The jejunal tissue used in paper I was obtained from patients (n=12) undergoing Roux-en-Y Gastric Bypass for morbid obesity. It is possible that the specimens were influenced by the surgical procedure or that they diverge in some aspect from specimens obtained from individuals with normal weight.

Cell culture

In paper I, Caco-2 cells (passage number 45) were cultured in a medium containing vitamins, amino acids and glucose (Dulbecco's Modified Eagle's medium supplemented with fetal bovine serum, non-essential amino acids and Penicillin-Streptomycin). The cells were first cultured in flasks before being seeded onto culture inserts and allowed to grow until confluent (cells covering the insert), after which the medium was changed (FBS-free and to the lower compartment insulin, transferrin and selenium) to establish cell polarity and structural differentiation (46, 47). After that, the cells were cultivated for approximately ten days before performing Ussing chamber experiments.

Cell cultures offer the advantage of performing numerous experiments under highly uniform conditions without the large variability impacts seen in human specimen settings. Caco-2 cells are a human epithelial colorectal adenocarcinoma cell line, but resemble small intestinal enterocytes when cultured under specific conditions. However, precautions have to be taken regarding e.g. cell clone type, passage time, medium, supplements, cultivation time and cell growth supporting material. Mechanisms occurring in Caco-2 cells are, of course, not

equivalent to mechanisms occurring in the human small intestine, but can give valuable insights into various regulatory steps. In paper I, the cell culture experiments were primarily used to validate the Ussing method and not to elucidate physiological mechanisms.

Histology

Experienced histologists blinded to the experimental protocol examined samples of the tissue specimens to confirm normal appearance (I, II and III) or signs of reflux disease (II). The tissue specimens were fixed, embedded in paraffin, sections mounted on glass slides and examined with white light microscopy. After Ussing chamber experiments, the epithelial thickness, edema, cell damage and amount of debris were investigated as well as changes in the jejunal epithelial surface area (I). To look for reflux related morphological changes, the DIS, PL and BCL thickness were investigated in paper II.

Reverse transcriptase polymerase chain reaction – RT-PCR

Reverse transcriptase polymerase chain reaction was used in paper II to investigate gene transcripts (*m*RNA) belonging to various RAS components. This method is based on that DNA is transcribed into messenger RNA (*m*RNA) that in turn is translated into proteins. The *m*RNA levels indicate the activity of a gene and hence the probability of production of a specific protein. However, *m*RNA levels do not directly correspond to functional protein levels since *m*RNA: can be rapidly degraded; can be spliced into various variants and; the protein can be post-translationally modified.

The RT-PCR process was initiated with the conversion of *m*RNA into complementary DNA (cDNA) using the enzyme reverse transcriptase and specific starter primers. Electrophoresis (1.5% agarose gel containing Tris acetate/EDTA and ethidium bromide) of the products was performed and compared to molecular weight standard. The cDNA was then replicated during thermal cycling, where strands were separated so selective primers could bind and amplify the strands with the aid of DNA polymerase. The new strands were in turn used as templates and hence the cDNA was exponentially amplified. The samples' cDNA amounts were compared to known standard curves and consequently the initial *m*RNA levels could be determined. The primer sequences and PCR product sizes of the present analyze are shown in paper II, Table I.

Western blot – WB

The quantitative amounts of the specific proteins were estimated by the antibody-based method western blot (WB; immunoblotting), which was used in papers II and III. Tissue specimens were first solubilized by sonication with ultrasound energy in buffer containing proteinase blockers. Cell debris was removed by centrifugation and the supernatant's protein content was analyzed using the Bradford method (48). Diluted samples were loaded onto a porous gel and the proteins were separated according to size by gel electrophoresis in an electrical field. The proteins on the gel were transferred (blotted) to a membrane to make the proteins detectible to the antibodies. The membrane was then incubated with the specific primary antibody and also with a secondary antibody to enhance the signal. The secondary antibody was conjugated with alkaline phosphatase that, upon reaction with the reagent CDP-Star, creates a chemoluminescent light (CDP-Star allow for development for up to 24 hours) that in turn can be captured by a camera. Consequently, the specific protein of interest was detected and the intensity of the protein bands was analyzed using a software program.

WB is a semi-quantitative analysis, meaning that the protein levels will not be given in absolute values but in relation to the intensity of the other analyzed samples. Hence, the specific RAS-peptides in the different groups could be graded according to each other. WB is based on each antibody's specificity, which can be verified by analyzing for example a positive sample known to contain the protein of interest (e.g. kidney and liver in papers II and III) or by pre-incubating the antibody with a control antigen impeding the antibody from binding to the target protein. It is also important to compare the detected protein with a molecular weight standard so the correct size of the target protein can be confirmed. Another aspect to consider is that equal amounts of each sample have to be loaded onto the gel and that proteins are completely transferred to the membrane. In papers II and III this was controlled for by normalizing the target protein amount to the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a so-called house keeping protein required for basic cellular functions with a constant expression despite any pathophysiological condition. Also the intra assay coefficient of variation for the WB analysis was calculated in the laboratory and got a value of 4.6%, which was established by analyzing the same sample in 10 different wells on the gel.

Enzyme-linked immunosorbent assay – ELISA

Enzyme-linked immunosorbent assay (ELISA) is also an antibody-based method and was used in paper II to detect the levels of angiotensin II. The tissue specimens were prepared in the same way as for WB, and the analysis was performed using a commercial kit. The samples were put into separate wells on a plate where the potential AngII could bind to immobilized antibodies. The plate was incubated with another antibody labelled with acetylcholinesterase that enzymatically reacts with a chromogen, creating a yellow compound. The compound was colorimetrically detected, where the intensity of the colour corresponds to the amount of AngII in the samples. The absolute amount in each sample was determined by comparison with a standard curve with known AngII concentrations.

It is a great advantage that the absolute AngII content can be determined, since this means that a sample from one analysis can be compared with a sample from another. However, the kits have been developed for use in culture media and blood samples, and not for solid tissue. In addition, the used AngII kit also had cross reactivity for AngIII and AngIV (to a lesser extend than for AngII). Consequently, it cannot be completely ruled out that some of the detected AngII in paper II could in fact have been AngIII or IV. Precautions have to be taken also when it comes to the variation in and between the ELISA kits. According to the manufacturer the intra and inter assay coefficient of variations are between 2 and 14.5%, depending on amount peptide quantified. An intra assay coefficient has also been tested in the laboratory and got a value of 3.9%, which was established by analyzing double samples of the standard curve.

Immunohistochemistry – IHC

Immunohistochemistry was used in papers II and III to find the intraepithelial location of the investigated proteins. The tissue specimens were fixed and embedded in paraffin before sections were cut and put on glass slides. The paraffin was removed from the tissue and the specific antigen was revealed by boiling in citrate buffer, after which incubation with the primary antibody was performed. To enhance detection signal, a secondary antibody conjugated with an enzyme was used that in turn reacted with a chromogen, resulting in a brown colour that was detected with white light microscopy.

As with all antibody-based methods the reliability of the results depends on the specificity of

the antibody. As with WB, it can be beneficial to use a positive tissue control section that is known to contain the targeted protein. In the present studies, this was only done for new antibodies that had never been used in the laboratory before. Another way to check for specificity is (as with WB) to use a pre-absorbed control antigen so that the antibody-control antigen complex cannot bind any antigen in the tissue. This was not done in the present studies because it had already been done previously in the laboratory for some antibodies and sometimes no appropriate control peptide exists. A way to preclude unspecificity of the secondary antibody is to omit the primary antibody and instead incubate the sections with buffer or normal IgG. Such negative control sections were done in both paper II and paper III. A limitation of the method is the difficulties to determine the position of the immunoreactivity precisely. To increase the resolution and contrast there are other preferable methods such as confocal or electron microscopy. However, those techniques were not employed in the present studies.

Ussing chamber experiments

Ussing chamber experiments are attractive because functionality can be assessed concomitant with structural studies. In paper I, the epithelial electrical resistance (R_{ep}) was examined by square wave current pulse analysis, also called the Ussing pulse method (UPM). In papers II and III the UPM was used to characterize endoscopic biopsies from healthy individuals and patients with reflux disease (Figure 7).

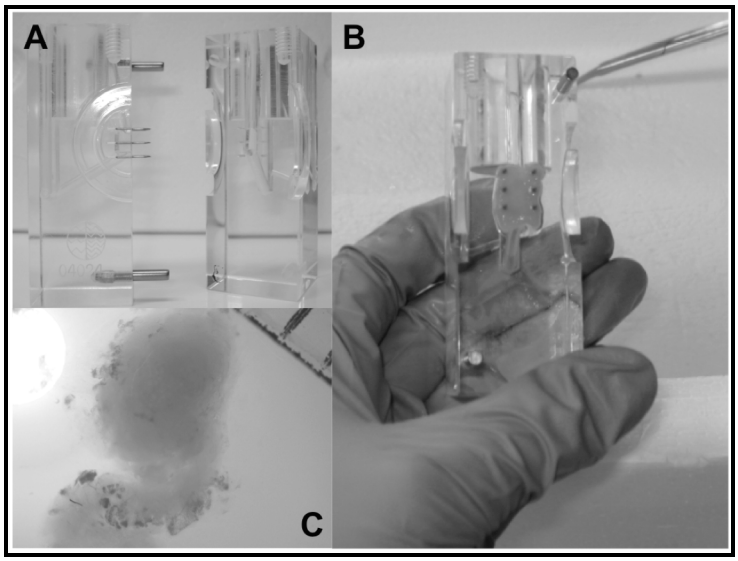

Figure 7. Ussing chamber and tissue specimens
An Ussing chamber (A) where the tissue specimen is mounted on pins (B) and clamped between two block-halves so that the luminal and "serosal" tissue side face separate reservoirs. Panel C shows a jejunal biopsy on an insert that allows direct mounting into the Ussing chamber. Part of the insert opening appears in the upper left corner and a scale bar (1 mm) in the upper right corner in C.

The Ussing chamber assesses the epithelial electrical characteristics reflecting various physiological properties. The epithelial cells actively transport ions to maintain basal cellular functions. The Ussing chamber assessed epithelial electrical current (I_{ep}) estimates the net transcellular ion transports (see Figure 2), while the electrical epithelial resistance (R_{ep}) estimates the paracellular tightness (see Figure 2). The I_{ep} and R_{ep} will cause a charge gradient (= potential difference; PD) across the mucosa. The PD in the Ussing chamber can quite easily be recorded by using a sensitive voltmeter. The transepithelial ion current can be assessed by the short circuit current technique (Scc), applying a known current until the PD becomes zero (short circuit current; I_{sc}), after which the resistance can be calculated according to Ohm's law (PD = R x I). The major disadvantage with this approach is that it is not known if the Scc-condition affects the endogenous ion transport and if the epithelium is completely short-circuited (49, 50) that in turn can give rise to false estimations of R_{ep}. Another approach is to instead first assess R_{ep} and then calculate I_{ep}, using instant PD. This principle was chosen for the present investigations. The classical way of assessing transepithelial resistance is based on Ohm's law using the PD obtained when applying a known current across the mucosa (the current clamp method). One problem with current clamping is that it measures the total 'transmural resistance' (R_{trans}) and does not discriminate the resistance of the subepithelial tissue (R_{subep}) from the epithelial resistance (R_{ep}). The UPM is based on square wave analysis, where short lasting current pulses charges the epithelial capacitors, i.e. cells get polarized in an electrical field (Figure 8). The subsequent depolarization is dependent exclusively on the *epithelial* resistance, or more correctly, on the epithelial conductance (=1/R). Furthermore, it is mainly the paracellular resistance that determines the total resistance of the tissue (51). The reason for this is because the transcellular resistance is much higher than the resistance existing between cells. As electrical conductivity assumingly reflects the intercellular tightness it also reflects the paracellular permeability. However, the latter cannot be taken for granted because the electrical conductivity relates to the transport of small-sized ions of the tissue fluids and may be unrelated to the passage capacity of larger molecules. Theoretically, R_{ep} should be a better estimate of paracellular permeability than R_{trans}. This is elucidated in further detail in paper I.

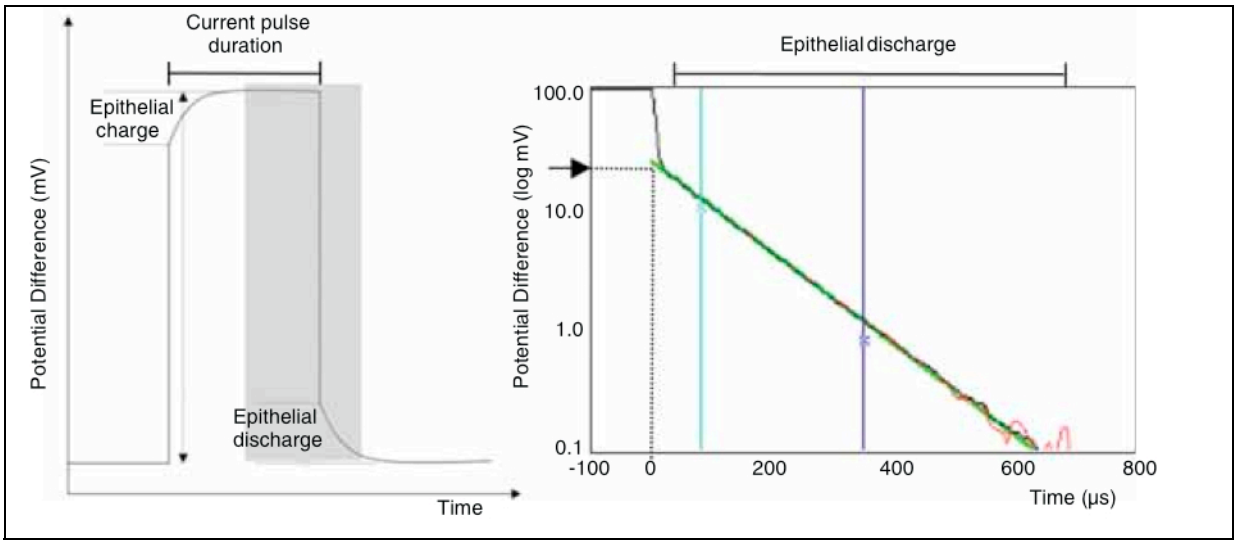

Figure 8. UPM measurement
An applied current generates a voltage response and charges the epithelial capacitor, which gradually is discharged when the current ends (left panel). The discharge rate depends on the epithelial electrical resistance (Rep). The voltage decline is shown in the grey area (left panel) and in the right panel. The Rep is assessed from the voltage response (arrow) and the amplitude of the applied current. Abbreviations; mV: millivolt, µs: microsecond.

In the present studies, only tissue specimens that possessed satisfying electrical parameters at baseline were included. In pilot experimentations, it was observed that esophageal mucosal specimens had to have a lumen negative PD >1 mV for resected tissue and >0.3 mV for endoscopic biopsies in order to be viable and reactive. Furthermore, in the present studies several Ussing chambers were run in parallel and untreated time control preparations were always included. The viability of the time control preparation was usually checked at the end of the study period by adding the sodium channel blocker amiloride (resulting in a current reduction of at least 20%).

Statistics

Differences in gene activity (II), protein expression (II and III) and dilated intercellular spaces (II) were either ordinal data or were not considered to be normally distributed. For that reason these parameters were analyzed with the non-parametric methods Kruskal-Wallis and Mann-Whitney U-test for independent variables, and Wilcoxon's signed rank test for related variables. Due to confirmed normal distribution, large groups or ratio data differences in electrical Ussing parameters (I, II and III), probe permeability (I), BCL (II) and PL (II) were analyzed parametrically using the Student's t-test for paired or unpaired values. Associations between R_{ep} and P_{app} (I) were tested by Pearson correlation. Spearman's correlation was considered for non-linear associations. Repeated measurements were taken into consideration,

although no adjustment for the risk of mass significance was performed. In paper I, a special strategy was developed, where the difference in the electrical Ussing parameters was expected to be largest at the end of the time curve. The preceding time point was statistically analyzed only if the last time point was significant, and so on. A p-value of ≤ 0.05 was considered significant. Individuals were denoted n and preparations/observations N.

VII. RESULTS AND COMMENTS

1. First aim: to confirm and further map the presence of RAS in the distal human esophageal mucosa of healthy subjects

The gene activity, protein expression and location of representative RAS components in the human esophageal mucosa were investigated by the use of RT-PCR, WB, ELISA and IHC (II and III). Endoscopic biopsies were collected from healthy volunteers at the distal esophagus, within 1 cm from the GEJ in the 3 o'clock position.

AngII formation pathways

Transcription activity and protein expression of the "classical" RAS components: AGT, renin, ACE, the AT1R and AT2R were detected in the esophageal mucosa (II), demonstrating that a local RAS was present. Interestingly, by using ELISA it was also possible to establish the presence of the main effector peptide AngII. The intraepithelial protein locations are summarised in Table I and shown in Figure 9. Several of the components were present around the papillae, in the blood vessel wall structures and in the basal epithelium, for example AGT, renin and ACE. Moreover, several components were evenly distributed across the epithelium, thus also in the superficial layers. The latter was true for the AngII receptors, suggesting that AngII mediated effects can be exerted even close to the esophageal lumen. The protein locations imply that AngII can be locally produced in the mucosa in addition to the infusion by the blood.

Moreover, the results indicated that AngII could be generated by alternative pathways, by actions of CMA, CatG and CatD. However, CMA and CatG were only detected by IHC (Figure 9) and not by WB. This could be due to diverse antibody behaviour at different methods, e.g. due to various tissue preservations, buffer solutions and antigen retrievals. Strong IHC staining was observed for CMA, which was surprising since the occurrence of mast cells are sparse in the esophagus. However, the correctness of this finding is supported by that no immunostaining was observed in the negative controls and by previous control of the antibody's specificity for MC in esophageal achalasia specimens (Casselbrant, unpublished results).

Table I. Location of RAS proteins in the esophageal epithelium

Protein	Stratum superficiale	Stratum spinosum	Stratum basale	Peripapillary
ACE	+	+	+	+ ves
AGT	-	+	+	+ ves
AP-A	-	-	-	-
AP-B	+ cyt	+ mem, around nuc	+	+
AP-M	+ cyt	+ mem, nuc	+	+ ves
AT1R	+	+ mem, cyt	+ mem, possibly cyt	+
AT2R	+	+ mem, cyt	+ mem, cyt	+
AT4R	+ cyt	+ mem, in nuc	+	+
CatD	+ cyt	+ cyt	+ cyt	+ cyt
CatG	+	+ nuc, cyt	+ nuc	+
CMA	+	+	+ cyt, around nuc	+
MasR	+	+ cyt, around nuc	+	+
NEP	+	+ mem, possibly cyt	+	+
Renin	+	+ mem	+ nuc	+ ves

+ immunoreactivity
- no immunoreactivity
Abbreviations; ACE: angiotensin-converting enzyme, AGT: angiotensinogen, AP-A, AP-B and AP-M: aminopeptidase A, -B and -M, AT1R and AT2R: angiotensin II type 1 and type 2 receptor, AT4R: angiotensin IV receptor, CatD and CatG: Cathepsin D and -G, CMA; mast cells chymase, cyt: cytosolic, MasR: mas oncogene receptor, mem: membranous, NEP: neprilysin, nuc: nuclear, ves: vessels.

Other angiotensins

The results showed that the esophageal mucosa also probably expresses angiotensins other than AngII. The Ang1-7 forming enzyme NEP and the receptor MasR were present, and the data also indicated that AngII could be converted to AngIII and AngIV since the formations enzymes AP-A, AP-B and AP-M were observed (Figure 9 and WB data in II and III). AP-A was not detected by IHC, which could be due to usage of an old secondary antibody or due to other technical reasons such as to low primary antibody concentration or to that the antigen retrieval was not optimal. Also the AT4R receptor was present, suggesting that AngIV mediates biological actions through the receptor in the esophagus.

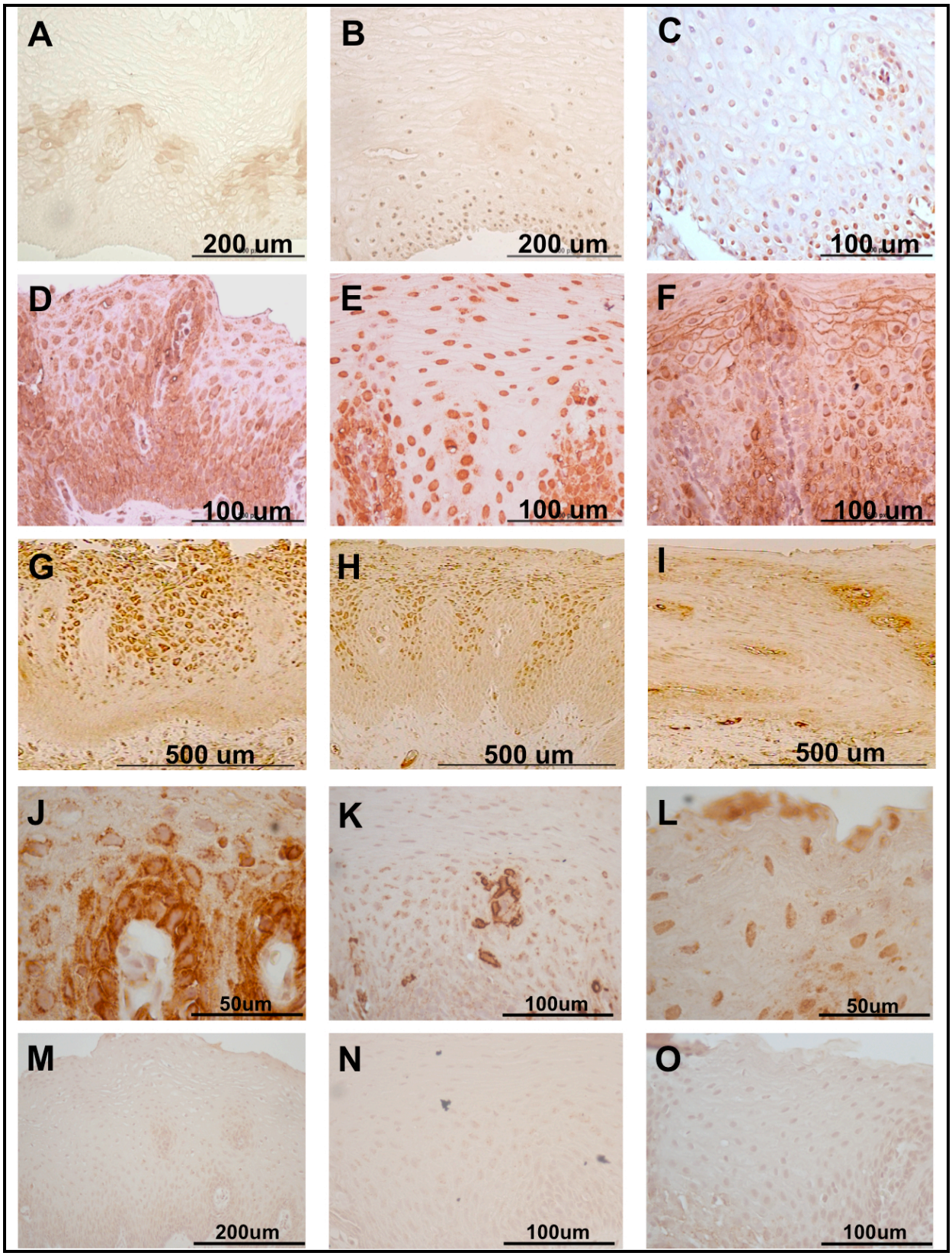

Figure 9. Immunoreactivity of RAS components in the esophageal mucosa
The panels show immunohistochemical staining of: angiotensinogen (A), renin (B), cathepsin G (C), chymase (D), Mas oncogene receptor (E), neprilysin (F), angiotensin II type 1 receptor (G), angiotensin II type 2 receptor (H), angiotensin-converting enzyme (I), aminopeptidase B (J), aminopeptidase M (K), the angiotensin IV receptor (L) and negative controls (M, N, O). Haematoxylin-eosin background staining in sections C, D, E and F.

1st Conclusion

All ‘classical’ RAS components were present in the distal esophageal mucosa as were enzymes for alternative AngII generating pathways. Expression of the enzymes necessary for AngII breakdown was observed, implying that angiotensin 1-7, III and IV also are present in the human esophagus.

2. Second aim: to investigate the distribution of RAS components in the mucosae from patients diagnosed with ERD

RT-PCR, ELISA and WB were used to investigate if the gene activity or protein expression of various RAS components were altered in GERD (II and III).

Gene transcription activity

The gene transcripts of ACE and the AT1R were significantly higher in the mucosal break area, as was the ACE levels in the normal mucosa of ERD patients, compared with the esophageal mucosa of healthy individuals (Table II) (II). Although that changes in gene transcript levels not always correspond to tissue functionality, the observed picture strongly supports that the local mucosal RAS was altered in relation to reflux disease.

Table II. Gene transcripts in squamous mucosae

	Healthy 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 normalised to total RNA and are given as median values and range; n = number of individuals. Significant differences from control are indicated with asterisks (*=p≤0.05 and ** =p≤0.01)

Protein expression

Although gene transcription of ACE and the AT1R differed between healthy and reflux-diseased mucosae the corresponding protein levels were reasonably similar (II). The primary explanation to this is that gene transcription and protein expression are processes with different time resolution. For example, a gene transcript signal can occur (and disappear) before the corresponding protein is detectable in a tissue.

In paper II, it was observed that the protein level of the MasR was markedly reduced in the mucosal break area (Figure 10 A). Considering that the MasR is ascribed anti-inflammatory signalling, it can be speculated that the decreased MasR level can participate partly in the inflammatory picture of ERD.

Interestingly, the AT2R protein levels were higher in the normal epithelium of ERD, compared with within the mucosal break area, as well as with the mucosa from healthy subjects (Figure 10 B) (II). This receptor is described to promote anti-inflammatory processes and to be tissue protective (52), so it is possible that the elevated AT2R levels are part of a mucosal protective response, counteracting the more hostile luminal environment associated with ERD.

The AT4R expression was lower in the mucosal break area than in the normal epithelium of ERD patients and a similar trend was observed compared with healthy individuals (p-value 0.055; Figure 10 C) (III).

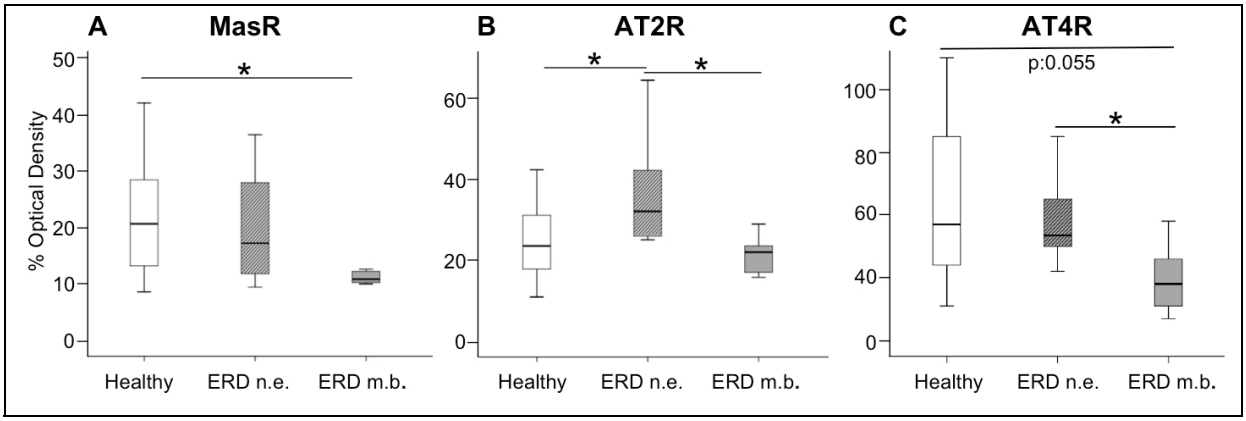

Figure 10. Protein expression of the MasR, AT2R and AT4
The MasR (A), AT2R (B) and AT4R (C) in healthy individuals (Healthy) and in endoscopically normal epithelium (ERD n.e.) and mucosal break area (ERD m.b.) of ERD patients. The y-axis displays the optical density of the protein amounts in relation 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≤0.05).

2nd Conclusion

Gene transcription activity and protein expression of various RAS components are altered in the mucosa of ERD patients.

3. Third aim: to establish an *in vitro* method for assessment of the epithelial electrical resistance in human esophageal mucosa

It was considered of interest to investigate the functional responses of some RAS receptors and the Ussing chamber technology was chosen for this investigation since it allows study of epithelial properties and pharmacological effects in small sized mucosal specimens. However, first the Ussing pulse method (UPM) was tested to see if the transepithelial permeability was reflected by the assessed *epithelial* resistance, which in turn makes optimal I_{ep} calculations possible. The applicability of the technique was also examined for very small esophageal biopsies. The mucosae were obtained endoscopically, which makes specimens available not only from patients but also from healthy individuals, serving as the proper control group.

Can UPM discriminate epithelial resistance from subepithelial resistance?

Theoretically, analysis of the mucosal response to square wave current pulses (the Ussing Pulse Method) can be used to estimate R_{ep}. The UPM was tested on three different types of epithelia and in line with the theory the esophageal, jejunal and Caco-2 cell preparations all displayed lower R_{ep} than R_{trans}, the latter obtained by conventional current clamping (Table III). The R_{ep} was highest in the stratified esophageal epithelium (280 $\Omega x cm^2$) and lowest in the single layer columnar lined jejunal epithelium (~10 $\Omega x cm^2$).

Table III. Electrical parameters in the jejunum, Caco-2 cells and esophagus

		UPM assessed			CC assessed	
	PD (-mV)	**R_{ep}** ($\Omega*cm^2$)	**I_{ep}** ($\mu A/cm^2$)	**C_{ep}** ($\mu F/cm^2$)	**R_{trans}** ($\Omega*cm^2$)	**R_{subep}** ($\Omega*cm^2$)
Jejunum (n=8, N=50)	5.6±0.1	9.2±0.6	690±33	14±1.0	72±3	63±3
Caco-2 (N=36)	0.6±0.1	143±6	4.7±0.5	19±2	568±16	424±19
Esophagus (n=14, N=42)	5.5±0.4	280±29	39±7.2	1.4±0.4	401±37	121±6

Abbreviations; CC: current clamping, Cep: epithelial electrical capacitance, Iep: epithelial electrical current, n: number of patients, N: number of preparations, PD: potential difference, Rep: epithelial electrical resistance, Rsubep: subepithelial electrical resistance, Rtrans: transmural electrical resistance, UPM: the Ussing pulse method. Values are given as means±SEM.

The various epithelia were exposed to hypoxia in an attempt to selectively lower the epithelial resistance (Figure 11). The R_{ep} of the jejunal preparations and of the Caco-2 cells decreased upon hypoxia (Figure 11 A and C) while the resistance from the subepithelial layers was relatively constant (I). This observation also supports the theory that the UPM measures the

epithelial resistance exclusively.

Does UPM assessed R_{ep} reflect permeability of molecular probes with known size?

The transepithelial permeation of two probes (FSS, 376-Mw and FD4, 4000-Mw) was recorded during the hypoxia experiments in the Ussing chambers. R_{ep} and probe permeability had a linear relationship in jejunum and Caco-2 cells, where the probe permeability increased with decreased resistance (Figure 11 B and D). This observation implies that R_{ep} not only reflect the conductivity of small ions, but also the passage of larger molecules. However, the esophageal R_{ep} did not decrease upon hypoxia (Figure 11 E), although the cellular viability clearly decreased, as displayed by the marked reduction in PD and I_{ep} (I). In accordance with the unchanged resistance, also the permeability of the molecular probes was low. The relatively stable esophageal R_{ep} during hypoxia could be due to cell swelling and diminished intercellular spaces that would have counteracted a possible resistance decrease. However, when the esophageal epithelium was challenged with the bile salt deoxycholic acid (DCA) and trypsin, the R_{ep} decreased and the probe permeability increased (Figure 11 F and G), which is in line with a previous experiment performed in the rabbit esophagus (53).

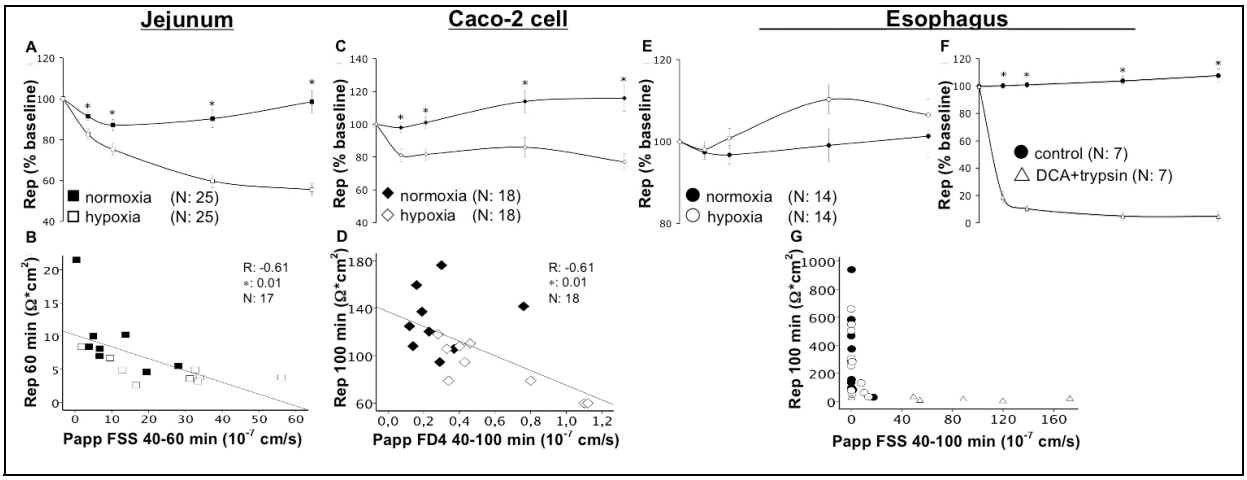

Figure 11. Challenge of the epithelial integrity

The epithelial electrical resistance (Rep) in jejunal (A), Caco-2 cells (C) and esophageal (E) specimens during oxygenated control situation and during hypoxia. The esophageal epithelium was also challenged by 5mM deoxycholic acid (DCA) and 0.04mM trypsin (F). Panel B, D and G demonstrate the relationship between Rep and the apparent permeability coefficient (Papp) for fluorescein sodium salt (FSS) or fluorescein isothiocyanate-dextran 4000-Mw (FD4). Rep data are presented in percentage of baseline and plotted as mean ±SEM. An asterisk denotes a significant difference ($p \leq 0.05$) between exposure groups and N denotes number of chamber observations.

Can UPM be used in small aperture Ussing chambers for endoscopic biopsies?

The UPM validation presented above was performed on surgically resected mucosae or on large areas of Caco-2 cell layers. However, to be useful in clinical research, the technique should be applicable also on small sized endoscopically acquired mucosal samples. UPM applied on esophageal mucosal endoscopic biopsies in mini Using chambers was indeed successful, provided that the specimens exhibited an initial lumen negative PD of at least 0.3 mV (8% of the biopsies were excluded). Furthermore, the mini Ussing preparations discriminated between mucosae obtained from healthy individuals and individuals with erosive reflux disease (ERD). As shown in Table IV, the ERD epithelium outside the mucosal break had almost half the R_{ep} value and more than double the I_{ep} value than the mucosa from healthy individuals. In addition, the PD was significantly higher in ERD patients (II). Taken together, these results showed that ERD patients had alterations in their epithelial properties, indicating events of disease. The lower tissue resistance of ERD patients is in line with a previous study (1) and also with the observed tissue alterations in relation to GERD in paper II, where the ERD patients had dilated intercellular spaces, which probably results in lower epithelial resistance. It was noted that the electrical parameters differed between the conventional and the small aperture mini Using chamber, with the PD and R_{ep} being lower in the latter (Table III and IV), which e.g. can be due to patient selection or larger impact of edge damage effects in the biopsies.

Table IV. Electrical parameters in esophageal biopsies

Data from paper II	**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, Rep: epithelial electrical resistance, Iep: 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≤0.05.

3rd Conclusion

Square wave current pulse analysis can be used in Ussing chambers for assessment of epithelial electrical resistance that in turn reflects transepithelial molecular permeability.

4. Fourth aim: to investigate *in vitro* mucosal effects of some RAS components of particular interest

The functionality of the AT2R and AT4R was tested with particular interest because of the observed alterations in expression patterns related to ERD. Endoscopic esophageal biopsies from healthy volunteers and of ERD patients were mounted in mini Ussing chambers. After baseline measurements, either the selective AT2R agonist C21 (II) or the peptide AngIV (III) was added to the serosal compartment.

Healthy mucosa

AT2R stimulation and addition of AngIV resulted in increased ion currents and more lumen negative PD, with magnitude of about 8-10% from baseline at dose $1x10^{-6}$M (Figure 12). AngIV, which was applied in separate doses, displayed a dose-response relationship. As shown in the present investigation the esophageal epithelium expressed the AT4R, which likely mediated the observed effects of AngIV. However, to prove such an interaction a pharmacological tool interfering with AngIV and the AT4R is needed. Apparently, such compounds are becoming available and can be used in future studies (37, 54). The C21 effect was truly AT2R mediated since concomitant addition of the AT2R antagonist PD123319 blocked the I_{ep} and PD increase (II). No changes in the resistance occurred upon AT2R stimulation (II) which confirms data from a previous study (3). Now, for the first time, such a result was displayed also for AngIV (III).

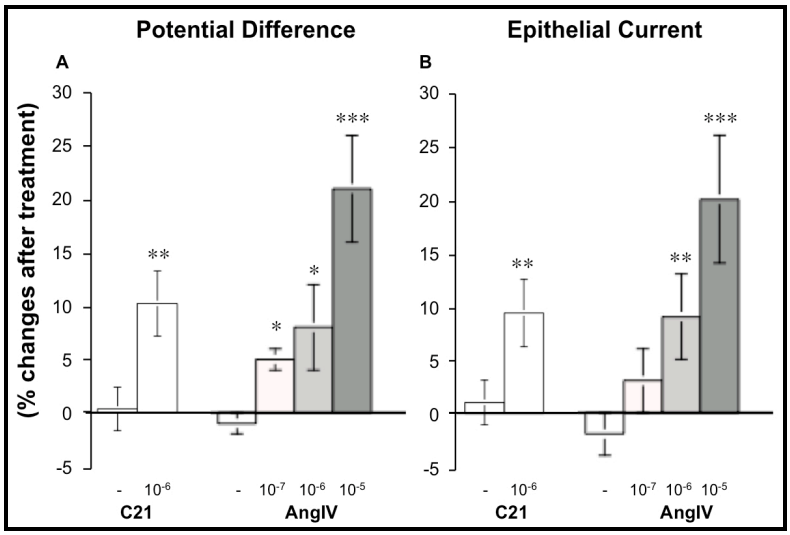

Figure 12. AT2R and AngIV effects in the healthy mucosa

Biopsies from healthy volunteers were mounted in Ussing chambers where the AT2R and AngIV effects were examined. The left panel shows the changes in the esophageal PD (A) and the right panel shows the changes in the I_{ep} (B). Data are mean±SEM of % change from baseline. Significant differences from time controls (-) are denoted by p-value: *≤0.05, **≤0.01 and ***≤0.001.

ERD mucosa

In contrast to the healthy mucosa, the I_{ep} did not increase significantly upon AT2R stimulation in ERD mucosa, despite higher receptor expression (Figure 13). This implies that the AT2R has a reduced capability to increase the net transepithelial current in ERD patients.

When the actions of AngIV were investigated in the normal mucosa of ERD patients a similar response was observed as in the mucosa of healthy individuals. Both the PD and I_{ep} increased in a dose-dependent manner. However, the increase was not as great as in the healthy mucosa (I_{ep} is shown in Figure 13).

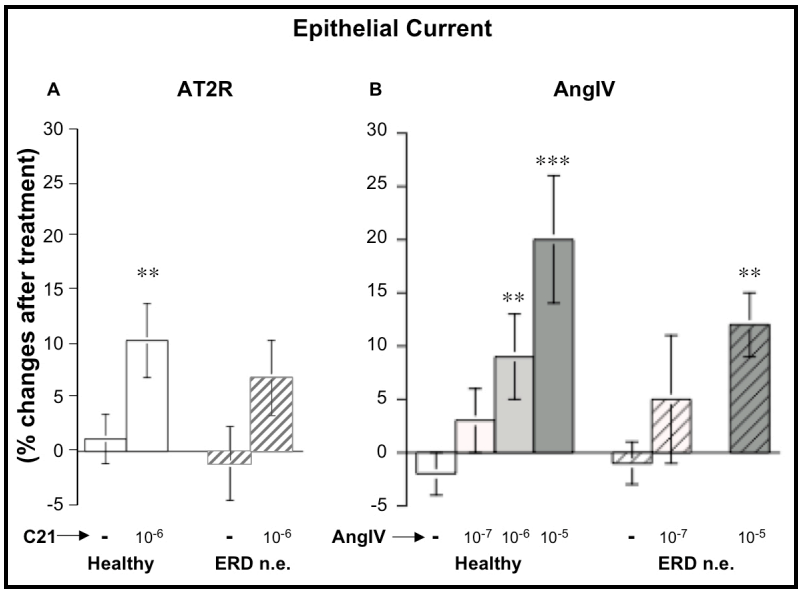

Figure 13. AT2R and AngIV effects on I_{ep} in healthy individuals and ERD

Biopsies from healthy volunteers (Healthy) and from endoscopically normal epithelium of ERD patients (ERD n.e.) were mounted in Ussing chambers. The % changes from baseline after AT2R stimulation (A) and after AngIV addition (B) are shown. Data are expressed as mean±SEM. Significant differences from the time controls (-) are denoted by p-value: **≤0.01 and ***≤0.001

4th Conclusion

Both the AT2R agonist C21 and the peptide AngIV stimulate the net transport of ions in the esophageal epithelium and these responses are attenuated in ERD patients.

VIII. CONCLUSIONS

1. All ‘classical’ RAS components were present in the distal esophageal mucosa as were enzymes for alternative AngII generating pathways. Expression of the enzymes necessary for AngII breakdown was observed, implying that angiotensin 1-7, III and IV also are present in the human esophagus.

2. Gene transcription activity and protein expression of various RAS components are altered in the mucosa of ERD patients.

3. Square wave current pulse analysis can be used in Ussing chambers for assessment of epithelial electrical resistance that in turn reflects transepithelial molecular permeability.

4. Both the AT2R agonist C21 and the peptide AngIV stimulate the net transport of ions in the esophageal epithelium and these responses are attenuated in ERD patients.

IX. GENERAL DISCUSSION

RAS in the human esophagus

The main purpose of the thesis was to investigate the expression of the renin angiotensin system in the human esophageal mucosa. Several interesting observations were done. First, the present data confirm earlier findings of the RAS components: ACE, AT1R, AT2R and CatD (3, 55), being present in the esophageal epithelium. Second, for the first time the main effector peptide AngII was detected in the mucosa, as were the components AGT, renin, CatG and CMA. This, in turn indicates that AngII formation can occur through non-classical pathways. Third, the results imply presence of the additional angiotensins: Ang1-7, AngIII and AngIV, since their formation enzymes (NEP, AP-A, AP-B and AP-M) and receptors (the MasR and the AT4R) were present, in addition to the substrate AngII. Hence, the results suggest that AngII can be supplied both by the blood circulation and by local tissue production since the component expressions were localized to several tissue compartments, i.e. the vessel walls, in close proximity to the papillae, in the entire epithelium and also within cells.

Potential involvement of RAS in reflux disease

The thesis shows that several of the investigated RAS components were altered in relation to ERD that in turn implies that the system might be involved in the pathogenesis of the disease. It is plausible that RAS can respond to noxious influences with activation or modulation of inflammation, proliferation and apoptosis in the esophagus. It is possible that the increased gene transcription of ACE and AT1R, as well as the lowered protein expression of the MasR (anti-inflammatory), that were observed in relation to ERD in the present investigation, are reflecting a pro-inflammatory signal. Although a lot of details remain to be elucidated, the potential involvements of RAS in GERD is strongly supported by a recent study, demonstrating that AT1R antagonists supported the healing of reflux esophagitis, induced by proton pump inhibition (56). It is reasonable to believe that the beneficial effect, not only was due to inhibition of the AT1R by a selective AT1R antagonist, but also due to that AngII stimulated the unopposed AT2R. Interestingly, in the present investigation, the protein expression of the AT2R was increased in the endoscopically normal epithelium of reflux patients. This receptor is described to promote anti-inflammatory processes and to be tissue protective (6, 52). Thus, it is very appealing to speculate that the present up-regulation of the

AT2R in ERD might be a mucosa protective adaptation to reflux attacks.

The Ussing pulse method - UPM

The Ussing pulse method, using square wave current pulse analysis, was evaluated for assessment of the epithelial electrical resistance and current, both being factors reflecting the mucosal barrier properties.

The present validation showed that the UPM allowed measurements of the *epithelial* resistance (i.e. reciprocal to conductance), omitting the resistance from the subepithelial compartment. The esophageal epithelium exhibited higher R_{ep} and lower P_{app} compared with the jejunal and Caco-2 cell monolayers. The results also indicated that simultaneous probe measurements could be omitted in the future since UPM assessed R_{ep} reflected the molecular transepithelial permeability. However, unlike the columnar intestinal epithelia, the squamous esophageal epithelium was in the basal state quite impermeable to medium-sized molecular probes. Only after challenging the mucosal viability with noxious substances, the R_{ep} was reduced and the molecular probe permeability was increased substantially. Still, as R_{ep}, *per definition,* is reciprocal to paracellular electrical conductance (see Methodological considerations) the esophageal epithelial resistance appears to be a good estimate of the passage capacity of small-sized ions, for example hydrogen ions, making it very useful also during conditions with intact epithelial integrity.

Furthermore, the UPM can be valuable when examining small biopsies acquired during clinical routine endoscopies. In the present study, the esophageal ERD biopsies exhibited lower R_{ep} and higher I_{ep} at baseline than the biopsies obtained from healthy individuals. This confirms pathophysiological changes and epithelial disease in ERD, as has previously been suggested (1).

The present electrical baseline parameters deviated from values obtained in other Ussing chamber studies. Besides patient cohorts, therapy and surgical procedures, this is likely due to different Ussing chamber techniques. The UPM technique is based on the assumption of a capacitor and resistor coupled in parallel and has not been used as frequent as e.g. the short circuit current technique (Scc). The estimations are certainly dependent on whether the parameters are assessed (UPM: PD and R_{ep}, Scc: PD and I_{sc}) or calculated (UPM: I_{ep}, Scc:

R_{trans}). It is likely that R_{ep} is more sensitive than R_{trans} (57) and there are indications that ion currents are underestimated at Scc conditions (49, 50). The present results showed that the electrical parameters also diverged between the conventional and the mini Ussing chambers. These deviations are likely due to the relative sizes of the investigated tissue specimens (conventional chamber: 0.29 cm^2, mini chamber: 0.018 cm^2), since the impact of edge damages increases with smaller area due to a larger edge to surface ratio. In theory, the PD is independent of the area but the mounting process damages the tissue and consequently the PD is lowered (17, 58, 59).

In summary, despite some principle differences, compared with for example Scc based measurements and the well known edge effect of small aperture Ussing setups, the UPM application was considered to be a rather user-friendly technique, having a time resolution suitable for investigating esophageal endoscopic biopsies.

Actions of RAS in the esophagus

Based on its widespread expression, it appears very likely that RAS exerts regulatory actions in the human esophagus. This concept was strengthened by the present results, and by previously reported observations of AngII being a potent stimulator of esophageal muscle contractions *in vivo* (35), and of its influence on the epithelial electrical parameters *in vitro* (3). The present results confirm that the AT2R influences I_{ep} but not R_{ep} in healthy mucosa (3), which in the present study was shown to be the case also for AngIV. However, the ERD mucosa did not respond with increased I_{ep} upon AT2R stimulation, despite higher receptor levels as shown by WB. The ERD mucosa responded with increased I_{ep} upon AngIV addition. However, the response was small compared to healthy mucosa. These results imply that the capability of the AT2R and possibly also the AT4R is altered in relation to reflux disease. The mechanisms behind these alterations remains to be elucidated but could be due to functional defects, to faster endogenous peptide cleavage or to receptor internalization. An alternative explanation could be that the already elevated I_{ep} at baseline in the ERD preparations reduced the capability to further increase the net transepithelial current. The baseline shift could in turn be due to endogenous AngII, stimulating the receptors or to other, so far unknown, stimulatory mechanisms.

The AT2R is reported to influence duodenal HCO_3^- secretion in the rat (60) and to block

Na^+/H^+ exchangers in the mouse (61). The AT4R is described to affect the intracellular Na^+ and Ca^{2+} concentrations and also the activity of the Na^+/K^+ ATPase pump in the kidney proximal tubule cells (62, 63). Na^+/H^+ exchangers and Cl^-/HCO_3^- transporters are of particular interest in the esophagus, where sodium absorption is the main ion transport event occurring. The consequent electrochemical gradient is used to regulate the intracellular pH, by absorbing HCO_3^- or extruding H^+ from the cell cytosole. Increased H^+ extrusion protects the tissue from cellular damage, but can in the long-term lead to cytosolic alkalinization and luminal acidification (64). It has been shown that GERD patients have induced expression of the basolateral Na^+/H^+ exchanger NHE-1 and also that the Na^+/K^+ ATPase pumps have increased activity (64). These GERD associated alterations could be an explanation to the present observed increase of the basal net currents in ERD. It is also possible that RAS components, e.g. the AT2R and AT4R, influence the activity of the described channels and, hence, affect the intracellular acid regulation. This remains to be elucidated, but it is apparent that tuned ion transport processes are vital for optimising the barrier properties in order to protect the mucosa from reflux attacks.

Plasticity of RAS

RAS is a potent regulatory system, exerting effects on the intracellular, tissue, organ and systemic level (7, 65). The plasticity and complexity of RAS is spectacular with various pathways, with compensatory and feedback component production and with various components having counteracting effects (6, 7). In addition, several of the angiotensins have affinity for various receptors and, some components can act as both receptors and enzymes (e.g. the AT4R and ACE) (4, 7, 37). Other important determinants for activity are the rate of delivery or formation, as well as elimination of angiotensins, that in turn are dependent on the actual expression pattern of the RAS associated enzymes.

The detection of RAS components does not necessarily mean that RAS is active in a tissue. RAS enzymes can e.g. act on other substrates than angiotensins and RAS receptors might bind other ligands. One well-known example is ACE that, in addition to the formation of AngII, also participates in the kinin-kallikrein system with the inactivation of bradykinin. Hence, it is possible that components included in RAS are involved in "RAS independent processes" such as submucosal gland secretion (e.g. CMA), hormone and neurotransmitter degradation (e.g. ACE, NEP, AP-M and AT4R), peptide signalling (e.g. NEP), inflammation

(e.g. CMA and CatG) as well as cellular differentiation, proliferation and carcinogenesis (e.g. CMA CatD, AP-M) (7, 37). However, the result of the present thesis, with presence of an extensive RAS, including the key mediator AngII, and with observed *in vitro* actions of RAS receptors, strongly indicate that the system exerts physiological effects in the human esophageal mucosa.

Future perspectives

GERD is a disease affecting the daily life of many people and the prevalence is increasing. The introduction of PPIs improved the quality of life for many patients, but the treatment fails to completely reduce symptoms in some cases and, thus, there are still individuals acquiring insufficient treatment. The pathophysiology behind GERD is complex, involving various mechanisms such as reflux amount, muscle and sphincter tension, processing of sensory information and state of the mucosal barrier. This thesis has focused on the renin angiotensin system and its role in GERD and particularly the system's potential impact on the epithelial barrier properties. The studies were undertaken in an attempt to connect the bioscientific possibilities with the still unmet clinical needs regarding GERD. Examining endoscopic biopsies in mini Ussing chambers with the user-friendly UPM may facilitate future studies concerning GI and esophageal diseases. Exploration of RAS in the esophagus has the potential to reveal new diagnostic and therapeutic targets. The present results revealed that many RAS components, not least the receptors, are regulated and act differently in the mucosa of reflux patients. Many agonists/antagonists interfering with RAS are already commercially available and more are under development. It is possible that some of the RAS components, for example the AT2R, could be an interesting pharmaceutical target for treating GERD, e.g. by reducing inflammation or by strengthening the esophageal mucosal barrier properties and hence improve the ability to withstand a noxious refluxate.

X. REFERENCES

1. 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 Jun;106(6):1039-47.

2. Barlow WJ, Orlando RC. The pathogenesis of heartburn in nonerosive reflux disease: a unifying hypothesis. Gastroenterology. 2005 Mar;128(3):771-8.

3. Casselbrant A, Edebo A, Hallersund P, Spak E, Helander HF, Jonson C, et al. Angiotensin II receptors are expressed and functional in human esophageal mucosa. Am J Physiol Gastrointest Liver Physiol. 2009 Nov;297(5):G1019-27.

4. Garg M, Angus PW, Burrell LM, Herath C, Gibson PR, Lubel JS. Review article: the pathophysiological roles of the renin-angiotensin system in the gastrointestinal tract. Aliment Pharmacol Ther. 2012 Feb;35(4):414-28.

5. Fandriks L. The renin-angiotensin system and the gastrointestinal mucosa. Acta Physiol (Oxf). 2010 Jan;201(1):157-67.

6. Volpe M, Musumeci B, De Paolis P, Savoia C, Morganti A. Angiotensin II AT2 receptor subtype: an uprising frontier in cardiovascular disease? J Hypertens. 2003 Aug;21(8):1429-43.

7. Fyhrquist F, Saijonmaa O. Renin-angiotensin system revisited. J Intern Med. 2008 Sep;264(3):224-36.

8. Yazaki E, Sifrim D. Anatomy and physiology of the esophageal body. Dis Esophagus. 2012 May;25(4):292-8.

9. DeNardi FG, Riddell RH. The normal esophagus. Am J Surg Pathol. 1991 Mar;15(3):296-309.

10. Al Yassin TM, Toner PG. Fine structure of squamous epitheilum and submucosal glands of human oesophagus. J Anat. 1977 Jul;123(Pt 3):705-21.

11. Holloway RH. The anti-reflux barrier and mechanisms of gastro-oesophageal reflux. Baillieres Best Pract Res Clin Gastroenterol. 2000 Oct;14(5):681-99.

12. Long JD, Orlando RC. Esophageal submucosal glands: structure and function. Am J Gastroenterol. 1999 Oct;94(10):2818-24.

13. Marcinkiewicz M, Grabowska SZ, Czyzewska E. Role of epidermal growth factor (EGF) in oesophageal mucosal integrity. Curr Med Res Opin. 1998;14(3):145-53.

14. Orlando RC. The integrity of the esophageal mucosa. Balance between offensive and defensive mechanisms. Best Pract Res Clin Gastroenterol. 2010 Dec;24(6):873-82.

15. Orlando RC. Review article: oesophageal tissue damage and protection. Review article: reflux and its consequences--the laryngeal, pulmonary and oesophageal manifestations. Conference held in conjunction with the 9th International Symposium on Human Pepsin

(ISHP) Kingston-upon-Hull, UK, 21-23 April 2010. Aliment Pharmacol Ther. 2011 Apr;33 Suppl 1:8-12.

16. Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol. 2009 Nov;9(11):799-809.

17. Tobey NA, Argote CM, Vanegas XC, Barlow W, Orlando RC. Electrical parameters and ion species for active transport in human esophageal stratified squamous epithelium and Barrett's specialized columnar epithelium. Am J Physiol Gastrointest Liver Physiol. 2007 Jul;293(1):G264-70.

18. Yamamura H, Ugawa S, Ueda T, Nagao M, Joh T, Shimada S. Epithelial Na+ channel delta subunit is an acid sensor in the human oesophagus. Eur J Pharmacol. 2008 Dec 14;600(1-3):32-6.

19. Goldman A, Chen H, Khan MR, Roesly H, Hill KA, Shahidullah M, et al. The Na+/H+ exchanger controls deoxycholic acid-induced apoptosis by a H+-activated, Na+-dependent ionic shift in esophageal cells. PLoS One. 2011;6(8):e23835.

20. Sarosiek J, McCallum RW. Mechanisms of oesophageal mucosal defence. Baillieres Best Pract Res Clin Gastroenterol. 2000 Oct;14(5):701-17.

21. Dunne DP, Paterson WG. Acid-induced esophageal shortening in humans: a cause of hiatus hernia? Can J Gastroenterol. 2000 Nov;14(10):847-50.

22. Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. Am J Gastroenterol. 2006 Aug;101(8):1900-20; quiz 43.

23. Ness-Jensen E, Lindam A, Lagergren J, Hveem K. Changes in prevalence, incidence and spontaneous loss of gastro-oesophageal reflux symptoms: a prospective population-based cohort study, the HUNT study. Gut. 2012 Oct;61(10):1390-7.

24. Kang JY. Systematic review: geographical and ethnic differences in gastro-oesophageal reflux disease. Aliment Pharmacol Ther. 2004 Oct 1;20(7):705-17.

25. Wang A, Mattek NC, Holub JL, Lieberman DA, Eisen GM. Prevalence of complicated gastroesophageal reflux disease and Barrett's esophagus among racial groups in a multi-center consortium. Dig Dis Sci. 2009 May;54(5):964-71.

26. Vieth M, Fiocca R, Haringsma J, Delarive J, Wiesel PH, Tam W, et al. Radial distribution of dilated intercellular spaces of the esophageal squamous epithelium in patients with reflux disease exhibiting discrete endoscopic lesions. Dig Dis. 2004;22(2):208-12.

27. Vieth M, Peitz U, Labenz J, Kulig M, Naucler E, Jaspersen D, et al. What parameters are relevant for the histological diagnosis of gastroesophageal reflux disease without Barrett's mucosa? Dig Dis. 2004;22(2):196-201.

28. Lundell LR, Dent J, Bennett JR, Blum AL, Armstrong D, Galmiche JP, et al. Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. Gut. 1999 Aug;45(2):172-80.

29. Bredenoord AJ. Mechanisms of reflux perception in gastroesophageal reflux disease: a review. Am J Gastroenterol. 2012 Jan;107(1):8-15.

30. Quigley EM. Non-erosive reflux disease, functional heartburn and gastroesophageal reflux disease; insights into pathophysiology and clinical presentation. Chin J Dig Dis. 2006;7(4):186-90.

31. Ronkainen J, Aro P, Storskrubb T, Johansson SE, Lind T, Bolling-Sternevald E, et al. Prevalence of Barrett's esophagus in the general population: an endoscopic study. Gastroenterology. 2005 Dec;129(6):1825-31.

32. Kountourakis P, Ajani JA, Davila M, Lee JH, Bhutani MS, Izzo JG. Barrett's Esophagus: A Review of Biology and Therapeutic Approaches. Gastrointest Cancer Res. 2012 Mar;5(2):49-57.

33. Hvid-Jensen F, Pedersen L, Drewes AM, Sorensen HT, Funch-Jensen P. Incidence of adenocarcinoma among patients with Barrett's esophagus. N Engl J Med. 2011 Oct 13;365(15):1375-83.

34. Altan E, Blondeau K, Pauwels A, Farre R, Tack J. Evolving pharmacological approaches in gastroesophageal reflux disease. Expert Opin Emerg Drugs. 2012 Sep;17(3):347-59.

35. Casselbrant A, Edebo A, Wennerblom J, Lonroth H, Helander HF, Vieth M, et al. Actions by angiotensin II on esophageal contractility in humans. Gastroenterology. 2007 Jan;132(1):249-60.

36. Resende MM, Mill JG. Alternate angiotensin II-forming pathways and their importance in physiological or physiopathological conditions. Arq Bras Cardiol. 2002 Apr;78(4):425-38.

37. Vauquelin G, Michotte Y, Smolders I, Sarre S, Ebinger G, Dupont A, et al. Cellular targets for angiotensin II fragments: pharmacological and molecular evidence. J Renin Angiotensin Aldosterone Syst. 2002 Dec;3(4):195-204.

38. Williams B. Angiotensin II and the pathophysiology of cardiovascular remodeling. Am J Cardiol. 2001 Apr 19;87(8A):10C-7C.

39. Naseem RH, Hedegard W, Henry TD, Lessard J, Sutter K, Katz SA. Plasma cathepsin D isoforms and their active metabolites increase after myocardial infarction and contribute to plasma renin activity. Basic Res Cardiol. 2005 Mar;100(2):139-46.

40. Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. Immunol Rev. 2007 Jun;217:141-54.

41. Ramaha A, Patston PA. Release and degradation of angiotensin I and angiotensin II from angiotensinogen by neutrophil serine proteinases. Arch Biochem Biophys. 2002 Jan 1;397(1):77-83.

42. Rykl J, Thiemann J, Kurzawski S, Pohl T, Gobom J, Zidek W, et al. Renal cathepsin G and angiotensin II generation. J Hypertens. 2006 Sep;24(9):1797-807.

43. Wright JW, Krebs LT, Stobb JW, Harding JW. The angiotensin IV system: functional implications. Front Neuroendocrinol. 1995 Jan;16(1):23-52.

44. Hallersund P, Elfvin A, Helander HF, Fandriks L. The expression of renin-angiotensin system components in the human gastric mucosa. J Renin Angiotensin Aldosterone Syst. 2011 Mar;12(1):54-64.

45. 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(3):232-8.

46. Zucco F, Batto AF, Bises G, Chambaz J, Chiusolo A, Consalvo R, et al. An inter-laboratory study to evaluate the effects of medium composition on the differentiation and barrier function of Caco-2 cell lines. Altern Lab Anim. 2005 Dec;33(6):603-18.

47. Beaslas O, Torreilles F, Casellas P, Simon D, Fabre G, Lacasa M, et al. Transcriptome response of enterocytes to dietary lipids: impact on cell architecture, signaling, and metabolism genes. Am J Physiol Gastrointest Liver Physiol. 2008 Nov;295(5):G942-52.

48. 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 May 7;72:248-54.

49. Hemlin M, Jodal M, Lundgren O, Sjovall H, Stage L. The importance of the subepithelial resistance for the electrical properties of the rat jejunum in vitro. Acta Physiol Scand. 1988 Sep;134(1):79-88.

50. Reims A, Strandvik B, Sjovall H. Epithelial electrical resistance as a measure of permeability changes in pediatric duodenal biopsies. J Pediatr Gastroenterol Nutr. 2006 Nov;43(5):619-23.

51. Anderson JM, Van Itallie CM. Physiology and function of the tight junction. Cold Spring Harb Perspect Biol. 2009 Aug;1(2):a002584.

52. Fandriks L. The angiotensin II type 2 receptor and the gastrointestinal tract. J Renin Angiotensin Aldosterone Syst. 2010 Mar;11(1):43-8.

53. 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 Oct;57(10):1366-74.

54. Andersson H, Demaegdt H, Johnsson A, Vauquelin G, Lindeberg G, Hallberg M, et al. Potent macrocyclic inhibitors of insulin-regulated aminopeptidase (IRAP) by olefin ring-closing metathesis. J Med Chem. 2011 Jun 9;54(11):3779-92.

55. Breton J, Gage MC, Hay AW, Keen JN, Wild CP, Donnellan C, et al. Proteomic screening of a cell line model of esophageal carcinogenesis identifies cathepsin D and aldo-keto reductase 1C2 and 1B10 dysregulation in Barrett's esophagus and esophageal adenocarcinoma. J Proteome Res. 2008 May;7(5):1953-62.

56. Miwa H, Hongo M, Kusano M. Combination of angiotensin II receptor blockers promotes proton pump inhibitor-based healing of reflux esophagitis. J Gastroenterol. 2012 Mar;47(3):249-55.

57. Fromm M, Krug SM, Zeissig S, Richter JF, Rosenthal R, Schulzke JD, et al. High-resolution analysis of barrier function. Ann N Y Acad Sci. 2009 May;1165:74-81.

58. Dobson JG, Jr., Kidder GW, 3rd. Edge damage effect in in vitro frog skin preparations. Am J Physiol. 1968 Apr;214(4):719-24.

59. Clarke LL. A guide to Ussing chamber studies of mouse intestine. Am J Physiol Gastrointest Liver Physiol. 2009 Jun;296(6):G1151-66.

60. Johansson B, Holm M, Ewert S, Casselbrant A, Pettersson A, Fandriks L. Angiotensin II type 2 receptor-mediated duodenal mucosal alkaline secretion in the rat. Am J Physiol Gastrointest Liver Physiol. 2001 Jun;280(6):G1254-60.

61. Tsutsumi Y, Matsubara H, Masaki H, Kurihara H, Murasawa S, Takai S, et al. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. J Clin Invest. 1999 Oct;104(7):925-35.

62. Handa RK. Characterization and signaling of the AT(4) receptor in human proximal tubule epithelial (HK-2) cells. J Am Soc Nephrol. 2001 Mar;12(3):440-9.

63. Kotlo K, Shukla S, Tawar U, Skidgel RA, Danziger RS. Aminopeptidase N reduces basolateral Na+ -K+ -ATPase in proximal tubule cells. Am J Physiol Renal Physiol. 2007 Oct;293(4):F1047-53.

64. Siddique I, Khan I. Regulation of Na/H exchanger-1 in gastroesophageal reflux disease: possible interaction of histamine receptor. Dig Dis Sci. 2003 Sep;48(9):1832-8.

65. Kumar R, Thomas CM, Yong QC, Chen W, Baker KM. The intracrine renin-angiotensin system. Clin Sci (Lond). 2012 Sep;123(5):273-84.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to all individuals that have contributed to this thesis and by whom I have been supported by during my years as a Ph.D. student. I could not have done it without you!

Especially, I would like to thank my main supervisor, Professor **Lars Fändriks**, for his sharing of knowledge, stimulating discussions and excellent guidance, particularly at hard times.

My assistant supervisor, Dr. **Anna Casselbrant**, for her brilliant and close collaboration, guiding me in the scientific research and laboratory practicals.

Sören Lundberg for his technical support, regarding the Ussing pulse method and for his patience when explaining electrotechnology.

Christina Ek for her brilliant laboratory work and for being a great colleague.

All upper GI-surgeons and physicians who made these studies possible, when willing to make the extra efforts required collecting research specimens. Especially thanks to **Anders Edebo** for his support and patience when collecting endoscopic biopsies and for being prepared to try new solutions when problems arose. Thanks to **Hans Lönroth, Claes Jönson, Erik Johnsson, Hans Axelsson, Per Björklund, Cecilia Engström, Srdjan Kostic, Almantas Maleckas, Hanns-Ulrich Marschall, Torsten Olbers, Ulrika Smedh** and my co-authors **Svein Olav Bratlie** and **Michael Vieth**.

The nurses at the Department of Gastrosurgical Research, **Niclas Björnfot, Eva-Lotta Eén, My Engström, Diana Lustgarten, Eva Peras** and **Maria Wållberg**, for the great help in assembling the study individuals and assisting upon endoscopy. I would also like to thanks the **nurses at GEA**.

To all **healthy volunteers** and **patients**, willing to contribute with valuable tissue specimens, even in times of severe difficulties.

My co-author **Herbert Helander** for his enthusiasm and his excellent contributions and

sharing of knowledge regarding morphology and histology.

Gunilla Pervik, Sylvi Abramson, Ann-Sofie Nord and **Lhinn Nilsson Nyman** for your secretarial services, making everything run smoothly.

Erik Elias, Peter Hallersund, Kamuran Inci, Anna Laurenius, Mantas Malinauskas, Mihai Oltean, Josefine Rössberger, Emma Spak, Ville Wallenius and **Malin Werling** for being supportive colleagues and for interesting scientific discussions.

To all my **friends** for making me happy.

To all my **relatives** and my **family** for just being there, unconditionally. Especially thanks to my parents, **Anna** and **Beng-Olof,** to my sister **Maria** and my brother **Robert**. Also thanks to young **Ville** and **Sigge**, making me forget about everything else when I am with you.

And finally, many thanks to **Tobias** for giving me strength and support during this time, as well as for wanting to be by my side.

The studies in the thesis received financial support from the Swedish Research Council, the University of Gothenburg, the Sahlgrenska University Hospital (ALF-grants) and the Foundation of Wilhelm and Martina Lundgren.