The Department of GastroResearch
Institute of Surgical Sciences

The Nitric Oxide and Renin-angiotensin II Systems in the Human Esophagus

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The Sahlgrenska Academy
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II. Casselbrant A, Pettersson A, Fändriks L. Oesophageal intraluminal nitric oxide facilitates the acid-induced oesophago-salivary reflex. Scand J Gastroenterol 2003;38:235-38

III. Casselbrant A, Edebo A, Helander HF, Vieth M, Fändriks L. Expression of the renin-angiotensin system (RAS) in the human esophagus. *In manuscript*

IV. Casselbrant A, Edebo A, Wennerblom J, Lönroth H, Fändriks L. Actions by angiotensin II on esophageal contractility in man. *In manuscript*

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ABSTRACT

Casselbrant A. 2005. The Nitric Oxide and Renin-angiotensin II Systems in the Human Esophagus. Pages 1–41. Department of GastroResearch. Institute of Surgical Sciences, The Sahlgrenska Academy at Göteborg University, SE-413 45 Göteborg, Sweden.

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Examinations of esophageal mucosa and motor activity *in vivo* were performed on healthy volunteers. Specimens of the esophageal musculature with confirmed normal appearance were obtained from patients undergoing esophagogastric resection. Juxtamucosal NO formation was assessed using a tonometric technique. Salivary volume and titrable alkalinity were used to calculate alkaline secretion. Esophageal smooth muscle contractions in response to angiotensin II were studied using an organ-bath. Contractile events of the distal esophagus *in vivo* were assessed using multiple recordings of hydrostatic pressure and potential difference along a nasogastric catheter. Immunohistochemistry, PCR and the Western blotting were used to detect NO synthases and components of RAS.

The results demonstrate that two sources exist for the esophageal luminal NO formation; chemical reduction of salivary nitrite and enzymatic degradation of L-arginine in the epithelium, both dependent on the presence of acid in the esophageal lumen. Salivary alkaline secretion increased markedly following intraluminal acid exposure and data suggest that intraluminal NO facilitates initiation of the acid-induced esophago-salivary reflex. Mucosal biopsies and muscular tissue both indicate the existence of a local renin-angiotensin system in the normal human esophagus. Angiotensin II stimulates the human distal esophageal musculature *in vitro* via the AT_1 receptor subtype, and administration of the AT_1 receptor antagonist candesartan reduces the amplitude of swallows-induced peristaltic contractions and the length of the high-pressure zone *in vivo*.

In conclusion, the investigations show that both juxtamucosal NO formation and a local RAS exist in the human esophagus, and that both these regulatory systems are functional during physiological conditions.

Key-words: acid, nitric oxide, nitric oxide synthase, salivary secretion, renin-angiotensin, AT_1 receptor, AT_2 receptor, mucosa, smooth muscle, contractility, peristaltic, high-pressure zone,
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ABBREVIATIONS

ACE	angiotensin converting enzyme
Ang II	angiotensin II
AT_1	angiotensin II type 1 receptor
AT_2	angiotensin II type 2 receptor
CO_2	carbon dioxide
cGMP	cyclic guanosine monophospate
EDRF	endothelium-derived relaxing factors
eNOS	endothelial nitric oxide synthase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GERD	gastro-esophageal reflux disease
H^+	hydrogen ion
HCl	hydrochloric acid
HCO_3^-	bicarbonate ion
HPZ	high-pressure zone
iNOS	inducible nitric oxide synthase
LES	lower esophageal sphincter
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
PD	potential difference
RAS	renin angiotensin system
TLESR	transient lower esophagus sphincter relaxation

CONTENTS

BACKGROUND

The esophagus

The esophagus is a foot-long tube that passes through the thoracic cavity, penetrates the diaphragm, which separates the thoracic cavity from the abdominal cavity, and joins the stomach a few centimetres below the diaphragm. The principal function of esophagus and its sphincters is to transport food and fluid from the pharynx to the stomach, and to prevent the reflux of gastric contents into the esophagus, pharynx and respiratory tract. Striated muscles surround the upper third of the human esophagus, the mid part contains both striated and smooth muscles, whereas the lower third consists of smooth muscle only. Esophageal peristaltic contractions are initiated by swallowing and are accompanied by relaxation of the upper and subsequently the lower esophageal sphincter (LES), to allow the passage of a food bolus into and through the esophagus. The swallow-induced esophageal propagated contraction is termed primary peristalsis, and takes about 9 sec to reach the stomach. The central and the enteric nervous systems jointly control the peristaltic and sphincter functions. Afferent fibbers from the receptors in the esophageal wall signal to the swallowing centre which can alter the efferent activity e.g. if food does not reach the stomach during the initial peristaltic wave, the distension of the esophagus activates receptors that initiate reflexes causing repeated contractile waves, so called secondary peristalsis.

The lower esophageal sphincter (LES)

The most distal part of the esophagus constitutes the lower esophageal sphincter (LES) with a length of 2-4 cm. This esophageal segment generates a high- pressure zone (HPZ), preventing gastro-esophageal reflux. The ability of the LES to maintain a mechanical barrier between the stomach and the esophagus is aided by the fact that its distal part lies below the diaphragm and is subject to the same abdominal pressures as is the stomach. If the pressure in abdominal cavity is raised, the pressure on both the gastric contents and the terminal segment of the esophagus are raised together, preventing the formation of a pressure difference that could force stomach contents into the esophagus. The LES generates pressure up to 80 to 90 mm Hg, with a resting pressure of 12 to 18 mm Hg. It is important to note that in addition to the esophageal musculature also other anatomical properties contribute to the sphincter completed. For example, the gastroesophageal valve created by the angle of His as well as the gastric sling musculature and particularly the crural diaphragm (Boeckxstaens 2005).

Reflux

Reflux of contents from the stomach into the esophagus is a normal physiological phenomenon that occurs at least a few times daily in most people when the intragastric pressure transiently overcomes the competence of the esophagogastric junction. In healthy individuals, 94% of all reflux episodes are related to so-called transient lower esophageal sphincter relaxation (TLESR) (Dent et al 1980, Schoeman et al 1995, Sifrim et al 1996). These sphincter relaxations are not swallow-induced and they are absent during sleep (Dent et al 1980). The TLESRs are believed to be a normal physiological event involved in the mechanism of belching (Sifrim et al 1996). The regulation of spontaneous TLESRs is still unclear. Some investigators suggest that they are triggered by a subthreshhold pharyngeal stimulus, whereas others believe that gastric distension is the major triggering factor. Whatever the initial stimulus, spontaneous TLESRs are eliminated by blocking the vagal nerve, demonstrating the nature of an extrinsic reflex (Sifrim et al 1996).

Gastroesophageal reflux disease

Gastroesophageal reflux disease (GERD) is a term that describes a disorder caused by the retrograde flow of gastric (acidic) content from the stomach into the esophagus, regardless of whether or not signs of esophagitis are found. It is one of the most common upper gastrointestinal disorders, and early recognition of symptoms associated with GERD is important for the diagnosis and management of the disease. The pathogenesis of GERD is multifactorial, involving TLESRs as well as other lower esophageal sphincter pressure abnormalities. GERD is associated with a decrease in LES pressure, which can depend on anatomical abberations (i.e. hiatal herniation) or be provoked by factors such as foods (fat, chocolate, etc), alcohol, smoking and medications. These factors have also been shown to increase TLESRs. As a result, reflux of food, acid, bile, pepsin and pancreatic enzymes occurs, leading to esophageal mucosal injury, which in the long term can progress to Barrett's metaplasia and, in a minority of patients, to esophageal adenocarcinoma (Castell et al 2004).

The esophageal muscular layers and mucosa

The wall of the esophagus is, as well as the rest of the gut, principally organized into two muscular layers separated by a neuronal network (myenteric plexus). In the outer layer, the musculature is orientated in the axial direction (longitudinal muscle) whereas the inner layer is orientated transversally (circular muscle). In contrast to the gastric and intestinal parts of

the gut, which is constituted entirely of smooth muscle, the human esophagus contains also striated muscle fibbers, predominantly in the cranial part.

The layers of the esophagus include an epithelial lining, lamina propria, muscularis mucosa, submucosa, muscularis propria (circular and longitudinal muscular layer) and adventitia. The luminal surface is covered by a non-keratinized squamous epithelium and can be separated structurally into three parts: a basal proliferating cell layer: stratum basalis; a mid-zone layer of viable metabolically active squamous cells: stratum spinosum; and a most superficial dead-cell layer: stratum corneum (Frierson 1990). The lamina propria is curving in the epithelium and forms the connective tissue papillae.

Barriers of the esophageal mucosa

The esophageal mucosa faces an environment that can be quite aggressive. After swallowing food boluses, there is mechanical wear and tear, as well as heating (or even burning) or cooling. During reflux episodes, gastric acidic contents will exert a chemical challenge. The ability of the epithelium to tolerate such stress depends on the balance between the intensity of noxious stimulation and the protective mechanisms. Noxious substances are gastro-duodenal contents including bile and pancreatic enzymes, but most important is refluxed gastric acid. It follows that most research is focused on effects and prevention of acid reflux. It is convenient to divide the esophageal barrier against refluxed acid into three compartments, the pre-epithelial barrier, the epithelial barrier, and the sub-epithelial barrier.

The pre-epithelial barrier

The pre-epithelial barrier consists of a mucus-bicarbonate layer and mechanical inhibition of reflux as well as luminal clearance mechanisms.

Mucus bicarbonate layer. Unlike the gastric and duodenal mucosa where the surface epithelial cells secrete bicarbonate (HCO_3^-), the esophageal epithelial cell do not secrete HCO_3^-. Instead, the submucosal-glands, in human located primarily in the proximal and middle esophagus are a source for HCO_3^- and mucin movement into the lumen (Namiot et al 1994, Goldstein et al 1994). These protective secretions are of particular value during sleep when esophageal clearance mechanisms such as swallow-induced peristalsis and salivary secretion are inoperative (Abdulnour-Nakhoul et al 2004). Since noxious substances act on the luminal side of the esophageal mucosa, the mucus bicarbonate layer is the first line of

defence, but in contrast to the gastric and duodenal epithelia, the mucus layer in the esophagus is quite thin (Quigley et al 1987).

Esophageal clearance. There are two major factors affecting the time for clearance of acid out of the esophagus: esophageal peristalsis and salivary function. In the normal esophagus both the mechanical distension and chemical stimulus by refluxate trigger primary and secondary peristaltic motor activity. One peristaltic wave clears the esophageal lumen of most refluxate (Bremner et al 1993, Anggiansah et al 1994) but does not restore esophageal pH to a normal level. The restoration of pH is step-wise and related to repeat swallows, whereby each swallow exposes the mucosa to salivary bicarbonate thus eliminating the remaining acid by dilution and chemical neutralisation (Helm et al 1984, Helm et al 1982, Abdulnour-Nakhoul et al 2004).

Salivary secretion. Human saliva is produced by three pairs of major salivary glands, the parotid, the submandibular and sublingual, and minor salivary gland structures scattered in various regions of the oral cavity, such as the lingual, labial, buccal, palatine, molar and incisive glands. Their histology reveals two distinct types of glandular morphology: predominantly mucous (located on the palate and tongue), predominantly serous (parotid glands) or mixed type salivary glands (submandibular, sublingual, glands within lips and cheeks (Sarosiek et al 2000). Secretion of salivary organic components is predominantly mediated through the adrenergic transmission, whereas water and electrolytes are mediated through cholinergic as well as non-adrenergic non-cholinergic (NANC) pathways mainly conveyed in cranial nerve VII or IX (Sarosiek et al 2000). In the awake state, salivary flow is continuous (about 0.5 mL /min) and carried into the esophagus by spontaneous swallows that occur about once a minute, but virtually ceases during sleep. Salivary secretion is greatly influenced by gustatory stimulation, olfactory sense, thinking of food, sensation of hunger, mastication, and by intra-esophageal mechanical and chemical stimulation mediated by esophageal-salivary reflex pathway.

The epithelial barrier

The structural elements of the esophageal epithelium, which are of importance for the barrier, consist of apical membranes, the intracellular junctions, and intercellular glycoconjugate material (Orlando 1994). Tight junctions hold the cells together and seal them in such a way that leakage of large molecules between the cells is restrained. In contrast, electrolytes and

small water-soluble molecular may cross these junctions. The intercellular glycoprotein matrix complements the barrier function of tight junctions (Orlando 2000). The buffering ability in the intercellular space depends on bicarbonate diffusion from blood, and protection against intracellular acidosis depends on the presence of basolateral membrane bicarbonate transport via mechanisms such as the Na^+-dependent Cl^-/ HCO^-_3 exchanger (Goldstein et al 1994, Tobey 1995). When the buffering capacity is overwhelmed, hydrogen ions enter the cells and inhibit vital cellular enzymes, including Na^+-K^+ ATPase, which results in swelling and cell death (Orlando 1994).

The sub-epithelial barrier

The sub-epithelial defence, acting in the submucosa, depends on the submucosal blood flow that is responsible for the delivery of protective agents including oxygen, metabolic substrates or nutrients, and bicarbonate. It also removes potentially noxious agents such as carbon dioxide and hydrogen ions that have entered the epithelium following reflux, along with metabolic by-products and cellular debris (Tobey 1995, Orlando 2000). Perfusion of the esophagus with acid leads to an increase in esophageal blood flow in rabbit and cat, and this might prevent interstitial and intracellular acidosis by increasing the delivery of bicarbonate to the mucosa (Tobey 1995). However, this phenomenon could not be confirmed in healthy human subjects (Bove et al 2005).

VARIABLES STUDIED IN THE PRESENT THESIS

The regulatory principles of esophageal functions are not fully elucidated. Local mediators as well as central neural sensory-motor controls are operation alone or in combination. This thesis deals with two regulatory systems, the juxtamucosal nitric oxide (NO) generation and the renin-angiotensin system (RAS), both briefly described below.

Nitric oxide

Nitric oxide (NO) is a small lipophilic and easily diffusible gas molecule. NO in biological systems has a short half life (3-4 seconds), so that although NO is freely diffusible, its actions are limited to only a few cell diameters from its site of synthesis. NO, classified as a free radical, is highly reactive with other molecules, preferably other radicals and oxygen and can form reactive nitrogen species, e.g. NO_2 and N_2O_3 (Beckman et al 1996, Grisham et al 2000). In the body, the actions of NO are restricted due to its binding to the iron of haeme in blood haemoglobin. Binding to metal ions in proteins is in fact a point of action for NO in many

signalling systems, e.g. when NO binds to guanylate cyclase in endothelial cells, leading to increased intracellular production of cyclic guanosine monophospate (cGMP), resulting in relaxation of the smooth muscle in the vessel walls (Anggard 1994). In the body NO was first recognised as the endothelium-derived relaxing factors (EDRF) (Ignarro et al 1987, Palmer et al 1987) and was soon found to be an important signalling molecule in other biological systems as well (Lowenstein et al 1992).

Nitric oxide synthase

NO is formed by degradation of L-arginine, a reaction catalysed by a family of enzymes called nitric oxide synthases (NOS). NOS consists of three main isoforms, two constitutive forms and one inducible (Moncada et al 1991, Nathan 1992). The constitutive iso-forms (cNOS) are mainly found in nerves (nNOS) (Garthwaite et al 1988), and endothelium (eNOS) (Palmer & Moncada 1989), and have been shown to require calcium for activation (Cho et al 1992, Snyder 1995, Griffith et al 1995). The third type, the inducible NOS (iNOS) is often expressed in response to a challenge e.g. an infection, first identified in macrophages, and has shown to be calcium-independent for activation (Marletta et al 1988, Stuehr et al 1991, Cho et al 1992). The general view is that the two constitutive forms typically produce NO in low (picomolar) concentrations that exert signalling effects during physiological conditions. The iNOS, on the other hand, produces NO in high (nanomolar) concentrations, over longer periods, and is mostly associated to pathophysiological conditions (Palmer et al 1992).
The NOS classification is, however, becoming a bit simplistic as there have been reports of additional NOS isoforms (Radomski et al 1991, Palmer et al 1992, Shirato et el 1998) and the expression of iNOS has been found during non-stimulated conditions in epithelia of paranasal sinuses, airways, duodenum, jejunum and kidneys (Nathan et al 1994, Lundberg et al 1995, Holm et al 2001, Snygg et al 2000, Guo et al 1995).

Non-enzymatic NO production

Non-enzymatic NO production occurs in the upper gut when luminal pH is < 3 (pKa for NO is about 3.2-3.4). Swallowed nitrite reacts with gastric acid and large quantities of NO are formed intraluminally (Benjamin et al 1995, Lundberg et al 1994, Åneman et al 1996). Such nonenzymatic production of NO in the stomach contributes to the defence against swallowed pathogens and may also be important in gastric functional regulation, e.g. gastrin release (McKnight et al 1997, Weitzberg et al 1998, Holm et al 2000). The salivary nitrate originates from dietary factors or the endogenous nitrogen turnover and is taken up from the plasma and

secreted by the salivary glands. In the oral cavity nitrate is rapidly reduced to nitrite by the bacteria flora (Tannenbaum 1976). It follows that the rate of gastric non-enzymatic NO formation varies considerably with the luminal nitrite load, which in turn is dependent on the diet and salivary secretion (Dykhuizen et al 1996, Duncan et al 1997, Vallance et al 1997).

NO in esophagus

The enzymatic L-arginine/NO pathway of deeper parts of esophageal tissue has been proposed to have a role in the neurogenic control of peristalsis (Yamato et al 1992, Konturek et al 1997), and LES relaxation (Murray et al 1991). NOS immunoreactive nerve fibres have been shown in the esophageal myenteric and, submucous plexa, as well as in free nerve endings entering the epithelium, particularly so in the lower esophagus (Rodrigo et al 1997). Epithelial NOS has been little investigated in the esophagus but has been associated with pathological conditions such as cell transformation, e.g. Barrett's metaplasia/dysplasia and adenocarcinoma (Wilson et al 1998). Furthermore, weak iNOS immunoreactivity has been reported present in the basal and parabasal layers also of normal human esophageal mucosal (Tanaka et al. 1999). Although, well characterised in the stomach, neither the existence, nor functional consequences of non-enzymatic NO formation in the reflux-acidified esophagus have been studied.

The renin-angiotensin system (RAS)

The renin-angiotensin system (RAS) is one of the body's most powerful regulators of arterial pressure and body fluid volumes. Reduced intake of salt and water increases the activity of the RAS in order to enhance sodium and water retention via direct actions on the kidneys and via stimulation of other hormone systems, e.g. aldosterone. Increased sodium intake inversely decreases the activity of the RAS to promote sodium excretion.

RAS cascade- classical view

Renal release of renin is stimulated by reduction of the renal perfusion pressure sensed by renal vascular baroreceptors in the juxtaglomerular apparatus of the afferent arteriole. Renin release is also stimulated by increased renal sympathetic nerve activity, regulated by cardiopulmonary and aortic baroreceptors and by decreased sodium delivery to the macula densa of the distal tubules (Laragh 1992). Angiotensinogen, mainly secreted by the liver, is the primary substrate for the enzyme renin and converts angiotensinogen into the decapeptide Angiotensin I (Ang I). Ang I has some potential direct actions, such as mild vasoconstrictor

properties and stimulation of catecholamine production and central nervous system (Pelayo et al 1981). Ang I is, however, mainly considered as a prohormone. The angiotensin-converting enzyme (ACE) is a large, zinc dependent dipeptidyl carboxypeptidases that converts Ang I into Angiotensin II (Ang II), which is the main effectors of most known RAS actions. ACE is expressed mainly in endothelial cells throughout the vasculature, as well as in a circulating form (Laragh 1992). ACE is not selective for Ang I and catalyses also the degradation of, for example, bradykinin, enkephalins, neurotensin and substance P (Corvol et al 1995). In addition to the key mediator octapeptide Ang II, other angiotensin I fragments have also been shown to be biologically active, e.g. Angiotensin (1-7), Angiotensin III and IV (Cesari et al 2002).

Angiotensin II receptors

Two main Ang II receptor types have been defined in humans, the AT_1 receptor and AT_2 receptor. The AT_1 receptor is a 359 amino acid molecule with a molecular mass of 41 kDa (Volpe et al 2003). This receptor subtype is expressed in most organs and is ascribed the majority of the biological effects of Ang II; the homeostasis of arterial blood pressure, maintenance of electrolyte and water balance, thirst, hormone secretion, renal function and cellular growth in the cardiovascular system (Volpe et al 2003).

The AT_2 receptor is a 363 amino acid molecule with a molecular mass ranging from 60 to 140 kDa (due to prominent glycosylation), and has only a 33 % sequence homology with the AT_1 receptor (De Gasparo et al 2000, Volpe et al 2003). This receptor subtype is predominantly expressed in foetal tissue, but also in adult tissue, e.g. in brain, adrenal glands, uterus, ovaries, myometrium, vascular endothelium, and in pathological conditions such as stroke, skin wounds and vascular lesions (Volpe et al 2003). It follows that the distributions of AT_2 receptor in adults is far from being homogeneous, and may differ according to age, species, tissue type and pathophysiological state (Volpe et al 2003, Ewert et al 2003). The AT_2 receptor inhibits vascular and perivascular cell growth and proliferation, promotes apoptosis in a wide variety of cell types, and promotes cell differentiation in neuronal cell types (Volpe et al 2003). Thus, from several studies in adult individuals, it appears that the AT_2 receptor mediates opposing effects to those by the AT_1 receptor, probably in order to modulate the responses upon Ang II stimulating (De Gasparo et al 1999). One example of this is that AT_1 mediated vasoconstriction is counteracted by AT_2 receptor induced vasodilatation. Both the AT_1 and AT_2 receptors are members of the G protein-coupled receptor family. AT_2

intracellular signalling pathways have yet to be fully defined, but evidence so far indicates distinct differences compared to AT_1 signalling (Volpe et al 2003, Jackson 2001).

Locally expressed RAS

The traditional view of the renin-angiotensin system is that of a classical endocrine system with its key components associated to the renal, hepatic and lung circulations (Jackson 2001). However, data have accumulated during the two last decades suggesting that this is an oversimplification and should include also local (tissue-based) renin-angiotensin systems (Jackson 2001). Many tissues, including the brain, pituitary, blood vessels, heart, kidney and adrenal gland, express mRNAs for renin, angiotensinogen and ACE, and cultured cells from these tissues produce renin, angiotensinogen, and ACE (Jackson 2001). In addition to the potential of being a local system with dynamic expression and paracrine actions, also other formation pathways for angiotensins have been proposed. For example, expression of chymase results in Ang II formation independent on ACE (Volpe et al 2003). The biological significance of these unconventional angiotensin formation pathways are still not fully evaluated.

RAS in the esophagus

There exist some reports on the presence of Ang II receptors in the opossum and rat lower esophageal sphincter (Mykhopadhyay AK et al 1978, Fan YP et al 2002), but no information existed on presence of RAS within esophageal mucosa or the wall musculature in man.

THE THESIS

As described above the transport of food and the protection against gastroesophageal reflux are primary features of the esophagus. Regulation of muscular activity and mucosa-protective functions are thus pivotal for optimal esophageal functionality. Local mediators as well as central neural reflexes are operational alone or in combination.
The general aim of the thesis was to investigate aspects of two previously less studied regulatory systems in the normal human esophagus: luminal NO formation and the renin-angiotensin system.

The specific aims were:

- to investigate luminal NO levels during baseline and acidic conditions and to clarify the sources of such NO formation

- to clarify whether the signalling molecules NO and CO_2 participate in the acid induced esophago-salivary reflex

- to elucidate the expression and distribution of renin-angiotensin system components in the mucosa and muscular layer

- to investigate possible contractile actions by Angiotensin II on distal longitudinal and circular smooth muscle preparations *in vitro* and to clarify if endogenous Angiotensin II takes part in normal distal motor activity *in vivo*

METHODOLOGICAL CONSIDERATIONS

Subjects (paper I-IV)

Healthy subjects. Healthy volunteers (n= 39, 18–62 y, 9 females) participated in the studies. The subjects were *Helicobacter pylori*-negative as tested by the ^{13}C-urea breath test (Hamlet et al 1999) to avoid the risk for interference with the L-arginine/NO pathway in the esophagogastric mucosa (von Bothmer et al 2002, Elfvin et al 2005). Some subjects participated at several occasions.

Patients. Muscular specimens were obtained from patients (n= 11, 48-86 y, 5 females) undergoing radical surgery due to malignancy in the esophagogastric junction or proximal esophagus (usually superficial adenocarcinoma).

Ethics. All participants had given informed consent and the study had been approved by Ethical Committee of Göteborg University and was performed in accordance with the Declaration of Helsinki.

Intubation procedures (paper I, II, IV)

After an overnight fast, the subjects were supplied with a nasogastric catheter of various designs (se below). The lower esophageal sphincter (LES) was identified using a manometric assembly and the catheter was placed with an infusion site positioned 15 cm above the LES. In some experiments one pH glass electrode was also inserted via the other nostril and positioned a few cm above the LES and was connected to a computer, which recorded and displayed pH online.

NO measurements (paper I)

The nasogastric catheter was supplied with a silastic balloon (tonometer) 5 cm above LES to measure luminal NO. The gas-permeable balloon was inflated with 5 mL of room air. Equilibration between NO in the intra-esophageal atmosphere and the air of the inflated balloon was allowed for 10 minutes. The equilibration of NO in the tonometric balloon is rapid reaching more than 90% of the surrounding NO concentration within 5 min (Snygg et al 2000). The equilibrated gas was transferred into a gastight syringe and immediately injected into the sample line of a chemiluminescence NO-analyser (Modified Seres NOX 4000, Seres, Aix-en-Provence, France). The detection limit for NO was 1 ppb and calibrations were

performed with known concentrations of NO in N_2 (AGA, Stockholm, Sweden). The NO levels obtained by this tonometric technique thus mainly reflect NO formation in the luminal compartment and by the mucosa located close to the luminal compartment. During standardised conditions the technique exhibited an inter-sample variability of ±5% (Snygg et al 2000).

Salivary samplings and bicarbonate measurement (paper II)

The subjects were supplied with a dental suction device and instructed not to swallow. The saliva was collected in 10-minute fractions and the volume of each fraction recorded. Salivary buffering capacity (titratable alkalinity) was measured by titration with HCl to pH 2 (Radiometer GK2320C, Copenhagen, Denmark) during vigorous stirring to remove the carbon dioxide formed from the reaction with HCO_3^-. The alkaline secretion was defined as the product of volume secretion over 10 min and titratable alkalinity.

Bernstein-test (paper I, II, III)

A Bernstein-test is an artificial lowering of the pH of the distal esophagus by perfusion of an acidic solution during 15-30 min. The test is positive if these procedures produce heartburn symptoms and was originally used to diagnose GERD (Bernstein & Baker 1959). In the present thesis a Bernstein-like procedure was used to allow investigation of acid induced functional changes.

Histology (paper III)

To assure that the mucosal biopsies and muscular tissue had a normal appearance, the morphological analysis was performed by an experienced histopathology's. Paraffin sections of formaldehyde fixed tissues were mounted on slides and stained with haematoxylin-eosin. In coded sections, morphometry of the esophageal epithelium was carried out using the total thickness of epithelium, length of papillae, and thickness of basal cell layer in relation to reference data.

Western blot analysis of iNOS (paper I)

Esophageal mucosal biopsies were taken by endoscopy, and immediately frozen in liquid nitrogen and stored at –80°C. The frozen specimens were sonicated in phosphate buffer

containing EDTA, CHAPS and proteinase blockers. After centrifugation the supernatant was analysed for protein content by the method of Bradford (Bradford MM 1976) and stored at –80°C for further analysis. Electrophoresis was run on a 3–8% tris-acetate gel on which one lane was loaded with prestained molecular weight standards, and one lane was loaded with lysate from IFN-γ stimulated mouse macrophage cells (RAW264.7) and served as positive control to iNOS. After electrophoresis, the proteins were transferred to a polyvinyl difluoride membrane, which was incubated with a polyclonal antibody against iNOS. An alkaline phosphatase conjugated secondary goat anti-rabbit antibody and CDP-Star as a substrate, were used to identify immunoreactive proteins by chemiluminescence and photographed. Data were presented as optical density per microgram of protein.

Immunohistochemistry (paper I, III)

Mucosal biopsies or muscular tissues were immediately fixed in 4% (w/v) formaldehyde in phosphate buffered saline (pH 7.4). Detailed descriptions are given in each paper. Endogenous peroxidase was quenched by 5 min incubation in peroxidase blocking solution. Endogenous biotin in the tissue was blocked using Biotin Blocking system (Dakopatts AB, Älvsjö, Sweden). The slides were pre-incubated with serum block and then incubated with primary polyclonal antibodies against iNOS, eNOS, nNOS, AT_1, AT_2 receptors or angiotensin-converting enzyme (ACE). Control sections were incubated with normal rabbit or goat IgG 0.4μg/μL, or in some case using preabsorbed antigen instead of the primary antibody. Human suprarenal gland and smooth muscle served as a positive control to AT_2 receptors and iNOS, respectively. Immunoreactivity was detected by the ImmunoCruz™ Staining system. After being washed, the slides were incubated with biotinylated secondary antibody and the complex was detected using horseradish peroxidase (HRP)-streptavidin. The colour was developed using 3,3'-diaminobenzidine.

Reverse transcriptase polymerase chain reaction (rtPCR) (paper I, III)

Mucosal biopsies or muscular tissues were immediately placed in extraction solution and snap frozen in liquid nitrogen. Frozen tissue was homogenised and total RNA was extracted using phenol-chloroform extraction and ethanol precipitation. The RNA concentration was quantified by absorbance measurement at 260 nm and the integrity was assessed by absorbance measurement at 280 nm. Reverse transcription from 2.5 μg of total RNA was

carried out using the SUPERSCRIPT™ First-Strand Synthesis System (Invitrogen, Lidingö, Sweden) with Oligo (dT) Primers. Resulting cDNA was stored at – 20°C until use.

Polymerase chain reaction (PCR) for iNOS (paper I)

Amplification of cDNA was performed using the Amplimer Set for human inducible nitric oxide synthase primers included positive control. The cycles used were 94°C for denaturation, 62°C for annealing and 72°C for elongation and samples were then cooled. Electrophoresis of the products was performed on 1.5 % agarose gel containing Tris acetate/EDTA and ethidium bromide. A molecular weight standard was used as a molecular size standard. Visualisation of PCR products was achieved using ultraviolet light and images were captured.

Real time PCR for RAS components (paper III)

Light Cycler Q-PCR (Roche Diagnostics, Mannheim, Germany) performed the PCR reaction using SYBR Green I as marker and in accordance to the manufacturer's instructions. $MgCl_2$ concentration was optimised to 4 mM to obtain the highest signal intensity and lowest background. The primer sequences, Q-PCR conditions, PCR products sizes and reference are shown in paper III. Sample concentration was determined from a standard curve for each pair of primers. The quantification was performed by the software supplied by Roche Diagnostics (Mannheim, Germany).

Measurements of isometric tension (paper IV)

Resected esophageal tissue was transferred immediately to ice-cold oxygenated Krebs solution. The muscular layers were separated from the mucosa and circular and longitudinal smooth muscle strips of the distal part of the esophageal body were cut to a size of approximately 2 x10 mm. Each muscle strip was secured at the end with silk sutures and transferred to a 10 ml tissue bath containing oxygenated Krebs solution at 37°C (Letica Automated Organ Bath, AD Instruments Pty, Hastings, UK). The other end of the strip was attached to a force transducer for isometric recording of muscular activity (PowerLab®, AD Instruments Pty Ltd, UK). The strips were prestretched to a tension of 1 g and allowed to equilibrate for 30 min to develop a stable spontaneous tone. After the equilibration period, bethanechol, a non-selective muscarinic agonist resistant to cholinesterase degradation (de Godoy et al 2002) was added at a concentration of 10^{-4} M to obtain a reference contraction. The strips were washed prior to addition of either the selective AT_1 receptor antagonist losartan ($10^{-7}$and 10^{-5} M), or the selective AT_2 receptor antagonist PD123319 ($10^{-7}$and 10^{-5}M).

A baseline period of 15 min was allowed. Ang II was subsequently added at stepwise increasing concentrations (10^{-10}M to 10^{-5}M) every 5 min in a cumulative manner. The strips were washed and the experiment was finalised by another addition of bethanechol. Smooth muscle contractions were expressed as percent of the maximal contraction obtained with bethanechol (mean of the contractile response before and after the experiment).

Multiple manometry/potential difference recordings *in vivo* (paper IV)

The subjects were supplied with a nasogastric catheter (Ch12) with 15 sideholes at 1 cm interdistance (customised E57, Dentsleeve International LTD, Mississauga, Canada). The catheter was fixed to one nostril and positioned so that it straddled the esophagogastric junction leaving 4-5 side holes in the gastric lumen and the rest in the distal esophagus (Fig 1, left panel). Each side hole was connected to a pressure transducer that was separately fed with a low flow (3 mL h^{-1}) of 150mM NaCl. In addition, each pressure recording line (electrically isolated from each other) was used as an electrode for electrical potential recording via an Ag/AgCl bridge connected to a high-impedance voltmeter with a reference electrode positioned subcutaneously. Thus, both the intraluminal hydrostatic pressure and transmucosal electrical potential difference (PD) at each side-hole was displayed on-line and stored for later analysis on a Macintosh personal computer (Apple Computers, CA, USA) using specially designed software (Labview, National instruments, Austin, USA). The side hole recordings were plotted as a function of the distance from the nostril thus allowing simultaneous identification of 1. / the pressure profile along the distal esophagus and esophagogastric junction; and 2. / the position of the step-up in PD from esophageal mucosal values (typically 12-15mV) to that of gastric mucosa (typically 25-50mV) (Fig 1, right panel). The PD step-up allowed a continuous identification of the position of the esophagogastric mucosal transition zone in relation to the nostril, a changed distance indicating axial movements of the esophagus. In addition, the subjects were supplied with a dental suction device and instructed to avoid swallowing. Submental electromyography was used to identify voluntary and involuntary swallowing episodes and was recorded and stored as described above.

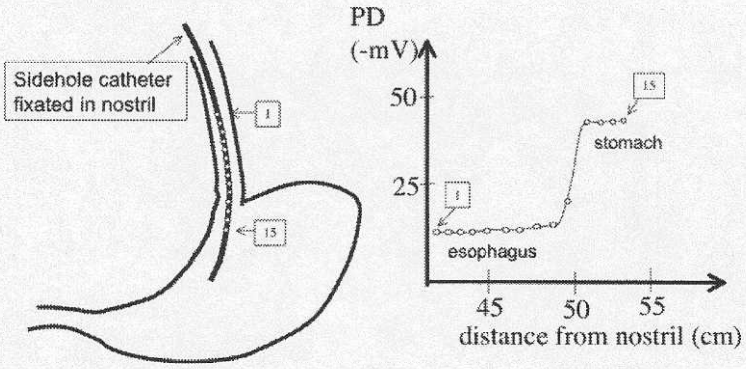

Figure 1

Left panel: Illustration of the multiple manometry and potential difference (PD) catheter used in the *in-vivo* study. The hydrostatic pressure and PD were recorded at 15 side holes (interval: 1 cm) straddling the esophagogastric junction. Right panel: The potential difference (PD) recordings were displayed as a function of distance from nostril. A step-up of PD values indicates the transition from esophageal squamous to gastric columnar mucosa (corresponding to the endoscopic Z-line). Since the nasogastric catheter is fixed to one nostril, movements of the PD step-up along the x-axis indicate axial movements of the esophagus. (The simultaneous esophageal manometric recordings are not shown in the figure.)

Principle behaviour of transmucosal potential difference

All the epithelia in the digestive tract, including that of the esophagus, exhibit a transmucosal electrical potential difference (PD). Even if different ions are implicated in the generation of PD, its principal source seems to be the transport of chloride and sodium ions from one side of the epithelia to the other. The result of these phenomena is a separation of charges across the epithelium so that the luminal surface has excess negative charges and the serosal surface has excess positive charges. In the esophagus this separation of charges is achieved by active transport of sodium from the lumen to serosal surface. Since the active transport of sodium is relatively fast compared with the passive diffusion of chloride ions, the net result is a greater number of positive charges on the serosal surface and a greater number of negative charges on the luminal surface. In the stomach additional ions are involved in the generation of PD e.g potassium, sodium, bicarbonate and its principal source seems to be the active transport of chloride ions (Schwartz et al 1987, Scarpignato et al 1995). Disruption of the mucosal barrier by the so-called "barrier breakers" such as ethanol, aspirin, and bile is associated with a decrease in PD (Scarpignato et al 1995). Presence of H^+ in the lumen will also influence PD in a biphasic manner: an initial increase in PD is followed by a return towards the baseline within 30 min (Scarpignato et al 1995). The first in PD change is considered to be a result of

increased H^+ ion diffusion from the lumen to serosa and the fall in PD reflects the back diffusion of ions (Orlando et al 1981, Jacobson et al 2002).

Drugs and chemicals (paper IV)

The following chemicals were used in the *in vitro* study: Angiotensin II, bethanechol chloride and the AT_2 receptor antagonist PD123319 (Sigma Chem. Inc., St Louis, MO, USA); AT_1 receptor antagonist losartan (Merck, Whitehouse Station, NJ, USA) were all dissolved in Krebs solution. In the human study 16 mg of the angiotensin II type 1 receptor antagonist candesartan (Atacand®, AstraZeneca, Mölndal, Sweden) was ingested 10-12h prior to investigation to obtain optimal plasma concentration and AT_1 receptor inhibition (Gleiter et al 2002, Azizi et al 2004).

Statistics (paper I-IV)

Differences in NO formation between groups in paper I were identified using ANOVA and Fisher's PLSD. Significant differences for multiple dependent groups of data in paper II were identified using Friedman`s two-way analysis of variance and contrasted by the Wilcoxon signed rank test. In papers III and IV, significant differences between multiple independent groups of data were identified using the Kruskal-Wallis and contrasted by the Mann-Whitney U test. In paper IV, significant differences for dependent group of data were identified using the Wilcoxon signed rank test. Calculations were processed by Statview 4.1 (SAS Institute Inc., Cary, NC, USA). A p-value $\gamma 0.05$ was considered to be statistically significant. Unless otherwise stated, values are given as means ± SEM.

For tension recording studies in paper IV, the amplitude of contraction was standardised as the percentage of the contraction induced by $1x10^{-4}$ M bethanechol. Differences between groups were determined based on EC_{50} values defined as the concentration Ang II producing 50% of the maximal response as interpolated from the individual concentration response relationship, in the presence or absence of the antagonists. Individual values of contractile events *in vivo* were calculated as means of the number of recorded events (usually 3 wet swallows, 6-8 dry swallows and 2-4 TLESRs). Basal values were defined as 2-10 min stable conditions without swallowing or TLESR activity recorded at least 3 times during the course of an examination and presented as mean of these recordings.

RESULTS AND COMMENTS

INVESTIGATION OF NITRIC OXIDE SYSTEM (PAPERS I AND II)

Measurement of luminal NO formation (paper I)

Swallowing of nitrite-containing saliva into the acidic stomach results in formation of large quantities of NO. Therefore, there are good reasons for believing that similar high NO levels occur also in the esophagus when swallowed saliva meets an acid reflux.

The nitrite concentration in saliva is highly dependent on nitrate intake (Tannenbaum et al 1976). Dietary precautions had to be taken into account before assessing the intraluminal esophageal NO forming capacity. The volunteers were instructed to avoid nitrate-rich food (vegetables, spicy, smoked or pickled food) during the three days preceding the examination. During baseline conditions intraluminal pH was neutral and no intra-esophageal NO was detected. Furthermore, the absence of NO formation was independent of whether the saliva was swallowed or whether it was diverted from the oral cavity by a suction device (Fig 2, panel a).

During esophageal infusion of acid over 30 min, giving an intraluminal pH of around 1, the NO levels rose dramatically to around 12 000 ppb. This high rate of NO formation in the presence of acid fell by 95% following the deviation of saliva (Fig 2, panel a). Thus, these experiments showed convincingly that intraluminal NO formation was saliva and acid dependent.

In a separate series of experiments, the subjects ingested a solution containing 200 mg potassium nitrate 40 min before instrumentation. The amount of nitrate is comparable to eating a large lettuce meal and the procedure has previously been shown to raise the salivary concentration of nitrite to sub-maximal physiological levels (Weitzberg et al 1998). NO formation was almost doubled during acid perfusion compared to the experiments without the nitrate load. Compared to the baseline experiments with low nitrate intake, the nitrate-loaded individuals showed some NO production also during pH-neutral conditions (Fig 2b), although this difference did not reach statistical significance. When the saliva was diverted, esophageal NO levels were negligible during pH-neutral conditions. During acid perfusion, however, NO increased to approximately 5000 ppb (Fig 2b), i.e. fivefold that observed without a nitrate load (Fig 2a).

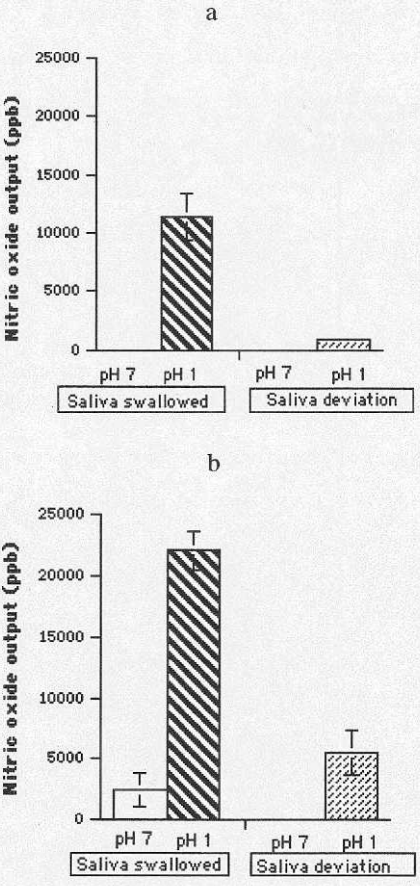

Figure 2
Panel a. The esophageal NO formation in healthy volunteers (n=5) after three days of low nitrate diet. Measurements were performed during baseline (pH 7) and after 30 min of acid perfusion (pH 1). The procedure was repeated during salivary deviation on a second study day. The subjects were then supplied with a dental suction device and were instructed not to swallow. Panel b. Nitric oxide output after an acute nitrate load (200 mg potassium nitrate solution 40 min before the examination, n=5). NO formation was measured during baseline (pH 7) and after 30 min of acid perfusion (pH 1), without or with salivary deviation.

Taken together the experiments demonstrated that a nitrate rich diet contributes to an increased esophageal NO formation capacity, provided that the lumen is acidified. However, it was obvious from the experiments that NO also occurred to some degree during salivary deviation and acid conditions. It was suspected that the salivary deviation was insufficient and that small amounts of saliva reached the acidified part of the esophageal lumen.

In order to avoid acid-dependent NO formation completely, the lumen was rinsed by infusion of saline and pH was allowed to return to above 5 as confirmed by simultaneous pH-metry. In these experiments, 30 min after the termination of the acid perfusion a substantial NO response persisted in most cases. This was not the case following control perfusion with saline. The interpolation of these results was that some juxtamucosal NO formation still occurs also when non-enzymatic NO formation cannot, by definition, take place.

Mucosal biopsies were taken after the experiments and immunohistochemistry revealed a distinct staining for iNOS in the esophageal squamous epithelial cells in all biopsies, whereas immunoreactivity for eNOS and nNOS was not observed. Western blot analysis of the esophageal mucosal tissue confirmed the presence of a 130 kD protein corresponding to iNOS. Furthermore, additional support for the presence of mucosal iNOS was obtained using RT-PCR, which detected iNOS mRNA in all the biopsies tested. These findings indicate that some NO is formed upon luminal acid exposure also by the L-arg/NO pathway in the esophageal epithelium.

In conclusion, it is evident that intra-esophageal NO formation is a prominent phenomenon provided that the lumen is acidified. The bulk of luminal NO originates from reduced nitrite, but a small portion is very probably a product of degradation of L-Arg by epithelial iNOS.

Regulatory function of luminal NO and CO_2 (paper II)

Mucosal irritation by acid activates the oral salivary glands via the esophago-salivary reflex. It has been suggested that impairment of this mucosa protective reflex contributes to gastro-esophageal disease (Sonnenberg et al 1982). In paper II, we explored some aspects of the triggering stimuli that elicit the esophago-salivary reflex. One obvious stimulus for such alkaline salivation is the acid itself. However, there are theoretically two acid-dependent molecules with messenger potential in the esophageal lumen: carbon dioxide (CO_2) formed when refluxed hydrogen ions (H^+) meet bicarbonate in swallowed saliva; and, as discussed above, NO that is formed when salivary nitrite reacts with acid-reflux:

$$HCO_3^- + H^+\gamma \quad CO_2 + H_2O$$
$$NO_2^- + H^+\gamma \quad \gamma \quad NO$$

We investigated whether the signalling molecules NO and CO_2 participated in the regulation of salivary volume secretion and neutralising capacity in response to luminal acid exposure (modified Bernstein-test).

Salivary secretion is greatly influenced by gustatory stimulation, olfactory sense, thinking of food, sensation of hunger, mastication, and by intra-esophageal mechanical and chemical stimulation and it was not surprising that baseline salivary volume secretion and titratable

alkalinity differed markedly between individuals, ranging between 3.6 and 51 mL/10 min, and 6.0 to 36 mEq/L respectively. It follows that the calculated alkaline secretion ranged between 4.2 and 143 μEq/min (median = 20.0 μEq/min, 48 measurements).

Intra-esophageal acidification induced a prompt increase in both salivary volume and alkalinity. Furthermore, salivary volume secretion increased significantly more when nitrite was added in comparison with acid alone, particularly during the first 10 min. Additions of bicarbonate alone, or together with nitrite, tended to decrease the salivary volume secretory response. Importantly, the alkalinity of the saliva was not altered by addition of nitrite or bicarbonate. It follows that the salivary alkaline secretion increased upon exposure due to increased volume secretion only. The initial part of this response was significantly larger in the presence of nitrite compared to acid alone. This was not observed when bicarbonate was added or nitrite and bicarbonate were given in combination (Fig 3). The addition of bicarbonate to the acid-solution will somewhat increase the pH in the solution an effect that reduces the acid load and possibly the stimulus. However, the addition of nitrite also neutralises hydrogen ions when NO is formed, but in this case a significant salivary stimulation occurred. The stimulus thus appears to be related to NO rather than to pH.

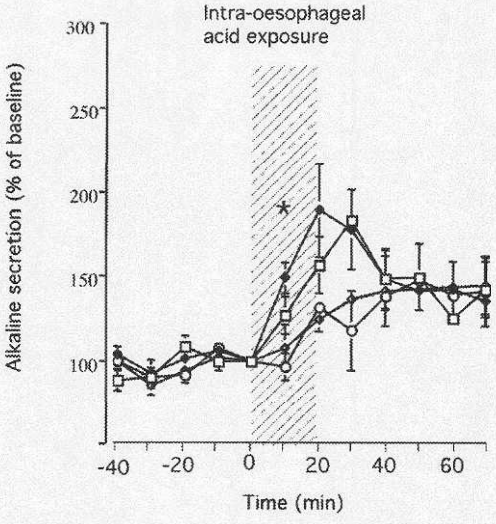

Figure 3
The salivary alkaline secretion in response to a single (20 min) intraluminal acid exposure with 100mM(□) acid (n=9), (◆) acid+nitrite (n=9), (□) acid+bicarbonate (n=8), (◆) acid+ nitrite + bicarbonate in combination (n=8) in human esophagus. The data shown is expressed as mean values ± SEM and represents the mean percentage change from baseline. *P≤0.05, significant difference between groups (Wilcoxon signed rank test).

The continuous infusion of acid used in the Bernstein test (20 min) does not mimic the short lasting pH-deflections that occur upon endogenous gastro-esophageal reflux. We therefore tested whether the salivary responses to two separate acid exposures differed from the one obtained after a long-lasting acidification of similar total duration. Unlike the comparable first 10 min expression in the previous experiments, the short (10 min) acid exposure stimulated

neither salivary volume secretion nor titratable alkalinity (n=7). The reason for this difference is not known. However, when nitrite had been added to the acid solution, volume secretion increased transiently with a peak reaching +22 ± 2.6% compared to baseline, but titratable alkalinity remained largely constant. It follows that the first 10 min acidification raised salivary alkaline secretion only in the presence of nitrite ($p<0.05$) (Fig 4).

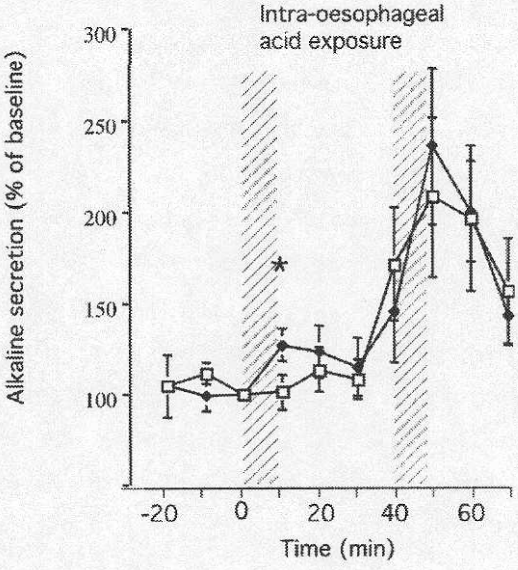

Figure 4
The salivary alkaline secretion in response to a repeated 10 + 10 min intra-esophageal acidification with 100mM(□) acid (n=7) or (◆) acid+ nitrite (n=7) in a human esophagus. The data shown are expressed as mean values ± SEM and represents the mean percent change from baseline. *P≤0.05, significant difference between groups (Wilcoxon signed rank test).

This confirming the finding that presence of nitrite accelerates the induction of the esophago-salivary reflex. Upon a second intraluminal acidification performed 30 min after the first one, salivary alkaline secretion increased markedly, regardless of the presence of nitrite (Fig 4). The accumulated salivary alkaline secretion during 70 min following a single 20 min intra-esophageal acid exposure ranged between 107 to 398 μEq. In quantitative terms this response was similar to the one obtained when the acidification was divided into two separate periods of each 10 min. In conclusion, nitrite (NO formation) accelerated the induction of the esophago-salivary reflex. The absolute magnitude over time of the salivary alkaline response was independent on if acid expression was continued or intermittent (10+10 min).

INVESTIGATION OF RENIN ANGIOTENSIN II SYSTEM (PAPERS III AND IV)

Expression of the renin-angiotensin system (paper III)

In paper III, we explored the presence of RAS components in the normal human esophageal body. Only volunteers with no history of even mild gastroesophageal reflux disease and with normal mucosal appearance at endoscopy and histological analyses were included. The muscular specimens of the distal esophageal body were taken from patients with malignant disease. Precautions were taken not to include tissue in close relation to the pathological process as assessed macroscopically and confirmed by normal histological appearance.

Real-time qPCR analysis of the esophageal specimens, mucosa and muscular tissue, all showed existence of RNA indicating transcription of angiotensinogen, renin, ACE, AT_1 receptor and AT_2 receptor genes.

Immunohistochemistry performed on mucosal biopsies revealed a distinct staining for the AT_1 receptor in the epithelium and most obvious in the stratum basale and stratum spinosum. AT_1 receptor immunoreactivity was also detected in the blood vessel walls supplying the epithelium and in the lamina propria. Immunostaining for AT_2 receptors was detected in the epithelium and most distinctly in stratum spinosum and in blood vessel walls supplying the epithelium in the lamina propria. Immunoreactivity for ACE was found in the capillary walls located at the tip of the papillae and in the blood vessel walls in the lamina propria. Immunohistochemistry performed on muscle tissue samples revealed a distinct staining for AT_1 receptor in the muscular bundles and in the wall of the small blood vessels close to the muscle cells. Immunoreactivity for AT_2 receptor was faint in the muscle cells, but was observed in blood vessels wall. ACE immunostaining in the muscular tissue was also found in the vessels wall.
The results strongly indicated the existence of a local renin-angiotensin system in the normal human esophageal mucosa and wall musculature. The localisation of ACE and Ang II receptors indicated an Ang II-formation in both the mucosa and the musculature.

Angiotensin II and esophageal muscle (paper IV)

In paper IV, we focused on potential muscular action by Ang II, particularly contractile actions and related receptor pharmacology.

Ang II caused a concentration dependent rise in tension of both longitudinal and circular smooth muscle preparations *in vitro*. The maximal increase in longitudinal smooth muscle tone was 17.9 ± 3.6% (EC_{50} value = 1.2 ± 0.4 x10^{-7} M Ang II) and for circular smooth muscle 23.4 ± 4.3% (EC_{50} value = 1.0 ± 0.4 x10^{-7} M Ang II) of the bethanechol reference contraction. The AT_1 receptor antagonist losartan at 10^{-5} M abolished Ang II induced contractions. At a concentration of 10^{-7} M losartan caused a significant rightward shift of the concentration-response curve in both the longitudinal (EC_{50} value = 7.3 ± 1.0 x10^{-7} M; $P<0.01$) and the circular (EC_{50} value = 1.4 ± 0.6 x10^{-6} M; $P<0.01$) smooth muscle as compared with Ang II alone (Fig 5a). In contrast, the AT_2 receptor antagonist PD123319 (10^{-7}M or 10^{-5}M) caused no significant modification of concentration response curves by Ang II, neither in the longitudinal (EC_{50} value = 2.0 ± 0.7 x10^{-8} M) nor in the circular (EC_{50} value = 1.4 ± 0.5 x10^{-8} M) muscle preparations (Fig 5b). When losartan and PD123319 were added in combination the response curve was generally similar to when losartan was given alone (data not shown in figure).

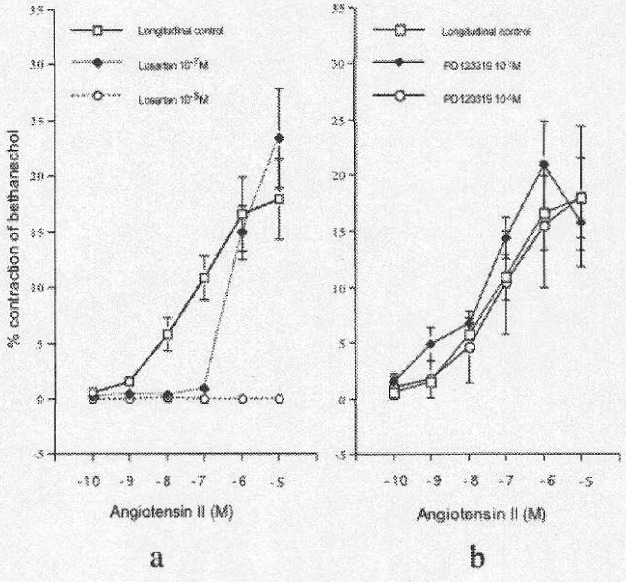

Figure 5

Concentration-response curve showing the contractile effect of Ang II (□) in the human longitudinal (18 strips from 9 patients) smooth muscle. Panel a: treatment with the AT_1 receptor antagonist losartan 10^{-7} M (◆) and 10^{-5} M (□) (5 and 7 strips from 3 and 4 patients, respectively). Panel b: treatment with AT_2 receptor antagonist PD123319 10^{-7} M (◆) and 10^{-5} M (□) (5 and 7 strips from 3 and 4 patients, respectively). Data are plotted as means±SEM.

The *in vitro* experiments provided evidence that Ang II mediates AT_1 receptor dependent contractions in both longitudinal and circular smooth muscle of the distal human esophagus. These results evoked the question if endogenous Ang II takes part in distal esophageal motor activity *in vivo*. The latter was assessed by recording esophageal contractile events in healthy

volunteers with or without pre-administration of candesartan. This compound was ingested as a single dose of 16 mg 10-12 h before recordings to obtain an optimal AT_1 receptor interference (Azizi et al 2004, Gleiter et al 2002). We measured the following contractile events: the amplitude of primary (swallow induced) peristaltic wave; swallow-associated axial shortening of the esophagus; the tonic LES pressure and length of the HPZ; and the reinforced contraction in LES that occurs after a transient lower esophageal sphincter relaxation (TLESRs elicited by air distension of the stomach).

Voluntarily induced swallowing induced a typical propulsive contraction that peaked at the distal part of the esophagus immediately after that the high-pressure-zone had vanished and a retraction of the PD step-up had occurred. By comparison, peak-pressures were significantly lower following dry swallows in presence of candesartan ($p<0.05$), and tended to be lower also following wet swallows, but that difference did not attain statistical significance. The length of oral retraction of the PD step-up was similar independent of treatment with candesartan (Table 1).

The LES was visualized as a 2-4 cm high-pressure zone (HPZ), and the length of the HPZ was significantly shorter in presence of candesartan ($p<0.05$). The basal pressure of the HPZ was 16-20 mmHg above intragastric pressure and tended to be lower after candesartan, but that difference did not attain statistical significance (Table 1). Insufflation of 500 ml air into the stomach induced transient relaxations of the LES allowing gas evacuation (belching). The TLESR was followed by a profound contraction that after 10-30 s was reduced to a sustained HPZ with more moderate intraluminal pressure. The peak pressure of the "post belch" contraction" in the LES was of a similar order of magnitude with or without candesartan treatment (Table 1).

Table 1. Contractile activity in distal esophagus of healthy volunteers (n=7) at two separate study days in the absence (control) or presence of an angiotensin II type I receptor antagonist (candesartan; Atacand® 16 mg *p.o.* administered 10-12 h prior to recordings).

	Control	Candesartan
Primary peristalsis, manometry		
Peak pressure (mm Hg)		
Dry swallow	80.9 ± 6.5	-24 % ± 7.4 (#)
Wet swallow	87.4 ± 12	-12 % ± 13.7
Primary peristalsis, axial movements		
PD step up retraction (cm)		
Dry swallow	2.3 ± 0.3	+8 % ± 17.4
Wet swallow	2.3 ± 0.3	-13 % ± 17.4
LES		
LES basal tone (mm Hg)	18.3 ± 3.7	-27.6 % ± 14.2
Basal HPZ length (cm)	3.1 ± 0.14	-18.2 % ± 5.5 (#)
Post-TLESR contraction (mmHg)	65.3 ± 6.1	+7 % ± 10.9

Definitions: PD= potential difference; HPZ=high-pressure zone; LES= lower esophageal sphincter; TLESR= transient lower esophageal sphincter relaxation.

These *in vivo* results indicate that Ang II is involved in esophageal contractions via the AT_1 receptor. Furthermore, our data suggest a differential involvement of Ang II in the co-ordination of distal esophageal contractile events influencing primarily the circumferential contractions that create peristaltic pressure-waves in the esophageal body and the HPZ in the LES region.

CONCLUSIONS

The following conclusions were drawn from Paper I to IV:

- two sources exist for esophageal luminal NO formation, both dependent on the presence of acid in the esophageal lumen: 1) non-enzymatic chemical reduction of salivary nitrite, a mechanism related to dietary intake of nitrate, 2) enzymatic NO-generation by iNOS expressed in the epithelium

- esophageal intraluminal NO facilitates the acid-induced esophago-salivary reflex whereas, intraluminal CO_2 seems to have negligible effect

- there exists a local renin angiotensin system in normal human esophageal mucosa and wall musculature

- Angiotensin II mediates an AT_1 receptor dependent contractile effect on the smooth muscle of the distal human esophagus

- Angiotensin II exert differential effects in the co-ordination of distal esophageal contractile events influencing primarily the circumferential contractions that create peristaltic pressure-waves in the esophageal body and the HPZ in the LES region

GENERAL DISCUSSION

The results demonstrate that two sources exist for the esophageal luminal NO formation; chemical reduction of salivary nitrite and enzymatic degradation of L-arginine in the epithelium, both dependent on the presence of acid in the esophageal lumen. Salivary alkaline secretion increased markedly following intraluminal acid exposure and data suggest that intraluminal NO facilitates initiation of the acid-induced esophago-salivary reflex. Mucosal biopsies and muscular tissue both indicate the existence of a local renin-angiotensin system in the normal human esophagus. Angiotensin II stimulates the human distal esophageal musculature *in vitro* via the AT_1 receptor subtype, and administration of the AT_1 receptor antagonist candesartan reduces the amplitude of swallows induced peristaltic contractions and the length of the high-pressure zone *in vivo*.

Juxtamucosal NO formation

Nonenzymatic NO production occurs in the stomach when luminal pH is < 3 (pKa about 3.2-3.4). Swallowed nitrite reacts with gastric acid and large quantities of NO and other nitrogen species, e.g. NO_2 and N_2O_3 are formed intraluminally (Benjamin et al 1994, Lundberg et al 1994, Åneman et al 1996, McColl 2004). The present investigation (I) shows that such non-enzymatic intraluminal NO formation occurs also in the esophagus in case of acid reflux. The results also suggest that NO is locally produced by NO-synthases within the squamous epithelium in response to acid exposure. Epithelial NOS has been little investigated in the esophagus and has been associated with pathological conditions such as cell transformation, e.g. Barrett´s esophagus and adenocarcinomas (Wilson et al 1998). Furthermore, Tanaka et al. (1999) have shown that weak iNOS immunoreactivity is normally expressed in the basal and parabasal layers of esophageal mucosal squamous epithelium. Recently Bove et al (2005) also described juxtamucosal enzymatic NO formation in ferret esophagus and suggested a function related to epithelial integrity.

The finding of salivary (nitrate) dependent NO formation in the acidified esophagus is of particular interest. Nitrate is a natural component (an essential plant nutrient) of all cereals, fruit and vegetables, and green plants organs (e.g. spinach leaves) usually contain the highest concentrations (Duncan et al 1997). That study also showed a dietary dependent NO formation related to nitrate intake. The role of this chemically derived NO formation in the stomach is probably to take part in the luminal gastric defence against swallowed pathogens

(Dykhuizen et al 1996) and to be an acidity-signalling factor for mucosal functions e.g. gastrin release (Holm et al 2001).

The present study (II) demonstrated that intra-esophageal NO formation may have a signalling function in the mediation of salivary volume in response to luminal acidity. Salivary secretion has previously been shown to increase after acid exposure (Helm et al 1984, Dutta et al 1992). In the present study it was observed that the addition of nitrite in the acid solution accelerated the onset of the initial 10 min part of the salivary secretion response. This initial part of acid-exposure is the most relevant with regard to real gastro-esophageal reflux, which seldom exceeds a few minutes.

The general opinion regarding the induction of the esophago-salivary reflex is that the presence of acid on the surface of the esophageal mucosa results in the diffusion of hydrogen ions through the thin mucus-buffer layer into the mucosa. The lowered intramucosal pH then stimulates chemoreceptors of the submucosal nerve plexus. The chemoreceptors will convey the stimulatory signal via the afferents to the CNS and activate a reflex stimulation of salivary secretion (Sarosiek et al 2000). NO is a good signalling molecule, and it may be speculated that juxtamucosal NO activates neural reflexes or the release of other agents from the epithelium. NO may actually be an intermediate messenger between luminal acidity and mucosa protective mechanisms. Such activation of mucosal protection in response to luminal acidity has previously been reported regarding duodenal mucosal alkaline secretion in rat and pig duodenum as well as the acid dependent inhibition of gastrin release from the human antrum (Holm et al 1997, 1998, 2000, 2001). The topographical organisation of the nitric oxide synthase in the epithelium in combination with luminal non-enzymatic NO, may create particular conditions for NO gradients through the mucosa (fig 6).

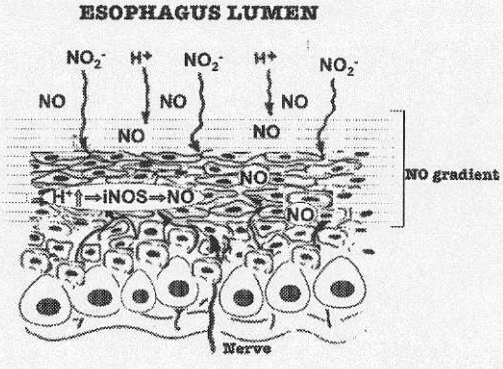

Figure 6.
Hypothetical depiction of NO gradient through the esophagus epithelium. Intra-epithelial NO formation by the L-arg/NO pathway may be activated by H^+ diffusion and salivary (nitrate) dependent NO formation, immediately formed upon luminal acidification create a NO concentration gradient through the epithelium serving for induction of the esophago-salivary reflex via afferent nerve.

If NO is a main contributor to the activation of a subepithelially located afferent receptor it is important to consider the kinetics and short distance of action of this molecule. Acid induced intra-epithelial NO formation by the L-arg/NO pathway have some lag time due to restricted H^+ diffusion in the epithelium, but could then be quite long lasting, thus persisting also after that the luminal stimulus (H^+) has been eliminated. Non-enzymatic NO formation, on the other hand, is instantly formed upon luminal acidification. Theoretically it creates a marked concentration gradient through the epithelium even before tissue pH is lowered. It follows that the esophago-salivary reflex is initiated before that the epithelium is acidified.

Simultaneously with non-enzymatic NO formation also CO_2 is generated in the neutralising reaction between hydrogen ions and bicarbonate; the latter being present in both the saliva and submucosal glands secretion. Previous studies have shown that CO_2 acts as a mediator of the acid-induced increase in duodenal mucosal alkaline output in rats (Holm et al 1998). In the present study (II), a CO_2-containing acid solution exposing the esophageal mucosal had no clear-cut effect on the salivary response. Also the combined presence of bicarbonate and nitrite in the acid solution was without effect, suggesting an inhibitory action of CO_2 on the initiation of the esophago-salivary reflex. Recently it was shown that NO and CO_2 may act in an opposing direction. Holm et al (2001) showed that acid-induced inhibition of meal-stimulated gastrin release is partly dependent on NO generated from swallowed salivary nitrite, whereas CO_2 instead seems to potentiate gastrin release. The present experiments on CO_2 cannot be regarded as conclusive and further studies are needed to fully understand the relative effects of CO_2 and NO in the esophagus.

In the situation of gastro-esophageal reflux disease (GERD), patients often report aggravated symptoms related to certain food ingredients, several of them having high nitrate levels (Nebel et al 1976). A variety of factors have been shown to decrease the lower esophageal sphincter, including fatty foods and alcohol (Nebel et al 1976). Based on the finding in (I) and (II) it is tempting to suggest that juxtamucosal NO formation plays a role in GERD. Bove et al (2003) showed that nitrate supplementation had no significant effect neither on the sphincter resting tone nor on motor function in healthy volunteers or in GERD patients. However, the acute nitrate load, used by Bove et al (2003) increased the nitrite in saliva only marginally from basal value of approximately 2 μM to 12 μM, and additional studies with repeated nitrate dosing are desirable.

Nitric oxide in high doses is known to be mutagenic due to the fact that NO reacts with oxygen to form N_2O_3 which can damage DNA directly or indirectly via generation of N-nitroso compounds (McColl 2004). The major factor preventing the formation of N-nitroso compounds from nitrite entering the acidic stomach is ascorbic acid, which is actively secreted in human gastric juice as well as being present in most ingested foods. Ascorbic acid has also been suggested to be present in saliva (McKnight et al 1997).
Although the harmful and potentially carcinogenic activity of N- nitroso compounds cannot be dismissed, epidemiological evidence for this association is principally lacking (Duncan et al 1997, Dukhuizen et al 1996, McColl 2004). It seems unlikely that luminal NO formation *per se*, being a physiological event in the stomach and at least occasionally in the esophagus, should contribute significantly to carcinogenesis. To be carcinogenic it is reasonable to assume that an inflammation has to be simultaneously present with generation of oxygen radicals like superoxide. NO and superoxide can form peroxynitrite which in turn is highly cytotoxic and mutagenic (Grisham et al 2000).

RAS in the esophagus

The present investigation (III) also demonstrated the existence of a local renin-angiotensin system in human esophageal mucosa and wall musculature.
The traditional view of the renin-angiotensin system is that of a classical endocrine system with its key components associated to the renal, hepatic and lung circulations (Jackson 2001). However, data have accumulated during the last decades suggesting that there exist also local (tissue-based) renin-angiotensin systems (Jackson 2001). The expression analyses in the present study showed gene transcripts for angiotensinogen, renin, ACE, AT_1 and AT_2 receptor both in the esophageal mucosa and wall musculature. These data strongly suggest that a local RAS is operational in the human esophagus.

The biological actions of RAS are largely related to the effects of the octapeptide angiotensin II (Ang II) and its binding to specific receptors, the AT_1 and AT_2 receptors (de Gasparo et al 2000). Furthermore, many actions mediated by AT_1 receptors are inhibited by concomitant binding of Ang II to the AT_2 receptor (if present) (de Gasparo et al 2000).

Ang II has been shown to exert potent contractile effects via AT_1 receptors in different gastrointestinal smooth muscles such as rat and opossum lower esophageal sphincter and in guinea pig ileum, gastric fundus and gall bladder (Mukhopadhyay et al 1978, Leung et al

1993, Fan et al 2002, de Godoy et al 2004). The present *in vitro* experiments (IV) demonstrated that Ang II has a marked contractile effect both on longitudinal and circular smooth muscles of the distal esophageal body. Furthermore, sensitivity to losartan indicates mediation through the AT_1 receptor. On the other hand, the AT_2 receptor antagonist PD123319 had no significant effect on Ang II-induced contraction. That finding is supported by our previous morphological evaluation demonstrating that only the AT_1 receptor subtype is localised in the muscle cells.

However, it should be noted that AT_2 receptor expression might vary according to tissue conditions. For example hypoxia and inflammation promote AT_2 expression and may differ according to age, species, tissue type and pathophysiological state (De Gasparo et al 1999). It remains to be tested if there exists conditions where AT_2 receptor expressions are altered and if this is associated with an altered response to Ang II.

The control of esophageal motility has a complex organisation involving extrinsic innervation and local reflex arcs. In addition to classical excitatory (e.g. acetylcholine) and inhibitory (e.g. VIP, nitric oxide) neurotransmitters a number of co-transmitters have been postulated to be involved (Wörl J 2005). Also, the mechanisms of peristaltic control are different between striated and smooth muscle, yet the peristaltic contraction sweeps from one to the other muscle type without a detectable break (Wörl et al 2005).

Half of the muscle mass in the esophagus and, in the entire digestive tract, is composed of a longitudinal muscle layer, yet its role in gastrointestinal tract motility and bolus movement is not well understood, partly because of the difficulty in recording its function. Recently studies using ultrasound imaging have defined longitudinal muscular contractions on the basis of the law of mass, as an increase in muscular thickness without a concomitant luminal pressure increase (Nicosia et al 2001, Mittal et al 2005). In the present *in vivo* experiment (IV) esophageal motor activity was assessed by combined intraluminal multiple manometry and transmucosal PD-measurements. The methodology using the PD-step up (the difference in PD between esophageal and gastric mucosa) to measure the longitudinal muscular contraction seems to be of great value. However, occasional presence of H^+ in the esophageal lumen will influence PD, so the results obtained during acid reflux must be interpreted with care.

Interestingly, both the primary peristaltic contraction and the length of the HPZ were inhibited by pre-treatment with the AT_1 receptor blocker candesartan. This compound was ingested as a single dose of 16 mg 10-12 h before recordings. This time and dose was chosen based on previously reports on plasma concentration and functional AT_1 receptor interference (the latter assessed as effects on renin-release) (Gleiter et al 2002, Azizi et al 2004). Although distinct effects on esophageal circular muscle activity were discovered, it appears unlikely that candesartan and related compounds influence esophageal motor functions to any considerable degree. However, studies on long-term administration of these commonly prescribed drugs focusing on esophageal disorders have so far not been conducted.

Interactions between juxtamucosal NO and RAS in the esophagus?

The RAS interacts with NO-dependent regulation on several levels. For example effects of AT_2 receptor activation in several organ system have been reported to lead to bradykinin release in turn activating the L-arg./NO pathway (Siragy et al 1996, Siragy et al 1997, Berry et al 2001, Gohlke et al 1998). In the kidney NO formation may also be stimulated directly via AT_2 stimulation, independent of bradykinin (Volpe et al 2003). In the small intestine AT_2 receptor activation increases fluid and sodium absorption and this process is dependent on NO and subsequent cGMP formation (Jin et al 1999). Both bradykinin and mucosal NO formation have been shown to regulate duodenal mucosal alkaline secretion (Chen et al 1997, Holm et al 2001). Previous studies in our group have shown that duodenal mucosal alkaline secretion can be stimulated by the AT_2 receptor agonist CGP42112A and inhibited by AT_2 receptor antagonist PD123319 (Chen et al 1997, Ewert et al 2003). Furthermore, AT_2 receptors were reported to be located in the duodenal mucosa/submucosa (Johansson et al 2001). AT_2-mediated duodenal alkaline secretion was proposed to be mediated via bradykinin, via BK2 receptor situated in the duodenal crypt epithelium although a direct link to NO was not reported (Ewert et al 2003).

It can thus be speculated that interactions between AT_2 receptors and L-arg/NO pathway occur also in the esophagus. Immunoreactivities to both AT_1 and AT_2 receptors were found in the wall of small blood vessels supplying the epithelium and the muscle layers (III), suggesting involvement of Ang II in the regulation of local microcirculation, an action where NO is involved in (Anggard 1994, Sandler et al 1993). AT_1 and AT_2 receptors were also found in the epithelium (III) where NOS-dependent liberation of NO also was found (I). Ang II stimulates growth and/or proliferative responses via the AT_1 receptor in various tissues,

actions that are counterbalancing by the AT_2 receptor (de Gasparo et al 2000). In addition, the AT_2 receptor promotes apoptosis in a wide diversity of cell types (Volpe et al 2003), an ability that NO also has (Shen et al 1998). Endogenous NO formation contributes to low intercellular permeability, by affecting the intercellular tight junctions (Salzman et al 1995) and, as mentioned above, is involved in the alkaline neutralisation by the duodenal mucosa (Holm et al 2001). It may thus be speculated that Ang II via AT_1 and AT_2 receptors exert tissue remodelling (cellular growth/differentiation/ion transport/tight junction/apoptosis), an action most likely also involving the L-Arg/NO pathway in human esophageal epithelium.

Epilogue

The present thesis is the result of an integrative physiological approach and points at the existence of two regulatory systems, juxtamucosal nitric oxide (NO) generation and the renin-angiotensin system (RAS) in normal human esophagus. Further studies are needed to elucidate the functional potential of these regulatory systems during various physiological conditions. Since gastroesophageal reflux disease (GERD) is very common and has great input on quality of life, the pathophysiology for this disorder is of much interest. Research concerning presence and function of the nitric oxide system and RAS, in relation to GERD is strongly recommended.

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