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Sources of Intra-Oesophageal Nitric Oxide Production Following Intraluminal Acid Exposure

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Background: The aim of the present study was to assess luminal nitric oxide (NO) levels in the oesophagus during baseline and acidic conditions and to clarify the sources of such oesophageal NO formation. **Methods:** Healthy volunteers received an intra-oesophageal infusion of either HCl (100 mM) or NaCl (150 mM) on two separate study days. After a low nitrate diet, nitrate load or no dietary restrictions/pretreatment, direct intraluminal measurements of NO formation were performed using a tonometric technique. Endoscopy was performed and mucosal biopsies were taken and analysed by means of immunohistochemistry, Western blot and RT-PCR. **Results:** No intra-oesophageal NO was detected during baseline conditions with pH neutrality. During the infusion of HCl the NO levels rose dramatically to around 12000 ppb. This high rate of NO formation fell by 95% following deviation of saliva. NO formation after an acute nitrate load was almost doubled during acid perfusion compared to control. Immunohistochemistry demonstrated distinct staining for iNOS in the oesophageal squamous epithelial cells, and Western blot and RT-PCR confirmed the presence of iNOS. **Conclusion:** Two sources exist for intra-oesophageal NO formation, both dependent on the luminal acidity: 1) chemical reduction of salivary nitrite, a mechanism related to dietary intake of nitrate, and 2) NO formation within the oesophageal mucosal epithelium by enzymatic degradation of L-arginine. In the latter case, the NO synthase has antigenic characteristics, indicating the inducible isoform, although a functional behaviour suggests an unconventional subtype.

Key words: Nitrate; nitric oxide synthase; nitrite; non-enzymatic nitric oxide; oesophagus; salivary

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Nitric oxide (NO) has been shown to be involved in the regulation of several gastrointestinal functions such as motility (1), blood flow (1) and epithelial permeability and mucosal protection (2). NO also plays a role as a cytotoxic factor in host defences (3, 4). Theoretically, there are two sources of NO production in the upper gut: enzymatic degradation of L-arginine and non-enzymatic chemical reduction of nitrite in an acidic environment. Enzymatic NO production involves specific enzymes, collectively called nitric oxide synthase (NOS). Two isoforms of NOS (eNOS and nNOS) are constitutive and can rapidly be activated by changes in intracellular Ca^{2+} to produce NO for several minutes (5).

The inducible isoform of NOS (iNOS), when expressed, produces NO for several days and in large quantities, even at low Ca^{2+} levels (5). Especially during inflammatory states, iNOS-mediated NO production by macrophages, for example, is important in several pathophysiological conditions.

Non-enzymatic NO production occurs in the upper gut when luminal pH is <3. Absorbed dietary nitrate, secreted by the salivary glands, is rapidly reduced to nitrite by oral

bacteria (6). Swallowed nitrite reacts with gastric acid and large quantities of NO are formed intraluminally (3, 7, 8). Such non-enzymatic 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 (9–11). The rate of gastric non-enzymatic NO formation varies with the luminal nitrite load, which in turn is dependent on the diet and salivary secretion (12–14).

There is therefore a large NO-forming capacity in the acidic stomach after the swallowing of nitrite-containing saliva. There are good reasons for believing that similar high NO levels occur in the oesophagus when saliva meets an acid reflux. The aim of the present study was to assess the luminal NO levels in the oesophagus during baseline and acidic conditions and to clarify the sources of such oesophageal NO formation.

Materials and Methods

Subjects

Sixteen healthy volunteers (mean age 36 years, range 18–

60 years, 4 women) participated in the study. The subjects underwent a full examination, including past medical history and a physical examination, and all were *Helicobacter pylori*-negative as tested by the ^{13}C-urea breath test (15). The project was approved by the Ethics Committee of Sahlgrenska University Hospital and informed consent was obtained from each subject.

Technical description

After an overnight fast, the subjects were supplied with a double-lumen nasogastric catheter (Ch12) with a silastic balloon at the distal end. Using a manometric assembly and 'station pull-through', the lower oesophageal sphincter (LES) was identified and the tip of the catheter was placed 5 cm above the high pressure zone. The manometric assembly also included an infusion site positioned 15 cm above the LES. Simultaneous recording was performed for pH during the studies. One pH glass electrode (Type 440-M3, Ingold AG, Urdorf, Switzerland) was inserted via the other nostril and placed 10 cm above the LES. The intraluminal pH electrode was connected to a computer (Power Macintosh 7100/80, Apple Computer Inc., Cupertino, Calif., USA), which recorded and displayed the pH online. To minimize oesophageal expression to swallowed nitrate/nitrite, the subjects were supplied with a dental suction device and were instructed not to swallow. They were randomized to receive oesophageal infusion of either hydrochloric acid or saline on two separate study days. The second examination was performed between 2 and 30 days after the first one.

NO tonometry catheter and chemiluminescence analysis

The gas-permeable silastic balloon of the tonometer was inflated with 5 ml of room air. Equilibration between NO in the intra-oesophageal atmosphere and the air of the inflated balloon was allowed for 10 min (16). The equilibrated gas was then transferred into a gas-tight 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).

Oesophageal tissue specimens

At the end of some experiments, the assembly was removed, and an endoscopy was performed and 8–10 biopsies were taken in the distal oesophagus 5 and 6 cm above the gastro-oesophageal junction (Z line). Six to eight biopsies were immediately frozen in liquid nitrogen and stored at $-80\,°C$ and the rest of the biopsies were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (pH 7.4) for 60 min at $4\,°C$. The fixed biopsies were then frozen in liquid nitrogen and embedded in OCT compound (Sakura Finetek USA Inc., Torrance, Calif., USA).

Immunohistochemistry

The frozen mucosal tissue was cut into 8–10 µm sections and mounted on slides. The slides were first pre-incubated with serum block (ImmunoCruz Staining System, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and then incubated with primary antibodies against different isoforms of NOS overnight at $4\,°C$. Polyclonal antibodies against iNOS, eNOS and nNOS which had been raised in rabbits (Affinity BioReagents, Boulder, Col., USA) were used at dilutions of 1:800, 1:500 and 1:800, respectively. As control, sections were incubated overnight with normal rabbit IgG (Immuno-Cruz Staining System, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) instead of the primary antibody. 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. Immunostaining was scored arbitrarily with regard to localization using a Nikon Microphot FXA (Nikon Corporation, Tokyo, Japan).

Western blot analysis of iNOS

The frozen specimens were sonicated in a PE buffer (10 mM potassium phosphate buffer, pH 6.8 and 1 mM EDTA) containing 10 mM 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane sulphonate (CHAPS: Boehringer Mannheim, Mannheim, Germany), aprotinin (1 µg/ml, Boehringer Mannheim), leupeptin (10 µg/ml, Boehringer Mannheim), pepstatin (10 µg/ml, Boehringer Mannheim) and Petablock (1 mg/ml, Boehringer Mannheim). The homogenate was then centrifuged ($10,000\,g$ for 10 min at $4\,°C$) and the supernatant was analysed for protein content by the method of Bradford (17) and stored at $-80\,°C$ for further analysis. Samples, diluted in SDS sample buffer, were heated at $70\,°C$ for 10 min before they were loaded (10 µg of total protein/lane) on a NuPAGE 3%–8% Tris-acetate Gel (NOVEX, San Diego, Calif., USA). One lane per gel was loaded with prestained molecular weight standards (SeeBlue, NOVEX, San Diego, Calif., USA). After electrophoresis, the proteins were transferred to a polyvinyl difluoride membrane (Amersham, Buckinghamshire, UK). The membrane was then incubated with a specific polyclonal antibody against iNOS (Affinity BioReagents, Boulder, Col., USA). An alkaline phosphatase conjugated secondary goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and CDP-Star (Tropix, Bedford, Mass., USA), as a substrate, were used to identify immunoreactive proteins by chemiluminescence. Finally, the membranes were exposed in an LAS 1000 CCD camera (Fujifilm, Tokyo, Japan) and immunoreactive bands were quantified using the software Image Gauge (Fujifilm, Tokyo, Japan).

Reverse transcriptase polymerase chain reaction

Frozen tissue was homogenized in RNAse-free tubes

containing ice-cold RNA STAT-60 (Nordic BioSite AB, Stockholm, Sweden), using a Polytron tissue homogeniser (IKA Laboratechnik, Staufen, Germany). Total RNA was extracted according to the methods supplied by the manufacturer, following phenol-chloroform extraction and ethanol precipitation. First-strand cDNA was reverse-transcribed from 5 μg of total RNA in a total volume of 20 μl with 200 units of superscript preamplification system (Life Technologies, Täby, Sweden) and Oligo(dt) primers (Life Technologies, Täby, Sweden). Amplification by PCR was carried out in a total volume of 25 μl containing 2 μl of each RT sample, 0.3 μM of each specific primer, DEPC-treated water and High Fidelity PCR Master mix (Boehringer Mannheim Scandinavia AB, Bromma, Sweden). PCR primers, using the Amplimer Set for human iNOS included positive control (Clontech Laboratories, Inc., Palo Alto, Calif., USA), were based on the human hepatocyte iNOS cDNA sequence (18). The sense primer was 5′-CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG-3′ and the antisense primer was 5′-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3′. The cycles used were: 94 °C for 5 min for 1 cycle, 94 °C for 30 s/ 62 °C for 30 s/ 72 °C for 40 s for 35 cycles, followed by an additional 7-min cycle period at 72 °C. Electrophoresis of the 259 bp amplified products was performed on 1.5% agarose gel containing Tris acetate/EDTA and ethidium bromide. A molecular weight standard (Roche Diagnostics Scandinavia AB, Bromma, Sweden) was used as a molecular size standard. The gel was exposed in a LAS 1000 CCD camera (Fujifilm, Tokyo, Japan) and the bands were quantified using the software Image Gauge (Fujifilm, Tokyo, Japan).

Experimental protocol

The experimental procedures were performed after some dietary precautions. The subjects were (1) asked to avoid nitrate-rich food for 3 days prior to the study day (baseline conditions) or (2) given 200 mg potassium nitrate in solution (nitrate load) 40 min before instrumentation or (3) received no dietary restrictions/pretreatment. The experimental protocol continued for 75 min and started with 5 min of baseline. During a 10-min control period the balloon was inflated for assessment of the baseline intraluminal NO concentrations. Over the next 30 min, the oesophageal segment was exposed to either HCl (0.1 M) or saline (150 mM) infused at a rate of 2 ml/min. After the exposure, the oesophageal mucosa was allowed to recover for 20 min and then NO was measured again. At the end of the experiment the assembly was removed, and in some experiments endoscopy was performed after a further 30 min.

Statistics

Significant differences were identified using ANOVA and Fisher's PLSD as processed by Statview 4.1 (Abacus Concepts Inc.). A *P*-value ≤ 0.05 was considered to be statistically significant. Unless otherwise stated, values are given as mean $\pm s_{\bar{x}}$ (standard error of the mean).

Results

Baseline conditions and effect of acid exposure

Dietary precautions had to be taken into account before assessing the intraluminal oesophageal NO-forming capacity. The volunteers were instructed to avoid nitrate-rich food (vegetables, spicy, smoked or pickled food) during the 3 days preceding the examination. No intra-oesophageal NO was detected during these baseline conditions with pH neutrality. Furthermore, the absence of NO formation was independent of whether the saliva was swallowed or whether it was diverted from the oral cavity by the suction device (Fig. 1, panel A). During oesophageal infusion of HCl (0.1 M, 2 ml/ min) 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% ($P < 0.001$) following the deviation of saliva (Fig. 1, panel A).

Effects of an acute nitrate load

In a separate series of experiments, the subjects ingested a solution containing 200 mg KNO_2 40 min before instrumentation. The amount of NO_2–is comparable to eating a large lettuce, and the procedure has previously been shown to raise the salivary concentration of nitrite to submaximal physiological levels (10). NO formation was almost doubled ($P < 0.01$) 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. 1B), although this difference did not reach statistical significance. When the saliva was diverted, oesophageal NO levels were negligible during pH-neutral conditions. During acid perfusion, however, NO increased to approximately 5000 ppb (Fig. 1B), i.e. 5-fold that observed without a nitrite load (Fig. 1A).

NO formation during pH neutrality and salivary deviation

It was obvious from the experiments described above that NO also occurs in the oesophageal lumen during salivary deviation, particularly during acid conditions. It may be that the salivary deviation was insufficient and that small amounts of saliva reached the acidified part of the oesophageal lumen. Accordingly, in order to avoid such acid-dependent NO formation, the lumen was rinsed through an infusion of saline and pH was allowed to return to >5. In these experiments, too, 30 min after termination of the acid perfusion and with a confirmed intraluminal pH >5, a substantial NO response persisted in most cases (Table I). This was not the case following control perfusion with saline.

Origin of oesophageal NO formation during pH neutrality and salivary deviation

Immunohistochemistry performed on biopsies taken after oesophageal perfusion revealed a distinct staining for iNOS in
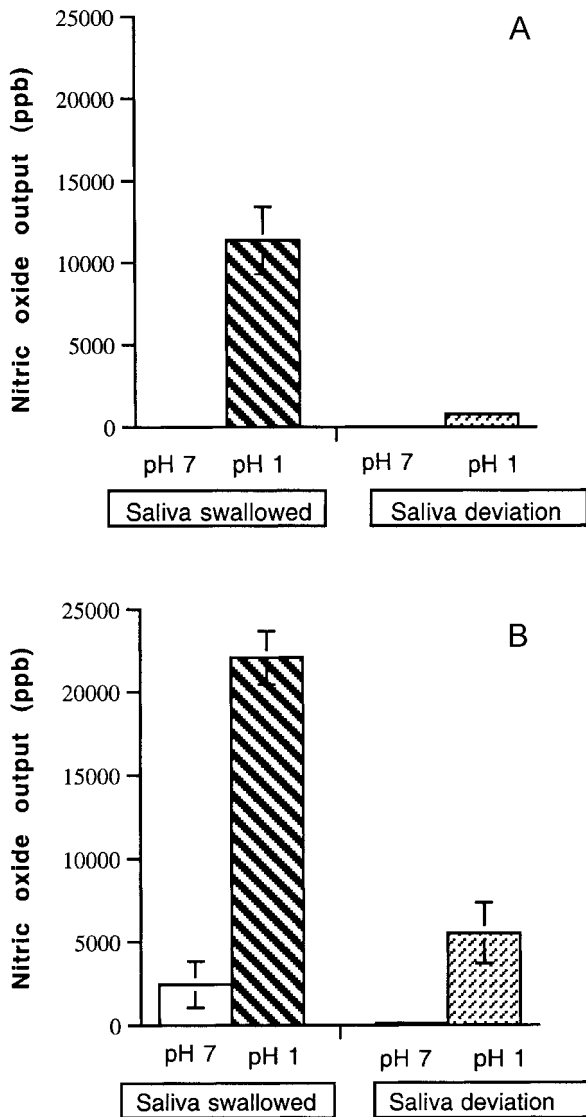
Table I. NO formation during pH neutrality and salivary deviation in the human oesophagus by use of the tonometric technique after perfusion with saline or hydrochloric acid

Subject	Saline	HCl
1	0	88
2	0	410
3	0	339
4	0	441
5	0	0
6	0	510

Six healthy volunteers participated and received 30 min of oesophageal infusion of 0.1 M acid or 150 mM saline on two separate study days. After the exposure, the mucosa rested for 10 min before being rinsed with buffered saline for 10 min to reach pH 5.0. NO formation was measured during baseline and 20 min after the exposure of acid or saline. The NO outputs in the baseline were all zero. The subjects were supplied with a dental saliva device, and instructed not to swallow.

Fig. 1. A. The oesophageal NO formation in healthy volunteers ($n = 5$) after 3 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. 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.

the oesophageal squamous epithelial cells in all biopsies, whereas immunoreactivity for eNOS and nNOS was not observed. Negative control was dark in all specimens (Fig. 2A).

Western blot analysis of the oesophageal mucosal tissue confirmed the presence of a 130 kD protein corresponding to iNOS (Fig. 2B). Furthermore, additional support for the presence of mucosal iNOS was obtained using RT-PCR, which detected iNOS mRNA in all the biopsies tested (Fig. 2C).

Discussion

The present study in humans demonstrates that intra-oesophageal NO formation is a prominent phenomenon associated with luminal acid exposure. The oesophageal NO formation was assessed using intraluminal tonometry, a method previously described for intestinal use by Snygg et al. (16). By definition, the tonometric approach allows the determination of NO levels in the close vicinity of the gas-equilibrating balloon. The NO levels obtained by this technique thus mainly reflect NO formation in the luminal compartment and by the mucosa located close to the luminal compartment.

It is now well established that swallowed nitrite, when entering the stomach, reacts with gastric acid and that large quantities of NO (and other nitrogen oxides) are formed intraluminally. The role of this chemically derived intragastric NO formation is probably to take part in the luminal gastric defence against swallowed pathogens (3, 7, 9, 12, 14, 19) and to be an acid-signalling factor for mucosal functions (11).

In the present experimental study, we found high levels of intraluminal NO in the exogenously acidified oesophagus, strongly indicating a similar phenomenon upon physiological and/or pathological gastro-oesophageal reflux episodes. The study also shows a dietary dependency related to nitrate intake. After an acute (physiological) nitrate load, the intraluminal formation of NO reached extremely high levels, with recorded values in the order of 25 ppm. A substantial part of this NO formation could be prevented by salivary deviation. Interestingly, during nitrate load, NO formation increased also in the absence of luminal acidity, indicating the existence of other nitrite-reducing agents, e.g. ascorbic acid.

During baseline conditions with salivary deviation, no NO formation occurred until the mucosa was challenged with acid. This acid-dependent NO formation was small in comparison with the situation when saliva was allowed to be swallowed and may be explained as due to activation of
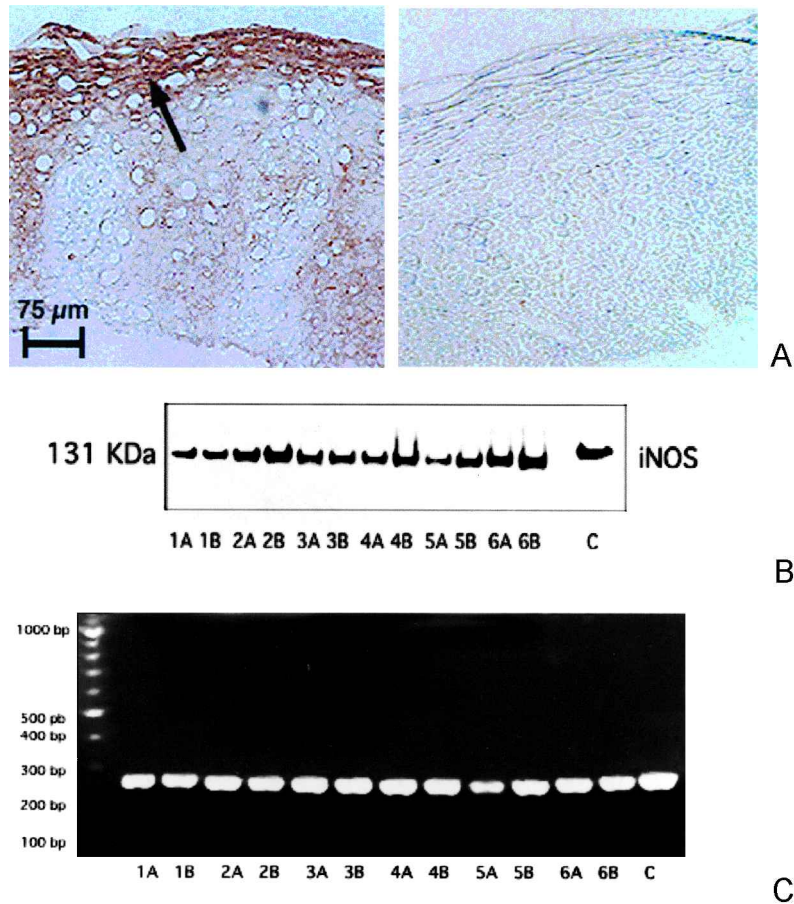
Fig. 2. A. Representative photomicrograph of immunostaining for iNOS in the biopsies from human oesophageal mucosa. Immunostaining is localized (see arrow) mainly to the upper layer (stratum corneum) and the mid-zone layer (stratum spinosum) in the non-keratinized squamous epithelium (left). Negative control section incubated overnight with normal rabbit IgG instead of the primary antibody (right). B. Western blot analysis of iNOS from oesophageal tissue of six healthy volunteers (lane 1A–6B). Lane (C) positive control of mouse macrophage cell line (RAW264.7) stimulated with IFN-γ and LPS. C. Representative gel electrophoresis of products from RT-PCR using a primer against human iNOS cDNA. Lane 1A–6B shows duplicate RT-PCR analysis of iNOS mRNA expression in oesophageal tissue from six healthy volunteers. Lane (C) is a positive human iNOS control and (D) is the size market. PCR analysis revealed single bands of predicted size of 259 bp.

NO-producing enzymes in the surface mucosa. The enzymatic L-arginine/NO pathway of deeper parts of oesophageal tissue has previously been investigated with regard to the control of peristalsis and vascular tone (20–22) as well as NANC-mediated relaxation in the lower oesophageal sphincter (20, 23). Epithelial NOS has been little investigated in the oesophagus and has been associated with pathological conditions such as cell transformation, e.g. Barrett's oesophagus and adenocarcinomas (24). However, Tanaka et al. (25) have shown that weak iNOS immunoreactivity is normally expressed in the basal and parabasal layers of oesophageal mucosal squamous epithelium. This location was confirmed in the present study by means of immunohistochemistry, Western blot and RT-PCR for iNOS mRNA.

The fact that the oesophageal mucosa contains considerable amounts of immunoreactivity for iNOS but that no NO formation could be detected at baseline pH is intriguing. The general opinion is that the inducible NO synthase isoform continuously catalyses the degradation of L-arginine to citrullin and NO (5). Apparently this is not the case in the oesophageal epithelium. This suggests that the inducible NO synthase in oesophageal epithelium may differ from what is described in other cell systems. There are studies indicating that iNOS can be regulated even at the level of enzyme activity (26), and several authors have discussed the possibility of more than one isoform of inducible NOS existing, depending on the characteristics and expression (18, 27).

It has been reported that constitutively expressed iNOS serves physiological roles in various uninflamed tissues, e.g. the epithelium of paranasal sinuses, airways and kidneys (5, 28, 29). The functional role(s) for intra-oesophageal NO

formation remains to be elucidated. Because of the short transit time for swallowed pathogens in the oesophagus, NO-related luminal antimicrobial defence is unlikely to be of any major significance. However, as NO is an exceptional signalling molecule, it may be speculated that it mediates changes in the functional state of the oesophagus. Several investigators have demonstrated different effects of NO in other epithelia, e.g. the maintenance of low intestinal epithelial permeability (2, 30) and the mediation of the bicarbonate response to mucosal acid exposure in duodenum (31). NO is eliminated by blood haemoglobulin and so has a short range of action in vascularized tissues. Direct actions by the luminally or epithelially derived NO on, for example, oesophageal peristalsis or the function of the LES are unlikely. However, it may well be that juxtamucosal NO elicits neural reflexes or the release of other agents from the epithelium, which in turn influences functions at distance, e.g. LES pressure. Several studies have indicated a role for NO as a chemical link in the generation of pain by both nociceptive and antinociceptive responses (32, 33). Of particular interest is the situation of gastro-oesophageal reflux disease (GORD), where patients often report aggravated symptoms related to certain food ingredients, several of them having high nitrate levels (34).

In summary, two prominent sources exist for oesophageal luminal NO formation, both dependent on the presence of acid in the oesophageal lumen: 1) acidity will induce chemical reduction of salivary nitrite, a mechanism related to dietary intake of nitrate, 2) NO formation is induced in the oesophageal mucosal epithelium by enzymatic degradation of L-arginine. In the latter case, the NO synthase has antigenic characteristics indicating the inducible isoform, but functional behaviour suggesting an unconventional subtype. Further studies are needed to elucidate the functional role of luminal oesophageal NO formation.

Acknowledgements

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