EMERGING LEADERSHIP LECTURE

Involvement of luminal nitric oxide in the pathogenesis of the gastroesophageal reflux disease spectrum

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Abstract

Over the last 3 decades, the incidence of esophageal adenocarcinoma has dramatically increased in Western countries; a similar increase may be observed in Asian countries in the near future. Esophageal adenocarcinoma arises from a sequential gastroesophageal reflux disease (GERD) spectrum from reflux erosive esophagitis, to Barrett’s esophagus, and finally to esophageal adenocarcinoma. At present, gastric acid and bile are assumed to be primarily involved in the etiology of the GERD spectrum. We reported in 2002 that, at the gastroesophageal junction in humans, abundant amounts of nitric oxide (NO) are generated luminally through the entero-salivary re-circulation of dietary nitrate. Since then, we have carried out a series of experiments to demonstrate that NO diffuses into the adjacent epithelium at cytotoxic levels. This diffusion results in disruption of the epithelial barrier function, exacerbation of inflammation, acceleration of columnar transformation in the esophagus (Barrett’s esophagus) via the induction of caudal-type homeobox 2, and the shifting of carcinogenic N-nitroso compound formation from the luminal to epithelial compartment. These results suggest that, in addition to conventionally recognized causative factors, luminal NO could also be involved in the pathogenesis of the GERD spectrum. In addition, we recently showed that there is a prominent gender-related difference in NO-related cytotoxicity in the esophagus and that estrogen attenuated the esophageal tissue damage via the estrogen receptor in female rats. The role of estrogen in attenuating the esophageal tissue damage in NO-related esophageal damage could explain the well-recognized male predominance in the GERD spectrum in humans.

Introduction

Over the last 3 decades, the incidence of esophageal adenocarcinoma has dramatically increased, especially in Western countries.1 It is known that esophageal adenocarcinoma arises from a sequential gastroesophageal reflux disease (GERD) spectrum from erosive reflux esophagitis, which progresses to Barrett’s esophagus, and finally to esophageal adenocarcinoma. Exposure of the esophageal mucosa to the refluxed gastroduodenal contents is an important contributing factor to the sequential GERD-related esophageal disorder.7 To date, gastric acid and bile acid have been the most extensively studied with respect to identifying the exact pathogenic stimuli within the reflux material that propels the progression of the GERD-related disease spectrum.2–5 In humans, reflux of both acid and bile occur simultaneously in the majority of reflux episodes with a graded increase in the severity across the GERD spectrum, suggesting a synergistic activity of acid and bile in progression of the disease.6,7 However, only 10% of patients with GERD are diagnosed with Barrett’s esophagus, whereas others only suffer from squamous esophagitis.1,6 Furthermore, Barrett’s esophagus advances to high grade dysplasia or esophageal adenocarcinoma in only a small fraction (0.3–1.0%) of patients.9 Taken together, these data suggest that factors other than acid or bile reflux are pivotal for progression of the GERD-related disease spectrum.

A series of recent studies have suggested a high concentration of luminal nitric oxide (NO) at the human gastroesophageal (GE) junction after nitrate ingestion is a potential pathogenic stimulus responsible for various diseases occurring at that site.10,11 In this review, we have outlined the influence of NO, particularly NO derived exogenously from dietary nitrate, on each stage of the GERD-related disease spectrum.

Generation of NO at the GE junction of humans

NO is an inorganic compound consisting of nitrogen and oxygen, and it is ubiquitously generated by nitric oxide synthase (NOS) in various kinds of cells in mammals. Despite being a simple molecule, NO is an important radical that mediates a wide range of physiologic and pathologic events in mammals including humans. In general, NO is known to have both cytoprotective and cytotoxic
To the circulating nitrate in the blood is re-secreted into the mouth by the salivary glands. Bacteria on the dorsum of the tongue then reduce about 30% of this nitrate to nitrite. Under fasting conditions, the salivary nitrate concentration is approximately 50 μM, which increases to as high as 2 mM after ingesting food with high nitrate content such as green lettuce. When salivary nitrite enters the stomach, the combination of the acidity and ascorbic acid content of the gastric juices converts the nitrite to NO.

\[ \text{NO}_2^- + \text{H}_2\text{O} \rightarrow \text{NO}_3^- + \text{H}_2\text{O} \]

Furthermore, because NO is generated at the site where salivary nitrite first encounters gastric acid, the site of luminal NO generation could shift to the distal esophagus in cases with GE reflux. Therefore, luminal NO may also be involved in the pathophysiology of various diseases occurring in the lower esophagus as well as the GE junction.

**Effect of the exogenous NO generated luminally on the integrity of the adjacent epithelium of the GE junction**

Membranes in tissues are not barriers to the diffusion of NO because of its gaseous and lipophilic properties. Therefore, NO produced in the lumen can readily diffuse into the surrounding epithelium where it may be accumulated to a level sufficient to exert some influence on the integrity of the tissue. In addition, since NO is known to have dual cytoprotective and cytotoxic effects within tissues, depending on the gas level, determination of the NO level is required to evaluate its function in tissues. We developed an animal model in which high concentrations of NO were generated luminally at the GE junction by co-administration of physiological concentrations of nitrite plus ascorbic acid. In this study, because NO is an unstable free radical in tissue, an Fe-diethylthiocarbamate complex was employed as a trapping agent to form a relatively stable radical adduct. Then, the concentration of NO in the gastric tissue of rats was measured by quantifying the resultant radical adduct using electron paramagnetic resonance spectroscopy. In the rat model, the high level of effects within tissues depending on the NO level. For example, NO generated at low concentrations by constitutive NOS (cNOS) is cytoprotective by modulating neuromuscular and vascular functions. On the other hand, higher concentrations of NO generated by inducible NOS (iNOS) are cytotoxic by affecting immune and inflammatory responses. Sustained generation of NO by iNOS has been implicated in the etiology of the mutagenesis related to chronic inflammation and GERD-related esophageal carcinogenesis. That is, NO derived from iNOS is involved in the pathogenesis in the disease spectrum because iNOS is expressed even at the early phase of esophageal inflammation, the expression is increased stepwise with progression of the disease spectrum.

However, the highest concentrations of NO occurring in the body are not the result of enzymatic synthesis, but rather from chemical reactions derived from dietary nitrate within the lumen of the stomach (Fig. 1). The modern diet contains substantial quantities of nitrate, mainly derived from nitrogen fertilizer usage and other intensive farming practices. In particular, dietary nitrate is contained in potatoes and other root crops, green leafy vegetables, and cereal. Ingested nitrate as an ingredient in food is absorbed from the small intestine into the bloodstream. When salivary nitrite enters the stomach, the combination of the acidity and ascorbic acid content of the gastric juices converts the nitrite to NO.

\[
\begin{align*}
\text{NO}_2^- & \rightleftharpoons \text{HNO}_2 \\
2 \text{HNO}_2 & \rightleftharpoons \text{N}_2\text{O}_3 + \text{H}_2\text{O} \\
\text{AA} + \text{N}_2\text{O}_3 & \rightarrow \text{DHAA} + 2\text{NO} + 2\text{H}_2\text{O}
\end{align*}
\]

Nitrite, NO is involved in the pathophysiology of various diseases occurring in the lower esophagus as well as the GE junction. In the rat model, the high level of NO occurring in the body are not the result of enzymatic synthesis, but rather from chemical reactions derived from dietary nitrate within the lumen of the stomach (Fig. 1). The modern diet contains substantial quantities of nitrate, mainly derived from nitrogen fertilizer usage and other intensive farming practices. In particular, dietary nitrate is contained in potatoes and other root crops, green leafy vegetables, and cereal. Ingested nitrate as an ingredient in food is absorbed from the small intestine into the bloodstream. When salivary nitrite enters the stomach, the combination of the acidity and ascorbic acid content of the gastric juices converts the nitrite to NO.

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Figure 1  Entero-salivary recirculation of dietary nitrate. Of all the nitrate absorbed from the diet or produced endogenously, 25% is taken up by the salivary glands and secreted into the mouth. Bacteria on the dorsum of the tongue convert 10–90% of this nitrate in saliva to nitrite. When saliva is swallowed and meets acidic gastric juice, it is converted to nitrosating species and by further reacting with ascorbic acid in gastric juice to nitric oxide. In patients with reflux disease this chemistry occurs within the esophagus where gastric refluxate mixes with saliva. (Citation from ref. 11).

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Nitric oxide and esophagus

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Exogenously derived NO detected in the mucosal layer at the GE junction of nitrite-administered rats was at a level comparable with the level of endogenously derived NO from iNOS. Meanwhile, exogenous NO affected some important cell functions in the adjacent tissue, including NO consumption of glutathione, a major antioxidant in the protection against cell damage, and/or NO inhibition of mitochondrial aconitase, an important enzyme for cell respiration. These results from animal model studies indicate that exogenous NO can induce cytotoxic effects in living cells in adjacent tissues. In addition, by employing a unique endogenous characteristic signal comprised of NO-mediated degradation of iron–sulfur cluster-containing proteins, we also confirmed that diffusion of NO from the lumen into the adjacent tissue could occur in humans as well.

The majority of salivary nitrite is rapidly converted to NO at the human GE junction, and the gas rapidly diffuses into the adjacent epithelium; therefore, only a small amount of nitrite is left in the more distal stomach. Accordingly, luminal concentrations of NO in the distal stomach become even lower compared with those at the GE junction. Since NO has dual effects on the tissue depending on the level of the gas, a relatively low concentration of NO, as seen in the distal stomach, could function as a protective mediator to maintain the gastric mucosal integrity. In contrast, a high cytotoxic concentration of local NO at the gastric cardia may be involved in the pathology of mucosal injury at that site. In fact, intragastric topical application or parental administration of NO is reported to have some beneficial effects on the stomach.

**Effects of NO on inflammation at the GE junction and lower esophagus in humans**

Because exogenous NO arises from an acidic lumen and diffuses into the adjacent tissue at the GE junction, the surface of the epithelium at the GE junction is exposed to the highest concentration of exogenous NO. Employing an *ex vivo* chamber system, we have demonstrated that luminal NO impairs the adjacent gastric barrier function primarily by disrupting the tight junction of the surface epithelium. Similarly, the esophageal barrier function is also susceptible to NO generated in the esophageal lumen, for example luminal NO-induced dilation of the intercellular space in the squamous epithelium. The gut barrier function is known to play a primary role in the maintenance of the mucosal structure and function in the presence of potentially damaging agents such as gastric acid or bile acid. Therefore, disruption of the mucosal barrier function may represent the earliest effect of NO-derived stress on the epithelium at the GE junction; persistent abnormalities in the barrier function at the human GE junction could be involved in the perpetuation of chronic inflammation at that site (e.g. carditis in the GE junction and erosive esophagitis in the esophagus). Thus, exogenous luminal NO may be an important initial event in disrupting the epithelial barrier function around the GE junction, acting synergistically with refluxed acid and pepsin.

Once gastric acid, bile, and possibly luminal NO have disrupted the initial resistance of the esophageal mucosa to damage, activated inflammatory cells are accumulated at the disrupted site and become one of the main sources of superoxide (O$_2^-$), an important oxygen-derived free radical. Although NO possesses a multi-

\[
\text{NO} + \text{O}_2^- \rightarrow \text{ONOO}^-
\]

We demonstrated that exogenous luminal NO (sodium nitrite plus ascorbic acid) administration to an established rat acid-reflux esophagitis model for a week greatly exacerbated the pre-existing esophageal tissue damage of reflux esophagitis. Thus, diffusion of luminal NO into the adjacent superoxide-enriched inflamed tissue of the reflux esophagitis could lead to the production of the highly toxic agent peroxynitrite, which could be responsible for exacerbation of the esophageal damage. Subsequently, a biological cascade is initiated by the additional generation of superoxide and superoxide from infiltrated inflammatory cells, leading to the further generation of peroxynitrite at that site (Citation from ref.47).

**Figure 2** Exacerbation of esophageal damage by exogenous luminal nitric oxide exposure. Nitric oxide generated within the lumen of the lower esophagus by the chemical reaction of salivary nitrite with ascorbic acid in the presence of refluxed acid. Diffusion of the luminal nitric oxide into the adjacent superoxide-enriched inflamed tissue of the reflux esophagitis could lead to the production of the highly toxic agent peroxynitrite, being responsible for the exacerbation of the esophageal damage. Subsequently, a biological cascade is initiated by the additional generation of superoxide and superoxide from infiltrated inflammatory cells, leading to the further generation of peroxynitrite at that site (Citation from ref.47).
microbiome in relation to inflammation and metaplasia in the distal esophagus. Further studies are warranted to investigate how the diversity of microbiomes in the oral cavity as well as the esophagus affect the exogenous luminal NO production at the GE junction or at the distal esophagus by modulating the conversion of nitrate to nitrite.

One important observation concerning esophageal adenocarcinoma is its strong male predominance (male : female ratios of 3:1). The male-predominant gender difference consistently exists across the GERD spectrum, although the ratios become higher with progression toward the later stages. Meanwhile, reflux symptoms or non-erosive reflux disease in general affects more women than men. These epidemiological data allow us to hypothesize that the esophageal epithelium is more vulnerable in men, or more resistant in women, to the refluxed gastroduodenal contents, than in their respective counterparts. Identification of the causative luminal factors for inducing the gender-related difference would be clinically relevant to predict the actual etiologic factors involved in the pathogenesis of reflux esophagitis in humans. Employing chronic rat reflux esophagitis models of both sexes, we found that there was a striking male-predominant, gender-related difference in esophageal tissue damage in the presence of exogenous NO and that estrogen attenuated the esophageal tissue damage via the estrogen receptor. Further, we found a potential role of esophageal mast cells in the mediation of the suppression of the immune system under estrogen administration. Interestingly, the gender-related difference in the esophagitis model was more prominently observed when exogenous NO was administered compared with exogenous acid (pH 1.8), suggesting that gender-related differences may be specifically potentiated in the presence of exogenous NO as the aggravating agent. These results indicated that gender-related differences in the susceptibility of the esophageal epithelium to damage by exogenous NO might be at least partially responsible for prominent gender-related disease differences in GERD in humans.

Effect of exogenous NO generated luminally on the function of the lower esophageal sphincter (LES)

The LES is a bundle of muscles at the lower end of the esophagus, and it plays a primary role in preventing reflux of gastric contents into the esophagus. It is well known that NO endogenously derived from cNOS localized to non-adrenergic, non-cholinergic nerves mediates the relaxation of the smooth muscle cells, including those of the LES. An in vitro study using muscle strips from the LES of an opossum demonstrated that a low concentration of NO (nM) was sufficient to induce relaxation of the muscle. Therefore, the high concentration range (μM) of NO formed in the lumen at the human GE junction may be sufficient to penetrate the epithelium, and then affect the inner smooth muscle cells of the LES, leading to relaxation of the muscle and reflux of the acidic gastric contents into the esophagus. There have been several studies that addressed the potential effect of exogenous NO generated luminally on the function of the LES. A previous report demonstrated that superphysiological levels of NO generated luminally at the human GE junction affected the LES, leading to a significant increase in the transient relaxation of the LES. However, a more recent study demonstrated that NO generated luminally, at more physiological conditions, did not affect LES function. Similarly, we have demonstrated that dietary nitrate ingestion did not affect the gastric motility such as gastric emptying.

Effect of NO on the development of Barrett’s esophagus

Barrett’s esophagus is a metaplastic change in which the normal squamous epithelium is replaced with columnar epithelium, and it is considered to be a premalignant condition associated with an increased risk of developing esophageal adenocarcinoma. Because the development of Barrett’s esophagus is a chronic adaptive protection against a hostile luminal environment, it is possible that imposing NO-derived nitrosative stress could enhance the Barrett’s esophagus formation. Thus, employing an established rat Barrett’s esophagus model in which both gastric and duodenal contents could reflux into the esophagus, we investigated whether continuous exogenous NO (sodium nitrite plus ascorbic acid) exposure could facilitate the development of Barrett’s esophagus. We found that exogenous NO exposure clearly accelerated the emergence and increased the area of Barrett’s esophagus in the rat model, suggesting that exogenous luminal NO in the esophagus could promote columnar transformation of the esophagus. Since it is widely accepted that the development of Barrett’s esophagus is an adaptive response to chronic injury of the esophageal mucosa, the increase in esophageal inflammation due to NO exposure could be at least partially responsible for the subsequent development of Barrett’s esophagus. In addition, because NO is a well-known bioactive molecule, there is another possibility that NO exposure might directly affect the epithelial transformation without being involved in the inflammatory response.

To investigate the potential molecular regulatory mechanisms by which NO could affect Barrett’s esophagus formation, Vaninetti et al. have demonstrated that stimulation of NO enhanced bile acid-induced caudal type homeobox 2 (CDX-2), a transcription factor involved in intestinal epithelial phenotype, in the normal human esophageal cell line Het1A. We extended this study by showing a potentially direct role of NO in the induction of CDX-2 expression through activation of an epidermal growth factor receptor in the esophageal squamous cell-line KYSE-30. These in vitro studies suggest that higher concentrations of NO, whether derived from iNOS or exogenously from dietary nitrate, could induce CDX-2 expression, leading to formation of Barrett’s esophagus in the presence or absence of co-stimulation by bile acid. Further investigation of the precise molecular mechanism by which NO exposure facilitates the columnar transformation of squamous esophagus is warranted for therapeutic intervention to prevent the progression of Barrett’s esophagus.

Effect of NO on the development of esophageal adenocarcinoma

A variety of carcinogenic effects exerted by high concentrations of NO are well recognized, for example a high concentration of NO can directly exert a mutagenic and carcinogenic effect through the formation of higher oxides of nitrogen such as N₂O₃ which can damage DNA directly via deamination of bases and indirectly by forming N-nitroso compounds. N₂O₃ is also known to inactivate DNA repair enzymes such as O²-alkylguanine DNA damage enzyme.
alkyltransferase.79 A considerable amount of research has focused on NO-related Barrett’s esophagus carcinogenesis. While some of the research assumed exogenous NO to be a putative source of NO-related carcinogenesis, others have considered endogenous iNOS to be the main source. Both endogenous luminal NO and endogenous NO from iNOS may be involved in the carcinogenesis because iNOS is overexpressed in Barrett’s esophagus as well as esophageal adenocarcinoma.15-17 and exogenous luminal NO diffuses into the adjacent tissue to a similar level as iNOS-derived high concentrations of NO.10,27

Results of a bench-top model study suggested that exogenous luminal NO might contribute to local generation of carcinogenic N-nitroso compounds due to its diffusion into the adjacent epithelium,71 which was also confirmed in humans.72 The N-nitroso compounds possess carcinogenic properties due to their ability to alkylate DNA.43 One such compound (N-methyl-N-nitro-N-nitrosoguanidine) is widely used as a carcinogen in an animal model of gastric cancer.43 To investigate the direct interaction of NO with DNA, Clemens et al.73 demonstrated that physiological, luminal concentrations of NO could cause DNA damage in the form of double-strand DNA breaks in Barret’s esophagus, high-grade dysplasia, and adenocarcinoma cells. These data suggest that NO can be a specific mutagen for Barrett’s esophagus carcinogenesis and may play a role in the accumulation of genetic abnormalities in the development of esophageal adenocarcinoma. Further, the same researchers74 showed that physiological concentrations of NO enhanced invasiveness in high-grade dysplasia and esophageal adenocarcinoma cell lines through modulation of matrix metalloproteinase expression, a family of enzymes known to be crucial in the process of extracellular matrix remodeling and invasion. These data suggest that NO may be also involved in promoting the progression of dysplastic lesions to invasive carcinoma in addition to its DNA-damaging effects at the initial stage of carcinogenesis. Another recent report linking NO to esophageal carcinogenesis demonstrated a role of NO in regulating intracellular pH. Goldman et al.75 showed NO mediated inhibition of the activity of the sodium/hydrogen exchanger that can pump protons out of cells to maintain intracellular pH. Subsequently, intracellular acidification induced NO exposure that led to DNA damage, toxicity, and neoplastic development.

In addition, there have been several animal model studies that investigated the involvement of NO in the pathogenesis of esophageal carcinogenesis. Accumulating previous animal model studies demonstrated that administering nitrite with ascorbic acid or other anti-oxidants to rats induced tumors in their forestomach, which is contiguous with the esophagus and lined by squamous epithelium.76-79 The tumors did not occur if only nitrite was administered. Hence, the combination of nitrite and anti-oxidants generated a high concentration of NO, which had a mutagenic effect on the epithelium.79 Kuroiwa et al.80 extended these studies by showing that administration of exogenous NO (sodium nitrite plus ascorbic acid) to an acid-type reflux model of rat induced esophageal squamous cell carcinoma, although the treatment failed to induce the development of Barrett’s esophagus or esophageal adenocarcinoma. This study may indicate that NO alone in the absence of refluxed duodenal contents is incapable of causing columnar transformation of the esophagus, although the treatment might elicit squamous carcinogenesis. In another study, Kumagai et al.81 demonstrated that triproline (a nitrite scavenger) inhibited the development of esophageal adenocarcinoma by gastroduodenal reflux in rats, suggesting the involvement of reactive nitrogen species such as NO, peroxynitrite, and nitroso compounds in the pathogenesis of esophageal adenocarcinoma.

**Epidemiological studies**

Since the 1980s, numerous studies have shown that the incidence of esophageal adenocarcinoma has been increasing rapidly in many western countries; however, the precise cause for the cancer endemic remains unclear.82 In terms of a mass survey, a 20-fold increase in use of chemical nitrogenous fertilizers and associated increased dietary nitrate exposure post-war may be potentially responsible for the marked increase in the incidence of the esophageal adenocarcinoma10,11,20

Thus far, epidemiological studies have not shown an association of nitrate intake with esophageal adenocarcinoma.83,84 Additionally, a recent epidemiological study has demonstrated a paradoxical association between nitrate intake and Barrett’s esophagus depending on gender, that is, total nitrate intake was inversely associated with Barrett’s esophagus in men, while it was positively associated with the pre-malignant condition in women.85 These studies suggest that individual susceptibility to esophageal adenocarcinoma does not depend on nitrate intake. However, other co-factors are also closely involved in the chemical reaction of luminal NO generation in the lower esophagus. For example, a *Helicobacter pylori*-negative healthy stomach is required to continuously supply sufficient amounts of gastric acid86,87 and ascorbic acid88,89 to the reaction, or reflux is required to shift the site of NO generation to the lower esophagus,25 although the absence of *H. pylori*90 and GE reflux91 are well-recognized risk factors for GERD-related esophageal disorders. Theoretically, oral intake of vitamin C (ascorbic acid) may also be involved in the chemical reaction. Thus, these co-factors may confound the potential association of dietary nitrate with esophageal adenocarcinoma or Barrett’s esophagus. Otherwise, genetic susceptibility to the NO-related chemical insult may be important to determine the progression of the GERD-related esophageal disorders, considering that only a small portion of people eventually suffer from more advanced complications of GERD such as Barrett’s esophagus and esophageal adenocarcinoma.

**Conclusion**

A cytotoxic concentration of NO is generated luminally at the human GE junction. Recent studies, including ours, suggest that in addition to conventionally recognized causative factors such as gastric acid and bile, luminal NO could also be involved in the pathogenesis of GERD-related esophageal disorders.

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