Novel mechanism of nitrosative stress from dietary nitrate with relevance to gastro-oesophageal junction cancers

Katsunori Iijima, Jeanette Grant1, Kenneth McElroy1, Valerie Fyte1, Tom Preston2 and Kenneth E.McColl1,3

Department of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan, 1Section of Medicine, Western Infirmary, 44 Church Street, Glasgow G11 6NT, UK and 2Scottish Universities Environmental Research Centre, East Kilbride, Glasgow G75 0QF, UK

Introduction

For many years there has been interest in the potential for generation of carcinogenic N-nitroso compounds from dietary nitrate and nitrite within the upper gastrointestinal tract. In patients with atrophic gastritis and associated achlorhydria this may occur due to colonization of the gastric lumen by denitrifying bacteria (1). In patients with healthy acid-secreting stomachs, it may occur due to the acidification of nitrite producing nitric acid and nitrosating species (2). In the developed world, the incidence of atrophic gastritis and associated cancer of the mid and distal stomach is falling and increasing interest is focusing on the aetiology of cancer of the gastro-oesophageal junction which occurs in patients with normal gastric acid secretion (3).

The main source of nitrite entering the acid-secreting stomach is swallowed saliva, which contains substantial concentrations of nitrite derived from the entero-salivary recirculation of dietary nitrate. Nitrate is absorbed from the small intestine and 25% of this is taken up by the salivary glands and secreted into the mouth (4–10). Bacteria on the dorsum of the tongue then reduce ~30% of this nitrate to nitrite (4–10). Under fasting conditions, the median salivary nitrite concentration is ~50 μM and this rises to 200 μM after ingesting a typical salad portion containing 2 mmol nitrate (11). We have recently reported that the nitrite concentration in the distal oesophagus is similar to that in saliva (12).

When these upper alimentary secretions encounter acidic gastric juice, the nitrite in them is converted to nitrous acid and various nitrosating species, including N2O3 and NO+ (3,13). Saliva and gastric juice also contain substantial concentrations of thiocyanate, which in the presence of acidified nitrite forms the nitrosating species NOSCN (14–16). These nitrosating species formed by the acidification of nitrite can react with N-nitrosatable molecules to form N-nitroso compounds (3,13). The latter are potentially carcinogenic due to their ability to alkylate DNA (2). The most widely used animal model of gastric cancer utilizes the carcinogen N-methyl-N'-nitro-N-nitrosoguanidine, which is an N-nitroso compound (17).

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 (18–21). Ascorbic acid effectively competes with N-nitrosatable compounds for reaction with the nitrosating species (22–26). In the reaction between ascorbic acid and nitrosating species the former is oxidized to dehydroascorbic acid and the latter reduced to nitric oxide (22–26).

This reaction between nitrite and ascorbic acid at acidic pH is very rapid (27), resulting in the great majority of salivary nitrite being converted to nitric oxide within a few seconds of encountering acidic gastric juice (12,28,29). Consistent with this, we have recently shown that the intraluminal concentration of nitric oxide generated by the reaction in healthy volunteers is maximal at the gastro-oesophageal junction and cardia, where the nitrite in saliva first encounters gastric acid (30). In patients with severe reflux disease and Barrett’s oesophagus, the highest luminal nitrite oxide concentration occurs within...
the oesophagus, where the refluxing gastric acid first encounters saliva (31). At these anatomical locations substantial luminal concentrations of nitric oxide are generated for several hours following nitrate ingestion, in some subjects in excess of 50 μM (30).

Though the reaction between ascorbic acid and salivary nitrite provides protection against the intraluminal generation of N-nitroso compounds, it could in the process induce nitrosative stress within the surrounding epithelium as a result of its generation of such high local concentrations of nitric oxide. The nitric oxide produced in the lumen will readily diffuse into the surrounding epithelium (31) and in that neutral pH and lipid-rich environment react with oxygen to reform the nitrosating species N₂O₃ within the epithelial cells (32,33). The latter can damage DNA directly by causing deamination of the nucleic acid components cytosine, adenine and guanidine and indirectly via the formation of N-nitrosocompounds (33). The nitrosating species can also damage DNA repair enzymes and irreversibly inactivate DNA repair proteins (33–41). This possibility of luminally generated nitric oxide inducing nitrosative stress within the adjacent epithelium is of clinical significance as high luminal nitric oxide concentrations occur at the gastro-oesophageal junction and within Barrett’s oesophagus, which are both sites of particularly high incidence of epithelial mutagenesis and neoplasia (42–51).

The aim of our studies was to investigate the effects of the luminal nitrite chemistry known to occur at the human gastro-oesophageal junction on the adjacent epithelium.

Materials and methods

A benchtop model of the gastrooesophageal lumen and adjacent epithelium was developed. The lipid membranes of the epithelial cells act as a barrier to penetration by hydrogen ions due to their hydrophobic properties but are highly permeable to gases such as nitric oxide or oxygen. The epithelial cells also contain buffer, which neutralizes any hydrogen ions managing to traverse the lipid membranes. We simulated these epithelial properties by using a thin tube made of silastic³ (Dow Corning Corp., Midland, MI) filled with phosphate buffer, pH 7.4. The silastic wall of the tube acts as the lipid epithelial cell wall, being relatively impermeable to hydrogen ions or other polar molecules and highly permeable to nitric oxide and oxygen (52–54).

**Apparatus**

The model was constructed using a transparent plastic cylinder (Delrin®; DuPont UK Ltd, Hemel Hempstead, Herts, UK), which was 7 cm tall and 2.2 cm in internal diameter, with a wall thickness of 2 mm (Figure 1). Its base was sealed and its top left open. Vertical fenestrations were cut in the cylinder wall extending for 1.5 cm above the base to within 3.4 cm of the cylinder top. They were 0.3 cm in width and spaced 1.5 cm apart. Silastic tubing with an internal diameter of 500 μm and wall thickness of 250 μm was then wound round the cylinder in a single layer completely occluding the fenestrations. The silastic tube was filled with 15 mM phosphate buffer, pH 7.4. The volume of fluid contained within the coil of silastic tubing was 250 μl.

The cylinder with its silastic tubing attached was then placed in a glass dish containing water maintained at 37°C by a heating plate. The water completely covered the outer aspect of the silastic tubing wound round the cylinder. The cylinder itself was filled with 20 ml of HCl to a level above the fenestrations. A plastic magnetic stirrer was placed in the cylinder. A nitric oxide sensor was also placed in the solution in the cylinder to allow constant monitoring of the nitric oxide concentration. Preliminary studies were performed to confirm that the cylinder was completely sealed by the silastic tubing.

The lumen of the cylinder acted as the lumen of the upper gastrointestinal tract. The surrounding layer of silastic tubing represented the epithelium. The lumen of this silastic tubing, filled with pH 7.4 phosphate buffer, represented the cytoplasm of the epithelial cells separated from the lumen by the thin hydrophobic silastic membrane, representing the cell membrane. The water in the water bath in contact with the outside of the silastic tubing represented the deeper mucosal layers and their blood supply, able to deliver oxygen and remove nitric oxide. Sampling from the cylinder lumen and aspirating the contents of the silastic tubing allowed us to examine the chemistry occurring in the lumen and within the surrounding epithelial cells, respectively.

**Terminology**

The lumen of the cylinder representing the lumen of the upper gastrointestinal tract is referred to as the luminal compartment. The lumen of the silastic tubing represents the cytoplasm of the epithelial cells and is referred to as the epithelial compartment.

**Studies of nitrosative chemistry within the luminal and epithelial compartments**

Conditions were created within the luminal compartment reproducing those occurring within the lumen where salivary nitrite encounters gastric juice. For this, the luminal compartment was filled with HCl of varying pH containing 1 mM thiocyanate, with and without 2 mM ascorbic acid (representing gastric juice). The secondary amine morpholine was also added to allow assessment of nitrosation by measuring formation of nitrosomorpholine. The experiment was started by adding nitrite to the luminal compartment to simulate nitrite in saliva entering the stomach. A further bolus of nitrite was added after 15 min.

The epithelial compartment contained phosphate buffer, pH 7.4, and in some experiments, ascorbic acid or glutathione was also added. Morpholine was added to allow measurement of nitrosation. The pH of the epithelial compartment was adjusted to 7.4 before each experiment and confirmed to be at the same pH after each experiment.

**Nitrite determination**

Samples for nitrite determination were rendered alkaline immediately after collection by addition of NaOH. They were stored at 4°C and analysed the same day on 96-well microplates using a modified Griess reaction as previously described (11). Colorimetric analysis was performed 15 min after the addition of the Griess reagents using a 540 nm filter.

![Diagrammatical cross-section of the benchtop model for studying nitrite chemistry at the gastro-oesophageal junction.](https://academic.oup.com/carcin/article-abstract/24/12/1951/2390381)
Nitrile determination
Nitrile was assayed by reducing it to nitrite with a cadmium column (Nitralyzer™ nitrate reductor; World Precision Instruments Inc., Sarasota, FL) and measuring the nitrite as above.

Ascorbic acid, total vitamin C measurements
An aliquot of 0.5 ml of sample was added to two test tubes. Each contained 0.5 ml of 2% metaphosphoric acid/0.5% sulfamic acid and one also contained 6 mg/ml dithiothreitol (DTT). The sulfamic acid was added to remove any remaining nitrite. The purpose of the DTT treatment was to regenerate ascorbic acid from any dehydroascorbic acid in the sample, thereby allowing quantification of total vitamin C levels. Upon collection samples were placed in liquid nitrogen before storage at −80°C. They were stored for no more than 4 weeks. Ascorbic acid was measured by high performance liquid chromatography as previously described (11), based upon the method of Sanderson and Schorah (55).

N-nitrosomorpholine measurements
An aliquot of 1 ml of a solution of 0.08 M HCl and 5% (w/v) sulphamic acid in saturated NaCl was pipetted into screw cap vials. Immediately before the experiments, 10 µl of a 0.01% N-nitroso-dibutylamine (in methanol) internal standard was added to each vial and mixed well. For each time point, 1 ml of sample was withdrawn from the luminal compartment and 200 µl of sample was aspirated from the epithelial compartment and pipetted into the appropriate vial along with 0.5 ml of extraction solvent mix (45:55 dichloromethane:diethyl-ether) and mixed well to allow transfer of the nitrosoamines into the organic phase. The vials were then left to stand to allow the layers to separate out. The upper (organic solvent) layer was then transferred to a tapered vial and 0.5 ml of fresh solvent added to the original vial and the procedure repeated. The resulting 1 ml of solvent in the tapered vial was then concentrated to ~50 µl by blowing with a stream of nitrogen gas. These vials were sealed with crimp caps and stored at −20°C until measurement by gas chromatography–mass spectrometry.

Nitric oxide measurements
The aqueous dissolved nitric oxide concentration was monitored with an isolated dissolved nitric oxide meter (ISO-NO Mark II; World Precision Instruments Inc.). The nitric oxide electrode was calibrated as follows. An aliquot of 20 ml of 0.1 mol/l H2SO4/KI solution was placed in a vial and the nitric oxide sensor placed in it. Aliquots of 200 µl of sodium nitrite solution were added to produce final concentrations of 20, 40, 60, 80 and 100 µmol/l. Under these conditions the concentration of nitric oxide in the solution equals the concentration of nitrite. The electrode response was linear up to a nitric oxide concentration of 100 µmol/l. The sensitivity of the electrode was 2.00–2.72 nM nitric oxide/pA.

Glutathione assay
Glutathione assay was performed by HPLC based on the method of Winters et al. (56) using the compound N-(1-pyrenyl)maleimide (NPM), which reacts with free sulfhydryl groups to form fluorescent derivatives. An aliquot of 100 µl of sample was added to 100 µl of acetonitrile and 200 µl of a solution of NPM in acetonitrile and the procedure followed by addition of 50 µM nitrite 15 min later. At 30 min after first addition of nitrite, samples were taken from both the luminal and epithelial compartments for N-nitrosomorpholine determination. Data represent means ± SE of at least three separate experiments.

RESULTS

N-nitrosation in the luminal compartment
In the absence of ascorbic acid in the luminal compartment
The addition of nitrite to the acidic luminal compartment containing thiocyanate, but no ascorbic acid, resulted in the N-nitrosation of morpholine within that compartment (Figure 2). N-nitrosomorpholine formation was maximal at pH 2.5 (Figure 3). The amount of N-nitrosomorpholine generated increased with the amount of nitrite added and with the morpholine concentration (Table I). This addition of nitrite to the acidic luminal compartment in the absence of ascorbic acid produced only very low levels of nitric oxide in this compartment. The maximal nitric oxide concentration recorded was 1 µM, which occurred after adding 100 µM nitrite and at the lowest pH studied of 1.5.

In the presence of ascorbic acid in the luminal compartment
The addition of ascorbic acid to the luminal compartment completely prevented the formation of N-nitrosomorpholine within this compartment, under all conditions studied (Figure 2). In the presence of ascorbic acid the nitrite was converted to nitric oxide. At pH 1.5, virtually all the added nitrite appeared as nitric oxide within the few seconds response time of the nitric oxide sensor (Figure 4). The concentration of nitric oxide then fell slowly by ~50% over the subsequent 15 min when addition of a further dose of nitrite equivalent to 50% of the initial dose restored the nitric oxide concentration. The concentration of nitric oxide generated by adding nitrite to the luminal compartment containing ascorbic acid progressively fell as the pH was raised to 2.5, 3.5 and 4.5 (Figure 4).

N-nitrosation induced in the adjacent epithelial compartment
In the absence of ascorbic acid in the luminal compartment
When nitrite was added to the acidic luminal compartment in the absence of ascorbic acid in either compartment, nitrite...
appeared in the epithelial compartment. Addition to the luminal compartment at pH 1.5 of 100 μM nitrite at time 0 plus 50 μM nitrite at time 15 min produced a nitrite concentration of 200 μM in the epithelial compartment at 30 min. At this pH, the concentration of nitrite detected in the epithelial compartment was directly proportional to the amount of nitrite added to the acidic luminal compartment.

The addition of nitrite to the acidic luminal compartment in the absence of ascorbic acid in either compartment resulted in very limited N-nitrosation of the morpholine present in the epithelial compartment (Figure 2). N-nitrosomorpholine was only detected with the higher doses of nitrite added to the luminal compartment, with the luminal compartment at its lowest pH of 1.5 and with the highest concentration of morpholine in the epithelial compartment (Table I). The maximum concentration of N-nitrosomorpholine detected in the epithelial compartment in these experiments was only 30% of that occurring simultaneously in the luminal compartment (Figure 2). Additional experiments indicated that N-nitrosomorpholine could not itself pass between the luminal and epithelial compartments.

In the presence of ascorbic acid in the luminal compartment. When nitrite was added to the acidic luminal compartment containing ascorbic acid, it resulted in the accumulation of substantial concentrations of nitrite in the epithelial compartment. The concentration of nitrite detected in the epithelial compartment was proportional to the amount of nitrite added to the luminal compartment and also increased with decreasing pH of the luminal compartment.

Addition to the luminal compartment at pH 1.5 of 100 μM nitrite at time 0 plus 50 μM nitrite at time +15 min produced a nitrite concentration of 1500 μM in the epithelial compartment at 30 min. The nitrite accumulated in the epithelial compartment showed an excellent correlation with the mean nitric oxide concentration in the luminal compartment (Figure 5). The concentration of nitrate was also measured in the epithelial compartment and was always less than 5% of the nitrite concentration.

Table I. N-nitrosomorpholine formed in the luminal and epithelial compartments when nitrite was added to the luminal compartment containing HCl, pH 1.5, 1 mM thiocyanate and 2 mM ascorbic acid

<table>
<thead>
<tr>
<th>Total nitrite added (μM)</th>
<th>Morpholine concentration (mM)</th>
<th>In the presence of ascorbate in the lumen</th>
<th>In the absence of ascorbate in the lumen</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>N-nitrosomorpholine in lumen (μM)</td>
<td>N-nitrosomorpholine in epithelial compartment (μM)</td>
</tr>
<tr>
<td>37.5 5</td>
<td>u.d.</td>
<td>23.5 (0.5)</td>
<td>3.3 (0.5)</td>
</tr>
<tr>
<td>75 5</td>
<td>u.d.</td>
<td>40.3 (1.1)</td>
<td>3.8 (0.2)</td>
</tr>
<tr>
<td>112.5 5</td>
<td>u.d.</td>
<td>91.7 (5.4)</td>
<td>5.2 (0.9)</td>
</tr>
<tr>
<td>150 5</td>
<td>u.d.</td>
<td>137 (5.6)</td>
<td>6.2 (2.0)</td>
</tr>
<tr>
<td>150 0.1</td>
<td>n.a.</td>
<td>3.2 (0.4)</td>
<td>u.d.</td>
</tr>
<tr>
<td>150 0.5</td>
<td>n.a.</td>
<td>15 (0.0)</td>
<td>u.d.</td>
</tr>
<tr>
<td>150 1</td>
<td>n.a.</td>
<td>44.7 (9.8)</td>
<td>2.1 (0.3)</td>
</tr>
</tbody>
</table>

In the top four experiments 5 mM morpholine was present in both compartments. In the bottom three, morpholine was only present in either the epithelial or the luminal compartment, at the concentrations indicated. Data represent means (± SE) of at least duplicate experiments. u.d., undetected; n.a., not available.
high concentrations of \( N \)-nitrosomorpholine within the epithelial compartment containing only phosphate buffer pH 7.4 and morpholine. The concentration of \( N \)-nitrosomorpholine detected in the epithelial compartment was directly proportional to the amount of nitrite added to the luminal compartment (Table I) and progressively increased as the pH of the luminal compartment was lowered from pH 4.5 to 1.5 (Figure 3). The concentration of \( N \)-nitrosomorpholine formed in the epithelial compartment also correlated directly with the mean nitric oxide concentration generated in the luminal compartment (Figure 6) and with the nitrite accumulated in the epithelial compartment (Figure 6). It also correlated directly with the morpholine concentration added to the epithelial compartment (Table I).

The concentration of \( N \)-nitrosomorpholine generated in the epithelial compartment when nitrite was added to the luminal compartment at pH 1.5 containing 2 mM ascorbic acid was substantially higher (>20 times) than the maximum concentrations generated in the luminal compartment under optimal conditions for luminal nitrosation (Figure 2 and Table I). One can also express the results in terms of the total amount of \( N \)-nitrosomorpholine generated in the two compartments, which depends upon the volume of the compartment as well as the concentration of \( N \)-nitrosomorpholine within it. The volume of the epithelial compartment was 20 times less than the volume of the adjacent luminal compartment. Consequently, the amount of \( N \)-nitrosomorpholine generated within the epithelial compartment when nitrite was added to the luminal compartment at pH 1.5 containing ascorbic acid was equivalent to that generated in the luminal compartment in the absence of ascorbic acid.

**Mechanism of nitrosation in the epithelial compartment in the presence versus absence of ascorbic acid in the luminal compartment**

The concentration of \( N \)-nitrosomorpholine generated within the epithelial compartment showed the same correlation with the mean nitric oxide concentration in the luminal compartment irrespective of whether ascorbic acid was added to the luminal compartment (Figure 6). However, the correlation between the concentration of \( N \)-nitrosomorpholine generated in the epithelial compartment and nitrite accumulated in the epithelial compartment was different in the presence versus absence of ascorbic acid in the luminal compartment (Figure 6). In the presence of ascorbic acid in the luminal compartment, the ratio of nitrosomorpholine generated in the epithelial compartment to nitrite accumulated in the epithelial compartment was approximately 10 times higher than that observed in the absence of ascorbic acid in the luminal compartment.

**Effect of adding ascorbic acid or glutathione to the epithelial compartment on generation of \( N \)-nitrosomorpholine within the epithelial compartment**

We assessed the ability of adding ascorbic acid or glutathione to prevent nitrosation within the epithelial compartment. This was done by repeating the experiments generating nitric oxide in the luminal compartment with ascorbic acid plus nitrite and adding ascorbic acid or glutathione to the epithelial compartment containing phosphate buffer and morpholine. Adding 200 \( \mu \)M ascorbic acid to the epithelial compartment inhibited the nitrosation of morpholine in that compartment by ~40% (Figure 7). However, increasing the ascorbic acid concentration above 200 \( \mu \)M did not increase the inhibitory effect. The addition of 500 \( \mu \)M glutathione also inhibited the nitrosation of morpholine in the epithelial compartment by 40% (Figure 7). Again, however, further increasing the glutathione concentration did not increase the inhibitory effect. Ascorbic acid was more effective than glutathione at inhibiting nitrosation in the epithelial compartment as it achieved its maximal effect at a lower concentration (Figure 7).

We examined the effect of luminal conditions on the consumption of ascorbic acid and glutathione in the epithelial compartment. The addition of nitrite alone or ascorbic acid...
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Fig. 7. Inhibitory effect of ascorbic acid (A) and glutathione (B) added to the epithelial compartment on N-nitrosomorpholine production in the epithelial compartment. The solid line indicates the result when 100 μM nitrite plus 50 μM nitric acid 15 min later was added to the luminal compartment containing HCl, pH 1.5, 2 mM ascorbic acid and 1 mM thiocyanate. The broken line is when the experiment was repeated with 50 μM nitrite plus 25 μM nitric acid 15 min later. All points represent the mean (± SE) of triplicate experiments.

Fig. 8. Consumption of ascorbic acid (A) and glutathione (B) in the epithelial compartment by nitric oxide generated in the luminal compartment. An aliquot of 100 μM nitrite plus 50 μM nitric acid 15 min later was added to the luminal compartment containing HCl, pH 1.5, 1 mM thiocyanate and ±2 mM ascorbic acid (open bar, in the absence of ascorbic acid; closed bar, in the presence of ascorbic acid). The epithelial compartment contained 15 mM phosphate buffer, pH 7.4, and concentrations of ascorbic acid and glutathione as shown. The concentrations of ascorbic acid and glutathione remaining in the epithelial compartment were measured at +30 min. Data represent means (± SE) from triplicate experiments.

alone to the luminal compartment at pH 1.5 with 1 mM thiocyanate did not affect the concentration of ascorbic acid or morpholine in the epithelial compartment (Figure 8). However, adding the nitrite along with 2 mM ascorbic acid to the luminal compartment to generate a mean nitric oxide concentration of 70 μM within the lumen resulted in the ascorbic acid concentration in the epithelial compartment falling by ~120 μM. This fall in ascorbic acid concentration in the epithelial compartment was independent of the initial ascorbic acid concentration in that compartment (Figure 8). When 0.2 mM glutathione was present in the epithelial compartment, its concentration fell by 156 μM and was thus similar to that seen with ascorbic acid in the epithelial compartment. However, when the initial concentration of glutathione was 0.5 or 2 mM, the fall was greater at 340 and 460 μM, respectively.

Additional experiments to investigate the failure of ascorbic acid and glutathione to completely inhibit nitrosation in the epithelial compartment

The inability of ascorbic acid and glutathione to completely inhibit the nitrosation of morpholine by nitric oxide passing from the luminal to epithelial compartment suggested that nitrosation of morpholine might be occurring partly within the wall of the silastic tube, which would be inaccessible to these water-soluble antioxidants. In order to assess whether morpholine could enter the tube wall and be nitrosated within it, we performed experiments in which morpholine was added to the water in the water bath surrounding the coil, with no morpholine added to the solution within the tube. If morpholine could enter the tube and be nitrosated within the wall of the tube, then nitrosomorpholine should appear in the epithelial compartment when nitric oxide was generated in the luminal compartment. By adding the morpholine to the water in the water bath at pH 7 rather than the acid in the luminal compartment we ensured that adequate morpholine was in its unprotonated form and thus able to pass the tube wall. In this experiment, no N-nitrosomorpholine was detected in the epithelial compartment.

Discussion

For many years there has been concern about the potential formation of carcinogenic N-nitroso compounds within the lumen of the upper gastrointestinal tract due to the acidification of salivary nitrite on entering the stomach (2). We have extended previous work by studying not only the nitrosative stress produced within the acidic lumen but also that produced within the surrounding epithelium. Our benchtop model indicates that although ascorbic acid prevents acid-catalysed nitrosation within the lumen, in the process of doing so it may induce even greater nitrosative stress within the surrounding epithelium.

Our benchtop model was designed to reproduce the chemistry occurring as nitrite and thiocyanate in saliva encounter acidic gastric juice. The concentrations of the chemicals employed were based upon the levels measured in human subjects (11). We used hydrochloric acid in place of pure gastric juice as it produces less interference with the analytical assays and we have confirmed that hydrochloric acid and gastric juice behave in the same way with respect to nitrosative chemistry (27). In our benchtop model we simulate the interface between the lumen of the upper gastrointestinal tract and the adjacent epithelium by means of a thin silastic membrane. The latter, like the lipid epithelial cell wall, is highly permeable to gases such as nitric oxide and relatively impermeable to hydrogen ions. Soft polymers such as silastic have very similar permeability properties to the lipid bilayer of epithelial cells.
Nitrosation in the luminal compartment

Within the luminal compartment we confirmed the long recognized ability of acidified nitrite to react with secondary amines producing N-nitrosoamines (2). Acidification of nitrite produces nitrous acid (pK_a 3.23) and various nitrosating species such as N_2O_3 and NO^+. These nitrosating species react with the unprotonated amine forming the N-nitrosamine. The pK_a of morpholine is 8.3 and with decreasing pH less is in the nitrosatable unprotonated form. The optimum pH for acid-catalysed nitrosation of morpholine is 3.5, representing the most favourable balance between decreasing concentration of nitrosatable amine and increasing concentration of the nitrosatable species. Our experiments included 1 mM thiocyanate, representing the concentration of the anion present in saliva and gastric juice (14). Thiocyanate is a powerful catalyst of acid nitrosation and also lowers its pH optima to 2.5, as observed in our studies (15,16).

Within the luminal compartment we also confirmed the ability of ascorbic acid to inhibit acid-catalysed nitrosation (22–26). Ascorbic acid competes with secondary amines for the nitrosating species, reducing the latter to nitric oxide and in the process the ascorbic acid is oxidized to dehydroascorbic acid (Fig. 2). In the acidified luminal compartment containing ascorbic acid, the addition of nitrite did not produce any N-nitrosomorpholine but led to the rapid appearance of nitric oxide.

Nitrosation in the epithelial compartment

We extended previous work by studying nitrosation induced in the surrounding epithelial compartment as a consequence of the nitrosative chemistry occurring within the luminal compartment.

When nitrite was added to the acidic luminal compartment without ascorbic acid, it resulted in the accumulation within the epithelial compartment of nitrite and low concentrations of N-nitrosomorpholine. The concentration of nitrite detected in the epithelial compartment with the luminal compartment at pH 1.5 was similar to the concentration of nitrite added to the luminal compartment. This could be explained partly by nitrous acid passing through the membrane into the epithelial compartment. In addition, some of the chemical species in equilibrium with nitrous acid, e.g. NO, NO_2, N_2O_3 and NOSCN, might pass through the membrane and be oxidized to nitrite. N-nitrosomorpholine was detected within the epithelial compartment only in the experiments with the lowest luminal pH and higher luminal concentrations of nitrite. The concentration of N-nitrosomorpholine generated in the epithelial compartment under these conditions was only 25% of that occurring in the luminal compartment. The silastic membrane is impermeable to N-nitrosomorpholine and the nitrosamine must therefore have been generated within the epithelial compartment. This nitrosation in the epithelial compartment must therefore have been the result of either nitrosating species passing through the membrane or by nitric oxide passing through and forming nitrosating species in the epithelial compartment. The subsequent studies in the presence of ascorbic acid in the luminal compartment (discussed below) support the latter mechanism.

When nitrite was added to the acidic luminal compartment containing ascorbic acid, substantial concentrations of nitrite appeared in the epithelial compartment. The concentration of nitrite in the epithelial compartment directly correlated with the mean nitric oxide concentration within the luminal compartment. This was consistent with the nitric oxide generated within the luminal compartment by reaction between the acidified nitrite and ascorbic acid passing through the membrane into the epithelial compartment. In the epithelial compartment the nitric oxide will react with dissolved oxygen to form the nitrosating species N_2O_3, which will then react with water in the neutral pH of the epithelial compartment to form nitrite (57). The nitrite formed in this way will be trapped within the epithelial compartment and thus provide an estimate of the total flux of nitric oxide from the luminal compartment into the epithelial compartment.

The addition of nitrite to the acidic luminal compartment containing ascorbic acid also generated high concentrations of N-nitrosomorpholine within the epithelial compartment. The concentrations of N-nitrosomorpholine produced in the epithelial compartment directly correlated with the mean nitric oxide concentration generated in the luminal compartment. This formation of nitrosomorpholine in the epithelial compartment can be explained by the nitric oxide passing into the epithelial compartment and reacting with oxygen to form N_2O_3. The latter, in addition to reacting with water to form nitric oxide, can also nitrosate amines and thus form N-nitrosomorpholine (33,34) (Figure 9).

The concentration of N-nitrosomorpholine generated within the epithelial compartment in the above way was 10- to 20-fold higher than that generated within the acidic luminal compartment under the most favourable conditions for acid-catalysed luminal nitrosation. This can largely be explained by the fact that the low pH necessary for formation of nitrosating species by acidification of nitrite means that very little of the amine is in its unprotonated nitrosatable form (morpholine pK_a = 8.3). This limitation to amine nitrosation does not apply when the N_2O_3 is formed by reaction between nitric oxide and oxygen at neutral pH. Although the concentration of N-nitrosomorpholine generated in the epithelial compartment when ascorbic acid was present in the luminal compartment was substantially higher than that generated in the luminal compartment in the absence of ascorbic acid, the total amount of N-nitrosomorpholine formed was similar. This is due to the fact that the volume of the luminal compartment was 20 times the volume of the adjacent epithelial compartment.

We did not examine other secondary amines, such as dimethylamine, which may be biologically more relevant. The pK_a of dimethylamine is 10 and one would anticipate a lower rate of nitrosation than morpholine at the pH of both the epithelium and the acidic lumen. However, the ratio of nitrosation between the epithelium and lumen should be unaltered.

These studies demonstrate an interesting paradox. Although ascorbic acid prevents nitrosation within the acidic lumen, in so doing it induces nitrosation within the surrounding epithelial compartment. This is due to the nitric oxide formed in the acidic luminal compartment by the reaction between ascorbic acid and nitrosating species readily diffusing through the hydrophobic barrier into the neutral environment of the epithelial compartment and there reacting with oxygen to reform nitrosating species. The ascorbic acid thereby shifts the nitrosative stress from the luminal compartment into the epithelial compartment (Figure 9).

The diffusion of nitric oxide from the luminal compartment into the epithelial compartment may also explain the small
concentration of N-nitrosomorpholine generated in the epithelial compartment when nitrite was added to the luminal compartment in the absence of ascorbic acid in the luminal compartment. Support for this comes from the finding that there was a linear relationship between the mean concentration of nitric oxide in the luminal compartment and concentration of nitrosomorpholine generated in the epithelial compartment irrespective of whether ascorbic acid was added to the luminal compartment. The ratio of nitrosomorpholine generated in the epithelial compartment to nitrite accumulated in the epithelial compartment was lower in the absence versus presence of ascorbic acid in the luminal compartment. This can be explained by the fact that in the absence of ascorbic acid in the luminal compartment there was diffusion into the epithelial compartment of nitrous acid or related species which did not produce nitrosation within the epithelial compartment. It appears that it is the passage of nitric oxide into the epithelial compartment that induces nitrosation.

Ascorbic acid and glutathione are the main molecules providing intracellular protection against nitrosative and oxidative stress. We therefore assessed their ability to prevent the nitrosation of morpholine within the epithelial compartment arising from the luminal generation of nitric oxide. Both molecules reduced the concentration of N-nitrosomorpholine formed in the epithelial compartment but could only achieve a maximal reduction of 40%. Their failure to completely prevent nitrosation within the epithelial compartment may be due to properties of the hydrophobic barrier. The nitric oxide passing from the acidic luminal compartment into the epithelial compartment is likely to react with oxygen while it is in the process of traversing the hydrophobic membrane, forming N$_2$O$_3$ within the membrane (58). Liu et al. have shown that the reaction between nitric oxide and oxygen to form N$_2$O$_3$ is 300 times faster within a wide range of hydrophobic media, including lipid cell membranes, than within aqueous solution (32). This is due to the fact that both nitric oxide and oxygen are more soluble in such media (32). This will increase the concentrations of both reactants in the hydrophobic media and consequently increase their rate of reaction, which is proportional to the oxygen concentration and the square of nitric oxide concentration. The highest concentration of the nitrosating species N$_2$O$_3$ arising from the NO produced in the acidic lumen will therefore occur within the hydrophobic membrane separating the two compartments (58). This could explain the inability of ascorbic acid and glutathione to prevent nitrosation within the epithelial compartment as they are unable to enter hydrophobic lipid compartments.

We investigated this possibility that morpholine was entering the silastic membrane and being nitrosated by the N$_2$O$_3$ formed within it. However, our experiments indicated that morpholine could not enter the silastic membrane. Consequently, we suspect that morpholine is being nitrosated at the interface between the hydrophobic membrane and the aqueous buffer, pH 7.4, at a location where the water-soluble antioxidants may have limited access to the nitrosating species generated within the membrane and emerging from it.

In summary, therefore, our studies indicate that although ascorbic acid may effectively prevent acid-catalysed nitrosation within the lumen, it may in the process induce substantial nitrosative stress within the surrounding epithelium as a consequence of its production of nitric oxide. This occurs due to the inability of nitric oxide to readily diffuse into the neutral pH and hydrophobic environment of the epithelial compartment and there react with oxygen to reform the nitrosating species N$_2$O$_3$. The hydrophobic barrier representing the epithelial cell lipid membrane may contribute to the phenomenon by enhancing the rate of regeneration of N$_2$O$_3$ and inhibiting the protective effect of ascorbic acid and glutathione. The effect of lipid-soluble antioxidants such as vitamin E require study.

It has been recognized for many years that potentially carcinogenic N-nitroso compounds may be formed within the lumen of the upper gastrointestinal tract by two distinct mechanisms. The first is by colonization of the achlorhydric stomach by denitrifying bacteria (1). The second is due to acidification of salivary nitrite in the absence of ascorbic acid (2). Our current studies indicate a third mechanism, namely nitrosative stress induced within the epithelium itself due to the diffusion of nitric oxide produced by the reaction.
between salivary nitrite and acidic gastric juice containing adequate ascorbic acid.

Our observations may be relevant to the high incidence of mutagenesis and neoplasia which occurs at the gastro-oesophageal junction, where saliva encounters gastric acid (42–51). We have demonstrated previously that intraluminal nitrosation in the healthy acid-secreting stomach is likely to be maximal at the gastro-oesophageal junction (12). This is due to the fact that the ratio of nitrite to ascorbic acid is highest at this location, where salivary nitrite enters the stomach in saliva (12). In addition, we have previously shown that luminal concentrations of nitric oxide are also highest at this site, due to the reaction between salivary nitrite and ascorbic acid in acidic gastric juice (30,31). Our current studies indicate that the nitric oxide may also contribute to local generation of N-nitroso compounds due to its diffusion into the adjacent epithelium. Consequently, nitrosation at this location may proceed irrespective of the availability of ascorbic acid.

The epidemiology of cancer at the gastro-oesophageal junction is distinct from that of cancer of the rest of the stomach. Although a diet high in antioxidants and vitamin C has been shown to be protective against cancer of the distal stomach, this is not the case for gastro-oesophageal junction cancer (59–61). Indeed, one large study indicated a trend towards increased cancer risk with increased vitamin C intake (60). Likewise, cancer of the gastro-oesophageal junction is more common in the middle than lower socio-economic classes, despite the former’s higher intake of antioxidants (62). The unusual epidemiology of cancer at this particular anatomical location might be explained by ascorbic acid merely shifting the nitrosative stress from the lumen into the epithelium rather than fully preventing it.

Dietary nitrate and gastro-oesophageal cancers


K.Iijima


