S-Nitrosoglutathione formation at gastric pH is augmented by ascorbic acid and by the antioxidant vitamin complex, Resiston

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\textbf{ABSTRACT}

\textbf{Context:} Exogenous nitrogen oxides must be made bioavailable to sustain normal physiology because nitric oxide synthase (NOS) deficient mice are viable. In the stomach, S-nitrosoglutathione (GSNO) is formed from ingested nitrite and high levels of airway glutathione (GSH) that are cleared and swallowed. However, gastric GSNO may be broken down by nutrients like ascorbic acid (AA) before it is absorbed.

\textbf{Objective:} To study the effect of AA on GSNO formation and stability.

\textbf{Materials and methods:} GSH and nitrite were reacted with or without 5 mM AA or Resiston (5 mM AA with retinoic acid and \(\alpha\)-tocopherol). GSNO was measured by reduction/chemiluminescence and HPLC. AA and reduced thiols were measured colorimetrically. O-Nitrosoascorbate and AA were measured by gas chromatography–mass spectrometry (GC–MS).

\textbf{Results:} GSNO was formed in saline and gastric samples (pH \(\sim 4.5\)) from physiological levels of GSH and nitrite. Neither AA nor Resiston decreased [GSNO] at pH > 3; rather, they increased [GSNO] (0.12 ± 0.19 \(\mu\)M without AA; 0.42 ± 0.35 \(\mu\)M with AA; and 0.43 ± 0.23 \(\mu\)M with Resiston; \(n = 4\) each; \(p < 0.05\)). However, AA compounds decreased [GSNO] at lower pH and with incubation >1 h. Mechanistically, AA, but not dihydroascorbate, increased GSNO formation; and the O-nitrosoascorbate intermediate was formed.

\textbf{Conclusions:} AA, with or without other antioxidants, did not deplete GSNO formed from physiological levels of GSH and nitrite at pH > 3. In fact, it favoured GSNO formation, likely through O-nitrosoascorbate. Gastric GSNO could be a NO-dependent source of bioavailable nitrogen oxides.

\textbf{Introduction}

Protein S-nitrosylation, the post-translational modification of a cysteine thiol by a nitric oxide (NO) group, is involved in a broad spectrum of cell signalling effects (Gow et al. 2002; Gaston, Singel, et al. 2006; Paige et al. 2008; Foster et al. 2009). In general, proteins and peptides that have been modified to form S-nitrosothiol bonds are involved in guanylate cyclase (GC)-independent signalling by nitrogen oxides, though S-nitrosylation also affects GC-dependent processes (Mayer et al. 1998). Disorders of S-nitrosylation are relevant to the pathophysiology of many diseases, such as cystic fibrosis, asthma, primary ciliary dyskinesia, sleep apnoea, Duchenne muscular dystrophy, etc. (Gow et al. 2002; Moya et al. 2002; Snyder et al. 2002; Gaston, Singel, et al. 2006; Colussi et al. 2008; Lim et al. 2008; Ozawa et al. 2008; Paige et al. 2008; Foster et al. 2009; Gonzalez et al. 2009; Marozkina and Gaston 2012; Marozkina et al. 2012). S-Nitrosothiols can be formed by NO synthase (NOS), by other metalloproteins, and by inorganic reactions (Mayer et al. 1998; Gow et al. 2002; Gaston, Singel, et al. 2006; Paige et al. 2008; Foster et al. 2009), but NOS knockout mice are viable (Huang 2000), suggesting that exogenous nitrogen oxides can be converted to bioavailable, physiologically sufficient nitrogen oxides.

Here, we have identified a reaction in the gastric mucosa that can lead to increased formation of the endogenous, clinically beneficial S-nitrosothiol, S-nitrosoglutathione (GSNO), in vivo. Surprisingly, this reaction is augmented, not inhibited, by ascorbic acid (AA) at gastric pH. GSNO was first identified in the airways (Gaston et al. 1993), but conditions would normally favour its formation in the lumen of the stomach (Gupta et al. 2016). Thus, we have focused on the reactions likely to occur in the stomach; these may also be relevant to chemistry, in the context of disease, in acidic airways and other organs (Gupta et al. 2016). Within the gastrointestinal tract, in locations other than the gastric lumen, GSNO is produced and plays a role in motility (Kirogolu et al. 2013), bile flow (Rodriguez-Ortigosa et al. 2010) and mucosal protection (Ohtake et al. 2009; Rodriguez-Ortigosa et al. 2010; Flamant et al. 2011; Savidge et al. 2011). In the proximal small bowel and ampulla of Vater, GSNO formed in the stomach will promote smooth muscle relaxation and gastric emptying (Kirogolu et al. 2013), and potentially will promote choleresis (Rodriguez-Ortigosa et al. 2010). It also has antimicrobial effects and can prevent mucosal injury (Ohtake et al. 2009; Flamant et al. 2011; Savidge et al. 2011). Importantly, GSNO absorption from the intestines appears to have systemic vascular benefits (Wu et al. 2016).
Typically, human stomach pH (3–4) is low and glutathione (GSH) levels are high. In the presence of ubiquitous nitrite, these conditions are ideal for inorganic synthesis of GSNO and other S-nitrosothiols (Carver et al. 2005). Airways can also have a low pH (Gaston, Kelly, et al. 2006), particularly in disease, and they also have high GSH levels; GSNO is formed (Gaston et al. 1993; Marozkina and Gaston 2015). Similar conditions exist in other organs (Gow et al. 2002; Broniowska et al. 2013), and under conditions of ischemia (Broniowska et al. 2013). GSNO exerts a range of effects relevant to host defence, ion channel regulation, smooth muscle relaxation, ciliary function and cell cycle regulation (Gaston et al. 1993; Snyder et al. 2002; Gaston, Singel, et al. 2006). High levels of GSNO formed inorganically in the stomach could augment many of these effects in the gut and/or after systemic absorption. Indeed, the GSNO catabolic enzyme, GSNO reductase, is upregulated in the stomach (Baraona et al. 2001), perhaps to prevent excessive accumulation or absorption of GSNO.

In the stomach, however, ingested nutrients produce a complex redox environment that could destabilize GSNO and related bioactive nitrogen oxides. It has been shown previously, that antioxidants such as AA can reduce GSNO to GSH and NO (Xu et al. 2000). Indeed, S-nitrosothiols are quite stable on the whole, and AA can, in a pH-dependent fashion, cause their rapid degradation through formation of unstable O-nitrosoascorbate intermediate (Aquat and Dasgupta 2004). Further, guinea-pig aorta model studies also show that AA interacts rapidly with nitrite which may result in decreased GSNO formation (Xu et al. 2000). Therefore, we hypothesized that antioxidant vitamins would lead to decreased GSNO levels with potential resulting adverse effects on GI and systemic physiology. We report, however, that AA can actually increase GSNO formation under certain physiological conditions. We tested the AA alone and AA in combination with other antioxidants (the ‘Resiston’ antioxidant complex) for their effect on the formation and stability of GSNO in vitro and in human gastric aspirates.

Materials and methods
Reagents
All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Synthesis of the ascorbate-NO adduct
Ascorbate-NO was made by reacting AA in citrate buffer (50 mM) with 1 μM sodium nitrite for 30 min at room temperature. Sample was lyophilized and reacted with 80 μL of bis(trimethylsilyl) trifluoroacetamide +10% trimethylchlorosilane (Regis, Morton Grove, IL) for 30 min at 75°C. This derivatization procedure (Dintzis et al. 1995) resulted in 2,3,5,6-tetrakis-(tri-methylsilyl) ether with or without –NO group.

Chemical assays
S-Nitrosoglutathione was measured both by HPLC (Welch et al. 1996) and by anaerobic reduction/chemiluminescence in 1 mM cysteine saturated with CuCl as previously reported (Fang et al. 1998). Ascorbic acid was measured using the Ascorbic Acid Assay Kit (BioVision, Milpitas, CA, Catalogue #K661-100) in accordance with manufacturer’s instructions using spectrophotometry at λ = 570 nm.

Results with S-Nitrosothiols were detected by reduction/chemiluminescence as described previously (Fang et al. 1998).

1. The pH effect on GSNO formation. Five hundred micromoles of GSH was incubated with 1 μM NaNO2 for 3 h at pH from 4 to 8. After pH optimization, 5 μM NaNO2 was incubated for 30 min in citrate buffer in presence or absence (control) 5 mM AA alone or 5 mM AA in combination with other antioxidants, Resiston. The composition of antioxidant vitamin complex Resiston is: vitamin A (retinol palmitate), 50,000 IU; vitamin E (α-tocopherol), 600 IU; vitamin C (AA), 2 g; β-carotenes, 20 mg. The composition has been proprietary (published Resiston drug information 2010). S-Nitrosothiols were detected by reduction/chemiluminescence as described previously (Fang et al. 1998).

2. Time course. Five hundred micromoles of GSH, 5 μM NaNO2 was incubated at pH 3.7 in citrate buffer in the presence or absence (control) of 5 mM AA alone or 5 mM AA in combination with other antioxidants, Resiston. S-Nitrosothiols were detected by reduction/chemiluminescence as above.

3. Biological samples. Discarded gastric aspirates (Institutional Review Board exemption) were mixed with physiological GSH 500 μM and NaNO2 5 μM in the presence or absence of AA or of the clinical antioxidant complex ‘Resiston’ (containing AA in a proprietary mixture with retinoic acid and α tocopherol, as previously described [published Resiston drug information 2010]); this is used as a cancer adjunctive therapy in Europe (Sukolinskii and Morozkina 1989).

Murine lung and liver assays for the presence of ascorbate
Murine lung and liver were blanched with intravascular ice-cold PBS, then homogenized in AA assay buffer containing Ascorbic Acid Probe and Ascorbic Acid Enzyme Mix (Catalogue #K661-100 from BioVision, Milpitas, CA), centrifuged to remove cellular debris and brought to a final volume of 120 μL/well in a 96-well plate for AA assay using spectrophotometry at λ = 570 nm.

Unreacted homogenate supernatant was used as the spectrophotometric control. Tissues were harvested in accordance with an approved IACUC protocol at the University of Virginia.

Statistical analysis
ANOVA followed by Student’s t-test or ANOVA on Ranks (if not parametrically distributed) was used for data analysis. All data are expressed as mean ± SD unless otherwise specified. Results with p < 0.05 were considered to be statistically significant.
Results

S-Nitrosoglutathione formation in vitro: effect of pH and time

Consistent with previous reports (Fang et al. 1998; Broniowska et al. 2013), GSNO formation from GSH and nitrite was pH-dependent (Figure 1(A,B)) and time-dependent (Figure 1(C)): at more basic pH, excess of OH– competes with GS– for NO+, affecting GSNO stability. As predicted (Smith and Dasgupta 2000; Xu et al. 2000), ascorbate decreased the yield of GSNO at pH <2. However, ascorbate and the ascorbate-based antioxidant actually increased GSNO formation at pH range from 3 to 5 during the first 60 min of co-incubation (Figure 1(B,C)). We hypothesized that the unstable intermediate, O-nitrosoascorbic acid, is produced in these conditions. Aerobic reduction/chemiluminescence method that was used for nitrosocompound detection could not completely distinguish between GSNO and O-nitrosoascorbate (Gow et al. 2002) and therefore the total amount of nitrosocompounds was measured; but the presence of the intermediate was confirmed by GC–MS (see below).

**Figure 1.** Ascorbate and an ascorbate-containing antioxidant mixture (Resiston) modify pH-dependent S-nitrosothiol formation. (A) GSNO formation after 3 h incubation of 500 μM GSH and 1 μM NaNO2 was pH-dependent. n = 3; p < 0.05. (B) 500 μM GSH, 5 μM NaNO2 was incubated for 30 min in citrate buffer in the presence or absence (control) of 5 mM AA or Resiston. S-Nitrosothiols were detected by 2/C14 C (copper chloride cysteine) assay as described previously (Fang et al. 1998). (C) Five hundred micromoles of GSH and 5 μM NaNO2 was incubated at pH 3.7 in citrate buffer in the presence or absence (control) of 5 mM AA or Resiston.

*S-Nitrosoglutathione formation in human stomach acid is not inhibited by ascorbic acid*

Next, we aimed to determine whether the observations in Figure 1 would be relevant to the stomach environment after exposure, as in oral ingestion, to AA. We studied the formation and stability of GSNO in human gastric secretion by adding AA and antioxidant vitamin complex with AA (‘Resiston’) to saline or to ex vivo gastric samples. Gastric aspirates or control saline samples were mixed with 500 μM GSH and 5 μM NaNO2 in the presence or absence of 5 mM AA or 5 g/L of the clinical antioxidant complex Resiston (containing 5 mM AA in mixture with retinoic acid and α-tocopherol).

Two grams of AA (0.011 M) is the amount in each dose of the pharmaceutical compound Resiston. Our calculation was based on the assumption that the stomach has a volume of 1–2 L after eating and drinking. Thus, the concentration would be 5–10 mM after ingesting Resiston. GSNO was formed in saline and gastric fluid (pH 4.5) (Giorgi et al. 1989; Flagg et al. 1994; Broniowska et al. 2013; Marozkina and Gaston 2015). Levels at baseline ex vivo were <10 nM (n = 4; Figure 2). With augmentation to pulmonary levels of GSH and nitrite (recapitulating...
swallowed material from lung clearance [Gaston et al. 1993]), S-nitrosothiol levels increased to 0.121 ± 0.19 μM (n = 4). Surprisingly, both AA and Resiston increased GSNO levels (levels were 0.417 ± 0.35 μM and 0.433 ± 0.23 μM, respectively, n = 4 each, p ≤ 0.05). A 3–4-fold increase of GSNO production in the presence of AA or Resiston was observed within 1 h of incubation. However, GSNO was lost at pH < 3 and at incubation time > 60 min (Figure 1(B,C)).

**Ascorbate is normally present in lungs and other tissues**

To determine whether endogenous AA levels were also high enough to contribute to GSNO stability, we measured concentrations in the murine lung and liver homogenates. The baseline AA level detected in the mouse liver was 0.154 ± 0.017 μM/g tissue (n = 10) and in the lungs, 0.238 ± 0.043 μM/g tissue (n = 10) (Figure 3). Concentrations were lower than AA concentrations detected in mouse lungs and liver by other researchers, though the AA concentrations in tissues are highly dependent on diet (Kratzing and Kelly 1982; Reidling et al. 2008; Iwama et al. 2012). These concentrations are not optimal to augment GSNO formation. This suggests that it may be a unique circumstance in the stomach, or under conditions in which the pH is low in the airways, gut and elsewhere (Benjamin et al. 1994; Ng et al. 2004; Hunt 2007) that leads to the augmented nitrosothiol formation.

**Ascorbate does not increase GSNO in mild acid by increasing the levels of reduced thiol substrate**

To determine whether the effect of AA to augment GSNO was because AA reduced GSSG to form GSH, we performed the reaction shown in Figure 5 in the presence of reduced and oxidized thiols. AA did not increase concentrations of reactant thiol (Figure 4).

To get insight of the mechanism behind augmented yield of nitrosocompounds in the presence of AA, we studied whether oxidized AA (dehydroascorbate) affects nitrosocompounds formation. We observed that, at pH 3.7, dehydroascorbate did not have pronounced effect on increase of nitrosocompound concentration in the presence of GSH and nitrite over one hour (Figure 5). This suggests that the active intermediate, ascorbyl radical, could stabilize NO⁺ (Figure 6). We therefore hypothesized that ascorbate increased the total amount of produced nitrosocompounds due to capture of nitrosonium NO⁺ by AA (or ascorbate) with formation of relatively stable compound O-nitrosoascorbic acid (or O-nitrosoascorbate) (Figure 6). O-Nitrosoascorbate is a short-lived, intermediate product of AA oxidation to dehydroascorbic acid (Figure 7) and apparently serves here as an NO⁺ donor to make GSNO. On the other hand, GSNO when formed, has a T₁/₂ of 10 h at pH 3 of 24 h (Nikitovic and Holmgren 1996). We acknowledge that some of our signal (Figure 2) may result from detection of unstable O-nitroascorbate, but this appears to be a minor and transient intermediate (Figure 7; also, see below).

**Figure 2.** S-Nitrosoglutathione formation in human gastric secretions. Gastric aspirates (pH 3.5–4.5) were mixed with 500 μM GSH and 5 μM NaNO₂ in the presence or absence of 5 mM AA or 5 mg/mL of the clinical antioxidant complex, Resiston (containing 5 mM AA in mixture with retinoic acid and tocopherol). GSNO was measured by anaerobic reduction/chemiluminescence in 1 mM cysteine saturated with CuCl₂ as previously reported (Fang et al. 1998). S-nitrosothiol levels measured at baseline were n = 4, with addition of normal pulmonary levels of GSH and nitrite (control) (n = 4), ascorbate (n = 5, p ≤ 0.05) and Resiston (n = 4, p ≤ 0.05).

**Figure 3.** Ascorbic acid is normally present in lungs and liver. Ascorbic acid in murine lung (n = 10) and liver (n = 10).

**Figure 4.** Ascorbate is not acting to keep thiols reduced in the presence of nitrite. Amount of thiols was detected by Elman’s reagent (Thermo Scientific, #22582, Waltham, MA) in the presence of 5 μM NaNO₂ and 5 mM ascorbic or 5 mM dehydroascorbic acid (n = 4, *p ≤ 0.001 compared to all groups with reduced thiols).
Ascorbic acid is modified by acidified nitrite

To add an NO group to form the nitrosoascorbate intermediate, we have reacted nitrite with ascorbate (concentrations as above) at pH 3.7 for 1 h. Addition of NO group to AA was verified using gas chromatography–mass spectrometry (Figure 7). From comparison of the peak areas, we found about 7% of the product with m/z 495 which is consistent with formation of O-nitrosoas- corbyl radical from parent mass of 464 (M + 31).

Conclusions and discussion

We conclude that strong biological reducing agents in the form of AA and antioxidant vitamins do not deplete the beneficial GSNO formed in human gastric contents at physiological gastric pH; in fact, they can augment it via transnitrosation from O-nitrosoascorbic acid to GSH. Dissociation energies of O–NO bonds are lower in comparison with S–NO bonds (Wu et al. 2016) and one can expect preferable GSNO formation in transnitrosation reactions. This suggests that GSNO formed in the stomach could be a source of bioavailable nitrogen oxides in the human gut under normal conditions (Ohtake et al. 2009; Rodriguez-Ortigosa et al. 2010; Flamant et al. 2011; Savidge et al. 2011; Kiroglu et al. 2013; Wu et al. 2016). Nitrile alone is reduced at low pH to NO radical, which can be inactivated by haemoglobin (Marozkina and Gaston 2015). S-Nitrosothiols, once formed, can transport bioactive nitrogen oxides in a regulated fashion to target tissues (Gupta et al. 2016; Wu et al. 2016). However, our data also suggest that this chemistry may be primarily observed in the stomach because other tissues have high pH and/or low ascorbate concentration. An exception could be the inflamed lung under the conditions of high ascorbate ingestion/levels (Flagg et al. 1994) and low pH (Gaston, Kelly, et al. 2006).

Our data suggest that the increased GSNO formation at pH range of 2.5–4.5 in the presence of ascorbate could most likely be explained by O-nitrosoascorbic acid formation that serves as a donor–acceptor for NO$^+$ (Figure 7). The AA does not appear to act through reduction of oxidized thiol (Figure 4). With regard to the possibility that GSNO could be formed in the stomach, our data confirm that GSNO is formed from physiological levels of GSH and nitrite in the aqueous solutions at different pH and in human gastric fluid. Reaction of nitrous acid HNO$_2$ with GSH is responsible for the GSNO formation and its rate increases with lower pH due to the protonation of nitrite ion:

$$\text{GSH} + \text{HNO}_2 \leftrightarrow \text{GSH} + \text{NO}^+ + \text{OH}^- \leftrightarrow \text{GSNO} + \text{H}_2\text{O}$$

One can see from Figure 1 that the GSNO formation in mixtures of GSH and nitrite can be detected at pH < 5. In accordance with this result, GSNO was formed in human gastric fluid (pH ~ 3.5) from physiological levels of GSH and nitrite (Figure 2). We believe that Resiston was superior to nitrosylated compound formation to AA alone in the short time frame, 20 min (Figure 2(C)), but Resiston and AA were equal in the longer period of time for nitrosylated compound formation (Figure 1(C)). We think that change in pH made Resiston and

Figure 5. S-Nitrosoglutathione formation in vitro can be augmented by ascorbic acid (but not dehydroascorbic acid) at low pH. Five micromoles of sodium nitrite was incubated with 5 mM AA or 5 mM dehydroascorbic acid in the presence of 0.5 mM GSH at pH 3.7 for 1 h. Nitrosocompounds concentration was measured by anaerobic reduction/chemiluminescence in 1 mM cysteine saturated with CuCl as previously reported (Fang et al. 1998) ($n = 3$), $p < 0.05$.

Figure 6. Proposed scheme. Ascorbic acid makes O-nitrosoascorbate, an intermediate containing NO$^+$ group at pH 3.7. Ascorbate anion exists in equilibrium with ascorbyl radical. NO$^+$ can be attached to the oxygen anion of ascorbyl radical at pH = 3.7. Alternatively, ascorbyl radical can acquire electron and form dehydroascorbic acid that can be converted to 2,3-diketo-1-gulonic acid.
AA alone equal to each other in term of formation of nitrosylated compound (Figure 1(A,B)). Both AA and Resiston significantly increased nitrosylated compound formation in gastric contents (Figure 2) compared to control (GSH and nitrite was added to the gastric contents) and baseline (gastric contents alone), but neither AA nor Resiston was superior to each other in this process. Note that even higher concentrations of AA might not be effective nitrosylated compound formation because of possible oxidative (pro-oxidant) properties of very high concentrations of the AA (Seo and Lee 2002).

In addition to the question of GSNO formation in human gastric secretions, we also addressed the stability of GSNO in this environment and whether this source of periodic GSNO formation (from swallowed GSH) can be physiologically relevant. S-Nitrosothiols can be reduced in the presence of electron donors:

\[ \text{GSNO} + \text{H}^+ + e^- \leftrightarrow \text{NO} + \text{GSH} \] (*)

Reduction potential for reaction (*) was found to be \( E_r = -0.98 \) V at pH 7.4 (Ford et al. 2002) and there are several reducing agents that can contribute in GSNO degradation process. For example, redox couple \( \text{Cu}^+/\text{Cu}^{2+} \) with reduction potential \( E_0 = 0.16 \) V.

\[ \text{Cu}^{2+}(\text{aq}) + e^- \rightarrow \text{Cu}^+(\text{aq}) \]

Additionally, \( \text{Cu}^+ \) ions can react with molecular oxygen producing superoxide anions and hydroxyl radicals that may also contribute in GSNO degradation.

Here, we have focused on the effect of AA on the GSNO concentration because it is an essential vitamin and reducing agent present in many foods, and is used ubiquitously in the nutraceutical industry as an orally ingested electron donor (‘nutritional antioxidant’). It is well known that presence of AA promotes decomposition of GSNO and other S-nitrosothiols at physiological pH; this reaction is widely used to measure S-nitrosothiol concentrations (Paige et al. 2008). Reaction of AA with S-nitrosothiol was studied earlier (Holmes and Williams 2000; Paige et al. 2008; Melzer et al. 2012) and two reaction pathways were identified. The first one dominates at low concentration of AA.
and is Cu²⁺ dependent and the second route becomes important at higher concentrations of AA and is [Cu²⁺]-independent. The first mechanism involves reduction of Cu²⁺ ions by AA to form Cu⁺ which further donates electron to GSNO resulting in NO and GSH production.

\[
2\text{Cu}^{2+} + \text{AA} \rightarrow 2\text{Cu}^+ + \text{DASC}
\]

\[
\text{Cu}^+ + \text{GSNO} + H^+ \rightarrow \text{Cu}^{2+} + \text{GSH} + \text{NO}
\]

At higher AA concentrations or in the presence of Cu²⁺ chelators, the reaction proceeds via intermediate step of O-nitrososacurbate formation in transnitrosation process yielding NO or NO⁻ (Figure 6)

\[
\text{GSNO} + \text{ASC}^{-} \rightarrow \text{ASCNO}^{-} + \text{GS}^{-} \quad (n)
\]

\[
\text{ASCNO}^{-} \rightarrow \text{ASC}^{-} + \text{NO}^{-} \quad (x)
\]

\[
\text{ASCNO}^{-} \rightarrow \text{DASC} + \text{NO}^{-} \quad (y)
\]

Rates of reactions (x), (y) and therefore the yield ratio of [NO⁻][NO] were found to depend on pH with dominant formation of NO⁻ at physiological conditions. Rate of O-nitrososacorbate formation showed dramatic dependence on pH, decreasing by two orders of magnitude when pH changed from 7.3 to 3.6 (Holmes and Williams 2000). This demonstrates that at low pH values degradation of GSNO by AA is less efficient. Moreover, AA can directly react with HNO₂ producing O-nitrososacorbate, i.e., this reaction can be a source of O-nitroso compounds (NO⁺ centred on O-atom of AA) in addition to S-nitroso compounds.

In basic pH, excess OH⁻ competes with NO⁺ and HONO is deprotonated to form nitrite: GSNO formation and stability are impaired. In acidic pH, the balance is reversed: HONO is stabilized, and NO⁻ transfers to GS⁻ is favoured: OH⁻ is the leaving group, which is depleted to form water to drive the reaction towards GSNO. However, HONO can also evolve NO: NO loss competes with GSNO formation. Our data suggest that 2-nitrosyl ascorbyl, perhaps in equilibrium with 2-nitrosyl ascorbate, stabilizes NO⁺, favouring transfer to GSH. Functionally, GSNO is favoured, though additional mechanistic work will be needed.

This work is important because high oral doses of AA and AA-containing antioxidant complexes are commonly used and have benefits in a number of diseases (Cameron and Pauling 1978; Sukolinskii and Morozkina 1989; Morozkina et al. 1991; Du et al. 2012; Rodrigo et al. 2014). Our data suggest that, rather than depleting gastric GSNO levels as we expected, these high exposure levels stabilize GSNO at gastric pH. This could actually augment gastric motility, vascular smooth muscle relaxation and host defense. Additional studies will be required, however, to determine the fate of GSNO under these conditions, and whether GSNO contributes to the beneficial effects of antioxidant complexes. The gastric chemistry of nitrogen oxides is also important because nitric oxide synthase (NOS) deficient mice are viable (Huang 2000). We have hypothesized that exogenous nitrogen oxides in the gut and lung can provide nitrogen oxides in a NOS-independent fashion. For example, the lung lining fluid normally contains near mM levels of reduced GSH and μM levels of nitrite; these are cleared from the airways to the larynx and swallowed. Under conditions of mild acidification in the gut and distal airways, inorganic formation of luminal S-nitrosogluthione (GSNO) would be predicted (Gaston et al. 1993; Fang et al. 1998; Gaston, Singel, et al. 2006; Morozkina and Gaston 2012, 2015). Our current data suggest that this NOS-independent source of stable nitrogen oxide bioactivity is stabilized, rather than broken down, by oral ingestion of AA given as an antioxidant therapy.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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