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Original Contribution

Dietary phenolic acids and ascorbic acid: Influence on acid-catalyzed nitrosative chemistry in the presence and absence of lipids

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ABSTRACT

Acid-catalyzed nitrosation and production of potentially carcinogenic nitrosative species is focused at the gastroesophageal junction, where salivary nitrite, derived from dietary nitrate, encounters the gastric juice. Ascorbic acid provides protection by converting nitrosative species to nitric oxide (NO). However, NO may diffuse into adjacent lipid, where it reacts with O_2 to re-form nitrosative species and *N*-nitrosocompounds (NOC). In this way, ascorbic acid promotes acid nitrosation. Using a novel benchtop model representing the gastroesophageal junction, this study aimed to clarify the action of a range of water-soluble antioxidants on the nitrosative mechanisms in the presence or absence of lipids. Caffeic, ferulic, gallic, or chlorogenic and ascorbic acids were added individually to simulated gastric juice containing secondary amines, with or without lipid. NO and O_2 levels were monitored by electrochemical detection. NOC were measured in both aqueous and lipid phases by gas chromatography–tandem mass spectrometry. In the absence of lipids, all antioxidants tested inhibited nitrosation, ranging from $35.9 \pm 7.4\%$ with gallic acid to $93 \pm 0.6\%$ with ferulic acid. In the presence of lipids, the impact of each antioxidant on nitrosation was inversely correlated with the levels of NO they generated ($R^2 = 0.95$, $p < 0.01$): gallic, chlorogenic, and ascorbic acid promoted nitrosation, whereas ferulic and caffeic acids markedly inhibited nitrosation.

The human gastroesophageal junction is an anatomical region of high and increasing incidence of epithelial metaplasia and neoplasia [1]. Adenocarcinoma at the gastroesophageal junction arises from this metaplasic mucosa, with the incidence of this cancer having risen markedly over recent decades [1–3]. The gastroesophageal junction is now the major site of adenocarcinoma of the human upper gastrointestinal tract [4]. Metaplasia and neoplasia at the gastroesophageal junction are thought to be due to the injurious effect of acid from the stomach refluxing onto the squamous mucosa of the distal esophagus [5,6].

Recent studies have indicated that this anatomical region is also an area of high luminal nitrosative stress arising from the chemistry that occurs when the nitrite-laden saliva encounters the acidic gastric juice [7–9]. The high concentrations of nitrite in saliva are due to the enterosalivary recirculation of ingested nitrate and its reduction to nitrite by buccal bacteria [10,11]. When nitrite in saliva encounters the acidic gastric juice, it is converted to nitrous acid (HNO_2) (Eq. (1)) and nitrosating species, including N_2O_3 (Eqs. (2) and (3)) and NO^+as well as NOSCN (Eq. (4)), formed from the reaction with thiocyanate, which is concentrated in saliva [12].

$$NO_2^- + H^+ \leftrightarrow HNO_2, \quad (1)$$

$$HNO_2 + H^+ \leftrightarrow NO^+ + H_2O \leftrightarrow H_2NO_2^+, \quad (2)$$

$$H_2NO_2^+ + HNO_2 \leftrightarrow N_2O_3 + H_2O + H^+, \quad (3)$$

$$HNO_2 + H^+SCN^- \leftrightarrow H_2O + NO^+ + SCN^- \leftrightarrow NOSCN + H_2O. \quad (4)$$

Nitrosating species (NOX; e.g., N_2O_3, NO^+, NOSCN) are able to react with secondary amines (R_2NH) and amides to produce potentially mutagenic *N*-nitroso compounds (Eq. (5)) [13–15]:

$$NOX + R_2NH \leftrightarrow R_2NNO + H^+ + X. \quad (5)$$

A major protective factor against acid nitrosation is ascorbic acid, which is present in the diet and actively secreted in gastric juice. Ascorbic acid competes with N-nitrosatable compounds for the nitrosating species, converting the latter to NO. In the process, ascorbic acid (ASC) is oxidized to dihydroascorbic acid (DHA) (Eq. (6)) [16,17]:

$$ASC + NOX \leftrightarrow DHA + 2NO + H^+ + X^- + H_2O. \quad (6)$$

Abbreviations: EDTA, ethylenediamine tetraacetic acid; GC–ITMS/MS, gas chromatography–ion-trap tandem mass spectrometry; NMOR, *N*-nitrosomorpholine; NPIP, *N*-nitrosopiperidine; SE, standard error.

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There are, however, a number of problems with the mechanism of inhibition of acid nitrosation by ascorbic acid, which converts nitrosating species to NO. The first is that NO is able to react with molecular oxygen (O_2) to re-form nitrite and nitrosating species (Eqs. 7 and 8):

$$2NO + O_2 \leftrightarrow 2NO_2, \quad (7)$$

$$NO_2 + NO \leftrightarrow 2N_2O_3. \quad (8)$$

This interaction between NO and O_2 is particularly rapid at high NO concentrations, because the rate of reaction is second order with respect to NO and first order with respect to O_2 [18]. Although ascorbic acid can reconvert the re-formed nitrosating species back to NO, this recycling process results in the rapid consumption of ascorbic acid, leading to its depletion and nitrosation resuming uninhibited [18,19]. For these reasons, the amount of ascorbic acid required to inhibit acid nitrosation in the presence of ambient O_2 concentrations is more than sixfold that required in the absence of O_2. Previous studies have indicated that up to 50 μM NO can be generated when nitrite meets gastric juice in the human gastrointestinal tract, in an environment also exposed to high concentrations of O_2 as air is swallowed along with saliva [7,9].

A second problem related to inhibition of acid nitrosation by ascorbic acid via NO formation has been recognized: NO is not very water soluble and preferentially diffuses to hydrophobic compartments and is therefore able to pass into the surrounding epithelium [20]. Benchtop models simulating the nitrite chemistry at the gastroesophageal junction have indicated that the NO can pass into surrounding neutral compartments and there react with O_2 to form N_2O_3, which can nitrosate at neutral pH [21,22]. Nitrosation at this pH is potentially more problematic as amines are more nitrosatable at neutral pH than at the pH of acidic gastric juice, because of their high pK_a. In addition, it is known that the reaction between NO and O_2 to form N_2O_3 is 400 times more rapid in lipid compartments than in aqueous compartments [20]. The epithelium at the gastroesophageal junction comprises lipid membranes and intracellular lipid structures, potentially providing a site for rapid generation of N_2O_3 from NO diffusing from the lumen. Furthermore, recent studies indicated that lipid antioxidants, such as vitamin E and β-carotene, are ineffective at preventing this nitrosative process within a lipid environment [23]. Although ascorbic acid may inhibit acid nitrosation within the lumen, in doing so it promotes nitrosation within the adjacent lipid epithelium, displacing the nitrosative stress to a biologically important site [22].

Ideally, one would wish to identify an antioxidant that would inhibit acid nitrosation without generating high concentrations of NO. In these studies, we have examined a range of dietary phenolics (aglycones), namely gallic acid, caffeic acid, ferulic acid, and 5-*O*-caffeoylquinic acid (chlorogenic acid) (Fig. 1), along with ascorbic acid, in an effort to identify dietary compounds that may more effectively inhibit acid-catalyzed nitrosation at the gastroesophageal junction.

Materials and methods

Dietary phenolics were tested along with ascorbic acid and negative controls (no antioxidant), at the acidic pH of the stomach, with respect to their ability to convert nitrite to NO, inhibit N-nitrosation in the presence or absence of O_2, and inhibit N-nitrosation in the presence versus the absence of lipid.

Nitrite conversion to NO: assay setup and measurements

All studies were carried out in closed benchtop systems simulating the acidic gastric environment. The setup consisted of a glass container fitted with an air-tight cap equipped with two ports for the NO and O_2 probes and a third port for sampling/addition of solutions. The system was kept in a foil pouch to limit light/UV exposure. The acidic aqueous phase (0.1 M HCl, pH 1.5) contained 1 mM EDTA[1] and 1 mM NaSCN. EDTA was included in the gastric juice to chelate any trace metals introduced in the in vitro setup that may otherwise interfere with the nitrosation reaction; NaSCN was added because it is a catalyst of nitrosation reactions, present in the saliva in concentrations up to 1.2 mM [7,24]. Caffeic, ferulic, gallic, chlorogenic, and ascorbic acids (either 250 μM or 2 mM) were added individually to the simulated gastric juice. Concentration choice was based on published total phenolic contents of common beverages, expressed in gallic acid equivalents (GAE), ranging from 0.9 mM GAE for white grape juice to 18 mM GAE for red wine, with most beverages tested comprising between 2 and 5 mM GAE [25]. Taking into account the diluent effect of gastric contents, a concentration of 250 μM phenolics was chosen as "low," and a concentration of 2 mM was chosen as "high." Negative controls (no antioxidant) were run in parallel experiments. Addition of nitrite (100 μM) at $t = 0$ initiated the reaction, which was incubated at 37°C for 60 min under gentle magnetic stirring. Where appropriate, O_2 was depleted from the benchtop system using a combination of sonication and helium/argon flushing to investigate the effect of O_2 on NO production and nitrosation. Dissolved NO in the aqueous phase was continuously monitored by electrochemical detection using a NO electrode and meter (ISO NO Mark II; World Precision Instruments, Sarasota, FL, USA), as previously described [22].

Oxygen measurement

Dissolved O_2 in the aqueous phase was continuously monitored with an O_2 electrode and meter (Mark II; World Precision Instruments), as previously described [22].

Inhibition of nitrosation: assay setup

All studies were carried out in a closed benchtop system simulating the acidic gastric environment, in the presence or absence of lipid. The system was fitted with a cap containing a single air-tight port for injection/sampling and was contained in a foil pouch. The acidic aqueous phase (0.1 M HCl, pH 1.5) contained 1 mM EDTA, 1 mM NaSCN, 5 mM morpholine and piperidine, two secondary amines with different pK_a (8.33 and 11.22, respectively) and lipid solubility (ClogP −0.55 and 0.52, respectively). Where applicable, the lipid phase (trioctanoin, 1:10 ratio) contained morpholine (5 mM) and piperidine (5 mM). Phenolics (caffeic, ferulic, gallic, or chlorogenic acids) were

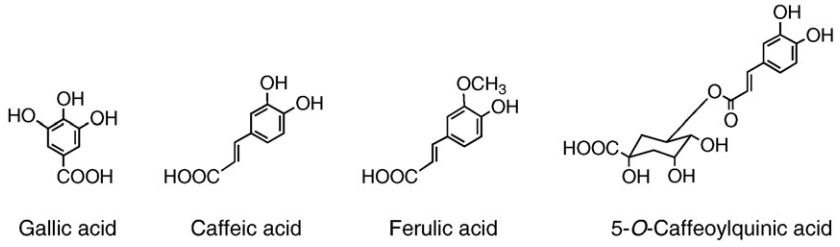

Fig. 1. Molecular structures of the four phenolic acids tested: caffeic acid, ferulic acid, gallic acid, and 5-*O*-caffeoylquinic acid (chlorogenic acid).