Temporal influence of ascorbic acid dose on the endogenous formation of N-nitrosoproline and N-nitrosothiazolidine-4-carboxylic acid in humans

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The temporal effect of ascorbic acid intake on the endogenous formation of N-nitrosoproline and N-nitrosothiazolidine-4-carboxylic acid was determined over 15 days in 10 ascorbate depleted men consuming a low nitrate and ascorbic acid (<23 μmol) diet. N-nitrosoproline precursors were given as follows: days 3–15, nitrate (5.24 mmol); days 2–15, L-proline (4.35 mmol) 30 min following the nitrate; days 5–11, ascorbic acid (2.62 mmol) 6, 5, 4, 3, 2, 1, and 0.5 hr before the proline, concurrent with the proline on day 12, and 0.5, 1 and 2 hr following the proline on days 13–15. Urine was analyzed for N-nitrosoamino and ascorbic acids. Gastric pH and residence time were measured on days 3–4 concurrently with the proline challenge. Excretion of N-nitrosoproline on nitrate and proline only days was 42.2 ± 16.0 nmol. Significant ($P < 0.05$) inhibition was observed when ascorbic acid was given 5 hr before proline (29.1 ± 10.5 nmol). These data suggest that the ascorbic acid content of the stomach due to secretion is sufficient to partially inhibit gastric nitrosation. Diets high in ascorbic acid could reduce gastric formation of N-nitroso compounds even if ascorbic acid is not consumed at the same time as the precursors. No correlation between gastric pH and gastric residence time with N-nitrosoproline and N-nitrosothiazolidine-4-carboxylic acid excretion was observed over the pH range observed. Several subjects consistently excreted high or low levels of N-nitrosoproline or N-nitrosothiazolidine-4-carboxylic acid compared to the mean, regardless of the ascorbic acid intake. This suggests that physiological differences between subjects may account for their endogenous nitrosation capacity.

Keywords: N-nitrosoproline; N-nitrosoamino acid; ascorbic acid; N-nitrosothiazolidine-4-carboxylic acid; gastric pH; endogenous formation

Introduction

Human exposure to N-nitroso compounds (NOC) may be important due to their potent carcinogenicity. Of nearly 300 NOC tested, 90% have demonstrated carcinogenicity in laboratory animals.[1] NOC may be endogenously formed from ingested precursors or from precursors which are themselves formed endogenously.[2,3] Dietary and physiological factors may play a role in the type and quantity of NOC formed.[4]

Ascorbic acid has been shown to quantitatively inhibit the nitrosation of secondary amines both in vitro[5] and in vivo[2] by competing with the precursors for nitrosating agents. Given the protective role that ascorbic acid may play in the prevention of gastric carcinogenesis, studies have investigated the concentration and stability of ascorbic acid in the human stomach. Rathbone et al.[6] and O'Connor et al.[7] recently reported that ascorbic acid is secreted and concentrated in the healthy stomach. These data raise the possibility that gastric secretion of ascorbic acid may provide partial inhibition of endogenous nitrosation.

Excretion of N-nitrosoproline (NPRO) can be taken as a measure of an individual's endogenous nitrosation capacity.[2] We and others[8,9] have observed that some subjects consistently form large amounts of NPRO endogenously, whereas others are consistently low responders. Physiological differences such as gastric

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pH[10] may exist between subjects and may account for these differences.

Several authors have shown that ascorbic acid is capable of inhibiting gastric NPRO formation when the ascorbic acid is given concurrently with the proline. It was our objective to determine if ascorbic acid would influence NPRO formation if given several hours prior to the proline. This may be of importance because ascorbic acid is not always consumed with each meal but might still inhibit gastric nitrosation. We also wanted to determine if differences in gastric pH or gastric residence time could account for the large inter-individual differences typical of endogenous NPRO formation studies.

Materials and methods

Chemicals

Sources of reagents were as follows: sodium nitrate, sodium azide, sodium sulfate, sodium chloride, dipotassium hydrogen phosphate, hexanes, methanol, ethanol, and anhydrous diethyl ether, Fisher Scientific Co. (Fair Lawn, NJ, USA); metaphosphoric acid, potassium hydroxide and sodium hyroxide, Mallinckrodt, Inc. (Raleigh, NC, USA); ascorbic acid donated by Hoffman-La Roche Co. (Nutley, NJ, USA); methylene chloride, Burdick and Jackson Laboratories (Muskegon, MI, USA); ammonium sulfamate, dithiothreitol, L-proline, Diazald, Aldrich Chemical Co. (Milwaukee, WI, USA); potassium dihydrogen phosphate, Diagnostics Procedure #555-A for creatinine analyses, Sigma Chemical Company (St. Louis, MO, USA); Test-Combination #409677 for ascorbic acid analyses; Boehringer Mannheim (Mannheim, Germany); NPRO, N-nitrosothiazolidine-4-carboxylic acid (NTCA) and N-nitrosopipecolic acid (NPIPC) were synthesized by the method of Lijinsky et al.[11] All chemicals were of reagent or analytical grade.

Urine collection and storage

All urine was collected directly into 1 L brown Nalgene bottles, containing 9 mmol sodium azide to prevent artifactual nitrosamine formation and microbiological activity.[12] Urine bottles were collected throughout the day, and stored at 5° C until the 24-hr collection was completed. NPRO and NTCA were determined stable under these conditions prior to the study. The 24-hr collections were then combined, the total volume and specific gravity determined, and a 0.5 L aliquot immediately frozen (−20° C) until analysis.

Urinary creatinine excretion was determined colorimetrically (Sigma Diagnostics Procedure #555-A) for each subject every day, to ensure that each subject complied with the 24-hr collection.

Total ascorbates analysis

The urinary ascorbic acid samples were stabilized using 20% (wt/vol) meta-phosphoric acid. Dehydroascorbic acid/L-ascorbic acid in urine were measured using Boehringer Mannheim Test-Combination #409677.[13]

N-nitrosoamino acid analysis

Urine (25 ml) to which 160 ng NPIPC was added (internal recovery standard) was adjusted to pH 8.5 (1 N NaOH), 4 g NaCl added, and extracted with 85 ml hexanes which were discarded. The pH was adjusted to 1 or lower (20% ammonium sulfamate in 3.6 N sulfuric acid), extracted (6 × 40 ml 10% methanol/methylene chloride), and the combined fractions dried (10 g anhydrous sodium sulfate), filtered, concentrated to 1–2 ml (vacuum evaporation, 40° C), and further concentrated to 0.5 ml under a stream of N_2. N-nitrosoamino acids (NAAs) were esterified by adding 1 ml ethereal diazomethane. The derivatized extract was concentrated (1 ml, N_2). Samples were analyzed by gas chromatography-thermal energy analyzer as previously described[14] except that a 2 m × 2 mm i.d. glass column packed with 3% OV-351 on 80-100 Chromosorb WHP support was used, and the column was temperature programmed (90° C 1 min hold, to 200° C at 8° C/min). NPRO and NTCA were quantified by comparison to external standards. The limit of detection was 0.3 ng NPRO/ml urine. The recovery of NPIPC averaged 98% ± 7% over the entire study, and the average recoveries of NPRO and NTCA in samples spiked with 160 ng of each NAA were 94% ± 5% and 89% ± 6%, respectively. Reported NPRO and NTCA values were not corrected for recovery.

Statistical analysis

All data were compared by analysis of variance with repeat measures. Adjusted data were compared by analysis of variance without using repeat measures. Statistically significant differences between the mean amounts of NPRO and NTCA excreted on days 5–15 relative to the control period (mean of days 3–4) were determined by the Least Significant Difference criterion. Differences were considered significant if $P < 0.05$.

Gastric pH and residence time

The Heidelberg pH Capsule System (Heidelberg International, Electro-Medical Devices, Inc.; Norcross, GA, USA) was used to measure gastric pH and gastric residence time by radiotelemetry. The Heidelberg capsule contains a radio transmitter, encapsulated for swallowing, which transmits pH values from the gastrointestinal tract. The signal is received by a belt-antenna receiver, from which the pH is recorded and displayed. Residence time was defined as the time the capsule resided in the stomach as measured by an abrupt increase in pH. The capsule was calibrated repeatedly in buffers at pH 1 and 7 (maintained at 37° C), until no further adjustments were necessary. The measuring accuracy is guaranteed by the manufacturer (Heidelberg International, Electro-Medical Devices,

Table 1 Composition of the diet

Breakfast	
41 g	Cereal with whole milk
59 g	Whole wheat toast
5 g	Margarine
19 g	Grape jelly
240 g	Whole milk
1–2 cups	Coffee or tea (optional, limit 2 cups)
Lunch	
115 g	Peanut butter/grape jelly/wheat bread
48 g	Shortbread cookies
368 g	Ginger ale
Dinner	
145 g	Chicken breast (boned and skinned)
178 g	*Rice
32 g	Roll
5 g	Margarine
240 g	Whole milk
86 g	Ice cream with chocolate sauce
Evening Snack	
100 g	*Bagel
29 g	*Cream cheese
368 g	*Ginger ale

* Subjects were allowed to choose extra portions of these items on days 1 and 2, and then continued with the same amount from day 3 until the end of the study.

Inc.; Norcross, GA, USA) to be ±0.5 pH units over 6 hr, within pH 1–8.

Subjects and diets

The ten subjects (healthy, non-smoking males; ages 21–38) were taking neither medications nor vitamin supplements prior to or during the study. A low nitrate (<0.50 mmol/day) and low ascorbic acid (<23 μmol/day) diet (*Table 1*) was the only food consumed. Composite diet samples (including water) were analyzed for nitrate, NPRO, and NTCA. Breakfast and dinner were consumed in the Cornell University Human Metabolic Unit. Lunch and the evening snack were packed for the subjects to take with them and eat at an assigned time. Distilled water was provided and was the only water consumed. All subjects completed a daily diary to record any experimental deviations.

Experimental protocol

The experimental protocol was as follows: day 1, diet only; days 2–15, diet plus L-proline (4.35 mmol in 25 ml distilled water, followed by 100 ml distilled water) at least 3 hr after the subjects last ate; days 3–15, sodium nitrate (5.24 mmol in 10 ml distilled water, followed by 100 ml distilled water) 30 min prior to the L-proline; days 5–15, ascorbic acid (2.62 mmol in 10 ml distilled water, followed by 100 ml distilled water). The times of ascorbic acid administration were 6, 5, 4, 3, 2, 1, and 0.5 hours before the L-proline challenge (days 5–11), concurrent administration with the L-proline (day 12) and 0.5, 1 and 2 hr following the L-proline challenge (days 13–15). Heidelberg Capsules were swallowed on day 3 or 4 immediately before the L-proline challenge in order to measure gastric pH and residence time.

Results

Ascorbic acid status

Urinary ascorbates (ascorbic acid + dehydroascorbate) were not detectable on day 4. On the first day ascorbic acid was given (day 5), an average of 0.06 ± 0.01 mmol was detected. On days 11–13, urinary ascorbates averaged 0.26 ± 0.21, 0.16 ± 0.13, and 0.30 ± 0.15 mmol, respectively. Since individuals are considered depleted when urinary ascorbates are not detected,[15-17] these data indicate that the body pools of ascorbic acid were less than saturated by day 4, and were resaturated quickly after one ascorbic acid supplementation on day 5.

NPRO and NTCA excretion

Ascorbic acid inhibited NPRO formation when dosed 6 hr before the proline, but was not significant. Statistically significant inhibition was observed when ascorbic acid was dosed 5 hr before the proline challenge (day 6; *Table 2*). NTCA excretion was not affected by ascorbic acid intake unless given one half-hour following the nitrate (*Table 3*). As expected, the optimum time to dose ascorbic acid for maximum inhibition of NPRO formation in vivo was concurrent with the proline dose (day 12). On days 8–10, one subject each day excreted large amounts of NPRO, thereby elevating the daily NPRO averages and standard deviations. If data from this subject are excluded on these days in the ANOVA test, the average NPRO excreted on days 8–10 is reduced considerably (*Table 2*), and statistically significant differences are observed on

Table 2 Average excretion of NPRO

Day	Ascorbic Acid[a] Treatment	Average[b] Excretion	Adjusted Average[c] Excretion
1	None	19.6 ± 9.7	—
2	None	12.1 ± 2.1	—
3	None	42.1 ± 12.8	—
4	None	42.3 ± 19.2	—
5	6 hr +	34.8 ± 10.5	—
6	5 hr +	29.1 ± 12.1*†	—
7	4 hr +	28.9 ± 14.1*†	—
8	3 hr +	39.3 ± 35.5	29.6 ± 18.5†
9	2 hr +	38.9 ± 25.5	31.3 ± 9.7†
10	1 hr +	33.1 ± 24.6	25.5 ± 6.3†
11	½ hr +	24.3 ± 10.2*†	—
12	Concurrent	19.0 ± 7.1*†	—
13	½ hr −	30.4 ± 10.8†	—
14	1 hr −	30.4 ± 9.2†	—
15	2 hr −	38.9 ± 14.5	—

[a] Time of ascorbic acid challenge relative to the L-proline challenge; (+) before, (−) after.
[b] Mean (nmol/day) ±1 SD.
[c] Mean (nmol/day) ±1 SD (n − 1); average with one subject not included in the average.
* Statistically significant inhibition ($P < 0.05$) relative to the average excretion on days 3–4 (42.2 ± 16.0 nmol/day).
† Statistically significant inhibition ($P < 0.05$) relative to the average excretion on days 3–4 (42.2 ± 16.0 nmol/day), using the adjusted average excretion on days 8–10 in the ANOVA test.

Table 3 Average excretion of NTCA

Day	Ascorbic Acid[a] Treatment	Average[b] Excretion	Adjusted Average[c] Excretion
1	None	22.6 ± 15.5	—
2	None	15.4 ± 8.0	—
3	None	45.0 ± 44.4	30.4 ± 8.4
4	None	30.1 ± 5.9	—
5	6 hr +	33.6 ± 8.1	—
6	5 hr +	34.6 ± 10.7	—
7	4 hr +	32.1 ± 13.1	—
8	3 hr +	48.9 ± 49.4	33.6 ± 9.3
9	2 hr +	54.9 ± 38.6	43.1 ± 11.7
10	1 hr +	29.9 ± 12.6	—
11	½ hr +	25.4 ± 7.5	—
12	Concurrent	23.6 ± 11.4*	—
13	½ hr −	35.4 ± 9.7	—
14	1 hr −	33.9 ± 11.4	—
15	2 hr −	36.5 ± 12.6	—

[a] Time of ascorbic acid challenge relative to the L-proline challenge.
[b] Mean (nmol/day) ±1 SD.
[c] Mean (nmol/day) ±1 SD (n − 1); average with one subject not included in the average.
* Statistically significant inhibition relative to the average excretion on days 3–4 (37.5 ± 25.1 nmol/day).

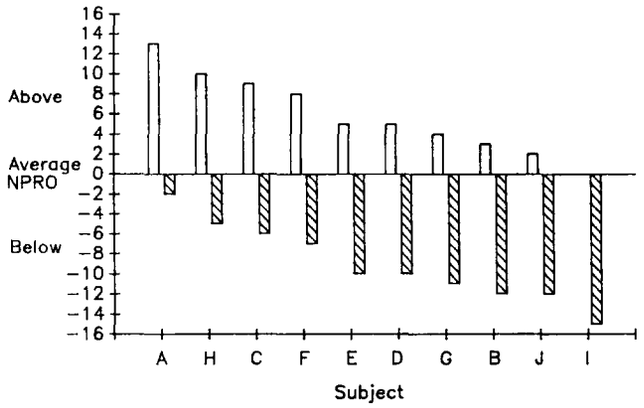

Figure 1 The number of days each subject excreted NPRO (nmol/day) above and below the average NPRO excreted by all subjects during the 15-day study.

days 6–14. The average daily excretion of NPRO and NTCA were correlated weakly (r^2 = 0.64). The decrease in mean NPRO excretion from day 1 to 2 is likely due to carry-over of NPRO from the free choice diet.

Individual variability

Significant intersubject differences in the ability to nitrosate proline and thiazolidine-4-carboxylic acid (TCA) endogenously were observed. Several subjects consistently excreted high or low levels of NPRO or NTCA throughout the study regardless of the protocol on any given day. For example, the amount of NPRO excreted by subject A was above the study average for 13 of the 15 days. Conversely, NPRO excretion from subject I was below the average throughout the

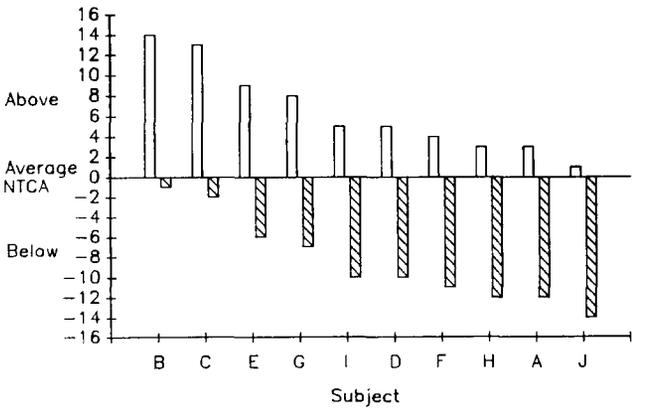

Figure 2 The number of days each subject excreted NTCA (nmol/day) above and below the average NTCA excreted by all subjects during the 15-day study.

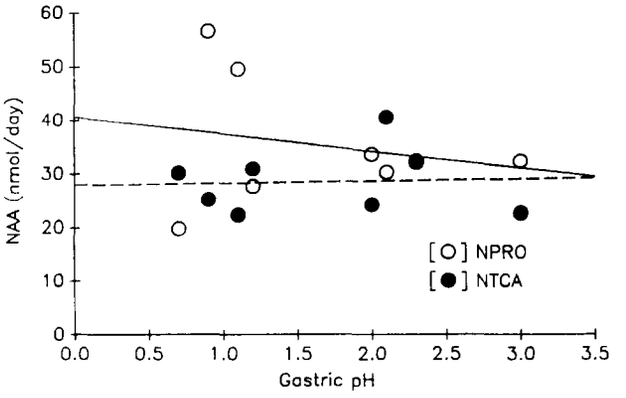

Figure 3 Linear regression analyses of NPRO (solid line; r^2 = 0.05) and NTCA excretion (dashed line; r^2 = 0.00) and gastric pH measured at the time of proline administration.

entire study (*Figure 1*). Similar data were seen with NTCA excretion (*Figure 2*), although not always for the same individual.

Gastric pH and residence time

Linear regression analyses failed to demonstrate a correlation between gastric pH and the excretion of NPRO or NTCA (*Figure 3*). There was likewise no correlation between gastric residence time with NPRO or NTCA excretion (*Figure 4*). Data from two subjects were not included due to deviations from the experimental protocol.

Discussion

Effect of ascorbic acid

While no ascorbic acid would be expected to remain in the stomach 5 hr after the ascorbic acid was given, significant inhibition of endogenous NPRO formation was observed. These results may be explained by recent reports of the secretion and concentration of ascorbic acid in the human stomach. O'Connor et al.[18] reported that the basal gastric concentration of

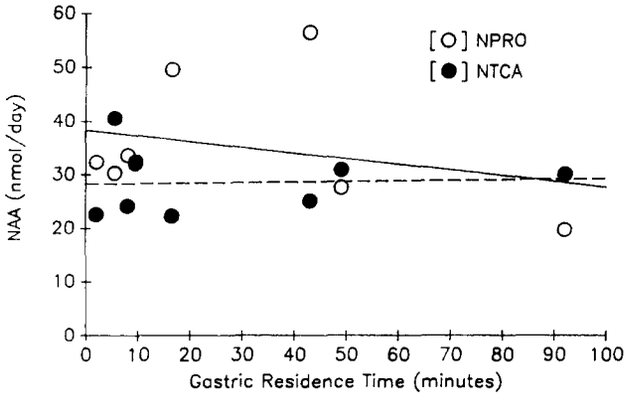

Figure 4 Linear regression analyses of NPRO (solid line; r^2 = 0.05) and NTCA excretion (dashed line; r^2 = 0.00) and gastric residence time, measured at the time of proline administration.

ascorbic acid was elevated and the mutagenic activity of gastric juice reduced in human subjects following oral supplements of vitamin C, and later reported that ascorbic acid is secreted in the stomach.[7] Rathbone et al.[6] found nearly three times more ascorbic acid in gastric juice of subjects with normal gastric histology than in the plasma. They estimated that approximately 0.34 mmol of vitamin C is secreted in the normal stomach daily. O'Connor et al.[7] found that the basal secretion of vitamin C increased from 0.42 mg/hr to 1.65 mg/hr 30–40 min post-pentagastrin treatment.

It is likely that the gastric ascorbate content of our subjects was low when the highest NPRO excretion was detected (days 3 and 4) because ascorbates were not detected in their urine. The ascorbate content of the gastric fluid would have been increased on days 5 through 15 when supplements were given.[18] These elevated gastric ascorbate levels would partially inhibit NPRO formation, regardless of the time ascorbic acid is consumed. These data are the first to show that ascorbic acid does not have to be taken concurrently with the proline to inhibit NPRO formation. It is likely that diets that are generally high in ascorbic acid would lower gastric nitrosation even if ascorbate-containing foods were not consumed at the same time as foods containing precursors.

Assuming that 5% of the ingested nitrate is reduced to nitrite and swallowed in the saliva,[19] 0.26 mmol of nitrite entered the stomach after each nitrate dose. Even though the majority of the nitrite enters the stomach within 12 hr of a bolus nitrate challenge,[20] inhibition of NPRO formation by the ascorbic acid that is secreted in the stomach (0.34 mmol/day) would be possible at these concentrations. Leaf et al.[9] found that ascorbic acid doses of as little as 0.05 mmol reduced NPRO formation by 6 nmol/day in humans.

Inhibition of endogenous NPRO formation was the greatest when the ascorbate and proline were taken concurrently (day 12), presumably when the concentration of ascorbic acid (basal + bolus dose remaining in the stomach) relative to nitrite was maximal. The average NPRO excreted on days 3 and 4 (no ascorbic acid challenge) was 42.2 nmol/day. Excretion on day 2 (proline only) was 12.1 nmol, which is the sum of dietary NPRO and NPRO endogenously formed when no nitrate was given. When corrected for the excretion on day 2, the average NPRO formed endogenously on days 3 and 4, and on day 12 were 30.1 nmol and 6.9 nmol, respectively. Thus, 77% of endogenous NPRO formation was inhibited when ascorbic acid was given concurrently with the proline challenge. Incomplete inhibition of NPRO formation may in part be due to the nitrosation of proline at sites other than the stomach.[8]

Excretion of NTCA was affected less by ascorbic acid intake than was NPRO. The optimum time to dose ascorbic acid for maximum inhibition of endogenous NTCA formation was ½ hour following the nitrate challenge (i.e., concurrent with the proline on day 12), and NTCA formation was also inhibited when ascorbic acid was challenged concurrently with the nitrate challenge (day 11). Since TCA is nitrosated 1,000-fold faster than proline,[21] TCA would be less sensitive than proline to the inhibitory affects of ascorbic acid.

The lowering of NPRO excretion on days 5 through 12 cannot be accounted for by the progressive accumulation of vitamin C in the body because excess absorbed ascorbic acid is eliminated rapidly in humans. A saturable body pool of ca. 8.5 mmol ascorbic acid exists in healthy adult men,[15-17] and the saturated pool cannot be further increased by additional consumption of vitamin C. When the body pool has been saturated (e.g., day 5), excess absorbed ascorbic acid is eliminated rapidly in the urine. The reabsorption of ascorbic acid in the proximal tubule is an active-transport process, and when the plasma vitamin C exceeds 0.75–1.0 mg/100 ml, the ability of the renal tubule to reabsorb vitamin C is exceeded.[22,23] Finally, if ascorbate were accumulated during these experiments, a reduction in NPRO excretion towards baseline between days 5 through 11 would be expected. No such trend was observed, although the large interindividual variation may prevent the observation of a subtle accumulation effect.

Gastric pH and residence time

There was no correlation of gastric pH as measured by the Heidelberg capsule with NPRO or NTCA excretion over the relatively narrow pH range (pH 0.7–3.0) of our subjects. The advantage of the Heidelberg pH capsule system is that the gastric pH is known at the time when proline nitrosation is most likely to occur because the capsule is swallowed immediately prior to the proline challenge. Given the small sample size and the large individual variability inherent to NAA excretion,[24] our data suggest, but do not prove, that differences in pHs < 3 cannot explain the interindividual differences in NPRO and NTCA excretion.

Individual variability

Large unexplained interindividual variability in response to the NPRO test have been observed and re-

viewed by others,[25] although no classification of individuals has been previously reported. The data in *Figures 1* and *2* suggest that one or more physiological variables (e.g., gastric pH, gastric residence time, salivary nitrate concentration, salivary nitrate reductase activity, and the concentration and presence of gastric catalysts and inhibitors) account for differences between each subject's potential for enhanced or limited endogenous nitrosation. We are investigating currently the effect of individual differences in oral nitrate-reductase activity on endogenous nitrosation.

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