Effect of Vitamins C and E on Endogenous Synthesis of N-Nitrosamino Acids in Humans: Precursor-Product Studies with $[^{15}\text{N}]$Nitrate

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ABSTRACT

The endogenous formation of nitrosoproline (NPRO) following administration of nitrate and proline is reported in ten healthy young adults. There was a relatively constant basal excretion of NPRO, 26 ± 10 (SD) nmol/day, in excess of amounts found in the diet. This basal synthesis of NPRO was not reduced by ascorbic acid (2 g/day) or $\alpha$-tocopherol (400 mg/day). A significant rise in the excretion of NPRO was observed following the administration of nitrate and proline, ranging from 29 to 318 nmol/24 h with a mean of 100 nmol/24 h. $[^{15}\text{N}]$Nitrate was used as a tracer to study the observed excess excretion of NPRO in urine. The data revealed that urinary NPRO excretion as a result of endogenous synthesis is not totally derived from ingested nitrate as its precursor. The ingestion of ascorbic acid and $\alpha$-tocopherol inhibited the incorporation of $[^{15}\text{N}]$nitrate into NPRO by 81 and 59%, respectively. An additional nitrosamine, N-nitrosothiazolidine-4-carboxylic acid, was present in the urine. It was found that N-nitrosothiazolidine-4-carboxylic acid increased 6-fold upon ingestion of nitrate. Ascorbic acid and $\alpha$-tocopherol blocked this nitrate induced synthesis.

INTRODUCTION

Substantial evidence has been assembled suggesting the possible health risk from exposure to N-nitroso compounds. Sander (1) originally proposed the concept of endogenous intragastric nitrosation, a process capable of generating carcinogenic N-nitroso compounds in humans. It has also been demonstrated that N-nitroso compounds are formed endogenously in animals given nitrite and a suitable amine (2, 3). However, the initial research on endogenous synthesis of N-nitroso compounds in humans was complicated by the presence of numerous artifacts of the analytical methods and the collection procedures (4-6). A method proposed by Ohshima and Bartsch (7) was the first potentially suitable procedure for estimating daily human exposure to endogenously formed N-nitroso compounds. The monitoring of urinary levels of NPRO2 after dosing with nitrate and proline was utilized in a human volunteer without adverse biological effects. Since this publication, a number of other investigators have used this procedure to demonstrate the endogenous formation of nitrosoproline in humans (8-10) as well as other nitrosoamino acids (11-14).

In our initial studies (10), we found that endogenous production of nitrosoproline could be demonstrated in human subjects ingesting a diet low in nitrate and NPRO. Upon administration of nitrate and proline, a significant rise in the excretion of NPRO was observed. The formation of NPRO even during low nitrate intakes could possibly be linked to the endogenous production of nitrate, previously reported by our group (15). We now report additional experiments in human subjects which have been conducted to extend our original observations and to observe the effect of N-nitrosation blocking agents such as ascorbic acid and $\alpha$-tocopherol. Experiments have been conducted to determine the extent of incorporation of $[^{15}\text{N}]$nitrate into NPRO in these same subjects using low resolution mass fragmentography in conjunction with capillary gas chromatography. The use of $^{15}\text{N}$ as a tracer allows a clear-cut distinction between intragastric synthesis of NPRO and other contributions to urinary excretion. Results will also be presented on the occurrence and modulation of rate of formation of another urinary compound, NTCA.

MATERIALS AND METHODS

Subjects and Diets. The subjects participating on these studies were MIT students, ranging in age from 19 to 27 years old. All subjects were nonsmokers and were judged to be healthy based on a thorough physical examination and routine blood and urine analysis. Throughout the study, subjects were fed a controlled, low nitrate diet consisting of an egg omelette and liquid soy and milk formula (15). During the first study (vitamin C study), powdered eggs were used in the diet, but then this was changed to fresh eggs for the second study (vitamin E study) because of the contamination of NPRO in the powdered eggs. The contribution of dietary NPRO was subtracted from total urinary excretion.

Chemicals. Standards of NPRO and $[^{15}\text{N}]$NPRO were synthesized according to the method of Lijinsky et al. (16) with minor modifications. L-Proline (500 mg; Sigma Chemical Co., St. Louis, MO) was dissolved in 25 ml water, and the pH was brought to 3.0 using HCl. Sodium nitrite (2.97 g) was added to the reaction mixture in the dark and the solution was stirred at room temperature for 18 h in the dark. $[^{15}\text{N}]$Nitrite, 99% $^{15}\text{N}$ atom % excess (KOR Isotopes, Cambridge, MA), was used for the synthesis of $[^{15}\text{N}]$NPRO. After completion of the reaction, the pH was taken to 1.0 with HCl. The solution was dried and extracted with methylene chloride. Preparation of NPIP was similar to that for NPRO except 500 mg L-pipelic acid (Sigma) were used instead of proline. N-Nitrosopipelic acid was prepared by dissolving 500 mg thiazolidine-4-carboxylic acid (Sigma) in 25 ml water and the pH was adjusted to 3.0 with HCl. Sodium nitrite (2.6 g) was added and the solution was stirred in the dark at room temperature for 18 h. The pH was then adjusted to 2.5 with HCl and the solution was evaporated to dryness using a rotary evaporator. The final nitrosated product was extracted with methanol:methylene chloride (1:1). The purity and identity of these compounds were verified by thin-layer chromatography and gas chromatography-mass spectrometry.

Analysis of Total Urinary NPRO and NTCA. Complete 24-h urine collections were preserved with absolute ethanol (100 ml), dibasic sodium phosphate (10 g), and sodium bisulfite (1.5 g) and stored frozen to prevent bacterial growth and antifungal nitrosamine formation.
N-Nitrosopippecolic acid (250 ng/100 µg methanol) was added as an internal standard to a 50-m1 aliquot of urine. This sample was eluted through a prewashed (water) column of strongly acidic cation exchange resin (2 x 10 cm; Dowex 50W, 50-X8-100, 50-100 mesh). The column was then washed with water (2 x 15 ml) and the combined eluates were concentrated to 5 ml using a rotary evaporator at 55°C. The concentrate was applied to a pretreated (15 ml ethyl acetate) Prep-Tube (Thermo Electron Corp., Waltham, MA). After 10 min, the Prep-Tube was washed with 100 ml ethyl acetate. The organic phase was collected and evaporated to dryness on a rotary evaporator at 35°C. The residue was extracted into another flask with méthylène chloride (3 x 10 ml) and then was applied to a pretreated (15 ml ethyl acetate) Prep-Tube (Thermo Electron Corp., Waltham, MA). After 10 min, the Prep-Tube was washed with 100 ml ethyl acetate. The organic phase was collected and evaporated to dryness on a rotary evaporator at 35°C. The residue was dissolved in methylene chloride (3 x 10 ml) and then the solvent was evaporated at 30°C. The residue was dissolved in méthylène chloride (1 ml) and treated with diazomethane at 0°C until a yellow color persisted. After 10 min, the solution was transferred to a 1-ml Reacti-Vial (Pierce, Rockford, IL) and concentrating bulb. The reaction flask was washed with an additional 2 ml of methylene chloride. Samples were very slowly evaporated using a gentle stream of nitrogen gas to a final sample volume of 0.8 ml.

The sample (1–5 µl) was analyzed by gas chromatography with thermal energy analysis (Thermo Electron Corp.). A 3% OV-225 column on 100/120 Supelcoport (Supelco, Bellefonte, PA) operating at 155°C was used for 5 min. After filtering through a Buchner filter funnel (Whatman No. 1 filter paper), the mixture was extracted with 100 ml n-hexane. The hexane extraction was performed for 5 min. After filtering through a Buchner filter funnel (Whatman No. 1 filter paper), the mixture was extracted with 100 ml n-hexane. The hexane layer was discarded and the methanol layer was concentrated to 5 ml with a rotary evaporator. This extract was placed on a Prep-Tube and then analyzed in a manner similar to that described above for urine samples.

Analysis of NPRO and NTCA in the Diet. The method of Sen et al. (17) was used to extract NPRO and NTCA from the diet samples. A 15-g aliquot of each diet component was mixed with 10 ml 1 N sulfuric acid containing 1% dissolved sulfamic acid, 100 ml methanol, and 5 g Celite for 5 min. After filtering through a Buchner filter funnel (Whatman No. 1 filter paper), the mixture was extracted with 100 ml n-hexane. The hexane layer was discarded and the methanol layer was concentrated to 5 ml with a rotary evaporator. This extract was placed on a Prep-Tube and then analyzed in a manner similar to that described above for urine samples.

$^{14}$N]-NPRO Analysis in Urine. Urine samples needed additional cleanup steps for mass fragmentography-isotope analysis. Samples were passed through an anion exchange column (10 x 1 cm; Dowex 1, 200–400 mesh, formate form) and NPRO was eluted with 60 ml 1 N HCl. Two further high pressure liquid chromatography steps were used to separate NPRO from other organic acids in urine. Samples were first chromatographed on a Spherisorb (5 µm) NH2 bonded phase column [Supelco; 250 x 5 mm; mobile phase 2 mM ammonium formate with 5% acetonitrile (pH 5)]; then through a LICHROMSorb (5 µm) C18 bonded phase column [Supelco; 150 x 5 mm; mobile phase 5 mM ammonium formate with 1% acetonitrile (pH 3)]. The flow rate through both columns was 1 ml/min and NPRO was detected at a wavelength of 245 nm. The eluate was collected ±1 min of the retention time of a NPRO standard. Samples were then methylated with diazomethane and analyzed by selected ion monitoring of m/z = 99 and m/z = 100 using a Hewlett-Packard GC/MS (HP 5995) with a 35-m glass capillary column (Carbowax 20M). A complete mass spectrum of the urine sample was used to identify the peak as NPRO.

Experimental Protocol. NPRO formation was studied during a control, low nitrate, and low NPRO diet and during two additional dietary periods to study the effect of nitrosation blocking agents. In the first study, six subjects were given the low nitrate diet and urine was collected for 5 consecutive days. The dietary intakes of ascorbic acid and α-tocopherol were 60 and 15 mg/day, respectively. On the sixth day of the study, following an overnight fast, subjects received 3.5 mmol sodium $^{15}$N nitrate (99% $^{15}$N) in 10 ml distilled water p.o., followed by 200 ml water. One hour later, 4.3 mmol l-proline in 10 ml water were administered. Urine was collected for the following 48 h. A break period of 1 week without the low nitrate diet followed but subjects ingested 2 g ascorbic acid (500-mg tablets, four times per day) during this period. Following the break period, subjects were placed back on the low nitrate diet but continued to consume the 2 g ascorbic acid daily for 1 week. After 5 days, the nitrate and proline were administered as described above with one 500-mg ascorbic acid tablet administered with the nitrate; three additional tablets were taken throughout the remainder of the day. A second study was conducted like the first one except that four subjects participated and one 400-mg α-tocopherol (acetate form) tablet per day was included in the diet during this dietary period. The $^{15}$N]nitrate and proline were administered as before with the tocopherol tablet given 0.5 h before the proline. The results of the two control periods of both studies have been combined under "Results" to increase the number of data points for base-line values.

RESULTS

Each subject excreted a relatively constant amount of NPRO per day over 5 days with the ingestion of the low nitrate diet (Chart 1). There was a basal excretion of 26 ± 10 (SD) nmol/day. This basal excretion represents the endogenous synthesis of NPRO since the amount of NPRO found in the diet has been subtracted from the total amount of NPRO found in the urine. A significant rise in the excretion of NPRO was observed following the administration of nitrate and proline, ranging from 29 to 318 nmol/24 h with a mean of 100 nmol/24 h (Chart 1).

The basal synthesis of NPRO was not significantly different during the high ascorbic acid or the α-tocopherol enriched diets, at 27 ± 8 and 39 ± 11 nmol/day, respectively. However, when ascorbic acid was administered with nitrate and proline, there was a significant decline in total NPRO production for four of the six subjects (Chart 2). Ascorbic acid reduced the mean nitrate induced synthesis of NPRO to 37 ± 19 nmol/24 h. Inclusion of α-tocopherol in the diet had a mixed effect in the four subjects studied (Chart 2). For two subjects, there was a significant decrease in the amount of NPRO synthesis after nitrate and proline intake, while the other two subjects had slightly enhanced levels compared to the control, low α-tocopherol diet.

The incorporation of $^{15}$N-nitrate into NPRO during the different dietary regimens is shown in Chart 3. Heterogeneity among the subjects was observed during the control period with respect to the amount of ingested $^{15}$N-nitrate which reacted with proline to form $^{15}$N[NPRO]. The amount of $^{15}$N[NPRO] synthesis after $^{15}$N-nitrate and proline intake ranged from 4 to 187 nmol/24 h. $^{15}$N[NPRO] in urine accounted for between 10 and 60% of total NPRO synthesis (sum of $^{15}$N[NPRO] + $^{15}$N[PRO]).

In contrast, it was found that with the inclusion of ascorbic acid in the diet the amount of $^{15}$N[PRO] synthesis was significantly reduced, ranging from 0 to 9 nmol/24 h. Every subject showed significant inhibition with respect to the incorporation of $^{15}$N-nitrate into NPRO. The concentration of $^{15}$N[NPRO] in urine

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Chart 2. Effect of vitamins C and E on nitrate and proline induced nitrosoproline synthesis. The control diet for ten subjects contained 60 mg ascorbate and 15 mg α-tocopherol. Four 500-mg ascorbate tablets were given daily to six subjects for the high vitamin C diet and 400 mg α-tocopherol acetate were added to the daily diet of four subjects for the high vitamin E diet.

Chart 3. Effect of vitamins C and E on [15N]nitrosoproline synthesis following [15N]nitrate and proline administration. except for one subject was not influenced by the inclusion of ascorbic acid in the diet.

In general, the results observed during the α-tocopherol period were qualitatively similar to that seen with ascorbic acid. The level of [15N][NPRO] during this period ranged from 9 to 236 nmol/24 h but three of four subjects had significantly less [15N][NPRO] produced compared to the control period.

Upon analysis of urine samples for NTCA, a background level of this nitrosoamino acid, as shown in Chart 4, was observed. The diet contained no detectable level of NTCA; detection limit, 3 nmol/kg diet. The mean excretion of NTCA was 4 ± 5 nmol/day. Not every subject excreted detectable levels of NTCA in the urine on the low nitrate diet, but upon ingestion of nitrate every subject excreted NTCA. Overall there was a 6-fold increase in NTCA synthesis following nitrate administration, 25 ± 16 nmol/24 h as shown in Chart 4.

Administration of 2 g ascorbic acid in the diet did not eliminate the endogenous background level of urinary NTCA. However, ascorbic acid completely blocked nitrate induced synthesis of NTCA (Chart 4). The high vitamin E diet appeared to block the endogenous background level of NTCA since NTCA was detected only on the first day of the dietary period; however, vitamin E was not as effective as ascorbic acid with respect to the inhibition of nitrate induced synthesis of NTCA. It is of interest that the lack of effective blocking by vitamins C and E was in the same subject suggesting a role for other physiological factors.

DISCUSSION

An estimate of endogenous nitrosation in humans using the NPRO test has been obtained in ten apparently healthy young adults. There was a wide individual variation in the formation of nitrosoproline following nitrate and proline intake. Although the pH of gastric juice was not measured in these subjects, it can be speculated that there were differences among the subjects. Since the nitrosation of proline is highly pH dependent (18), with a pH optimum of 2.5, different levels of gastric acidity could influence the amount of NPRO formed. However, gastric acidity is one of many factors which will determine endogenous nitrosation. Other factors to be considered would be the conversion of nitrate to nitrite by the microflora in the mouth (19), the level of gastric nitrosation catalysts such as thiocyanate (20), and the level of unknown natural protective factors (20). All the subjects consumed the same diet, yet profound differences in the synthesis of NPRO were observed, suggesting that additional factors must be considered when estimating the endogenous nitrosation in population groups.

Previous studies (10) and the present experiments have shown NPRO excretion even in the absence of additional nitrate and proline uptake. Dietary intake of NPRO does not account for the levels observed in the urine. We have suggested that NPRO must be synthesized from endogenous nitrate in the body. [15N]Nitrate was used as a tracer to study the observed excess excretion of NPRO in urine. The data revealed that urinary NPRO excretion as a result of endogenous synthesis is not totally derived from ingested nitrate as its precursor since all subjects continued to excrete unlabeled [15N][NPRO] after [15N]nitrate and proline intake. This is further evidence for the existence of endogenous nitrosation in humans which takes place even in the
absence of dietary precursors. The ingestion of ascorbic acid and \( \alpha \)-tocopherol inhibited the incorporation of \([\text{\textsuperscript{15N}}]\)nitrate into NPRO. The mean inhibition of \([\text{\textsuperscript{15N}}]\)NPRO synthesis was 81% by ascorbic acid, with four of six subjects having greater than 95% inhibition. \( \alpha \)-Tocopherol blocked \([\text{\textsuperscript{15N}}]\)NPRO synthesis by an average of 59% in three of four subjects, but in the remaining subject there was no inhibition. Although \( \alpha \)-tocopherol was not as effective a nitration inhibitor as was ascorbic acid under the protocol used in these experiments, this may be partially explained by the different dosing regimens which involved 10 times the level of administration of ascorbic acid on a molar basis.

Ascorbic acid and \( \alpha \)-tocopherol did not reduce the amount of endogenous NPRO appearing in the urine during the control dietary period. In addition, although the formation of \([\text{\textsuperscript{15N}}]\)NPRO was blocked by ascorbate and tocopherol, the level of \([\text{\textsuperscript{15N}}]\)NPRO during the study periods was unchanged. The \([\text{\textsuperscript{15N}}]\)NPRO appears to represent the intragastric nitrosation of ingested proline from administered \([\text{\textsuperscript{15N}}]\)nitrate. Therefore either NPRO synthesis from endogenous precursors in the body occurred in the stomach at times when ascorbate or tocopherol were not present or there is synthesis of NPRO at an additional site which is not accessible to these blocking agents.

An additional nitrosamino acid was present in the urine of our subjects. As reported previously by Ohshima et al. (11) and Tsuda et al. (13), this nonvolatile nitrosamine was identified as NTCA. Although this nitrosamine was not detectable in the urine of all subjects ingesting the control diet, it was found that NTCA increased 6-fold upon ingestion of nitrate. Ascorbic acid and \( \alpha \)-tocopherol blocked this nitrate induced synthesis, suggesting that the site of synthesis may be confined to the stomach. Ohshima et al. (12) has also found that ascorbic acid significantly decreases NTCA synthesis.

The results of this study show that endogenous nitrosation takes place in humans from both exogenous and endogenous precursors. Nitrosation blocking agents such as ascorbic acid and \( \alpha \)-tocopherol are clearly effective inhibitors with regard to nitrosation of meal derived precursors. These experiments show the usefulness of the NPRO test with regard to estimating endogenous nitrosation and reducing this exposure in humans by dietary manipulation. Additionally measurement of another nitrosamino acid, NTCA, may contribute to the identification of high risk population groups. We conclude that the measurement of urinary nitrosamino acids in various population groups with different dietary and social customs can be used to identify the multitude of factors regulating endogenous nitrosation.

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