Urinary Excretion of N-Nitrosamino Acids and Nitrate by Inhabitants of High- and Low-Risk Areas for Esophageal Cancer in Northern China: Endogenous Formation of Nitrosopropine and Its Inhibition by Vitamin C

Shih-Hsin Lu, Hiroshi Ohshima, Hua-Ming Fu, Yuan Tian, Fung-Ming Li, Maria Blettner, Jürgen Wahrendorf, and Helmut Bartsch


ABSTRACT

A total of 238 samples of 24-h urine were collected from inhabitants of high-risk (Lin-xian) and low-risk (Fan-xian) areas for esophageal cancer in northern China, according to three protocols: (a) from undosed subjects; (b) from subjects who had ingested 100 mg L-proline three times a day 1 h after each meal; and (c) from subjects in Lin-xian who had ingested 100 mg ascorbic acid together with 100 mg L-proline three times a day 1 h after each meal. As an index of individual exposure to N-nitroso compounds or their precursors, ingested in food and/or formed endogenously, the levels of four urinary N-nitrosamino acids and nitrate were determined. The amounts of N-nitrosopropine, N-nitrosothiazolidine 4-carboxylic acid, N-nitrososarcosine, and nitrate excreted in the 24-h urine of undosed subjects in Lin-xian were significantly higher than those in Fan-xian, indicating a higher exposure of the inhabitants in the high-risk area to N-nitroso compounds and their precursors. Ingestion of L-proline resulted in a marked increase in urinary N-nitrosopropine levels in inhabitants from both areas, suggesting that endogenous nitrosation may occur to a larger extent when appropriate amine precursors are ingested in foods. Intake of moderate doses of ascorbic acid by high-risk subjects effectively reduced the urinary levels of N-nitrosamino acids to those found in undosed subjects in the low-risk area. If N-nitroso compounds formed in vivo are among the causative factors for esophageal cancer in this area of northern China, ascorbic acid appears to be effective in lowering the body burden of these carcinogenic compounds, thus offering a rational basis for long-term intervention studies in this area.

INTRODUCTION

EC occurs frequently in some provinces in northern China, and Lin-xian county of Henan province has the highest age-adjusted mortality rates, 151/100,000 in men and 115/100,000 in women (2). Extensive epidemiological and clinical investigations that have been undertaken to identify the causative factors of EC in these areas have suggested that intake of alcohol and tobacco, which accounts for 70% of the risk in populations of Europe and America (3), appears to play a minor role in China but that other environmental factors may be more important (1, 4). Of the suspected etiological factors in the environment, N-nitrosamines and their precursors have received the most attention (1, 4).

Earlier studies demonstrated that a relatively high content of the precursors of N-nitrosamines (nitrite, nitrate, and secondary amines) and of some preformed N-nitrosamines was present in various foods collected in Lin-xian county, including pickled vegetables, wheat, corn, and millet (5-8). In particular, the amount and frequency of consumption of pickled vegetables (commonly eaten in Lin-xian) were shown to be positively correlated with the rate of mortality from EC (9).

With the objective of assessing individual exposure to N-nitroso compounds and their precursors, samples of 24-h urine were collected from 238 inhabitants of Lin-xian (high-risk area) and Fan-xian (low-risk area), a county located 150 km east of Lin-xian with age-adjusted EC mortality rates of 35/100,000 in men and 16/100,000 in women (2). The urine samples were analyzed for nitrate and for several N-nitrosamino acids, such as NPRO and NTCA, as an exposure index. The potential for endogenous nitrosation was estimated by measuring urinary excretion of NPRO after intake of L-proline, with or without ascorbic acid (12). The results described below indicate that subjects living in the high-risk area for EC could be exposed to greater amounts of N-nitroso compounds through both ingestion of preformed compounds in foods and endogenous formation than those in the low-risk area. This exposure of high-risk subjects could be effectively reduced, however, to the lower level seen in subjects of the low-risk area by dietary supplementation with ascorbic acid, a well-known inhibitor of N-nitrosation.
MATERIALS AND METHODS

Chemicals. NPRO, NSAR, and NPIC were synthesized according to the method of Lijinsky et al. (13). NTCA and NMTCA were prepared as described previously (14, 15). Their purity was ascertained by thin-layer chromatography and gas chromatography coupled with either a thermal energy analyzer, a flame ionization detector, or a mass spectrometer. Diazomethane was prepared by action of potassium hydroxide on N-nitroso-N-methyl-p-toluenesulfonamide (Merck, Darmstadt, Federal Republic of Germany) in diethyl ether. Other chemicals used were: l-proline (Sigma Chemical Co., St. Louis, MO), ascorbic acid (vitamin C), ammonium sulfamate, sulfuric acid, N-1-naphthylethlenediamine dichlorohydrochloride, ethyl acetate, methanol (Merck), and dichloromethane (Farmitalia Carlo Erba, Milan, Italy). Cadmium powder for reducing nitrate to nitrite was obtained from Wako Pure Chemical, Osaka, Japan.

Study Subjects and Collection of Urine Samples. Samples of 24-h urine were collected from 238 healthy subjects living in Lin-xian and Fan-xian. The sex and age distribution of the study subjects are shown in Table 1. Urine specimens were collected according to three protocols: (a) for undosed specimens, in order to determine the background levels of N-nitrosamino acids and nitrate, 24-h urine samples were collected from 44 subjects (22 male, 22 female) in Lin-xian and 40 subjects (19 male, 21 female) in Fan-xian; (b) for proline specimens, 24-h urine samples were collected from 50 Lin-xian subjects (24 male, 26 female) and 55 Fan-xian subjects (25 male, 31 female) who had ingested 100 mg l-proline three times a day 1 h after each meal; (c) for proline plus vitamin C specimens, 24-h urine specimens were collected from 48 Lin-xian subjects (22 male, 26 female) who had ingested 100 mg l-proline together with 100 mg ascorbic acid three times a day 1 h after each meal. When l-proline was ingested alone or together with vitamin C both were dissolved in 10 ml distilled water. All urine samples were collected over 24 h in 2–3 liter polypropylene bottles containing 10 g NaOH. The samples were recovered from each subject immediately after a 24-h period and mixed thoroughly, and the volume was recorded. Two aliquots of 50 and 100 ml urine were then stored at −20°C prior to analyses for nitrate and N-nitrosamino acids, respectively. N-Nitrosamino acids and nitrate were stable under these storage conditions, and no appreciable artifact formation or degradation of the compounds was observed, even when urine samples to which nitrite had been added were stored at −20°C for 2 years. Study subjects were asked to complete a questionnaire for information on demography, food items eaten, and number of cigarettes or Chinese pipe tobacco smoked during the 24 h of urine collection.

Analysis of N-Nitrosamino Acids and Nitrate. NPRO and NSAR in urine were analyzed initially according to a previously reported method, using ethyl acetate as the extraction solvent (12). A 15-ml aliquot of urine, to which NPIC had been added as internal standard, was extracted three times with 25 ml ethyl acetate in the presence of 5 g NaCl and 3 ml 20% ammonium sulfamate solution in 3.6 N H2SO4. The combined ethyl acetate extracts were dried over anhydrous Na2SO4 and evaporated to dryness in a rotary evaporator at 50°C. Two ml of diethyl ether were then added to the residue, and the sample was derivatized with excess diazomethane. After concentration of the derivatized ethereal solution to about 0.5 ml, a 10-μl aliquot was analyzed by gas chromatography–thermal energy analysis under chromatographic conditions described elsewhere (15). The recoveries of NPRO and NSAR added to urine samples at 20 μg/liter were 85 and 82%, respectively.

Because the two sulfur-containing N-nitrosamino acids, NTCA and NMTCA, which were identified during the course of the study (14–17) were found to be partly decomposed during the working-up procedure described above (17), all urine samples were then reanalyzed with a modified extraction method, using methanol-dichloromethane (1:9, v/v) as extraction solvent. Thus, 7.5 ml of urine were extracted three times with 20 ml of methanol-dichloromethane in the presence of NPIC, 2.5 g NaCl, and 2 ml 20% ammonium sulfamate solution in 3.6 N H2SO4. The combined extracts were then dried and evaporated at 30°C. The residue was derivatized and analyzed as above. Under these conditions, the recoveries of NTCA, NMTCA, NPRO, and NSAR were 86, 90, 97, and 86%, respectively. Duplicate analyses of NPRO and NSAR using the two different extraction methods were found to give similar results, and the mean values were therefore used for further statistical analysis. In the case of NTCA and NMTCA, the results obtained from the second analysis were used. All values were corrected for the recovery of the N-nitrosamino acids as described above. Nitrate and nitrite were determined in urine according to the method of Sen and Donaldson (18). The nitrate value is expressed as NO3− (mg) per 24-h urine per person. When the concentrations of compounds were measured at or below their detection limits, the corresponding values for their limit of detection were used for statistical analyses.

Statistical Analysis. The differences between the five study groups were investigated by nonparametric techniques for descriptive and inferential purposes. Medians and their 95% confidence intervals were used for the first purpose and the Wilcoxon test was used for the latter (19). This approach was chosen because the distributions of the measured levels of N-nitrosamino acids were skewed and values at or below the limit of detection were included for some compounds. Correlations among the variables, with possible adjustment for the influence of some of them, were calculated after a logarithmic transformation of the values obtained from the chemical analyses. Covariant analysis was carried out to study the effect of smoking habits and food items on the amounts of urinary N-nitrosamino acids and nitrate. Calculations were carried out using the statistical package BMDP (20) and GLIM (21).

RESULTS

The ages of the study subjects (median values and range) in the five study groups are summarized in Table 1. The numbers of persons who reported smoking and consumption of certain food items during the period of urine collection are also given. Lin-xian subjects (groups LA, LB, and LC) had a median age of 53 years, while the median age for Fan-xian subjects (groups FA and FB) was 40 years. Although the Lin-xian subjects were older, there was no significant effect of age on the amounts of urinary N-nitrosamino acids and nitrate excreted (details not shown).

Medians (with 95% confidence intervals) of the volume of the 24-h urine samples and the amounts of the N-nitrosamino acids (μg/day) or nitrate (mg/day) that were detected in the 24-h urine samples of the subjects in the five groups are listed in Table 2, together with P values of the statistical comparisons. For the different variables the degree of within-group variation as illustrated by the confidence intervals should always be carefully considered. The median volume of 24-h urine (1.7 liters) excreted by all Lin-xian subjects was greater (P < 0.001) than that by all Fan-xian subjects (1.2 liters). Because the volume of 24-h urine showed some correlation (r = 0.4; P < 0.001) with the amounts of NPRO, NTCA, and nitrate excreted, both the concentrations (μg or mg/liter) and the amounts excreted daily (μg or mg/day) were used for further statistical analyses of the comparison (data not shown). However, because the comparisons yielded similar results for NSAR, NPRO, NMTCA, and NTCA, only the daily amounts of these nitrilo compounds are shown in Table 2 and in Figs. 1–3. Although the amount of nitrate excreted in the 24-h urine of all Lin-xian subjects together was significantly greater than that of all Fan-xian subjects together, the concentration (mg/liter) of urinary nitrate did not differ between the two areas. No significant correlation between the volume of the urine and the concentration of urinary N-nitrosamino acids or nitrate was observed.
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Table 1
Characteristics of study populations

<table>
<thead>
<tr>
<th>Smoking habit (no. of subjects)</th>
<th>Food items consumed (no. of subjects)</th>
</tr>
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<tbody>
<tr>
<td>Chinese cigarette pipe</td>
<td>Corn, Wheat, Pickles, Cabbage, Radish, Meat</td>
</tr>
<tr>
<td>Nonsmoker</td>
<td></td>
</tr>
</tbody>
</table>

| Lin-xian | |
|----------| |
| Group LA (undosed) | |
| Male | 22 | 44 (25-71) | 15 | 0 | 7 | 19 | 19 | 1 | 3 | 0 | 0 |
| Female | 22 | 64 (26-89) | 1 | 0 | 21 | 18 | 19 | 1 | 4 | 0 | 0 |
| Group LB (proline) | |
| Male | 24 | 54 (18-72) | 11 | 0 | 13 | 20 | 22 | 0 | 12 | 1 | 3 |
| Female | 26 | 36 (17-65) | 1 | 0 | 25 | 22 | 22 | 0 | 10 | 1 | 2 |
| Group LC (proline + vitamin C) | |
| Male | 22 | 59 (24-70) | 7 | 2 | 13 | 17 | 21 | 0 | 0 | 0 | 0 |
| Female | 26 | 61 (27-77) | 0 | 0 | 26 | 24 | 26 | 0 | 0 | 0 | 0 |

| Fan-xian | |
|----------| |
| Group FA (undosed) | |
| Male | 19 | 35 (20-64) | 9 | 9 | 1 | 14 | 11 | 0 | 9 | 6 | 1 |
| Female | 21 | 39 (21-73) | 0 | 1 | 20 | 15 | 13 | 0 | 12 | 9 | 3 |
| Group FB (proline) | |
| Male | 25 | 50 (20-75) | 9 | 11 | 5 | 15 | 14 | 0 | 8 | 7 | 3 |
| Female | 31 | 38 (18-74) | 0 | 1 | 30 | 21 | 19 | 0 | 10 | 9 | 3 |

Fig. 1. Urinary excretion of NPRO in subjects from high- and low-risk areas for esophageal cancer in northern China. L, Lin-xian; F, Fan-xian; (A), undosed; (B), praline; (C), praline plus vitamin C; m, median value.

Figs. 1–3 show the frequency distributions of the amount of urinary NPRO, the sum of the four N-nitrosamino acids, and the amount of nitrate, respectively, found in the 24-h urine of the subjects in the five groups. As shown in Table 2, the levels of NPRO, NTCA, and NSAR detected in the urine of Lin-xian subjects (group LA) who had ingested neither praline nor ascorbic acid were higher than those of undosed Fan-xian subjects; the NMTCA levels did not differ significantly. Consequently, the sum of the four N-nitrosamino acids was higher for subjects from Lin-xian than from Fan-xian.

Intake of L-proline (100 mg three times a day) resulted in an increase in the urinary NPRO excretion in subjects from both areas (groups LB and FB). Thus, the median NPRO levels after L-proline intake increased from 5.7 to 8.3 in Lin-xian subjects and from 1.7 to 4.4 in Fan-xian subjects, compared with specimens from undosed (groups LA and FB) subjects. Intake of L-proline did not affect the amounts of urinary NTCA, NMTCA, or NSAR in inhabitants from either areas.

Intake of moderate doses of ascorbic acid together with L-proline resulted in a significant decrease in the urinary excretion of each N-nitrosamino acid in Lin-xian subjects (group LC). In that group, the median of the sum of the four N-nitrosamino acids was reduced to 6.6 µg/day from the respective values of 21.2 (group LA) and 18.0 (group LB) µg/day.

The median value of urinary nitrate (mg/day) was higher in Lin-xian subjects than that in Fan-xian (P < 0.001) but was not different among the groups within one county. Nitrite levels were below the detection limit (<0.1 mg/liter) in all urine specimens analyzed.

In Table 1 the number of cigarette smokers, smokers of
Chinese pipes, and nonsmokers are given. Smoking (both types combined) is mainly reported by the males: among 77 smokers, 73 were male and 4 were female; among 161 nonsmokers, 39 were male and 122 were female. The proportion of smokers (both types combined) in the different subgroups are roughly comparable. The effect of smoking (measured as yes-no variable) on the urinary levels of N-nitrosamino acids was analyzed after adjusting for the effects of the treatments (intake of praline or ascorbic acid) and of the counties. In accordance with the results of other studies (15, 22, 23) smokers tended to excrete greater amounts of NPRO, NTCA, and NMTCA (and the sum of the four N-nitrosamino acids) than did nonsmokers (P < 0.01). However, all comparisons reported in Table 2 observed similar trends when the analyses were confined to smokers or nonsmokers.

Major food items consumed by subjects during the period of urine collection are summarized in Table 1, and their possible effect on urinary levels of N-nitrosamino acids and nitrate was examined. No clear influence of the type of food consumed, i.e., wheat, corn, pickled vegetables, or/and cabbage, could be found. It was noted, however, that there was no great variation of food consumption among the study subjects within one county.

Correlations among the variables were examined after logarithmic transformation of the values obtained from chemical analyses. The amount of urinary NPRO was correlated with that of NTCA and NMTCA in all study subjects as well as in each of the study groups. After adjusting for effects of group, age, sex, and volume of urine, the partial correlation between NPRO and NTCA was 0.55 (P = 0.001) and that between NPRO and NMTCA was 0.45 (P = 0.001). Similarly, a correlation was observed between the urinary levels of NTCA and NMTCA (ρ = 0.36, P = 0.001). However, it should be kept in mind that many of the values for NMTCA were at the limit of detection.

The correlation coefficient between urinary levels of NPRO and of nitrate in undosed (groups LA and FA) subjects in Lin-xian and Fan-xian combined was 0.39 (P < 0.01), with the slope from a linear regression analysis for log [μg NPRO/person/day] versus log [mg nitrate/person/day] being 0.52 (Fig. 4A1). Similarly, linear regression analyses of urinary NPRO, following ingestion of praline (groups LB and FB in Lin-xian and Fan-xian; Fig. 4B1) or of praline plus ascorbic acid (group LC in Lin-xian; Chart 4C1), yielded correlation coefficients of 0.57 and 0.63 (P < 0.001), and the respective slopes for log [μg NPRO/person/day] versus log [mg nitrate/person/day] were 0.76 and 0.67, respectively (Fig. 4B1 and C1). The coefficients of correlation between the sum of the four N-nitrosamino acids and nitrate in the above groups were 0.46 (group A), 0.60 (group B), and 0.68 (group C); the respective slopes for linear regression analyses of log [μg nitrosamino acid/person/day] versus log [mg nitrate/person/day] were 0.56, 0.72, and 0.69, respectively (Fig. 4A2, B2, and C2).

### DISCUSSION

As an approach to assessing individual exposure to N-nitroso compounds of subjects living in high- and low-risk areas for EC in northern China, urine samples were collected and analyzed.
for N-nitrosamino acids (NPRO, NTCA, NMTC, and NSAR) and nitrate. These N-nitrosamino acids are all excreted rapidly and nearly quantitatively in the urine of rats after p.o. administration (15, 24-26); therefore, their levels in human urine provide a valuable index of exposure to N-nitroso compounds. Urinary excretion of nitrate, which can be converted to nitrite in vivo, has also been shown to reflect the ingestion or biosynthesis of nitrates (27, 28). We also estimated the potential for endogenous nitrosation by measuring urinary levels of NPRO following intake of an amino acid L-proline. NPRO has not been reported to be mutagenic or carcinogenic (29, 30) and has not been found to undergo metabolism in rats to alkylate DNA or proteins (25, 26) but is eliminated almost quantitatively in the urine (24-26). Thus the urinary excretion of NPRO following ingestion of L-proline with or without nitrate has been validated as a marker of endogenous nitrosation in humans (12, 22, 23, 31).

Results of the present study demonstrate that the inhabitants of a high-risk area for EC excrete higher levels of NPRO, NTCA, NSAR, and nitrate in their urine than those in a low-risk area. The median level (µg/day) of all four N-nitrosamino acids found in the urine of high-risk subjects was about four times greater than that of low-risk subjects. Intake of proline resulted in a marked increase in the urinary excretion of NPRO in subjects in both areas, implying that endogenous nitrosation of proline had occurred. However, ingestion of proline together with ascorbic acid by Lin-xian subjects reduced the urinary levels of NPRO and other N-nitrosamino acids, indicating that ascorbic acid effectively inhibits endogenous nitrosation. The urinary level (median value) of all four N-nitrosamino acids (µg/person/day) after intake of ascorbic acid was 6.6 (Lin-xian, group LC), compared to 21.2 in undosed subjects (Lin-xian, group LA). On the basis of previous experiments, we can assume that endogenous synthesis of N-nitrosamino acids was inhibited to a large extent, but not totally, by the doses of ascorbic acid; recently, Wagner et al. (32) have shown that a fraction of urinary NPRO, following ingestion of proline and [15N]nitrate, results from endogenous synthesis not involving ingested nitrate as a precursor and is refractory to inhibition by ascorbic acid. However, if the difference between the values (LA - LC) reflects the amount of N-nitrosamino acids formed endogenously, the subjects in Lin-xian could be exposed daily to 14 µg of endogenously formed N-nitrosamino acids and to about 7 µg of preformed compounds in foods. Although in Fan-xian urine samples were not collected after dosing with ascorbic acid, the median level of the four N-nitrosamino acids excreted by undosed (group FA) subjects was 5.6 µg/person/day, suggesting that the amount of endogenously formed N-nitroso compounds is even lower. These results suggest that in high-risk subjects the potential for endogenous...
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nitrosation is more than twice as high as that in low-risk subjects. However, the urinary levels of N-nitrosamino acids (μg/person/day) showed large interindividual variations (Fig. 2), i.e., from 3 to 95 in Lin-xian (group LB) versus 1 to 57 μg in Fan-xian (group FB). This variation may be due in part to individual differences in daily intake of nitrosating agents (nitrite/nitrate) and dietary inhibitors or catalysts of endogenous nitrosation. Although many pairwise comparisons were made in the statistical analysis of these data (Table 2), the results point strongly and consistently to the direction outlined above; thus, there is little likelihood that the results are due only to chance because of the multiple comparisons made.

Our findings with regard to urinary nitrate in Lin-xian subjects and earlier studies in this area on nitrate/nitrite levels in drinking water, foods, and saliva (see Introduction) show that the daily intake of nitrosating agents is higher in these high-risk subjects than in subjects in Fan-xian. As shown in Fig. 4, urinary levels of N-nitrosamino acids show some correlations with that of nitrate, and our previous study (12) showed that NPRO formation in vivo in humans, following ingestion of nitrate and proline, is strongly dependent on the dose of nitrate. Consistent with this earlier observation, the levels of N-nitrosamino acids in the urine of Lin-xian subjects, whose intake of nitrate is higher, are higher than those in the low-risk area. In addition, nutritional surveys conducted in northern China revealed that inhabitants of Lin-xian consume few fresh vegetables and little fruit (33) and that the blood levels of various vitamins, such as vitamins C and A and riboflavin, were lower than those seen in inhabitants of low-risk areas (34). Fresh vegetables and fruit have been reported to contain high concentrations of vitamin C and various other phenolic compounds, such as chlorogenic acid and tannins; all these dietary constituents are generally inhibitors of N-nitrosation (35, 36). Our data and the results of nutritional surveys thus suggest that inhabitants of the high-risk area ingest a higher level of nitrosating agents and fewer nitrosation inhibitors, leading to more endogenous nitrosation in their stomachs.

Although the consumption of pickled vegetables in the study area has been associated with an increased risk for EC (9), the effect of this food item on urinary excretion of N-nitrosamino acids could not be evaluated in this study, because only two subjects were reported to have consumed it during the period of urine collection.

In order to study further seasonal and geographical variations in exposure to N-nitroso compounds, more urine samples are being collected at different seasons and in other high-risk areas for EC in Henan province of northern China.

The results of the present study, together with previous environmental studies (see "Introduction"), provide some indication that N-nitroso compounds are involved in the causation of EC in the high-risk area, but identification and characterization of N-nitroso compounds present in foods and of their precursor amino compounds are still required. Only recently, such studies identified a new nitroso compound and nitrosating agent, Roussin's red methyl ester. Acta Chem. Sin., 38: 95-102, 1980.

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