Urinary Excretion of Nitrosamino Acids and Nitrate by Inhabitants of High- and Low-Risk Areas for Nasopharyngeal Carcinoma in Southern China

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Abstract

The hypothesis that endogenous synthesis of nitrosamines from dietary precursors is a risk factor for nasopharyngeal carcinoma (NPC) in China was tested by applying the nitrosoproline (NPRO) test to subjects living in high- and low-risk districts for NPC in Zangwu county, Guangxi region, in southern China. Samples of 12-h urine were collected from 77 subjects: (*a*) before any treatment; (*b*) after ingestion of proline; and (*c*) after ingestion of proline together with vitamin C. NPRO, other nitrosamino acids, and nitrate were measured as indices of exposure to preformed and endogenously formed nitrosamines or their precursors. The NPRO level after proline intake was significantly increased in subjects from the high-risk area ($P = 0.012$) and markedly reduced after ingestion of ascorbic acid ($P = 0.007$), but such an effect was not seen in subjects from the low-risk area. Levels of *N*-nitrosothiazolidine-4-carboxylic acid and the sum of nitrosamino acids in subjects in the high-risk area were significantly reduced by ascorbic acid ($P < 0.01$) but were not reduced in subjects from the low-risk area. The urinary nitrate level was about twice as high in subjects from the high-risk area. In subjects from high- and low-risk areas combined, NPRO levels in any of the three dose groups were highly correlated with nitrate levels ($P = 0.0001$). These results demonstrate a higher potential for endogenous nitrosation in subjects living in the high-risk area of NPC and suggest the occurrence of nitrosation inhibitors in the diet consumed in the low-risk area. Thus, in addition to infection by Epstein-Barr virus and genetic predisposing factors, dietary habits that may entail higher nitrosamine exposure appear to play a role in NPC etiology.

Introduction

NPC[3] exhibits wide variations in incidence throughout the world; it is most common in China and southeast Asia, and among Magrebian Arabs in north Africa and Eskimos in the Arctic (1). Risk factors for NPC that have been identified include genetic predisposition (HLA haplotypes) (2, 3), infection by EBV, and environmental factors, especially food consumption habits (4–7). Earlier studies have shown that some samples of Cantonese-style salted fish contain relatively high levels of volatile nitrosamines (8, 9), some of which induce tumors in the nasal cavities of experimental animals (10, 11). Recent studies in high-risk areas for NPC in Tunisia, southern China, and Greenland revealed that these widely different populations all commonly consume preserved foods (12).[4] A study in Guan-Xi in southern China has shown that consumption before the age of 2 years of a number of preserved foods, such as Cantonese-style salted fish, is strongly associated with an increased risk for NPC (6). However, similar Japanese dried fish and vegetables were also reported to contain relatively high concentrations of volatile nitrosamines, but the incidence of NPC in Japan is very low.

We have searched, therefore, for additional hitherto unknown environmental risk factors for NPC and analyzed food extracts from high-risk areas for the presence of substances that activate EBV. Cantonese-style soft salted dried fish and *harissa*, a Tunisian spice mixture, were found to contain agents with EBV-inducing activity (13). Furthermore, our previous studies revealed that certain preserved food items contained high levels of precursors that, upon nitrosation *in vitro*, yielded volatile nitrosamines and direct-acting mutagens (14), suggesting that *in vivo* nitrosation could occur after ingestion of precursors and nitrosating agents in the diet. In the present study, we applied the NPRO test to inhabitants of high- and low-risk areas for NPC in southern China to compare their endogenous nitrosation potential. The excretion of urinary nitrosamino acids and of nitrate was used as an index of individual exposure to nitroso com-

Received 8/24/92.

[1] G. B. has been supported by the Ligue Nationale Française Contre le Cancer and by a special training award from the International Agency for Research on Cancer.

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[3] The abbreviations used are: NPC, nasopharyngeal carcinoma; NPRO, nitrosoproline; EBV, Epstein-Barr virus; NTCA, *N*-nitrosothiazolidine-4-carboxylic acid.

[4] Y. M. Zheng, P. Tuppin, A. Hubert, D. Jeannel, W. J. Pan, Y. Zeng, and G. de Thé. Environmental and dietary risk factors for nasopharyngeal carcinoma: a case-control study in Zangwu country, Guangxi region, China, submitted for publication.

Table 1 Characteristics of study subjects

	n	Median age (SE) (years)	No. of Smokers	No. of Nonsmokers
High-risk district				
Male (M)	18	40.5 (1.2)	12	6
Females (F)	19	39.0 (1.4)	0	19
Low-risk district				
Males	20	48.0 (2.9)	15	5
Females	20	44.0 (3.5)	1	19
Comparison between low- and high-risk areas				
High-risk (M + F)	37	40.0 (0.9)	12	25
Low-risk (M + F)	40	47.0 (2.9)	16	24
P value of comparison		0.004	Nonsignificant	

pounds or their precursors, ingested in food or formed endogenously (15).

Materials and Methods

Subjects and Sample Collection. Samples of 12-h urine were collected in the spring of 1990 from 77 healthy subjects living in two villages in Zangwu county in the Guangxi region of southern China with contrasting incidence rates for NPC. The high-risk district had an incidence of NPC of 28/100,000/year, while for the low-risk district the corresponding figure was 2.9/100,000/year (16). These figures are taken from the local cancer registry, which was established in 1976. Characteristics of the study subjects who were selected at random are shown in Table 1. Three urine specimens were collected (for 12 h overnight starting 1 h after the evening meal) on three consecutive days from each subject according to the following protocols: (*a*) urine samples (groups H1, L1) were collected before dosing in order to determine the background levels of nitrosamino acids and nitrate; (*b*) proline specimens (groups H2, L2) were collected after subjects had ingested 300 mg L-proline 1 h after the evening meal; (c) proline plus vitamin C specimens (groups H3, L3) were collected after subjects had ingested 300 mg L-proline together with 300 mg ascorbic acid 1 h after the evening meal. Analyses of nitrosamino acids and nitrate were performed as reported by Lu *et al.* (17). Study subjects were asked to complete a questionnaire to obtain information on demography, food items, beverages consumed, and number of cigarettes smoked during the urine collection period.

Statistical Analyses. Because the distribution of the variables was skewed, nonparametric tests were used. Descriptive analysis included median and its standard error (SE in Tables 1–3) as proposed in the BMDP software (18). To compare the distribution of the concentration of nitrosamino acids and of nitrate excreted by inhabitants from high- and low-risk areas, a Wilcoxon rank-sum statistical test was used. In each area the differences between samples 1, 2, and 3 were compared using the Wilcoxon signed-rank test for comparison of matched samples. Correlation between continuous variables was calculated using natural logarithmic transformation (to normalize the variables) or Box-Cox transformation (19) when the log transformed variables were still not normal. All the tests were two-sided, and the level of significance chosen was 5%.

Results

Urinary Levels of Nitrosamino Acids. The nitrosamino acids analyzed were NPRO, NTCA, *N*-nitrososarcosine, and *N*-nitroso-2-methylthiazolidin-4-carboxylic acid. The latter two are not individually listed but are included in the sum levels of all four nitrosamino acids. Table 2 summarizes urine volume, levels of nitrosamino acids (μg/12 h/person), nitrates (mmol/12 h/person), and creatinine (mmol/12 h/person) that were detected in the six sets of urine samples. The volumes of the 12-h urine samples showed no difference between the two areas. Median urinary levels of NPRO, NTCA, and the sum of

Table 2 Median (SE of median) for volume of 12-h urine and for amounts of *N*-nitrosamino acids, nitrate, and creatinine detected in urine

Urine sample from	Excretion of *N*-nitrosamino acids (μg/12 h) NPRO	NTCA	Sum[a]	Urine volume (ml/12 h)	Creatinine (mmol/12 h)	Nitrate (mmol/12 h)
High-risk district[b]						
H1	3.90 (0.87)	21.30 (4.30)	33.30 (5.25)	350 (40.41)	3.18 (0.40)	0.87 (0.14)
H2	7.60 (2.86)	39.30 (10.71)	68.50 (13.42)	300 (28.87)	4.14 (0.35)	2.01 (0.31)
H3	3.40 (0.84)	18.70 (3.78)	28.40 (4.50)	350 (28.87)	3.65 (0.40)	1.13 (0.24)
P (H1 vs. H2)[c]	0.012	0.073	0.067	0.530	0.006	0.004
P (H2 vs. H3)	0.007	0.004	0.007	0.812	0.105	0.030
Low-risk district[b]						
L1	3.30 (0.80)	23.26 (4.11)	31.30 (7.79)	300 (28.87)	2.94 (0.37)	0.62 (0.10)
L2	3.17 (1.24)	13.78 (2.26)	20.10 (4.59)	300 (37.53)	2.82 (0.30)	0.39 (0.08)
L3	2.44 (0.80)	13.33 (3.00)	18.70 (4.62)	250 (28.87)	2.68 (0.33)	0.45 (0.07)
P (L1 vs. L2)	0.485	0.008	0.035	0.458	0.553	0.526
P (L2 vs. L3)	0.936	0.687	0.545	0.662	0.936	0.643
Comparison between areas						
P (H1 vs. L1)	0.930	0.839	0.819	0.751	0.693	0.027
P (H2 vs. L2)	0.090	<0.001	0.002	0.437	0.038	<0.001
P (H3 vs. L3)	0.495	0.737	0.340	0.078	0.210	<0.001

[a] Sum also includes *N*-nitrososarcosine and *N*-nitroso-2-methyl-thiazolidine 4-carboxylic acid (values not shown).
[b] Samples from undosed subjects (1), proline-dosed subjects (2), and proline- and vitamin C-dosed subjects (3).
[c] *P* values of comparison are also listed, with significant values underlined.

Table 3 Medians (SE of median) for volume of 12-h urine and amounts of *N*-nitrosoamino acids and nitrate detected in urine (expressed per mmol creatinine levels)

Urine sample from	*n*	Excretion of *N*-nitrosamino acids (μg/mmol creatinine)			Nitrate (mmol/mmol creatinine)
		NPRO	NTCA	Sum[a]	
High-risk district[b]					
H1	37	1.40 (0.30)	7.79 (1.72)	11.00 (2.17)	0.41 (0.07)
H2	37	1.83 (0.47)	9.85 (1.65)	15.30 (3.35)	0.46 (0.13)
H3	37	0.94 (0.21)	4.51 (0.62)	7.20 (0.69)	0.41 (0.09)
P (H1 vs. H2)[c]		0.225	0.531	0.656	0.307
P (H2 vs. H3)		0.018	0.018	0.032	0.422
Low-risk district[b]					
L1	39	1.09 (0.16)	8.44 (1.54)	10.60 (1.36)	0.16 (0.02)
L2	40	1.25 (0.45)	4.23 (1.69)	8.25 (2.28)	0.15 (0.04)
L3	40	1.21 (0.18)	5.78 (0.87)	8.15 (1.96)	0.21 (0.03)
P (L1 vs. L2)		0.413	0.046	0.120	0.597
P (L2 vs. L3)		0.872	0.619	0.773	0.350
Comparison between areas					
P (H1 vs. L1)		0.835	0.759	0.666	0.002
P (H2 vs. L2)		0.335	0.019	0.040	<0.001
P (H3 vs. L3)		0.771	0.318	0.714	0.005

[a] See Footnote a in Table 2.
[b] Urine from (1) undosed, (2) proline-dosed, and (3) proline plus vitamin C-dosed subjects.
[c] *P* values of comparison are also listed, with significant values underlined.

nitrosamino acids in the samples from undosed subjects did not differ between subjects from the high- and low-risk areas. Intake of proline (300 mg) increased urinary NPRO excretion by the subjects in the high-risk area from 3.9 to 7.6 μg/day ($P = 0.012$) but did not significantly change NPRO levels among subjects in the low-risk area. Intake of ascorbic acid together with proline (300 mg) by the high-risk subjects significantly decreased the urinary level of NPRO, NTCA, and the sum of nitrosamino acids ($P < 0.01$). This inhibiting effect was not significant in subjects from the low-risk area. The marked reduction of urinary excretion of nitrosamino acids after intake of ascorbic acid indicates that endogenous formation of these compounds was inhibited by ascorbic acid; this decrease provides an indication of an individual's nitrosation potential *in vivo*, which was found to be higher in subjects living in the high-risk area.

Since, for logistic reasons, no 24-h urines could be collected, we determined the creatinine concentration in the urine and expressed the levels of nitrosamino acids per mmol of creatinine (Table 3). However, as shown before, endogenously formed NPRO is almost totally excreted within 12 h (20). Creatinine concentrations in the urine did not show any consistent difference between subjects from the two areas (Table 2). Comparisons of urinary nitrosamino acid levels, expressed per mmol of creatinine levels, led to essentially the same conclusions as for the uncorrected values in Table 2, although some comparisons were not statistically different. For example, NPRO levels after proline intake did not increase when expressed per creatinine (H1:H2 in Table 2 *versus* Table 3). A correlation between creatinine and NPRO excretion has been observed earlier and could be attributable to an increased meat or protein intake (21). However, the major findings presented in Table 2 are confirmed in Table 3: after proline intake, NTCA levels and the sum of nitrosamino acids were significantly higher in subjects from the high-risk area (by roughly a factor of 2). After ascorbic acid intake, the urinary levels of NPRO, NTCA, and the sum of nitrosamino acids were significantly reduced in subjects living in the high-risk area, while no such reduction was seen in the subjects in the low-risk area.

The effect of smoking on urinary excretion of nitrosamino acids was examined. In the group 1 samples (undosed subjects), the amounts of either NPRO ($P < 0.01$), NTCA ($P < 0.01$), and the sum of nitrosamino acids ($P < 0.001$) were higher in smokers than in nonsmokers. In group 2 (proline specimens) and group 3 (proline plus vitamin C specimens), there were no statistical differences between smokers and nonsmokers. The proportions of smokers in high- and low-risk areas were similar (Table 1).

The urinary levels of nitrate per 12 h or those corrected for creatinine are listed in Tables 2 and 3. The nitrate levels excreted in subjects from the high-risk area in all three dose groups were significantly higher than those from the low-risk area. This approximately 2-fold difference remained significant when nitrate levels were expressed per mmol of creatinine (Table 3). After Box-Cox transformation of data on urinary volumes from undosed subjects and those receiving proline, the urinary volume was positively correlated with log NPRO ($P < 0.01$); this positive correlation, however, became nonsignificant when the proline values were corrected for creatinine. The Pearson correlation coefficients (each at $P = 0.0001$) between urinary levels of NPRO and of nitrate (both log transformed) were 0.47 in undosed specimens (combined groups H1 and L1; 0.67 in proline specimens [combined groups H2 and L2 (Fig. 1)], and 0.42 in proline plus vitamin C specimens (combined groups H3 and L3). Similarly, in undosed specimens (groups H1 and L1 combined), log [sum of nitrosamino acids] *versus* log nitrate were correlated ($r = 0.33$ and $P < 0.01$; Fig. 2). The linear regression lines (shown in Figs. 1 and 2) had slopes of 0.75 and 0.38, respectively. Recorded food items consumed by subjects during the day of urine collection included meat, salted and fresh fish, vegetables, and

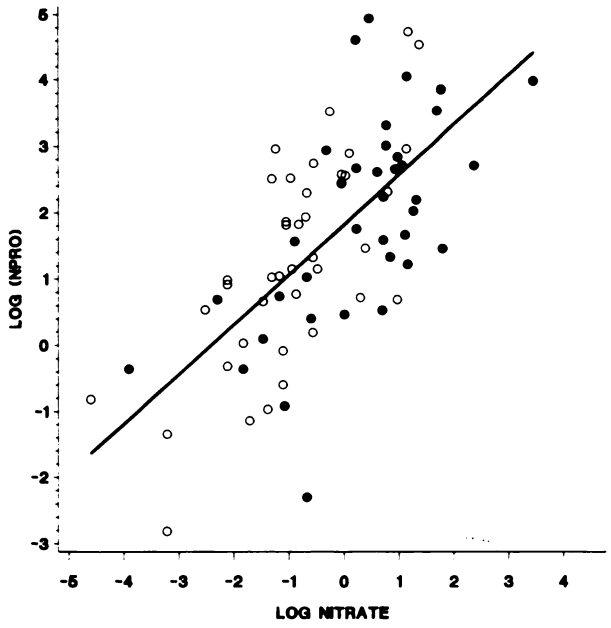

Fig. 1. A plot of log (NPRO) *versus* log (nitrate) concentration in the urine in subjects dosed with proline from high-risk (●) and low-risk (○) districts for NPC in southern China ($r = 0.67$, $P < 0.001$). The corresponding linear regression line is shown.

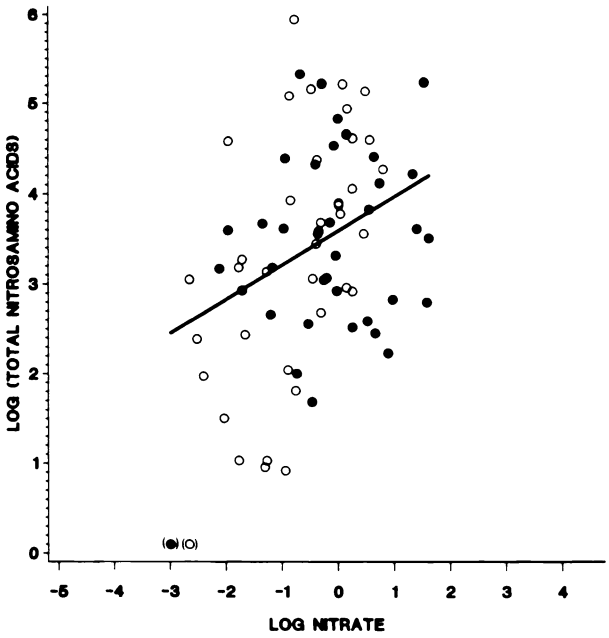

Fig. 2. A plot of log (sum of nitrosamino acids) *versus* log (nitrate) concentration in the urine of undosed subjects from high-risk (●) and low-risk (○) districts for NPC in southern China ($r = 0.33$, $P < 0.01$). The corresponding linear regression line is shown. Two outlying points (in parentheses) with very low levels of nitrosamine acids were excluded for this analysis.

fruits. Each type of nutrient was poorly represented, and the relationship between the levels of nitrosamino acids and the amount of any type of food item could not be adequately analyzed.

Discussion

The NPRO test has previously been applied to subjects living in high- and low-risk areas for stomach cancer in Japan, Poland, and Costa Rica and for esophageal cancer in northern China (22). In general, the nitrate exposure and potential for endogenous nitrosation, measured by the increased levels of urinary NPRO after intake of proline, were much higher in the high-risk populations. Furthermore, low-risk subjects may ingest sufficient amounts of protective agents to suppress endogenous nitrosation. Results from this study provide for the first time an indication that intragastrically formed *N*-nitroso compounds (or other nitrite-derived mutagens/carcinogens) may be risk factors for NPC in southern China. The roughly 2-fold higher nitrosation potential that we have observed in the high-risk subjects appears attributable to their higher nitrate intake and lack of nitrosation inhibitors that are probably present in the diet of subjects living in the low-risk area. Our study was not designed to enable us to pinpoint a particular food item that could inhibit nitrosation. Vegetables and fruits contain not only nitrate but other constituents as well, such as vitamin C and phenolic compounds which generally inhibit *N*-nitrosation. The subjects from the high-risk area, after intake of vitamin C, had a drastically lowered level of nitrosamino acids, and no such decrease was observed in subjects from the low-risk area. These results strongly suggest that nitrosation of dietary precursor compounds ingested by high-risk subjects may generate mutagens or carcinogens that play a role in the etiology of NPC. This hypothesis fits with the earlier suggestion by Ho (23), who proposed that risk factors for NPC involve an interaction between genetically determined susceptibility, early infection by the Epstein-Barr virus, and exposure to chemical carcinogens through the consumption of traditional preserved food, especially salted fish in southern China, from the weaning period onward. Since then, several case-control studies (4–7)[4] have been conducted in southern Chinese living in different locations, and all of the results support Ho's hypothesis, that diet plays a role in NPC etiology.

We have previously screened preserved food items that are frequently consumed in areas endemic for NPC in southern China, Tunisia, and Greenland for the presence of mutagens and volatile nitrosamines, before and after nitrosation (9, 14). The level of preformed volatile nitrosamines (*N*-nitrosodimethylamine, *N*-nitrosopiperidine, and *N*-nitrosopyrrolidine) ranged from nondetectable up to 500 μg/kg wet weight and was highest in hard salted and dried fish from China. After chemical nitrosation, 9 of 15 samples (aqueous food extracts) showed increased mutagenicity, and the levels of volatile nitrosamines were elevated in 12 of 15 samples; the highest level of *N*-nitrosodimethylamine was found in nitrosated extracts of hard salted and dried fish from China (1200 μg/kg wet weight) and the highest *N*-nitrosopyrrolidine level in a nitrosated *harissa* sample (about 3800 μg/kg wet weight). These two food items are among those that recent studies in high-risk areas have implicated as NPC risk factors. Some volatile nitrosamines induce tumors in the nasal cavity of experimental animals (10, 11) and are therefore candidates for involvement in NPC etiology.

Our observation that nitrosation potential is increased in subjects living in a high-risk area for NPC implies that volatile nitrosamines could be formed *in vivo* at high exposure levels in subjects who ingest dietary nitrosamine precursors from early childhood onward. In fact, Yu *et al.* (5) have shown in a case-control study on young NPC patients that consumption of Cantonese-style salted fish during childhood, especially between 1 and 10 years of age, is associated with NPC; the relative risk for weekly as compared to rare consumption, at the age of 10, was 37.7. It was estimated that over 90% of NPC cases in Hong Kong Chinese under the age of 37 could be linked to consumption of salted fish during childhood. The nature and levels of nitrosamines and nitrite-derived mutagens formed after nitrosation of high-risk food items such as salted fish have not been characterized, apart from the three volatile nitrosamines mentioned above. Two animal bioassays have demonstrated the carcinogenic effects of Cantonese-style salted fish: Huang and Ho (24) reported that 4 of 10 female Wistar albino rats which consumed steamed Cantonese-style salted fish developed carcinoma (both adenocarcinoma and squamous cell carcinoma) of the nasal and paranasal regions, and Yu *et al.* (25) found nasal cavity tumors (squamous cell carcinoma, undifferentiated and spindle-cell carcinomas) in 3 of 74 Wistar-Kyoto rats fed powdered diet containing salted fish.

In order to identify additional risk factors for NPC, we have further analyzed food items consumed by inhabitants from areas of high NPC risk for the presence of substances that activate EBV *in vitro* (13). We found that aqueous extracts of Cantonese-style salted and dried fish, and *harissa*, a Tunisian spice mixture, can induce EBV early antigen in latently infected Raji cells (13, 26). This activity may be important, since it is known that IgA antibodies against EBV viral capsid antigen and early antigens reflect *in vivo* EBV activation; these are regularly present in NPC patients and appear in subjects before tumor development (27). However, since EBV-inducing activity did not parallel either mutagenicity or the level of volatile nitrosamines in the food extracts investigated (13), we are now undertaking the isolation and characterization of EBV-inducing substances in food items that have been associated with NPC risk.[5]

In conclusion, our results support the hypothesis that diet plays a role in NPC etiology, as indicated by case-control studies (reviewed in Ref. 28); the latter consistently demonstrated that consumption of Chinese-style salted fish is strongly related to risk for nasopharyngeal cancer. Nitrite-derived carcinogens (formed from dietary precursors) and EBV-inducing substances are also implicated as risk factors.

Acknowledgments

We thank Dr. J. Cheney for editorial help and Mrs. M. Wrisez for typing the manuscript.

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