Uptake and Metabolism of Carcinogenic Levels of Tobacco-specific Nitrosamines by Sudanese Snuff Dippers

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Abstract

It was recently reported that toombak, a type of snuff used in the Sudan, contained unusually high levels of tobacco-specific nitrosamines. To estimate the internal dose of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK) received by individuals who use this type of tobacco, urine from a group of users was analyzed for 2 metabolites of NNK, 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its O-glucuronide, NNAL-Gluc. NNK is a strong lung carcinogen believed to contribute to human lung cancer. NNAL is also a lung carcinogen. NNAL and NNAL-Gluc were analyzed by gas chromatography with a nitrosamine selective detector. The average levels detected were 0.39 ± 0.14 (SD) nmol/ml urine (n = 7) and 0.88 ± 0.50 nmol/ml urine (n = 7), respectively. In a 24-h period, these individuals would excrete 0.12 to 0.44 mg of these 2 metabolites (expressed per mg NNAL). Therefore, assuming chronic toombak use, the minimum daily dose of NNK to which these users were exposed was 0.12–0.44 mg. This is the highest documented uptake of a nonoccupational carcinogen. Two diastereomers of NNAL-Gluc were present in all urine samples analyzed. Previously, these two diastereomers were identified in the urine of an NNK-treated patas monkey but only one was detected in the urine of NNK-treated rats. The level of the 4-hydroxy-1-(3-pyridyl)-1-butanol releasing hemoglobin adduct was also quantified in these individuals. This adduct is believed to be a measure of NNK activation. The levels ranged from 68 to 323 fmol/g hemoglobin [mean, 148 ± 104 (SD)]. The wide range of adduct levels which were observed suggests that despite similar levels of NNK exposure, there are significant differences in the ability of individuals in this population to activate NNK, as well as potential differences in their cancer risk.

Introduction

Biochemical markers have been suggested as a means by which to more accurately assess the carcinogenic exposure of individuals and, therefore, their cancer risk. This approach has been used most successfully for human exposure to aflatoxin, a strong liver carcinogen (1). The accuracy of the marker which was used, the N7-guamine adduct of aflatoxin, was demonstrated in two populations with very high exposure to aflatoxin. A correlation was demonstrated between the level of aflatoxin in the food consumed and the level of the aflatoxin guanine adduct in the urine of the same individual (2). Exposure levels ranged from 0.1 to 10 μg aflatoxin/day.

It was recently reported that a type of smokeless tobacco used in the Sudan contains extraordinarily high levels of tobacco-specific nitrosamines. This tobacco (“toombak” in the local language) is used as an oral snuff. It is prepared by mixing sun-dried tobacco leaves, primarily Nictiana rustica, with sodium carbonate. Approximately 1 g of this mixture is used at a time and left in the mouth for a period ranging from a few minutes to several hours, until it becomes bland, at which time it is replaced with fresh toombak. The level of NNK in this tobacco ranged from 0.62 to 7.87 mg/g and accounted for up to 50% of the tobacco-specific nitrosamines present (3). The highest level of NNK previously reported in other smokeless tobacco products was 0.014 mg/g and was less than 15% of the total tobacco-specific nitrosamines (4). Idris et al. (5) estimated, based on the average level of NNK in toombak (2.31 ± 1.55 mg/g), that a user of this tobacco would be exposed to 11.5 mg NNK per day or 0.16 mg/kg/day. This assumes the quantity of snuff used daily in the Sudan is half that in the United States, or 5 g/day. This is probably an underestimation. The level of tobacco-specific nitrosamines in the saliva of toombak users ranges from 0.7 to 30.6 μg/ml (5), or 10–100 times the levels found in saliva of users of other types of smokeless tobacco (6–8). These levels are consistent with an unusually high level of exposure to these carcinogenic nitrosamines.

NNK induces lung tumors in animals whether it is administered by i.p. or s.c. injection or given in the drinking water (9, 10). Rats treated with a total dose as low as 1.8 mg/kg develop lung tumors. Users of toombak would be exposed to this dose in less than 2 months. If the internal dose of the carcinogen were to be even 1/100 of the reported amount, this would be the highest documented

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3 The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane; NNAL, 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanol; NNAL-Gluc, 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanol-O-glucuronide; NNN, N'-nitrosonomicotine; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanol; isoNNAL, 4-(methyl nitrosamino)-4-(3-pyridyl)-1-butanol; NNK-N-oxide, 4-(methyl nitrosamino)-1-(3-pyridyl)-N-oxide; diol, 4-hydroxy-4-(3-pyridyl)-1-butanol; HPLC, high performance liquid chromatography; GC-TEA, gas chromatography-thermal energy analyzer; a nitrosamine selective detector; PAH, polynuclear aromatic hydrocarbons; TMS, trimethylsilyl.
Biochemical Markers for NNK in Toombak Users

Fig. 1. Metabolism of NNK to NNAL-glucuronides and the HPB-releasing hemoglobin adduct. The absolute configurations of NNAL-Gluc (I) and NNAL-Gluc (II) have not been determined.

Materials and Methods

Chemicals. [5-3H]NNAL-Gluc (II) was collected from patas monkey urine (18). Unlabeled NNAL-Gluc (I) and NNAL-Gluc (II) were collected from NNK-treated rats and monkeys, respectively. [3,3'-D2]HPB and the tetrafluorobenzoate of HPB were synthesized (16, 19). NNAL, isoNNAL, and nitrosoguvacoline were synthesized and provided to us by Dr. Shantu Amin (20–22). Bis-trimethylsilyltribluoracet-imide/1% trimethylchlorsilane was obtained from Regis Chemical Co. (Morton Valley, IL). β-Glucuronidase, type IXA was obtained from Sigma Chemical Co. (St. Louis, MO). 3-Acetylpymidine, pentafluorobenzyl chloride, and 2-pyridylcarbinol were obtained from Aldrich Chemical Co. (Milwaukee, WI). The last was converted to its acetate by treatment with acetic anhydride and trimethylamine in methylene chloride.

Collection of Samples. Urine and blood samples were obtained immediately following the use of toombak from 7 men who were chronic toombak-users. Collections were made at the Khartoum Cancer Hospital under the supervision of Dr. Idris. A 100-ml urine sample and a 10-ml blood sample were collected. Urine was frozen immediately at −20°C. The blood sample was centrifuged at 1000 X g and the RBC pellet was washed 3 times and then frozen at −20°C. The samples were packed in dry ice and hand carried by Dr. Idris from Khartoum, Sudan to the American Health Foundation in Valhalla, New York.

Analysis of Urine for NNAL and NNAL-Gluc. Urine was analyzed for NNAL and NNAL-Gluc as described previously (14) with minor modifications. Briefly, a 0.5-ml aliquot of urine was extracted with ethyl acetate. Fifty ng of isoNNAL was added as an internal standard to the organic layer containing NNAL which was dried and further purified by HPLC. The aqueous layer was treated with β-glucuronidase, spiked with 50 ng isoNNAL, extracted with methylene chloride, dried, and further purified by HPLC. After collection of the NNAL fraction from HPLC, which nonoccupational exposure of any population to a known carcinogen.

Twenty-eight % of the 526,000 estimated cancer deaths in the United States in 1993 were due to lung cancer (11). Greater than 80% of these cancers are due to the use of tobacco and NNK is considered to be one causalistic agent for lung cancer (10, 12). If NNK is a lung carcinogen in humans, then one would expect Sudanese toombak users to have a high risk of developing lung cancer. This is the ideal population in which to establish whether NNK is a lung carcinogen, and if, like in animals, induction of lung tumors occurs without direct exposure to the lung. While an increased occurrence of oral cancer in the Sudan has been attributed to the use of toombak (13), the relative risk of developing lung cancer has not been investigated. It is critical to determine the internal dose of NNK received by this population in order to accurately assess the relationship of lung cancer incidence to NNK exposure. Therefore, our goal in this study was to validate a biochemical marker for NNK exposure in toombak-users to be used in a future molecular epidemiological study of lung cancer in the Sudan.

Recently, the urinary levels of two metabolites of NNK were proposed as biochemical markers for NNK uptake in individuals exposed to tobacco (14). These metabolites, NNAL and NNAL-Gluc, have been quantified in the urine of active smokers and non-smokers exposed to sidestream smoke (14, 15). NNK and NNN, another tobacco-specific nitrosamine, are metabolized by α-hydroxylation to a common diazohydroxide (Fig. 1) which reacts with DNA and hemoglobin. The adduct formed has been quantified in hemoglobin of United States snuff dippers and cigarette users as released HPB (16). An HPB-releasing DNA adduct has also been quantified in smokers (17).

In this paper we describe the quantitation of NNAL and NNAL-Gluc in a group of toombak users. In addition, the level of the HPB-releasing hemoglobin-adduct in these individuals was determined.
easily collect the regions containing NNAL-Gluc (I) and other compounds in the urine sample. This allowed us to collect a 50 ng/pl diol and 50 ng/pl NNK-N-oxide fraction was used for analysis by GC-TEA.

Aliquots of each sample were injected with 200 μl of the marker mix. Two fractions were collected, one from the top of the diol peak to 4 min past (44.8 min) and the second from 44.8 min to the end of the peak for the E-isomer of NNK-N-oxide (Fig. 2). To confirm the accuracy of our collections, one urine sample was spiked with [5-3H]NNAL-Gluc (II) and the retention time of the radioactive peak was shown to be within fraction 2, as determined by combined UV and radioflow detection. The HPLC fractions containing each diastereomer were treated with β-glucuronidase (5000 units) overnight at 37°C, isoNNAL was added as an internal standard, and the released NNAL was analyzed by GC-TEA as described previously (14).

**Analysis of HPB-releasing Hemoglobin Adduct.** A hemoglobin solution was prepared from the frozen RBC pellet (16). Briefly, the cells were lysed in an equal volume of HPLC grade H2O (Burdick and Jackson, Muskegon, MI). The resulting solution was stirred briskly and mixed on a vortex mixer; a 40% volume of 0.67 M sodium phosphate buffer (pH 7) was added and the solution was centrifuged for 25 min at 20,000 × g. The supernatant was dialyzed against 4 l-I volumes of H2O over a 24-h period. The hemoglobin solution was frozen at 20°C in 5-ml aliquots until analysis.

The amount of HPB-releasing adduct in this hemoglobin was analyzed as described previously (14, 15) with modifications. HPB was released from hemoglobin by treatment with 0.1 N NaOH; the sample was neutralized and the protein was precipitated by the addition of 2 M NaH2PO4.

In the original method of analysis, the HPB present in the supernatant was partially purified by multiple extractions. The filtrates were replaced by the use of a 1000-mg C18 SepPrep column (Fisher Scientific, Springfield, NJ). After application of the supernatant to the column it was washed with an equal volume of H2O, followed by 1 ml of methanol to elute HPB. The methanol was added to 4 ml of H2O and extracted three times with an equal volume of methylene chloride. The methylene chloride layer was dried and analyzed using a 30-m, 0.25-mm Intraline methylsilicone (DB-17) bonded phase column (0.15 μm film thickness, J&W Scientific, Folsom, CA) was used, and injection was by an autosampler (Hewlett-Packard Model 7673B).

**Analysis of Urinary Cotinine.** Urinary cotinine analysis was by radioimmunoassay and was carried out in the Clinical Biochemistry Facility of the American Health Foundation (24, 25). The levels of cotinine reported include some specific nicotine exposure (25).

Urine and blood samples were collected from 7 individuals who had used toombak within the last hour. Urine was analyzed for NNAL and NNAL-Gluc as a measure of NNK exposure, and hemoglobin was analyzed for the HPB-releasing adduct as a reflection of NNK and NNN α-hydroxylation, i.e., activation. Urinary cotinine levels were also analyzed for each as a measure of tobacco exposure, specifically nicotine exposure (25).

The level of free NNAL present in the urine of one of these individuals relative to that in a cigarette smoker is...
Users cigarettes spiked with sample prepared from 20 ml of urine from a toombak user spiked with 50 ng 1.5., at 0.4 mm is the injection standard nitrosoguvacoline; the correlates with the amount of total NNAL plus NNAL-

to a 1 pack/day United States cigarette smoker's exposure of daily would be exposed to 1 900-3600 pg NNK compared the users at the same time urine and blood samples were

toombak was analyzed from 6 samples provided to us by

toombak users are presented in Table 2. In all cases both diastereomers in toombak-users, a reverse phase HPLC system which clearly separated NNAL-Gluc (I) and NNAL-Gluc (II) was developed. This separation is illustrated in Fig. 2. The NNAL-Gluc standards were coincjected with diol and NNK-N-oxide to determine if these two metabolites could be used as retention time markers. NNAL-Gluc (I) eluted just past diol and the second isomer of NNAL-Gluc (II) coeluted with the earlier eluting isomer (E) of NNK-N-oxide. Therefore, diol and NNK-N-oxide were added to each urine sample and two fractions were collected as indicated in Fig. 2. Fractions 1 and 2 from HPLC analyses were each treated with 3-glucuronidase and analyzed for released NNAL by GC-TEA. The amount of each glucuronide was quantified in all 7 toombak users. The data are presented in Table 2. In all cases both diastereomers were present and the amount of NNAL-Gluc (II) was always greater. The NNAL-Gluc (II):NNAL-Gluc (I) ratio ranged from 2.7 to 1.3 (average, 1.9 ± 0.5).

Discussion

The data presented in this paper confirm a very high exposure of Sudanese snuff dippers to NNK. The average levels of NNAL and NNAL-Gluc present in the urine of these individuals were 0.39 and 0.88 nmol/ml, respectively. The mean estimated daily excretion of both metabolites was 270 µg. The 24 h average excretion of these two metabolites in a group of United States smokers was 4 µg (14). The 68-fold difference in the urinary excretion of NNAL and NNAL-Gluc by these two groups is consistent with the large differences in their exposure to NNK. For four of these smokers, information was available on the number of cigarettes smoked and the NNK in the mainstream smoke of these cigarettes. From this one can calculate that the NNAL and NNAL-Gluc excreted by these individuals accounted for 40–100% of the NNK dose. The amount of toombak used daily by the individuals in the present study was not reported. The average amount of NNK in the toombak used was 270 µg/g. Therefore, if 10 g of toombak were used daily, NNAL and NNAL-Gluc in the urine would account for 10% of the NNK dose. This is comparable to the percentage of NNAL-glucuronide plus NNAL excreted by the patas monkey following administration of 16–18 µg NNK/kg body weight (18).

Fig. 3. GC-TEA traces of urinary NNAL from a Sudanese toombak user and a United States smoker. I.S. injection standard nitrosoguvacoline and internal standard iso-NNAL. (A) Chromatogram obtained by injecting 6% of a sample prepared from 20 ml of urine from a toombak user spiked with 50 ng iso-NNAL. (B) Chromatogram obtained by injecting 50% of a sample prepared from 40 ml of urine from a 2 pack/day smoker of United States cigarettes spiked with 20 ng iso-NNAL. TMS, trimethylsilyl.
Hemoglobin (Hb) adducts were analyzed as HPB released on base treatment (details in “Materials and Methods”).

dCotinine values include some 3'-hydroxycotinine.

Total is expressed as nmol or mg NNAL released/liter urine.

Urine was analyzed before and after β-glucuronidase treatment for NNAL by CC-TEA (details given in “Materials and Methods”).

Sample NNAL-Gluc contained NNAL-Gluc (II) and NNAL-Gluc (I) in a 1.9:1 ratio as mentioned previously, NNK-treated rats excreted detectable NNAL-Gluc (I) only. The urine of toombak users contained NNAL-Gluc (II) and NNAL-Gluc (I) in a 1:9:1 ratio. This suggests that the patas monkey is a better model in which to study NNK metabolism.

Detectable levels of the HPB-releasing hemoglobin adduct were measured in all 7 toombak users. Previously this adduct was measured in the blood from 22 United States snuff dippers immediately following chewing. It was detected in 20 of 22 individuals and ranging from 19 to 1930 fmol/g hemoglobin. The average was 517 ± 538 fmol/g hemoglobin. This is higher than the levels detected in the toombak users, and somehow surprising given their higher NNN and NNK exposure levels, which has been documented in this study. The adduct is believed to be a measure of activation of NNK or NNN, not exposure, and therefore may vary significantly among individuals as well as among populations. The two small groups we are comparing are from two very different populations. The United States group consisted of young white men while the toombak users are black North Africans.

In the patas monkey, the average excretion of NNAL-Gluc in a 24-h urine sample accounted for 22% of the urinary metabolites or about 6% of the dose. NNAL was not detected in the same urine sample. Both diastereomers of NNAL-Gluc were quantified in monkey urine; 4 times more NNAL-Gluc (II) was excreted than NNAL-Gluc (I). As mentioned previously, NNK-treated rats excreted detectable levels of NNAL-Gluc (I) only. The urine of toombak users contained NNAL-Gluc (II) and NNAL-Gluc (I) in a 1:9:1 ratio. This suggests that the patas monkey is a better model in which to study NNK metabolism.

NG. In a recent study of urinary aflatoxin levels in The Gambia the total level of aflatoxin metabolites ranged from 0.2 to 20 ng/mg creatinine (2). It has been estimated that this population has one of the highest exposures to aflatoxin (32). The exposure to NNK in the Sudan is more than 10 times the level of aflatoxin exposure in the Gambia. Aflatoxin is a strong liver carcinogen and an association between human liver cancer and aflatoxin has been established (reviewed in Ref. 32). Lung cancer is estimated to cause 149,000 deaths in the United States alone and a much greater number worldwide (10). The majority of the latter deaths are due to the use of tobacco products. A major lung carcinogen in tobacco products is NNK. Therefore, tobacco exposure, and potentially NNK exposure, are major causes of cancer worldwide. While the easiest means of significantly reducing the incidence of these cancers is to eliminate the use of tobacco, this is not going to happen in the near future. Therefore, it is important to understand the etiology of tobacco-related cancers. This requires the accurate assessment of carcinogen exposure.

The Sudanese population which uses toombak provides a unique opportunity in which to study whether NNK exposure leads to the induction of lung cancer in humans. It is estimated that 42% of adult males in the Sudan use toombak and only a small percentage of these individuals smoke. This group is exposed to high levels of NNK, the internal dose of which is documented in this publication, but has little exposure to other lung carcinogens, specifically PAH, present in tobacco smoke. Therefore, in a future molecular epidemiology study of toombak-users, one may be able to establish NNK as a human lung carcinogen in much the same way aflatoxin has been identified as a human liver carcinogen.

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