Advances in Brief

Metabolites of the Tobacco-specific Nitrosamine 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone in Smokers’ Urine

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Abstract

Metabolites of the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a potent pulmonary carcinogen, have been quantified in the urine of 11 smokers. They were not detected in nonsmokers’ urine. The metabolites, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and its glucuronide, were detected in quantities of 0.23-1.0 and 0.57-6.5 µg/24 h, respectively. The results of this study provide the first evidence for metabolites of tobacco-specific nitrosamines in human urine.

Introduction

It has been estimated that up to 90% of lung cancer deaths in the United States are attributable to cigarette smoking (1). The tobacco-specific nitrosamine NNK (Fig. 1) is believed to play an important role in the induction of lung cancer in smokers because it is a potent pulmonary carcinogen in rats, mice, and hamsters inducing tumors at total doses similar to the estimated doses to which smokers are exposed (2, 3). NNK may also be involved in oral and pancreatic cancer associated with the use of tobacco products (3, 4). Although the metabolism of NNK has been extensively studied in laboratory animals, relatively little is known about its uptake and metabolism in humans. We believe that such information is critical to an understanding of mechanisms of cancer induction in humans.

In rodents and monkeys, identified pathways of NNK metabolism include α-hydroxylation, pyridine-N-oxidation, carbonyl reduction to NNAL, and conjugation of NNAL to the diastereomeric glucuronides NNAL-Gluc(I) and NNAL-Gluc(II) (Fig. 1) (2, 5, 6). Previous studies using cultured human tissues or microsomes have shown that NNK is metabolized by α-hydroxylation and carbonyl reduction (7, 8). Hemoglobin and DNA adducts resulting from α-hydroxylation of NNK, NNAL, or the related carcinogen NNN have been detected in the blood of smokers or snuff-dippers (9, 10). In this article, we present the first evidence for the presence in smokers’ urine of NNK metabolites: NNAL; NNAL-Gluc(I); and NNAL-Gluc(II). Quantitation of NNK metabolites in human urine should greatly facilitate our understanding of its role in tobacco-related cancers.

Materials and Methods

Chemicals. [5-3H]NNAL was obtained by NaBH₄ reduction of [5-3H]NNK (Chemsyn Science Laboratories, Lenexa, KS). [5-3H]NNAL-Gluc(II) was isolated from the urine of a patas monkey treated with [5-3H]NNK (6). NNAL, iso-NNAL, nitrosoguacoline, and 4-(methylamino)-4-(3-pyridyl)-1-butanone were synthesized (11-13). Bis-trimethylsilyl trifluoroacetamide/1% trimethylchlorosilane was obtained from Regis Chemical Co. (Morton Grove IL). β-Glucuronidase, type IXA, sulfatase, type VIII, and saccharic acid 1.4-lactone were obtained from Sigma Chemical Co. (St. Louis, MO). 3-Acetylpyridine and 2-pyrydylcarbinol were obtained from Aldrich Chemical Co. (Milwaukee, WI). The latter was converted to its acetate by treatment with acetic anhydride and triethylamine in CH₂Cl₂.

Apparatus. HPLC was performed using a Millipore, Waters Division system as previously described (9) and a 3.9 x 300-mm Bondclone 10 C18 column (Phenomenex, Torrance, CA) with UV detection at 254 nm. Solvent A was H₂O and solvent B was methanol. The solvent program was 15% B in A for 10 min, then to 45% B in A over 30 min, then back to initial conditions in 5 min, and then held for 25 min prior to the next injection.

GC-TEA was performed with a Model 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) interfaced with a Model 610 Thermal Energy Analyzer (Thermedics Inco, Woburn, MA) and a Model D-2000 integrator (Hitachi Instruments, Danbury CT) (14). The gas chromatograph was equipped with a 2-mm x 12-ft glass column filled with 3% XE-60 on GasChrom Q, 100/200 mesh (Alltech/Applied Sciences, Deerfield, IL). The oven temperature was programmed as follows: 150°C for 3 min; then 6°C/min to 220°C; and then held for 15 min. The injection port temperature was 230°C and the flow rate was 33 ml/min argon.

GC-MS-SIM was carried out with a Hewlett-Packard Model 5988A instrument, operated in the positive chemical ionization mode with a methane pressure of 0.88 torr, an ionizing energy of 107 eV, and a source temperature of 200°C. For electron impact experiments, the ionizing energy was 70 eV and the source temperature was 200°C. The analyses were performed by splitless injection on a 0.25-mm x 30-m Econacap SE 54 column (film thickness, 0.25 µm; Alltech/Applied Sciences), with a 0.32-mm x 1-m retention gap. The carrier gas was He (head pressure, 12 psi) and the oven temperature was programmed as follows: 100°C for 1 min; then 8°C/min to 180°C; and then held for 30 min.

Volunteers. Eleven smokers and 7 nonsmokers ranging in age from 20 to 65 years were recruited. The protocol for collection of urine was approved by the American Health Foundation Institutional Review Board for protection of human subjects.

Analysis of Urine by GC-TEA. Twenty-four-hour urine samples were collected in 3 liter amber specimen containers (Baxter Scientific Products Division, McGaw Park, IL), to which 10 ml of a solution of 20% ammonium sulfamate (Sigma) in 3.6 N H₂SO₄ had been added to inhibit artifactual nitrosation. Samples were stored at room temperature during collection. Aliquots of 100 ml were adjusted to pH 7 with 10 N NaOH. To this was added 0.5 ml of an aqueous solution of [5-3H]NNAL-Gluc(II) (2.1 Ci/mmol; 21,000 dpm) as internal standard. The resulting solution was extracted 3 times with equal volumes of ethyl acetate. The aqueous portion (A-1) was saved. A solution of [5-3H]NNAL (2.1 Ci/mmol; 100,000 dpm) in 50 µl of methanol was added to the combined ethyl acetate extracts, as standard for un conjugated NNAL. The ethyl acetate layers were dried (Na₂SO₄) and concentrated to dryness by rotary evaporation. The residue (Fig. 2, Fraction 1) was dissolved in two 0.5-ml aliquots of H₂O, which were combined and set aside for subsequent HPLC purification and analysis for NNAL. Twenty µl of "antifoam B emulsion" (Sigma) were added to the aqueous portion, A-1, which was then concentrated by rotary evaporation (water bath temperature, 35°C) to approximately 70% of its initial volume. The purpose of this step was removal of most of the ethyl acetate, traces of which inhibited β-glucuronidase activity. β-Glu
A centrifuge tube. The collected material was concentrated to dryness on a Model SVC200H Speedvac concentrator (Savant Instruments, Farmingdale, NY) at approximately 35°C. The residue was transferred to a 100-ul glass-lined microcentrifuge tube. The collected material was concentrated to dryness on a Model SVC200H Speedvac concentrator (Savant Instruments, Farmingdale, NY) at approximately 35°C. The residue was transferred to a 100-ul glass-lined microvial (#225195; Wheaton, Millville, NJ) with three 50-ul portions of methanol. The methanol was removed by Speedvac evaporation.

To the residue was added 5 ul of CH₃CN containing 30 ng of iso-NNAL and 20 ng of nitrosoguvacoline, which were used as silylation and injection standards, respectively. After thorough mixing, 5 ul of bis-trimethylsilyl trifluoroacetamide containing 1% trimethylchlorosilane were added, the vial was capped, and the mixture was heated at 40°C for 1 h. Then 5 ul were analyzed by GC-TEA. The remaining 50% of the sample was washed into a scintillation vial with CH₃CN and methanol for determination of recovery of radioactivity.

Amounts of NNAL in the samples were determined by integration of the peak corresponding in retention time to NNAL-TMS (see Fig. 3) and comparison to a standard curve. Efficiency of silylation was generally quantitative, as determined by integration of the peak corresponding in retention time to iso-NNAL-TMS. Results were corrected for recovery of internal and injection standards.

Some urine samples were analyzed for NNK, [5-3H]NNAL, 114,000 dpm, was added to the ethyl acetate extract of urine. This was analyzed by HPLC as described above and the regions corresponding in retention time to NNAL and NNK were collected. The NNK fraction was concentrated to remove methanol and treated with 5 mg NaCNBH₃. Excess reducing agent was quenched with 2 N HCl and the mixture was neutralized and then extracted 3 times with CH₂Cl₂. This was concentrated, and the residue was silylated and analyzed as above for NNAL-TMS.

Preparation of Urine for Analysis by GC-MS-SIM. Morning void (approximately 800 ml) from smokers 2 and 3 (Table 1) was combined. Fraction 2 was obtained essentially as described above, with allowances for the larger scale being made. Reverse-phase HPLC was carried out with a 7.8- x 300-mm Bondclone 10 C18 column using the program described above, with a flow rate of 4 ml/min. The fraction containing NNAL was further purified by normal phase HPLC with a 4.6- x 250-mm Microsorv-MV 5-µm silica column (Rainin Instrument Co., Woburn, MA) with elution by solvents A (50/50:CHCl₃/hexane) and B (20% isopropl alcohol in solvent A). The solvent program proceeded from 70 to 50% A in 15 min and was then held for 25 min. The flow rate was 1 ml/min. Eluent was collected from 24 to 45 min, concentrated to 35°C, and treated with 5 mg NaCNBH₃. Excess reducing agent was quenched with 2 N HCl and the mixture was neutralized and then extracted 3 times with CH₂Cl₂. This was concentrated, and the residue was silylated and analyzed as above for NNAL-TMS.

Results

The analytical method is summarized in Fig. 2. Fraction 1, obtained by extraction with ethyl acetate, contained unconjugated NNAL. The aqueous portion of the urine was treated with β-glucuronidase to release NNAL from NNAL-Gluc(I) and NNAL-Gluc(II). The released NNAL was enriched by partitioning to give Fraction 2. Each fraction proceeded from 70 to 50% A in 15 min and was then held for 25 min. The flow rate was 1 ml/min. Eluent was collected from 24 to 45 min, concentrated to dryness, and silylated.

The identity of NNAL in Fraction 2 of smokers’ urine was confirmed by GC-MS-SIM. Major peaks in the chemical ionization spectrum of standard NNAL-TMS are m/z 282 (M + 1; relative intensity, 3.86). The identity of NNAL in Fraction 2 of smokers’ urine was confirmed by GC-MS-SIM. Major peaks in the chemical ionization spectrum of standard NNAL-TMS are m/z 282 (M + 1; relative intensity, 3.86).

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### Table 1 NNAL and NNAL-Gluc in smokers’ urine

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Cigarettes/day</th>
<th>NNAL (nmol/24 h)</th>
<th>NNAL-Gluc (nmol/24 h)</th>
<th>Cotinine (mg/24 h)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>36</td>
<td>2.2 (0.46)*</td>
<td>11 (4.4)*</td>
<td>15.3</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>29</td>
<td>1.8 (0.37)</td>
<td>14 (5.5)</td>
<td>20.0</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>20</td>
<td>1.7 (0.35)</td>
<td>17 (6.5)</td>
<td>6.82</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>25</td>
<td>2.1 (0.43)</td>
<td>4.4 (1.7)</td>
<td>3.39</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>20</td>
<td>3.3 (0.69)</td>
<td>10 (3.9)</td>
<td>7.31</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>29</td>
<td>4.8 (1.10)</td>
<td>10 (3.9)</td>
<td>8.80</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>25</td>
<td>1.1 (0.23)</td>
<td>11 (4.2)</td>
<td>8.22</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>25</td>
<td>3.3 (0.70)</td>
<td>1.5 (0.57)</td>
<td>2.28</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>12</td>
<td>4.2 (0.87)</td>
<td>3.6 (1.4)</td>
<td>5.92</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>20</td>
<td>1.4 (0.29)</td>
<td>8.6 (3.3)</td>
<td>2.71</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>15</td>
<td>1.3 (0.28)</td>
<td>7.2 (2.8)</td>
<td>4.22</td>
</tr>
</tbody>
</table>

* Values include some 3'-hydroxycotinine.

** Numbers in parentheses, µg.

* Levels of NNK in mainstream smoke of the cigarettes used by these volunteers were available. These were multiplied by cigarettes/day to obtain estimated daily dose of NNK, as follows (nmol): 4, 16.4; 6, 22.7; 9, 7.7; 10, 13.0.

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The possibility that NNAL or NNAL-Gluc could have formed artifactually during analysis of the urine samples was considered although the amine precursors to NNAL and NNAL-Gluc are not known to be constituents of smokers' urine. Urine was collected from a smoker and divided into three 50-ml aliquots, each of which was mixed with ammonium sulfamate/H$_2$SO$_4$ as described in “Materials and Methods.” The first aliquot was treated with 0.5 mg of 4-(methylamino)-4-(3-pyridyl)-1-butanol, the amine precursor to iso-NNAL, and was allowed to stand for 24 h at room temperature. It was then analyzed for iso-NNAL. The purpose of this experiment was to determine the extent of nitrosation, under our collection and analysis conditions, of a secondary amine that is readily nitrated and is structurally related to the secondary amine precursor to NNAL, which was not available. The yield of iso-NNAL in this experiment was 0.01%. The second 50-ml aliquot was treated with 4.5 mg NaNO$_2$, which is equivalent to the amount of NaNO$_3$ typically present in 50 ml of urine (15). [Urine samples from noninfected individuals do not normally contain nitrite (15)]. The third aliquot was not treated. These two aliquots were analyzed by GC-TEA after silylation. NNAL-TMS was detected in all three fractions, which is consistent with the presence of NNAL-Gluc(I) and (II) as well as unconjugated NNAL in smokers' urine.

Discussion

The data presented here clearly demonstrate that NNAL and NNAL-Gluc are present in smokers' urine. The amounts detected are in the expected range based on the levels of NNK in cigarette mainstream smoke. These data confirm the uptake of NNK by smokers and allow one to estimate its dose. This will be important in further studies evaluating the relationships of NNK to tobacco-induced cancer. The present results do indicate that total uptake of NNK in a lifetime of smoking is comparable to the lowest doses of this carcinogen which induce lung tumors in rodents (4).

It is possible that some of the NNAL and NNAL-Gluc in urine may come from NNAL in cigarette smoke. The presence of NNAL in cigarette smoke has not been reported, although it has been tentatively identified in snuff products, at levels which appear to be considerably lower than those of NNK (16). NNAL has also been detected in the saliva of Sudanese snuff-dippers (17). NNAL, like NNK, is a potent pulmonary carcinogen in the rat and mouse (18-20). It also induces ductal tumors of the pancreas in the rat (20).
The failure to detect NNK was not unexpected based on metabolism studies which have shown that little if any of this nitrosamine is excreted unchanged in laboratory animals (5, 6, 12). The detection of NNAL-Gluc in smokers’ urine is consistent with our recent studies of NNK metabolism in the patas monkey, which showed that NNAL-Gluc(1) and (II) were prominent urinary metabolites (6).

Previous studies have shown that hemoglobin adducts, known to result from metabolic activation of NNK, NNAL, and NNN, are elevated in approximately 20% of smokers (9). This presumably relates to higher levels of α-hydroxylation of these nitrosamines in some smokers. The data in Table 1 indicate that the ratio of NNAL to NNAL-Gluc varies from 0.1 to 2.3. It will be important to determine whether the ratio of free to conjugated NNAL influences levels of hemoglobin and DNA adducts in smokers.

Acknowledgments

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References

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