Short Communication

A Metabolite of the Tobacco-specific Lung Carcinogen 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone in the Urine of Hospital Workers Exposed to Environmental Tobacco Smoke[1]

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Abstract

We analyzed the urine of nonsmoking hospital workers exposed to environmental tobacco smoke for [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glucosiduronic acid (NNAL-Gluc), a metabolite of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Samples were collected three times on a single day from nine workers. Quantitative analysis was carried out by combined gas chromatography-nitrosamine-selective detection. The identity of NNAL-Gluc was confirmed by combined gas chromatography-tandem mass spectrometry. The results demonstrated the presence of NNAL-Gluc in the urine of the exposed subjects. The mean level of NNAL-Gluc ± SD was 0.059 ± 0.028 pmol/ml urine (23 pg/ml urine); range, 0.005–0.11 pmol/ml urine. Levels of NNAL-Gluc per milliliter of urine correlated with those of cotinine ($r = 0.51$; $P = 0.029$). These results demonstrate for the first time that NNAL-Gluc, a metabolite of the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, is present in the urine of nonsmokers exposed to environmental tobacco smoke under field conditions.

Introduction

Several groups have concluded that ETS[3] causes lung cancer in nonsmokers (1–5), but limited data are available on levels of lung carcinogens or their metabolites and adducts in exposed nonsmokers. Polycyclic aromatic hydrocarbons and the tobacco-specific nitrosamine NNK are the most likely causes of lung cancer in smokers, and these compounds can plausibly be implicated as potential causes of lung cancer in nonsmokers as well (6). In one study, polycyclic aromatic hydrocarbon albumin adducts were higher in the children of smoking mothers than in controls (7). Another study demonstrated that two metabolites of NNK, NNAL and its *O*-glucuronide (NNAL-Gluc), were present in the urine of nonsmokers exposed to relatively high levels of ETS in a chamber (8). Uptake of NNK by nonsmokers is of particular interest, because in rodents NNK induces mainly adenocarcinoma of the lung, the same type of tumor seen in most ETS-exposed nonsmokers (9).

Our goal in this study was to analyze the urine of nonsmokers exposed to ETS under field conditions. We used GC-TEA to analyze NNAL-Gluc in the urine of hospital workers occupationally exposed to ETS. The identity of NNAL-Gluc was confirmed by GC-MS/MS.

Materials and Methods

Subjects and Urine Collection. Subjects were employees of a long-term care hospital that has several smoking lounges for patients. Employees of the hospital are obliged to perform their duties in these areas, which are not ventilated separately. The study was undertaken as part of an effort to investigate employee exposure to carcinogens in ETS.

Nine nonsmoking employees, chosen to represent a spectrum of possible ETS exposures, were asked to avoid ETS exposure outside the workplace for at least 4 days before sample collection. They performed the collections toward the end of the workweek. They gave urine samples on arrival at work, during the work day, and on leaving work. The samples were stored at 4°C during the workday and at −20°C thereafter. The workers filled out a brief questionnaire concerning their perceived exposure to ETS in the workplace. Nonsmoking employees of our research group gave urine samples as negative controls.

Samples from exposed subjects were shipped frozen to the American Health Foundation, where the initial steps of the analysis were carried out. They were analyzed in five sets of four to five samples each. A negative control urine sample was analyzed with each set. The analyses were completed at the University of Minnesota Cancer Center.

Analysis for NNAL-Gluc. The workup of the urine was carried out essentially as described previously (8), except for the following changes: (*a*) the initial sample size was 50 ml of urine; (*b*) 2 ng of *iso*-NNAL were added as internal standard; and (*c*) β-glucuronidase hydrolysis was performed with 25,000 units of enzyme. For quantitation, GC-TEA analysis was performed with a 30 m × 0.32-mm i.d. DB-1701 column (0.25-μ

Received 8/5/97; revised 12/10/97; accepted 12/18/97.
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[2] To whom requests for reprints should be addressed, at the University of Minnesota Cancer Center, Box 806 Mayo, 420 Delaware Street SE, Minneapolis, MN 55455.
[3] The abbreviations used are: ETS, environmental tobacco smoke; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL-Gluc, [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glucosiduronic acid; GC-TEA, combined gas chromatography-nitrosamine-selective detection; GC-MS/MS, combined gas chromatography-tandem mass spectrometry; *iso*-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; TMS, trimethylsilyl ether; i.d., inside diameter.

Table 1 NNAL-Gluc and cotinine in the urine of hospital workers

Subject/ sample[a]	Sex	Job	Ever smoked? (years since stopping)	NNAL-Gluc		Cotinine		Cotinine × 10^{-3}
				pmol/ml[b]	pmol/mg creatinine	pmol/ml[c]	pmol/mg creatinine	NNAL-Gluc
1-A	M	Cleaner	Yes (10)	0.053	0.038	85	61	1.6
1-B				0.064	0.034	110	61	1.7
1-C				0.051	0.049	110	100	2.1
2-A	M	Cleaner	No	0.11	0.035	200	65	1.8
2-B				0.046	0.034	220	160	4.8
2-C				0.039	0.028	200	140	5.1
3-A	F	Clerk	Yes (10)	0.034	0.036	120	120	3.5
3-B				0.013	0.024	97	190	7.5
3-C				0.055	0.056	160	160	2.9
4-A	F	Clerk	No	0.052	0.047	180	160	3.5
4-B				0.077	0.050	400	260	5.2
4-C				0.088	0.052	430	250	4.9
5-A	M	Doctor	No	NA[d]	NA	NA[e]	NA	
5-B				0.005	0.003	ND[f]	ND	
5-C				NA	NA	ND	ND	
6-A	F	Nurse	No	NA	NA	28	94	
6-B				NA[e]	NA	28	17	
6-C				0.071	0.031	100	45	1.4
7-A	F	Nurse	No	NA	NA	34	65	
7-B				0.029	0.021	NA[e]	NA	
7-C				NA	NA	ND	ND	
8-A	M	Orderly	Yes (5)	0.11	0.046	470	190	4.3
8-B				0.062	0.042	550	370	8.9
8-C				0.064	0.050	400	320	6.3
9-A	M	Orderly	Yes (4)	0.065	0.058	NA[e]	NA	
9-B				0.090	0.062	97	67	1.1
9-C				0.061	0.041	100	68	1.6
Mean ± SD				0.059 ± 0.028 (n = 21)	0.041 ± 0.014 (n = 21)	170 ± 160 (n = 24)	120 ± 100 (n = 24)	3.8 ± 2.3 (n = 18)

[a] Three urine samples were collected from each subject: A, on arrival at work; B, during the workday; and C, on leaving work.
[b] To convert to pg/ml, multiply by 385.
[c] To convert to ng/ml, multiply by 0.176.
[d] NA, not analyzed due to low RIA cotinine, unless noted otherwise.
[e] Sample not analyzed due to technical difficulties.
[f] ND, not detected, <6 pmol/ml (1 ng/ml).

film thickness) from J & W Scientific (Folsom, CA) connected to a 2 m × 0.32-mm i.d. deactivated retention gap. The injection mode was splitless, the flow rate was 2.6 ml/min He, and the injection port temperature was 225°C. The temperature program was as follows: 80°C for 2 min; then 12°C per min to 175°C; and then 2°C per min to 210°C.

An aliquot of each sample was also analyzed by GC-MS/MS to confirm the identity of NNAL-Gluc. GC-MS/MS was performed on a Finnigan TSQ-7000 instrument operated in the positive ion chemical ionization mode using methane as the reagent gas (source pressure, 2500 mtorr; electron energy, 200 V; filament current, 200 μA). The instrument was tuned for maximum sensitivity by opening the resolution to a calibrant peak width of 2 atomic mass units at 10% peak height at the *m/z* regions of interest. Daughter ion scans were performed to monitor the transitions *m/z* 282→162 and *m/z* 282→132, operating Q3 in the selected ion monitoring mode at a scan rate of 0.5 scan/s and using Ar as the collision gas at a cell pressure of 0.75 mtorr. The gas chromatography was equipped with a 30 m × 0.32-mm i.d. DB-1301 column (0.25-μ film thickness) from J & W Scientific connected to a 2 m × 0.32-mm i.d. deactivated retention gap. The injection mode was splitless, the flow rate was 2.6 ml/min He, and the injection port temperature was 260°C. The temperature program was as follows: 80°C for 2 min; then 12°C per min to 155°C; and then 2°C per min to 200°C. The final temperature was held for 15 min.

Analysis for Cotinine and Creatinine. Initially, samples were screened for cotinine by RIA (10). Samples with low cotinine levels were not analyzed for NNAL-Gluc. The cotinine data in Table 1 were obtained by GC-MS, using [CD_3]cotinine as internal standard (11). Urinary creatinine was determined on a Kodak Ektachem 500 clinical chemistry analyzer.

Statistical Methods. Comparison of NNAL-Gluc levels in exposed and control samples was performed using Student's *t* test. Pearson correlations were used to evaluate the relationship between NNAL-Gluc and cotinine levels.

Results

The present GC-TEA method is about 20 times more sensitive than that reported previously for analysis of NNAL and NNAL-Gluc in the urine of ETS-exposed nonsmokers (8). The detection limit is approximately 4 fmol/ml urine, starting with a 50-ml sample. Previously, we used 24-h urine samples (8). The increase in sensitivity is due mainly to the use of capillary gas chromatography. As in the earlier work, *iso*-NNAL was used as internal standard. In this study, we analyzed NNAL-Gluc only, because we were unsure whether our sensitivity would have been sufficient for detection of free urinary NNAL.

NNAL was released from NNAL-Gluc by treatment of the urine with β-glucuronidase. After extractions and HPLC enrichment steps, it was silylated and quantified by GC-TEA. A

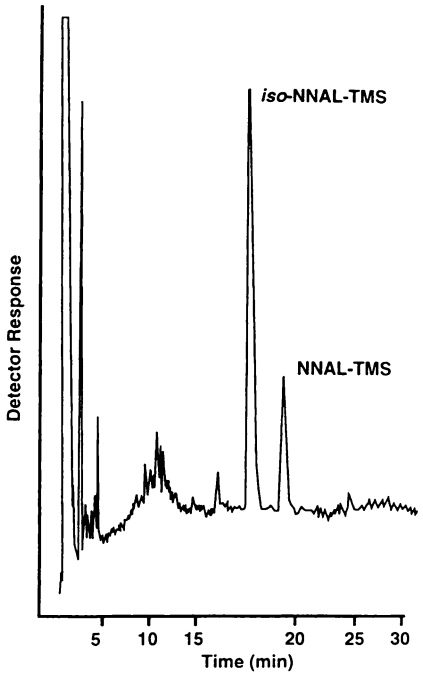

Fig. 1. Representative chromatogram obtained on GC-TEA analysis of NNAL-Gluc in the urine of subject 1-A (Table 1). *iso*-NNAL was the internal standard, detected as its trimethylsilyl derivative, *iso*-NNAL-TMS. NNAL was released from NNAL-Gluc by enzymatic hydrolysis and was detected as NNAL-TMS. The chart speed was changed from 2.5 to 10 mm/min at 18 min to facilitate integration. It was returned to 2.5 mm/min at 21 min.

typical GC-TEA trace is illustrated in Fig. 1. The indicated peaks are *iso*-NNAL-TMS and NNAL-TMS, which are the TMS derivatives of *iso*-NNAL and NNAL.

Aliquots of most samples were also analyzed by GC-MS/MS to confirm the identity of NNAL-TMS. In this analysis, *m/z* 282 [MH^+] of *iso*-NNAL-TMS and NNAL-TMS was allowed to enter the second quadrapole. Collision-induced dissociation gave *m/z* 162 [MH-HOTMS-NO]$^+$ and *m/z* 132 [MH-HOTMS-NO-CH_3NH)]$^+$. The *m/z* 132 fragment predominated in the spectrum of *iso*-NNAL-TMS, whereas the *m/z* 162 fragment was the major one in the spectrum of NNAL-TMS. Representative GC-MS/MS results are illustrated in Fig. 2. The retention times of the peaks corresponding to *m/z* 282→132 and *m/z* 282→162 were the same as those observed on analysis of standard *iso*-NNAL and NNAL, as were the ratios of the peak areas. These results confirm the identity of the NNAL-TMS peaks quantified by GC-TEA.

The results are summarized in Table 1. The mean level of NNAL-Gluc ± SD was 0.059 ± 0.028 pmol/ml urine (23 pg/ml urine); range, 0.005–0.11 pmol/ml urine. The mean level of NNAL-Gluc in the negative control samples was 0.012 ± 0.008 pmol/ml urine (n = 5), which was significantly less than in the subjects exposed to ETS (P = 0.0012). The mean level of cotinine in the exposed subjects was 170 ± 160 pmol/ml urine (30 ng/ml urine), range, not detected to 550 pmol/ml urine. NNAL-Gluc levels were relatively constant in the instances when three measurements were made over the course of a working day, suggesting that a steady-state level had been attained.

Levels of NNAL-Gluc and cotinine, expressed per milliliter of urine, were correlated (r = 0.51; P = 0.029; Fig. 3). No

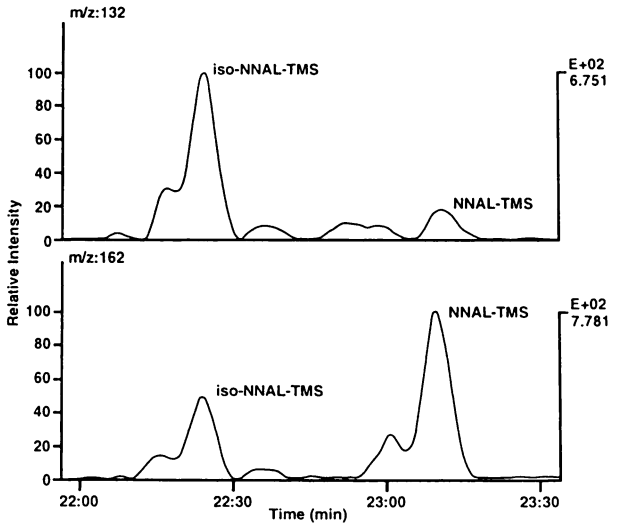

Fig. 2. Chromatogram obtained on GC-MS/MS analysis of NNAL-Gluc in the urine of subject 1-A (Table 1). *iso*-NNAL-TMS is the derivatized internal standard, and NNAL-TMS is the derivatized aglycone of NNAL-Gluc. *Upper panel*, selected ion monitoring of *m/z* 282→132; *lower panel*, *m/z* 282→162. The indicated peaks correspond in retention time and relative areas to standard *iso*-NNAL-TMS and NNAL-TMS

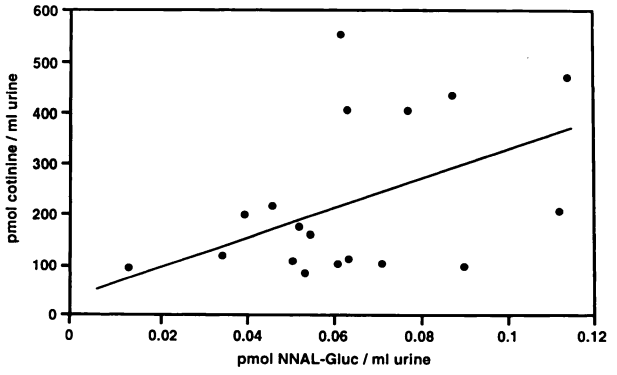

Fig. 3. Relationship between urinary cotinine and NNAL-Gluc in nonsmokers exposed to ETS (r = 0.51; P = 0.029).

correlation was observed when the NNAL-Gluc and cotinine levels were expressed per milligram of creatinine.

Discussion

These results clearly demonstrate the presence of NNAL-Gluc, a metabolite of the pulmonary carcinogen NNK, in the urine of nonsmokers exposed occupationally to ETS. This is the first report of an NNK metabolite in the urine of nonsmokers exposed to ETS in a field study. The results lend additional support to the proposal that ETS exposure causes lung cancer. Our data support regulations, now enforced in most work settings, that prohibit indoor smoking.

The ratio of cotinine:NNAL-Gluc in the present study was 3800, compared to 4600 in our previous study of nonsmokers exposed to ETS (8). In smokers, the ratio of cotinine:NNAL-Gluc was 3600 (12). Levels of NNAL-Gluc and cotinine, expressed per milliliter of urine, were correlated. In

previous studies, we and others have noted a correlation between levels of NNAL plus NNAL-Gluc and cotinine in nonsmokers and smokers (8, 12, 13). Collectively, these data indicate consistency among studies of NNAL-Gluc and cotinine in nonsmokers and smokers and suggest that NNAL-Gluc is an effective biomarker of lung carcinogen uptake, just as cotinine is a useful biomarker of nicotine uptake from ETS (14).

Levels of NNAL-Gluc were 69 times less than in smokers, and levels of cotinine were 84 times less than in smokers (12). These data indicate that carcinogen uptake in nonsmokers exposed to ETS is substantially less than in smokers, which is consistent with the epidemiological data showing lower risk for lung cancer (1–5).

The levels of cotinine determined in this study indicate that ETS exposure of our subjects was similar to that reported in ETS-exposed restaurant personnel (15, 16). In one study of restaurant personnel, the mean cotinine level was 56 ng/ml, compared to 30 ng/ml in our workers (15). Based on the observed correlations between cotinine and NNAL-Gluc levels, one can speculate that NNAL-Gluc levels in exposed restaurant personnel would be similar to those observed here. ETS levels have been divided into lower exposure venues and higher exposure venues (17). Smoking lounges, such as those in the present study, have been placed in the latter category (17). Consequently, the NNAL-Gluc values reported here may be typical of higher exposures to ETS, but this requires further study.

In ongoing work, we have observed that NNAL and NNAL-Gluc are excreted in urine for at least 4 months after smoking cessation. In the present study, four of the subjects were ex-smokers who stopped 4–10 years ago. Their cotinine levels were not different from those of the subjects who never smoked. There can be little doubt that urinary NNAL-Gluc in these subjects results from NNK uptake from ETS rather than persistent excretion from smoking 4–10 years earlier.

Although this was a small study, it demonstrates the feasibility of quantifying NNAL-Gluc in the urine of nonsmokers exposed to ETS. This biomarker will be useful in future studies examining the relationship of ETS exposure to lung cancer in nonsmokers. We are planning larger studies of this type.

Acknowledgments

We thank the participants for providing urine samples and Lyse Lemieux for helping with sample collection, Dominic Pullo and Sharon E. Murphy (University of Minnesota Cancer Center) for performing the GC-MS analyses of cotinine, the American Health Foundation Clinical Biochemistry Facility for carrying out the RIA analyses of cotinine, and Yin Liu (American Health Foundation, Valhalla, NY) for technical assistance.

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Cancer Epidemiol Biomarkers Prev 1998;7:257-260.