Presence of the Carcinogen *N*′-Nitrosonornicotine in the Urine of Some Users of Oral Nicotine Replacement Therapy Products

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Abstract

***N*′-nitrosonornicotine (NNN) is a strong carcinogen present in unburned tobacco and cigarette smoke. We here analyze data obtained in two studies, in which a biomarker of exposure to NNN—the sum of NNN and its pyridine-*N*-glucuronide, called total NNN—was quantified in the urine of people who had stopped smoking and used various nicotine replacement therapy (NRT) products. In 13 of 34 nicotine gum or lozenge users from both studies, total NNN at one or more time points after biochemically confirmed smoking cessation was comparable with, or considerably higher than, the baseline levels. For most of the subjects who used the nicotine patch as a smoking cessation aid, urinary total NNN at all post–quit time points was <37% of their mean baseline levels. These results indicate that endogenous formation of significant amounts of NNN may occur sporadically in some users of oral NRT. Given the carcinogenicity of NNN and the frequent use of nicotine gum as a smoking cessation aid, further studies are needed so that preventive measures can be developed.** [Cancer Res 2009;69(21):8236–40]

Introduction

Nicotine replacement therapy (NRT) products were developed to assist smokers in quitting, and are virtually free of toxicants and carcinogens that are abundant in tobacco and cigarette smoke (1). However, there is a concern about possible endogenous nitrosation of nicotine, directly or via its metabolite nornicotine (2), to form *N*′-nitrosonornicotine (NNN)—a human carcinogen (3) that is believed to be important in the induction by tobacco products of cancers of the esophagus and oral cavity (4). Because endogenous formation of *N*-nitrosamines commonly occurs in humans via the reaction of dietary precursors with nitrosating agents (5), it is biologically plausible that endogenous formation of NNN can occur in users of oral NRT, either in the acidic stomach (5), or via bacteria-mediated nitrosation of the nicotine metabolite nornicotine elsewhere in the body (6) (Fig. 1).

We analyzed a biomarker of exposure to NNN—the sum of NNN and its pyridine-*N*-glucuronide, called total NNN (7)—in the urine of people who had stopped smoking and used nicotine patch, nicotine gum, or nicotine lozenge. The sum of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its *N*- and *O*-glucuronides, called total NNAL, was also analyzed, as this is the commonly measured urinary metabolite of the related nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (8).

Materials and Methods

Subjects and study design. Urine of subjects recruited for two separate studies was analyzed. In the first study, called the Persistence of Biomarkers (POB) study (9), cigarette smokers continued smoking as usual over a 2-wk period during which two baseline 24-h urine samples were collected. After this period, subjects quit smoking and used either nicotine patch or nicotine gum or lozenge as a smoking cessation aid. On days 3, 7, 14, 21, 28, 42, and 56 after quitting, 24-h urine samples were collected and analyzed for total NNN and total NNAL.

The second study is called the Quit Nicotine (QuitNic) study (10). In this study, two baseline measurements were made during a 2-wk period while the recruited subjects smoked *ad libitum*. After this period, in one of the three treatment conditions, subjects stopped smoking and used 4 mg nicotine lozenges for 6 wk. Urine samples were collected at the end of weeks 2 and 6 of treatment, and total NNN and total NNAL were analyzed.

In both studies, the subjects were provided with the chosen or assigned cessation aid. Both studies were approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board: Human Subjects Committee.

Urine analyses. Total NNN was assayed essentially as previously described (11), except that β-glucuronidase was used to convert NNN-*N*-glucuronide to NNN (7), and liquid chromatography-electrospray ionization-tandem mass spectrometry was used for analysis. Urine samples with high total NNN after the quit date were reanalyzed by gas chromatography equipped with a thermal energy analyzer, to verify the identity and the amount of NNN (7). Total NNAL was analyzed as previously described (8). Negative controls (water blanks) were analyzed with each set of urine samples. Anatabine was analyzed as described elsewhere (12). Nitrate and nitrite content was assayed by ion chromatography (13). Creatinine was determined by using Vitros CREA slides.

Carbon monoxide. This was analyzed using the Bedfont Micro Smokerlyzer (Bedfont Scientific Limited). CO level of <6 was used to confirm abstinence.

Statistical analysis. For the POB study, both total NNN and total NNAL were analyzed on the natural log scale. The paired *t* test compared the initial change from baseline to day 3 and the repeated measures ANOVA evaluated the rate of change from day 3 to day 56. The percent of baseline was calculated for each time point and compared between total NNN and total NNAL using the paired *t* test. Due to a high degree of variability in the QuitNic study, we used the Wilcoxon signed-rank test to compare baseline to 2 and 6 wk for total NNN and total NNAL. A *P* value of <0.05 was considered statistically significant.

Results

Total NNN and total NNAL values in individual samples from both studies, as well as anatabine and nitrate and nitrite levels

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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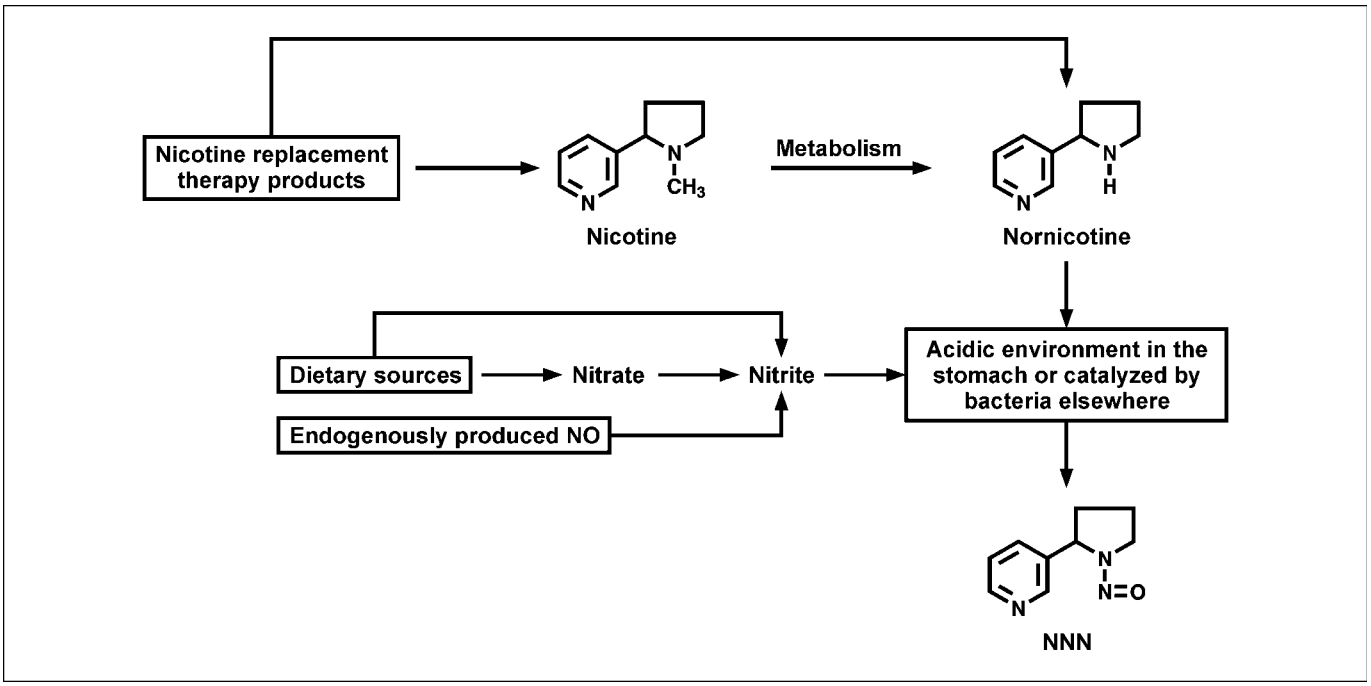

Figure 1. Hypothesized pathways of endogenous NNN formation in oral NRT users (adapted from ref. 15).

in selected samples from the POB study, are listed in Supplementary Tables S1 to S3 of Supporting Information.

POB study. The results of total NNN and total NNAL analyses in nine nicotine patch users and eight oral NRT users are summarized in Fig. 2.

In the nine nicotine patch users, after 3 days of abstinence from smoking, total NNN decreased from 63.1 (±99.8) pmol/24 h (baseline level) to 1.69 (±1.56) pmol/24 h (P = 0.001; Fig. 2*A*). These values for eight oral NRT users were 147 (±236) pmol/24 h and 2,280 (±6,290) pmol/24 h, respectively (P = 0.759; Fig. 2*C*). In nicotine patch users, the overall slight decrease in average urinary total NNN from day 3 to day 56 was not significant (P = 0.159). Among oral NRT users, there was a large variation in urinary total NNN levels from day 3 to day 56; at one or more time points during this period, six of eight subjects had levels of NNN in their urine similar to, or higher than, their baseline levels (Fig. 2*C*). For both nicotine patch and oral NRT users, the average decrease for total NNAL over this period was highly significant (P < 0.001; Fig. 2*B* and *D*).

QuitNic study. The results of total NNN and total NNAL analyses in the urine of 26 QuitNic study subjects are summarized in Table 1. In seven subjects, urinary total NNN at one or more time points following smoking cessation was similar to, or higher than, their mean baseline levels. The decrease in total NNN from baseline to week 2 and from baseline to week 6 in all QuitNic study subjects was not statistically significant (P = 0.938 and P = 0.844, respectively), but it was significant for total NNAL (P = 0.016 and P = 0.031, respectively).

Analysis of anatabine, nitrate, and nitrite. Urine of those POB study subjects, who after smoking cessation had elevated urinary total NNN, was analyzed for anatabine, nitrate, and nitrite (Supplementary Table S3). The levels of anatabine were either extremely low or nondetectable in samples collected after smoking cessation, and there was no overall correlation between urinary nitrite and total NNN in all samples.

Tests for artifactual NNN formation in urine. A set of 10 3-mL urine samples was selected to include both high- and low-total NNN samples from four different subjects. Neither addition of 500 ng nornicotine before overnight hydrolysis with β-glucuronidase, nor incubation of urine with 500 ng nornicotine for 24 hours at room temperature and subsequent hydrolysis with NaOH, had a significant effect on the measured total NNN in these samples.

Discussion

We report occasional significant increases in urinary biomarkers of the carcinogen NNN in some users of nicotine gum or lozenge, compared with baseline smoking levels in the same subjects. We made these observations in the course of analyzing data from two separate studies designed to monitor changes in urinary biomarkers of a number of tobacco carcinogens in people who stopped smoking. Our findings suggest that significant amounts of NNN are formed occasionally in some users of oral NRT products, most likely via endogenous nitrosation of nornicotine that is metabolically formed from nicotine or originally present in NRT products. Given the carcinogenicity of NNN, this presents a possible cancer risk in long-term users.

In 13 of 34 nicotine gum or lozenge users from both studies, total NNN at one or more time points after smoking cessation was comparable with, or considerably higher than, the baseline levels. Significant decreases in urinary total NNAL, exhaled CO, and urinary anatabine in these subjects confirmed their abstinence from tobacco products. NNN intake from NRT products could potentially contribute to the increase in urinary total NNN in our NRT users. Because these studies were not designed to specifically investigate the possible endogenous formation of NNN in users of oral NRT products, we did not analyze NNN in the nicotine gum or nicotine

lozenges that were given to our subjects. However, our previous study showed that NNN is virtually absent in this category of NRT products (1). Another potential contributor to the measured high total NNN levels could be artifactual NNN formation in the urine after its collection or during sample preparation, via nitrosation of nornicotine present in the urine. The lack of increase in total NNN in urine samples incubated with an excess amount of nornicotine, compared with nontreated aliquots from the same urine sample, does not support this hypothesis. Moreover, nitrate and nitrite levels measured in selected urine samples (Supplementary Table S3) did not correlate with total NNN levels in the same samples. Given the design of the studies, we were not able to test urine samples for the presence of bacteria. However, it is unlikely that bacteria-mediated artifactual NNN formation occurred exclusively in the urine of oral NRT users: for most of the subjects who chose to use nicotine patch as a smoking cessation aid, urinary total NNN at all post–quit time points was <37% of their mean baseline levels (Supplementary Table S1; Fig. 2).

Only one patch user showed a sudden large increase in urinary total NNN—17.9 pmol/24 hours at day 28 of nicotine patch use, compared with 4.3 pmol/24 hours at baseline (Supplementary Table S1, subject P7). This increase coincided with an increase in urinary nitrate, suggesting an overall increase in nitrosation potential at this time point. Subjects O2 and O6 (Supplementary Table S1) stood out among oral NRT users. In subject O2, after 7 days of smoking cessation and oral NRT use, urinary total NNN was 700 times higher than baseline. This increase was not accompanied by an increase in either urinary nitrate or nitrite. Subject O6, who at several time points after smoking cessation had ~30 times higher urinary total NNN than at baseline, also had elevated urinary total NNAL at the same time points, whereas anatabine was not

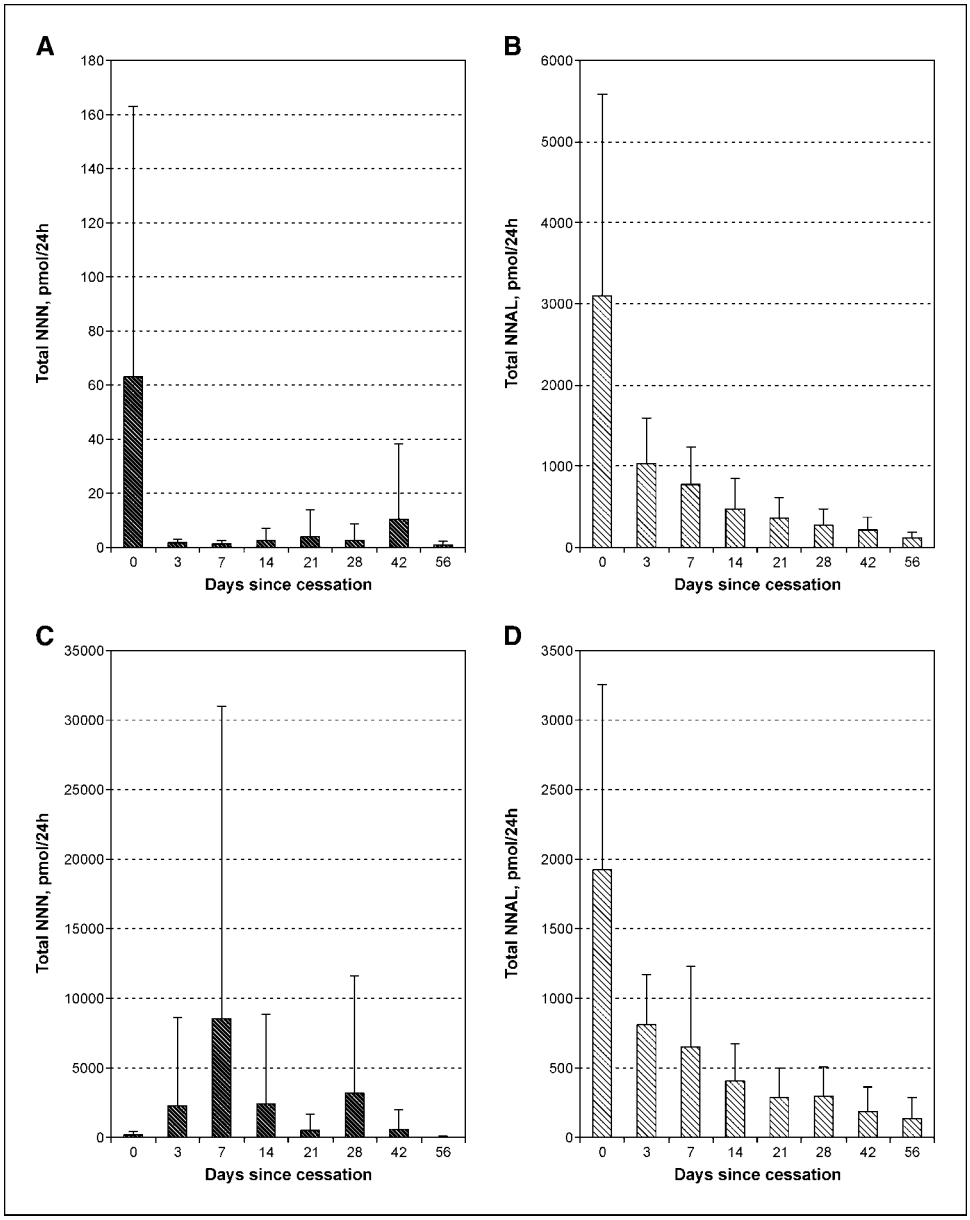

Figure 2. Total NNN and total NNAL in nine nicotine patch users and eight oral NRT users from the POB study: *A,* total NNN in nicotine patch users; *B,* total NNAL in nicotine patch users; *C,* total NNN in oral NRT users; *D,* total NNAL in oral NRT users. Each baseline value represents mean of two analyses. *Columns,* mean; *bars,* SD.

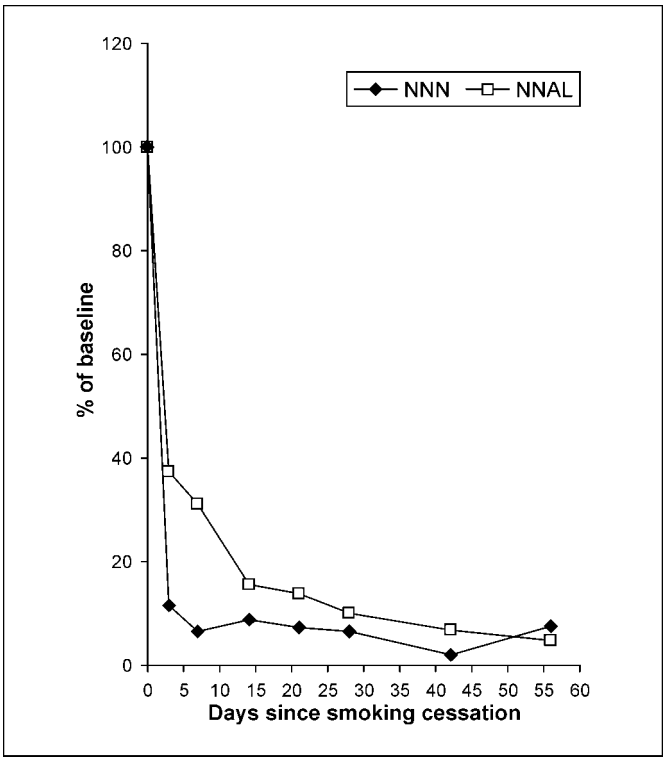

Figure 3. Total NNN and total NNAL as % of mean baseline levels in eight POB subjects (seven used nicotine patch and one used oral NRT) who did not have increased urinary NNN excretion during NRT use.

detected. This is the first indication that 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone also can be formed endogenously in humans. The sporadic nature of high total NNN concentrations observed here most likely results from the multiple factors that influence endogenous nitrosation including different dietary catalysts and inhibitors of nitrosation, timing of their consumption, and infections. There are also indications that the extent of endogenous nitrosation in humans might be dependent on variations in the atmospheric concentrations of NO_2 (14).

An interesting observation is that the QuitNic study participants with a sharp decline in urinary total NNN after smoking cessation also had lower average baseline total NNN levels when compared with the subjects whose urinary total NNN levels during nicotine lozenge use indicate endogenous nitrosation. These results suggest that some smokers, in addition to their exposure to NNN from cigarette smoke, probably form NNN endogenously, depending on host factors and/or dietary habits.

In subjects who did not have increases in urinary total NNN after smoking cessation, the levels of this biomarker dropped to 11% of the baseline value 3 days after quitting ($P = 0.015$; Fig. 3). It took an average of 4 weeks for total NNAL to decrease to the same 11% of the baseline value. These results support the idea of NNAL retention in the body, followed by slow release and reconversion to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, which, in turn, is again metabolized to NNAL. The decrease in urinary NNAL upon NRT use observed for most of the subjects in this study is consistent with previous studies (8, 15).

Major limitations of this investigation include the fact that neither of the two studies was designed to investigate endogenous formation of NNN in NRT users, and the lack of information on NNN in the NRT products. We also lacked of control group in which subjects did not use any NRT product after smoking cessation.

Despite these limitations, the presence of significant amounts of NNN in the urine of some oral NRT users is an alarming sign, especially in view of the reported increased use of NRT products and their over-the-counter availability (16, 17). In attempts to quit smoking, nicotine gum is one of the most frequently used NRT products (18), and some former smokers use these products for prolonged periods of time (19). These people, if susceptible to endogenous formation of NNN, can be continuously exposed to relatively high levels of this strong carcinogen and may eventually develop cancer.

In summary, we observed that significant amounts of NNN are excreted occasionally in some users of oral NRT, endogenous formation of this carcinogen being the most likely source. This presents a possible risk of cancer in long term users. Although use of NRT products significantly decreases exposure to a wide range of carcinogens and toxicants present in cigarette smoke and should be recommended as an aid for cessation, additional studies are urgently needed to understand the factors affecting endogenous NNN formation, and to develop preventive measures. The feasibility of preventing endogenous NNN formation in oral NRT users is

Table 1. Total NNN and total NNAL in the urine of QuitNic study participants

Subjects	No	pmol/mg creatinine					
		Total NNN			Total NNAL		
		Study week			Study week		
		Baseline*	2	6	Baseline	2	6
All	26	0.140 (±0.194)	0.080 (±0.142)	0.084 (±0.210)	1.20 (±0.688)	0.264 (±0.166)	0.154 (±0.112)
Subjects with decreasing total NNN	19	0.082 (±0.066)	0.022 (±0.027)	0.021 (±0.028)	1.29 (±0.763)	0.294 (±0.178)	0.163 (±0.124)
Subjects with elevated total NNN†	7	0.239 (±0.335)	0.232 (±0.210)	0.275 (±0.373)	0.81 (±0.433)	0.153 (±0.096)	0.109 (±0.065)

*Two baseline urine samples were collected and analyzed; each value represents mean of the two analyses.
†Levels at week 2 or week 6 were similar to or higher than baseline levels.

supported by the sporadic nature of the increases in urinary total NNN, the significant reduction in urinary total NNN in some oral NRT users after smoking cessation, and the overall knowledge of the major factors affecting endogenous nitrosation in humans.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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