Evidence for endogenous formation of tobacco-specific nitrosamines in rats treated with tobacco alkaloids and sodium nitrite

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Carcinogenic tobacco-specific nitrosamines are present in tobacco products and are believed to play a significant role in human cancers associated with tobacco use. Additional amounts of tobacco-specific nitrosamines could be formed endogenously. We tested this hypothesis by treating rats with nicotine and sodium nitrite and analyzing their urine. Initially, we treated groups of rats with (S)-nicotine (60 µmol/kg) and NaN₂O₃ (180 µmol/kg), (S)-nicotine alone, NaNO₂ alone or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 12 nmol/kg) by gavage twice daily for 4 days. We collected urine and analyzed for two metabolites of NNK; 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronide. We did not detect these metabolites in the urine of rats treated with nicotine alone or nicotine plus NaNO₂, indicating that endogenous conversion of nicotine to NNK did not occur. However, the urine did contain N'-nitrosonornicotine (NNN), N'-nitrosoanabasine (NAB) and N'-nitrosoanatabine (NAT). Analysis of the (S)-nicotine used in this experiment demonstrated that it contained trace amounts of nornicotine, anabasine and anatabine. In a second experiment, we used an identical protocol to compare the endogenous nitrosation of this (S)-nicotine with that of synthetic (R,S)-nicotine, which did not contain detectable amounts of nornicotine, anabasine or anatabine. NNN (0.53 × 10⁻³ % of nicotine dose), NAB (0.68 %) and NAT (2.1 %) were detected in the urine of the rats treated with the (S)-nicotine and NaNO₂. NNN (0.47 × 10⁻³ % of dose), but not NAB or NAT, was present in the urine of the rats treated with synthetic (R,S)-nicotine and NaNO₂. NNN probably formed via nitrosation of metabolically formed nornicotine. These results demonstrate for the first time that endogenous formation of tobacco-specific nitrosamines occurs in rats treated with tobacco alkaloids and NaNO₂. The potential significance of the results with respect to nitrosamine formation in people who use tobacco products or nicotine replacement therapy is discussed.

Introduction

The presence of carcinogenic tobacco-specific nitrosamines in tobacco products has been conclusively demonstrated (1–4). Seven tobacco-specific nitrosamines have been identified and quantified in tobacco products. Their structures are shown in Figure 1. Among these, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*), its metabolic reduction product 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and N'-nitrosonornicotine (NNN) are the most carcinogenic in laboratory animals and are believed to play a significant role in cancers of the lung, esophagus, oral cavity and pancreas caused by use of tobacco products (4). Human exposure to NNK, NNAL and NNN occurs upon use of tobacco products, including chewing tobacco, oral snuff and cigarettes. An important question is whether additional amounts of tobacco-specific nitrosamines are formed endogenously in people who use tobacco products.

The formation of nitrosamines occurs readily in laboratory animals upon administration of nitrite and secondary amines (5). Human exposure to nitrite occurs through the diet, via reduction of dietary nitrate, and from endogenously produced nitric oxide (6,7). Extensive studies have demonstrated that nitrosamine formation occurs in humans (8). Thus, nitrosoproline and related sulfur-containing nitrosamines can be quantified in human urine. Their levels increase upon ingestion of nitrate and proline and are reduced by nitrosation inhibitors such as ascorbic acid. Endogenous formation of nitrosoproline has been associated with a number of exposure scenarios linked to cancer risk, including cigarette smoking. It has been suggested that tobacco-specific nitrosamines could be formed endogenously from the tertiary amine nicotine, but no data have yet been presented in support of this hypothesis (1,2,4).

Nicotine can be nitrosated under mild conditions to produce NNK, NNN and 4-(methylnitrosamino)-4-(3-pyridyl)butanal (NNA) (9). However, the yields are low and the rate of...
nitrosation is markedly slower than that of the related secondary amines nornicotine or anabasine (10,11). Nitrosation of nicotine was performed in simulated saliva or gastric juice (12). One of the products of endogenous nitrosation could be 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC), formed by metabolism of NNA. This nitrosamine could also be produced by nitrosation of cotinine (13). Since iso-NNAC is excreted largely unchanged in the urine and feces of rats, it is a potential monitor of endogenous nitrosation of nicotine (13,14). However, a study of people who ingested nicotine or cotinine, with or without nitrate supplementation, provided no conclusive evidence that endogenous nitrosation had occurred (14).

One way to search for endogenous nitrosation of nicotine is to analyze for metabolites of NNK, NNN or NNA. NNK is metabolized to NNAL and its glucuronide, [4-(methyl-nitrosamino)-1-[3-pyridyl]-but-1-yl]-β-D-glucosiduronic acid (NNAL-Gluc)], in rats (15). NNAL and NNAL-Gluc are excreted in the urine. Previously, we developed a sensitive method to quantify NNAL and NNAL-Gluc in urine (16). This method was used in the present study to test the hypothesis that NNK could be formed endogenously in rats treated with nicotine and nitrite.

### Materials and methods

**Chemicals**

(S)-Nicotine was a gift from K.D.Brunnemann (American Health Foundation). (R,S)-Nicotine was synthesized by NaBH₄ reduction of myosmine followed by reaction with CH₃I (17). Anabasine, anatabine, nornicotine, myosmine, cotinine, NNK, NNN, Nₐ-N-nitrosoanabasine (NAB) and Nₐ-N-nitrosoanatamine (NAT) were synthesized as described (17–23).

**Animal experiments**

Male F344/CrlBR rats were obtained from Charles River Breeding Laboratories (Kingston, NY and Raleigh, NC). They were maintained on tap water and NIH-07 diet under standard conditions (24). In Experiment 1a, the mean weight of the rats (± SD) was 292 ± 42 g. There were four groups: (1) (S)-nicotine (60 µmol/kg body wt) plus NaNO₂ (180 µmol/kg body wt), five rats; (2) (S)-nicotine (60 µmol/kg body wt), three rats; (3) NaNO₂ (180 µmol/kg body wt), three rats; (4) NNK (12 µmol/kg body wt), three rats. Nicotine was dissolved in 0.17 M citric acid, pH 3. NaNO₂ was dissolved in distilled H₂O. NNK was dissolved in 0.17 M citric acid. All solutions (0.5 ml) were administered by gavage, twice daily for 4 days. In group 1, NaNO₂ was administered immediately after each dose of nicotine. The rats were housed in standard metabolism cages during the experiment and urine was collected continuously until 24 h after the last gavage. The collection vessel was cooled with dry ice/acetone and contained 0.5 ml 20% (w/v) ammonium sulfamate in 3.6 N H₂SO₄. The combined 4 day urine samples from each rat were analyzed. In Experiment 1b, the protocol employed for group 1 was repeated with five rats, mean weight 222 ± 4.8 g.

In Experiment 2, there were two groups of male F344/CrlBR rats, mean weights 215 ± 1.8 g. Group 1, three rats, was treated with (S)-nicotine plus NaNO₂ at the same doses as in Experiment 1, while group 2, three rats, was treated with (R,S)-nicotine plus NaNO₂ as in group 1. Experiment 2 was carried out for 5 days instead of 4. Urine on days 1–4 was collected as described above, except that the collection funnel was washed every 4 h and 5 min periods respectively. NA T and NAB eluted in fractions 6 and 7Male F344/CrlBR rats were obtained from Charles...
and (C) are due to the internal standard [5-3H]NNAL. See text for further details.

1 ml/min He and splitless injection was employed. Retention times of NNN, NA-T, and NAB were 23.17, 23.16, and 23.65 min respectively. Percentage recoveries of NNN, NA-T, and NAB were ~60–70% using this method. The data reported in Table I are isolated amounts, uncorrected for recovery.

Nicotine patch. The patches were purchased in pharmacies. They were rolled into 50 ml centrifuge tubes protected from light. The tubes were flushed with nitrogen for 30 s, then 4 ml degassed H2O was added. The mixture was shaken for 1 h. The aqueous extract was transferred to a 15 ml centrifuge tube and 1 g NaCl was added. The solution was flushed with N2 and 4 ml degassed CH2Cl2 was added. After shaking and vortexing, the aqueous layer was extracted again with CH2Cl2 and the extracts were combined and dried with Na2SO4. The extracts were concentrated to dryness, redissolved in 0.5 ml CH3CN and analyzed by GC-FID on a Hewlett-Packard Model 5830A instrument equipped with the DB-1701 column described above. The column was attached to a 2 m ×0.32 mm i.d. deactivated silica retention gap (a column inlet with a deactivated internal surface free of stationary phase). The following temperature program was used: 50°C for 2 min, then 2°C/min for 13 min to 128°C, then 2°C/min for 15 min to 158°C, then 6°C/min to 200°C, then maintained at 200°C for 10 min. The injection port temperature was 220°C and the carrier gas was He at 1 ml/min. Splitless injection was used. Typical retention times in minutes were as follows: nicotine, 18.0; nornicotine, 21.5; myosmine, 21.7; anabasine, 23.3; anatabine, 25.3; cotinine, 36.5.

Mass spectrometry (MS) analyses. Analyses were carried out on a Finnigan TSQ-700 instrument (San Jose, CA) interfaced to a Varian Model 3400 GC. Capillary GC conditions were the same as described above. Analyses of fractions with peaks corresponding in retention time to NNN, NA-T, and NAB were carried out by GC-tandem MS (GC-MS/MS) in the positive CI mode. M+1 ions were allowed to enter quadrupole 2 (Q2). The collision gas was argon, 0.88 torr. Q3 was scanned from m/z 100 to 183. Daughter ions were compared with those observed in standards. Analyses of the nicotine patches were performed by GC-MS in the positive CI mode, with methane as reagent gas. GC conditions were as described above.

**Results**

**Analysis of rat urine**

We designed the protocol in Experiment 1 to test the hypothesis that NNK could form endogenously in rats treated with nicotine and NaNO2. The end-point was detection of NNAL and NNAL-Gluc in the urine. The results are illustrated in Figure 2A–C. NNAL and NNAL-Gluc were detected in the urine of the rats treated with NNK. A GC-TEA trace of the conjugated NNAL fraction is shown in Figure 2A. The peaks marked NG and iso-NNAL-TMS are the injection and silylation standard respectively. A peak corresponding to NNAL-TMS was clearly evident. We obtained similar traces upon analysis of the free NNAL fraction. The mean total amounts of NNAL and NNAL-Gluc in the 4 day urine of the NNK-treated rats were 280 and 290 pmol respectively, corresponding to ~1% each of the NNK dose. In the rats treated with nicotine alone, a small peak corresponding to NNAL-TMS was evident in both the free and conjugate fractions. This is illustrated for the free NNAL fraction in Figure 2B. This peak could be accounted for entirely by the [3H]NNAL internal standard. None of the rats treated with nicotine had NNAL or NNAL-Gluc in their urine, with a detection limit of ~0.4 pmol. In the rats treated with NNK, NNAL and NNAL-Gluc were detected in the urine of these rats.

**Endogenous formation of tobacco-specific nitrosamines**

While NNN is one of the expected products of nicotine metabolism, various other nitrosamines were detected in the urine of rats treated with NNK and nicotine. Myosmine (0.35%) and cotinine (0.07%) were also present, but suggested the possible presence of other nitrosamines. Therefore, in Experiment 2, we compared nitrosation of nicotine with that of synthetic (R)-nicotine which did not contain detectable amounts of nornicotine, anabasine or anatabine (<0.0002%). The results are summarized in Table I. We found NAB (0.68% of nicotine dose) and NAT (2.1%) only in the urine of rats treated with (S)-nicotine which contained anabasine and anatabine. NNK, however, was pro-
duced in similar amounts both from (S)-nicotine which contained nornicotine \( (0.53 \times 10^{-3}\%) \) and from (R,S)-nicotine which did not contain detectable amounts of nornicotine \( (0.47 \times 10^{-3}\%) \). GC-MS/MS confirmed the identity of NNN produced from (R,S)-nicotine.

We investigated the potential for artefactual formation of nitrosamines during collection of the urine in Experiment 2. We collected urine separately for an additional day beyond the standard 4 day collection period. On the additional day, we did not add ammonium sulfamate to the collection vessel, did not cool the vessel and did not rinse the collection funnel. Analysis of this urine demonstrated that the nitrosamine levels were ~20% higher than on each of the first 4 days, indicating that artefact formation was minimal.

**Analysis of nicotine transdermal systems**

The above results demonstrated that even trace amounts of the secondary amines anabasine and anatabine were readily nitrosated. Therefore, we carried out qualitative analyses of the four leading brands of nicotine transdermal systems. These products each contained 20 mg nicotine. We analyzed for nornicotine, anabasine and anatabine. Three of the products contained detectable levels of these secondary amines, as confirmed by GC-MS. The amounts appear to be low: nornicotine \( (0.0005–0.004\% \text{ of nicotine}) \); anabasine \( (0.002–0.012\%) \); anatabine \( (0.01\%) \). Thus, a user of a 20 mg nicotine patch could be simultaneously exposed to ~0.3 \( \mu \text{g} \) nornicotine, 2.4 \( \mu \text{g} \) anabasine and 2.0 \( \mu \text{g} \) anatabine.

**Discussion**

The results provide the first evidence for endogenous formation of tobacco-specific nitrosamines in rats. Among these, NNN has considerable carcinogenic activity, producing tumors of the esophagus and nasal cavity in rats, respiratory tract tumors in mice and hamsters and tumors of the nasal cavity in mink \((25–34)\). NNN is extensively metabolized in the rat by \( \alpha \)-hydroxylation, leading to the formation of DNA adducts \((35–37)\). Only 3–5% of NNN is excreted unchanged in the urine of rats after doses of 3–300 mg/kg are given; no data are available at lower doses \((36)\). Therefore, the amount of NNN observed in the experiments described in Table I represents a minimum; the actual amount formed *in vivo* will be at least 20 times higher.

NNN formation could have occurred by direct reaction of nicotine with \( \text{NaNO}_2 \) in the stomach or by nitrosation of nornicotine that was produced metabolically from nicotine. The latter seems more likely. The reaction of nicotine with \( \text{NaNO}_2 \) is slow. It occurs in low yield and produces NNA and ine could also be concentrated in saliva \((49,50)\). Nitrite occurs in substantial quantities in tobacco and tobacco smoke \((43,44)\). Typical levels of anabasine, anatabine and nornicotine in tobacco are 0.1, 0.5 and 0.6 mg/g tobacco, while in tobacco smoke the corresponding amounts are 3–12, 4–14 and 27–88 \( \mu \text{g}/\text{cigarette} \).

The alkaloids of tobacco and tobacco smoke are one reactant in the nitrosation reaction; the other is the nitrosating species. In our experiments, conditions for nitrosation were optimized, but human exposure to nitrosating agents also occurs. One route of human exposure to nitrite is through the diet \((6)\). Reduction of dietary nitrate is another major source of nitrite \((6)\). Nitrite exposure in the upper gastrointestinal tract from these dietary sources has been estimated as 4 mg/day in the USA \((6)\). Salivary nitrite concentrations in healthy individuals are 6–10 mg/l, but can increase to 500 mg/l after ingestion of nitrate \((45)\). Endogenous formation of nitrosating agents occurs as a result of the nitric oxide synthase-mediated production of nitric oxide from arginine during infection and inflammation \((7,46)\). Moreover, tobacco smoke itself is a rich source of nitrogen oxides and several studies have demonstrated that urinary levels of nitrosoprine and other nitrosamino acids are ~2-fold greater in smokers than in non-smokers \((8)\). Nornicotine, anabasine and probably anatabine nitrosate more readily than proline \((5,11)\) and consequently the endogenous formation of NNN, NAB and NAT from the corresponding secondary amines is likely in smokers and may contribute significantly to their body burden of nitrosamines.

Nicotine replacement therapy is now widely used in smoking cessation programs and nicotine is being considered as a drug for other conditions \((47)\). Nicotine gum and nicotine transdermal systems (patches) are popular nicotine replacement systems. The four major brands of nicotine patches available in the USA were qualitatively analyzed for anabasine, anatabine and nornicotine. The results indicated that levels of these secondary alkaloids were low. However, nitrosation of nicotine to NNN via metabolically formed nornicotine could occur. Subjects using the nicotine patch concentrate nicotine in their saliva \((48)\). Humans, like rats, metabolize nicotine to nornicotine \((0.4–2.7\% \text{ of dose})\) and it is possible that nornicot ine could be stored in saliva \((49,50)\). Nitrile occurs in saliva. When saliva is swallowed, the stomach provides a favorable \( \text{pH} \) for nitrosation \((5,11)\). Nicotine and nornicotine are protonated in the stomach and poorly absorbed \((51)\). Therefore, conditions for nitrosation of metabolically formed nornicotine might be favorable in some nicotine patch users. This could be investigated by analyzing their urine for NNN and its metabolites.

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