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Brief Report

Nornicotine Nitrosation in Saliva and Its Relation to Endogenous Synthesis of *N*′-Nitrosonornicotine in Humans

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Abstract

Introduction: We recently reported that certain amounts of the carcinogen *N*′-nitrosonornicotine (NNN) can be formed endogenously from nicotine and/or nornicotine in some users of oral nicotine replacement therapy products. Although the acidic environment of the stomach creates the most favorable conditions for nitrosation, this reaction could also occur in the oral cavity in the presence of bacteria that catalyze nitrosation at neutral pH.

Methods: To test the hypothesis that endogenous formation of NNN could occur in the oral cavity, we investigated nitrosation of nicotine and nornicotine in human saliva. To specifically identify NNN as derived from precursors added to saliva, we incubated saliva samples with [pyridine-D_4]nicotine and [pyridine-D_4]nornicotine, with and without the addition of nitrite, and subsequently analyzed [pyridine-D_4]NNN by liquid chromatography–tandem mass spectrometry.

Results: Consistent with kinetic studies on nicotine and nornicotine nitrosation, incubation of saliva with [pyridine-D_4]nornicotine alone produced detectable amounts of [pyridine-D_4]NNN, whereas only traces of [pyridine-D_4]NNN were found in samples incubated with [pyridine-D_4]nicotine and sodium nitrite. Incubation of saliva samples from 10 nonsmoking volunteers with [pyridine-D_4]nornicotine resulted in the formation of [pyridine-D_4]NNN in 8 samples, with yields ranging from 0.003% to 0.051% of the added alkaloid.

Conclusion: Our results demonstrate that NNN can be formed from nornicotine in human saliva without deliberate addition of any other substance. Therefore, nornicotine, as present in tobacco or in nicotine replacement products, is a carcinogen precursor.

Introduction

Tobacco use causes about one third of all cancer deaths in the United States and is the leading preventable cause of death among Americans (United States Department of Health and Human Services, 2004). The tobacco-specific nitrosamines *N*′-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are among the most abundant and powerful carcinogens present in unburned tobacco and in smoke. Both NNN and NNK are formed from tobacco alkaloids during tobacco processing; therefore, human exposure to these carcinogens is believed to occur exclusively upon contact with tobacco products. However, we recently reported occasional significant increases in urinary NNN biomarkers in some users of oral nicotine replacement therapy (NRT) products such as nicotine gum or lozenge, compared with baseline smoking levels in the same subjects (Stepanov, Carmella, Briggs, et al., 2009; Stepanov, Carmella, Han, et al., 2009). NNN is believed to play an important role in the induction by tobacco products of cancers of the esophagus and oral cavity (Hecht, 1998), and along with NNK is classified by the International Agency for Research on Cancer as carcinogenic to humans (International Agency for Research on Cancer, 2007). We hypothesized that the observed occasional increases in urinary biomarkers of NNN in some NRT users are due to endogenous nitrosation of nicotine and/or nornicotine, the latter being metabolically formed from nicotine or originally present in NRT products. Furthermore, previous data suggest that endogenous formation of NNN might occur in some smokers (Stepanov, Carmella, Briggs, et al., 2009). This could contribute to the large interindividual variation in levels of urinary NNN biomarkers among smokers and to the remarkably strong association between the levels of urinary NNN biomarkers and risk of esophageal cancer in smokers (Yuan et al., 2011).

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In rats, treatment with nicotine or nornicotine and sodium nitrite resulted in endogenous formation of NNN (Carmella, Borukhova, Desai, & Hecht, 1997; Porubin, Hecht, Li, Gonta, & Stepanov, 2007). In humans, endogenous formation of *N*-nitrosamines occurs through the reaction of dietary precursors with nitrosating agents supplied by diet (Bartsch, Ohshima, Pignatelli, & Calmels, 1989; Marletta, 1988; Mirvish, 1995; Shepard, Schlatter, & Lutz, 1987). Saliva of oral NRT users and smokers contain nicotine and potentially nornicotine (Rose, Levin, & Benowitz, 1993), as well as nitrite (Granli, Dahl, Brodin, & Bockman, 1989; Marletta, 1988). While the acidic environment of the stomach creates the most favorable conditions for NNN synthesis from the precursors delivered with the swallowed saliva (Mirvish, 1975), this reaction can also occur in the oral cavity in the presence of bacteria that catalyze nitrosation at neutral pH (Jiebarth, Spiegelhalder, & Bartsch, 1997). Thus, some studies indicated that additional amounts of NNN could be formed in saliva of smokeless tobacco users (Hoffmann & Adams, 1981).

To test the hypothesis that endogenous formation of NNN can occur in the oral cavity of NRT or tobacco users, we investigated nitrosation of nicotine and nornicotine in human saliva. To specifically identify NNN as derived from the precursors added to saliva, we used [pyridine-D_4]nicotine and [pyridine-D_4]nornicotine and subsequently analyzed [pyridine-D_4]NNN by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Chemicals

[Pyridine-D_4]nicotine, [pyridine-D_4]nornicotine, and [pyridine-D_4]*N*′-nitrosonornicotine ([pyridine-D_4]NNN) were obtained from Toronto Research Chemicals (Toronto, Ontario, Canada), and [2,2′,3,4,5,6-^{13}C]NNN ([$^{13}C_6$]NNN) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA).

Subjects

Nonsmoking volunteers were recruited at the Masonic Cancer Center, University of Minnesota, and were asked to collect 2–5 ml of their saliva via expectoration into sterile polypropylene tubes. All subjects were Caucasian, between 23 and 46 years old, and 6 out of 10 were male. Collection of samples was approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board: Human Subjects Committee.

Saliva Incubation and [Pyridine-D_4] NNN Analysis

Freshly collected saliva was placed on ice and treated within 1 h of collection. As an initial test of nitrosation in saliva, 1 ml aliquots of pooled saliva from three nonsmokers were incubated for 30 min at 37 °C with either [pyridine-D_4]nicotine or [pyridine-D_4]nornicotine, with and without addition of sodium nitrite. The reaction was stopped by placing samples on ice and adding 20 µl 10 N NaOH (Mirvish, Wallcave, Eagen, & Shubik, 1972; Rao & McLennon, 1977). After addition of [$^{13}C_6$]NNN (internal standard), samples were promptly loaded on ChemElut cartridges and eluted with methylene chloride. This was followed by purification on Oasis MCX cartridges and BondElut cartridges as previously described for NNN analysis in urine (Stepanov, Carmella, Briggs, et al., 2009). The purified samples were analyzed for [pyridine-D_4]NNN by LC-MS/MS as previously described (Stepanov & Hecht, 2008), with selected reaction monitoring for *m/z* 182 → *m/z* 152 for [pyridine-D_4]NNN and *m/z* 184 → *m/z* 154 for [$^{13}C_6$]NNN.

Analysis of Nornicotine in Nicotine Gum and Lozenge

Nornicotine content in nicotine gum (Nicorette, 4 mg nicotine) and lozenges (Commit, 2 and 4 mg nicotine) was determined by gas chromatography–mass spectrometry as previously described (Stepanov, Jensen, Hatsukami, & Hecht, 2008).

Results

Optimization of Sample Purification

Various procedures for the postincubation sample purification were tested. Limiting sample purification to a single-step extraction with methylene chloride and concentration of the organic extract to dryness under a stream of N_2 led to ion suppression—a phenomenon that affects analyte ionization causing a loss in response by the mass analyzer—and artifactual formation of [pyridine-D_4]NNN. Ultimately, we subjected samples to the procedure described in the Materials and Methods section. No significant ion suppression was observed with this method. Because the procedure includes acidic conditions during MCX purification, which is favorable for nitrosation reactions, we conducted an additional experiment to test for potential artifactual formation of [pyridine-D_4]NNN during sample processing. A saliva sample was split into two aliquots, and [pyridine-D_4]nornicotine was added to one of the samples (aliquot A) prior to its incubation as described and to the other one (aliquot B) after its elution from ChemElut cartridges, prior to drying and acidification for the MCX step. Only a trace amount of [pyridine-D_4]NNN was detected in aliquot B, comprising 0.6% of the [pyridine-D_4]NNN yield in aliquot A.

Saliva Incubation with [Pyridine-D_4] Nicotine and [Pyridine-D_4]Nornicotine

Typical LC-MS/MS chromatograms obtained upon analysis of saliva incubated under various conditions are presented in Figure 1. [Pyridine-D_4]NNN was not detected in the blank saliva aliquot that was used as a negative control (Figure 1A). Incubation of saliva with 50 ng (0.33 nmol) of [pyridine-D_4]nornicotine alone produced 38 pg (0.21 pmol) [pyridine-D_4]NNN or 0.06% yield. Increasing the amount of [pyridine-D_4]nornicotine to 200 ng and adding 0.7 mg of sodium nitrite produced 5.5 ng [pyridine-D_4]NNN, a nearly 40-fold increase in yield. Only traces of [pyridine-D_4]NNN were detected in samples incubated with [pyridine-D_4]nicotine.

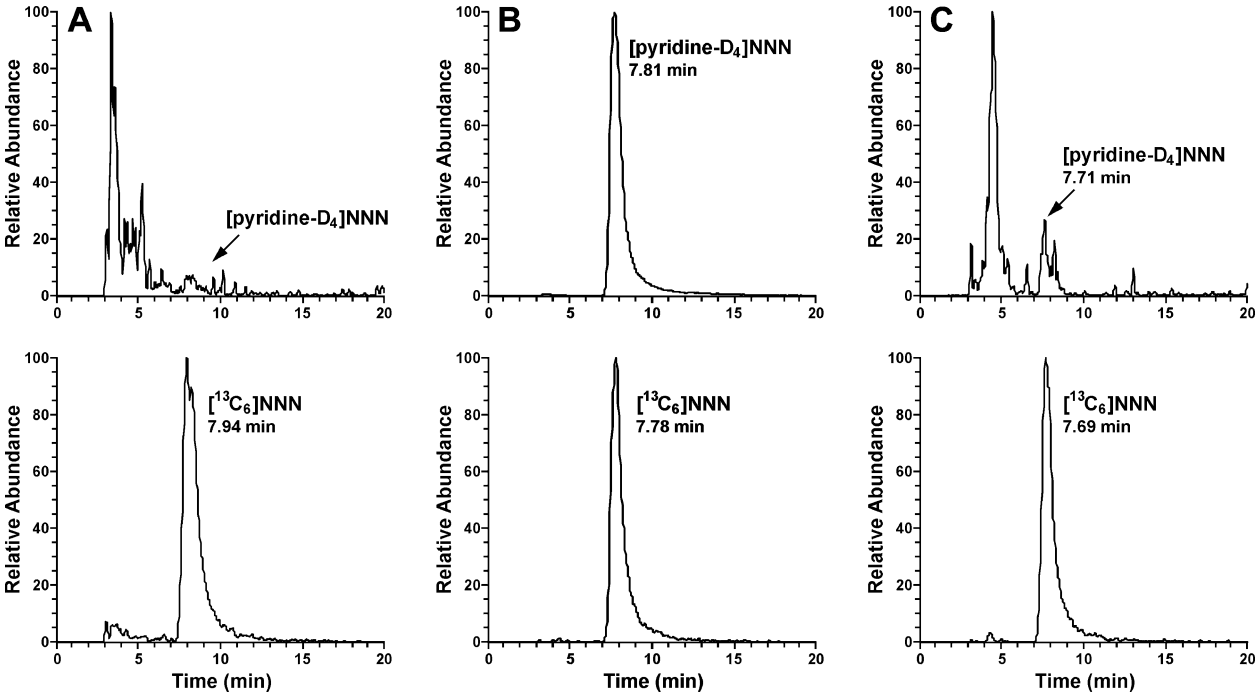

Figure 1. Examples of liquid chromatography–tandem mass spectrometry chromatograms obtained upon analysis of saliva incubated under various conditions: (A) Blank saliva (negative control), (B) saliva incubated with 50 ng [pyridine-D_4]nornicotine, and (C) saliva incubated with 5 μg [pyridine-D_4]nicotine and 0.7 mg $NaNO_2$.

[Pyridine-D_4]NNN Formation in Saliva of Nonsmoking Volunteers

Individual saliva samples from 10 nonsmoking volunteers were incubated with deuterium-labeled nicotine and nornicotine and analyzed for [pyridine-D_4]NNN. The results are summarized in Table 1. Formation of [pyridine-D_4]NNN was observed in 8 samples treated with [pyridine-D_4]nornicotine, yields ranging from 0.003% to 0.051% of the added alkaloid.

Nornicotine Analysis in Nicotine Lozenge and Gum

Commit lozenge (2 mg nicotine) contained 4.4 μg nornicotine/piece; Commit lozenge (4 mg nicotine) contained 5.5 μg nornicotine/piece; and Nicorette gum (4 mg nicotine) contained 9.5 μg nornicotine/piece. The amount of nornicotine averaged 0.2% of nicotine content in these products.

Table 1. Formation of [Pyridine-D_4]NNN Upon Incubation of Nonsmokers' Saliva With [Pyridine-D_4]nornicotine[a]

Subject	[pyridine-D_4]NNN formed, pg	% yield from [pyridine-D_4] nornicotine
1	3.20	0.005
2	LOD[b]	N/A[c]
3	4.29	0.007
4	LOD	N/A
5	7.25	0.012
6	30.26	0.051
7	2.25	0.004
8	2.08	0.003
9	6.75	0.011
10	7.85	0.013

[a]Saliva samples from individual nonsmokers were incubated with 50 ng (0.33 nmol) [pyridine-D_4]nornicotine for 30 min at 37°C.

[b]LOD, below the limit of detection (0.001 pmol [pyridine-D_4]NNN).

[c]N/A = not applicable.

Discussion

This study demonstrates for the first time that the minor tobacco alkaloid nornicotine can be easily nitrosated in human saliva to form NNN, a potent carcinogenic nitrosamine. Nornicotine is present in tobacco and cigarette smoke, and, as demonstrated here, in oral NRT products such as nicotine gum and lozenge. Nitrate is present in human saliva and is converted by oral microflora to nitrite. The findings of this study support our previous observation that NNN can be formed endogenously in users of oral NRT products and potentially in smokers and smokeless tobacco users.

The use of deuterium-labeled precursors allowed us to specifically identify NNN as formed from the precursors added to saliva prior to incubation, thus eliminating concerns that NNN measured in saliva of our nonsmoking volunteers could have other sources, for example, exposure to secondhand smoke. We also took particular precautions to ensure that artifactual nitrosation did not take place after the incubation during sample

preparation for LC-MS/MS analysis. The method used in this study is practically identical to the one that has been previously shown not to result in artifactual NNN formation (Stepanov & Hecht, 2005; Stepanov, Carmella, Briggs, et al., 2009).

Nitrosation of [pyridine-D_4]nicotine during saliva incubation experiments was minimal, which is in agreement with kinetic studies showing that nornicotine is nitrosated to form NNN much more efficiently than nicotine (Mirvish, Sams, & Hecht, 1977). These results also suggest that nornicotine, and not nicotine, is the major precursor of endogenously synthesized NNN in some users of oral NRT products.

There was significant interindividual variation in the amount of [pyridine-D_4]NNN formed upon incubation with [pyridine-D_4]nornicotine of saliva collected from nonsmoking volunteers (Table 1). Endogenous formation of *N*-nitrosamines in humans can be greatly affected by various dietary and host factors. For example, ascorbic acid and vitamin E inhibit endogenous nitrosation in humans, whereas some phenolic compounds can both inhibit and catalyze *N*-nitroso compound formation, depending on a variety of factors (Bartsch, Ohshima, & Pignatelli, 1988). The time of nicotine or nornicotine contact with nitrosating agents relative to the time of food consumption is also critical: under fasting conditions, the concentration of nitrite in saliva varies between 10 and 1000 μmol/L, increasing 2- to 5-fold for several hours after the ingestion of nitrate-containing food (McColl, 2005). In addition, certain oral microflora can catalyze nitrosation reactions at neutral pH (Jiebarth et al., 1997), and the use of antiseptic mouthwash reduces endogenous nitrosamine formation by 62%–74% (Shapiro, Hotchkill, & Roe, 1991). The potential role of oral microflora in the formation of [pyridine-D_4]NNN in this study is supported by the previous finding that NNN formation does not occur upon urine incubation with nornicotine (Stepanov, Carmella, Briggs, et al., 2009). Although the effect of diet, oral health status, and other factors on the salivary synthesis of [pyridine-D_4]NNN is outside the scope of this report, the observed interindividual variation in the capacity for oral nitrosation is in agreement with our previous observation that only some oral NRT users form NNN endogenously.

About 0.4%–0.8% of a nicotine dose is metabolized to nornicotine in humans (Benowitz, Jacob, Fong, & Gupta, 1994; Hukkanen, Jacob, & Benowitz, 2005), and it is possible that similar to nicotine, metabolically formed nornicotine could be concentrated in saliva (Rose et al., 1993). However, metabolism of nicotine to nornicotine, even though potentially a contributing factor in the endogenous NNN synthesis in NRT users, is not likely to be a major determinant of the effectiveness of this process. If endogenous formation of NNN depended on the enzyme-regulated metabolism of nicotine to nornicotine, significant interindividual, but not intraindividual, differences in urinary excretion of NNN would be observed in our previous studies (Stepanov, Carmella, Briggs, et al., 2009; Stepanov, Carmella, Han, et al., 2009).

The most likely sources of nornicotine in saliva are cigarette smoke or extraction from oral tobacco or NRT products. We here found that a single piece of nicotine gum or lozenge contains 100 to 200 times higher amount of nornicotine than the amount used in our incubation experiments. Users of nicotine gum extract up to 70% of its nicotine content (Benowitz, Jacob, & Savanapridi, 1987), and the same is likely true for nornicotine. Thus, chewing several pieces of nicotine gum per day for prolonged periods of time can potentially expose NRT users to significant amounts of orally synthesized NNN, depending on dietary habits, oral health status, and other factors. Conditions in the stomach are even more favorable for the nitrosation reactions (Mirvish, 1975). There are no data available on intragastric NNN synthesis in humans.

In summary, our results demonstrate that NNN can be formed in human saliva in the presence of nornicotine, supporting the hypothesis that NNN can be synthesized endogenously in humans. Oral synthesis of NNN could potentially contribute to the overall intake of this carcinogen by some smokers and smokeless tobacco users, affecting their risk of developing cancer. Removal of nornicotine from NRT products should be considered in order to protect consumers from being exposed to this potent carcinogen.

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Declaration of Interests

There are no competing interests.

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