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Endogenous Formation of *N*′-Nitrosonornicotine in F344 Rats in the Presence of Some Antioxidants and Grape Seed Extract

DIANA PORUBIN,[†] STEPHEN S. HECHT,[‡] ZHONG-ZE LI,[‡] MARIA GONTA,[†] AND IRINA STEPANOV*,[‡]

Department of Industrial and Ecological Chemistry, Moldova State University, 60 A. Mateevici Street, Chisinau, Moldova, and University of Minnesota Cancer Center, Mayo Mail Code 806, 420 Delaware Street Southeast, Minneapolis, Minnesota 55455

N′-Nitrosonornicotine (NNN) is one of the most abundant strong carcinogens in unburned tobacco and cigarette smoke and is classified by the International Agency for Research on Cancer as carcinogenic to humans. Human exposure to NNN mainly occurs upon use of tobacco products. It is also possible that additional amounts of NNN are formed endogenously. The goal of this study was to evaluate the inhibitory effect of some antioxidants, including ascorbic acid and grape seed extract (GSE), on endogenous NNN formation in rats treated with nornicotine and sodium nitrite by gavage twice daily for 3 days. The study included four groups of rats: (1) negative control group A, to which no chemical was administered; (2) negative control group B, treated with nornicotine alone (2.5 μmol per gavage); (3) positive control group, to which both nornicotine (2.5 μmol per gavage) and sodium nitrite (7.5 μmol per gavage) were administered; and (4) rats treated with nornicotine (2.5 μmol per gavage), inhibitor (7.5 or 37.5 μmol per gavage), and sodium nitrite (7.5 μmol per gavage). The mean ($\pm$SD) total amount of NNN in the 3-day urine of rats treated with both nornicotine and sodium nitrite was 4.78 $\pm$ 2.88 nmol. The order of inhibition of endogenous NNN formation in rats at the molar ratio [nitrite]:[inhibitor] 1:5 was as follows: ascorbic acid (91%) > dihydroxyfumaric acid (86%) $\approx$ catechin (85%) > resveratrol (no inhibition). Treatment of rats with grape seed extract did not produce statistically significant inhibition of endogenous nornicotine nitrosation. This is the first study that demonstrates endogenous NNN formation in rats treated with nornicotine and sodium nitrite and effective inhibition of this process by ascorbic acid, dixydroxyfumaric acid, and catechin.

KEYWORDS: ***N*′-Nitrosonornicotine; endogenous nitrosation; ascorbic acid; grape seed extract**

INTRODUCTION

Despite tremendous advances in medicine, cancer remains a major health concern in the world (*1*). Among the environmental factors that influence cancer risk, smoking and diet are considered to have a major role. Thus, smoking is responsible for 90% of lung cancer cases and is a major preventable cause of cancer death (*2*, *3*).

Tobacco-attributed cancers are in part associated with human exposure to tobacco-specific nitrosamines (TSNA), a group of carcinogens formed from tobacco alkaloids during the curing and processing of tobacco (*4*−*7*). The most carcinogenic of the commonly occurring tobacco-specific nitrosamines are *N*′-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (*4*). NNN induces tumors of the esophagus and nasal cavity in rats, the lung in mice, the respiratory tract in hamsters, and the nasal cavity in mink (*4*, *7*), while NNK induces lung tumors in rodents independent of the route of administration (*7*). A mixture of NNN and NNK produced oral cavity tumors in rats (*8*). According to the International Agency for Research on Cancer, NNN and NNK are carcinogenic to humans (*9*).

Human exposure to NNN mainly occurs upon smoking of cigarettes, use of smokeless tobacco products, and possibly through exposure to secondhand smoke. It is also possible that additional amounts of NNN and other TSNA are formed endogenously in people who use tobacco products. Extensive studies have demonstrated that endogenous formation of *N*-nitroso compounds (NOC) commonly occurs in humans, probably mainly in the acidic environment of the stomach through the reaction of nitrosating agents with a number of dietary NOC precursors (*10*, *11*). Human exposure to nitrite, the source of endogenous nitrosating agents, occurs through the diet, via reduction of dietary nitrate, and from endogenously produced nitric oxide (*12*, *13*) (**Figure 1**). It has been demonstrated that NNN is formed endogenously in F344 rats treated with nicotine and sodium nitrite, probably via nitrosation of metabolically formed nornicotine (*14*).

* To whom correspondence should be addressed. Phone: (612) 624-4998. Fax: (612) 626-5135. E-mail: stepa011@umn.edu.
[†] Moldova State University.
[‡] University of Minnesota Cancer Center.

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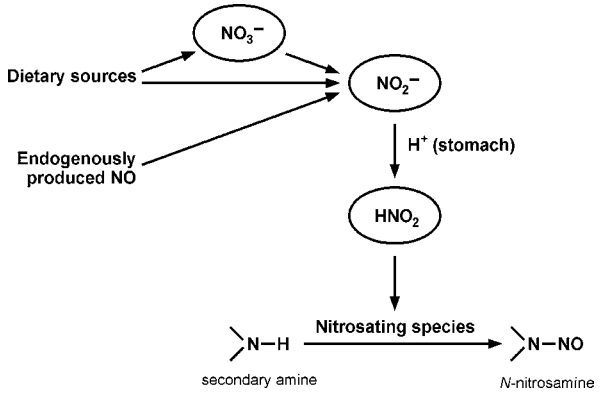

Figure 1. Major sources of endogenous nitrosating species.

The reduction of human exposure to endogenously formed NOC, as one method of cancer chemoprevention, is possible through the use of inhibitors of the nitrosation process. Thus, since the original discovery by Mirvish et al. (*15*), ascorbic acid and ascorbate were shown to inhibit in vitro and in vivo nitrosation by a rapid reduction of nitrous acid to NO (*16*−*18*). Phenolic compounds, which are present in high quantities in human foods and beverages derived from plants and fruits, are also potent blocking agents of nitrosation (*19*−*22*) and were shown to inhibit mutagenesis through the inhibition of NOC formation (*23*−*25*). Polyphenols synthesized by plants include a wide range of closely related compounds, such as flavonoids (catechins in tea leaves), isoflavonoids (genistein and daidzein in soybeans), and stilbenes (resveratrol in red grapes). Grape seed extracts (GSE) and red wine, which are rich in polyphenols, have been shown to possess antioxidant activity (*26*); inhibit aromatase enzyme activity (*27*); inhibit the growth of cancer cells in culture (*28*, *29*); and prevent disease in animal models of atherosclerosis (*30*), cataract formation (*31*), and skin cancer (*32*).

The goal of this study was to evaluate the inhibitory effects of some antioxidants and GSE on the process of endogenous NNN formation in rats. Since the reaction of nicotine with sodium nitrite is slow and occurs in low yield, producing NNN as well as other products (*33*, *34*), we used nornicotine as the NNN precursor. As a secondary amine, it undergoes nitrosation at a far greater rate than does nicotine (*35*), and it is a known metabolite of nicotine in the rat (*36*). The chemical structures of nornicotine, NNN, and the studied antioxidants are illustrated in **Figure 2**.

MATERIALS AND METHODS

CAUTION: NNN is carcinogenic and mutagenic and should be handled with extreme care, using appropriate protective clothing and ventilation at all times.

Chemicals and Grape Seed Extract. NNN and 5-methyl-*N*′-nitrosonornicotine (5-MeNNN) were synthesized as previously described (*37*). Sodium nitrite, ascorbic acid, dihydroxyfumaric acid, catechin, and resveratrol were purchased from Sigma Chemical Co. (St. Louis, MO). GSE, a blended powdered product containing about 55.6% grape seed extract, 27.8% grape skin extract, and 16.7% red wine extract, was ordered online from Whole Health Products, LLC (Golden, CO).

Animal Experiments. The study was approved by the University of Minnesota Research Subjects Protection Programs Institutional Animal Care and Use Committee. Male F344 rats were obtained from Charles River Breeding Laboratories (Kingston, NY). The rats were housed in standard metabolism cages and maintained under standard conditions (*38*) on tap water and NIH-07 diet.

Treatment and dosing of the rats was based on the experimental protocol that examined endogenous formation of TSNA in rats (*14*). Nornicotine, sodium nitrite, and ascorbic acid were dissolved in HPLC-grade water. Aqueous suspensions of dihydroxyfumaric acid, catechin, resveratrol, and grape seed extract were sonicated and vigorously vortexed prior to each administration. All solutions/suspensions (0.5 mL) were administered by gavage, twice daily for 3 days. There were four groups of rats: (1) negative control group A, to which no chemical was administered (two rats); (2) negative control group B, treated with nornicotine alone (2.5 μmol per gavage; 15 μmol total dose, three rats); (3) positive control group, to which both nornicotine (2.5 μmol per gavage; 15 μmol total dose) and sodium nitrite (7.5 μmol per gavage; 45 μmol total dose were administered 11 rats); and (4) rats treated with nornicotine (2.5 μmol per gavage; 15 μmol total dose), inhibitor (7.5 or 37.5 μmol per gavage; 45 or 225 μmol total dose, respectively), and sodium nitrite (7.5 μmol per gavage; 45 μmol total dose). There were 35 rats in group 4, the number of rats per dose of an inhibitor varying from two to nine. In the positive control group, sodium nitrite was administered immediately after each dose of nornicotine. In group 4, the reagents were administered in the following order: nornicotine, inhibitor, and sodium nitrite. The urine was collected continuously until 24 h after the last gavage. The collection vessel was cooled with dry ice and contained 0.5 mL of 20% (w/v) ammonium sulfamate in 3.6 N H_2SO_4. The combined 3-day urine sample from each rat was analyzed for total NNN.

Urine Analysis. NNN in the urine of rats was analyzed by a modification of the method developed for total NNN (NNN plus its *N*-glucuronide) in human urine (*39*). Four milliliters of urine was mixed with 700 μL of 10 N NaOH, and the mixture was incubated at 80 °C for 30 min, which converts any NNN-*N*-glucuronide to NNN. The pH of the base-treated urine was then adjusted to 7 with 1 N HCl and 1 M potassium phosphate buffer (pH = 7), and 50 ng of 5-MeNNN internal standard was added to each sample. The treated urine was applied to 10-mL ChemElut cartridges (Varian, Harbor City, CA) and eluted with 3 × 10 mL of CH_2Cl_2 into a clean 50-mL glass centrifuge tube. The combined eluants were concentrated to dryness (Speedvac concentrator). The dry residue was dissolved in 1 mL of H_2O, adjusted to pH 2−3 by adding 100 μL of 1 N HCl, and the mixture was applied to 60 mg Oasis MCX cartridges (Waters Corp., Milford, MA) activated with 5 mL of CH_3OH and equilibrated with 10 mL of H_2O. The cartridges were washed with 5 mL of 1 N HCl, 5 mL of CH_3OH, and 5 mL of H_2O:CH_3OH:NH_4OH (90:5). NNN and 5-MeNNN were eluted from the Oasis MCX cartridges with 5 mL of H_2O:MeOH:NH_4OH (45:50:5), and the eluant was concentrated to dryness. Residues were dissolved in 0.5 mL of CH_2Cl_2, purified by normal-phase extraction using Bond-Elut Silica cartridges (Varian, Harbor City, CA), and further analyzed by using gas chromatography with nitrosamine-selective detection (GC-TEA) as previously described (*39*).

Statistical Analyses. We used analysis of variance (ANOVA) to compare NNN levels in the urine of the rats from different groups. NNN excreted in group 4 (rats treated with inhibitor) was compared with that in the positive control group. The difference in outcome at different doses of the same inhibitor was also compared. All analyses were carried out in SAS 9.1. *P*-values were adjusted by the Bonferroni method for multiple comparisons. The significance level was set at 5%.

RESULTS

A total of 51 male F344 rats were used in this study. The average weight of the rats was 293.4 ± 27.6 g. The average volume of urine excreted by the rats in 3 days of treatment was 24.2 ± 5.4 mL.

The modified method for the NNN assay in urine produced clean GC traces and a good separation of NNN and 5-MeNNN. Typical GC-TEA traces from the urine of rats in the four groups are illustrated in **Figure 3**. The average percentage recovery of 5-MeNNN was 32%.

Control Groups. NNN was not detected in the urine of untreated rats (negative control group A, **Table 1**). Rats treated with nornicotine alone had traces of NNN detected in their urine (**Figure 3B**). The total amount of NNN in the 3-day urine of

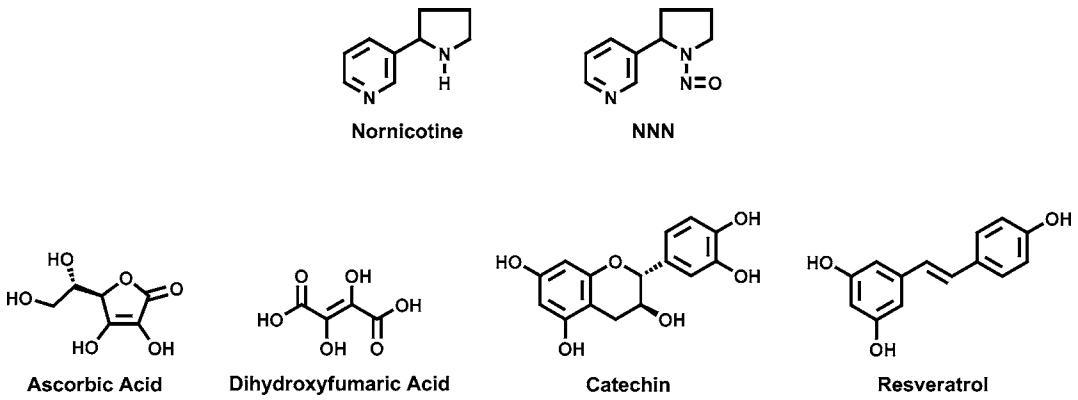

Figure 2. Structures of nornicotine, *N*′-nitrosonornicotine, and the antioxidants studied here.

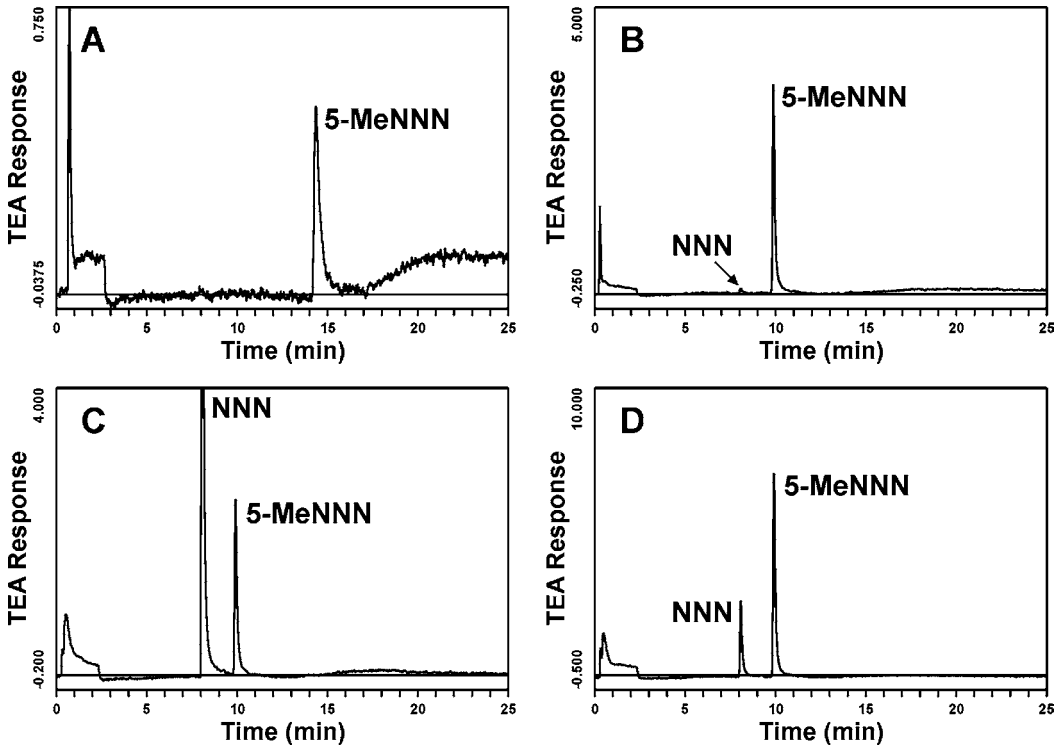

Figure 3. GC-TEA traces of NNN and 5-MeNNN in the urine of rats: (**A**) untreated rat (negative control group A), (**B**) rat treated with 15 μmol of nornicotine (negative control group B), (**C**) rat treated with 15 μmol of nornicotine and 45 μmol of sodium nitrite (positive control group), and (**D**) rat treated with 15 μmol of nornicotine, 225 μmol of ascorbic acid, and 45 μmol of sodium nitrite.

rats treated with both nornicotine and sodium nitrite was (mean ± SD) 4.78 ± 2.88 nmol, corresponding to 0.03% of the nornicotine dose.

Rats Treated with Inhibitors. The potential inhibitory activities of ascorbic acid, dihydroxyfumaric acid, catechin, resveratrol, and GSE were tested (**Table 1**). The molar ratio [nitrite]:[inhibitor] was established at 1:5 for ascorbic acid, dihydroxyfumaric acid, catechin, and resveratrol. Resveratrol showed no significant effect on endogenous nitrosation of nornicotine ($P = 1$; **Table 1**). The order of inhibition of endogenous NNN formation in rats by the rest of the compounds was as follows: ascorbic acid (91%) > dihydroxyfumaric acid (86%) > catechin (85%). Ascorbic and dihydroxyfumaric acids were also tested at a 1:1 nitrite/inhibitor ratio. Both compounds gave lower average excreted NNN as compared to the positive control group, showing 64% and 66% inhibition, respectively. However, the extents of inhibition were not statistically significant ($P = 0.59$ and 0.22, respectively, **Table 1**).

Two doses of GSE were tested: a total of 11.25 mg of GSE administered in 3 days (corresponding to approximately 7.5 mg/kg of body weight per gavage) and a total of 37.5 mg of GSE administered in 3 days (corresponding to approximately 25 mg/kg of body weight per gavage). Neither of these doses produced a consistent effect on endogenous nornicotine nitrosation. The average amount of excreted NNN by rats treated with the total GSE dose of 37.5 mg was 2.52 nmol, corresponding to 47% inhibition. However, this reduction was not statistically significant ($P = 0.36$, **Table 1**).

DISCUSSION

In this study, we investigated the effects of ascorbic acid, dihydroxyfumaric acid, catechin, resveratrol, and GSE on the extent of endogenous NNN formation in rats treated with nornicotine and sodium nitrite. Ascorbic acid, dihydroxyfumaric acid, and catechin acted as strong inhibitors when their molar ratio to nitrite was 5:1. The extents of inhibition of endogenous NNN formation were 91, 86, and 85%, respectively. Resveratrol and GSE had no significant effect on endogenous nornicotine nitrosation.

Table 1. NNN in the Urine of Rats Treated with Nornicotine, $NaNO_2$, and Potential Inhibitors of Nitrosation (Ascorbic Acid, Dihydroxyfumaric Acid, Catechin, Resveratrol, or Grape Seed Extract)

	total dose administered per rat, μmol[a]			mean (SD) NNN excreted in 3 days,	% reduction	
group	nornicotine	$NaNO_2$	inhibitor	nmol	of NNN	*P*-value[c]
			Control Groups			
negative control A	0	0	0	ND[b]	–	–
negative control B	15	0	0	0.06 (0.02)	–	–
positive control	15	45	0	4.78 (2.88)	–	–
			Inhibitor-Treated Group			
ascorbic acid	15	45	45	1.71 (0.81)	64	0.59
			225	0.44 (0.25)	91	0.001
dihydroxyfumaric acid	15	45	45	1.61 (0.23)	66	0.22
			225	0.68 (0.11(	86	0.04
catechin	15	45	225	0.72 (0.16)	85	0.04
resveratrol	15	45	225	3.06 (1.01)	36	1.00
GSE	15	45	11.3	3.68 (2.71)	23	1.00
			37.5	2.52 (0.90)	47	0.36

[a] Total dose of GSE is expressed in milligrams. [b] ND, not detected; detection limit of the method is 0.26 pmol/mL of urine. [c] All *P*-values are adjusted by the Bonferroni method for multiple comparisons.

We found that small amounts of NNN were formed in rats treated with nornicotine alone. A number of studies have shown that nitrite is formed in saliva of rats via microbial reduction of dietary nitrate on the posterior surface of the tongue (*40*, *41*). In our study, the presence of nitrates and/or nitrites in rats' diet and drinking water is probably responsible for the partial nitrosation of administered nornicotine. However, the amount of NNN excreted by these rats was very low and did not affect the results of our study.

The yield of NNN observed here is ~60 times higher than that reported in the previously published study (*14*), in which rats were treated with nicotine and sodium nitrite. This finding is not surprising, because, as a secondary amine, nornicotine undergoes nitrosation at a far greater rate than does nicotine (*35*). Taking into account that NNN is extensively metabolized in rats and only 3−5% of NNN dose is excreted unchanged in their urine (*42*), we can suggest that the actual amount of endogenously formed NNN is 20−30 times higher.

It is known that nornicotine is a nicotine metabolite in the rat; its amount in the urine corresponds to approximately 9% of the nicotine dose (*36*). Humans, like rats, metabolize nicotine to nornicotine (*43*). Subjects using the nicotine patch concentrate nicotine in their saliva (*44*), and it is possible that nornicotine could also be concentrated in saliva. After saliva containing nornicotine and nitrite is swallowed, the stomach provides favorable conditions for nitrosation (*35*, *45*). Moreover, it has been observed that endogenous synthesis of *N*-nitroso compounds occurs at a higher rate in smokers as compared to nonsmokers (*46*). Taken together, these data once again emphasize the need for NNN analysis in the urine of those people who use nicotine-containing stop-smoking aids.

The effective inhibition of endogenous nornicotine nitrosation by ascorbic acid observed here is in strong agreement with extensive studies that demonstrated its ability to inhibit intragastric NOC formation in experimental animals and in humans (reviewed in refs *16*, *17*). Partial inhibition of endogenous *N*-nitrosoproline formation also has been demonstrated in smokers who received a daily dose of 1 g of ascorbic acid followed by 300 mg of proline (*46*). Ascorbic acid was shown to inhibit nitrosation over a pH range of 2−5 through rapid reduction of nitrous acid to nitric oxide (NO) and formation of dehydroascorbic acid (*23*). It is possible that the same mechanism is involved in the inhibition of endogenous NNN formation by dihydroxyfumaric acid observed in this study. Dihydroxyfumaric acid can be formed through the oxidation of tartaric acid in the presence of iron(II) and hydrogen peroxide (*47*) and is found in wine (*48*). It was demonstrated that *N*-nitrosation of secondary amines in simulated gastric juice is effectively inhibited by dihydroxyfumaric acid (unpublished data), and the present study supports this finding. However, the potential health effects of dihydroxyfumaric acid are unknown.

Phenolic antioxidants were shown to inhibit or catalyze the formation of NOC, depending on their structure and reaction conditions (reviewed in *23*). Thus, catechin can form C-nitroso derivatives, which act as powerful nitrosating agents (*49*). In our study, we observed effective inhibition of endogenous nornicotine nitrosation in the presence of catechin. The effect of phenolic compounds on *N*-nitroso compound formation depends on pH, the nature of the nitrosated amine, and the relative concentrations of nitrite and phenolics (*23*). Thus, catalysis of *N*-nitrosation may occur with phenolics that can form C-nitroso derivatives at molar ratios of nitrite to phenolics > 1, while inhibitory effects are manifested when the molar ratio of nitrite to phenolic is <1. In the presence of high concentrations of phenolics, the nitrosating agent is either completely reduced to NO or converted into C-nitroso derivatives, so that *N*-nitrosation is blocked (*23*). In our study, the molar ratio of nitrite to catechin was 1:5, producing 85% inhibition of NNN formation in rats. Resveratrol, a polyphenolic phytoalexin from grape skin, has been the subject of numerous investigations of its cancer chemopreventive activity (*50*) attributed to its antioxidant properties (*51*, *52*). In our study, resveratrol had no inhibitory effect on endogenous NNN formation at [nitrite]:[resveratrol] ratio 1:5.

Administration of GSE along with nornicotine and nitrite did not produce statistically significant inhibition (**Table 1**). This may be due to the complex composition of the grape seed extract. Since the effect of different phenolic compounds on *N*-nitrosation depends on a number of factors, it is possible that some GSE polyphenols catalyze NNN formation through formation of powerful nitrosating agents while other components act as inhibitors through the reduction of nitrosating agents to nonreactive products. Thus, both catalysis and inhibition of endogenous N-nitrosation by different components of GSE might occur at the same time.

In summary, we present here the first study that demonstrates endogenous NNN formation in rats treated with nornicotine and sodium nitrite and effective inhibition of this process by ascorbic

acid, dixydroxyfumaric acid, and catechin. We also show that resveratrol and grape seed extract do not inhibit endogenous nitrosation of nornicotine in rats treated with nornicotine and sodium nitrite. Further studies are needed to investigate the possibility of nitrosation of metabolically formed nornicotine in people who use nicotine-containing stop-smoking aids.

ABBREVIATIONS

GSE, grape seed extract; 5-MeNNN, 5-methyl-*N*′-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NOC, *N*-nitroso compounds; TSNA, tobacco-specific nitrosamines.

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