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Tobacco-related-compound-induced Nitrosative Stress Injury in the Hamster Cheek Pouch

ABSTRACT

The nitric oxide radical (•NO) released from tobacco-related compounds induces DNA damage, protein modifications, and cellular toxicity through the formation of peroxynitrite ($ONOO^-$), the reaction product of •NO and the oxygen radical, superoxide. We hypothesize that tobacco-related compounds are cytotoxic and induce quantifiable DNA single-strand breaks in immortalized hamster cheek pouch (POII) cells, and that an amino acid marker of $ONOO^-$ injury, namely, 3-nitrotyrosine (3-NT), is detectable in hamster cheek pouch tissues chronically exposed to these compounds. We observed a dose-dependent decrease in POII cell viability with increasing tobacco-related compound concentrations, as well as a dose-dependent increase in DNA strand breaks. Semi-quantitative immunohistochemistry showed intense 3-NT immunoreactivity in hamster tissues treated with tobacco-related compounds compared with controls ($p < 0.005$). Our results suggest that tobacco-related compounds, including nicotine, are genotoxic, and that 3-NT is a quantifiable marker of $ONOO^-$ damage in intact hamster cheek pouch tissues.

KEY WORDS: tobacco-related compounds, protein nitrosation, DNA damage, oral mucosa.

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INTRODUCTION

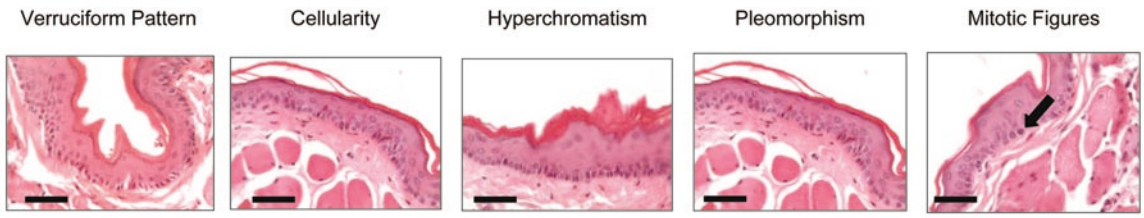
The free radical nitric oxide (•NO) controls numerous biological processes, including vascular smooth-muscle relaxation (Moncada *et al.*, 1988), signal transduction (Ignarro, 2002), and tumorigenesis (Xie *et al.*, 1995). Physiologic •NO concentrations are generated in the pico- to micromolar range by nitric oxide synthase (Nathan and Xie, 1994), which is disseminated locally, and by hemoglobin-mediated transport throughout the body (Stamler *et al.*, 1997).

At supra-physiologic concentrations, •NO cytotoxicity is mediated by peroxynitrite ($ONOO^-$), the reaction product of •NO and the oxygen radical, by superoxide ($O_2^{\bullet -}$), which is generated as a by-product of mitochondrial respiration, and by cellular oxidases (Beckman and Koppenol, 1996). The bioavailability of these radicals regulates $ONOO^-$ formation—that is, when •NO is high, or when $O_2^{\bullet -}$ scavenging by superoxide dismutase is impaired, as is the case in transformed and malignant cells (Oberley, 2001).

Lipid peroxidation, amino acid modifications to proteins, enzyme dysfunction, and DNA damage have been attributed to $ONOO^-$ (Szabó, 2003). Several of these so-called nitrosative lesions have also been used as endpoints or markers of oxidative stress injury from oxygen radicals, including $O_2^{\bullet -}$. While more specific biomarkers of nitrosative injury are being elucidated (O'Donnell *et al.*, 1999; Marshall *et al.*, 2000), the nitrosation of tyrosine residues (3-nitrotyrosine, 3-NT) is the most widely used marker of $ONOO^-$ injury (Viera *et al.*, 1999). In the oral cavity, basal 3-NT immunoreactivity has been demonstrated in the oral mucosa (Bentz *et al.*, 2000; Wang *et al.*, 2002), and in gingival tissues in experimental periodontitis (Lohinai *et al.*, 1998, 2001). Indeed, one such indigenous source in the oral cavity may be from oral bacteria (Duncan *et al.*, 1995).

The nitrosamine compounds, nitrosonornicotine (NNN) and 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are thought to be the major contributors to tobacco's carcinogenic activity. NNK causes DNA single-strand breaks in oral keratinocytes, and methylation (Hecht *et al.*, 1986). Alone, NNN and NNK do not commonly produce oral tumors (Chen *et al.*, 1994; Papageorge *et al.*, 1996); however, the application of smokeless tobacco enriched 10-fold with NNN or NNK, or the combination of these nitrosamines with nicotine, will produce oral tumors in rats (Chen *et al.*, 1994; Grasso and Mann, 1998). NNK together with hydrogen peroxide produces a significant number of cheek pouch tumors in hamsters, suggesting that NNK may act as an initiator of carcinogenesis, while hydrogen peroxide may act as the promoter (Padma *et al.*, 1989).

Numerous chemicals exert their pharmacologic effects *via* the generation of free radicals and related oxidants. We have recently shown that tobacco-related compounds release •NO in nano- to micromole quantities (Lam *et al.*, 2003). The •NO derived from these compounds may represent unrecognized sources of extracellular •NO in the oral cavity. We



Control (n = 4)	13.7 ± 5.6 (16.2)	19.7 ± 15.8 (23.4)	12.5 ± 9.6 (13.5)	6.0 ± 5.2 (8.1)	0.7 ± 0.6 (0.9)
Smokeless tobacco (n = 4)	25.7 ± 8.5 (27.5)	20.6 ± 19.5 (20.6)	34.8 ± 22.2 (35.0)	21.6 ± 34.4 (15.0)	2.0 ± 3.5 (3.8)
Nicotine (n = 2)	13.7 ± 2.1 (13.7)	29.6 ± 6.3 (29.4)	10.3 ± 8.5 (10.5)	3.0 ± 4.2 (3.2)	0.0 ± 0.0 (0.0)
NNN (n = 3)	12.4 ± 16.7 (12.3)	30.9 ± 26.7 (36.3)	25.6 ± 24.3 (29.2)	0.0 ± 0.0 (0.0)	3.3 ± 2.9 (5.9)
NNK (n = 4)	24.0 ± 15.6 (20.1)	20.5 ± 16.2 (24.3)	26.2 ± 23.6 (34.7)	4.4 ± 7.5 (4.9)	3.7 ± 3.8 (7.6)

Figure 1. Histopathologic changes in hamster cheek pouch epithelium following control, or tobacco-related-compound application. Mean incidence ± SD, and percentages (parentheses) are given. Scale bar represents 60 μm. Black arrow shows mitotic figure.

hypothesize that tobacco-related-compound-derived •NO favors the formation of $ONOO^-$ in the oral cavity, damages cellular macromolecules, and elevates 3-NT expression in oral tissues.

MATERIALS & METHODS

Cell Culture

Immortalized hamster cheek pouch (POII) cells (Schuster *et al.*, 1985) were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA),supplemented with 10% heat-inactivated fetal bovine serum, hydrocortisone, antimycotic, and antibiotics.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

The MTT assay was performed according to Mosmann (1983), with the use of a Beckmann 650 spectrophotometer (Beckmann/Coulter, Fullerton, CA, USA).

Comet Assay

The comet assay was performed under alkaline conditions (Trevigen, Gaithersburg, MD, USA). Cells were viewed by fluorescence microscopy at 490 nm. Tail moment analysis was performed with the use of Metamorph 6.0 (Universal Imaging Corp., Downingtown, PA, USA) and was defined as the length of the comet tail multiplied by the percentage of DNA in the tail, defined as (total intensity - head intensity)/total intensity. Twenty-five cells were analyzed from each slide for each treatment.

Animals

Thirty-day-old Syrian golden hamsters (*Mesocretus auratus*) were obtained from Charles River Laboratories (St. Constant, QC, Canada), and fed a commercial stock diet and tap water, *ad libitum*. Animals were killed by CO_2 asphyxiation. This protocol was reviewed and approved by the University of Alberta Health Sciences Animal Use and Welfare Committee.

Tobacco-related-compound Preparations

Copenhagen® smokeless tobacco (National Tobacco Co., Ltd., Pointe Claire, QC, Canada) was obtained locally. Racemic (+/-) nicotine was obtained from Sigma (St. Louis, MO, USA). NNN and NNK were obtained from the Midwest Research Institute (St. Louis, MO, USA). Extracts or solutions of each tobacco-related compound were made fresh in dimethylsulfoxide (DMSO) and mineral oil (USP), no more than 30 min prior to use. We used DMSO to facilitate dissolution and delivery of the compounds through the mucosa.

A 1:2 weight/volume (w/v) extract of smokeless tobacco was made in DMSO/mineral oil, incubated at 37°C for 20 min, and centrifuged for 5 min at 1000 x *g* for sedimentation of the tobacco. The extract was removed, vortexed, and mixed with the DMSO/mineral oil prior to use. Using an Agilent Technologies (Palo Alto, CA, USA) 6890 gas chromatograph coupled to a 5970 mass spectrometer, run in the selective ion monitoring mode, previous investigators have determined the efficiency of this extraction to be 24% ± 1% (Jacob *et al.*, 1981).

A 33-μM stock solution of nicotine was made in DMSO/mineral oil. A 4-μg/mL solution of NNN and a 1-μg/mL solution of NNK were made in the vehicle. A 50-μL quantity of each extract or solution was applied to the left cheek pouch of each animal with a number 4 sable brush, 3 times *per* wk for 10 mos. Control animals received 50 μL of the DMSO/mineral oil only.

In all instances, water was withheld for 1 hr following application of the extract or solution.

Histopathology and Immunohistochemistry

Hamster cheek pouch tissues were excised and prepared for hematoxylin and eosin (H/E) and immunohistochemical staining. H/E sections were reviewed, blinded, by an oral and maxillofacial pathologist (EP). High-power fields of each specimen were viewed sequentially and scored for the presence or absence of epithelial verruciform morphology (the presence of pointed epithelial or keratin surface projections), hypercellularity (increased cellularity), nuclear hyperchromatism (increased nuclear hematoxylin staining intensity), pleomorphism (abnormally shaped nuclei and cells), as well as the number of mitotic figures (Neville *et al.*, 2002).

Immunohistochemistry was performed according to Lam *et*

al. (2000), with the Vectastain ABC system (Vector Laboratories, Burlingame, CA, USA) and metal-enhanced diaminobenzidine (Pierce, Rockford, IL, USA). The 3-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, USA) was applied at 1:100 volume/volume. Image analysis was performed on non-hematoxylin counter-stained sections.

Semi-quantitative Digital Imaging Analysis

Immunostained sections were digitally captured *via* a Photometrix CoolSNAP camera (Roper Industries Inc., Duluth, GA, USA) and a Leica DM-IRB inverted microscope (Leica Microsystems Inc., Richmond Hill, ON, Canada). We used MetaMorph (Universal Imaging Corp., Downingtown, PA, USA) software to analyze the digitized immunostained sections for mean gray level values, a measure of 3-nitrotyrosine intensity, according to Lam *et al.* (2000).

Statistical Analysis

Statistical analysis of staining intensities was performed by one-way analysis of variance and *post hoc* Tukey test (Systat Inc., Evanston, IL, USA) software. The null hypothesis was rejected at the 0.05 level of significance.

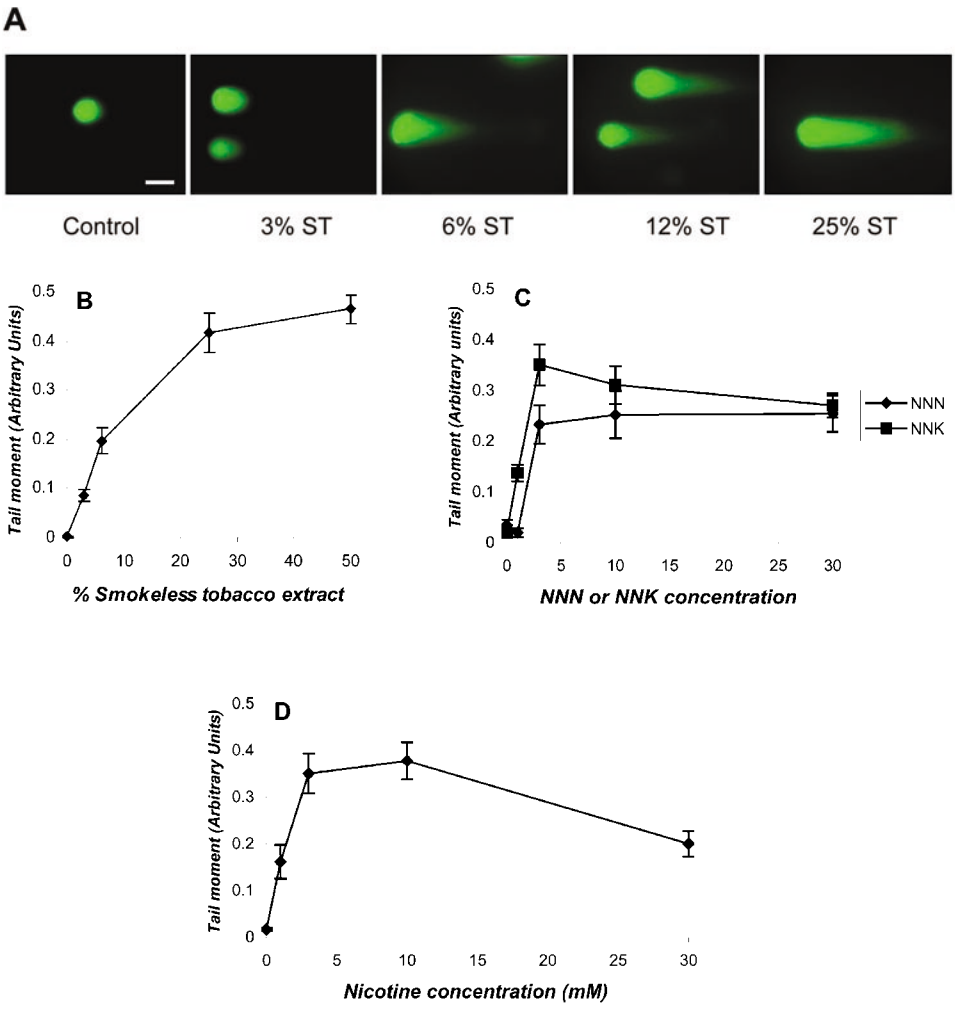


Figure 2. Comet assay confirms DNA single-strand breaks induced by tobacco-related compounds. **(A)** Dose-dependent increase in comet tail moment with increasing smokeless tobacco extract. Each entity represents 1 POII cell. POII tail moment increases with increasing concentrations of smokeless tobacco extract **(B),** NNN and NNK **(C),** and nicotine **(D).** Each point is the mean ± standard error of 25 individual cells.

RESULTS

Histopathologic Findings

Histopathologic findings in tobacco-related-compound-treated hamster cheek pouch mucosa were consistent with mild epithelial dysplasia. Tissue morphology data were summarized (Fig. 1). The verruciform pattern was seen in 27.5% of high-power fields in smokeless-tobacco-extract-treated animals. Also, these animals showed the highest frequency of hyperchromatism and pleomorphism. Animals treated with NNN showed the highest frequency of increased cellularity, while animals treated with NNK showed the highest number of mitoses.

Cell Viability

A dramatic reduction in POII viability was seen following 24-hour tobacco-related-compound treatment. The lethal doses to 50% of cells (LD_{50}) were calculated to be 2.5% weight/volume (w/v) for smokeless tobacco, 7.4 mM for nicotine, 2.5 mg/mL for NNN, and 2.2 mg/mL for NNK. The LD_{50} with pure $ONOO^-$ was 0.2 mM.

Comet Assay

The comet assay, performed under alkaline conditions, measures DNA single-strand breaks (Fig. 2A). Each experiment was optimized for incubation time. Maximal tail moments for smokeless tobacco extract were identified after 30 min, and after 5 and 15 min for NNN and NNK, respectively (Figs. 2B, 2C). Nicotine, which has not previously been reported to be genotoxic, induced DNA strand breaks in 40 sec (Fig. 2D). At time points over 1 min, we observed repair of nicotine-induced DNA damage, as evidenced by a loss of tail moment (data not shown).

Nitrotyrosine Expression in the Hamster Cheek Pouch

Compared with DMSO/mineral-oil-treated control tissues, tobacco-related-compound-treated tissues showed generally wide distribution of 3-nitrotyrosine immunoreactivity throughout all cell layers (Figs. 3A, 3B-3E). Background basal cell 3-nitrotyrosine immunoreactivity in control tissues is believed to reflect endogenous nitric oxide synthase activity (Lohinai and Szabó, 1998; Bentz *et al.*, 2000; Lohinai *et al.*, 2001). Image analysis of immunostained sections showed 3-nitrotyrosine immunoreactivity to be strongest in nicotine-

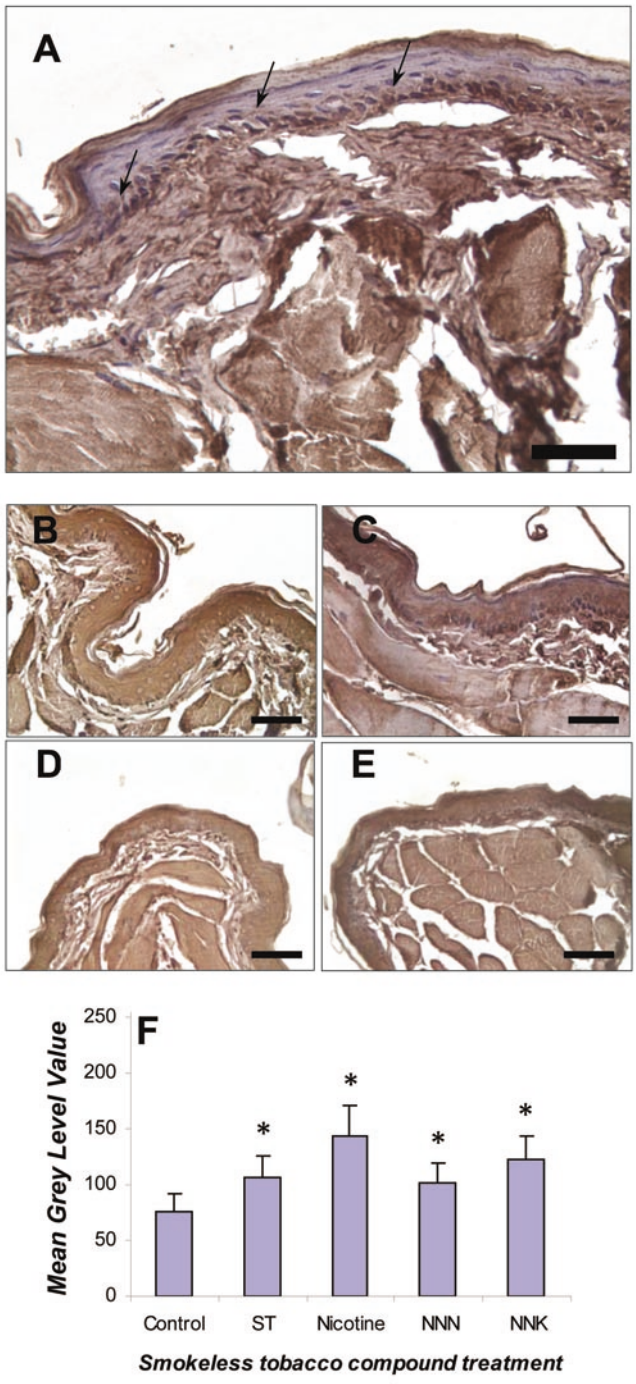


Figure 3. 3-Nitrotyrosine immunoreactivity increases in hamster cheek pouch tissues with tobacco-related-compound application. **(A)** Tissue treated with DMSO/mineral oil; **(B)** smokeless tobacco extract; **(C)** nicotine; **(D)** NNN; and **(E)** NNK. Control epithelium shows 3-nitrotyrosine immunoreactivity confined to basal cells (arrows). All tobacco-related-compound treatments resulted in full-thickness 3-nitrotyrosine immunoreactivity. Scale bar represents 70 μm. **(F)** Mean gray level values ± SD of 3-nitrotyrosine intensity from 15 50 x 50 pixel regions of interest in 2 to 5 animals (* $p < 0.005$ compared with control).

treated tissues (mean gray level value, 143.2 ± 27.1 arbitrary units [AU]) (Fig. 4). Control tissue values were considerably lower at 76.2 ± 15.6 AU. Values for NNK-, NNN-, and

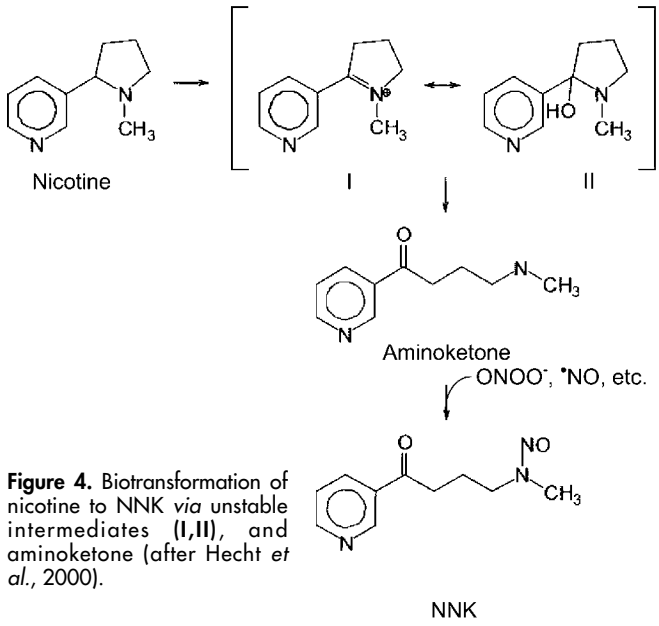


Figure 4. Biotransformation of nicotine to NNK *via* unstable intermediates **(I,II)**, and aminoketone (after Hecht *et al.*, 2000).

smokeless-tobacco-treated tissues were 123.1 ± 20.1, 101.4 ± 17.9, and 105.8 ± 20.1, respectively. These differences were significant at $p < 0.005$ compared with control tissues.

DISCUSSION

The role of oxygen free radicals and related oxidants in human disease has been widely investigated. Under physiologic conditions, chain-breaking and preventive anti-oxidants control the concentration and distribution of reactive species, limiting oxidative injury in biological systems. In comparison, reactive nitrogen-centered species and nitrosative stress injury are less well-characterized. This may be due, in part, to an elementary understanding of biological nitrosation mechanisms, and the limited number of specific biomarkers of nitrosative injury. Ischiropoulos (1998) suggests that the major putative mechanism of nitrosation at physiologic pH is uncatalyzed nitrosation by $ONOO^-$, the reaction product of $NO^\bullet$ and $O_2^{\bullet-}$.

While we failed to observe tumor formation in tobacco-related-compound-treated hamster mucosa, we did identify mild dysplastic changes. These findings have been reported previously (Chen *et al.*, 1994; Papageorge *et al.*, 1996; Grasso and Mann, 1998). When H/E sections were blindly reviewed by an oral and maxillofacial pathologist (EP), many of the cellular and tissue changes mirrored those seen in human biopsies.

Under alkaline conditions, the comet assay detects DNA single-strand breaks. Strand breaks confer a 'comet tail'-like appearance on each cell after low-voltage electrophoresis, and, unlike DNA laddering studies (Bagchi *et al.*, 2002), the comet assay is quantifiable. All tobacco-related compounds, including nicotine, are genotoxic in POII cells. The time-course, however, appears to be compound-dependent. While these assays have been used to characterize oxidative cellular damage, such assays are not specific for nitrosative stress injury caused by $ONOO^-$.

Conditions that favor the formation of $ONOO^-$ and/or the stability of $NO^\bullet$ and $O_2^{\bullet-}$ in biologic systems may determine the extent of nitrosative stress injury. 3-Nitrotyrosine

immunoreactivity has been used extensively as a marker of this injury (Viera *et al.*, 1999). Our previous work with the $O_2^{\bullet-}$ scavenger, manganese superoxide dismutase, demonstrated immunolocalization of this enzyme to the spinous and granular cell layers of the hamster cheek pouch, and not the basal layer (Lam *et al.*, 2000). Therefore, when expression of this enzyme is low (*i.e.*, in undifferentiated, transformed, or tumor cells), the potential for nitrosative stress injury should be elevated. Indeed, this is what was observed. In control hamster tissues that received only mineral oil/DMSO, we observed 3-nitrotyrosine localized primarily to the mucosal basal layer, corroborating the work of Bentz *et al.* (2000), Lohinai *et al.* (2001), and Lohinai and Szabó (1998) in the human and the rat. Sporadic 3-nitrotyrosine staining was also seen in some granular layer cells, and in the stratum corneum. This may reflect nitrosation of keratin.

Semi-quantitative immunohistochemistry showed that nicotine produced the highest intensity of 3-nitrotyrosine immunoreactivity, followed by NNK, smokeless tobacco extract, and NNN. Since the nitrosamine metabolites are also found in very low (*i.e.*, microgram) quantities in smokeless tobacco compared with nicotine, which is found in milligram quantities, we observed somewhat less 3-nitrotyrosine immunoreactivity in hamster tissues treated with smokeless tobacco extract.

Given the structure of nicotine, it would seem implausible for it to behave as an $^{\bullet}NO$ donor, or to induce changes similar to those seen with the other tobacco-related compounds. A small proportion of nicotine, approximately 11%, is hydroxylated in the 2′ position of the 5-membered pyrrol ring (Fig. 4). Opening of the ring intermediate results in the formation of an aminoketone, 4-(methylamino)-1-(3-pyridyl)-1-butanone, a direct precursor of NNK (Hecht *et al.*, 2000). Nitrosation by $^{\bullet}NO$, $ONOO^-$, or related intermediaries at neutral pH yields NNK, the putative $^{\bullet}NO$ donor. In this way, NNK may be formed from nicotine.

Our studies suggest that tobacco-related compounds induce cytotoxicity and DNA damage, and cause nitrosative stress injury in intact tissues. Future work will include modulation of $ONOO^-$ formation in cells by the scavenging of $O_2^{\bullet-}$ in cells and animals.

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