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Metabolism of tobacco-specific *N*-nitrosamines by cultured human tissues*

(carcinogen/organ culture/α-carbon hydroxylation)

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ABSTRACT ***N'*-Nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are present in cigarette smoke and snuff and are carcinogens in laboratory animals. In tobacco smokers, the buccal mucosa, trachea, esophagus, bronchi, and peripheral lung are exposed to smoke containing significant amounts of these *N*-nitrosamines. The results of the present study demonstrate that explants of these tissues as well as of the urinary bladder have the capacity to metabolize NNN and NNK by α-carbon hydroxylation. This metabolic pathway yields alkyldiazohydroxides, which are reactive and DNA-damaging electrophiles. The extent of α-carbon hydroxylation of NNN and NNK in human tissues was only 1/10th to 1/100th of that in animal tissues. Although the levels of α-carbon hydroxylation of NNN among different tissues of the same individual were similar, a 10-fold variation among individuals was observed. Reduction of the NNK carbonyl group was a major metabolic pathway observed with all human explants and may occur in the surface epithelia of the respiratory tract of smokers. These results provide further evidence that tobacco-specific *N*-nitrosamines could play a role in cancers related to the smoking and chewing of tobacco.**

Since the pioneering work of Wynder and Graham (1) and Doll and Hill (2) relating higher risks of bronchiogenic carcinoma to cigarette smoking, other epidemiologic surveys have observed, among smokers, an increased risk of cancer at various sites, including the oral cavity, larynx, esophagus, and urinary bladder (3). Case-control studies have indicated that long-term tobacco chewing (4) or snuff dipping (5) are important factors in the etiology of oral cancer.

Carcinogenicity assays of various smoke fractions or substances have indicated that *N*-nitrosamines could play a role in tobacco carcinogenesis (3, 6). In this respect, *N'*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are particularly important because of their relatively high levels in cigarette smoke (7) and snuff (8) and their carcinogenic potency in animals (9).

Most *N*-nitrosamines are considered procarcinogens and require hydroxylation on the carbon next to the *N*-nitroso group to become carcinogenic derivatives. The resulting α-hydroxy-*N*-nitrosamines have relatively short half-lives (10) and decompose to electrophilic intermediates that react immediately with DNA. Thus, DNA alkylation is likely to occur in the same tissues that have the enzymatic capacities to bioactivate *N*-nitrosamines by α-carbon hydroxylation. Metabolism studies with mice, hamsters, and rats (9, 11) have shown that NNN and NNK are activated by tissues that are susceptible to their carcinogenic effects. If NNN and NNK play a role in human carcinogenesis, similar bioactivation would have to occur in smokers and snuff dippers, most likely in the oral and respiratory tissues.

The aim of the present study was to determine the capacity of cultured human tissues to metabolize NNN and NNK and to compare the results with their metabolism by those animal tissues that develop tumors after exposure to these carcinogens.

MATERIALS AND METHODS

Chemicals. Syntheses of NNN, NNK, and their metabolites have been described (12, 13). [2'-^{14}C]NNN (51.7 mCi/mmol; 1 Ci = 3.7×10^{10} Bq) was obtained from New England Nuclear and purified as reported (14). [*Carbonyl*-^{14}C]NNK (4.2 mCi/mmol) was prepared from [*carboxyl*-^{14}C]nicotinic acid (California Bionuclear, Sun Valley, CA) and purified (>99%) by TLC [2-mm silica gel, CH_2Cl_2/CH_3OH, 100:5 (vol/vol), three migrations, R_f = 0.30–0.47 (unpublished data)].

Explant Culture. Grossly normal tissues were taken from 18 individuals at autopsy within 2–6 hr of death. The age varied from 39 to 95 years and the mean age was 65. Among 7 female individuals, 1 was a smoker, 4 were nonsmokers, and 2 had unknown smoking histories. Among 11 male individuals, 3 were smokers, 3 were nonsmokers, and 5 had unknown smoking histories.

Buccal mucosae were obtained from the inner cheek, tracheal specimens from the lower one-third of the trachea, esophageal specimens from the middle portion of the esophagus, and bronchial specimens from the main stem bronchus and primary order bronchi. Peripheral lung and bladder specimens were obtained as described (15, 16). By using aseptic conditions, muscle and connective tissues were removed from the esophagus, bladder, and buccal cavity specimens leaving the epithelial layer intact, and the bronchial specimens were trimmed of adherent lung tissues. Representative samples of each tissue were fixed, embedded, sectioned, and examined by light microscopy as described (15, 16).

Specimens from all tissues were cut into explants ranging from 0.2-cm^2 (peripheral lung) to 0.5-cm^2 (buccal mucosa, trachea, esophagus, bronchus, urinary bladder) pieces and were placed in rocking organ culture as described (15, 16). After 48 hr, the medium was replaced with fresh medium containing either [2'-^{14}C]NNN or [*carbonyl*-^{14}C]NNK (1 μCi of each radiolabeled *N*-nitrosamine per ml). Controls consisted of tissues heated in boiling water for 30 min prior to the addition of radiolabeled *N*-nitrosamines. Incubations of medium with radio-

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Abbreviations: NNN, *N'*-nitrosonornicotine; NNN-1-N-oxide, *N'*-nitrosonornicotine-1-*N*-oxide; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAl, 4-(methylnitrosamino)-1-(3-pyridyl)butan-1-ol.

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labeled *N*-nitrosamines but without the tissues served as medium controls. After incubation for 24 hr, the media were collected and stored immediately at −20°C until analysis of metabolites by HPLC. The tissues were collected and stored at −20°C for analysis of DNA content.

HPLC Analyses. The HPLC apparatus and chromatographic conditions used to analyze 1-ml aliquots of the media have been described (12). With this method, 70–100% of the radioactive compounds present in the media were characterized. The chromatographic systems were the following: In system A, the elution program was solvent 1 for 10 min, followed by a linear gradient to 60% solvent 2 in 60 min. Solvent 1 was 0.06 M sodium acetate at pH 4.5. Solvent 2 was CH_3OH/H_2O (1:1). System B was the same as system A except that the pH of solvent 1 was adjusted to 6.0 with 1 M NaOH. In system C, separation was achieved by paired-ion chromatography under isocratic conditions with 0.005 M tetrabutylammonium phosphate in CH_3OH/H_2O (81:19). In system D, the elution program was a linear gradient from solvent 1 to 60% solvent 2 in 10 min. Solvents 1 and 2 were the same as in system B. Two μBondapak C_{18} columns (23.9 mm × 30 cm, Waters Associates) connected in series were used in systems A, B, and D, but only one column was necessary in system C. The flow rate was 1 ml/min for all systems.

Each radioactive metabolite was compared to reference compounds in two different chromatographic systems. The hydroxy acid **7** was characterized in systems A or B and C. *N'*-Nitrosonornicotine-1-*N*-oxide (NNN-1-N-oxide) (**1**) and keto acid **6** were identified in systems A and D and 4-(methylnitrosamino)-1-(3-pyridyl)butan-1-ol (NNAl) was chromatographed in systems A and B. For quantitation, the keto acid **6** was purified with system A and rechromatographed in system D.

Quantitation of DNA. DNA in explant homogenates was quantitated by the diphenylamine method (17). To determine if explant DNA had been alkylated by NNN and NNK metabolites the DNA was isolated from selected bronchial and peripheral lung explants by using hydroxylapatite chromatography (15).

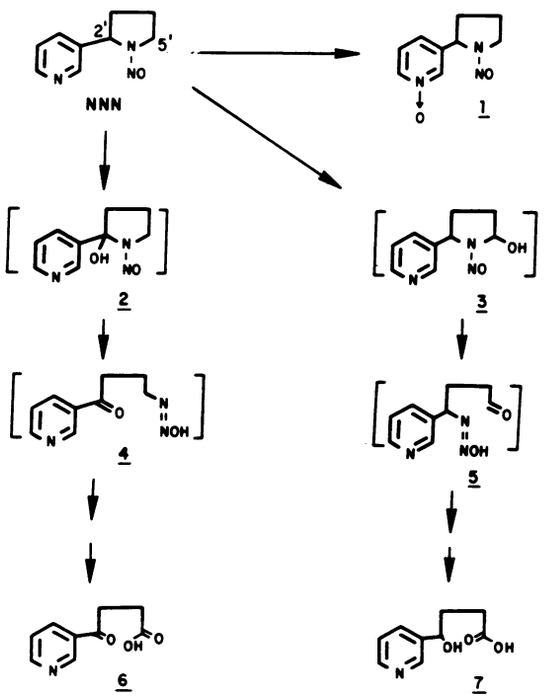

FIG. 1. Metabolic transformations of NNN. Structures in brackets are hypothetical intermediates.

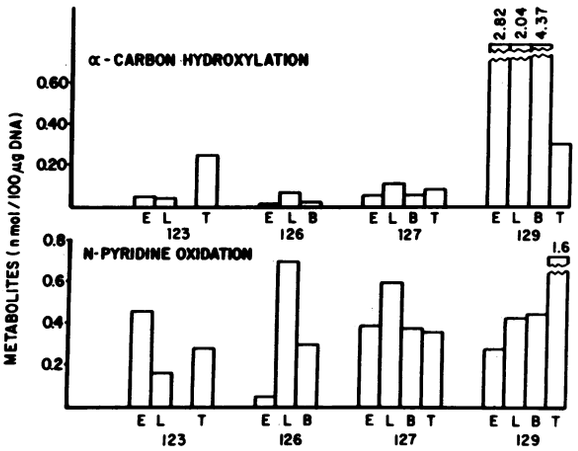

FIG. 2. Comparison of the metabolism of [2'-^{14}C]NNN by explants of various tissues from the same individual and explants of various individuals. Individuals 123 and 129 were nonsmokers, 127 was a smoker, and 126 had unknown smoking history. E, esophagus; L, peripheral lung; B, bronchus; T, trachea.

RESULTS

Metabolism of NNN by cultured human tissues is illustrated in Fig. 1. Pyridine *N*-oxidation yields NNN-1-N-oxide (**1**). Hydroxylation of the 2'-carbon of NNN gives the unstable α-hydroxynitrosamine **2**. This unstable intermediate rearranges to the diazohydroxide **4**, which could either react with nucleophiles by an S_n2 mechanism or yield an alkyldiazonium ion or carbocation. By reacting with water, these reactive species form the intermediate 4-hydroxy-1-(3-pyridyl)-1-butanone. The levels of keto acid **6**, an oxidation product of this intermediate, were low and could be measured with only a few explants.

By a similar mechanism, hydroxylation of the 5'-carbon of NNN gives 5'-hydroxy-NNN (**3**). This intermediate rearranges to diazohydroxide **5** and ultimately yields the hydroxy acid **7**. Interestingly, the amounts of **7** formed by three different tissues from case 129 were remarkably high (Fig. 2). The ratio **6**:**7** was found to be 0.1 with peripheral lung explants from case 126. For case 129, the ratio was 3.3 with peripheral lung and 0.1 with both the urinary bladder and esophagus. However, in

Table 1. Formation of metabolites of [2'-^{14}C]NNN by cultured human tissues

Tissue	Cases, no.	Metabolite, nmol/100 μg of DNA	
		1*	7*
Buccal mucosa	3	0.2 ± 0.2	0.03 ± 0.03
Trachea	3	0.7 ± 0.7	0.2 ± 0.1
Esophagus	9	0.2 ± 0.2	0.4 ± 0.9
Bronchus	6	0.8 ± 1.1	0.9 ± 1.7
Peripheral lung	7	0.5 ± 0.2	0.4 ± 0.7
Urinary bladder	3	2.4 ± 3.4	1.1 ± 1.8
Control†	3	ND‡	ND‡

Tissue explants obtained within 6 hr of death were cultured for 24 hr with [2'-^{14}C]NNN (1 μCi, 3.9 nmol/ml of medium). Explants in each dish contained 50–500 μg of DNA. Values are given as mean ± SD.

* See Fig. 1 for structures.

† Bronchus explants were incubated in boiling water for 30 min prior to culture with [2'-^{14}C]NNN.

‡ ND, not detected; limit of detection was 0.01 nmol of hydroxy acid **7** and 0.3 nmol of NNN-1-N-oxide (**1**), which corresponds to three times the baseline radioactivity in chromatographic system A.

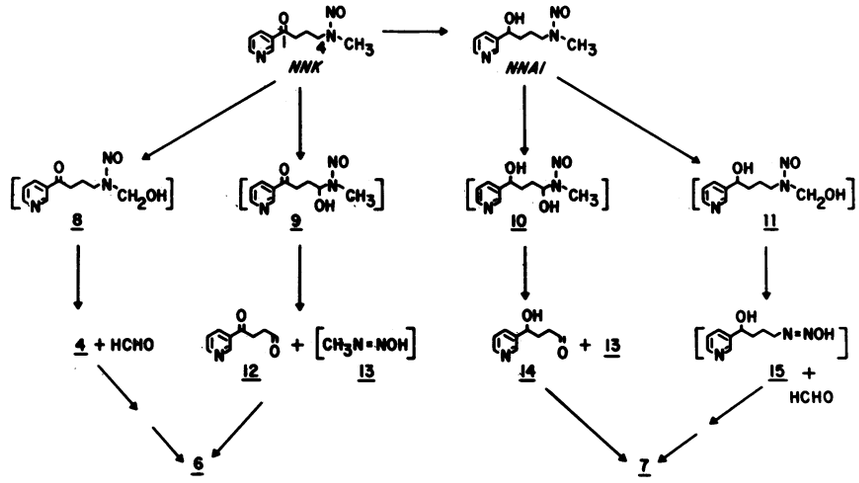

FIG. 3. Metabolic transformations of NNK and NNAl. Structures in brackets are hypothetical intermediates.

most cases, the keto acid **6** could not be detected (Table 1).

The metabolic pathways of NNK in human tissues are illustrated in Fig. 3. Carbonyl reduction of NNK gives NNAl. This metabolite was formed by all human explants and constituted >90% of the amount of radioactivity recovered by HPLC (Fig. 4). Its identity was confirmed by reoxidation to NNK by treatment with CrO_3. After 24 hr, the amount of NNAl present in the medium accounted for 50–80% of the initial amount of NNK and the ratio of NNAl:NNK varied from 30 to 400, depending on the case and the tissue. The rate of formation of NNAl, determined with bronchial explants from case 77, was linear during the first 12 hr and the half-life of NNK was 8 hr. Culture of bronchial explants with medium followed by incubation of the isolated medium with NNK did not result in NNAl formation. Thus, carbonyl reduction of NNK was probably not mediated by enzymes or factors released into the medium. NNAl was not detected when explants of bronchus and peripheral lung tissues were treated with boiling water.

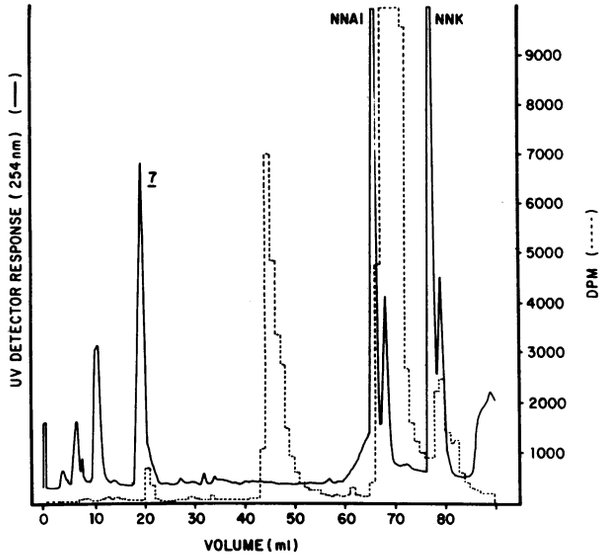

FIG. 4. HPLC profile of NNK and its metabolites formed by bronchus explants of individual 106 cultured with [*carbonyl*-^{14}C]NNK. Unlabeled NNK, NNAl, or **7** was added as a UV marker prior to chromatography with system B (see text). Under these conditions, radioactivity eluted later than the UV markers and the *E* and *Z* isomers of NNK and NNAl were partially separated.

Although α-carbon hydroxylation of NNK would yield the two α-hydroxy-NNK derivatives **8** and **9**, α-carbon hydroxylation of NNAl would give the α-hydroxy-NNAl derivatives **10** and **11** (Fig. 3). Rearrangement of **8** leads to the diazohydroxide **4**, which is also generated by 2′-carbon hydroxylation of NNN (Fig. 1). The potent methylating agent methyldiazohydroxide (**13**) is formed from **9**. As shown in Fig. 3, the keto acid **6** and hydroxy acid **7** are the end products of α-carbon hydroxylation of NNK and NNAl. Levels of **7** represented <0.5% of the final amounts of NNAl present in the media (Table 2). The metabolite eluting at 43–50 ml did not coelute with any known metabolites of NNK, including the pyridine-*N*-oxide of NNAl, and was not characterized. No differences in the metabolism of either NNN or NNK by explants from smokers and nonsmokers were observed (Fig. 2).

All explants remained morphologically normal during the period of culture. Treatment with boiling water destroyed the viability as well as the enzymatic capacities of the explants. No metabolism was observed during incubation of the medium with [2′-^{14}C]NNN and without explants. With tissues from three cases, maintaining the explants in culture for up to 5 days prior to exposure to NNN or NNK had no effect on the extent of metabolism.

Table 2. Formation of metabolites of [*carbonyl*-^{14}C]NNK by cultured human tissues

Tissue	Cases, no.	Metabolite, nmol/100 μg of DNA		
		NNAl*	7*	Unknown
Buccal mucosa	3	470 ± 273	0.26 ± 0.23	3.6 ± 6.2
Trachea	4	315 ± 157	0.24 ± 0.13	19 ± 21
Esophagus	10	210 ± 163	0.14 ± 0.12	3.7 ± 3.0
Bronchus	7	740 ± 581	0.86 ± 0.83	35 ± 23
Peripheral lung	7	705 ± 398	0.37 ± 0.33	20 ± 18
Urinary bladder	6	398 ± 302	0.19 ± 0.28	ND†
Control‡	2	ND†	ND†	ND†

Tissue explants obtained within 6 hr of death were cultured for 24 hr with [*carbonyl*-^{14}C]NNK (1 μCi, 238 nmol/ml of medium). Explants in each dish contained 30–600 μg of DNA. Values are given as mean ± SD.

* See Fig. 3 for structures.

† ND, not detected; limit of detection was 0.01 nmol of NNAl, 0.01 nmol of hydroxy acid **7**, and 0.04 nmol of the unknown metabolite (retention volume = 43–50 ml), which corresponds to three times the baseline radioactivity in chromatographic system B.

‡ Bronchus and peripheral lung explants were incubated in boiling water for 30 min prior to culture with [*carbonyl*-^{14}C]NNK.

No covalent binding of either [2'-^{14}C]NNN or [*carbonyl*-^{14}C]-NNK to DNA of bronchus or peripheral lung explants could be detected. The lower limit of detection was ≈0.05 pmol/μg of DNA.

DISCUSSION

It is estimated that 54 million Americans smoke cigarettes (18). Most individuals have started cigarette smoking by age 20 and have smoked >20 cigarettes per day (19). The mainstream smoke of a typical American filter cigarette contains about 310 ng of NNN and 150 ng of NNK (7). Assuming that 90% of these nitrosamines are absorbed by the oral cavity and respiratory tract tissues during smoking (20), an individual who has smoked 20 cigarettes per day for 40 years has been exposed to 81 mg of NNN and 39 mg of NNK.

The reactive electrophiles generated by α-carbon hydroxylation of NNN and NNK presumably initiate the carcinogenic process (9, 13). The results of the present study demonstrate that tissues of the human oral cavity and respiratory tract that are in contact with tobacco smoke during smoking can activate NNN and NNK to these reactive electrophiles. Target animal tissues also have the ability to metabolize NNN and NNK when they are cultured *in vitro*. During a 24-hr period, the mucosa covering the rat nasal septum can metabolize 100% of the NNN (10 nmol) added to the medium (11). The rat esophagus metabolizes 43% (14) and the mouse lung metabolizes 70% (9) of the initial amount of NNN. In contrast, human tissues such as the buccal mucosa, trachea, and bronchus, which are in contact with tobacco smoke during smoking, could metabolize only 1% of the amount of NNN added to the culture medium.

Harris *et al.* (21) reported a 75-fold variation among individuals in the ability of lung tissues to metabolize benzo(α)pyrene. Results obtained with NNN paralleled these observations. In the present study, levels of α-carbon hydroxylation of NNN by various tissues of the same individuals were generally low and fairly constant (Fig. 2). However, in most tissues from case 129, the levels of α-carbon hydroxylation were consistently high while levels of pyridine *N*-oxidation were comparable to those of other individuals. In most individuals, levels of keto acid **6** were either low or not detectable. However, in case 129, formation of **6** could be clearly demonstrated. The present study suggests that some individuals have a high capacity to activate NNN and NNK. Whether those individuals constitute a subpopulation with a high susceptibility to tobacco-related cancers warrants further study.

NNN was proposed to be an important etiological factor in the high mortality from oral and pharyngeal cancer among snuff dippers (5). NNN, NNK and *N'*-nitrosoanatabine are the only procarcinogens detected in appreciable amounts in snuff (8). The observation in the present study of α-carbon hydroxylation of NNN and NNK by cultured human oral mucosa further supports the hypothesis that tobacco-specific nitrosamines play a role in the etiology of oral cancer among snuff users.

NNK is extensively metabolized by cultured human tissues, with reduction to NNAl being the major metabolic pathway (Table 2). This extent of metabolism by cultured human tissues is the highest ever reported for an *N*-nitrosamine. However, <1% of the initial amount of NNK is α-carbon hydroxylated (Table 2; Fig. 4). The vast difference in these two enzyme activities suggests that NNAl is formed in the oral cavity and respiratory tract tissues and may be a major NNK metabolite circulating in the blood of smokers.

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