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OTHER ARTICLES

GENOTOXICITY STUDY ON NICOTINE AND NICOTINE-DERIVED NITROSAMINE BY ACCELERATOR MASS SPECTROMETRY

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ABSTRACT. We have studied DNA adduction with ^{14}C-labeled nicotine and nicotine-derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), by accelerator mass spectrometry (AMS) in mouse liver at doses equivalent to low-level exposure of humans. The dose ranges of nicotine and NNK administered were from 0.4 μg to 4.0×10^2 μg kg b.w.$^{-1}$, and from 0.1 μg to 2.0×10^4 μg kg b.w.$^{-1}$, respectively. In the exposure of mice to either nicotine or NNK, the number of DNA adducts increased linearly with increasing dose. The detection limit of DNA adducts was 1 adduct per 10^{11} nucleotide molecules. This limit is 1–4 orders of magnitude lower than that of other techniques used for quantification of DNA adducts. The results of our animal experiments enabled us to speculate that nicotine is a potential carcinogen. According to the procedure for ^{14}C-labeled-NNK synthesis, we discuss the ultimate chemical speciation of NNK bound to DNA. From the animal tests we derived a directly perceivable relation between tobacco consumption and DNA adduction as the carcinogenic risk assessment.

INTRODUCTION

Carcinogens are generally metabolically activated electrophilic species covalently bound to nucleophilic sites in DNA, forming adducts (Margison and O'Connor 1979). DNA adduction may be an early detectable and critical step in chemical carcinogenesis. However, the relation between adduct formation and exposure has primarily been established at carcinogen doses much higher than actual human exposure owing to the limitations of detection sensitivity. To date, the most sensitive technique for adduct detection was ^{32}P-postlabeling, which permitted a quantitative measurement at levels >1 adduct per 10^8–10^{10} nucleotides (Gupta 1985). Phillips *et al.* (1988) measured DNA adduction with unidentified mixed polycyclic aromatic compounds in cigarettes in human lung tumors using the ^{32}P-postlabeling method.

Felton *et al.* (1990) at Lawrence Livermore National Laboratory (LLNL) were the first to use AMS with a ^{14}C-labeled carcinogen, 2-amino-3,8-dimethyl-imidazo (4,5-f) quinoxaline (MeIQx), for the quantification of food carcinogen–DNA adducts, with a detection limit of 1 adduct per 10^{11}–10^{12} nucleotides. The introduction of this technique enabled dose-response studies to be carried out over several orders of magnitude, including environmental, low-level doses for many carcinogens. In recent years, Vogel and his colleagues at LLNL have presented more studies on DNA adduction, biodistribution and pharmacokinetics of xenobiotics with AMS (Vogel and Turteltaub 1992; Turteltaub *et al.* 1992; Creek *et al.* 1994; Frantz *et al.* 1995).

Nicotine, 3-(1-methy-2-pyrrolidinyl)-pyridine, is a major alkaloid in tobacco products, typically comprising 1–2 weight % of tobacco. The pharmacological effect of nicotine is a dominant factor for tobacco addiction. So far it is not clear whether nicotine itself is carcinogenic to humans. Nicotine was not mutagenic according to the Ames test, nor did nicotine administered by intraperitoneal injection at a maximum tolerable dose of 0.8 mg kg^{-1} increase urinary mutagenicity in Sprague-Dawley rats (Doolittle *et al.* 1991). However, it has been proposed, but not clearly demonstrated,

that nicotine could convert to carcinogenic nitrosamines in the human body (Hoffmann and Hecht 1985). 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent and predominant genotoxic carcinogen produced by the nitrosation of nicotine and is widely present in both mainstream and sidestream cigarette smoke as well as unburned tobacco. The content of NNK in cigarettes is roughly 1/10,000 of the content of nicotine. Chronic treatment of rodents with NNK resulted in the induction of tumors in the lung, liver, nasal cavity and pancreas (Hoffmann and Hecht 1985; Hecht, Young and Chen 1980). Figure 1 shows the molecular structure of ^{14}C-labeled NNK.

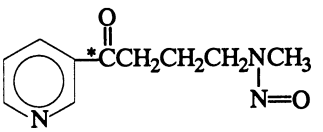

Fig. 1. Molecular structure of NNK with a single ^{14}C atom at 1-position (*)

Unfortunately, the genotoxicity of NNK in humans is difficult to determine accurately. The largest uncertainty results from the fact that studies with laboratory animals are generally carried out at doses well in excess of actual human exposure levels. Radioimmunoassay has been used to detect the methylated DNA adducts in rat lungs following a treatment with NNK at the lowest dose of 0.1 mg kg b.w.$^{-1}$ (Belinsky *et al.* 1990). However, this dose is *ca.* 3×10^3-fold higher than the actual daily exposure to NNK of smokers (Hoffmann and Hecht 1985).

In this study, we report sensitive measurements of DNA adduction with NNK in mice in a wide dose range beginning at a very low level just above the detection limit of our AMS. We affirm the genotoxicity of nicotine in mice at low dose levels with the aid of the high sensitivity of the AMS method.

Methods

^{14}C-nicotine [pyrrolidine-2-^{14}C] is a Dupont-NEN product with very high specific activity of 1.9 GBq mmol^{-1} (51.35 mCi mmol^{-1}), equivalent to ^{14}C in 82.14% of the molecules. We synthesized ^{14}C-NNK (6.59 MBq mmol^{-1}) by the procedure of Decker and Sammeck (1964). Nitrosation of ^{14}C-4-(methylamino)-1-(3-pyridyl)-1-butanone·2HCl forming ^{14}C-NNK was induced by sodium nitrite in the dark. We fed ^{14}C-nicotine and ^{14}C-NNK in 2% ethanol (10 μl g b.w.$^{-1}$) to mice (Kunming, 25–30 g) by stomach intubation (gavage). Doses of nicotine administered were from 0.4 μg to 4.0×10^2 μg per kg b.w., and doses of NNK from 0.1 μg to 2.0×10^4 μg per kg b.w. Mice were sacrificed at 24 h post administration and DNA adducts in the liver were isolated and purified as described by Gupta (1984). DNA purity was assayed by UV spectrometry, $A_{260nm}/A_{280nm} = 1.84 \pm 0.02$, with a standard ratio = 1.82 ± 0.05. Then we converted the purified DNA adducts to graphite samples to be used as ion source in the AMS. The conversion of DNA adducts to graphite was made after Vogel's protocol, consisting of oxidation of DNA to CO_2 by CuO and further reduction of CO_2 to graphite by Zn and TiH_2 in sealed tubes in a vacuum system (Vogel and Turteltaub 1992). The ^{14}C contents were measured by a protocol developed for the 2×6 MV EN Tandem AMS facility at the Institute of Heavy Ion Physics, Peking University. Details on this procedure can be found elsewhere (Chen *et al.* 1990). Our AMS allows measurements of the isotope ratio $^{14}C/^{12}C$ with a sensitivity of 7×10^{-15} and an instrument precision of 1%–4%. The isotope ratio was converted to the number of adducts based on some fundamental data (1 pMC = 5.9×10^{10} C gC^{-1}; 1 μg of DNA = 3240 pmol of nucleotide; carbon content in DNA = 30.0% ± 0.5%).

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RESULTS AND DISCUSSION

By careful handling of the samples (usually 1–3 mg) in disposable labware, the blank pMC of DNA from control mice was 1.19–1.29, proving that DNA isolation and graphite preparation were carried out without detectable ^{14}C contamination. The ^{14}C content in a core of a lump of Chinese anthracite was 0.07 pMC, used as a monitor of any contamination from the biological sample preparation system. We also used graphite from Chinese glucose prepared through the same sample preparation system as an internal standard.

We measured graphite samples from the DNA adducts in mice exposed to nicotine at different doses by AMS and calculated the numbers of DNA adducts. Each datum (o in the figures) was calculated from a mean value of 5–7 runs of AMS measurements. We have achieved a very low detection limit of 1 adduct per 10^{11} nucleotides for ^{14}C-labeled DNA adducts. This detection limit is 1–4 orders of magnitude lower than that of other techniques used for a quantitative assay of DNA adducts, such as ^{32}P-postlabeling, radioimmunoassay or fluorescence spectrometry (Gupta 1985).

Figure 2 shows the dose response of nicotine adducts in mouse liver. DNA adducts increase with increasing dose levels in this log / log presentation. The DNA adduction with toxic chemicals or carcinogens causes genetic changes in cells, and some fixed changes can cause DNA replication errors, resulting in mutations. In some cases, the mutations occur in genes controlling cell proliferation and replication, leading to cancers. It can be concluded that DNA adduction may be an early but critical step in chemical carcinogenesis. Therefore, DNA adducts have been widely accepted as a valid internal dosimeter for monitoring exposure to toxic chemicals and carcinogens (Beland and Poirier 1993; Farmer 1994).

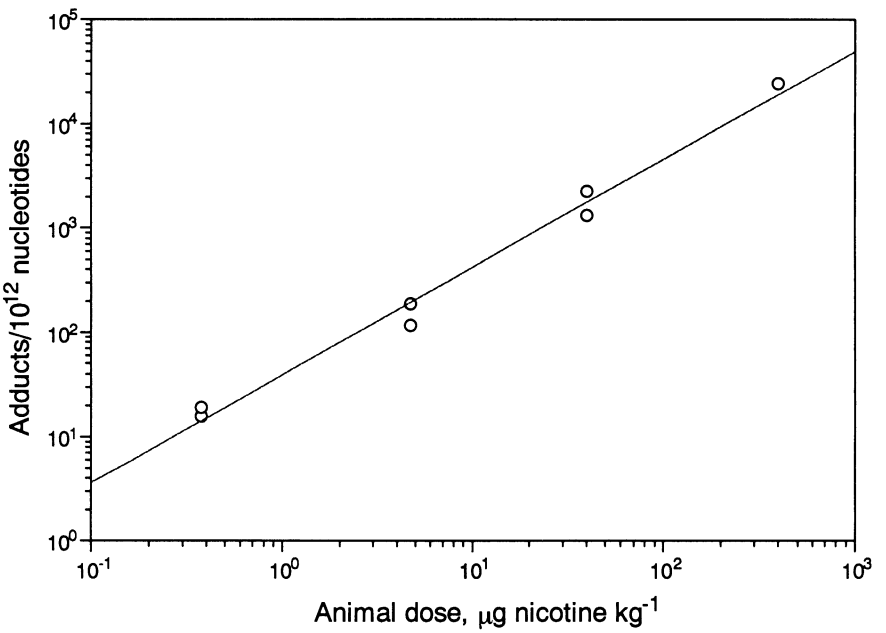

Fig. 2. Influence of nicotine exposure dose on DNA adduct levels. Regression results: $Y = a + bX$, $a = 1.59 \pm 0.07$, $b = 1.03 \pm 0.05$, r = 0.993

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Our findings concerning nicotine-DNA adduction and its good linear dependence on the exposure dose levels raises doubts about previous claims that nicotine is noncarcinogenic. Based on our observations in the preceding paragraph, we speculate that nicotine, besides being a source of conversion to carcinogenic nitrosamines, is likely a potential carcinogen *per se*.

In the case of NNK, we covered a much wider range of doses, namely from 0.1 μg to 2.0×10^4 μg per kg b.w. in measuring NNK-DNA adduction. Figure 3 shows the dependence of DNA adduction on the NNK exposure dose in mice. The lowest dose of NNK, 0.1 μg kg b.w.$^{-1}$, is 3 orders of magnitude lower than that accessible by other techniques (Belinsky *et al.* 1990).

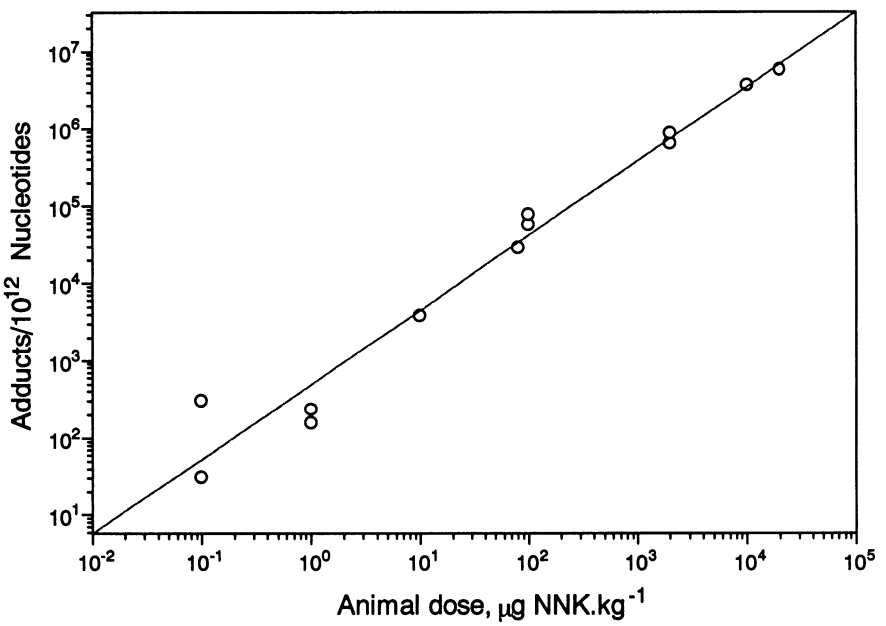

Fig. 3. Influence of NNK exposure dose on DNA adduct levels. Regression results: $Y = a + bX$, $a = 2.69 \pm 0.13$, $b = 0.96 \pm 0.05$, $r = 0.985$.

Although AMS provides no direct structural information on DNA adduction, in our study it yields some evidence on the mechanism of the adduction reaction. Hoffmann and Hecht (1985) proposed that two possible routes may lead NNK to damage DNA: (1) methylation of DNA by metabolite $CH_2N{=}NOH$ at the terminal of NNK; (2) reaction of DNA with the active metabolite 4-(3-pyridyl)-4-oxo-butyldiazohydroxide (Fig. 4). In our study only the ^{14}C-containing portion of NNK bound to the separated hepatic DNA could be detected by AMS, and the labeled NNK was synthesized with ^{14}C in the 1-position attached to the pyridine ring (Fig. 1). Hence, we have obtained an experimental finding to support route (2) in which the final active metabolite of NNK, ^{14}C-labeled-4-(3-pyridyl)-4-oxo-butyldiazohydroxide, combines with DNA. However, our result does not provide any positive or negative evidence for the existence of route (1).

In the animal tests, we created an exposure to nicotine and NNK *via* oral gavage that was similar to the ingestion exposure route in humans by snuff-dipping. Actually, only very few people dip snuff nowadays. Hoffmann and Hecht (1985) estimated that one snuff dipper ingested 10–20 mg of nicotine by consuming 1 g of fine-cut tobacco. We will assume the lower value of 10 mg of nicotine in

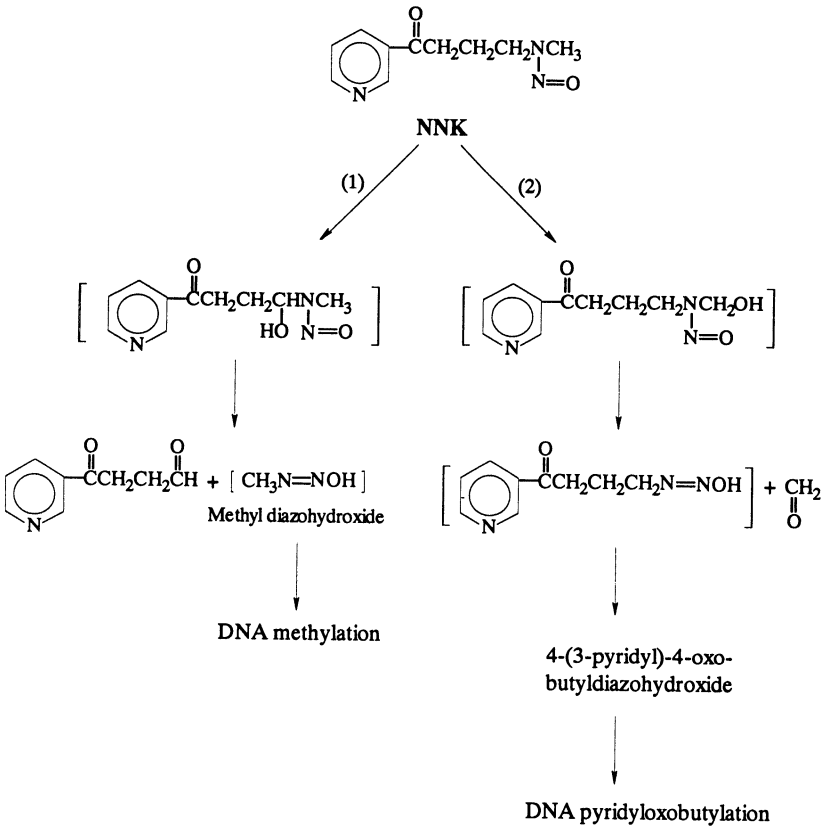

Fig. 4. Metabolic activation of NNK. Structures in brackets are likely intermediates that have not been isolated.

the following discussion. The exposure to NNK of a snuff-dipper consuming 10 g of tobacco was 16.1 μg, averaged from the leading five U.S. fine-cut tobaccos (Brunnemann, Scott and Hoffman 1982). Based on these estimated values, basic human physiological data (3×10^7 DNA/cell, 1.5×10^{13} cells/liver), and the data from the curves in Figure 2 and Figure 3 we can make a carcinogenic risk assessment in terms of a directly perceivable relation between the tobacco consumption and DNA adduction (Table 1).

Our findings show that AMS provides a very sensitive tool for the quantification of DNA adducts using ^{14}C tagged xenobiotics. Furthermore, it is evident that AMS would be a potential tool in biomedical research (Liu *et al.* 1994), particularly screening for genotoxicity and making risk assessment of drugs and chemicals at environmental exposure levels (Weisman 1996).

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TABLE 1. Exposure Dose Response of DNA Adduction in a Snuff-Dipping Person (70 kg b.w.)

Chemicals	Tobacco consumed (g)	Exposure dose (μg/kg b.w.)	Adducts / 10^{12} DNA	Adducts / liver cell	Adducts / liver	Equivalent consumption of cigarettes*
Nicotine	1	1.4×10^{2}†	7.2×10^{3}	2.2×10^{-1}	3.3×10^{12}	7‡
NNK	10	2.3×10^{-1}§	6.6×10^{1}	2.0×10^{-3}	3.0×10^{10}	111#

*By the exposure route of mainstream smoking inhalation.

†10 mg / 70 kg = 143 μg/kg

‡In the mainstream smoke of one brand of U.S. commercial filter cigarettes, the nicotine level is 1.5 mg per cigarette (Adams, O'Mara-Adams and Hoffman 1987), and the exposure of nicotine to a snuff-dipper consuming 1 g of fine-cut tobacco is 10 mg. Therefore, 10 mg / 1.5 mg = 6.66.

§16.1 μg / 70 kg = 0.23 μg/kg.

#The estimated exposure of a person to NNK of 20 cigarettes by the smoking inhalation route is 2.9 μg, equivalent to 0.145 μg per cigarette, and the exposure to NNK of 10 g tobacco by the snuff-dipping ingestion route is 16.1 μg (Hoffmann and Hecht 1985). Therefore, the equivalent consumption from smoking cigarettes is 16.1 μg/ 0.145 μg = 111.

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