Formation of 4-(Methylnitrosamino)-4-(3-pyridyl)butyric Acid in Vitro and in Mainstream Cigarette Smoke†

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During tobacco growing, processing, and smoking, nicotine, a major tobacco alkaloid, gives rise to five *N*-nitrosamines including 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC). In this study we have shown that iso-NNAC was not detected in the mainstream smoke (MS) of a blended U.S. cigarette (<1 ng/cigarette) but it occurs in minute amounts (3 ng/cigarette) in the MS of a French dark tobacco cigarette which is rich in nitrate and other nicotine-derived *N*-nitrosamines. In the sidestream smoke (SS) of the French cigarette, the biologically highly active tobacco-specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone was present in significantly higher concentration than in MS, as was the case with iso-NNAC, amounting to 7 ng/cigarette in comparison to 3 ng in the MS. Spiking of cigarettes with nicotine (10 mg/cigarette), nornicotine (1 mg), cotinine (2 mg), and cotinine acid (0.05 mg) did not significantly increase iso-NNAC in MS. The transfer rate for iso-NNAC, determined by spiking each of the blended U.S. cigarettes and the French cigarettes with 0.1 mg of this synthetic nitrosamino acid, was between 0.8 and 1.0%. These findings support the hypothesis that the presence of iso-NNAC in physiological fluids of cigarette smokers is indicative of endogenous formation of nicotine-derived tobacco-specific *N*-nitrosamines. Accordingly, the compound may serve as a biomarker of endogenous *N*-nitrosamine formation. In vitro nitrosation of nicotine at pH 7 and 37 °C does not give rise to iso-NNAC, while its major metabolites, cotinine and cotinine acid, do.

Nicotine gives rise to specific *N*-nitrosamines during tobacco growing, curing, processing, and aging. (Hecht and Hoffmann, 1988; Burton et al., 1989a; Djordjevic et al., 1989b; Andersen et al., 1989). Three of the tobacco-specific nitrosamines (TSNA) derived from nicotine (Figure 1), namely, *N'*-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), are highly carcinogenic in mice, rats, and hamsters; 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC) is not carcinogenic, and it is not known whether 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL) is carcinogenic (Hecht and Hoffmann, 1988; Rivenson et al., 1988, 1989). However, Brunnemann et al. (1987) found that iso-NNAL is genotoxic in primary rat hepatocytes. Nicotine is also a precursor for TSNA which are formed during tobacco chewing and snuff dipping (Wenke et al., 1984; Nair et al., 1985; Carmella et al., 1990). Compared to nonsmokers, cigarette smokers have an increased potential for the endogenous N-nitrosation of amines as was shown by an increased urinary excretion of *N*-nitrosoproline and *N*-nitrosothioproline (Hoffmann and Brunnemann, 1983; Ladd et al., 1984; Tsuda et al., 1986; Scherer and Adlkofer, 1986). However, there are no data which demonstrate unambiguously that endogenous nitrosation of nicotine, inhaled with tobacco smoke, does actually occur. Recently, we have identified the noncarcinogenic iso-NNAC in processed tobacco, though not in the mainstream smoke (MS) of blended cigarettes (Djordjevic et al., 1989a). This finding leads to the hypothesis that urinary excretion of iso-NNAC by cigarette smokers may be an indicator of endogenous formation of TSNA from inhaled nicotine. Iso-NNAC could be formed endogenously by direct oxidative nitrosation of nicotine via 4-(methylnitrosamino)4-(3-pyridyl)butanal (NNA, Figure 1) and/or by nitrosation of the major nicotine metabolite, cotinine, and its hydrolysis product, 4-(methylamino)-4-(3-pyridyl)butyric acid (cotinine acid).

Before exploring the endogenous formation of TSNA in cigarette smokers with iso-NNAC as a marker, we studied (i) the in vitro formation of iso-NNAC from nicotine, from cotinine, and from cotinine acid with sodium nitrite at pH 7, 37 °C; (ii) the occurrence of TSNA, including iso-NNAC, in tobacco, MS, and sidestream smoke (SS) of commercially available nonfilter cigarettes; and (iii) the transfer rate of iso-NNAC from tobacco into MS and its formation during smoking. For the latter test, we employed nonfilter cigarettes spiked with synthetic iso-NNAC and with the potential alkaloid precursors, respectively.

MATERIALS AND METHODS

Apparatus. TSNA and iso-NNAC were separated and quantified on a Hewlett-Packard Model 5890 gas chromatograph interfaced with a Model 610 thermal energy analyzer (TEA) (Thermo Electron Corp., Waltham, MA) with a modification described earlier (Brunnemann and Hoffmann, 1981) and a Model 3390A integrator (Hewlett-Packard, Paramus, NJ). Tobacco alkaloids were analyzed on a Hewlett-Packard Model 5890 gas chromatograph equipped with a thermionic N-P-specific detector (NPD) and interfaced with a Hewlett-Packard Model 3390A integrator. Nitrate and nitrite nitrogen analyses were done by using a Technicon Auto-Analyzer System II with a 50-mm flow cell in the colorimeter (Crutchfield and Burton, 1989). MS was generated with a Borgwaldt RM 20/CS 20-port smoking machine with rotating head (Heinrich Borgwaldt, Hamburg, FRG) modified as described previously (Hoffmann et al., 1983). The total particulate matter (TPM) of the MS was collected on 92-mm Cambridge filters (CF) (Djordjevic et al., 1989a). SS was collected on 44-mm CF by using the device described by Neurath and Ehmke (1964) and modified by Brunnemann and Hoffmann (1974), in conjunction with a single-port piston-type smoking machine (Borgwaldt). A purge flow rate of 1.5 L of air/min guaranteed that MS deliveries of tar were equivalent to those generated when the cigarette is smoked freely without the containment device required for the collection of SS. The Dub-

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