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N-METHYL-β-CARBOLINE-3-CARBOXAMIDE (FG 7142): AN ANXIOGENIC AGENT IN CIGARETTE SMOKE CONDENSATE AND ITS MECHANISM OF FORMATION

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Abstract

β-Carboline-3-carboxylic acid methylamide (FG 7142), an anxiogenic agent has been found in cigarette smoke condensate, but not in the cigarette itself. When a cigarette, except its filter portion, was immersed in 20 ml of potassium phosphate buffer, pH 7·4, then heated at 60°C for 2 days with or without presence of methylamine, FG 7142 was detected only in the mixture containing methylamine. Furthermore, when the mixtures of β-carboline derivatives and various amounts of methylamine hydrochloride were heated at 60°C for 5 days, FG 7142 was formed only in the mixtures containing methylamine and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-caroxylic acid (MTCA) or 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (TCCA). FG 7142 was also produced in the mixture of glucose, L-tryptophan and methylamine when heated at 200°C in a dry condition. These observations suggest that FG 7142 is formed through the smoking process and that methylamine in cigarette smoke may play an important role in the formation of FG 7142.

Keywords: β-Carboline, FG 7142, anxiogenic agent, cigarette smoke.

INTRODUCTION

β-Carboline-3-carboxylic acid methylamide (F 7142) is a representative of a class of non-benzodiazepine agents, which bind to the benzodiazepine receptor with high affinity, but have actions generally opposite to those of benzodiazepine agonists (Corda *et al.*, 1983; Petersen & Jensen, 1984; Corda & Biggio, 1986). FG 7142, classified as a partial inverse agonist at benzodiazepine receptors, produces a strong anxiety-like syndrome in experimental animals (File *et al.*, 1982; Petersen *et al.*, 1982; Crawley *et al.*, 1985; Kalin *et al.*, 1992). The anxiogenic effect of FG 7142 has also been found in man (Dorow *et al.*, 1983). Dorow and his coworkers reported that when administered to human volunteers, FG 7142 produced severe anxiety attacks in cases where plasma concentrations of the drug were high. Moreover, the effects on the central nervous system, vegetative and cardiovascular symptoms, and hormonal changes are the same as those described in patients with severe anxiety and panic attacks (Dorow *et al.*, 1983). In addition to the anxiogenic effect of FG 7142, the inverse agonists at benzodiazepine receptors show an anorectic effect on rats: when administered intraperitoneally to rats, FG 7142 brought about a reduction in food consumption (Cooper *et al.*, 1985; Cooper, 1987). Moreover, previous research demonstrated that low doses of FG 7142 improved performance in various learning and memory tests in animals if administered prior to training (Venault *et al.*, 1986; File & Pellow, 1988; Holmes & Drugan, 1991).

There are persistent reports that tobacco smoke can exert a 'tranquilizing' effect (Gilbert, 1979; Pomerleau & Pomerleau, 1987; Costall *et al.*, 1989). In many tests for anxiolytic activity, the effects of nicotine do not resemble those of established anxiolytic drugs such as diazepam (Tallman & Gallager, 1985; Balfour, 1991). It is likely that neural systems other than those which mediate the response to the benzodiazepines are responsible for the putative anxiolytic properties of nicotine (Balfour, 1991).

In contrast to the anxiolytic activity of nicotine, smoking offsets the sedative effects of benzodiazepines and neuroleptics such as diazepam and chlorpromazine (Swett, 1974; Miller, 1977; Pantuck *et al.*, 1982). This effect of smoking might be explained by the fact that smoking decreases the blood levels of many psychoactive drugs (Dawson *et al.*, 1984). However, it has been suggested that cigarette smoking does not enhance the overall rate of metabolism of chlorpromazine (Pantuck *et al.*, 1982). It may be possible that smoking alters the pattern of metabolism of the drug. However, we have to consider another possibility, that certain substances in tobacco smoke may reduce the actions of benzodiazepines and neuroleptics, since antagonists of benzodiazepine receptors such as norharman are reported to be present in cigarette smoke (Poindexter & Carpenter, 1962; Snook & Chortyk, 1984; Morin, 1984; Borea & Ferretti, 1986).

Tobacco smoke contains more than 3800 constituents (IARC, 1985). The formation and identification of biologically active agents in tobacco smoke has

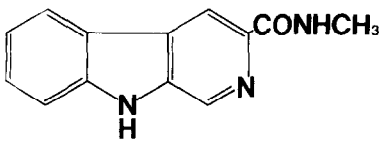

Fig. 1. Chemical structure of FG 7142.

been the subject of extensive laboratory studies. These agents include carbon monoxide, ammonia, volatile amines, volatile aldehydes, benzene, hydrogen cyanide, *N*-nitrosamine, nicotine, phenols, aromatic amines, polynuclear aromatic compounds, heterocyclic amines, and trace metals (IARC, 1985). As far as we know, there has been no report on the presence of FG 7142, an anxiogenic β-carboline derivative in tobacco smoke, although it has been reported that cigarette smoke contains β-carboline derivatives such as norharman and 1-methyl-β-carboline (harman) (Poindexter & Carpenter, 1962; Snook & Chortyk, 1984; IARC, 1985). In this investigation, it has been demonstrated that FG 7142 is present in cigarette smoke, but not in the cigarette itself. Moreover, the mechanism of formation of FG 7142 in cigarette smoke has been discussed. The chemical structure of FG 7142 is shown in Fig. 1.

MATERIALS AND METHODS

Extraction of FG 7142 in cigarette smoke

Commercially available cigarettes (A–C, Japanese brands; D,E, US brands; F,G, UK brands) were used in this study. The filter-tipped cigarettes were smoked by an automatic smoking machine (Filtrona Model 302) under standard conditions (puff frequency, 1 puff/60 s; puff volume, 35 ml/2 s; butt length 23 mm) (IARC, 1985). The cigarette smoke condensate from one cigarette was collected on a glass filter (Toyo Roshi Type GA 200, Tokyo Roshi, Tokyo, Japan) and extracted once with 100 ml of methylene chloride in an ultrasonic bath (Manabe *et al.*, 1990, 1991). The methylene chloride extract was condensed to ~20 ml with nitrogen gas. FG 7142 in the methylene chloride extract was further extracted with a disposable solid-phase column, the Mega Bond Elut SI column (Varian, Harbor City (VA, USA). The methylene chloride extract was applied on a Bond Elut SI column and eluted with 4 ml of methanol. The eluate was evaporated to dryness under a nitrogen stream. The eluate was redissolved in 4 ml of 20 mM H_3PO_4/20 mM $NH_4H_2PO_4$/acetonitrile (45/45/15, V/V/V) and filtered with a disposable filter unit (0·45 μm, Gelman Science Japan, Tokyo, Japan). One-fortieth of the filtrate (100 μl) was used for HPLC analyses owing to the high concentration of FG 7142.

In order to determine whether FG 7142 is present in the cigarette itself, the methylene chloride extract was prepared from US cigarettes (brand E). A cigarette sample was broken to pieces, and the broken cigarette except its filter portion was immersed in 100 ml of methylene chloride and left overnight at room temperature. Procedures which follow are similar to those described above.

In-vitro formation of FG 7142

In order to determine the mechanism of FG 7142 formation, the following experiments were undertaken. First, the role of methylamine on FG 7142 formation was studied because it was assumed that FG 7142 might be formed as a result of the reaction between β-carbolines which have the carboxylate moiety and methylamine, and also because FG 7142 was not detected in the cigarette itself although the anxiogenic β-carboline was detected in cigarette smoke. A cigarette (brand E) was shredded by hand and the filter removed. It was immersed in 20 ml of 50 mM potassium phosphate buffer, pH 7·4. Then, the mixture was heated at 60°C for 2 days with or without methylamine hydrochloride. FG 7142 formed in the mixtures was extracted with methylene chloride.

In order to determine whether β-carbolines bearing the carboxylate moiety could be the precursor(s) of FG 7142, various β-carboline derivatives including (–)-(1S,3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) and (–)-(3S)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (TCCA) were heated in the presence of methylamine. MTCA is formed as a result of the condensation reaction between L-tryptophan and acetaldehyde while TCCA is formed as a result of the reaction between L-tryptophan and formaldehyde. These reactions are known as Pictet–Spengler condensation. MTCA and TCCA were synthesized by the method of Brossi *et al.* (1973). The mixtures of β-carboline derivatives (0·5 mmol) and various amounts of methylamine hydrochloride in 10 ml of 50 mM potassium phosphate buffer, pH 7·4, were heated at 60°C for 10 days. Then, FG 7142 formed in the mixture was extracted with methylene chloride.

The results obtained by the experiments described above suggested to us that L-tryptophan, aldehyde (e.g. acetaldehyde) and methylamine could be the precursors of FG 7142. Moreover, our previous studies suggested that the mixture of amino acid (phenylalanine) and sugars is the source of aldehyde when heated (Manabe *et al.*, 1992, 1993). This reaction is well known as the Akabori amino acid reaction, i.e. aldehydes are formed by the oxidative decomposition of α-amino acids when heated with sugars. Therefore, mixtures of L-tryptophan (0·5 mmol), glucose (0·5 mmol) and various amounts of methylamine were heated in glass vials at 200°C in a dry condition. After heating at 200°C, the mixture was dissolved in 30 ml of 50 mM phosphate buffer, pH 7·4. Then, the solution was extracted with 30 ml methylene chloride. The methylene chloride extract was condensed to ~10 ml with nitrogen gas. The condensed extract was further extracted with disposable solid-phase column, as described above.

HPLC analyses

Reversed-phase HPLC analyses were performed by

means of a Hitachi 655A chromatograph (Hitachi, Tokyo, Japan). Partial purification was carried out using an ES-502C column (9·0 μm particle size, 7·6 × 100 mm, Showa Denko, Tokyo, Japan) under the following conditions: mobile phase, 20 mM H_3PO_4/20 mM $NH_4H_2PO_4$/acetonitrile (45/45/15, V/V/V); and flow rate, 1 ml/min at 50°C. Fluorescence was monitored at 454 nm, and the excitation wavelength was 279 nm. The fraction corresponding to FG 7142 was collected. Then, acetonitrile in this fraction was evaporated off with a centrifugal evaporator EC-57 (Sakuma Seisakusho, Tokyo, Japan). Final HPLC analysis was performed on a Kaseisorb LC ODS-300-5 column (5 μm particle size and 300 Å pore size, 7·5 × 250 mm; Tokyo Chemical Industries Ltd, Tokyo). The mobile phase was a linear gradient (0–30%) of acetonitrile in 10 mM H_3PO_4 over 30 min. The flow rate was 3·0 ml/min at 50°C. Fluorescence was monitored as described above.

Spectrometric analyses

Characterization of the fraction corresponding to FG 7142 was performed y spectrometric analyses. Cigarette smoke condensate collected from 100 cigarettes of brand E was processed as described above, and the fraction corresponding to FG 7142 was extracted with chloroform after the acetonitrile in the fraction had been evaporated and the pH adjusted to 7~8 with 28% ammonia water. UV absorbance and fluorescence spectra were measured with a Shimadzu UV 260 spectrophotometer (Shimadzu, Tokyo, Japan). Mass spectral analyses were conducted in the electron impact (EI) mode using the direct insertion probe on a Hitachi M-2500 mass spectrometer (Hitachi). Mass spectra were recorded with a Hitachi M-3001 mass data analysis system, employing a 70 eV ionization voltage, 100 μA ionization current and 4 kV accelerating voltage with a core temperature of 220°C.

RESULTS

HPLC analyses of FG 7142 in cigarette smoke

Typical charts of HPLC are shown in Fig. 2. A chromatogram of the first-step purification is shown in Fig. 2(A), where 1/40 of the extract from the mainstream smoke of a Japanese filter cigarette was analyzed owing to the high concentration of FG 7142. In the final-step analysis, a sharp peak corresponding to FG 7142 was clearly identifiable (Fig. 2(B)). The detection limit for FG 7142 was 15 fmol (3·4 pg). The fraction corresponding to FG 7142 was collected and examined by spectrometric analyses. The compound purified with HPLC was confirmed to be FG 7142 by its fluorescence spectrum (not shown), absorbance spectrum (Fig. 3) and mass spectrum (Fig. 4).

Recovery experiments were conducted as follows. The amount of FG 7142 in a cigarette smoke sample was determined. A sufficient amount of FG 7142 was then added to the condensate to double the amount present. The spiked sample was redetermined. The diff-

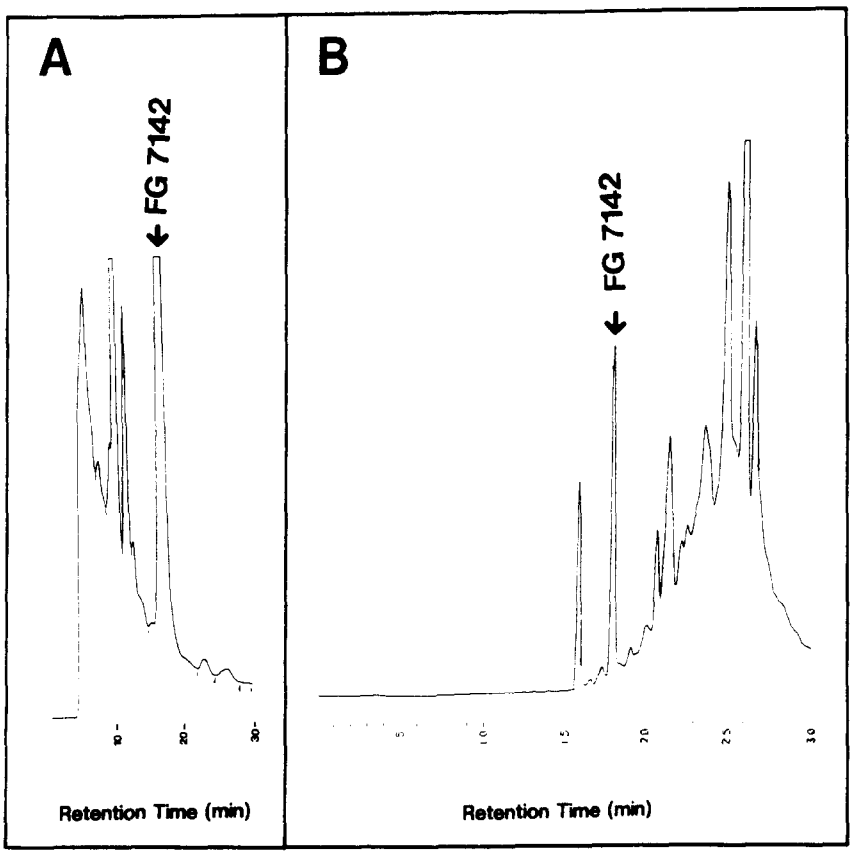

Fig. 2. HPLC chromatographic profiles of FG 7142 in cigarette smoke condensate (brand E). Fluorescence was monitored at 454 nm when excited at 279 nm. (A) Preparative chromatogram of cigarette smoke condensate on an ES-502C column. (B) Final chromatographic profile on an ODS-300-5 column.

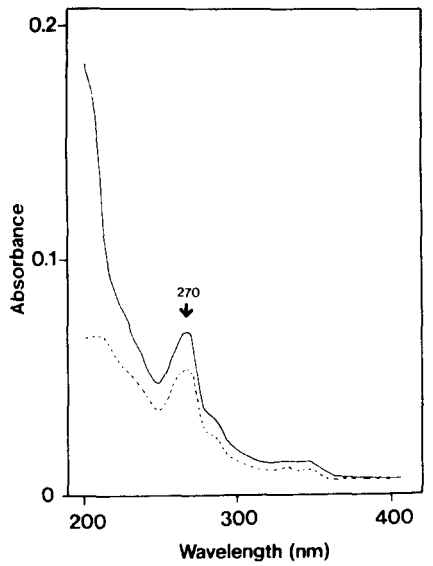

Fig. 3. Absorbance spectra of the fraction corresponding to FG 7142 in cigarette smoke condensate (——) and authentic FG 7142 (1·0 μM) (– – –). The compound purified with HPLC was dissolved in 1 ml methanol.

erence between the first and second determinations was divided by the added amount to give the recovery rate (70·4 ± 2·8%, mean ± SD, n = 5). Table 1 presents the corrected data for the recovery rate. The mean amount of FG 7142 in cigarette smoke was 4·04 ng (18 pmol) per cigarette.

In addition, FG 7142 was not detected in the extracts from any of the brands of cigarettes tested.

Effect of methylamine on FG 7142 formation

A cigarette (brand E) except for its filter portion was heated in 20 ml of 50 mM potassium phosphate buffer, pH 7·4 with various amounts of methylamine hydrochloride for 2 days. As shown in Table 2, FG 7142 was detected in the mixtures containing methylamine hydrochloride. The yield of FG 7142 in the mixtures was dependent on the amount of methylamine hydrochloride added. Among the mixtures studied, that containing 2 mmol methylamine hydrochloride showed the highest yield of FG 7142 . However, the amount of FG 7142 (7·1 pmol) formed in the mixture was lower than half of the mean amount of FG 7142 detected in cigarette smoke (18 pmol) (Table 1).

Formation of FG 7142 in the mixtures of β-carbolines and methylamine

Various β-carbolines including TCCA and MTCA were heated with or without methylamine hydrochloride at 60°C for 5 days. As shown in Table 3, FG 7142 was formed in the mixtures containing TCCA and MTCA in the presence of methylamine although FG 7142 was not detected in the mixture containing β-carolines which do not possess a carboxylate moiety. Furthermore, the yields of FG 7142 in the mixtures of methylamine and TCCA were higher than those in the mixtures of methylamine and MTCA.

Formation of FG 7142 in the mixtures of L-tryptophan, glucose and methylamine

The mixtures of L-tryptophan (0·5 mmol), glucose (0·5 mmol) and various amounts of methylamine hydrochloride were heated at 200°C in a dry condition. As shown in Table 4, FG 7142 was formed in the mixtures containing methylamine. The yields of FG 7142 in the

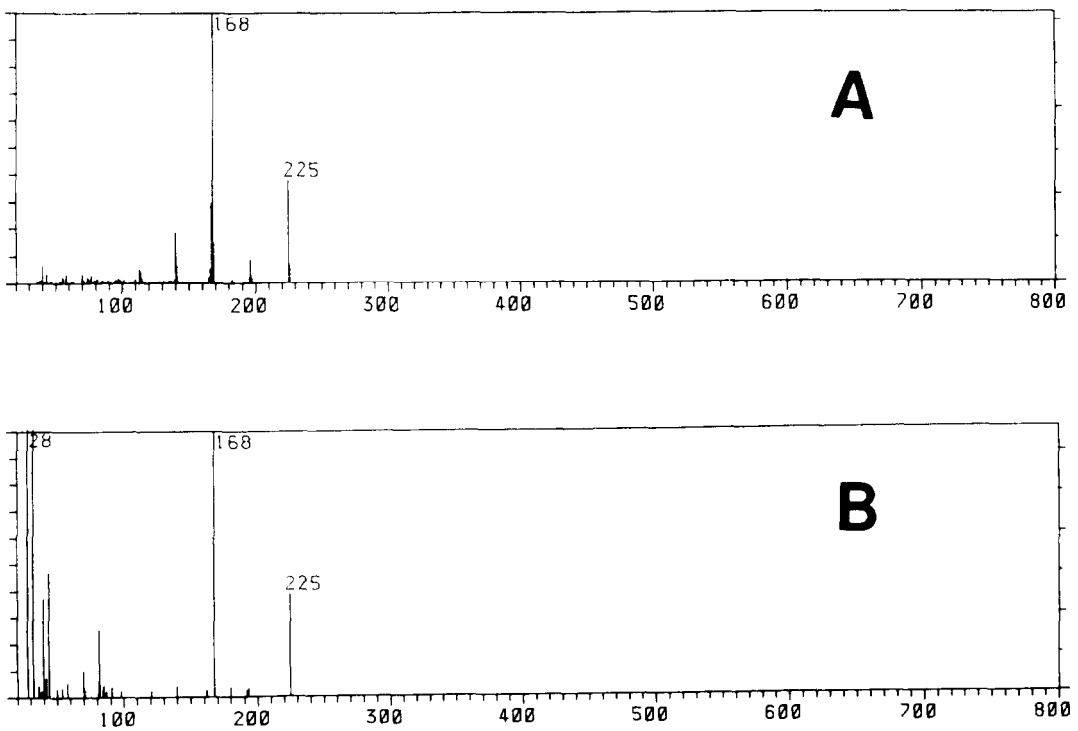

Fig. 4. Mass spectra of (A) FG 7142 fraction in cigarette smoke condensate collected from 100 cigarettes of brand E and (B) synthetic FG 7142.

Table 1. FG 7142 in a condensate of cigarette mainstream smoke

Tobacco product	n^a	FG 7142 (ng/cigarette)
Japenses filter cigarette		
A	5	3·82 ± 0·19[b]
B	5	4·50 ± 0·31
C	5	4·48 ± 0·19
US filter cigarette		
D	5	2·73 ± 0·27
E	5	4·17 ± 0·19
UK filter cigarette		
F	5	3·90 ± 0·29
G	5	4·70 ± 0·20
Mean ± SD		4·04 ± 0·65

[a]Number of cigarettes tested.
[b]Values were corrected for recovery rate (70·4%) and presented as mean ± SD.

mixtures were dependent on the amounts of methylamine added when assessed after heating for 60 min.

DISCUSSION

β-Carbolines are formed as a result of the Pictet–Spengler condensation between an indolamine (e.g. tryptophan) and aldehydes (e.g. acetaldehyde), a reaction that occurs non-enzymatically at room temperature. They are found in many plants, some of which have been used as hallucinogens and drugs (Airaksinen & Kari, 1981). They also occur as minor constituents in toacco smoke (IARC, 1985), although there has been no report on the presence of FG 7142 in tobacco smoke. In this experiment, it was demonstrated that FG 7142 was present in cigarette smoke (Table 1). However, it should be noted that FG 7142 was not detected in the cigarette itself. These results suggest that smokers are persistently exposed to FG 7142, an anxiogenic agent together with various chemical compounds. Moreover, our data indicate that FG 7142 is formed during the smoking process. It was speculated that precursors of FG 7142 may be present in tobacco leaves. Since FG 7142 has a carboxamide moiety in its chemical structure (Fig. 1), it is assumed that the introduction of the methylamino group into the carboxylate moiety in β-carbolines is essential for the formation of FG 7142. Methylamine is used in organic synthesis for introducing the methylamino group. Moreover, it is known that various volatile amines including methylamine are present in cigarette smoke. Among them, methylamine is relatively abundant and its content in cigarette smoke is reported to be ~5 μg (~0·16 μmol) per cigarette (IARC, 1985).

First, the effect of methylamine on the formation of FG 7142 was investigated by immersing a cigarette, except its filter portion, in 50 mM phosphate buffer, pH 7·4. As shown in Table 2, the addition of methylamine induced the formation of FG 7142 in a dose-dependent manner. This indicates that the precursor(s) of FG

Table 2. Effects of methylamine on the formation of FG 7142 in cigarettes immersed in a phosphate buffer

Amount of methylamine added (mmol)	FG 7142 (pmol)
0	ND[a]
0·2	1·4[b]
0·5	2·1
1	4·0
2	7·1

A cigarette (brand E) except its filter portion was immersed in 20 ml of 50 mM potassium phosphate buffer, pH 7·4, then heated at 60°C for 2 days with or without methylamine hydrochloride.
[a]Not detected.
[b]Each value represents the mean of two experiments and is corrected for recovery rate.

Table 3. Formation of FG 7142 in a mixture of a β-carboline derivative and methylamine during heating

β-Carbolines	Amount of methylamine added (mmol)	FG 7142 (pmol) heating time (days)	
		2	5
THBC	0	ND[a]	ND
	0·2	ND	ND
	1·0	ND	ND
TCCA	0	ND	ND
	0·2	0·5	241·1
	1·0	33·5	237·7
MTCA	0	ND	ND
	0·2	0·6	3·0
	1·0	1·2	4·2
Norharman	0	ND	ND
	0·2	ND	ND
	1·0	ND	ND
Harman	0	ND	ND
	0·2	ND	ND
	1·0	ND	ND
Harmine	0	ND	ND
	0·2	ND	ND
	1·0	ND	ND

The mixtures of β-carboline derivatives (0·5 mmol) and various amounts of methylamine hydrochloride in 10 ml potassium phosphate buffer, pH 7·4 were heated at 60°C for 5 days.
Abbreviations: THBC, 1,2,3,4-tetrahydro-β-carbolines; TCCA, 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid; MTCA, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid.
[a]Not detected.

Table 4. Formation of FG 7142 in the mixture of L-tryptophan (0·5 mmol), glucose (0·5 mmol) and methylamine when heated at 200°C under dry conditions

Amount of methylamine added (mmol)	FG 7142 (pmol) heating time (min)	
	60	90
0	ND[a]	ND
0·2	15·2[b]	195·8
0·5	61·8	743·4
1	119·1	1253·3
2	122·4	895·1

[a]Not detected.
[b]Each value represents the mean of two experiments and is corrected for recovery rate.

7142 are present in the extract of tobacco and that the precursors are β-carbolines which possess a carboxylate moiety. In order to confirm the hypothesis, β-carboline derivatives such as (–)-(3S)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (TCCA) and (–)-(1S,3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) were heated with or without methylamine. As shown in Table 3, FG 7142 was formed only in the mixture of methylamine and TCCA and that of methylamine and MTCA when heated at 60°C. This finding supports the hypothesis that FG 7142 is formed by introducing the methylamino group into the carboxylate moiety in β-carbolines such as TCCA and MTCA. Therefore, it is suggested that TCCA and MTCA are the precursors of FG 7142 in cigarette smoke, although the presence of these β-carbolines in tobacco and tobacco smoke has not yet been reported.

TCCA and MTCA are formed as a result of condensation between L-tryptophan and aldehydes such as formaldehyde and acetaldehyde. Such reaction occurs readily. Aldehydes in cigarette smoke are formed by nonenzymatic browning reactions, or during smoking from precursors such as polysaccharides, pectins, proteins and possibly, triglycerides in tobacco (Brunnemann & Hoffman, 1982; IARC, 1985). Aldehydes are relatively abundant in cigarette smoke. For example, contents of formaldehyde and acetaldehyde are 3~40 μg and 519~1144 μg per cigarette, respectively (IARC, 1985). Therefore, we cannot exclude the possibility that FG 7142 may be formed as a result of the reaction among tryptophan, aldehyde and methylamine during smoking, not only because aldehydes such as formaldehyde and acetaldehyde and methylamine are relatively abundant in cigarette smoke (IARC, 1985), but also because β-carbolines such as norharman and 1-methyl-β-carboline (harman) are thought to be formed during smoking (IARC, 1985). As shown in Table 4, FG 7142 was produced in the mixture of glucose, L-tryptophan and methylamine when heated at 200°C. Our previous studies suggest that the mixtures of amino acid (phenylalanine) and various sugars can be the sources of aldehyde when heated (Manabe *et al.*, 1992, 1993). Thus, the results suggest the possibility that FG 7142 may be formed as a result of the reaction among tryptophan, aldehyde and methylamine during smoking.

On the other hand, the possibility of potential artefactual formation of FG 7142 during collection of the smoke condensate may have to be considered, since all precursors are present in cigarette smoke. However, FG 7142 is detected in indoor air as well as in the air of the outdoor environment. Levels of this compound in indoor air were much higher than in outdoor air (unpublished data). Therefore, the possibility of potential artefactual formation of FG 7142 during collection of the smoke condensate may be ruled out.

The mean level of FG 7142 in cigarette mainstream smoke is approximately 4 ng (18 pmol) per cigarette (Table 1). Even when it is assumed that 20 cigarettes are smoked for a short period of time and that FG 7142 in cigarette smoke is absorbed completely from the airway and lungs, the plasma concentration of FG 7142 may be lower than 20 ng/l. This level is apparently lower than that which induces anxiety in humans, since severe anxiety was observed only in cases where plasma concentrations of FG 7142 were above 150 ng/ml (Dorow *et al.*, 1983). However, the possibility that FG 7142 in cigarette smoke may modulate the psychopharmacological effects of smoking on humans cannot be excluded. Further studies are needed to determine the exposure level of FG 7142 in smokers. In addition to this, it is important to determine whether or not FG 7142 in cigarette smoke is related to the pathogenesis of various symptoms found in smokers, since FG 7142 appears to have a broad range of effects, such as anxiogenic action, elevation of cortisol, growth hormone and prolactin in humans (Dorow *et al.*, 1983), anorectic action (Cooper, 1987) and improvement of performance in learning and memory tests in animals (Venault *et al.*, 1986; File & Pellow, 1988; Holmes & Drugan, 1991).

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