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Short Communication

FORMATION OF TETRAHYDRO-β-CARBOLINES IN HUMAN SALIVA

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Abstract—When human saliva obtained after cigarette smoking was incubated in the presence of tryptamine, the formation of 1,2,3,4-tetrahydro-β-carboline (TBC) and 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTBC) was observed in a short time. After incubation with tryptamine (2.5 μg/mL) for 10 min, the concentrations of TBC and MTBC formed were 3.27 ± 0.94 and 0.35 ± 0.17 ng/mL, respectively. The formation of TBC and MTBC in intact saliva and in saliva heated at 100° for 10 min was compared, but no significant difference was found. The analysis of foodstuffs showed that significant amounts of tryptamine were contained in various foods and beverages. The analysis of cigarette smoke solutions and immersion solutions of denture-base acrylic resins showed that ng-μg/mL levels of formaldehyde and acetaldehyde were contained in cigarette smoke and leached from dental resins. These results indicate that both precursors, tryptamine and aldehydes, coexist in oral environments and that their interaction to form TBC and MTBC potentially occurs in human saliva without participation of salivary enzyme.

Key words: tetrahydro-β-carboline; tryptamine; aldehyde; human saliva; condensation

Since tetrahydro-β-carbolines and related alkaloids have been identified in mammalian body fluids and tissues, their biological significance has attracted much concern with regard to the modulation of neurotransmission, alcoholism, and neuropsychiatric disorders based on their various neuropharmacological effects [1–4]. Although they are found in the human body, it has been debated whether substantial amounts of them are derived from diet or physiologically [5]. TBC‡ and MTBC (Fig. 1) are formed from tryptamine by condensing with either formaldehyde or acetaldehyde [4, 5]. While different tissues have been proposed for the *in vivo* formation of TBC and MTBC [2, 5], the oral cavity is also presumed to be one of the sites for the condensation to potentially occur because it is exposed to exogenous precursors via eating and smoking habits and wearing dental appliances [6–10]. Yu *et al.* [11] reported that serotonin interacts with components of cigarette smoke to form a cyanomethyl derivative of tetrahydro-β-carboline and that such an interaction readily proceeds in saliva collected after cigarette smoking. In the present study, we determined that TBC and MTBC are potentially formed in human saliva and that both precursors, tryptamine and aldehydes, coexist in oral environments.

Materials and Methods

Chemicals. TBC and MTBC were synthesized by the method of Hayashi *et al.* [12]. ETBC was synthesized by reacting TBC with ethyl iodide, as reported previously [13]. Formaldehyde, acetaldehyde, tryptamine, 5-methyltryptamine and fluorescamine were purchased from Kishida (Osaka, Japan), Merck (Darmstadt, Germany), Nacalai Tesque (Kyoto, Japan), Sigma (St. Louis, MO, U.S.A.) and Fluka (Buchs, Switzerland), respectively. All other reagents were of the highest quality available.

Sample preparation. Saliva collection was performed according to the guidelines of the Japanese Pharmacological Society. Informed consent was obtained from all subjects after the nature and consequences of their participation were explained.

Saliva was collected from 7 male subjects, aged 27–42 years, who expectorated saliva while smoking a single cigarette. Smokers' saliva (0.9 mL) was incubated with 0.1 mL of tryptamine (25.0 μg/mL) at 37°.

Saliva was also collected from the same 7 subjects without smoking. Each saliva sample was divided into two portions: one was used directly, and the other after heating at 100° for 10 min. Both intact and heated saliva (0.9 mL of each) were incubated similarly with 0.05 mL of tryptamine (50.0 μg/mL), while adding 0.05 mL of a mixture of formaldehyde (80.0 μg/mL) and acetaldehyde (4.0 mg/mL).

Cigarette smoke was bubbled through 5.0 mL of saliva or 0.1 M sodium phosphate buffer (pH 7.0) according to a previous method [14] under the following conditions: a puff duration of 3.0 ± 0.3 sec, an interval between puffs of 27 ± 0.3 sec, and a puff time of 4.0 min for each cigarette. An aliquot (0.05, 0.10, 0.20, 0.30, 0.40, and 0.50 mL) of bubbled saliva (smoke saliva) was incubated at 37° with tryptamine (2.5 μg/mL) in a total volume of 1.0 mL of each.

Analysis of TBC and MTBC. A 0.25-mL aliquot of the incubated solutions was mixed with 0.05 mL of ETBC (50.0 ng/mL) and 0.5 mL of 3.0 M potassium phosphate buffer (pH 8.5). To the mixture, 0.5 mL of a fluorescamine solution (5 mg/mL) in acetonitrile was added under vortex-mixing for 30 sec. Immediately after, 0.5 mL of an L-glycine solution (100 mg/mL) in 3.0 M potassium phosphate buffer (pH 8.5) was added under vortex-mixing for 30 sec. The mixture was extracted with 7.0 mL of ethyl acetate after adding 2.0 mL of 0.5 M NaOH, back-extracted to 1.0 mL of 0.2 M HCl, and then re-extracted with 7.0 mL of diethyl ether after adding 2.0 mL of 0.5 M NaOH according to a previous method [15]. After evaporating the extract to dryness, the resulting residue was analyzed by reversed-phase ion-pair HPLC with fluorometric detection, the detailed operating conditions of which have been described elsewhere [13, 15]. TBC and MTBC formed after incubation

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‡ Abbreviations: TBC, 1,2,3,4-tetrahydro-β-carboline; MTBC, 1-methyl-1,2,3,4-tetrahydro-β-carboline; and ETBC, 2-ethyl-1,2,3,4-tetrahydro-β-carboline.

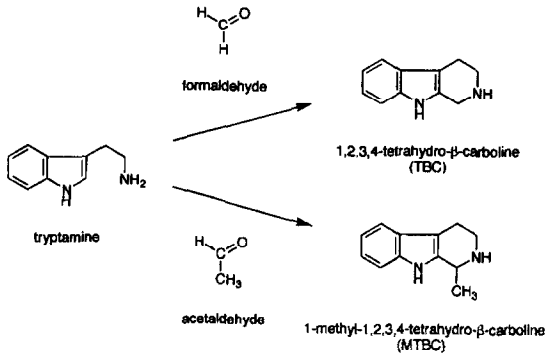

Fig. 1. Condensation between tryptamine and aldehydes to form 1,2,3,4-tetrahydro-β-carboline (TBC) and 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTBC).

were determined based on the calibration curves that were prepared by plotting the peak height ratios of standards to ETBC (linearity: 0.1 to 50.0 ng/mL). Their concentrations were corrected by the recovery obtained from spike experiments (104.8% for TBC and 98.9% for MTBC). The results were analyzed by an unpaired *t*-test to compare intact with heated saliva.

Analysis of tryptamine. Foods were minced and homogenized in glass homogenizers. Miso (soybean paste), cheese, and cocoa were homogenized in 0.1 N HCl (1.0 g/3.0 mL, 1.0 g/10.0 mL, and 1.0 g/6.0 mL, respectively). The homogenates were centrifuged at 12,000 *g* for 20 min. Beverages were filtered through a filter of 0.45 μm pore size. A 0.5-mL aliquot of the supernatants and filtrates was mixed with 0.05 mL of 5-methyltryptamine (2.0 μg/mL). The mixture was extracted and analyzed by HPLC according to a previous method [16], and then tryptamine was determined based on the calibration curve that was prepared by the peak height ratios of a standard to 5-methyltryptamine.

Analysis of aldehydes. Formaldehyde and acetaldehyde in the cigarette smoke solutions were determined by flow injection analysis [14] and enzymatic analysis using an acetaldehyde assay kit (Boehringer Mannheim-Yamanouchi, Tokyo, Japan), respectively.

Disks (8.5 ± 0.2 mm in diameter and 2.0 ± 0.2 mm in thickness) of denture-base acrylic resins were prepared from autopolymerized (Rebaron No. 3 pink; G-C Dental Industrial, Tokyo, Japan) and heat-polymerized resins (Acron No. 8 live pink; G-C Dental Industrial) as reported previously [17]. Each disk was immersed in 2.0 mL of 0.1 M sodium phosphate buffer (pH 7.0) at 37°. At specified time intervals, an aliquot of the immersion solutions was subjected to flow injection analysis for the determination of leached formaldehyde.

Results and Discussion

TBC and MTBC are formed by a condensation reaction between tryptamine and aldehydes that occurs either physiologically or chemically (Fig. 1). Initial studies were performed to assess the possibility for both precursors to coexist in the oral cavity. The analysis of foodstuffs proved that various foods and beverages contained tryptamine of ng–μg/g or ng–μg/mL levels (Table 1). In particular, kiwi, tomato, pineapple, soy sauce, ketchup and cocoa were abundant in tryptamine. Hence, tryptamine would be supplied to human saliva via dietary sources. The analysis of smoke solutions showed that cigarette smoke of all brands contained formaldehyde and acetaldehyde of μg/mL levels (Table 2). In the leaching experiments on denture materials, formaldehyde was leached from denture-base acrylic resins (Table 3). Acetaldehyde leaching was not detected because in dental acrylic polymers the oxidation of a residual methyl methacrylate monomer and the decomposition of an oxygen-methyl methacrylate copolymer form formaldehyde, but not acetaldehyde [9]. Formaldehyde is also known to be contained in various beverages [14]. In addition to tryptamine, aldehydes

Table 1. Tryptamine contained in foods and beverages

Sample	Tryptamine (μg/mL or μg/g)
Soy sauce	1.752*
Miso (soybean paste)	0.414
Ketchup	1.254
Cheese	0.073
Tomato	4.983
Pineapple	1.525
Kiwi	5.207
Plum	0.241
Prune	0.010
Banana	0.025
Eggplant	0.112
Pimiento	0.154
Cow's milk	0.037
Cocoa	0.706
Beer	0.321
Wine	0.001
Sake	0.003

* Each value is the mean of duplicate determinations.

would be supplied to human saliva by smoking and denture wearing. These results indicate that oral environments meet the prerequisite for the occurrence of an interaction between tryptamine and aldehydes, especially in the oral cavity of smokers and wearers of acrylic dental appliances.

TBC and MTBC were formed by incubating tryptamine in saliva through which cigarette smoke was bubbled (Fig. 2). While the incubation experiments were performed in duplicate by considering the volatility of aldehydes in smoke saliva, no significant difference was observed in the amount of TBC and MTBC formed. Their concentrations depended on the volume of added smoke saliva, indicating that the bubbled saliva trapped components that facilitated the formation of TBC and MTBC. From the smoke analysis described above, aldehydes appear to function as such components. There was about 100 times more acetaldehyde than formaldehyde in cigarette smoke. In smoke saliva, however, the concentrations of formaldehyde-derived TBC were several-fold higher than those of acetaldehyde-derived MTBC. This discrepancy may come from the higher reactivity of formaldehyde than acetaldehyde [15]. Different reactivity between formaldehyde and acetaldehyde is supported by the fact that the artifactual formation during analysis of formaldehyde-derived tetrahydro-β-carbolines occurs more easily than that of acetaldehyde-derived tetrahydro-β-carbolines [18, 19].

TBC and MTBC were also formed in smokers' saliva. The formation was enhanced with an increase in tryptamine concentrations (1.0 to 10.0 μg/mL) and incubation time (0.5 to 15 min). When incubating tryptamine (2.5 μg/mL) in smokers' saliva for 10 min, the mean concentrations (N = 7) and SD were

Table 2. Aldehydes contained in cigarette smoke*

Sample	Formaldehyde (μg/mL)	Acetaldehyde (μg/mL)
Brand A	5.08	325.79
Brand B	3.07	290.97
Brand C	3.30	357.45
Brand D	3.32	238.25
Brand E	5.23	259.89
Brand F	1.58	200.57
Brand G	2.03	229.94

* Cigarette smoke was bubbled through 5.0 mL of 0.1 M phosphate buffer (pH 7.0), and then the amount of formaldehyde and acetaldehyde in the smoke solutions was determined. Each value is the mean of duplicate determinations.

Table 3. Formaldehyde leached from denture-base acrylic resins

Sample	Formaldehyde (μg/mL)
Autopolymerized resins*	
10 min	0.134 ± 0.017†
30 min	0.269 ± 0.026
60 min	0.354 ± 0.053
1 day	0.820 ± 0.060
Heat-polymerized resins	
1 day	0.020 ± 0.001

* Disks prepared from denture-base acrylic resins were immersed in 2.0 mL of 0.1 M phosphate buffer (pH 7.0) at 37° for the time indicated.

† Mean ± SD (N = 3–8).

3.27 ± 0.94 ng/mL for TBC and 0.35 ± 0.17 ng/mL for MTBC. The concentrations increased almost linearly depending on an increase in incubation time. Neither TBC nor MTBC was found in saliva before incubation and without smoking (less than the detection limit of 0.1 ng/mL). The TBC and MTBC formed tended to be more in saliva through which smoke was bubbled than in smokers' saliva, which may be due to the difference in aldehyde trapping between bubbling and smoking.

A class of tetrahydro-β-carboline alkaloids were found originally in plants [20]. Since the related β-carbolines are known as pyrolysis products in tobacco smoke [5, 20], both cigarette smoke solutions and tobacco leaves homogenized in 0.1 N HCl were analyzed to assess the presence of TBC and MTBC in them. However, neither TBC nor MTBC was found in any samples. The smoke solutions were subjected to extraction and HPLC analyses similar to those of tetrahydro-β-carbolines, while the detection was performed at maximal excitation and emission wavelengths of β-carboline (norharman) and 1-methyl-β-carboline (harman). In such HPLC conditions, β-carbolines were separated from TBC, MTBC and ETBC, and no peak of any tetrahydro-β-carbolines (100 ng/mL of each) appeared on the chromatograms. Norharman and harman were identified in the smoke solutions by the retention times and spectral measurements of their peak fractions. Snook and Chortyk [21] reported that cigarette smoke contained norharman and harman of μg/cigarette levels. Since tetrahydro-β-carbolines were not contained in cigarette smoke in contrast to β-carbolines, TBC and MTBC found in smokers' saliva is ascribed to the result of an interaction between tryptamine and aldehydes as cigarette smoke components.

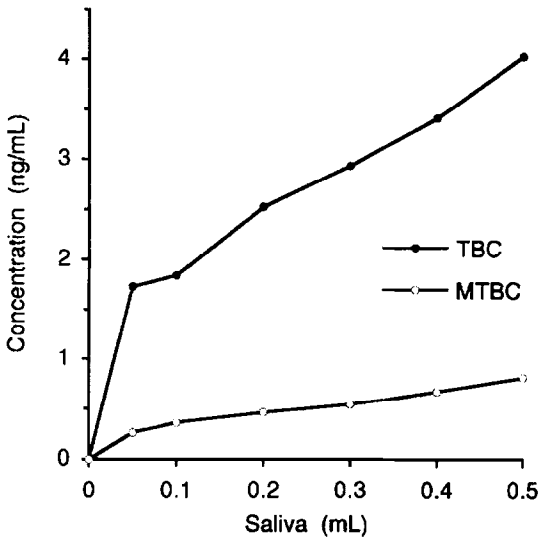

Fig. 2. TBC and MTBC formation in saliva. Smoke of a single cigarette was bubbled through 5.0 mL of saliva. Saliva samples of the volume indicated were incubated with tryptamine (2.5 μg/mL) at 37° for 10 min. Results are the means of duplicate determinations.

The formation was compared between intact and heated saliva. As the volatile components might be removed by heating, known amounts of aldehydes were added to the saliva samples (N = 7), followed by incubation at 37° for 10 min. The concentrations of formed TBC and MTBC did not show a statistically significant difference between intact and heated saliva. An enzymatically assisted reaction has been suggested for the *in vivo* formation of tetrahydro-β-carbolines [22, 23]. However, the result indicates that salivary enzyme(s) is not responsible for the formation of TBC and MTBC in human saliva.

Yu *et al.* [11] reported that biogenic amines form cyanomethyl tetrahydro-β-carboline by interacting with cigarette smoke components and that such an interaction readily occurs in saliva after cigarette smoking. However, they did not describe the origin of the precursors, nor did they deal quantitatively with the formation. The present study has proven that tryptamine and aldehydes are encountered in the oral cavity and that TBC and MTBC are formed at ng/mL levels in smokers' saliva.

When ingesting foodstuffs containing the equivalent of the tryptamine concentration in the incubation mixture, tryptamine would last for some time in saliva at concentrations sufficient to interact with aldehydes in the oral cavity. Whether the formation of TBC and MTBC actually occurs *in vivo* or not is evaluated by determining TBC and MTBC in saliva that is collected from smokers or denture wearers masticating foods and beverages. However, TBC and MTBC were also detected in certain foodstuffs containing tryptamine [24, 25]; therefore, TBC and MTBC formed in saliva must be strictly distinguished from TBC and MTBC in the diet. The fact that saliva after smoking only a single cigarette facilitated an interaction between tryptamine and aldehydes suggests at least the possibility that TBC and MTBC are formed in smokers' oral environments where all the precursors coexist.

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