Human Salivary Tea Catechin Levels and Catechin Esterase Activities: Implication in Human Cancer Prevention Studies

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Abstract
Because of the possible application of tea in the prevention of oral and esophageal cancers, the salivary levels of tea catechins were determined in six human volunteers after drinking tea. Saliva samples were collected after thoroughly rinsing the mouth with water. After drinking green tea preparations equivalent to two to three cups of tea, peak saliva levels of (−)-epigallocatechin (EGC; 11.7–43.9 μg/ml), EGC-3-gallate (EGCG; 4.8–22 μg/ml), and (−)-epicatechin (EC; 1.8–7.5 μg/ml) were observed after a few minutes. These levels were 2 orders of magnitude higher than those in the plasma. The elimination half-life ($t_{1/2}$) of the salivary catechins was 10–20 min, much shorter than that of the plasma. Holding a tea solution in the mouth for a few minutes without swallowing produced even higher salivary catechin levels, but taking tea solids in capsules resulted in no detectable salivary catechin level. Holding an EGCG solution in the mouth resulted in EGCG and EGC in the saliva and, subsequently, EGCG in the urine. The results suggest that EGCG was converted to EGC in the oral cavity, and both catechins were absorbed through the oral mucosa. A catechin esterase activity that converts EGCG to EGC was found in the saliva. The enzyme was likely of human origin, but the activity was not inhibited by common human esterase inhibitor. The present results suggest that slowly drinking tea is a very effective way of delivering rather high concentrations of catechins to the oral cavity and then the esophagus.

Introduction
Tea, which is made from the leaves of the plant Camellia sinensis, is the most popular beverage worldwide besides water. Many laboratories have demonstrated the inhibitory activities of tea and tea polyphenol preparations against tumorigenesis in animals, including those involving tumors in the skin, lung, liver, esophagus, forestomach, stomach, small intestine, and colon (1–4). The protective effect of tea consumption against human carcinogenesis, however, has not been convincingly demonstrated (1, 4, 5). For example, studies in northern Italy have suggested the protective effect of tea against oral, pharyngeal, and laryngeal cancer (6, 7). In a case-control study in Shanghai, frequent consumption of green tea was associated with a lower incidence of esophageal cancer, especially among nonsmokers and non-alcohol drinkers (8). However, many earlier studies suggested that tea drinking is a risk factor for esophageal cancer, although the hot temperature of tea may account for most of the risk (1, 2). The protective effect against gastric cancer by tea has been suggested in studies from Kyushu (Japan), Shanghai (China), northern Turkey, and central Sweden, but not in many other studies from different geographic areas (2, 4). In a prospective cohort study of postmenopausal women in Iowa, tea (mostly black tea) drinking was associated with a lower risk for digestive tract cancers and urinary tract cancers (9). On the other hand, in the Netherlands Cohort Study on Diet and Cancer, the consumption of black tea was not found to affect the risk for stomach, colorectal, lung, and breast cancers (10). Additional research, especially well-designed cohort and intervention studies, is needed to elucidate the relationship between tea consumption and human cancer risk.

In human trials, the method of delivery of the tea and the bioavailability of the putative effective agents in the target tissues are important issues. In many human studies, the agents are usually given in capsules or tablets, but these may not be the best methods of delivery in certain cases. Therefore, the experimental determination of blood and tissue levels of these compounds is important. We have previously developed methods and obtained data on blood and tissue levels of tea catechins [EGCG, EGC, and (−)-epicatechin (11)]. In preparation for preventive trials on oral and esophageal cancers, we determined the salivary levels of tea catechins. After drinking green tea, the salivary levels of EGCG, EGC, and (−)-epicatechin in human volunteers were found to be much higher than those observed in the blood, whereas the same amount of green tea solids in capsules produced a nondetectable level of salivary tea catechins. The enzymatic conversion of EGCG to EGC was observed in the oral cavity. This enzyme activity has not been reported previously, and we tentatively named it catechin esterase or EGCG esterase. Human saliva is known to contain esterases of the carboxylesterase type, which are sensitive to the inhibition by organophosphorus compounds (12). However, salivary EGCG esterase activity was not inhibited by bis-4-nitrophenyl phosphate. This article reports on our studies of

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1 The abbreviations used are: EGCG, (−)-epigallocatechin-3-gallate; EGC, (−)-epicatechin; HPLC, high-performance liquid chromatography; $t_{1/2}$, elimination half-life.
human salivary catechins and the characterization of salivary catechin esterase activity.

Materials and Methods

Chemicals and Reagents. The green tea solids and purified EGCG, EGC, (−)-epicatechin-3-gallate, and EC were obtained from the Thomas J. Lipton Co. The green tea solids were prepared by freeze-drying the water extracts of green tea leaves (1 g of powder is derived from 6 g of dry leaves). One g of green tea solids contained 89 mg of EGCG, 74 mg of EGC, 35 mg of (−)-epicatechin-3-gallate, and 29 mg of EC. ß-Glucuronidase (G-7896) and sulfatase (S-9754) were obtained from Sigma Chemical Co. (St. Louis, MO). Other reagents were of the highest grades available commercially. A stock solution of EGCG, EGC, and EC (10 mg of each/ml) were made in 1% ascorbic acid and stored in small aliquots at −80°C until use. The stock solutions were stable for at least 1 month.

Sample Collection. Six healthy adult volunteers (four males and two females) who did not smoke or drink alcoholic beverages, ranged in age from 30–50 years, and weighed 45–85 kg participated in the study. The subjects did not ingest tea or tea-related beverages for at least 2 days before the experiment and during the urine sample collection period. The volunteers brushed their teeth thoroughly after breakfast and drank the green tea solution (1.2 g of green tea solids in 200 ml of water) in a 2–3-min period between 9 and 10 a.m. They immediately rinsed their mouths vigorously with water 10 times in 2 min. In other experiments, the subjects held a tea or EGCG solution in their mouths for a specified period, voided the solution, and rinsed their mouths as described above. Saliva samples from the subjects were collected into conical tubes before the dose and at different time points after the dose. The salivary sample (1 ml) was mixed with 20 μl of ascorbate-EDTA solution [0.4 M NaH₂PO₄ buffer containing 20% ascorbic acid and 0.1% EDTA (pH 3.6)], and the mixture was stored at −80°C until analysis. Urinary samples were collected before the dose and during periods at 0–3, 3–6, 6–9, 9–12, 12–18, and 18–24 h after the dose. The volume of each urinary sample was recorded. Aliquots of 20 ml of each urinary sample were transferred into plastic bottles that contained 20 mg of ascorbic acid and 0.5 mg of EDTA and then stored at −80°C until analysis. Blood samples from the subjects were collected into heparin-containing blood collection tubes at 15 and 60 min after holding an EGCG solution in the mouth for 2 min. After centrifugation, 1 ml of each plasma sample was mixed with 20 μl of the ascorbate-EDTA solution, and the mixture was stored at −80°C until analysis.

Catechin Esterase Activity. For studying the catechin esterase activity, saliva samples (20 ml each time) were collected from volunteers and stored at −80°C. The samples were thawed after a few days and centrifuged at 16,000 × g for 5 min to remove particulates and mucous type matters. The supernatant was used as the enzyme source. The enzyme activity was assayed in a mixture containing 0.4 M sodium phosphate buffer (pH 6.8), EGCG, and saliva by incubation at 21°C and shaking for 30 min. The reaction was terminated by adding 1 ml of ethyl acetate. The ethyl acetate phase was separated and dried. The residues were redissolved in 1% ascorbic acid and analyzed by HPLC.

Sample Preparation for Catechin Analysis. The thawed saliva (100 μl) was mixed with 10 μl of ascorbate-EDTA solution, 40 μl of 4.5% HClO₄, and 350 μl of ice-cold water. A standard solution containing EGCG, EGC, and EC (500 ng of each in 50 μl) was added to a similar mixture containing the saliva collected before tea consumption. The mixture was vortex mixed for 4 min and centrifuged at 16,000 × g for 10 min, and the supernatant was then diluted, if necessary, before injection onto the HPLC.

For the analysis of urinary levels of catechins, our previous procedure was used (11). In brief, the thawed urine was incubated with a mixture of ß-glucuronidase and sulfatase in a pH 6.8 sodium phosphate buffer to convert the conjugated catechins to the free form. The reaction mixture was extracted twice with ethyl acetate, dried, redissolved in 10% acetonitrile aqueous solution, and injected onto the HPLC.

Analysis of Tea Catechins by HPLC. The conditions for HPLC using the NBS C₁₄ reversed-phase column (Niko Bioscience, Inc., Tokyo, Japan) were the same as those described previously (11), except that the column elution conditions were modified. The column was eluted at 35°C with 96% buffer A [30 mM NaH₂PO₄ buffer containing 1.8% acetonitrile and 0.1% tetrahydrofuran (pH 3.35)] and 4% buffer B [15 mM NaH₂PO₄ buffer containing 65% acetonitrile and 6.5% tetrahydrofuran (pH 3.45)] for 5 min, followed by 82% buffer A and 18% buffer B for 23 min at a flow rate of 1 ml/min. Over the next 10 min, the solvent was changed in a linear gradient to 2% buffer A and 98% buffer B and then maintained at 2% buffer A and 98% buffer B for another 8 min. After the run, the solvent was changed back to 96% buffer A and 4% buffer B for analysis of the next sample. The eluent was monitored by the ESA Model 5500 coulochem electrode array system with potential settings at −90, −10, 70, and 150 mV, and four chromatograms were obtained simultaneously.

Results

Salivary and Urinary Tea Catechin Levels after Ingestion of Green Tea. After drinking 200 ml of warm tea containing 1.2 g of green tea solids, the volunteers rinsed their mouths vigorously with water 10 times in 2 min. The saliva samples were collected as a function of time for 3 h. As shown in Fig. 1, HPLC peaks of EGC, EC, and EGCG were observed in the saliva samples. Consistent with previous results, EGC and EC, but not EGCG, were present in the urine samples. In both the saliva and urine samples, unknown peaks 2, 4, and 6 were observed and are suspected to be metabolites of tea components, because the peak size increased with time, with a maximal height at 1 h in saliva and at 6–9 h in urine, and decreased thereafter (Fig. 1B). The time-dependent salivary tea catechin levels of six subjects are shown in Fig. 2. The highest values (the initial data points) for EGC, EGCG, and EC were as follows: EGC, 11.7–43.9 μg/ml (mean ± SD, 30.1 ± 4.8 μg/ml); EGCG, 4.8–22.3 μg/ml (mean ± SD, 12.2 ± 2.2 μg/ml); and EC, 1.8–7.5 μg/ml (mean ± SD, 5.53 ± 0.83 μg/ml). The estimated t₁/₂ values for EGCG, EGCG, and EC were as follows: EGC, 7.1–14.1 min (mean ± SD, 10.6 ± 2.6 min); EGCG, 9.9–21.0 min (mean ± SD, 15.2 ± 4.5 min); and EC, 9.6–18.2 min (mean ± SD, 14.0 ± 3.4 min). The salivary catechin levels became undetectable after 3 h.

On the other hand, when 1.2 g of green tea solids were given in capsules taken with 200 ml of warm water, tea catechins were not detectable in the saliva samples collected from 0–7 h after the dose, although urinary excretion of EGC and EC was observed. The peak urinary concentrations of EGC and EC (in the 3–6 h urine) were 20 and 9.5 ng/ml, respectively, and the cumulative excretion levels were 4.2 and 1.8 mg, respectively (data not shown). These results suggest that the salivary catechins were not derived from systemically absorbed catechins.
Tea Catechin Levels in the Saliva, Urine, and Blood after Holding Green Tea or EGCG Solutions in the Mouth. In the first experiment, a subject held 60 ml of green tea solution containing 1.2 g of green tea solids in his/her mouth for 1 or 5 min without swallowing. After voiding the tea solution, the subject's mouth was rinsed, and the saliva samples were collected as described above. The results in Fig. 3 demonstrate that holding tea in the mouth for 5 min rather than 1 min resulted in salivary EGC and EGCG levels that were four to five times higher. With a 1-min holding time, the 7 mg/ml tea solution produced higher salivary levels of catechins than did the 1.5 mg/ml solution. The $t_{1/2}$ was 19.3–23.1 min for all three catechins. It was also noted that the EGC and EC (but not EGCG) levels at the second time-point were higher than those at the first time point. This result suggested a possible conversion of EGCG to EGC and EC in the oral cavity.

In a second experiment, saliva samples were collected as described above after two subjects held a 60-ml solution containing 96 mg of EGCG (1.6 mg/ml) in their mouths for 2 min. The expected EGCG level and elimination pattern were observed (Fig. 4). In addition, there was a time-dependent increase in salivary EGC level that peaked between 5 and 20 min, followed by a time-dependent decrease. The results suggest that EGCG was converted to EGC in the oral cavity. A suspected EC peak was also detected in the saliva, and it also appeared to show a time-dependent increase followed by a decrease, but the levels were much lower than those of EGC, and the identity of this peak has yet to be confirmed. Plasma EGCG levels at 15 min were 41.2 and 38.3 ng/ml for subjects 1 and 2, respectively, and at 60 min, the corresponding values were 24.4 and 16.6 ng/ml. EGC was not detected, but EC was observed at levels of 6.1–11.2 ng/ml in these plasma samples. The urinary excretion of EGC and EC by these two subjects is shown in Fig. 5. A total of 2000 and 700 mg of EGC were excreted by subjects 1 and 2, respectively, whereas only 56 and 15 mg of EC were excreted by subjects 1 and 2, respectively. It is noted that the catechin levels in the saliva and urine samples of subject 1 were higher than those of subject 2.

Fig. 1. Salivary and urinary tea catechin levels. After drinking 200 ml of warm tea containing 1.2 g of green tea solids, the subjects rinsed their mouths with water 10 times for 2 min. Saliva samples were collected as a function of time for 3 h, and urine samples were collected for 24 h. A, chromatograms of saliva and urine samples. EGC, EC, and EGCG peaks were observed in the saliva samples. EGC and EC peaks were observed in the urine samples. Possible metabolite peaks 2, 4, and 6 were also seen. B, the time-dependent appearance and disappearance of peaks 2, 4, and 6 in the saliva and urine samples.

Fig. 2. Tea catechin levels after the ingestion of green tea. After subjects drank 200 ml of green tea containing 1.2 g of green tea solids and thoroughly rinsed their mouths, saliva samples were collected as a function of time. The data points are the mean ± SD of six subjects.
than those of subject 2. The reason for this difference is not known.

Salivary Catechin Esterase Activity. The above results suggest that there was an esterase activity that converted EGCG to EGC. Such an activity was demonstrated by the results in Fig. 6. When 100 mg of EGCG were incubated with 0.2 ml of saliva in 1 ml of buffer, there was a time-dependent disappearance of EGCG accompanied by a corresponding formation of EGC (and perhaps also small amounts of EC). Additional studies suggested that the conversion was enzyme catalyzed. The reaction was linear with time up to 80 min and was linear with the amount of salivary protein present (Fig. 7). The optimal pH was between pH 6.5 and pH 7.0, and practically no activity was detected at pH 4 and pH 7.6. The enzyme appeared to be rather stable at room temperature and at 37°C, but it was inactivated by heating for 3 min in a boiling water bath. Kinetic studies indicated that the esterase had a $K_m$ of 90 $\mu$M EGCG and a $V_{max}$ of 515 pmol/min/mg protein (Fig. 8).

Additional Characterization of Catechin Esterase. To determine whether the esterase is a human enzyme or an enzyme derived from oral microorganisms, the volunteers were asked to clean their mouths thoroughly with a toothbrush and mouthwash solutions (Listerine; Warner-Lambert Co., Morris Plains, NJ) the night before the experiment and 1 h before the salivary sample collection. Such a cleaning procedure did not result in a significant change in the esterase activities. The EGCG esterase activities ranged from 254–594 pmol/min/mg among six adult volunteers. Saliva samples were also collected from two infants, a 2-month-old infant and a 5-month-old infant; their salivary EGCG esterase activities were 48 and 315 pmol/min/mg, respectively. EGCG esterase activity, however, was not detected in human plasma or in human liver samples, nor was...
the activity found in the saliva, plasma, and liver samples of rats.

Three commonly used esterase inhibitors, which are known to inhibit different types of esterases (13), were shown not to inhibit human salivary EGCG esterase activity (data not shown). The inhibitors and concentrations used were EDTA (0.1–1 mM) for arylesterase, bis-4-nitrophenyl phosphate (0.01–0.5 mM) for carboxylesterase, and physostigmine (0.05–1 mM) for cholinesterase. In the presence of physostigmine, an increase in EGCG esterase activity was observed, and at 1 mM, the activity was approximately doubled. The reason for this increase is not known. These results suggest that the EGCG esterase activity does not belong to any of these three types of esterases.

Discussion

The present studies demonstrate the presence of tea catechins in the saliva after drinking tea or holding a tea or EGCG solution in the mouth by human volunteers. After drinking green tea (1.2 g of tea solids in 200 ml), the highest salivary levels of EGC (which ranged from 11.7–43.9 μg/ml) and EGCG (which ranged from 4.8–22.3 μg/ml) were 2 orders of magnitude higher than the peak plasma values after drinking the same amount of tea (14, 15). However, the t1/2 of the salivary EGC and EGCG (10–20 min) were much shorter than those observed for plasma tea catechins (3–5 h; Ref. 15). Holding 60 ml of a slightly higher concentration of green tea (7 mg/ml) in the mouth without swallowing resulted in even higher salivary levels of tea catechins (Fig. 3). The presently observed salivary catechin levels are not due to residual tea solutions in the mouth because the mouth was rinsed thoroughly before saliva collection.

Because salivary catechins were not observed when tea was taken in capsules, the rather high level of salivary catechins could not come from systemically absorbed catechins. One possible explanation for this observation is that tea catechins are absorbed p.o. and then secreted from the salivary glands into the oral cavity. Alternatively, the catechins could be bound to the surface of the oral mucosa and redissolved in the saliva. The fact that holding a tea solution in the mouth for 5 min produced a much higher salivary catechin level than holding the same amount of tea in the mouth for 1 min favors the first possibility, because absorption is a time-dependent event, and physical binding takes place readily. The time-dependent oral conversion of EGCG to EGC (Figs. 3 and 4) and the formation of possible metabolites are also in agreement with the concept that tea catechins are absorbed p.o. and undergo biotransformation in the oral cavity. Catechin-related polyphenols are known to bind tightly to proline-rich salivary proteins (16). It is not clear whether the presently observed salivary proteins are in the free or bound form. It is interesting to note that at 120 min,
when the salivary levels of EGC and EGCG were almost nondetectable, sizable peaks of “metabolite” peaks 2 and 4 were observed in the same HPLC run (Fig. 1). The fact that these peaks were also observed in urine samples in a time-dependent manner again suggests that they are metabolites of tea catechins. Recent results indicated that these urinary peaks were also observed after the ingestion of EGCG or EGC by human volunteers, but the identities of these peaks remain to be determined.

Oral absorption and biotransformation may also account for the plasma EGCG, EGC, and EC and the urinary EGC and EC observed after holding an EGCG solution in the mouth for 2 min (Fig. 5). Another possible origin of the plasma catechins is the saliva that was swallowed, and the salivary catechins are then absorbed systemically. After drinking tea, it takes about 1.5 h to observe the peak plasma level of EGCG (15). If the swallowed saliva was the only source of the catechins, we would expect the peak plasma EGCG level to be observed at 1.5 h after holding the EGCG solution in the mouth. The fact that the 15 min plasma level of EGCG was about two times higher than that of the 60 min plasma suggests that oral absorption is responsible for the faster delivery of EGCG to the blood. In this experiment, most of the saliva secreted in the first 20 min was collected into test tubes for the experiment (Fig. 4), and only a small portion of the saliva was swallowed. This further favors the concept of oral absorption of tea catechins. The observation of the EC peak in the saliva after holding an EGCG solution in the mouth is rather surprising, because the conversion of EGCG or EGC to EC involves the removal of a phenolic group that is not easily accomplished by mammalian enzymes. The chemical identity of this “EC” peak needs to be determined.

The presently observed catechin esterase activity is rather intriguing. It is apparently responsible for the oral conversion of EGCG to EGC. The lack of this activity in the blood and liver is consistent with the observation that after an oral or i.v. administration of EGCG to rats, EGC is not observed in the blood (17). Similarly, the blood level of EGC was not observed after oral ingestion of EGCG (150 mg in 200 ml water). The origin of this esterase activity is still not clear. In addition to saliva secreted from the salivary glands, total saliva also contains epithelial cells, bacteria, and leukocytes. Esterase could be released from any of these types of cells. Esterase activities have been detected in human gingival epithelium and salivary glands (18–21). Esterase activity in oral microorganisms has also been described previously (22). Judging from the fact that extensive cleaning of the mouth with a toothbrush and mouthwash solutions did not significantly affect the esterase activity,
the esterase activity is likely to be due to human enzymes. The activity was not inhibited by the inhibitors of the three types of common human esterases (arylsterase, cholinesterase, and carboxylesterase) and may be due to an esterase that has not been described. The possible microbial origin of the presently observed catechin esterase, however, cannot be completely excluded.

The presently demonstrated salivary tea catechins and catechin esterase activity are important to our understanding of the bioavailability of tea catechins, especially in the oral cavity and the esophagus. In future human trials on the prevention of oral and esophageal cancer by tea, drinking a tea solution can obviously deliver much higher concentrations of tea catechins to the target tissues than using tea capsules. In addition to the initial exposure of the epithelial cells to tea during the drinking period, the levels of salivary catechins during the first couple of hours after drinking the tea are high enough to inhibit the growth of the preneoplastic cells in the oral cavity (23) and the esophageal epithelium, because we constantly swallow our saliva. Based on this analysis, the habit of drinking tea slowly may be a better practice, because it prolongs the contact time of tea with epithelial cells. Drinking tea slowly may also increase the bioavailability of tea catechin systemically because of the oral absorption of these compounds. In addition, the oral conversion of EGCG to EGC may increase the bioavailability of catechins, because the latter is more bioavailable. The bioavailability of EGC as compared to EGCG has been demonstrated in humans (15), and these two catechins have similar growth inhibitory effects against human lung tumor cells (24). For future prevention trials on oral and esophageal cancers, drinking tea slowly or using a tea mouthwash or lozenge would be effective ways to deliver tea catechins to the target tissues.

References

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