Tea Catechin Supplementation Increases Antioxidant Capacity and Prevents Phospholipid Hydroperoxidation in Plasma of Humans

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The effect of green tea catechin supplementation on antioxidant capacity of human plasma was investigated. Eighteen healthy male volunteers who orally ingested green tea extract (254 mg of total catechins/subject) showed 267 pmol of epigallocatechin-3-gallate (EGCg) per milliliter of plasma at 60 min after administration. The plasma phosphatidylcholine hydroperoxide (PCOOH) levels attenuated from 73.7 pmol/mL in the control to 44.6 pmol/mL in catechin-treated subjects, being correlated inversely with the increase in plasma EGCg level. The results suggested that drinking green tea contributes to prevent cardiovascular disease by increasing plasma antioxidant capacity in humans.

Keywords: *Tea catechin; antioxidant; lipid peroxidation; phosphatidylcholine hydroperoxide; human plasma*

INTRODUCTION

Green tea is consumed as a popular beverage in Japan and throughout the world. During the past decade, epidemiological studies have shown that tea catechin intake is associated with lower risk of cardiovascular disease (Hertog et al., 1993; Keli et al., 1996). In vitro biochemical studies have reported that catechins, particularly epigallocatechin-3-gallate (EGCg, for chemical structure see Table 1), help to prevent oxidation of low-density lipoprotein (LDL) (De Walley et al., 1990; Zhenhua et al., 1991; Miura et al., 1995). LDL oxidation has been recognized to be an important step in the formation of atherosclerotic plaques and subsequent cardiovascular disease (Steinberg et al., 1989).

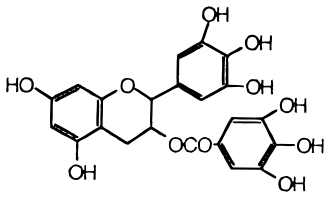
Previous metabolic studies have shown that EGCg supplement is incorporated into human plasma at a maximum concentration of 4400 pmol/mL (Lee et al., 1995; Unno et al., 1996; Nakagawa and Miyazawa, 1997a; Nakagawa et al., 1997). Such concentrations would be enough to exert antioxidant activity in the blood stream. The potent antioxidant property of tea catechin may be beneficial in preventing the oxidation of LDL. It is therefore of interest to examine whether tea catechin supplementation increases antioxidant capacity in humans.

In this study, the effect of green tea catechin supplementation on antioxidant capacity of plasma was investigated in humans by measuring plasma phosphatidylcholine hydroperoxide (PCOOH) levels as a marker of oxidized lipoproteins.

Table 1. Catechin Composition of Green Tea Extract

variable	% by wt	dose (mg)/subject
epigallocatechin gallate (EGCg)[a]	24.6	82
epicatechin gallate	11.4	38
gallocatechin gallate	11.1	37
epicatechin	10.0	33
epigallocatechin	8.1	27
gallocatechin	7.7	26
catechin	3.3	11
total	76.2	254

[a] The chemical structure of EGCg is



EXPERIMENTAL PROCEDURES

Chemicals. Green tea extract (Sunphenon DCF-1®) and EGCg (above 95% purity) were obtained from Taiyo Kagaku Co. (Yokkaichi, Japan). α- and γ-tocopherol were a gift from Eisai Co. (Tokyo, Japan). β-Carotene was purchased from Sigma Chemical Co. (St. Louis, MO). Lycopene was from Wako Pure Chemical Co. (Osaka, Japan). Other reagents and chemicals were commercially available extra-pure grade products.

Subjects and Protocol. Eighteen healthy male volunteers, aged 23−41 years, participated in this study. Body weight ranged from 52 to 81 kg (body mass index, 21.4 ± 2.3 kg/m^2). All subjects were generally healthy as evidenced by their medical history and a physical examination. All were nonsmokers. The subjects were employees of the laboratory of Taiyo Kagaku Co. and gave written informed consent to the experimental protocol which was approved by the local research ethics committee.

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10.1021/jf981195l CCC: $18.00
Published on Web 09/25/1999

All subjects avoided taking tea and tea-related beverages for 12 h prior to the experiment. After 12 h of fasting, each volunteer orally ingested a green tea extract tablet (Sunphenon DCF-1) containing 254 mg of catechin (82 mg EGCg, 38 mg epicatechin gallate, 37 mg gallocatechin gallate, 33 mg epicatechin, 27 mg epigallocatechin, 26 mg gallocatechin, and 11 mg catechin; Table 1). Blood was collected into heparinized tubes before and at 60 min after the catechin supplementation and centrifuged at 1000*g* for 15 min at 4 °C to separate plasma. Because one cup of green tea contains about 100−150 mg of catechin, the total amount of catechin (254 mg) ingested is comparable to two cups of green tea.

Plasma Catechin and Lipid Assay. Plasma EGCg was determined by a chemiluminescence detection−high performance liquid chromatography (CL−HPLC) method as previously reported (Nakagawa and Miyazawa, 1997a; Nakagawa et al., 1997; Miyazawa et al., 1999). Plasma phospholipid hydroperoxide (PCOOH) was analyzed with another CL−HPLC system as described by Miyazawa et al. (1992, 1994). This system enables the hydroperoxide-specific determination of lipid hydroperoxides in total lipids from various biological samples, with the high detection limit of 2 pmol. To analyze plasma PCOOH, plasma total lipids were quantitatively extracted with a chloroform−methanol mixture and were injected into the CL−HPLC instrument. During these analytical procedures, extraction solvent did not cause any artificial hydroperoxidation under the presence of butyl hydroxytoluene as antioxidant. The PCOOH concentrations of plasma samples were determined from a calibration curve made with authentic PCOOH standard. The recovery of PCOOH from plasma samples was more than 80%. Plasma thiobarbituric acid reactive substances (TBARS) were measured by the method of Simon et al. (1994) and expressed as malondialdehyde (MDA) equivalence. Plasma α-tocopherol, γ-tocopherol, β-carotene, and lycopene were determined by HPLC equipped with dual UV detectors (Nakagawa et al., 1996). Plasma phospholipids, total cholesterol, free cholesterol, and triacylglycerols were determined using phospholipid-C, cholesterol-E, free cholesterol-E, and triglyceride-E tests (Wako Pure Chemical Co.), respectively. Plasma cholesterol ester was calculated by subtracting free cholesterol from total cholesterol. The concentrations of total protein and albumin in plasma were separately measured with an A/G B test kit (Wako Pure Chemical Co.). Plasma globulin was calculated by subtracting albumin from total protein.

Plasma Lipid Peroxidation. Plasma samples spiked from four individuals showing the highest EGCg concentrations of all subjects were subjected to an in vitro peroxidation study: 100 mM $CuSO_4$ in Tris buffer (1 M, pH 7.0) was mixed in plasma and incubated at 37 °C in a shaking water bath in the dark (Simon et al., 1994).

Reaction of Catechin and Cupric Ion. Changes in the UV spectra (200−400 nm) of EGCg (100 μM) in 5 mM Tris (pH 7.0) were measured with or without $CuSO_4$ (100 μM) after 15 min of incubation. Ethylenediamine tetraacetic acid disodium salt (EDTA, 1.0 mg/mL final concentration) was also added after 15 min of incubation.

Statistical Analysis. The data were expressed as the mean and standard deviation (SD). Statistical comparisons were made with Student's *t*-test. All comparisons were made at the two-sided 0.05 significance level.

RESULTS

In Vivo Study. Although no chemiluminescence peak ascribed to tea catechin was detected in plasma before the supplementation (Figure 1A), plasma from the same subject after 60 min of catechin ingestion revealed an intense EGCg peak (10.7 min of retention time) together with epigallocatechin and gallocatechin gallate peaks (Figure 1A), and the catechins detected in plasma reflected roughly the initial composition of green tea extract (Table 1). On the other hand, PCOOH was clearly found in plasma prepared from subjects before catechin supplementation, and the PCOOH peak showed some reduction after 60 min of catechin ingestion (Figure 1B).

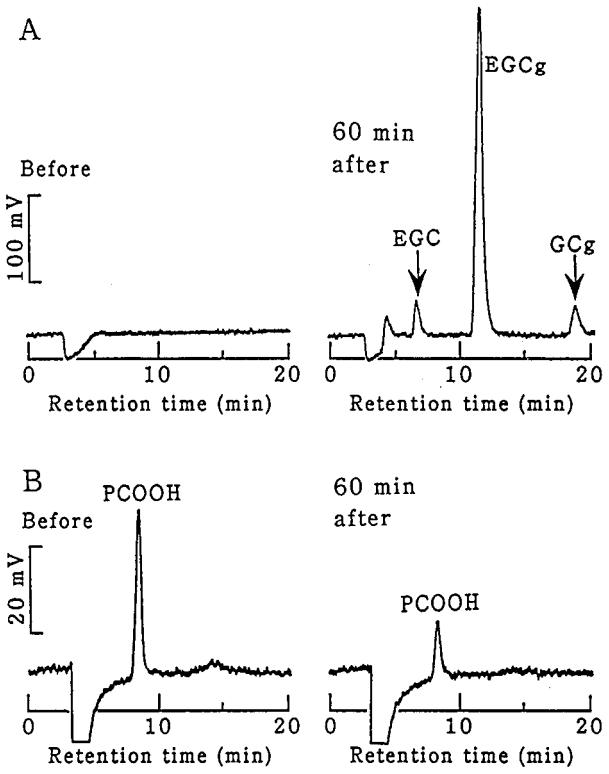

Figure 1. Typical chromatograms of plasma extract for determining catechin (A) and lipid hydroperoxide (B) concentrations. The methanol extracts from plasma before and 60 min after single oral administration of green tea extract (equivalent to 254 mg catechin) are analyzed in (A). EGCg, epigallocatechin-3-gallate; EGC, epigallocatechin; GCg, gallocatechin gallate. (B) Typical chromatograms of plasma PCOOH before and 60 min after green tea ingestion.

Plasma EGCg concentration increased from zero to 267 ± 126 pmol EGCg/mL plasma (mean ± SD, n = 18) at 60 min after catechin intake with rather large individual divergence (Figure 2A). Plasma PCOOH was 73.7 ± 32.4 pmol/mL before administration and was significantly decreased to 44.6 ± 8.9 pmol/mL after 60 min of catechin intake (Figure 2B); there was an inverse correlation (r = 0.52, $p < 0.05$) between the net changes in EGCg and PCOOH concentrations (Figure 2C). Catechin supplementation did not affect plasma TBARS (Figure 2D). No significant influence of catechin ingestion was observed on plasma phospholipids, total cholesterol, free cholesterol, cholesterol ester, triacylglycerols, α-tocopherol, γ-tocopherol, β-carotene, lycopene, albumin, globulin, or total protein levels (Table 2).

In Vitro Study. Catechin-enriched plasma obtained from 4 donors bearing the highest EGCg concentrations among the 18 subjects were subjected to an in vitro study, along with catechin-free (control) plasma. The plasma EGCg concentration was in the range from 315 to 579 pmol/mL (463 ± 112 pmol/mL, mean ± SD, n = 4). The initial values of plasma samples before peroxidation were as follows: PCOOH, 111 ± 40 pmol/mL in control and 46 ± 10 pmol/mL in catechin-rich plasma; TBARS, 4.32 ± 0.10 nmol of MDA equivalence/mL in control and 4.13 ± 0.15 nmol of MDA equivalence/mL in catechin-rich plasma; α-tocopherol, 25.6 ± 1.8 nmol/mL in control and 26.5 ± 2.9 nmol/mL in catechin-rich

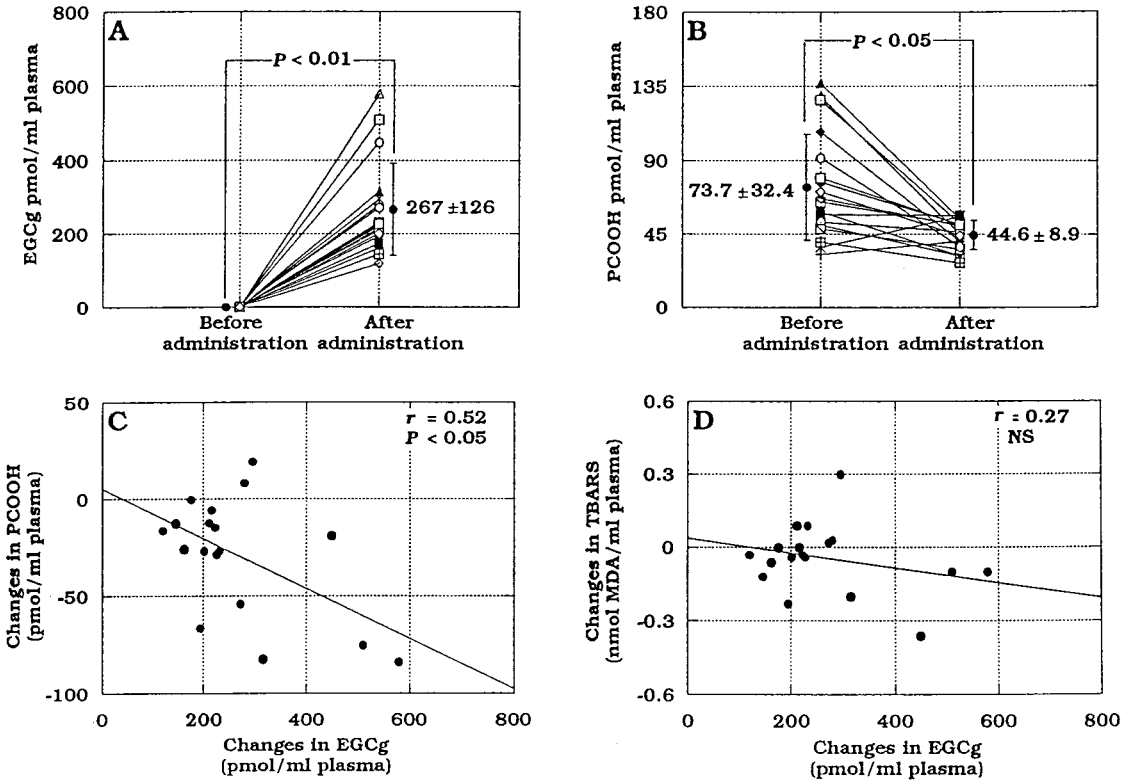

Figure 2. Relationships of plasma EGCg (A), PCOOH (B,C), and TBARS (D) before and 60 min after tea catechin administration in humans. Values are mean ± SD of 18 subjects. Green tea extract (equivalent to 254 mg catechin) was administered after 12 h of fasting. In (C) and (D), the data are plotted following the difference before and after catechin administration.

Table 2. Plasma Lipids, Antioxidants, and Proteins Before and After Catechin Intake[a]

variable	before (control) administration ($n = 18$)	60 min after administration ($n = 18$)	differences[b] compared with control	differences[b] significance
lipids				
phospholipids (mmol/L)	2.28 ± 0.44	2.23 ± 0.44	−0.05	NS[c]
total cholesterol (mmol/L)	4.45 ± 0.81	4.20 ± 0.72	−0.25	NS
free cholesterol (mmol/L)	1.31 ± 0.21	1.28 ± 0.23	−0.03	NS
cholesterol ester (mmol/L)	3.14 ± 0.63	2.93 ± 0.52	−0.21	NS
triacylglycerols (mmol/L)	0.72 ± 0.11	0.73 ± 0.11	0.01	NS
antioxidants				
α-tocopherol (μmol/L)	26.2 ± 3.2	26.3 ± 3.6	0.10	NS
(μmol/mmol cholesterol + TAG)[d]	5.11 ± 0.47	5.37 ± 0.61	0.26	NS
γ-tocopherol (μmol/L)	3.57 ± 0.78	3.60 ± 0.8	0.03	NS
(μmol/mmol cholesterol + TAG)[d]	0.70 ± 0.17	0.74 ± 0.19	0.04	NS
β-carotene (nmol/L)	820 ± 102	809 ± 112	−11	NS
(nmol/mmol cholesterol + TAG)[d]	161 ± 28	167 ± 32	6	NS
lycopene (nmol/L)	779 ± 102	781 ± 100	2	NS
(nmol/mmol cholesterol + TAG)[d]	154 ± 30	161 ± 30	7	NS
proteins				
albumin (g/100 mL)	4.80 ± 0.31	4.60 ± 0.38	−0.20	NS
globulin (g/100 mL)	1.94 ± 0.28	1.88 ± 0.32	−0.06	NS
total protein (g/100 mL)	6.74 ± 0.38	6.49 ± 0.35	−0.25	NS

[a] Mean ± SD. [b] Calculated as differences in the two groups compared. [c] Not significant. [d] Concentrations normalized for plasma lipids as follows: [antioxidant/(cholesterol + TAG concentration)], where TAG is triacylglycerols (Van Het Hof et al., 1997).

plasma; γ-tocopherol, 2.95 ± 1.06 nmol/mL in control and 3.06 ± 1.27 nmol/mL in catechin-rich plasma; β-carotene 787 ± 187 pmol/mL in control and 767 ± 215 pmol/mL in catechin-rich plasma; and lycopene, 728 ± 176 pmol/mL in control and 731 ± 175 pmol/mL in catechin-rich plasma. During Cu^{2+}-mediated peroxidation of control plasma, rapid PCOOH production (154% increase from 0 min) was observed during 45 min of incubation, and TBARS gradually increased over 180 min (272% increase from 0 min) (Figure 3A). On the other hand, the catechin-rich plasma showed significantly less production of PCOOH (69 ± 7% of control plasma, at 45 min of incubation) and of TBARS (72 ± 15% of control plasma, at 180 min of incubation) than control plasma (Figure 3A). Among plasma antioxidants, 95% of the EGCg was exhausted before tocopherols and carotenoids decayed (Figures 3B,C). The catechin-rich plasma showed slightly slower kinetics in depletion rates for endogenous antioxidant molecules than control plasma.

The direct reaction of EGCg with cupric ion was investigated because EGCg effectively suppressed Cu^{2+}-mediated plasma lipid peroxidation. After the addition of a stoichiometric amount of Cu^{2+} to EGCg solution,

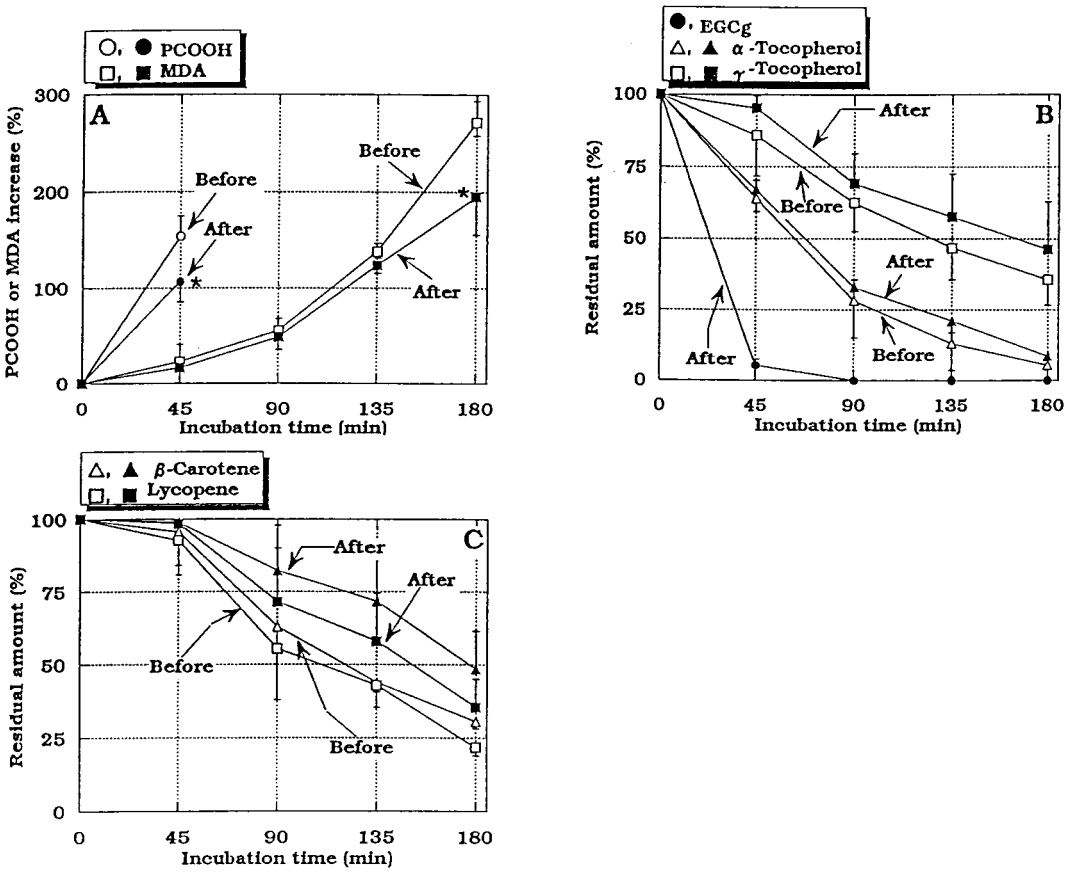

Figure 3. Production of PCOOH and TBARS (A), and decay of EGCg, tocopherols (B), and carotenoids (C) in plasma during Cu^{2+}-mediated lipid peroxidation. Before: plasma before catechin administration; after: plasma 60 min after catechin ingestion. The plasma from 4 individuals bearing the highest EGCg concentration among the 18 subjects was examined. Sample plasma solutions containing 4.76 μmol Cu^{2+}/mL were incubated at 37 °C in the dark. Values are mean ± SD of four subjects. *PCOOH and *TBARS indicate values significantly lower than those before catechin supplementation ($p < 0.05$).

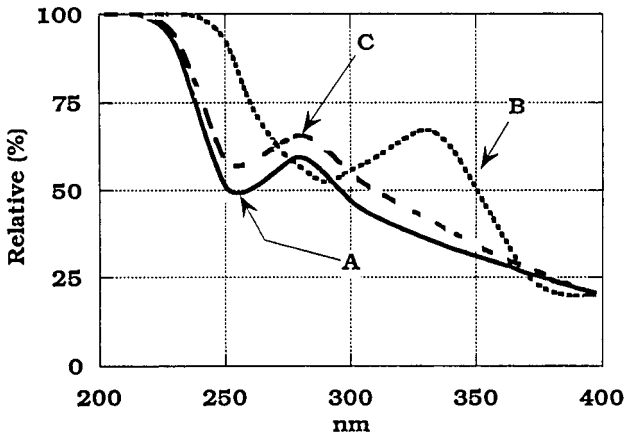

Figure 4. Changes in UV spectra of EGCg during reaction with Cu^{2+}. The spectra were recorded for (A) EGCg (100 μM) in 5 mM Tris at pH 7.0; (B) EGCg (100 μM) incubated with $CuSO_4$ (100 μM) in 5 mM Tris at pH 7.0 for 15 min; and (C) EDTA (1.0 mg/mL final concentration) mixed with reactant (B).

the absorption maximum of EGCg at 279 nm changed to 327 nm (Figure 4). Furthermore, excess addition of EDTA resulted in restoration of the initial spectrum of EGCg (λ_{max} 279 nm).

DISCUSSION

In the present study, we confirmed that EGCg is actually incorporated into human plasma after a single oral intake of green tea catechin (254 mg of catechin of which 82 mg is EGCg), and that as a result plasma PCOOH levels decreased just 60 min after the ingestion. We corroborated that catechin-incorporated plasma is highly resistant to Cu^{2+}-dependent in vitro lipid peroxidation. Such findings indicate that catechin ingestion helps to increase the antioxidant capacity of human plasma, thereby reducing the risk of cardiovascular disease by preventing oxidative modification of plasma lipoproteins (Hertog et al., 1993; Keli et al., 1996).

To date numerous in vitro studies have shown that tea catechin is a potent hydrophilic antioxidant that scavenges oxygen radicals (Rice-Evans, 1995; Salah et al., 1995) and chelating metal ions (Morel et al., 1994). Among catechins, EGCg shows the strongest antioxidant activity in vitro (Katiyar et al., 1994).

In contrast to in vitro studies, several papers have been published on in vivo effects of tea catechin in humans. Serafini et al. (1996) reported that in men a single dose equivalent to 300 mL (2 cups) of green tea increases resistance against radical initiator-induced peroxidation in plasma at 30−50 min after ingestion. Ishikawa et al. (1997) found that daily consumption of 750 mL (5 cups) of black tea for 4 weeks lowers the susceptibility of LDL to oxidative modification. However, recently, Van Het Hof et al. (1997) reported that daily consumption of 900 mL (6 cups) of green tea for 4 weeks had no effect on the resistance of LDL to oxidation. Thus, there is no consensus as to the in vivo effect of tea catechin and in vivo antioxidant mechanism.

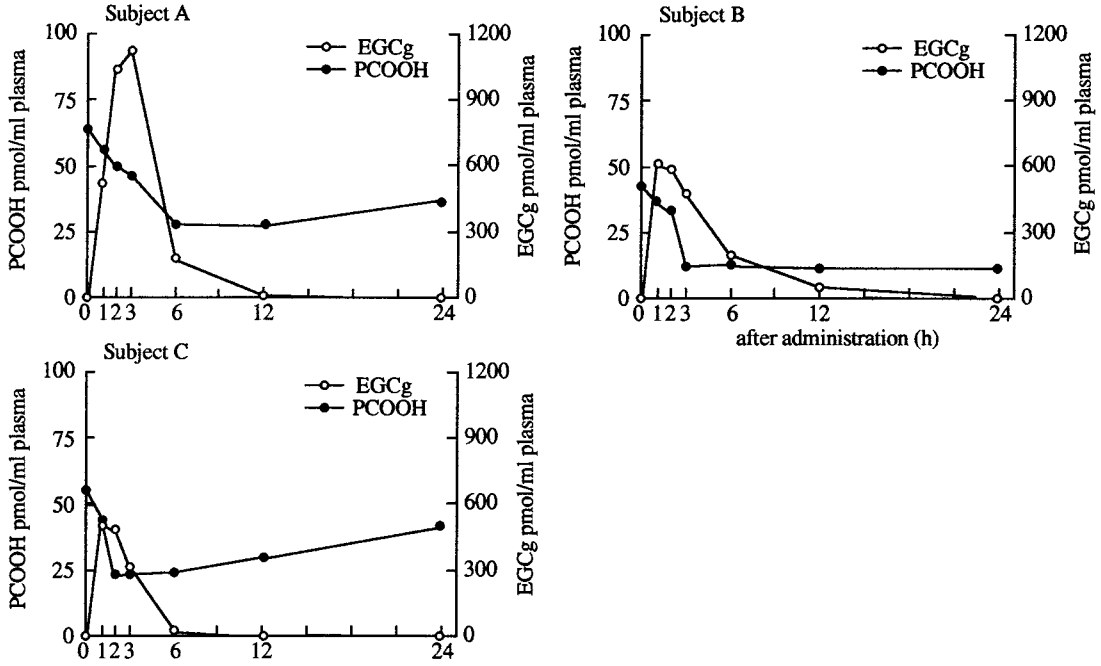

Figure 5. Time-course changes of human plasma EGCg and PCOOH concentrations after a single oral administration of tea catechin. After 12 h of fasting, each healthy male volunteer received green tea extract (equivalent to 762 mg of catechin; composed of 246 mg EGCg, 114 mg epicatechin gallate, 111 mg gallocatechin gallate, 99 mg epicatechin, 81 mg epigallocatechin, 78 mg gallocatechin, and 33 mg catechin) with a single oral administration.

To clarify these controversies regarding the antioxidant effects of tea catechin in vivo, the effects of catechin need to be evaluated taking into account the following points: the dose of tea catechin in humans, the concentration of tea catechin to be incorporated into human plasma, the metabolic pathway of the catechin, the identification of an oxidative stress marker pertinently reflecting in vivo lipid peroxidation, and whether such an oxidation marker is reduced in concentration as the result of interaction with supplemented tea catechin.

In this study, each human volunteer received a single oral dose of 254 mg total catechins (Table 1). This dosage is comparable to that of 2 cups of green tea drink in humans; Japanese people who consume tea regularly usually ingest such an amount of tea catechin daily. Thus, 254 mg was considered a suitable dosage for studying the antioxidant properties of tea catechin in humans. No harmful effect was found clinically or biochemically in any of the subjects taking the supplement; Yamane et al. (1996) reported that serum aspartate aminotransferase, creatinine, total cholesterol, and iron levels are not changed after consumption of tea catechin at concentrations up to 1000 mg/day for 3 months.

In the present study, a substantial level of catechin (EGCg) was confirmed to be present in human plasma after 60 min of ingestion, though the basal level was below the detection limit (<2 pmol/mL) (Figure 1A and 2A). These results suggest that in humans the half-life in kinetics of tea catechin metabolism is relatively short, as was reported in rats (Unno and Takeo, 1995; Nakagawa and Miyazawa, 1997a, 1997b). In the study by Van Het Hof et al. (1997), human plasma antioxidant activity and oxidation marker were measured after overnight fasting, i.e., at least 12 h after their most recent tea consumption. Therefore, it is likely that the plasma catechins disappeared, even though subjects consumed 900 mL (6 cups) of green tea daily for 4 weeks. This would explain the lack of effect of tea catechin at this dosage. We presently observed that the plasma EGCg concentration was increased to 267 pmol/mL (122 ng/mL) at 60 min after an ingestion of catechin (containing 82 mg EGCg) (Figure 2A). The concentrations of endogenous plasma antioxidant molecules such as α-tocopherol, γ-tocopherol, β-carotene, and lycopene show no change before and after the ingestion (Table 2). This is advantageous for elucidation of the antioxidant contribution of EGCg.

Regarding the metabolites, Lee et al. (1995) reported glucuronide and sulfate conjugates of catechins in human plasma after green tea ingestion, in which a phenolic hydroxyl group of the catechins is supposed to be substituted for glucuronide or sulfate. Because the antioxidant activity of catechins is closely dependent on the number of phenolic hydroxyl groups (Chen and Ho, 1995), catechins in the free form, but not conjugates, would be most important to the antioxidant action. Because of their high polarity, glucuronide and sulfate conjugates would be excreted rapidly from the blood stream. It is possible that the antioxidative contribution of these metabolites in blood plasma is less than that of the free form.

To evaluate the peroxidizability, we measured PCOOH as a marker of oxidative injury of plasma lipoproteins. Because phosphatidylcholine is generally located as a major constituent on the outer surface of the lipid monolayer of the lipoprotein particle, the occurrence of PCOOH directly reflects an increase of oxidative challenge in monolayer membranes of lipoprotein in plasma. PCOOH is the predominant membranous lipid hydroperoxide in human plasma (Figure 1B and 2B) as in our previous reports (Miyazawa et al., 1993, 1994). Because PCOOH is a primary oxidation product of phosphatidylcholine, an increase in PCOOH reflects stimulation of in vivo peroxidation of plasma lipoprotein phospholipid (Miyazawa, 1993; Miyazawa et al., 1993, 1994). As evidenced here, tea catechin supplementation clearly lowers plasma PCOOH concentrations in humans (Fig-

ure 2B). Further, such decreases in PCOOH level correlated with plasma EGCg increase (Figure 2C). Generally, PCOOH level is individually rather stable throughout the day, keeping certain levels between 50 and 100 pmol PCOOH/mL plasma in a healthy donor. Thus, the rapid decline of plasma PCOOH level after the catechin ingestion (Figure 2B) suggests the increase of the plasma antioxidant capacity resulting from EGCg absorption. These results indicate that the EGCg incorporated into plasma prevents spontaneously occurring membrane phospholipid peroxidation of the plasma lipoproteins. Accordingly, the beneficial effect of daily tea catechin intake in reducing the risk of cardiovascular disease (Hertog et al., 1993; Keli et al., 1996) could be mediated in part through protection of oxidative modification of plasma lipoproteins.

Although several epidemiological studies have reported that increased tea consumption is associated with reduction of serum lipids (cholesterol) (Kono et al., 1992; Stensvold et al., 1992; Imai and Nakachi, 1995), we found no such lipid-lowering effects (Table 2), consistent with results by Van Het Hof et al. (1997). Tea consumption may not strongly associate with plasma lipids metabolism in humans.

In the study of in vitro peroxidation caused by $CuSO_4$, the catechin-incorporated plasma showed significantly less production of PCOOH and TBARS (Figure 3A). The reactivity of EGCg with oxygen radicals and the chelating activity with Cu^{2+} (Figure 4) would be important for such reduced production of PCOOH and TBARS. The effect of plasma endogenous antioxidants, such as ascorbic acid, tocopherols, and carotenoids, on Cu^{2+}-mediated oxidation plasma or the synergistic reaction between these antioxidants and EGCg should be considerable (Esterbauer et al., 1992; Meydani et al., 1994; Laranjinha et al., 1995; Nardini et al., 1995; Pietta and Simonetti, 1998).

In addition to antioxidant activity, tea catechins are known to have anticarcinogenic effects (Kono et al., 1988; Yu and Hsieh, 1991). Recently, we reported that green tea catechin clearly prevents dimethylhydrazine-induced colonic carcinogenesis in rats, in which both the formation of PCOOH and 8-hydroxydeoxyguanosine are significantly suppressed in the colon mucosal cells relative to control rats (Matsumoto et al., 1996). This also indicated that the potent antioxidant effect of tea catechin helps to prevent colonic carcinogenesis. Other biological functions of tea catechin may include inhibition of hydrolytic and oxidative enzymes (phospholipase A_2, cyclooxygenase, and lipoxygenase) relating to anti-inflammatory activity (Laughton et al., 1991).

In addition to the present results, we recently examined the relationships of plasma EGCg and PCOOH levels before and 1, 2, 3, 6, 12, and 24 h after tea catechin administration in healthy volunteers (Figure 5). After 12 h of fasting, each subject received green tea extract (762 mg of total catechin of which 246 mg is EGCg; just 3-fold dosage as that given in Table 1) with a single oral administration. The plasma EGCg reached maximum at 1−3 h after the oral intake, and gradually decreased. On the contrary, plasma PCOOH levels attenuated rapidly during the initial 1−6 h after the oral intake, and then they increased somewhat to the basal PCOOH level. The results strongly supported our conclusions that drinking green tea contributes to a high plasma antioxidant capacity in humans. The taking of tea catechin as an antioxidative nutrient can be recommended as a way to reduce the risk of cardiovascular disease.

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Received for review November 2, 1998. Revised manuscript received May 5, 1999. Accepted July 7, 1999.

JF981195L