Absorption and Urinary Excretion of (−)-Epicatechin after Administration of Different Levels of Cocoa Powder or (−)-Epicatechin in Rats

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(−)-Epicatechin is a major polyphenol component of cocoa powder. The absorption and urinary excretion of (−)-epicatechin following administration of different levels of either cocoa powder (150, 750, and 1500 mg/kg) or (−)-epicatechin (1, 5, and 10 mg/kg) were evaluated in rats. Both the sum of plasma (−)-epicatechin metabolites at 1 h postadministration and peak plasma concentrations increased in a dose-dependent fashion. The sum of (−)-epicatechin metabolites in urine, excreted within 18 h postadministration, also increased with dose. Moreover, the sum of (−)-epicatechin metabolites excreted in urine reached the same level in both (−)-epicatechin and cocoa powder administration groups for equivalent amounts of (−)-epicatechin. These results suggest that, in the dose range examined in this study, bioavailability of (−)-epicatechin following administration of either (−)-epicatechin or cocoa powder shows dose dependence and that the various compounds present in cocoa powder have little effect on the bioavailability of (−)-epicatechin in cocoa powder.

Keywords: *(−)-Epicatechin; cocoa powder; absorption; excretion; rat*

INTRODUCTION

Cacao beans (*Theobroma cacao*) are used worldwide as an ingredient of chocolate and cocoa powder. Cocoa powder is rich in polyphenols such as (−)-epicatechin, (+)-catechin, and their oligometric procyanidins (*1*, *2*). Arts et al. have reported that chocolate contributes 20% of the total catechin intake in the Dutch population and that chocolate may thus represent an important dietary source of catechins, especially among the younger generation (*3*). Epidemiological studies have suggested a negative correlation between polyphenol consumption and the incidence of coronary heart disease (*4*, *5*). Moreover, it was recently reported that cocoa consumption reduces the platelet activation and microparticle formation induced by ADP or epinephrine in humans (*6*). More recently, we reported that daily intake of cocoa powder increases the resistance of low-density lipoproteins to oxidation in humans (*7*). These reports suggest that daily intake of cacao products such as chocolate and cocoa may contribute beneficially to human health.

It has been reported that the polyphenol-rich fraction of cocoa powder is particularly important in its beneficial contribution, likely due to its antioxidative activity (*8*−*11*). For instance, procyanidin in cocoa powder alters eicosanoid synthesis in humans (*12*), stimulates endothelium-dependent relaxation via activation of endothelial nitric oxide synthase in the rabbit aortic ring (*13*), and protects against peroxynitrite-dependent oxidation and nitration in vitro (*14*). These studies indicate that polyphenol-containing cocoa powder has numerous physiological effects.

(−)-Epicatechin is one of the major polyphenol components of cocoa powder (*3*). Rein et al. have reported that (−)-epicatechin can be detected in human plasma after the intake of 80 g of chocolate (*15*). We have also shown that, in humans, (−)-epicatechin from chocolate and cocoa is absorbed from the digestive tract and excreted in urine (*16*). Richelle et al. and Wang et al. have both reported that following intake of different levels of chocolate, the plasma level of (−)-epicatechin increases with chocolate intake level (*17*, *18*).

Cacao products such as chocolate or cocoa consist of a mixture of various nutritional components, including carbohydrates, proteins, fats, and polyphenols. Serafini et al. have reported that milk protein could negatively affect the bioavailability of catechins (*19*). However, it has also been reported that the addition of milk does not affect the absorption of catechins from cocoa (*20*). Aside from these conflicting results, little information is available regarding the effect of different nutritional components on the bioavailability of (−)-epicatechin in cocoa powder. The purpose of this study was to investigate the dose dependence of absorption and urinary excretion of (−)-epicatechin following administration of either cocoa powder or (−)-epicatechin. In addition, we were able to observe what effect the total composition of cocoa powder has on the bioavailability of (−)-epicatechin. Moreover, we examined the antioxidative effects of both cocoa powder and (−)-epicatechin on the oxidation of plasma that is induced by either a water-soluble radical generator or copper ions.

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MATERIALS AND METHODS

This study was approved by the Animal Committee of Meiji Seika Functional Foods Research and Development Laboratories. All animals received humane care under institutional guidelines.

Chemicals. (−)-Epicatechin and sulfatase type H-5 were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical or HPLC grade. The fermented and dried cacao beans were imported from Ecuador and made into cocoa powder by roasting, cracking, and compressing the beans at Meiji Seika Kaisha Ltd. (Tokyo, Japan). The general composition of cocoa powder was as follows (units/100 g): 3.7 g of moisture, 22.3 g of protein, 13.5 g of fat, 12.8 g of carbohydrate, 26.2 g of fiber, 7.9 g of minerals, 7.62 g of total polyphenol, 678 mg of (−)-epicatechin, 199 mg of (+)-catechin, 262 mg of procyanidin B2, 177 mg of procyanidin C1, and 286 mg of cinnamtannin A2. Total polyphenol, (−)-epicatechin, (+)-catechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 contents in cocoa powder were measured according to methods described in previous papers (*2, 21, 22*).

Purification of 3′-*O*-Methyl-(−)-epicatechin from Rat Urine as a Standard. Sprague−Dawley male rats ($n = 5$) were used at 10 weeks of age. Rats were deprived of food for 12 h before administration of (−)-epicatechin and were operated on under diethyl ether inhalation anesthesia for collection of urine. A plastic tube was attached to the penis of each animal. (−)-Epicatechin was suspended in deionized water (50 mg/mL) and was administered orally to rats at a dose of 500 mg/kg. All urine samples excreted within 24 h postadministration were collected under chilled conditions using an ice bath. Urine (20 mL) was extracted three times with ethyl acetate. The ethyl acetate phase was concentrated to dryness in vacuo and dissolved in 3 mL of 70% methanol. One hundred microliters of solution was injected into a reversed-phase semipreparative HPLC column (Deverosil ODS-HG-5, 5 μm, 250 × 20 mm; Nomura Chemical Co., Aichi, Japan). The column was then eluted at room temperature with a linear gradient of solvent A starting from 10% methanol containing 0.05% acetic acid to 45% methanol containing 0.05% acetic acid in 30 min at a flow rate of 15 mL. The eluted compounds were monitored at a wavelength of 220 nm. Each fraction of methylated (−)-epicatechin was collected and used for HPLC-MS and NMR analyses.

HPLC-MS and NMR Analyses. HPLC-MS analyses of methylated (−)-epicatechin were performed using an HP1100 series HPLC according to a previously published method (*22*). NMR spectra were obtained by a JEOL JNM-JSX 400 spectrometer, using CD_3OD as solvent. As the internal chemical shift standard, the proton and carbon peaks of deuterated methanol were set at 3.35 and 49 ppm, respectively.

Measurement of (−)-Epicatechin and Its Metabolites in Plasma and Urine. Sprague−Dawley male rats ($n = 30$) were obtained at 7 weeks of age from Clea Japan Inc. (Tokyo, Japan). Rats were kept at 23 °C and 55% relative humidity under a 12 h dark/light cycle with free access to pelleted food (Oriental Yeast Co. Ltd., Tokyo, Japan) and deionized water for 1 week. Rats were deprived of food for 12 h before administration of (−)-epicatechin or cocoa powder and were operated on under diethyl ether inhalation anesthesia for collection of blood and urine. A polyethylene tube was implanted into the femoral artery and sutured, and a plastic tube was attached to the penis of each animal. Thereafter rats were placed in restraining cages (Natsume Seisakusho Co. Ltd., Tokyo, Japan) with free access to deionized water. After waiting for recovery from anesthesia, rats were divided into six groups of five according to body weight. (−)-Epicatechin was suspended in deionized water (1, 5, and 10 mg/10 mL). Similarly, three levels of cocoa powder suspension were prepared (150, 750, and 1500 mg/10 mL in deionized water). (−)-Epicatechin contents for the 150, 750, and 1500 mg doses of cocoa powder were approximately 1, 5, and 10 mg, respectively (exactly 1.02, 5.08, and 10.16 mg, respectively). Each suspension was vortexed and was administered orally by direct stomach intubation, with three groups receiving (−)-epicatechin at a dose of 1, 5, and 10 mg/kg of body weight, and the other three groups receiving cocoa powder at a dose of 150, 750, and 1500 mg/kg of body weight. Blood samples were collected from the cannulated femoral artery into heparinized tubes before administration and at 1, 2, 4, 8, and 18 h postadministration. The volume of all urine samples excreted between 0 and 18 h postadministration was measured; all urine samples were collected under chilled conditions using an ice bath. Plasma was obtained by centrifugation of blood samples at 1400*g* for 10 min at 4 °C. Plasma and urine samples were stored at −80 °C under nitrogen gas until used for analysis.

(−)-Epicatechin and its metabolites in rat plasma were determined by HPLC-MS according to the method of Piskula and Terao (*23*). Conjugates of nonmethylated or 3′-*O*-methylated forms were hydrolyzed to the nonconjugated form by sulfatase type H-5. The amount of conjugate of nonmethylated or 3′-*O*-methylated forms in the samples was calculated as the amount after enzymatic hydrolysis minus the amount before hydrolysis in nonmethylated or 3′-*O*-methylated forms (*23*). Urine samples were filtered and diluted optimally with saline for analysis and analyzed as described above. HPLC-MS analyses of (−)-epicatechin in plasma and urine extracts were performed using an HP 1100 series HPLC (Hewlett-Packard, Palo Alto, CA) as described previously (*22*).

Measurement of Lipid Peroxide in Plasma Oxidized by 2,2′-Azobis(2−amidinopropane) Dihydrochloride (AAPH) or $CuSO_4$ Solution. Sprague−Dawley male rats ($n = 35$) were used at 7 weeks of age. Rats were kept with free access to pelleted food and deionized water for 1 week. Rats were deprived of food for 12 h before administration and divided into seven groups of five according to body weight. A single dose of either (−)-epicatechin (1, 5, or 10 mg/kg of body weight) or cocoa powder (150, 750, or 1500 mg/kg of body weight) was administered to six of the seven groups, and deionized water was administered orally to a control group (10 mL/kg). Blood samples were collected from the abdominal vein into heparinized tubes under diethyl ether anesthesia 60 min postadministration. The accumulation of lipid peroxide in plasma oxidized by 25 mmol/L AAPH for 120 min or by 500 μmol/L $CuSO_4$ for 180 min was measured according to the method of Baba et al. (*22*).

Calculations and Statistics. All data are presented as means with standard errors. For (−)-epicatechin metabolites in plasma data, a three-way ANOVA [time, dose and (−)-epicatechin source] was performed. For (−)-epicatechin metabolites in urine data, a two-way ANOVA [dose and (−)-epicatechin source] was performed. For accumulation of lipid peroxide in oxidized plasma data, Tukey's test following a one-way ANOVA was performed. Significance was recognized at $P < 0.05$. All statistical analyses were performed using SPSS for Windows 7.5.1 software (SPSS Japan Inc., Tokyo, Japan).

RESULTS

Structural Elucidation of 3′-*O*-Methylated (−)-Epicatechin by NMR Analysis. MS data for methylated (−)-epicatechin showed a prominent $[M - H]^-$ product ion at *m*/*z* 303, which was assigned to the molecular ion. NMR data of methylated (−)-epicatechin was as follows: ^{1}H NMR (in CD_3OD) δ (*J* in Hz) assignment 4.86 (*s*, H-2), 4.18 (*m*, H-3), 2.88 (*dd*, H-4δ), 2.74 (*dd*, H-4δ), 5.95 (*d*, H-6), 5.92 (*d*, H-8), 7.12 (*d*, H-2′), 6.78 (*d*, H-5′), 6.89 (*dd*, H-6′), 3.86 (*s*, OMe); ^{13}C NMR (in CD_3OD) δ (*J* in Hz) assignment 80.0 (C-2), 67.6 (C-3), 29.4 (C-4), 100.1 (C-4′), 158.0 (C-5), 96.5 (C-6), 157.7 (C-7), 95.9 (C-8), 157.4 (C-8a), 132.3 (C-1′), 111.9 (C-2′), 148.6 (C-3′), 147.0 (C-4′), 115.7 (C-5′), 120.6 (C-6′), 56.4 (OMe). A cross-peak between C-3′ and the methyl proton was observed by a heteronuclear multiple-bond connectivity experiment. These results also demonstrated that methylated (−)-epicatechin was 3′-*O*-methyl-(−)-epicatechin.

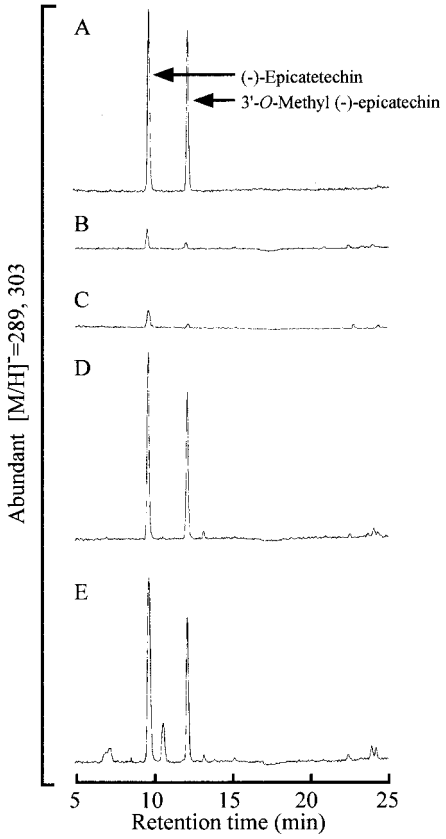

Figure 1. Typical HPLC-MS chromatograms for rat plasma obtained at 60 min after administration of (−)-epicatechin (B, D) or cocoa powder (C, E) with (D, E) or without (B, C) enzymatic treatment. Sulfatase (type H-5) was used as the enzyme. Standards for (−)-epicatechin and 3′-*O*-methyl-(-)-epicatechin are shown (A). Molecular ion $[M - H]^-$ peaks detected at about 10 and 12 min were identified as (-)-epicatechin and 3′-*O*-methyl-(-)-epicatechin, respectively.

Identification of Nonmethylated and 3′-*O*-Methylated Forms by HPLC-MS. Typical HPLC-MS chromatograms of rat plasma obtained at 60 min after administration are shown in Figure 1. At *m*/*z* 289 and 303 in the LC-MS analysis, a peak was detected at 10 and 12 min in plasma at 60 min postadministration. No peak was detected in plasma hydrolyzed with sulfatase H-5 before administration of (−)-epicatechin or cocoa powder (data not shown). The peak detected at 10 min showed the same retention time as the (−)-epicatechin standard; thus, it was identified as (−)-epicatechin. The peak eluted at 12 min showed the same retention time as the 3′-*O*-methyl-(−)-epicatechin standard; thus, it was identified as 3′-*O*-methyl-(−)-epicatechin.

Plasma Concentration of (−)-Epicatechin and Its Metabolites. The plasma concentrations of (−)-epicatechin and its metabolites after administration of either (−)-epicatechin or cocoa powder are shown in Tables 1 and 2. Statistical analysis data using three-way ANOVA [(−)-epicatechin source, dose, and time] is shown in Table 3. No (−)-epicatechin metabolites were detected in plasma prior to administration of (−)-epicatechin or cocoa powder. Following the administration of either substance, most of the (−)-epicatechin absorbed was present as a conjugate of nonmethylated or 3′-*O*-methylated forms in plasma. The sum of (−)-epicatechin metabolites in plasma reached the peak at 1 h after administration of either (−)-epicatechin or cocoa powder. The peak concentrations of the nonmethylated form after administration of 1, 5, and 10 mg/kg (−)-epicatechin were 0.97, 3.21, and 4.41 μmol/L, respectively. The peak concentrations of the 3′-*O*-methylated form after administration of 1, 5, and 10 mg/kg (−)-epicatechin were 1.00, 3.05, and 4.50 μmol/L, respectively. Similarly, the peak concentrations of the nonmethylated form after administration of 150, 750, and 1500 mg/kg cocoa powder were 0.35, 2.12, and 5.08 μmol/L, respectively. The peak concentrations of the 3′-*O*-methylated form after administration of 150, 750, and 1500 mg/kg cocoa powder were 0.12, 1.05, and 2.49 μmol/L, respectively.

Urinary Excretion of (−)-Epicatechin and Its Metabolites. (−)-Epicatechin and its metabolites found in urine excreted within 18 h after administration of (−)-epicatechin or cocoa powder are shown in Table 4. Conjugates of nonmethylated or 3′-*O*-methylated forms were present in urine postadministration. Moreover, nonconjugates of nonmethylated and 3′-*O*-methylated forms were also detected in urine. The sums of (−)-epicatechin metabolites in urine excreted within 18 h after administration of 1, 5, and 10 mg/kg (−)-epicatechin were 397, 1870, and 3003 nmol, respectively. Similarly, in the case of administration of 150, 750, and 1500 mg/kg cocoa powder, the sums of (−)-epicatechin metabolites in urine were 415, 1523, and 3074 nmol, respectively. The sum of (−)-epicatechin metabolites in urine excreted after administration of 1 mg/kg (−)-epicatechin was similar to that after administration of 150 mg/kg cocoa powder, the (−)-epicatechin content of which is ∼1 mg. Similarly, the sum of (−)-epicatechin metabolites in urine excreted after administration of 5 mg/kg (−)-epicatechin was similar to that after administration of 750 mg/kg cocoa powder, which contains ∼5 mg of (−)-epicatechin. Finally, the sum of (−)-epicatechin metabolites in urine excreted after administration of 10 mg/kg (−)-epicatechin was similar to that after administration of 1500 mg/kg cocoa powder, which contains ∼10 mg of (−)-epicatechin. The sums of (−)-epicatechin metabolites excreted in urine within 18 h postadministration were 48.0 ± 8.5, 44.2 ± 4.5, and 34.4 ± 5.0% of dose in 1, 5, and 10 mg/kg (−)-epicatechin administration groups, respectively. Similarly, in the case of 150, 750, and 1500 mg/kg cocoa powder administration groups, 52.3 ± 4.2, 35.7 ± 1.3, and 34.6 ± 3.3% were excreted in urine postadministration, respectively.

Lipid Peroxide Accumulation of Plasma Induced by a Radical Generator or Copper Ion. Figure 2shows a profile of the accumulation of lipid peroxide in plasma obtained at 60 min postadministration, which was oxidized by treatment with either 25 mmol/L AAPH for 120 min or 500 μmol/L $CuSO_4$ for 180 min. In the case of oxidation by 25 mmol/L AAPH, the accumulation of lipid peroxide of plasma obtained after administration of (−)-epicatechin was significantly lower than that after administration of deionized water. Furthermore, in the case of oxidation by 500 μmol/L $CuSO_4$, lipid peroxide accumulation in plasma was significantly lower after administration of 5 mg/kg (−)-epicatechin than after administration of deionized water. Administration of cocoa powder, however, showed only a tendency to depress the accumulation of lipid peroxide in plasma oxidized by AAPH or $CuSO_4$.

DISCUSSION

Cacao products such as cocoa and chocolate are rich in polyphenols such as (−)-epicatechin, (+)-catechin, and

Table 1. Nonmethylated Metabolite (Nonconjugate and Conjugate) Concentration of (−)-Epicatechin in Plasma after Administration of (−)-Epicatechin or Cocoa Powder[a]

	(−)-epicatechin			cocoa powder		
time (h)	1 mg/kg	5 mg/kg	10 mg/kg	150 mg/kg	750 mg/kg	1500 mg/kg
			Nonconjugate (μmol/L)			
before	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	0 ± 0	0.02 ± 0.01	0.25 ± 0.12	0.05 ± 0.03	0.19 ± 0.05	0.34 ± 0.06
2	0 ± 0	0 ± 0	0.04 ± 0.03	0 ± 0	0.02 ± 0.01	0.19 ± 0.08
4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.03 ± 0.02
8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
18	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
			Conjugate (μmol/L)			
before	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	0.97 ± 0.14	3.20 ± 0.30	4.16 ± 0.50	0.30 ± 0.04	1.93 ± 0.03	4.74 ± 0.42
2	0.67 ± 0.06	2.04 ± 0.24	2.84 ± 0.26	0.17 ± 0.04	1.17 ± 0.05	3.57 ± 0.47
4	0.31 ± 0.18	1.00 ± 0.22	1.09 ± 0.45	0.04 ± 0.02	0.40 ± 0.08	1.73 ± 0.12
8	0 ± 0	0.21 ± 0.11	0.15 ± 0.12	0 ± 0	0 ± 0	0.21 ± 0.11
18	0 ± 0	0.20 ± 0.11	0 ± 0	0 ± 0	0 ± 0	0.02 ± 0.01
			Total[b] (μmol/L)			
before	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	0.97 ± 0.14	3.21 ± 0.29	4.41 ± 0.50	0.35 ± 0.04	2.12 ± 0.05	5.08 ± 0.43
2	0.67 ± 0.06	2.04 ± 0.24	2.84 ± 0.26	0.17 ± 0.04	1.20 ± 0.06	3.76 ± 0.48
4	0.31 ± 0.18	1.00 ± 0.22	1.09 ± 0.45	0.04 ± 0.02	0.40 ± 0.08	1.76 ± 0.13
8	0 ± 0	0.21 ± 0.11	0.15 ± 0.12	0 ± 0	0 ± 0	0.21 ± 0.11
18	0 ± 0	0.20 ± 0.11	0 ± 0	0 ± 0	0 ± 0	0.02 ± 0.01

[a] Values are mean ± SEM, $n = 5$ per group. [b] Total is the sum of nonconjugate and conjugate in nonmethylated forms.

Table 2. 3′-*O*-Methylated Metabolite (Nonconjugate and Conjugate) Concentration of (−)-Epicatechin in Plasma after Administration of (−)-Epicatechin or Cocoa Powder[a]

	(−)-epicatechin			cocoa powder		
time (h)	1 mg/kg	5 mg/kg	10 mg/kg	150 mg/kg	750 mg/kg	1500 mg/kg
			Nonconjugate (μmol/L)			
before	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	0 ± 0	0 ± 0	0.03 ± 0.02	0 ± 0	0.02 ± 0.01	0.03 ± 0.02
2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.02 ± 0.01
4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
18	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
			Conjugate (μmol/L)			
before	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	1.00 ± 0.02	3.05 ± 0.15	4.48 ± 0.23	0.12 ± 0.04	1.03 ± 0.05	2.45 ± 0.18
2	0.74 ± 0.04	2.32 ± 0.26	3.51 ± 0.55	0.03 ± 0.02	0.77 ± 0.04	2.33 ± 0.40
4	0.73 ± 0.03	1.92 ± 0.26	1.83 ± 0.33	0.02 ± 0.01	0.38 ± 0.13	1.60 ± 0.24
8	0.30 ± 0.17	1.04 ± 0.28	0.70 ± 0.27	0 ± 0	0.47 ± 0.12	0.69 ± 0.26
18	0 ± 0	0 ± 0	0.03 ± 0.02	0 ± 0	0 ± 0	0 ± 0
			Total[b] (μmol/L)			
before	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	1.00 ± 0.02	3.05 ± 0.15	4.50 ± 0.22	0.12 ± 0.04	1.05 ± 0.05	2.49 ± 0.16
2	0.74 ± 0.04	2.32 ± 0.26	3.51 ± 0.55	0.03 ± 0.02	0.77 ± 0.04	2.35 ± 0.40
4	0.73 ± 0.03	1.92 ± 0.26	1.83 ± 0.33	0.02 ± 0.01	0.38 ± 0.13	1.60 ± 0.24
8	0.30 ± 0.17	1.04 ± 0.28	0.70 ± 0.27	0 ± 0	0.47 ± 0.12	0.69 ± 0.26
18	0 ± 0	0 ± 0	0.03 ± 0.02	0 ± 0	0 ± 0	0 ± 0

[a] Values are mean ± SEM, $n = 5$ per group. [b] Total is the sum of nonconjugate and conjugate in 3′-*O*-methylated forms.

Table 3. Three-Way ANOVA of (−)-Epicatechin Metabolites in Plasma after Administration of Different Levels of Cocoa Powder or (−)-Epicatechin in Rats

	three-way ANOVA[a], $P <$						
	(−)-epicatechin source (E)	dose (D)	time (T)	E × D	E × T	D × T	E × D × T
nonconjugate in nonmethylated form	0.004	0.001	0.001	NS	0.02	0.001	NS
conjugate in nonmethylated form	0.03	0.001	0.001	0.001	NS	0.001	0.02
total nonmethylated forms[b]	NS	0.001	0.001	0.001	NS	0.001	0.01
nonconjugate in 3′-*O*-methylated form	NS	0.003	0.001	NS	NS	0.003	NS
conjugate in 3′-*O*-methylated form	0.001	0.001	0.001	0.002	0.001	0.001	0.03
total 3′-*O*-methylated forms[c]	0.001	0.001	0.001	0.002	0.001	0.001	0.03

[a] NS, not significant, $P > 0.05$. [b] Total nonmethylated forms is the sum of nonconjugate and conjugate in nonmethylated forms. [c] Total 3′-*O*-methylated forms is the sum of nonconjugate and conjugate in 3′-*O*-methylated forms.

procyanidins (*1*−*3*, *21*). Wollgast et al. recently reviewed the consumption of chocolate and chocolate confectionary in the European Union and reported ranges from 1.3 kg/year per capita in Portugal to 8.8 kg/year per capita in Germany (*24*). Moreover, it has been reported that daily catechin intake is 50 mg/day in the Dutch population and that chocolate is an especially important source of dietary catechins for children (*25*). (−)-Epi-

Table 4. Nonmethylated and 3′-*O*-Methylated Metabolite (Nonconjugate and Conjugate) Concentration (Nanomoles) of (−)-Epicatechin in Urine Excreted within 18 h after Administration of (−)-Epicatechin or Cocoa Powder[a]

	(−)-epicatechin			cocoa powder			two-way ANOVA,[b] $P <$		
	1 mg/kg	5 mg/kg	10 mg/kg	150 mg/kg	750 mg/kg	1500 mg/kg	epicatechin source	dose	interaction
nonconjugate in nonmethylated form	150 ± 46	857 ± 72	1231 ± 439	127 ± 23	512 ± 142	1067 ± 448	NS	0.005	NS
nonconjugate in 3′-*O*-methylated form	197 ± 47	653 ± 84	743 ± 277	103 ± 33	244 ± 83	687 ± 203	NS	0.005	NS
conjugate in nonmethylated form	17 ± 15	124 ± 41	479 ± 181	83 ± 17	395 ± 136	1064 ± 514	NS	0.016	NS
conjugate in 3′-*O*-methylated form	33 ± 14	236 ± 41	550 ± 118	102 ± 42	373 ± 66	257 ± 123	NS	0.001	0.03
total[c]	397 ± 35	1870 ± 101	3003 ± 212	415 ± 18	1523 ± 120	3074 ± 218	NS	0.001	NS

[a] Values are mean ± SEM, $n = 5$ per group. [b] NS, not significant, $P > 0.05$. [c] Total is the sum of nonconjugate and conjugate in nonmethylated or 3′-*O*-methylated forms.

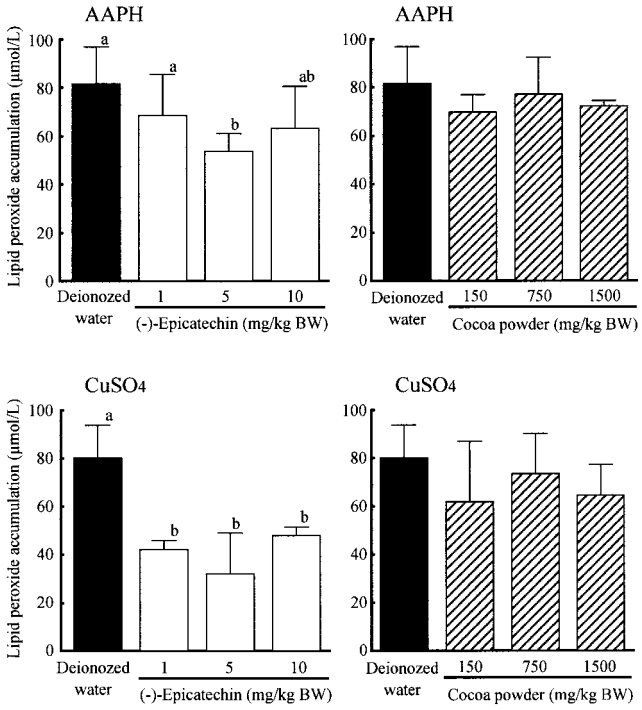

Figure 2. Accumulation of lipid peroxide in plasma obtained at 60 min after administration of deionized water, (−)-epicatechin, and cocoa powder, oxidized by 25 mmol/L AAPH or 500 mmol of $CuSO_4$. Values are expressed as the mean ± SEM, $n = 5$ per group. Means with different letters are significantly different at $P < 0.05$ (ANOVA and Tukey's test).

catechin is one of the major components of polyphenol in cocoa powder (*3*). Previously, we had reported that (−)-epicatechin was absorbed and excreted in urine following the intake of chocolate and cocoa in humans (*16*) and that administration of cocoa powder enhanced antioxidative activity in rat plasma (*22*). In this study, we investigated the absorption and urinary excretion of (−)-epicatechin after administration of different levels of cocoa powder or (−)-epicatechin. In addition, we observed the effect of the total composition of cocoa powder on the bioavailability of (−)-epicatechin. We further examined the effect of cocoa powder or (−)-epicatechin administration on the production of lipid peroxide in plasma oxidized by AAPH or $CuSO_4$.

As shown in Tables 1 and 2, the sum of nonmethylated and 3′-*O*-methylated (−)-epicatechin metabolites in plasma reached the maximum peak level 1 h after administration of cocoa powder or (−)-epicatechin. In cocoa powder administration groups, the peak concentration of total (−)-epicatechin metabolites in plasma increased with dose in both nonmethylated and methylated forms. Similarly, in the case of (−)-epicatechin administration groups, the peak concentration of (−)-epicatechin metabolites in plasma also increased with dose for both nonmethylated and methylated forms. Yang et al. reported that after intake of 1.5 and 3.0 g of decaffeinated green tea, the peak of plasma concentration of (−)-epicatechin is positively correlated with the administered dose of tea (*26*). It was reported elsewhere that after intake of different levels of chocolate, plasma levels of (−)-epicatechin were positively correlated with the dose of chocolate (*17*, *18*). In this study, we found a dependence of (−)-epicatechin plasma concentration on the dose of (−)-epicatechin administered (alone or in cocoa powder). This result suggests that the saturation of (−)-epicatechin absorption does not occur in the dosage range in this study. However, the sums of (−)-epicatechin metabolites excreted in urine within 18 h postadministration were 48.0, 44.2, and 34.4% for 1, 5, and 10 mg/kg (−)-epicatechin administration groups, respectively. Similarly, in the case of 150, 750, and 1500 mg/kg cocoa powder administration groups, 52.3, 35.7, and 34.6% of the (−)-epicatechin were excreted in urine, respectively. These results indicate that the bioavailability of (−)-epicatechin gradually decreases with increasing dose level. Future studies may be able to clarify the mechanism of (−)-epicatechin absorption.

Previously, it was reported that the bioavailability of catechins was influenced by other components such as milk protein in foods. For example, Serafini et al. reported that intake of green tea and black tea enhances the level of the total plasma antioxidative activity but that the addition of milk to both teas inhibits the increase in plasma antioxidative activity in vivo (*19*). On the other hand, Hof et al. reported that, in humans, intake of black tea with or without milk results in similar levels of catechins in blood, measured either by peak or by area under the curve (*20*). In this study, the sums of nonmethylated and 3′-*O*-methylated (−)-epicatechin excreted in urine within 18 h postadministration were similar between (−)-epicatechin and cocoa powder administration groups (Table 4). In addition to (−)-epicatechin, cocoa powder consists of various components such as fat, carbohydrate, protein, and other polyphenols. These results suggest that the total composition found in cocoa powder has no effect on the bioavailability of (−)-epicatechin as measured by urinary excretion. However, in this study, the sum of 3′-*O*-methylated (−)-epicatechin in plasma after administration of cocoa powder was lower than that after administration of (−)-epicatechin (Tables 2 and 3). Absorption of (−)-epicatechin in cocoa powder may be

slow compared with that of (−)-epicatechin alone, resulting in lower plasma levels.

As shown in Figure 2, the production of lipid peroxide in plasma induced by AAPH or $CuSO_4$ was significantly reduced by administration of (−)-epicatechin compared with administration of deionized water. Administration of cocoa powder also showed a trend of suppressing of lipid peroxide production induced by AAPH or $CuSO_4$. We previously reported that administration of cocoa powder reduced the production of lipid peroxide and the consumption of α-tocopherol in rat plasma oxidized by AAPH or $CuSO_4$ (*22*). In a human study, Rein et al. reported that intake of chocolate resulted in increased antioxidant capacity and decreased thiobarbituric acid reactive substances in plasma (*15*). Moreover, it was reported that oral administration of (−)-epicatechin suppressed the production of lipid peroxide and the consumption of α-tocopherol in oxidized plasma (*27*). These results suggest that administration of cocoa powder enhances the antioxidative activity of rat plasma and that (−)-epicatechin may mediate this effect.

We previously reported that the (−)-epicatechin in cocoa powder is absorbed into the blood stream and that its main components were glucuronide and/or sulfate in nonmethylated and *O*-methylated forms postingestion in both rats and humans (*16*, *22*). In this study, (−)-epicatechin absorbed and found in the plasma was mainly present as conjugate in nonmethylated and 3′-*O*-methylated forms following oral administration of either cocoa powder or (−)-epicatechin (Tables 1 and 2). Piskula and Terao have reported that, in rats, the enzymatic activity for conjugation and methylation are found in the intestinal mucosa, the liver, and the kidneys (*23*). These results suggest that after administration of cocoa powder or (−)-epicatechin, (−)-epicatechin is absorbed and metabolized by enzymes for conjugation and methylation in the body, existing as metabolite forms in plasma.

In this study, absorbed (−)-epicatechin was present mainly as metabolites such as conjugates of nonmethylated and methylated forms in plasma. However, nonconjugates of nonmethylated and methylated (−)-epicatechin excreted in urine were 87.5, 80.5, and 66.0% of total (−)-epicatechin metabolites in 1, 5, and 10 mg/kg (−)-epicatechin administration groups, respectively (Table 4). Similarly, in the case of 150, 750, and 1500 mg/kg cocoa powder administration groups, nonconjugates of nonmethylated and methylated (−)-epicatechin excreted in urine were 55.4, 49.6, and 57.5% of the total (−)-epicatechin metabolites, respectively (Table 4). In our previous study, (−)-epicatechin was excreted abundantly in urine as nonconjugated form after intake of chocolate and cocoa (*16*). These results suggest that deconjugation such as deglucuronidation or desulfation happens in the body before the metabolites are excreted into the urine. This deconjugation may be of physiological significance for the availability of dietary catechins, although it is not elucidated yet.

In conclusion, for the dose range of this study, the bioavailability of (−)-epicatechin following oral administration of either (−)-epicatechin or cocoa powder showed dose dependency in rats. In addition, we have shown that the (−)-epicatechin present in cocoa powder was absorbed as efficiently as (−)-epicatechin administered alone.

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