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Bioavailability of (-)-Epicatechin Upon Intake of Chocolate and Cocoa in Human Volunteers

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We evaluated the levels of (-)-epicatechin (EC) and its metabolites in plasma and urine after intake of chocolate or cocoa by male volunteers. EC metabolites were analyzed by HPLC and LC/MS after glucuronidase and/or sulfatase treatment. The maximum levels of total EC metabolites in plasma were reached 2 hours after either chocolate or cocoa intake. Sulfate, glucuronide, and sulfoglucuronide (mixture of sulfate and glucuronide) conjugates of nonmethylated EC were the main metabolites present in plasma rather than methylated forms. Urinary excretion of total EC metabolites within 24 hours after chocolate or cocoa intake was 29.8 ± 5.3% and 25.3 ± 8.1% of total EC intake. EC in chocolate and cocoa was partly absorbed and was found to be present as a component of various conjugates in plasma, and these were rapidly excreted in urine.

Keywords: Chocolate; Cocoa; (-)-Epicatechin; Human; Bioavailability

INTRODUCTION

Flavonoids are present in various vegetables, fruits and teas. There are many reports of epidemiological studies indicating that there is a negative correlation between flavonoid intake and the incidence of cardiovascular disease.[1–4] Cacao products are rich in polyphenols such as (-)-epicatechin (EC), catechin and procyanidins.[5–8] It has been reported that chocolate is a major source of catechins. In a Dutch study, it was found that chocolate contributed almost 20 % of total catechins intake.[9] In a clinical study, cocoa powder supplementation was found to delay the oxidation of low-density lipoprotein.[10] However, there have been few reports concerning the metabolism of antioxidative polyphenols upon human intake of cacao products. In this study, we evaluated the absorption of these compounds after chocolate or cocoa intake using EC and its metabolites in human plasma and urine as indices.

MATERIALS AND METHODS

Subjects

Five healthy male volunteers were enrolled in this study and their characteristics were as fol-

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lows: age: 31 ± 1 y (range: 30 – 33 y), body weight: 63 ± 3 kg (range: 60 – 68 kg), body mass index: 22.5 ± 1.3 kg/m^2(range: 20.4 – 23.9 kg/m^2). Before this study was started, informed consent was obtained from each of the subjects. This study was approved by the Human Studies Committee of the School of Medicine, Tokushima University, and was performed under their guidelines.

Materials

EC, D-saccharic acid 1,4-lactone, β -glucuronidase type VII-A, sulfatase type VIII, and sulfatase type H-5 were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were available products of analytical or HPLC grade. The chocolate and cocoa used in this study were prepared by Meiji Seika Kaisha Ltd. (Saitama, Japan) and the composition of each is shown in Table I.

Experimental procedure

This study was carried out based on a cross-over (chocolate or cocoa intake) design with 6-day intervals. Blood samples were drawn from the intermediate cubital vein as a control after a 12-hour fasting period, and were drawn at 1, 2, 4, 8, and 24 hours after chocolate or cocoa intake. Urine samples were collected for 0 to 8 hours and 8 to 24 hours post-intake. The same foods and drinks were served to all subjects from the day before chocolate or cocoa intake until 24 hours after intake. To avoid the effect of polyphenols derived from the other foods and drinks consumed, the subjects were given rice, raw fish and water in the experimental period.

TABLE I Composition of chocolate and cocoa used in this study

	Chocolate	*Cocoa*
	units/serving	
Cocoa powder	35 g	35 g
Sucrose	31 g	31 g
Cocoa butter	30 g	
Total energy	540 kcal	270 kcal
Carbohydrate (excluding fiber)	37.6 g	36.6 g
Fat	34.5 g	5.15 g
Protein	7.58 g	7.39 g
Fiber	9.50 g	9.04 g
Ash	2.78 g	2.84 g
Caffeine	0.14 g	0.12 g
Theobromine	0.84 g	0.86 g
α -Tocopherol	576 μ g	66 μ g
γ -Tocopherol	7.97 mg	1.06 mg
δ -Tocopherol	192 μ g	ND
Total Polyphenol	2.74 g	2.73 g
(-)-Epicatechin	760 μ mol	760 μ mol
(+)-Catechin	214 μ mol	214 μ mol
Procyanidin B2	159 μ mol	159 μ mol
Procyanidin C1	72 μ mol	72 μ mol
3-Galactosyl-ent-epicatechin-(2 α →7, 4 α →8)-epicatechin	10.6 μ mol	10.6 μ mol
Cinnamtannin A2	87 μ mol	87 μ mol

Measurement of (-)-epicatechin in plasma and urine by HPLC

The levels of EC and its metabolites in plasma were determined by HPLC according to the method of Piskula et al. and Silva et al..[11,12] The levels of glucuronide, sulfate, and sulfoglucuronide conjugates of nonmethylated or methylated EC, were measured after treatment with β -glucuronidase type VII-A, sulfatase type VIII, or sulfatase type H-5. The amounts of each EC metabolite in plasma were calculated as the amount of EC detected after enzymatic treatment minus the amount of EC detected without enzymatic treatment. Urine samples were filtered and diluted 10-fold with saline, and HPLC measurements were performed as described above.[11,12]

HPLC-MS analysis of EC and its metabolites in plasma and urine

Plasma obtained 60 min after chocolate or cocoa intake, and urine collected for the period 0 to 8 hours after intake were analyzed by HPLC/MS after treatment with the enzymes described above.[11,12] The HPLC/MS used was the HP1100 system (Hewlett Packard, Palo Alto, CA, USA), and the conditions were as follows; column: CAPCELL PACK-UG 120, 150 mm × 2.0 mm I.D. (Shiseido, Tokyo, Japan), solvent: (A) 0.03 % formic acid (B) acetonitrile, elution: 10–50 % linear gradient of B in A (0–30 min), flow rate: 0.2 ml/min. The conditions for LC-MS analysis in the negative ion mode, scan mode were as follows: capillary voltage, 4500 V; fragmentor, 90 V; nebulizing pressure, 40 psig; drying gas temperature, 320 °C; drying gas flow, 8 l/min.

Statistical analysis

Data are presented as means and standard deviations. Data were analyzed by Student's paired t-test. Significance was recognized at $P<0.05$. All statistical analyses were performed using SPSS for Windows Ver. 7.5.1 software (SPSS Japan Inc., Tokyo, Japan).

RESULTS

Identification of nonmethylated and methylated (-)-epicatechin in plasma and urine by HPLC and LC/MS

In the plasma and urine samples obtained after chocolate or cocoa intake, two peaks were detected, at 12 and 26 min, after enzymatic treatment of the samples. The first peak showed the same retention time as the EC standard, and it showed a molecular ion $[M-H]^-$-peak at m/z 289 in the LC-MS analysis, thus it was identified as EC. The second peak eluted at 26 min showed a $[M-H]^-$-peak at m/z 303, and it was identified as methylated EC on the basis of agreement in terms of molecular weight.[11,12]

Levels of (-)-epicatechin and its metabolites in plasma

The profiles of EC and its metabolites in plasma after chocolate and cocoa intake are shown in Figure 1 and Table II. EC and its metabolites were not detected in plasma before intake. The maximum level of total EC metabolites reached 4.77 ± 0.94 μmol/l at 2 hours after chocolate intake, and 4.92 ± 0.94 μmol/l at 2 hours after cocoa intake. At one hour after intake, the plasma level of total nonmethylated EC in the cocoa group was significantly higher than that in the chocolate group. The concentrations of free nonmethylated EC 1 and 2 hours after cocoa intake were significantly higher than those in the case of chocolate intake. The concentrations of total methylated EC in the chocolate group were significantly higher than those in the cocoa group at 2 and 4 hours after intake. There were no significant differences in plasma levels of other EC-related compounds. At 24 hours

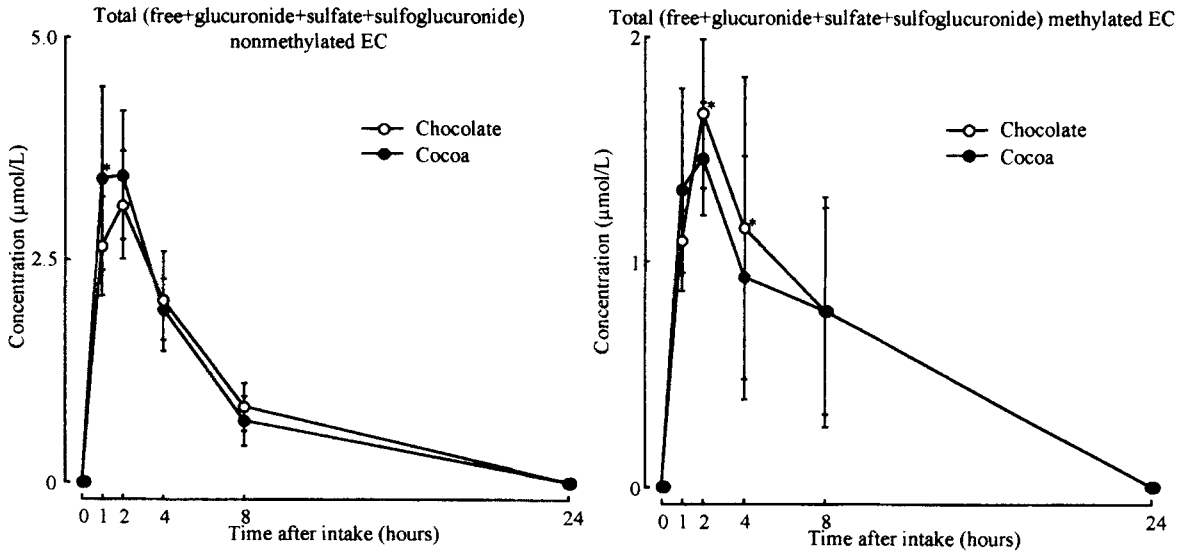

FIGURE 1 Profiles of total (-)-epicatechin in human plasma after chocolate or cocoa intake. Data shows the sum of free, glucuronide, sulfate, and sulfoglucuronide of nonmethylated or methylated forms concentration in plasma before and at 1, 2, 4, 8, and 24 hours post-intake. Values are means with their standard deviations (n=5). Significantly different from the value between chocolate and cocoa (*$P<0.05$)

post-intake, all EC metabolites had disappeared from plasma. Free methylated EC and glucuronide conjugates of methylated EC were not detected.

Levels of (-)-epicatechin and its metabolites in urine

The urinary excretion of EC and its metabolites after chocolate and cocoa intake is shown in Figure 2 and Table III. Urinary excretion of total EC metabolites within 24 hours after chocolate and cocoa intake was 29.8 ± 5.3% and 25.3 ± 8.1% of total EC intake (Table III). About 80 % of the total EC metabolites were excreted within 8 hours after either chocolate or cocoa intake. There were no significant differences in any of the metabolites excreted in urine at any point comparing chocolate and cocoa intake. Sulfoglucuronide and sulfate conjugates of nonmethylated and methylated EC and free nonmethylated EC were the main metabolites present in urine after either chocolate or cocoa intake. After intake, free nonmethylated EC was detected, but free methylated EC was not present in urine (Figure 2).

DISCUSSION

In a previous study, Richelle et al. reported that EC in black chocolate was absorbed in human plasma, and its concentration reached a maximum 2 to 3 hours after intake.[13] However, in that study EC metabolites were not detected in detail. In the present study, we evaluated the metabolites of EC in human plasma and urine after ingestion of chocolate and cocoa. It has been reported that the major EC-related compounds were sulfate, glucuronide, and sulfoglucuronide conjugates of nonmethylated EC in rats.[11] Our findings show that similar metabolites are produced in humans (Table II). The main metabolites of EC in urine after chocolate or cocoa intake were sulfoglucuronide and sulfate conjugates of nonmethylated and methyl-

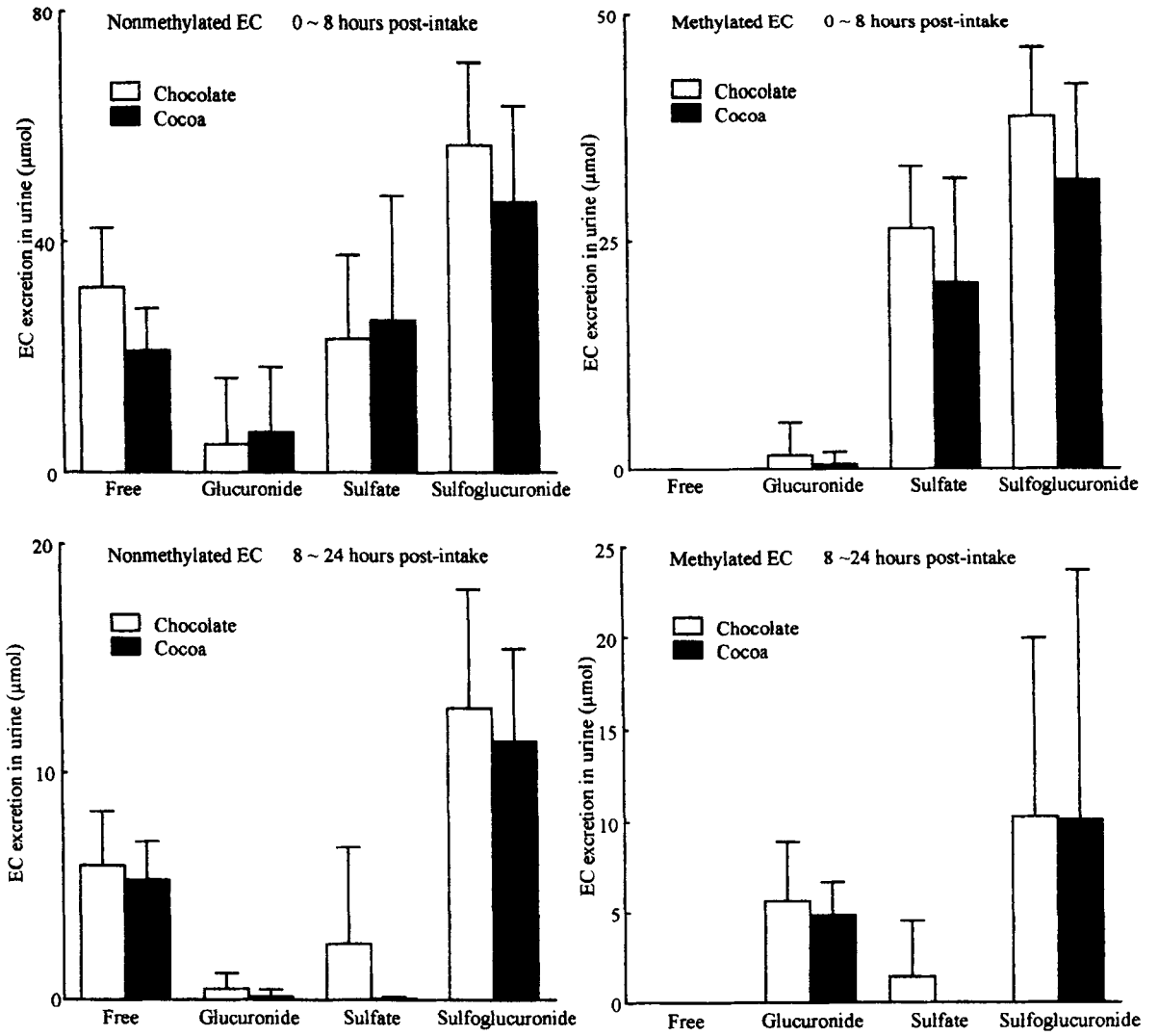

FIGURE 2 Profiles of (-)-epicatechin (EC) and its metabolites in human urine after chocolate and cocoa intake. Data shows the EC and its metabolites excreted in urine for 0 to 8 hours and for 8 to 24 hours post intake. Values are means with standard deviations (n=5). There were no significant differences in urinary excretion of EC metabolites between chocolate and cocoa intake ($P<0.05$)

ated EC (Figure 2). Unexpectedly, free nonmethylated EC was detected at a high level in urine as compared with the plasma level. In a previous human study, the major EC metabolite detected in both plasma and urine after intake of one cup of decaffeinated green tea (containing 110 μmol of EC) was the sulfated form.[14] After intake of 120 mL of red wine (containing 120 μmol of (+)-catechin), which is also rich in catechins and procyanidin, similar to cacao, the concentration of total catechin in human plasma was shown to reach a maximum at 1 hour post-intake[15], and the sulfate form of non-methylated catechin represented about 20 % of the total nonmethylated forms. These results suggest that the profile and total amount of cate-

chin metabolites might be affected by the experimental conditions such as the dose administered, the components of other foods or drinks consumed, etc.

TABLE II Plasma concentration of EC and its metabolites after chocolate or cocoa intake

	Nonmethylated EC (μ mol/L)							
	Free		*Glucuronide*		*Sulfate*		*Sulfoglucuronide*	
	chocolate	*cocoa*	*chocolate*	*cocoa*	*chocolate*	*cocoa*	*chocolate*	*cocoa*
Before intake	0	0	0	0	0	0	0	0
1 hour after intake	0.10±0.03[a]	0.22±0.06	0.52±0.15	0.81±0.20	0.84±0.25	1.09±0.24	1.19±0.44	1.28±0.76
2 hours after intake	0.15±0.04[a]	0.22±0.02	0.78±0.28	0.91±0.21	1.11±0.43	1.14±0.21	1.07±0.24	1.19±0.57
4 hours after intake	0.07±0.02	0.15±0.06	0.56±0.11	0.66±0.16	0.77±0.31	0.70±0.38	0.64±0.29	0.43±0.34
8 hours after intake	0	0	0	0	0.12±0.27	0	0.73±0.05	0.69±0.28
24 hours after intake	0	0	0	0	0	0	0	0

	Methylated EC (μ mol/L)							
	Free		*Glucuronide*		*Sulfate*		*Sulfoglucuronide*	
	chocolate	*cocoa*	*chocolate*	*cocoa*	*chocolate*	*cocoa*	*chocolate*	*cocoa*
Before intake	0	0	0	0	0	0	0	0
After 1 hour intake	0	0	0	0	0.54±0.14	0.82±0.36	0.55±0.07	0.51±0.32
After 2 hour intake	0	0	0	0	0.95±0.27	1.00±0.34	0.71±0.14	0.46±0.12
After 4 hour intake	0	0	0	0	0.63±0.37	0.53±0.36	0.52±0.32	0.41±0.25
After 8 hour intake	0	0	0	0	0.13±0.30	0	0.65±0.31	0.78±0.51
After 24 hour intake	0	0	0	0	0	0	0	0

Values are means ± SD, n = 5.

a. Significantly different compared with cocoa ($p < 0.05$).

TABLE III Total EC metabolites excretion in urine after chocolate or cocoa intake[a]

	Total EC metabolites excretion[b]	
	Excretion volume (μ mol)	*Excretion recovery (%)*[c]
0 ~ 8 hours		
Chocolate	188 ± 33	24.7 ± 4.4
Cocoa	159 ± 53	20.9 ± 6.4
8 ~ 24 hours		
Chocolate	39.5 ± 19.1	5.2 ± 2.6
Cocoa	33.4 ± 14.6	4.4 ± 1.8
0 ~ 24 hours		
Chocolate	226 ± 39	29.8 ± 5.3
Cocoa	192 ± 63	25.3 ± 8.1

[a] Values are masns ± SD, n=5. There were no differences in total EC excretion volume or recovery at any point between chocolate and cocoa ($P<0.05$).
[b]Total EC metabolites mean the sum of free, glucuronide, sulfate, and sulfoglucuronide conjugates of nonmethylated and methylated forms.
[c]Excretion recovery is the ratio to EC intake.

The percent EC recovered in analysis of urine after chocolate and cocoa intake was 29.8 ± 5.3 % and 24.1 ± 8.1% of total EC intake, and about 80 % of the total metabolites were excreted within 8 hours after intake (Table III). It has been reported that the elimination half-life of catechins is between 3 and 5 hours, whereas that of quercetin is 24 hours in humans.[16] This suggests that the absorption of catechins, including EC, is faster than that of quercetin, and that catechins are quickly excreted in urine and bile in humans. Moreover, cacao products are known to contain a high amount of procyanidins which are oligomers of EC. The EC and its metabolites detected in plasma and urine in this study might be derived from procyanidin in cacao. There have been no reports concerning the absorption of procyanidin oligomers in humans.

The intake of cocoa butter per serving of chocolate and cocoa in this study was 34.5 g and 5.15 g, respectively (Table I), but there was no difference in the profiles or the percent recovery of EC metabolites comparing chocolate and cocoa intake. Whether the fat consumed had an effect on EC absorption in this study is not clear.

In conclusion, EC in chocolate and cocoa was absorbed and was present as various conjugates of nonmethylated and methylated EC in plasma, and these were rapidly excreted in urine.

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