Catechins and Their Oligomers Linked by C4 → C8 Bonds Are Major Cacao Polyphenols and Protect Low-Density Lipoprotein from Oxidation *In Vitro*

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***In vitro* effects of catechins and their oligomers linked by C4 → C8 bonds are major antioxidative components of chocolate and cocoa. Their effects on the susceptibility of human low-density lipoprotein (LDL) to oxidation were evaluated. The strength of the antioxidative activity was measured using copper ions as the radical generator as compared by weight varied in the following order: (+)-catechin > procyanidin B2 ≧ (−)-epicatechin ≧ procyanidin C1 > cinnamtannin A2. Using 2,2′-azobis (4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) as the radical generator, the order was (−)-epicatechin ≧ procyanidin B2 ≧ procyanidin C1 > (+)-catechin ≧ cinnamtannin A2. It is suggested that these compounds contribute to the activity of cacao products to protect LDL from oxidation.**

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Key words: catechin; procyanidin; LDL; copper; MeO-AMVN; cacao

A protective role of plant polyphenols against atherosclerosis has been suggested by findings in several studies. According to the results of epidemiological studies, plant polyphenol consumption is associated with a reduced risk of coronary heart disease (CHD) (1–3). Polyphenolic substances derived from tea (4) or grape seeds (5) have been shown to be effective in prevention of atherosclerosis in hypercholesterolemic rabbits. These studies suggested that the antiatherosclerotic effect of these compounds is the result of inhibition of low-density lipoprotein (LDL) oxidation.

Cacao beans, the seed of *Theobroma cacao,* are known to contain various polyphenolic substances (6–8). (−)-Epicatechin, (+)-catechin, and their oligomers linked by C4 → C8 bonds such as procyanidin B2, procyanidin C1, and cinnamtannin A2 have been determined to be the major antioxidative components of cocoa and chocolate (9). The chemical structures of these compounds are shown in Figure 1. The isolation and determination in cacao products of these compounds were carried out by the method described in a previous report (9). The amounts of these compounds in commercially available products, six dark chocolates and six cocoa powders, are shown in Table I. We also confirmed the presence of other polyphenolic substances in cacao products as minor components, such as clovamide, dideoxyclovamide, quercetin-glucopyranoside, and quercetin-arabinopyranoside (7). The polyphenol fraction derived from cacao beans has been found to display various useful physiological activities such as an oxidative stress-reducing effect in vitamin E-deficient rats (10), anti-ulcer activity (11), and antimutagenic effects against heterocyclic amines (12). We have also reported that administration of cocoa powder or its polyphenolic fraction to human volunteers or to hypercholesterolemic rabbits reduces the susceptibility of LDL to oxidation (13–15). In the present study, we examined the effects of catechins and procyanidins isolated from cacao beans on the susceptibility of human LDL to oxidation induced by copper ions or the lipophilic azo initiator MeO-AMVN *in vitro.*

Materials and Methods

Chemicals. Catechin was purchased from Extrathese (Genay, France), epicatechin was obtained from Sigma (St. Louis, MO), and MeO-AMVN was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Procyanidin B2,

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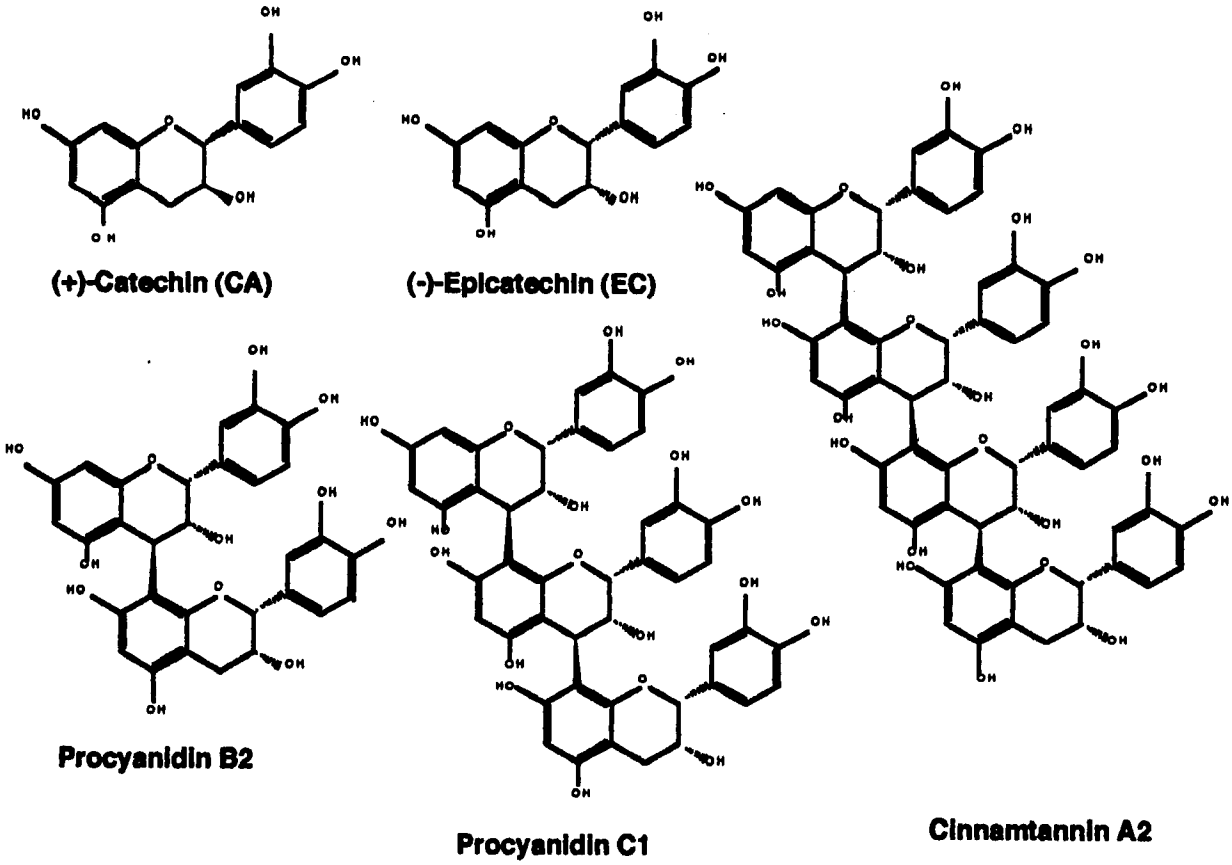

Figure 1. The chemical structures of polyphenols in chocolate and cocoa.

TABLE I. Polyphenol Content in Commercially Available Dark Chocolate and Cocoa Powder[a]

	Dark chocolate $n = 6$	Cocoa powder $n = 6$
(+)-Catechin (w/w, %)	0.028 ± 0.006	0.093 ± 0.023[b]
(−)-Epicatechin (w/w, %)	0.087 ± 0.030	0.101 ± 0.025
Procyanidin B2 (w/w, %)	0.037 ± 0.012	0.040 ± 0.015
Procyanidin C1 (w/w, %)	0.026 ± 0.011	0.024 ± 0.011
Cinnamtannin A2 (w/w, %)	0.054 ± 0.020	0.033 ± 0.019

[a] Each value represents the mean and standard deviation of six samples.
[b] Significant difference between dark chocolate and cocoa powder $P \leq 0.05$.

procyanidin C1, and cinnamtannin A2 were isolated from cacao liquor as previously described (9). Other chemicals were commercially available products of analytical or HPLC grade. Six kinds of dark chocolates and cocoa powders were purchased at a local store.

LDL Oxidation. LDL oxidation was assayed by the methods of Esterbauer (16) and Hirano and Kondo (17) with slight modification. LDL was isolated from the plasma of healthy human subjects by single-spin density gradient centrifugation (417,000*g*, 40 min, 4°C) (18). The fractions obtained were dialyzed against a 2,000-fold volume of nitrogen-purged 10 m*M* phosphate-buffered saline (pH 7.4) at 4°C overnight. The protein concentration was determined by the bicinchonic acid method (19). Each compound was dissolved in methanol the concentration and added to LDL. The methanol concentration was 0.001 (w/w, %). The final concentrations of test compounds were 0.125, 0.25, 0.5, 1.0, and 2.0 μg/ml. A mixture consisting of the human LDL fraction (200 μg of protein/ml) and 10 μM $CuCl_2$ or 750 μM MeO-AMVN as the initiator of radical formation was incubated at 37°C. MeO-AMVN was dissolved in acetonitrile, and the final concentration of acetonitrile in the reaction mixture was 0.001 (w/w, %). The kinetics of LDL oxidation were determined by monitoring the change in absorbance at 234 nm for 420 min due to conjugated diene formation. Each analysis was performed six times, and the mean and standard deviation are shown.

Statistical Analysis. The data are expressed as the mean ± the standard deviation. Analyses were done by using SPSS for Windows, ver. 7.5 (SPSS Co. Ltd., Tokyo, Japan). When ANOVA revealed $P \leq 0.05$, the data were further analyzed by Tukey's multiple range test. Differences were considered statistically significant at $P \leq 0.05$.

Results

LDL Oxidation Induced by Copper Ions. The effects of catechins and procyanidins in suppressing the oxidation of LDL induced by copper ions were evaluated. The typical pattern of conjugated diene formation at 234 nm in either the presence or absence of epicatechin is shown in

Figure 2a. Likewise, all of the other cacao polyphenols tested markedly prolonged the lag time. Table II shows the relationship between the final concentration of each component and the lag time. There was a good correlation between them (r^2 = 0.968–0.986) within the concentration range used in this study. The addition of more than 0.25 μg/ml of the addition of all of the polyphenols significantly prolonged the lag time compared with no addition. At a concentration of 2 μ/ml, there was a significant difference between (+)-catechin and procyanidin B2. (−)-epicatechin, procyanidin C1, and cinnamtannin A2. Cinnamtannin A2 was significantly less effective compared with the other compounds at less than 1.0 μg/ml. The concentration of each compound required to prolong the lag time by 2-fold compared with that in the case of the control with no addition was calculated by the least squares method, and the results are shown in Table III. The strength of the antioxidative activity compared by weight varied in the following order: (+)-catechin > procyanidin B2 ≧ (−)-epicatechin ≧ procyanidin C1 > cinnamtannin A2. When concentrations were expressed in terms of morality, the strength was cinnamtannin A2 ≧ procyanidin C1 > procyanidin B2 > (+)-catechin > (−)-epicatechin.

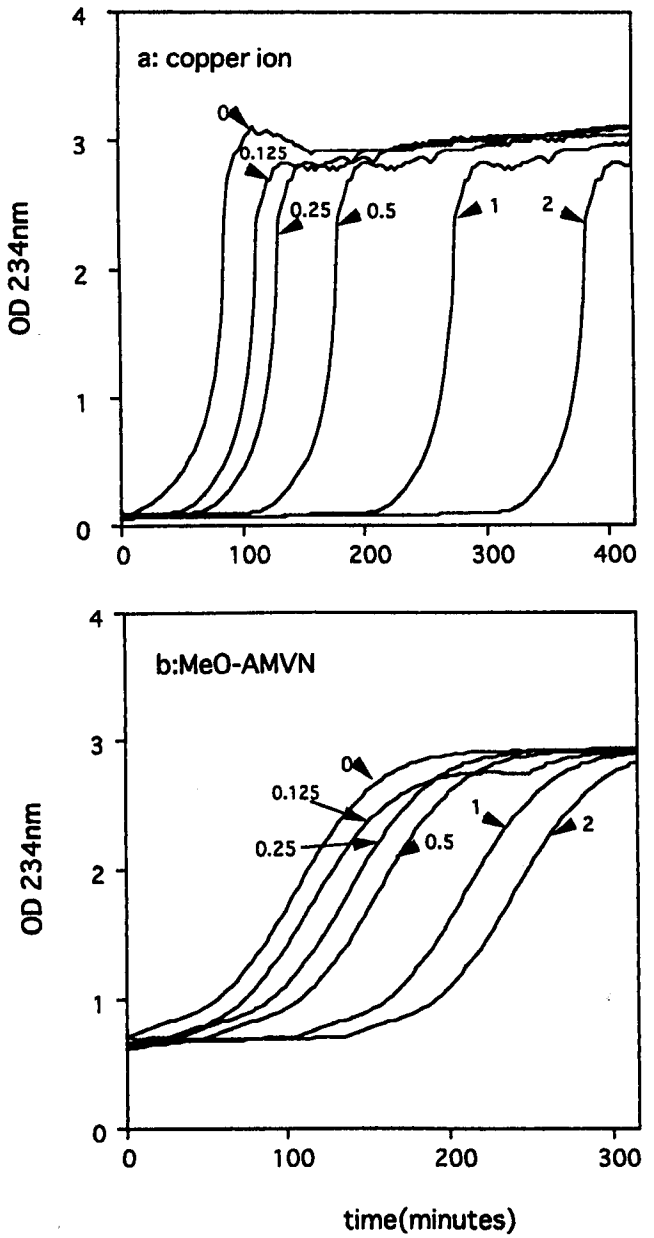

Figure 2. Typical patterns of conjugated diene production induced by 10 μM $CuCl_2$ or 750 μM MeO-AMVN (a) at 234 nm (b) in the presence or absence of (−)-epicatechin. The numbers shown in the figure are the final concentrations of (−)-epicatechin in the mixture (micrograms per milliliter).

LDL Oxidation Induced by MeO-AMVN. The effects of catechins and procyanidins in suppressing the oxidation of LDL induced by MeO-AMVN were evaluated. Likewise, the typical pattern of conjugated diene formation is shown in Figure 2b. Table II shows the relationship between the final content of each component and the lag time. There was a good correlation between them (r^2 = 0.931–0.991) within the concentration range used in this study. More than 0.125 μg/μl of the addition of (−)-epicatechin, (+)-catechin, and procyanidin B2 significantly prolonged the lag time compared with no addition. Likewise. the lag time was significantly prolonged by more than 0.5 μg/μl of the addition of procyanidin C1, and more than 1.0 μg/μl cinnamtannin A2. There were significant differences between (−)-epicatechin, procyanidin B2, procyanidin C1, and (+)-catechin, cinnamtannin A2 at the concentration of 2 μg/ml. The concentration of each compound required to prolong the lag time 2-fold as compared with that in the case of the control with no addition was calculated by the method of least squares, and the results are shown in Table III. The strength of the antioxidative activity to be compared by weight varied in the following order: (−)-epicatechin ≧ procyanidin B2 ≧ procyanidin C1 > (+)-catechin cinnamtannin A2. When concentrations were expressed in term of molarity, the strength was procyanidin C1 ≧ cinnamtannin A2 > procyanidin B2 > (−)-epicatechin > (+)-catechin.

Discussion

Recent evidence suggests that LDL oxidation plays an important role in the pathogenesis of atherosclerosis (20–22). A finding in a famous epidemiological study, the so-called French paradox, is that low mortality from coronary heart disease was negatively correlated with the consumption of saturated fat (23). According to the results of recent research, there is an association between red wine consumption and a risk of occurrence of CHD. One of the types of components of red wine effective in preventing CHD was suggested to be polyphenols such as procyanidins and catechins (24, 25). However, there have been few studies examining which components are effective, or how these compounds contribute to the beneficial effect. We have previously reported the effects of daily intake of cacao powder on LDL oxidative resistance in healthy human volunteers (14). The volunteers consumed 12 g of cacao powder after breakfast, lunch, and dinner for 2 weeks. The susceptibility of their LDL to oxidation induced by copper ions or MeO-

TABLE II. Effect of Polyphenols from Cacao Beans in the Extension of the Lag Time of Conjugated Diene Formation in LDL Oxidation Induced Copper Ion and MeO-AMVN[a]

	Polyphenol concentration (μg/ml)				
	0.125	0.25	0.5	1.0	2.0
Induced by copper ion					
No addition	47.2 ± 4.7 b	45.4 ± 0.8 b	45.0 ± 4.5 b	46.1 ± 1.9 b	46.9 ± 2.3 b
(−)-Epicatechin	50.1 ± 2.9 b	60.9 ± 6.5 c	100.6 ± 11.0 d	203.4 ± 39.8 c	305.5 ± 20.8 c
(+)-Catechin	49.2 ± 3.0 b	58.7 ± 3.7 cd	89.2 ± 17.2 c	231.4 ± 26.8 c	375.8 ± 26.7 d
Procyanidin B2	46.1 ± 4.5 b	64.0 ± 3.5 c	97.3 ± 23.2 c	215.9 ± 32.2 c	308.1 ± 23.0 c
Procyanidin C1	47.8 ± 4.6 b	58.2 ± 3.3 cd	91.9 ± 23.2 c	212.4 ± 28.7 c	303.9 ± 31.2 c
Cinnamtannin A2	46.8 ± 3.9 b	52.7 ± 4.0 d	75.2 ± 12.4 b	145.4 ± 31.0 d	272.3 ± 28.7 c
Induced by MeO-AMVN					
No addition	51.8 ± 2.3 b	52.7 ± 2.4 b	53.4 ± 2.1 b	48.9 ± 3.5 b	50.1 ± 1.5 b
(−)-Epicatechin	59.2 ± 2.7 cd	68.8 ± 7.6 d	109.7 ± 1.7 d	135.4 ± 6.5 d	178.8 ± 15.2 d
(+)-Catechin	57.2 ± 1.5 ce	61.3 ± 3.3 c	81.4 ± 5.6 c	96.8 ± 12.7 c	139.2 ± 9.4 c
Procyanidin B2	61.3 ± 3.2 d	61.7 ± 2.0 c	87.1 ± 9.5 c	135.6 ± 13.0 c	177.0 ± 27.2 d
Procyanidin C1	54.9 ± 1.1 be	57.6 ± 2.6 b	78.5 ± 5.7 c	124.7 ± 7.1 c	175.3 ± 22.3 d
Cinnamtannin A2	51.9 ± 1.8 b	55.5 ± 3.4 b	58.5 ± 2.6 b	96.0 ± 3.6 b	132.1 ± 20.2 c

Note. Values in a row not sharing the same letters are significantly different at $P \leqq 0.05$.
[a] Each value represents the mean and standard deviation (n = 6).

TABLE III. Polyphenol Concentration Required to Prolong the Lag Time 2-Fold Relative to the No Addition[a]

	MW	Copper ion[b]		MeO-AMVN[c]	
		μg/ml	μM	μm/ml	μM
(+)-Catechin	290	0.366	1.262	1.190	4.103
(−)-Epicatechin	290	0.393	1.355	0.704	2.427
Procyanidin B2	578	0.382	0.661	0.758	1.311
Procyanidin C1	866	0.399	0.460	0.872	1.007
Cinnamtannin A2	1154	0.526	0.455	1.346	1.166

[a] The value was calculated using at least squares analysis.
[b] A mixture consisting of human LDL fraction (200 μg protein/ml) and 10 μM $CuCl_2$.
[c] A mixture consisting of human LDL fraction (200 μg protein/ml) and 750 μM MeO-AMVN.

AMVN was significantly decreased compared with the control group. In addition, when hypercholesterolemic rabbits were fed a high-fat, high-cholesterol diet containing 1% cacao liquor polyphenol fraction (containing 50% total polyphenols prepared by the method described in a previous report [10]) for about 10 days, the oxidative resistance of their LDL was significantly increased compared with that before intake (13).

In this study, we evaluated the antioxidative activity of the main polyphenolic components in chocolate and cocoa, including (−)-epicatechin, (+)-catechin, and their oligomers linked by C4 → C8 bonds by examining their effect on the susceptibility of LDL to oxidation induced by copper ions or MeO-AMVN *in vitro.* Copper ions are known to induce the formation of radicals in the aqueous phase, whereas MeO-AMVN is a lipid soluble azo-compound that generates radicals in lipid bilayers. Using copper ions as the radical generator, (+)-catechin was most effective in extension of the lag time, followed by procyanidin B2, (−)-epicatechin, and procyanidin C1, and cinnamtannin A2 was less effective to be compared by weight relatively. When expressed on a molar basis, the antioxidant activity increased as the degree of polymerization increased. Hence, they prolonged the lag time in the following order: cinnamtannin A2 > procyanidin C1 > procyanidin B2 > catechins. Using the lipophilic azo-compound MeO-AMVN as the radical generator, (−)-epicatechin and procyanidin B2 were the most potent, showing similar effectiveness, followed by procyanidin C1, while (+)-catechin and cinnamtannin A2 were less effective compared by weight. Likewise, when expressed on a molar basis, the antioxidant activity increased as the degree of polymerization increased. Hence, they prolonged the lag time in the following order: cinnamtannin A2 ≧ procyanidin C1 > procyanidin B2 > catechins. According to these results, the number of hydroxyl groups in chemicals was important to show antioxidative activity. However, it seems to be the only factor. This is because, in the present study, we found no correlation between the degree of polymerization of the procyanidins and inhibition of LDL oxidation in either of the experiments when compared by weight. This is partly due to the characteristics of each polyphenolic compound, such as their effectiveness in reduction of radical species or reactive oxygen species, their affinity in interaction with proteins in the LDL particle, their affinity in interaction with the LDL bilayer, their metal chelating activity, etc. In general, these compounds show high affinity in interaction with proteins. da Silva *et al.* (26) reported that interactions between procyanidins and proteins such as gelatin, casein, proteins in dried blood, or arabinogalactan-protein increased with the degree of polymerization of the procyanidins. In addition, the radical scavenging activity of these compounds seems be the most important in terms of the mechanism of protecting LDL from oxidation. Recent research has shown that

LDL oxidation in blood is caused by some radical species produced enzymatically or induced by metallic substances (27–29). There have been several previous reports concerning the radical scavenging ability of catechins and their oligomers. da Silva *et al.* (30) reported the effects of procyanidins in scavenging superoxide radicals (O_2^-) generated by the xanthine-xanthine oxidase system and hydroxyl radicals (OH·) generated in Fenton's reaction. According to their report, the order of potency in terms of O_2^- scavenging activity is procyanidin C1 > procyanidin B2 > (−)-epicatechin > (+)-catechin, and the order in terms of OH· scavenging activity is (−)-epicatechin > (+)-catechin > procyanidin C1 > procyanidin B2. Arteel *et al.* (31) reported that among epicatechin oligomers, the tetramer was particularly efficient in protecting against oxidation by peroxynitrite. However, with respect to the other radical species or active forms of oxygen that are thought to be generated by copper ions and azo-compounds, the scavenging effects of these procyanidins remain obscure. In this way, the degree of polymerization seems to affect the affinity for interaction with the LDL particle, metal chelating ability, and radical scavenging activity. However, these have not been well studied.

Recently, there have been several reported studies on the effects of polyphenols derived from cacao on LDL oxidation *in vitro*. Lottio *et al.* (32) suggested that procyanidin oligomers showed potent antioxidative activity in studies focusing on liposomes and LDL. Bearden *et al.* (33) reported similar activity in cacao polyphenols. There are several differences in the methods used in this study and their studies, especially in that individual compounds were used in our study, whereas fractions containing several procyanidin oligomers that showed the same molecular weight were used in their studies. As described above, in the present study, the antioxidative activities differed significantly between (+)-catechin and (−)-epicatechin, which were the stereo isometric compounds. (+)-Catechin was the most effective antioxidant when LDL oxidation was induced by copper ions, and in the case of oxidation induced by MeO-AMVN, (−)-epicatechin showed the strongest antioxidative activity among procyanidin oligomers compared by weight. On the other hand, the monomer did not show the strongest antioxidant activity in the previous studies. In this way, the antioxidative activity was closely dependent on the chemical structures. Therefore, it is difficult to discuss the efficacy of antioxidative ability by the previous results using crude fractions. Teissedre *et al.* (34) examined the inhibitory effects of several polyphenolic substances from red wine on human LDL oxidation induced by copper ions. They reported that (+)-catechin, (−)-epicatechin, and procyanidin C1 were more effective than procyanidin B2, other flavonols (myricetin, quercetin, or rutin), phenolic acids (caffeic acid, ellagic acid, or sinapic acid), or α-tocopherol at a concentration of the same molarity. Their experimental conditions differed from those in the present study, especially in that the polyphenolic substance/LDL ratio was markedly higher than that in this study, and they evaluated the activity by monitoring hexanal production in LDL. The order of effectiveness of the compounds was slightly different comparing the results of these two studies, and this discrepancy was likely due to the differences in experimental conditions.

In addition, it has been reported that catechins are absorbed, distributed in blood, and excreted in urine as several conjugated forms (35, 36). Recently, it has been reported that epicatechin and its metabolites reach high concentrations in plasma after an intake of 36 g of cocoa powder in human volunteers (37). The maximum total concentration of epicatechin metabolites in plasma 2 hr after intake was about 0.5 μg/ml in this clinical research. We used procyanidin dosage ranging from 0.125–2.0 μg/ml in this *in vitro* study. This concentration is almost the same as the plasma concentration of (−)-epicatechin-relative substances after 36 g of cocoa powder are consumed. On the other hand, it has been reported that a daily intake of 36 g of cocoa powder reduced the susceptibility of LDL to oxidation in human volunteers (14). Taken together, these findings suggest that the polyphenols in cocoa powder are absorbed and distributed in blood at an effective concentration, and that they enhance the oxidative resistance of LDL.

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