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In Vitro Antioxidative Activity of (−)-Epicatechin Glucuronide Metabolites Present in Human and Rat Plasma

MIDORI NATSUME[a,*], NAOMI OSAKABE[a], AKIKO YASUDA[a], SEIGO BABA[a], TAKASHI TOKUNAGA[a], KAZUO KONDO[b], TOSHIHIKO OSAWA[c] and JUNJI TERAO[d]

[a]*Healthcare R&D Laboratories, Meiji Seika Kaisha Ltd., 5-3-1 Chiyoda, Sakado-shi, Saitama 350-0289, Japan;* [b]*Institute of Environmental Science for Human Life, Ochanomizu University, Ohtsuka, Bunkyo-ku, Tokyo 112-8610, Japan;* [c]*Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi, 464-8601, Japan;* [d]*Department of Food Science, Graduate School of Nutrition and Biosciences, The University of Tokushima, 3 Kuromoto-cho, Tokushima 770-8503, Japan*

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Recently we identified four conjugated glucuronide metabolites of epicatechin, (−)-epicatechin-3′-*O*-glucuronide (E3′G), 4′-*O*-methyl-(−)-epicatechin-3′-*O*-glucuronide (4′ME3′G), (−)-epicatechin-7-*O*-glucuronide (E7G) and 3′-*O*-methyl-(−)-epicatechin-7-*O*-glucuronide (3′ME7G) from plasma and urine. E3′G and 4′ME3′G were isolated from human urine, while E7G and 3′ME7G were isolated from rats that had received oral administration of (−)-epicatechin (Natsume *et al.* (2003), *Free Radic. Biol. Med.* **34**, 840–849). It has been suggested that these metabolites possess considerable *in vivo* activity, and therefore we carried out a study to compare the antioxidant activities of the metabolites with that of the parent compound. This was achieved by measuring superoxide scavenging activity, reduction of plasma TBARS production and reduced susceptibility of low-density-lipoprotein (LDL) to oxidation. (−)-Epicatechin was found to have more potent antioxidant activity than the conjugated glucuronide metabolites. Both (−)-epicatechin and E7G had marked antioxidative properties with respect to superoxide radical scavenging activity, plasma oxidation induced by 2,2′-azobis-(2-aminopropane) dihydrochloride (AAPH) and LDL oxidation induced by copper ions or 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN). In contrast, the other metabolites had light antioxidative activities over the range of physiological concentrations found in plasma.

Keywords: (−)-epicatechin metabolites; Antioxidative activity; Superoxide radical; Plasma oxidation; Low-density-lipoprotein(LDL)

INTRODUCTION

Epidemiological evidence indicates that consumption of plant polyphenols is associated with reduced risk of coronary heart disease (CHD),[1−4] with recent reports suggesting that catechins reduce this risk as effectively as flavonols.[4] Studies in postmenopausal women have also shown that there is a strong inverse correlation between CHD risk and intake of (+)-catechin and (−)-epicatechin. Gallated catechin does not, however, show this association.[5] Major sources of catechins in the diet are black tea, apples and chocolate[4−7] with studies in rats demonstrating that (−)-epicatechin ingested orally is absorbed from the intestinal tract and metabolized to glucuronidated or sulfated conjugates in the plasma.[8] We have shown in both humans and rats that following ingestion of cocoa powder, (−)-epicatechin is absorbed from the intestinal tract and is distributed as various conjugated and/or methylated forms in the blood before being excreted in the urine.[9,10] We have also isolated and identified the (−)-epicatechin glucuronide metabolites that include (−)-epicatechin-3′-*O*-glucuronide (E3′G), 4′-*O*-methyl-(−)-epicatechin-3′-*O*-glucuronide (4′ME3′G) in human plasma, and (−)-epicatechin-7-*O*-glucuronide (E7G) and 3′-*O*-methyl-(−)-epicatechin-7-*O*-glucuronide (3′ME7G), in rat plasma.[11]

Despite the possibility that flavonoid metabolites may have similar biological activities to the parent compound, there are only a limited number of reports on the antioxidative activities of the metabolites.[12,13] Therefore, the aim of the present study was to examine in greater detail the antioxidative activity of the glucuronide metabolites

*Corresponding author. Tel.: +81-49-284-7596. Fax: +81-49-284-7598. E-mail: midori_natsume@meiji.co.jp

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of (−)-epicatechin present in human and rat plasma.

MATERIALS AND METHODS

(−)-Epicatechin and superoxide dismutase (SOD) were purchased from Sigma Chemical Co. (St Louis, MO). The (−)-epicatechin human metabolites, (−)-epicatechin-3′-*O*-glucuronide (E3′G), and 4′-*O*-methyl-(−)-epicatechin-3′-*O*-glucuronide (4′ME3′G) were prepared from human urine, while the epicatechin rat metabolites, (−)-epicatechin-7-*O*-glucuronide (E7G) and 3′-*O*-methyl-(−)-epicatechin- 7-*O*-glucuronide (3′ME7G) were prepared from rat urine according to methods described in our previous study.[11] The structures of these compounds are shown in Fig. 1. The purity of the epicatechin metabolites was measured by monitoring UV absorbance at 220 nm using HPLC. Each solution was applied to a Capcellpak UG120 column (Shiseido, Tokyo, Japan; 150 mm × 2.0 mm I.D., 5 μm). The solvents used were (A) 0.03% formic acid in H_2O and (B) CH_3CN. Elution was achieved using a linear gradient from 10 to 50% (v/v) B over 30 min at a flow rate of 0.2 ml/min. The purity of epicatechin metabolites was as follows: E3′G; 97.8%, 4′ME3′G; 92.4%, E7G; 94.6%, 3′ME7G; 90.0%. 2, 2′-azobis-(2-aminopropane) dihydrochloride (AAPH), and 2, 2′-azobis(4-methoxy-2, 4-dimethylvaleronitrile) (MeO-AMVN) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Assay of Superoxide Scavenging Activity

Superoxide scavenging activity was measured by the superoxide dismutase (SOD) assay kit-WST (Dojindo Laboratory, Kumamoto, Japan).[14] A 20 μl aliquot of test samples dissolved in 1% (v/v) aqueous methanol was mixed with a WST working solution (200 μl) and enzyme working solution (20 μl). Blanks were prepared by substituting 1% (v/v) aqueous methanol for the sample solution [blank 1], dilution buffer for the enzyme working solution [blank 2] and 1% (v/v) aqueous methanol solution and dilution buffer for the sample solution and enzyme working solution. Following

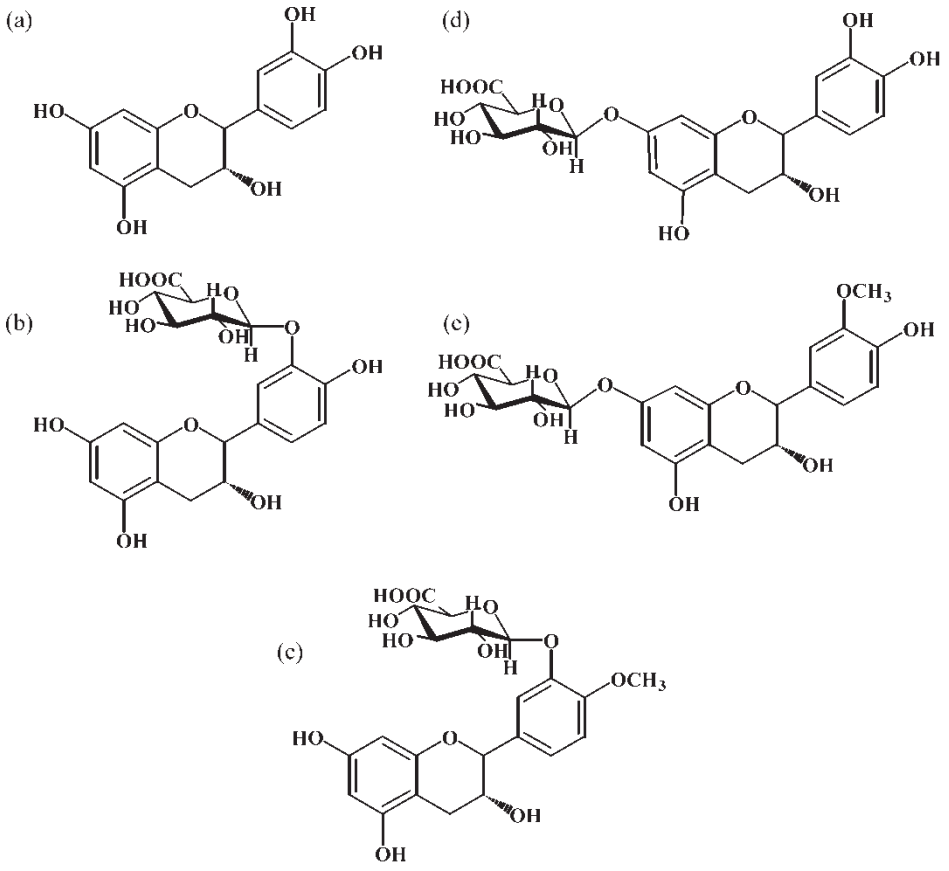

FIGURE 1 Structure of (−)-epicatechin and its glucuronide metabolites isolated from human and rat urine. (a) (−)-epicatechin; (b) (−)-epicatechin-3′-*O*-glucuronide (E3′G); (c) 4′-*O*-mehyl-(−)-epicatechin-3′-*O*-glucuronide (4′ME3′G); (d) (−)-epicatechin-7-*O*-glucuornide (E7G); (e) 3′-*O*-methyl-(−)-epicatechin-7-*O*-glucuonide (3′ME7G).

incubation at 37°C for 20 min, the absorbance (A) at 450 nm of the reacted solutions was measured using a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA). Superoxide scavenging activity (inhibition rate %) was calculated as

$$\{[(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})]/(A_{blank1} - A_{blank3})\} \times 100.$$

Each analysis was performed in triplicate with the data being expressed as the mean and standard deviation.

Plasma Oxidation Model

Inhibition of human plasma oxidation by (−)-epicatechin and its metabolites was determined by a modification of the method of Baba *et al.*[10] Briefly, 10 μl of human plasma, 10 μl of (−)-epicatechin-related compound dissolved in 10% v/v aqueous ethanol and 10 μl of AAPH (final concentration 25 μM) were added to 470 μl of PBS and incubated at 37°C for 60 min. After incubation, the production of thiobarbituric reactive substances (TBARS) in the reaction mixture was measured according to the method of Ohkawa *et al.*[15] Inhibition of TBARS production caused by each compound was calculated as the percentage of that measured in a reaction mixture containing no added (−)-epicatechin related compounds. Each analysis was performed 4 times, with the data expressed as the mean and standard deviation.

Low-density-lipoprotein (LDL) Oxidation Model

LDL oxidation was evaluated by a modification of the method of Esterbauer[16] and Hirano and Kondo.[17] LDL was isolated from human plasma by single-spin density gradient centrifugation (417,000*g*, 40 min, 4°C). The fraction obtained was dialyzed overnight against a 2000 fold volume of nitrogen-purged 10 mM phosphate-buffered saline (pH 7.4) at 4°C with the protein concentration then being determined by the bicinchonic acid method. The reaction mixture consisted of human LDL fraction (200 μg of protein/ml), various concentrations of (−)-epicatechin related compounds dissolved in 10% v/v ethanol and either 10 μM $CuCl_2$ or 750 μM Me-AMVN dissolved in acetonitrile as the initiators of radical formation. The ethanol concentration in the reaction mixture was 0.001%. The kinetics of LDL oxidation was determined by monitoring for 42 min the changes in absorbance at 234 nm due to formation of conjugated dienes. Each analysis was performed 4 times with the data expressed as mean and standard deviation.

Statistical Analysis

Analyses were carried out using SPSS statistical software with the data expressed as mean ± standard error. When ANOVA showed a p value < 0.05, the data were analyzed further using Scheffe's multiple range test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Comparison of Superoxide Scavenging Activity

The relationship between the concentration of (−)-epicatechin and its metabolites and percentage inhibition of superoxide scavenging activity is summarized in Fig. 2. The addition of more than 2.0 μM of either (−)-epicatechin or E7G resulted in greater than 50% inhibition of WST-1 formazan synthesis. However, the addition of other metabolites at a concentration up to 4.0 μM did not inhibit WST-1 formazan synthesis.

Comparison of (−)-Epicatechin and its Metabolites Antioxidative Effect on Plasma Oxidation

The effects of the antioxidative activities of (−)-epicatechin and its metabolites on the production of plasma TBARS induced by 25 mM AAPH are shown in Fig. 3. (−)-Epicatechin at a concentration of 2.15 μM caused 33% inhibition of TBARS production, while a concentration of 34.5 μM resulted in approximately 80% inhibition of oxidation. All the metabolites tested in the series of experiments were less effective inhibitors of TBARS production than (−)-epicatechin. For example, at a concentration of 42.9 μM, E7G and E3′G caused 61 and 20% inhibition of TBARS production, respectively, while 4′ME3′G at a concentration of 41.6 μM caused only 12% inhibition compared with (−)-epicatechin. There were significant differences in inhibitory activity between (−)-epicatechin and its metabolites over the entire concentration range of 2.15–42.9 μM, with antioxidative activity being graded as follows; (−)-epicatechin > E7G > E3′G > 4′ME3′G.

Comparison of (−)-Epicatechin and its Metabolites Antioxidative Effect on LDL Oxidation

The effectiveness of (−)-epicatechin and its metabolites to suppress LDL oxidation induced by either MeO-AMVN or copper ions was evaluated. The relationship between the final concentration of each metabolite and lag time is shown in Fig. 4 and Table I. Compared with controls, addition of (−)-epicatechin at a concentration greater than 0.125 μg/ml (0.43 μM) caused a significant increase in the lag time of oxidation induced by the radical

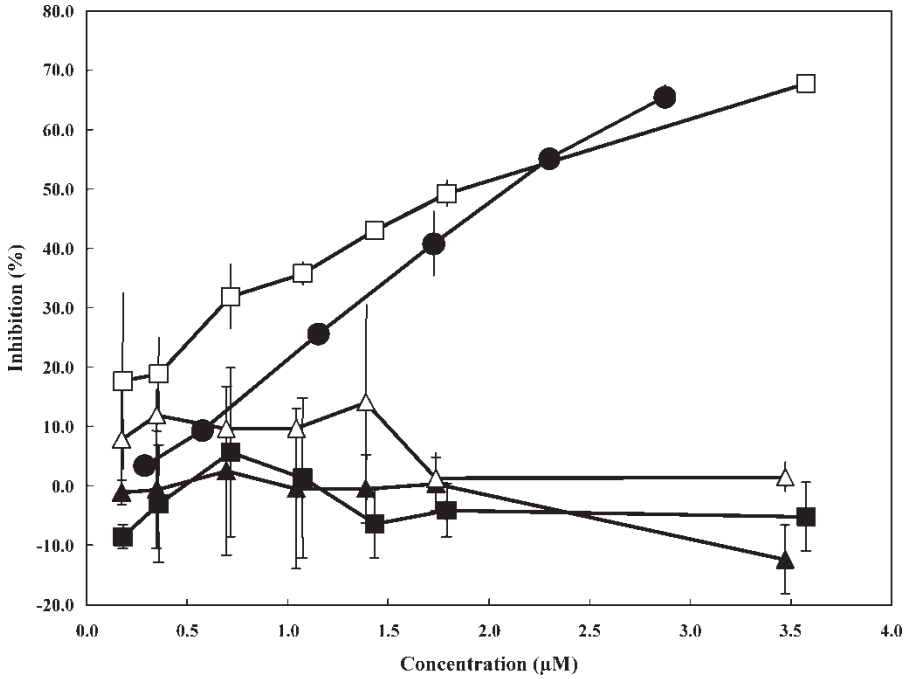

FIGURE 2 Superoxide anion radical scavenging activity of (−)-epicatechin and its metabolites. Superoxide scavenging activity was measured by the superoxide dismutase (SOD) assay kit-WST. The concentration range of each of the test samples was 0.18–3.57 μM. The data are representative of three experiments. (−)-epicatechin (closed circles), E3′G (closed squares), 4′ME3′G (closed triangles), E7G (open squares) and 3′ME7G (open triangles).

initiators. The concentration of each compound required to prolong the lag time two-fold compared with controls was calculated using the least squares method with the results summarized in Table II. The antioxidative activity of the compounds in response to free radicals generated by MeO-AMVN, standardized for molality, varied in strength according to the following order, (−)-epicatechin≧ E7G ≫ E3′G > 4′ME3′G. The order and relative activities of the compounds were identical against radicals initiated by copper ions.

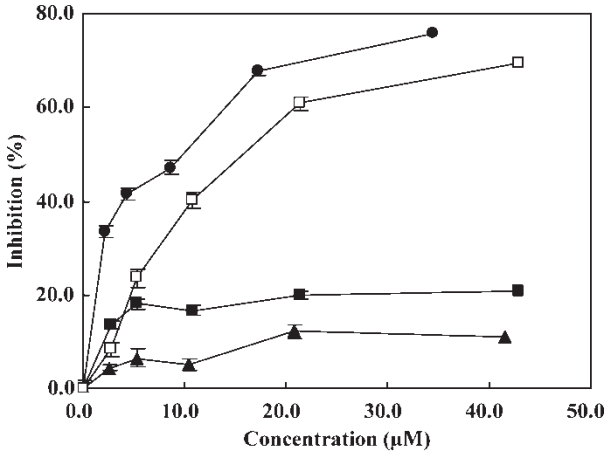

FIGURE 3 Antioxidative activities of (−)-epicatechin and its metabolites on plasma TBARS production induced by 25 mM AAPH. The concentration range of the tested compounds was 0–42.9 μM. Each analysis was performed 4 times, and the mean and standard deviation are shown. (−)-epicatechin (closed circles), E3′G (closed squares), 4′ME3′G (closed triangles), and E7G (open squares).

DISCUSSION

Polyphenols have been shown to have multiple *in vivo* biological actions that include suppression of inflammation,[18] LDL oxidation,[19–21] and the development of CHD.[22,23] It has been suggested that these effects are attributable to the antioxidant and chelating properties of polyphenols. This study had the objective of assessing the antioxidative activity of (−)-epicatechin and its metabolites. This was achieved by measuring *in vitro* superoxide radical scavenging activity, and inhibition of plasma and LDL oxidation. The study also incorporated a comparison of the activities of (−)-epicatechin and its four major metabolites, E3′G and 4′ME3′G present in human plasma, and E7G and 3′ME7G present in rat plasma.[11]

We found that both (−)-epicatechin and E7G had similar superoxide radical scavenging activity causing approximately 50% inhibition. In contrast, no other metabolite had an effect on superoxide scavenging activity over the concentration range 0.1 to 4 μM. Our findings are consistent with other studies that have shown flavonoids such as (−)-epicatechin[24] and (−)-epicatechin-5-*O*-glucuronide[13] have superoxide scavenging activity.

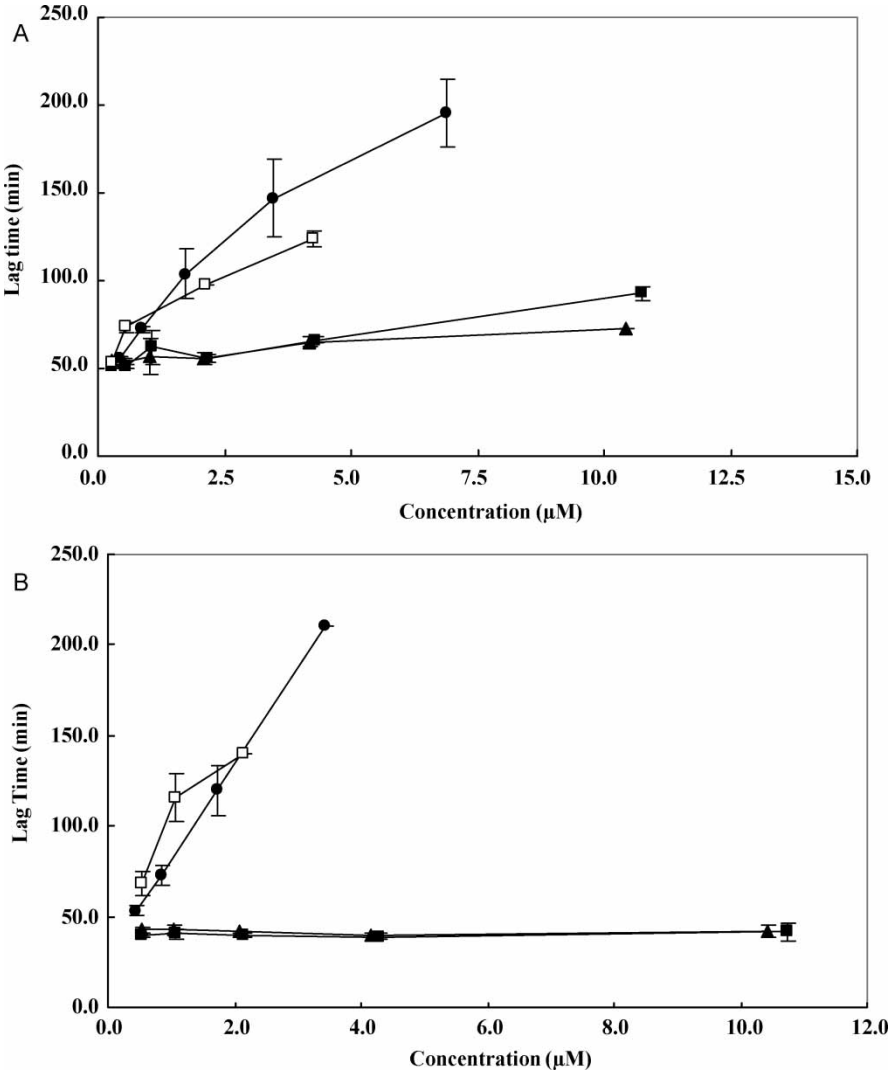

FIGURE 4 Effects of (−)-epicatechin and its metabolites on the susceptibility of LDL to oxidation induced by (a) MeO-AMVN and (b) copper ions. The concentration range of each of the tested compounds was 0–17.2 μM. Each analysis was performed 4 times and the mean and standard deviation are shown. (−)-epicatechin (closed circles), E3′G (closed squares), 4′ME3′G (closed triangles), and E7G (open squares).

TABLE I The effect of (−)-epicatechin and its metabolites on lag time of conjugated diene formation in LDL oxidation induced by MeO-AMVN or copper ions

	(−)-Epicatechin metabolite concentration (μg/ml)					
	0.125	0.25	0.50	1.0	2.0	5.0
Induced by MeO-AMVN						
No addition	48.7 ± 1.0 a	47.9 ± 1.0 a	48.2 ± 2.8 a	46.4 ± 2.4 a	51.1 ± 4.1 a	51.8 ± 0.6 a
(−)-Epicatechin	55.9 ± 1.1 b	72.3 ± 1.5 b	103.8 ± 13.9 b	147.1 ± 22.1 b	195.3 ± 19.0 b	NT
E3′G	51.7 ± 2.1 ab	50.9 ± 1.2 a	62.1 ± 9.9 a	55.6 ± 2.4 a	66.2 ± 1.9 c	93.0 ± 4.1 b
4′ME3′G	54.0 ± 1.7 ab	53.0 ± 2.6 a	56.6 ± 10.1 a	55.6 ± 3.1 a	65.0 ± 2.8 c	72.8 ± 0.2 c
E7G	53.5 ± 1.4 ab	73.5 ± 3.0 b	NT	97.9 ± 0.0 c	124.3 ± 4.5 d	NT
Induced by copper ion						
No addition	37.8 ± 2.0 a	37.4 ± 1.1 a	37.5 ± 1.6 a	35.3 ± 0.8 a	37.1 ± 0.7 a	35.3 ± 1.4 a
(−)-Epicatechin	53.2 ± 2.7 b	72.8 ± 5.5 b	119.5 ± 14.0 b	210.0 ± 0.0 b	NT	
E3′G	NT	40.0 ± 1.2 a	40.2 ± 2.4 a	39.7 ± 0.9 c	39.0 ± 1.3 b	41.3 ± 4.7 b
4′ME3′G	NT	42.6 ± 1.8 a	42.9 ± 1.9 a	41.7 ± 0.6 c	39.6 ± 1.1 b	41.5 ± 3.4 b
E7G	NT	68.3 ± 6.2 b	115.6 ± 13.5 b	140.1 ± 0.0 d	NT	NT

Values in a row not sharing the same letters are significantly different at $p < 0.05$; Each value representes the mean and standard deviation ($n = 4$); NT: not tested.

TABLE II The concentration of (−)-epicatechin metabolites required to prolong lag time two-fold relative to controls

	MeO-AMVN		Copper ion	
	μM	Ratio to (−)-epicatechin	μM	Ratio to (−)-epicatechin
(−)-Epicatechin	1.96	1.0	0.80	1.0
E3′G	12.14	6.2	133.80	168.1
4′ME3′G	21.76	11.1	350.74	440.5
E7G	2.02	1.0	1.74	2.2

Bors *et al.* demonstrated that the *O*-dihydroxy (catechol) structure in the B ring of flavonoid was the most important structure for radical scavenging activity,[25] and it is interesting to note that all the metabolites without scavenging activity in our study had glucuronidated and/or methylated derivatives in the B ring.

It has been reported that the addition of catechins to human plasma prevents the accumulation of TBARS induced by either AAPH or 2, 2′-azobis (2, 4-valeronitrile).[26,27] Our study examined whether (−)-epicatechin and its metabolites had similar effects. We found that (−)-epicatechin and E7G had relatively high activity compared with the other metabolites and from the data we were able to calculate the concentration of these compounds required to cause 50% inhibition of TBARS production. In contrast, the addition of E3′G and 4′ME3′G over the concentration range 2.6 to 42.9 μM resulted in less than 50% inhibition of AAPH-induced TBARS production in plasma. Baba *et al.*[28] reported that 1 to 5 mg/kg of (−)-epicatechin administered orally to rats caused significant reductions in the level of plasma lipid peroxide induced by either copper ions or AAPH, and that the plasma collected 1 h after ingestion contained 1.0 to 4.5 μM of (−)-epicatechin metabolites.[28] Similar studies showed that E7G formed almost 60% of (−)-epicatechin metabolites found in plasma.[9] In our study, a concentration of E7G from 10 to 30 μM caused a significant reduction in production of lipid peroxides. These results indicate that E7G acts as an antioxidant in the plasma of rats following oral administration of (−)-epicatechin.

Oxidative modification of LDL is thought to play a crucial role in the initiation of atherosclerosis[29] with enhanced uptake of oxidized LDL by scavenger receptors on macrophages[30] leading ultimately to the formation of lipid-laden foam cells, a hallmark of early atherosclerosis.[31] There is considerable evidence that oxidized LDL is a chemotactic agent for monocytes and T-lymphocytes resulting in increased production of inflammatory cytokines[32] and growth factors,[33] promotion of procoagulant activities,[34] and impairment of arterial vasotumor responses.[35] Numerous studies, including our own, have investigated the effectiveness of a variety of antioxidants to increase the resistance of LDL to oxidation.[36–39] In an earlier study[39] we showed that LDL collected from subjects who had ingested cocoa was significantly less susceptible to oxidation induced by copper ions and MeO-AMVN compared with controls. (−)-Epicatechin is one of the polyphenolic components found in cacao products with its concentration being approximately 8 to 9%.[40] In the present study, we evaluated the *in vitro* antioxidative activity of (−)-epicatechin and its metabolites by measuring the compounds ability to suppress LDL oxidation induced by either copper ions or MeO-AMVN. Using the lipophilic azo-compound MeO-AMVN as a radical generator and copper ions as an oxidizing agent, we showed (−)-epicatechin was the most potent antioxidant, followed by E7G, whereas both E3′G and 4′ME3′G had light activity. The inhibitory effects of quercetin and its metabolites on copper ion-induced lipid peroxidation in human plasma LDL have been examined by Moon *et al.*[12] who found that quercetin- 4′-*O*- glucuronide was less effective than quercetin -3-*O*-glucuronide or quercetin. Similar results were reported by Manach *et al.* who showed that the conjugated derivatives, quercetin glucuronide and quercetin-3-*O*-sulfate, were not as potent as quercetin for preventing copper ion-induced oxidation of human LDL.[41] These results suggest that the *O*-dihydroxy (catechol) structure in the B ring of a flavonoid is the most important factor for protecting LDL against oxidation. In addition, this structure provides radical scavenging activity[25] and the ability to chelate metals.[42]

Kondo *et al.* in a study in humans found that administration of 36 g cocoa powder also resulted in increased resistance of LDL to oxidation.[43] We have shown previously that administration of an equivalent amount of cocoa powder results in a plasma concentration of (−)-epicatechin and its glucuronide metabolites of 0.22 and 0.81 μM, respectively.[10] In a review of the pharmacokinetic values for flavonoids[44] the maximum plasma levels of epicatechin ranged from 0.04 to 0.7 μM. However, in our study, human epicatechin glucuronide (E3′G) at a concentration of 1.0 μM did not prolong oxidation lag time. As numerous studies have shown that (−)-epicatechin metabolites are present in human plasma, not only as glucuronides, but also as sulfate, sulfoglucuronide, and methylsulfate conjugates[45] it is possible that these conjugated forms also contribute to the antioxidative effect on LDL. On the other hand, Shimoi *et al.*[46] suggested that flavonoid glucuronides may be degraded to intact forms at the site of inflammation by leukocyte-derived glucuronidase. According to this hypothesis, flavonoids present as glucuronide conjugated forms in the blood may have

an antioxidative effect as an aglycon under the inflammatory conditions associated with chronic disease. As mentioned previously, we have shown that following the administration of cocoa, the plasma concentrations of (−)-epicatechin and its glucuronide metabolites were 0.22 and 0.81 μM, respectively.[10] Therefore, if all the glucuronidated epicatechin was degraded by glucuronidase, the concentration of free epicatechin in the plasma would be 1 μM. Halliwell[47] reported that antioxidant action was not observed at concentrations less than 1 μM. On the other hand, Deprez *et al*.[48] reported that polyphenols such as catechins and catechin oligomers were degraded by incubation with human colonic microflora under anaerobic conditions, resulting in the formation of several phenylacetic acid metabolites. Taken together these results suggest that a number of catechin metabolites may contribute to antioxidant activity in plasma after administration of catechin rich foods.

In summary, the results of this study suggest that there are a variety of epicatechin conjugates present in blood plasma and that some of these have antioxidative activity.

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