Antioxidant Effects of Polyphenols in Chocolate on Low-Density Lipoprotein both In Vitro and Ex Vivo

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Summary  Cacao is rich in polyphenols such as (−)-epicatechin, and a colored component of cacao (cacao-red) is polyphenol, which is an antioxidant. These properties stimulated an investigation of the effects of cacao liquor polyphenols (CLP) on low-density lipoprotein (LDL) oxidation. The 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (AMVNCH₃O)-induced oxidizability of LDL was assessed by monitoring the absorbance at 234 nm. In vitro, 0.1–0.5 mg/dL CLP prolonged the oxidation lag time of LDL in a dose-dependent manner. Compared with the controls, it was prolonged 1.7-fold in the presence of 0.1 mg/dL CLP, 2.9-fold at 0.2 mg/dL, 3.8-fold at 0.3 mg/dL, 5.4-fold at 0.4 mg/dL, and 6.4-fold at 0.5 mg/dL. Furthermore, we enlisted 13 male volunteers to consume 35 g delipidated cocoa. Venous blood samples were taken before and at 2 h and 4 h after consuming the cocoa. The oxidation lag time of LDL before cocoa ingestion was 59.0±6.3 min, but it was prolonged at 2 h after cocoa (68.3±6.0 min); before returning to the initial lag time (61.7±5.7 min) before consumption. Thus we have shown that cocoa inhibited LDL oxidation both in vitro and ex vivo.

Key Words  cacao liquor polyphenol (CLP), LDL oxidizability, lag time, atherosclerosis, antioxidant

Oxidized low-density lipoprotein (LDL) is taken up by macrophages via scavenger receptors, leading to foam cell formation (1), and is thus considered to bring about atherosclerosis. Therefore inhibiting LDL oxidation is known to reduce the formation of atherosclerotic lesions. Dietary flavonoids, among other agents, protect LDL against oxidative modification (2). For example, quercetin, a major member of the flavonoid family, inhibits the oxidation of LDL in vitro (3, 4).

Flavonoids are widespread in the plant kingdom and are found in vegetables, fruits, and beverages such as tea and wine. Recent epidemiological evidence suggests that the protective function of flavonoids against coronary heart disease (CHD) (5–7) is attributed in part to their antioxidant properties; flavonoids are scavengers of superoxide anions (8), singlet oxygen (9), and lipid peroxyl radicals (10).

Cacao liquor is rich in polyphenols including (−)-epicatechin, a prominent catechin that polymerizes to form complex tannins during fermentation and is the major substrate for enzymatic browning during drying (11). A previous study (12) reported that in vitro, cacao liquor extract, abundant in flavonoid polyphenols, was a potent antioxidant for LDL oxidation, and further we suggested that chocolate might have an antioxidant effect ex vivo (13). In this present study, we extended our previous work by investigating the effect of the cacao liquor polyphenols (CLP) component on LDL oxidation both in vitro and ex vivo.

MATERIALS AND METHODS

Preparation of polyphenols from chocolate. CLP was purified from Ghana cacao beans as previously described (14, 15). In brief, cacao powder was separated from cacao butter with n-hexane and extracted twice with 80% ethanol. The ethanol extract was evaporated in vacuo, and the polyphenols redissolved in ethanol and separated by reverse-phase adsorption chromatography, using a Diaion HP-2MG column (35 mm × 310 mL; Mitsubishi Kasei Co., Japan). Fractions rich in polyphenol were eluted with 80% ethanol/H₂O and lyophilized. This powder was used as the CLP source in the present study.

Clinical study protocol. Thirteen healthy male consenting volunteers (average age 40.0±4.0, confirming
Table 1. Composition of chocolate before and after delipidation.

|                  | Before | After |
|------------------|--------|-------|
| Protein<sup>a</sup> | 11.6   | 22.7  |
| Fat<sup>a</sup>   | 54.5   | 11.0  |
| Water<sup>a</sup> | 1.0    | 2.0   |
| Ash<sup>b</sup>   | 3.2    | 6.3   |
| Starch<sup>a</sup>| 6.1    | 11.9  |
| Fiber<sup>a</sup>| 16.9   | 33.1  |
| Vitamin A<sup>b</sup> | 20.0 | 39.0 |
| Thiamin<sup>b</sup>| 0.17   | 0.33  |
| Riboflavin<sup>b</sup> | 0.13 | 0.25  |
| Vitamin B<sub>6</sub><sup>b</sup> | 0.085 | 0.166 |
| Vitamin C<sup>a</sup> | <1     | <2    |
| Vitamin E<sup>a</sup> | 13.4  | 26.2  |
| Tannin<sup>a</sup>   | 3.31   | 6.47  |
| Epicatechin<sup>a</sup>| 0.14   | 0.27  |
| Catechin<sup>a</sup> | 0.031  | 0.061 |
| Quercetin<sup>a</sup> | 0.0013 | 0.0026 |
| Caffeine<sup>a</sup> | 0.09   | 0.18  |
| Theobromine<sup>a</sup> | 1.3 | 2.5   |

<sup>a</sup> g per 100 g. <sup>b</sup> mg per 100 g.

* Vitamin E means all of α-, β-, γ-, and δ-tocopherol.

...to guidelines of the Human Ethics Committee of National Institute of Health and Nutrition, Japan) consumed 35 g of delipidated chocolate after an overnight fast. Table 1 shows the composition of cacao liquor and delipidated chocolate. Tannin, epicatechin, catechin, and quercetin are a part of CLP. Venous blood samples were taken before and then at 2h and 4h after consuming the chocolate. The subjects were allowed nothing but water during the experiment.

**Isolation and preparation of LDL.** Plasma was separated from blood collected in EDTA-containing tubes by centrifugation at 2,000 × g for 10 min at 4°C. LDL was isolated from 1.1 mL plasma by a single-spin density gradient ultracentrifugation (417,000 × g, 40 min, 4°C), using the fixed angle TLA-100.4 rotor (Beckman Instruments Inc., CA). Plasma (1.1 mL) was added to ultracentrifuge tubes (3.9 mL) followed by 0.3575 g KBr to raise the density to 1.21 g/mL. After dissolving, the sample was overlaid with 2.8 mL of d=1.006 g/mL saline-EDTA buffer (16).

The protein content of LDL was determined by the BCA protein assay (Pierce Laboratories Inc., IL) using bovine albumin as the standard. Before the start of the oxidation experiments, LDL samples were diluted with PBS to give final concentrations of 70 μg/mL LDL protein.

**Oxidation modification of LDL.** The oxidation of LDL was performed as previously reported (17). Briefly, oxidation was initiated by the addition of freshly prepared 2,2′-azobis (4-methoxy-2,4-dimethylvaleronitrile) (AMVN-CH₃O; Wako Pure Chemical, Japan) solution at final concentrations of 200 μM, using acetonitrile as the solvent. The kinetics of the LDL oxidation was determined by monitoring the change in absorbance of conjugated diene formation at 234-nm in a Beckman Model DU 650 spectrophotometer. The change in 234-nm absorbance was recorded at 5 min intervals at 37°C.

The 234-nm absorbance curve was divided into three phases: a lag phase, a propagation phase, and a decomposition phase. Lag time and propagation rate were measured as previously described (18). The lag time was defined as the interval between the intercept of the tangent of the slope of the curve in the propagation phase with the baseline and was expressed in minutes. The propagation rate of conjugated dienes was determined from the linear portion of the curve, using the extinction coefficient for conjugated dienes at 234 nm (29,500 L/mol/cm).

**Analyses.** The total cholesterol, HDL-cholesterol, and triacylglycerides in plasma were determined enzymatically by kit methods (Kyowa Medex Co., Japan). VLDL- and LDL-cholesterol contents were calculated by using the Friedewald formula (19). Plasma nonesterified (free) fatty acids were also determined by using a commercially available assay kit (Nippon Shoji Kaisha Ltd., Japan). Plasma apolipoproteins were quantified by immunoturbidimetry, using monospecific polyclonal antibodies (Daiichi Pure Chemicals Co., Ltd., Japan). These assays were performed on the COBAS MIRA autoanalyzer apparatus (F. Hoffmann La Roche, Switzerland).

Plasma fatty acids were analyzed after methylation, using HCl-methanol. Pentadecan acid (15:0) was used as an internal standard as described in a previous paper (20). The fatty acid content was measured by gas-liquid chromatography (Perkin Elmer Japan Auto System GC) on an Rscot Sillier 5CP capillary column (0.25 mm φ × 50 m) as described previously (20).

α-Tocopherol (α-toc) was quantified by high-performance liquid chromatography (HPLC), using the method of Ueda and Igarashi (21). After adding 0.8 mL distilled water and 1.0 mL ethanol to 0.2 mL plasma, the samples were extracted with 5.0 mL n-hexane and centrifuged at 2,000 × g for 5 min at 4°C. Four milliliters of the n-hexane layer were transferred to another tube and evaporated to dryness, the samples being redissolved in 1.0 mL n-hexane containing PMC (2,2,5,7,8-pentamethyl-6-hydroxycroman) as an internal standard. Five microliter aliquots were injected into the chromatograph port (Shimadzu LC-3A, Japan). A mixture containing 2 μg/mL α-toc, β-toc, γ-toc, and δ-toc were used as standards. A nucleosil 5NH2 column (4.0 mm φ × 150 mm) was used at a flow rate of 1.0 mL/min with 99% n-hexane and 1% (v/v) 2-propanol as the mobile phase. Fluorescence was measured with a fluorometric detector (Shimadzu RF535, Japan). The excitation wavelength was set at 295 nm, and the emitted fluorescence was measured at 325 nm.

**Statistical methods.** The results in tables are presented as means ± SE. Comparisons between data of lag time obtained before and 2 h or 4 h after consumption were analyzed by Wilcoxon’s signed-rank test for matched pairs, using Statview J4.02 software (Abacus Concepts Inc., CA). The level of significance was set at...
RESULTS

In vitro effects of CLP on LDL oxidizability

Figure 1 describes the production of conjugated dienes from LDL unsaturated fatty acids. Compared with the control, the lag time was prolonged dose dependently by adding CLP, being 1.7-, 2.9-, 3.8-, 5.4-, and 6.4-fold in the presence of 0.1, 0.2, 0.3, 0.4, and 0.5 mg/dL CLP, respectively.

CLP contained 3.5% (-)-epicatechin, 1.5% (+)-catechin, 0.8% quercetin, 0.1% clovaide, and the tannin fraction comprised of 35% cacao liquor polyphenols (CLT) included caffeine and coumarin acid with small amounts of other compounds. Based on this composition, an experiment was performed to investigate the effects of CLP components on LDL oxidizability. Compared with the control, it was prolonged 4.0-, 1.3-, or 1.2-fold in the presence of 200 μg/dL CLP, 7 μg/dL (-)-epicatechin, and 70 μg/dL CLT, respectively, whereas 3 μg/dL (+)-catechin and 1.6 μg/dL quercetin had no effect (Fig. 2). These data suggest that the antioxidant effect resides in the major fractions present in CLP.

Clinical study on effects of chocolate to LDL oxidation

Thirteen LDL samples were analyzed from blood taken before and 2 h and 4 h after delipidated chocolate had been consumed. As shown in Table 2, the oxidation lag time was 59.0±6.3 min before chocolate ingestion, but it was significantly prolonged to 68.3±6.0 min at 2 h after chocolate (p<0.005), before returning to the initial lag time (61.7±5.7 min) at 4 h. Lag times for individual subjects are given in Table 3. In 11 of the 13 subjects, a prolongation of the oxidation lag time was observed after chocolate ingestion, and LDL oxidizability was not effectively changed in two subjects. No significant changes were found for propagation rates of

| Subject No. | Lag time (min) Before | After 2 h | After 4 h |
|-------------|----------------------|----------|----------|
| 1           | 61.7                 | 75.3<sup>a</sup> | 58.8     |
| 2           | 55.5                 | 55.4     | 49.9<sup>b</sup> |
| 3           | 122.3                | 125.7<sup>a</sup> | 117.9    |
| 4           | 66.8                 | 79.5<sup>b</sup> | 71.2     |
| 5           | 72.6                 | 82.4<sup>a</sup> | 76.5     |
| 6           | 66.7                 | 69.8<sup>a</sup> | 64.9     |
| 7           | 51.9                 | 53.7     | 54.8<sup>a</sup> |
| 8           | 53.0                 | 70.3<sup>b</sup> | 54.1     |
| 9           | 59.6                 | 58.9<sup>b</sup> | 59.5     |
| 10          | 47.1                 | 74.9<sup>a</sup> | 44.1     |
| 11          | 36.0                 | 46.6     | 68.9<sup>a</sup> |
| 12          | 40.7                 | 50.9<sup>a</sup> | 49.8     |
| 13          | 32.6                 | 44.4<sup>a</sup> | 32.3     |

Table 2. Effects of delipidated chocolate on LDL oxidation lag time and propagation rate.

| Lag time (min) | Before | After 2 h | After 4 h |
|----------------|--------|-----------|-----------|
| 59.0±6.3       | 68.3±6.0<sup>***</sup> | 61.7±5.7  |
| Propagation rate (nmol diene/min/mg protein) | 4.5±0.5 | 4.2±0.4 | 4.3±0.4 |

An asterisk indicates a significant difference between the before and after administration in the same row (*** p<0.005, Wilcoxon’s signed-rank test for matched paired). Values are means±SE, n=13.
conjugated dienes at 2 h and 4 h after chocolate ingestion (Table 2).

The levels of plasma lipids, lipoproteins, and apolipoproteins are shown in Table 4. No significant changes were found in plasma total cholesterol and triacylglycerides following chocolate ingestion. Although HDL-cholesterol was significantly higher after 2 h \( (p<0.05) \), the levels remained within normal range. Plasma fatty acids, especially palmitic, stearic, and oleic acid, which are the major fatty acids of cacao butter, increased, being especially significant for stearic acid (Table 5).

The proportion of each component in chocolate increased more after delipidation, as shown in Table 1. Incidentally, we determined the level of vitamin E, \( \alpha \)-tocopherol, in the plasma in order to confirm whether antioxidants besides CLP potentially influenced LDL oxidizability. However, chocolate ingestion had no significant effect on plasma \( \alpha \)-tocopherol levels (Table 5).

**DISCUSSION**

Chocolate, which has been enjoyed by people worldwide since before Christ, is rich in polyphenols such as \((-\)-epicatechin and quercetin. To investigate the potential antioxidant effect of these polyphenols, we prepared a CLP extract enriched in them that included 3.5\% \((-\)-epicatechin, 1.5\% \((+)-catechin, 0.8\% quercetin, 0.1\% clovamide, and the tannin fraction (35\%) of cacao liquor polyphenols (CLT), as well as caffeine, coumarin acid, and other ingredients. We wished to determine if CLP inhibited the formation of conjugated dienes during the peroxidation of LDL initiated by AMVN-CH_3O, which is a lipid-soluble radical generator. As shown in Fig. 1, LDL oxidizability was inhibited in a dose-dependent manner by CLP, and Fig. 2 suggests that the maximal antioxidant effects of CLP result from an interactive effect of each flavonoid.

Flavonoids can act as chain-breaking antioxidants by scavenging chain-propagating peroxyl radicals because of the presence of phenolic hydrogens responsible for the peroxyl radical-scavenging activity (22). The antioxidant activity of flavonoids is mainly determined by three structural groups: 1) the \( o \)-dihydroxyl (catechol) structure in the B ring, which is the obvious radical target site for all flavonoids; 2) the \( 2,3 \)-double bond in conjunction with the \( 4 \)-oxo function, which is responsible for electron delocalization; and 3) the additional presence of both \( 3 \)- and \( 5 \)-hydroxyl groups responsible for maximal radical scavenging potential and strongest radical absorption. Our data support the suggestion...
that these structural features of flavonoids may contribute to a reduction in LDL oxidizability.

The biological activity of flavonoids is not fully understood, and little is known about the absorption and metabolism of flavonoids, though some reports suggest that they are poorly absorbed by humans (23). On the other hand, Hackett et al. (24) showed that significant amounts of catechin are absorbed from the gut and that the catechin subsequently found in blood is largely conjugated in the form of glucuronides. In fact, Nakagawa et al. (25) recently reported that green tea extract increased epigallocatechin gallate (EGCG) level in the plasma, leading to an increase in the antioxidant capacity of human plasma. Recent studies have indicated that the administration of red wine, which is also rich in flavonoids, rapidly increased the “antioxidant activity” of serum (26), suggesting that flavonoids are absorbed in sufficient quantities and remain the activity to inhibit oxidation in the serum after absorption. In this study, 13 human volunteers consumed 35 g of delipated chocolate (39 g cacao powder), including 2.7 g CLP (tannin, epicatechin, catechin, and quercetin, as shown in Table 1) equivalent to six cups of hot chocolate containing 6.5 g cacao powder per cup, or 75 g of bitter chocolate. Our data appear to strengthen the hypothesis that circulating CLP protects LDL against oxidative modification in humans.

However, we were unable to detect catechins in the plasma (data not shown) by the method of Unno et al. (27) after the ingestion of 35 g of delipated chocolate, which may indicate that the absorption of only small amounts of flavonoids in chocolate inhibit LDL oxidation and further, that the antioxidant effects of CLP seem to be a collective interaction of each of the polyphenols in CLP, including (-)-epicatechin, (+)-catechin, and quercetin (Fig. 2), accounting for the observed antioxidant effect of CLP on LDL oxidizability in vitro. This suggestion may be partly supported by the previous report that the increase of catechin (EGCG) levels in human plasma inversely correlated phosphatidylcholine hydroperoxide production (25).

As shown in Table 3, chocolate, although effective in most subjects, did not inhibit LDL oxidizability in 2 of the 13 individuals. This result may reflect a difference in the individual absorption of CLP. More studies are necessary to identify if other antioxidative compounds are present in CLP and to assess the potential biological properties of flavonoids, including absorption, metabolism, and mechanisms involved in antioxidation.

We demonstrated that chocolate inhibits LDL oxidation both in vitro and ex vivo. Previous reports, including the Zutphen Elderly Study (5, 6) and a cohort study in Finland (7), have indicated that flavonoid-rich foods may protect against CHD. Our results, which provide direct evidence that a small daily intake of chocolate is enough to inhibit LDL oxidation ex vivo suggests that chocolate, in part, may reduce the risk of atherosclerosis. The Zutphen Elderly Study reported that high intakes (≥30 mg daily) of flavonoids predicted a half-CHD mortality compared with low intakes (≤19 mg daily) (6). One daily cup of chocolate, which contains over 30 mg flavonoid, may therefore make a significant contribution to a reduction in CHD risk. The evidence supports the suggestion that we should seriously consider the consumption of antioxidant beverages and foods in moderate quantities to reduce oxidative damage caused by various oxidative stresses.

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