

Continuous intake of polyphenolic compounds containing cocoa powder reduces LDL oxidative susceptibility and has beneficial effects on plasma HDL-cholesterol concentrations in humans^{1,2}

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ABSTRACT

Background: Cocoa powder is rich in polyphenols such as catechins and procyanidins and has been shown in various models to inhibit LDL oxidation and atherogenesis.

Objective: We examined whether long-term intake of cocoa powder alters plasma lipid profiles in normocholesterolemic and mildly hypercholesterolemic human subjects.

Design: Twenty-five subjects were randomly assigned to ingest either 12 g sugar/d (control group) or 26 g cocoa powder and 12 g sugar/d (cocoa group) for 12 wk. Blood samples were collected before the study and 12 wk after intake of the test drinks. Plasma lipids, LDL oxidative susceptibility, and urinary oxidative stress markers were measured.

Results: At 12 wk, we measured a 9% prolongation from baseline levels in the lag time of LDL oxidation in the cocoa group. This prolongation in the cocoa group was significantly greater than the reduction measured in the control group (−13%). A significantly greater increase in plasma HDL cholesterol (24%) was observed in the cocoa group than in the control group (5%). A negative correlation was observed between plasma concentrations of HDL cholesterol and oxidized LDL. At 12 wk, there was a 24% reduction in dityrosine from baseline concentrations in the cocoa group. This reduction in the cocoa group was significantly greater than the reduction in the control group (−1%).

Conclusion: It is possible that increases in HDL-cholesterol concentrations may contribute to the suppression of LDL oxidation and that polyphenolic substances derived from cocoa powder may contribute to an elevation in HDL cholesterol. *Am J Clin Nutr* 2007; 85:709–17.

KEY WORDS Cocoa, LDL oxidative susceptibility, HDL cholesterol, catechins

INTRODUCTION

Evidence indicates that oxidation of LDL has a pathogenic role in the development of atherosclerosis (1). Uptake of oxidized LDL by macrophages and smooth muscle cells leads to the formation of fatty streaks, a key event in early atherosclerosis. These vascular lesions accumulate large amounts of lipids, such as cholesterol ester. In addition, oxidized LDL induces the expression of adhesion molecules in monocytes, such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, and also increases production of growth factors by smooth muscle

cells and fibroblasts (2, 3). These findings suggest that inhibition of LDL oxidation may prevent atherosclerotic lesions.

Prospective studies, such as the Framingham Heart Study, Multiple Risk Factor Intervention Trial, Coronary Primary Prevention Trial, Lipid Research Clinics Prevalence Mortality Follow-up Study, and the Prospective Cardiovascular Münster study all reported a negative correlation between plasma HDL cholesterol and cardiovascular disease (4, 5). It has been proposed that HDL may inhibit LDL oxidation by various mechanisms (6). There is also clinical evidence of this suppressive effect of HDL: a study conducted in 270 patients with coronary heart disease showed a negative correlation between plasma concentrations of HDL cholesterol and oxidized LDL (7). These results suggest that one of the protective mechanisms of high HDL concentrations is to cause inhibition of LDL oxidation. Prospective studies have shown a negative correlation between the consumption of plant polyphenols and mortality from both coronary and ischemic heart diseases (8, 9), with studies conducted in both rats and humans reporting that intake of these polyphenols suppressed oxidation of LDL (10).

Cacao beans are used as an ingredient in cocoa and chocolate and are known to be rich in polyphenols, such as catechin, epicatechin, procyanidin B2 (dimer), procyanidin C1 (trimer), cinnamtannin A2 (tetramer), and other oligomeric procyanidins (11). A Dutch study revealed chocolate is a major source of catechins, especially in the younger population (12). In previous studies, we showed intake of polyphenolic-rich fractions derived from cocoa powder increased the resistance of LDL to oxidation and suppressed the formation of atherosclerosis in hypercholesterolemic rabbits (13). Studies we carried out in healthy human subjects also showed intake of dairy cocoa powder enhanced the resistance of LDL to oxidation (14, 15). To further delineate the role of cocoa powder in atherogenesis protection, we examined

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the effects of cocoa intake on plasma concentrations of oxidized LDL and lipids and the urinary oxidative stress markers 8-oxo-7,8-dihydro-2'-deoxyguanosine and lipid hydroperoxide-derived protein modification in normocholesterolemic and mildly hypercholesterolemic human subjects.

SUBJECTS AND METHODS

Materials

Cocoa powder was prepared by roasting, cracking, and compressing fermented and dried cacao beans imported from Ecuador. The general composition of the cocoa powder was as follows (units/100 g): 23.0 g protein, 11.5 g fat, 23.1 g carbohydrate, 26.9 g fiber, 7.7 g minerals, 377 mg epicatechin, 135 mg catechin, 158 mg procyanidin B2, 96.1 mg procyanidin C1, 2192 mg theobromine, and 470 mg caffeine. The catechin, epicatechin, procyanidin B2, and procyanidin C1 content in the powder was analyzed by an HPLC method (11). Other reagents used in the study were commercially available products of analytic and HPLC grade.

Subjects

Twenty-five healthy Japanese male subjects participated in the study. The study was approved by and performed under the guidelines of the ethics committee of Tomisaka Hospital, and informed consent was obtained from each of the subjects before commencement of the study. All subjects were of normal body weight and were nonsmokers with no evidence of chronic disease. None of the subjects consumed >25 mL alcohol/d or were taking other medications, antioxidants, or vitamin supplements. The study group had a mean (\pm SEM) age of 38 ± 1 y, a mean body weight of 64 ± 1 kg, and a mean body mass index (BMI) of 22.1 ± 0.2 kg/m². The concentration ranges of plasma total, LDL, and HDL cholesterol in the subjects were 4.65–6.41 mmol/L, 2.46–4.92 mmol/L, and 0.75–2.60 mmol/L, respectively.

Experimental design

The subjects were divided into 2 groups according to BMI, and plasma total, LDL, and HDL cholesterol concentrations and were then instructed to consume one of the following test drinks daily for 12 wk: 12 g sugar/d (control group) or a mixture of 26 g cocoa powder and 12 g sugar/d (cocoa group). The cocoa powder was consumed as a beverage after the addition of hot water, with the test drinks being consumed twice each day: before noon and during the afternoon. At baseline and at 12 wk, the subjects fasted for 12 h, and then blood samples were collected from the intermediate cubital vein into a tube containing EDTA-2Na. At the same times during the study, 24-h urine samples were collected from 0900 the day before the blood collection until 0900 of the day of the collection. Body weight, blood pressure, and heart rate were also measured at the beginning and end of the study. Home deliveries of food were made to each subject to ensure that the same foods were consumed in the 3 d before collection of the blood and urine samples. In addition, to maintain their normal diets, the subjects kept complete dietary records throughout the study. The 3-d food records were analyzed with the Excel Food-Frequency Questionnaire (Kenpakusha, Tokyo, Japan) on days 1–3, 26–28, 54–56, and 80–82 of each dietary period. The subjects were also requested to avoid all other cacao products and to lead their usual lifestyle throughout the study.

Plasma LDL oxidative susceptibility

Plasma LDL oxidative susceptibility was measured as the lag time of conjugated diene production formed by a radical generator. The lag time was determined by using methods described previously (16, 17). LDL was isolated from plasma by single-spin density gradient centrifugation ($417\,000 \times g$, 40 min, 4 °C) by using an ultracentrifuge (Optima TLX; Beckman Instruments, Inc, Palo Alto, CA). The density gradient was adjusted to 1 mL plasma by the addition of 0.325 g potassium bromide. The protein concentration of the LDL fraction was measured by using the Micro BCA protein assay reagent kit (Pierce, Rockford, IL), followed immediately by the assay of LDL oxidation. Isolated LDL samples were diluted with phosphate-buffered saline (PBS; pH 7.4) to a concentration of 100 μ g LDL protein/mL, followed by incubation for 250 min at 37 °C with the radical generator 2–2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (200 μ mol/L). The formation of conjugated dienes was monitored continuously every 3 min by the change in absorbance at 234 nm with the use of a spectrophotometer (DU800; Beckman Instruments Inc, Palo Alto, CA). The lag time in this reaction, expressed in minutes, provided an assessment of LDL oxidation and was calculated by determining the point of intersection of the baseline and propagation phase of the absorbance curve.

Plasma lipids and oxidative LDL

Plasma VLDL-, LDL-, and HDL-cholesterol concentrations at baseline and at 12 wk were measured by a rapid electrophoresis scanning automated system (Helena Laboratories, Saitama, Japan) with the use of agarose-gel electrophoresis (18). Triacylglycerol was assayed by a standard laboratory technique (BML Inc, Tokyo, Japan).

The monoclonal antibody mAb-4E6 was used to quantify the concentration of oxidized LDL in plasma at baseline and at 12 wk (19). This assay was carried out by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Merco-dia Oxidized LDL ELIZA; Merco-dia AB, Uppsala, Sweden), according to the manufacturer's instructions.

Urinary oxidative stress markers

The following variables were measured as markers of urinary oxidative stress at baseline and at 12 wk: 8-oxo-7,8-dihydro-2'-deoxyguanosine, *N*^ε-(hexanoyl)lysine, dityrosine, bromotyrosine, and dibromotyrosine. Urine 8-oxo-7,8-dihydro-2'-deoxyguanosine concentrations were measured by using a commercially available ELISA kit (8OHdG check, JaICA; Nikken SEIL Co, Shizuoka, Japan) according to the manufacturer's instructions, whereas quantification of *N*^ε-(hexanoyl)lysine in urine was carried out by using liquid chromatography–tandem mass spectrometry (LC–MS) as described previously (20). Quantification of oxidized modified forms of tyrosine was also carried out by using liquid chromatography–tandem mass spectrometry LC–MS (Y Kato, N Dozaki, T Nakamura, et al, unpublished observations, 2004).

Urinary catechin and epicatechin

The amounts of catechin and epicatechin in the urine samples collected at baseline and at 12 wk were analyzed by LC-MS according to methods described previously (21, 22). These earlier reports showed that ingested catechin and epicatechin are present in plasma and urine primarily as various metabolites, such as glucuronide conjugated forms, sulfate conjugated forms,

or both. In our study, we therefore measured catechin and epicatechin metabolites in urine after hydrolysis treatment with glucuronidase and sulfatase (Sulfatase type H-5; Sigma, St Louis, MO) (23). The sum of each catechin or epicatechin metabolites was calculated to determine the total amounts of catechin and epicatechin excreted in the urine.

Safety measurements

The following variables were measured in the blood samples collected at baseline and at 12 wk: plasma total protein, albumin, glucose, uric acid, urea nitrogen, creatinine, free fatty acids, phospholipids, total bilirubin, aspartate aminotransferase, alanine aminotransferase, γ -glutamyltranspeptidase, alkaline phosphatase, lactate dehydrogenase, sodium, potassium, chloride, and calcium. Urine samples collected at baseline and at 12 wk were used for qualitative analysis of proteinuria, glucosuria, urobilinogen, and occult blood. All these variables were assayed by using standard laboratory techniques (BML Inc, Tokyo, Japan).

Statistics

The data were expressed as means \pm SEMs. The change from baseline (12 wk – baseline) in the control and cocoa groups were compared by using repeated-measures analysis of variance and unpaired *t* tests to assess whether a significant group \times time interaction had occurred. A mixed model analysis was used to examine the interaction between 2 risk factors with time and the risk factors acting as the independent variables. If a significant interaction was found, separate correlations were calculated at baseline and 12 wk using Pearson's correlation analysis. A *P* value $<$ 0.05 was considered statistically significant. All the statistical analyses were performed by using SPSS for WINDOWS version 12.0J (SPSS Japan Inc, Tokyo, Japan).

RESULTS

Subject characteristics and dietary records

Mean BMI, systolic blood pressure, diastolic blood pressure, and heart rate at baseline in the control and cocoa groups were 22.1 ± 0.3 and 22.1 ± 0.4 kg/m², 117 ± 2 and 124 ± 3 mm Hg, 79 ± 2 and 77 ± 2 mm Hg, and 71 ± 4 and 77 ± 2 beats/min, respectively. Mean BMI, systolic blood pressure, diastolic blood pressure and heart rate at 12 wk in the control and cocoa groups were 21.5 ± 0.3 and 21.6 ± 0.4 kg/m², 120 ± 3 and 122 ± 2 mm Hg, 77 ± 2 and 75 ± 2 mm Hg, and 72 ± 3 and 77 ± 3 beats/min, respectively. No significant differences were observed in any of these variables between the 2 groups (BMI, *P* = 0.730; systolic blood pressure, *P* = 0.221; diastolic blood pressure, *P* = 0.934; and heart rate, *P* = 0.961). The baseline values of plasma biochemical variables, lipids, oxidized LDL concentrations, LDL susceptibility, and urinary oxidative stress markers did not differ significantly between the 2 groups. No subject reported any adverse events resulting from cocoa intake at the interviews conducted throughout the study. No significant differences in daily mean energy and nutrient intake were observed between the 2 groups during the 3-d periods that dietary records were collected (Table 1).

Plasma LDL oxidative susceptibility

Changes in the susceptibility of LDL to oxidation expressed as lag time are shown in Table 2. In the control group, we observed

TABLE 1

Daily energy and nutrient intakes of the participants¹

	Control group (<i>n</i> = 12)	Cocoa group (<i>n</i> = 13)
Energy		
(MJ)	8.58 \pm 0.38	8.68 \pm 0.36
(kcal)	2050 \pm 91	2076 \pm 85
Protein (g)	74.0 \pm 3.1	74.7 \pm 3.1
Total fat (g)	72.5 \pm 4.3	67.3 \pm 3.2
Saturated (g)	18.4 \pm 1.5	16.1 \pm 1.1
Monounsaturated (g)	25.3 \pm 1.5	22.5 \pm 1.2
Polyunsaturated (g)	15.8 \pm 0.8	15.4 \pm 0.6
Carbohydrate (g)	267 \pm 13	279 \pm 13
Cholesterol (mg)	373 \pm 27	338 \pm 25
Ascorbic acid (mg)	76.5 \pm 11.3	78.8 \pm 9.1
Vitamin E (mg) ²	9.1 \pm 0.7	9.4 \pm 0.5

¹ All values are $\bar{x} \pm$ SEM. Values were calculated from four 3-d food records per subject. These data include contributions from both test drinks. No significant differences were observed between the groups (repeated-measures ANOVA).

² α -Tocopherol equivalents.

a 19.8% reduction in lag time at 12 wk compared with baseline. In contrast, in the cocoa group we found a 9.4% prolongation in lag time. The changes were significantly different between the 2 groups (*P* $<$ 0.001).

Plasma lipids and oxidative LDL

The profiles of plasma total, VLDL, LDL, and HDL cholesterol and of triacylglycerol at baseline and at 12 wk are summarized in Table 3. Plasma total, VLDL-, LDL-, and HDL-cholesterol concentrations at baseline were not significantly different in the 2 groups. In the cocoa group, there was a 23.4% increase in HDL cholesterol at 12 wk compared with baseline concentrations. This increase in the cocoa group was significantly greater (*P* $<$ 0.001) than that measured in the control group (5.1%). The concentrations of LDL cholesterol at 12 wk were reduced by 12.6% and 4.5% in the cocoa and control groups, respectively. However, there was no significant difference in the magnitude of these reductions between the 2 groups. Also, no significant difference in total and VLDL cholesterol and triacylglycerol concentrations was observed between the 2 groups.

The plasma concentrations of oxidized LDL at baseline and at 12 wk are shown in Table 2. No significant difference in oxidized LDL concentrations was observed between the 2 groups.

Urinary oxidative stress markers

The concentrations of 8-oxo-7,8-dihydro-2'-deoxyguanosine, N^ε-(hexanoyl)lysine, dityrosine, bromotyrosine, and dibromotyrosine in urine at baseline and at 12 wk are shown in Table 4. At 12 wk, there was a 23.6% reduction in dityrosine from baseline concentrations in the cocoa group. This reduction in the cocoa group was significantly greater (*P* $<$ 0.05) than the reduction in the control group (-1.1%). A nonsignificant trend of decreasing N^ε-(hexanoyl)lysine concentrations was observed in the cocoa group compared with the control group (group \times time interaction, *P* = 0.06).

Urinary catechin and epicatechin

The 24-h urinary excretion of catechin and epicatechin at baseline and at 12 wk are shown in Figure 1. After 12 wk, we

TABLE 2Plasma oxidized LDL and LDL oxidative susceptibility in the 2 groups at baseline and at 12 wk¹

	Baseline	Week 12	Change (week 12 – baseline)	
			Value	P ²
Plasma oxidized LDL (U/L plasma)				
Control group	73.7 ± 8.8 ³	70.9 ± 4.1	–2.8 ± 6.6	
Cocoa group	70.8 ± 5.1	57.7 ± 3.2	–13.2 ± 4.3	NS
Plasma oxidized LDL (U · mmol LDL cholesterol ^{–1} · L plasma ^{–1})				
Control group	20.9 ± 2.5	22.2 ± 2.1	1.27 ± 1.67	
Cocoa group	20.7 ± 1.6	19.8 ± 1.5	0.95 ± 1.12	NS
Lag time (min) ⁴				
Control group	58.6 ± 3.8	47.0 ± 2.3	–11.6 ± 3.8	
Cocoa group	57.4 ± 3.1	62.8 ± 1.8	5.4 ± 2.9 ⁵	<0.001

¹ n = 12 and 13 for the control and cocoa groups, respectively. The control group received 0 g cocoa; the cocoa group received 26 g cocoa powder/d. Baseline values did not differ significantly between the 2 groups.

² Unpaired t test. Statistical analyses with the use of repeated-measures ANOVA provided essentially the same results.

³ $\bar{x} \pm$ SEM (all such values).

⁴ LDL oxidative susceptibility was measured by the lag time of conjugated diene production formed with the radical generator 2,2'-azobis (4-methoxy-2,4-dimethylvaleronitrile).

⁵ Significantly different from the control group, P < 0.05.

observed an approximate 8-fold increase in catechin excretion and about a 10-fold increase in epicatechin excretion in the cocoa group. These increases resulted in the urinary excretion of both catechin and epicatechin being significantly higher at 12 wk in the cocoa group than in the control group (P < 0.001 for both).

Safety measurements

The concentrations of plasma biochemical variables at baseline and at 12 wk are summarized in **Table 5**. No significant differences were observed between the 2 groups for any of these variables. Other variables, including plasma total protein, albumin, urea nitrogen, free fatty acids, phospholipid, sodium, potassium, chloride, calcium, and all other urinary variables listed

in the Methods section were within the normal range in all the subjects during the study (data not shown).

Correlation between plasma and urinary factors

The relation between plasma oxidized LDL (in U/L plasma) and LDL and HDL cholesterol at baseline and at 12 wk is shown in **Figure 2**. A negative correlation was observed between plasma oxidized LDL and HDL cholesterol at 12 wk (r = –0.460, P = 0.021), whereas the correlation between plasma oxidized LDL and LDL cholesterol was not significant.

The correlations between plasma oxidized LDL (in U/L plasma) and HDL cholesterol and urinary concentrations of catechin and epicatechin are shown in **Figure 3**. A negative correlation was

TABLE 3Plasma total cholesterol, VLDL-cholesterol, LDL-cholesterol, HDL-cholesterol, and triacylglycerol concentrations in the 2 groups at baseline and 12 wk¹

	Baseline	Week 12	Change (week 12 – baseline)	
			Value	P ²
Total cholesterol (mmol/L)				
Control group	5.27 ± 0.14 ³	5.17 ± 0.19	–0.09 ± 0.15	
Cocoa group	5.28 ± 0.16	5.09 ± 0.17	–0.19 ± 0.10	NS
VLDL cholesterol (mmol/L)				
Control group	0.39 ± 0.05	0.38 ± 0.05	–0.01 ± 0.03	
Cocoa group	0.41 ± 0.06	0.35 ± 0.04	–0.06 ± 0.05	NS
LDL cholesterol (mmol/L)				
Control group	3.52 ± 0.19	3.36 ± 0.23	–0.16 ± 0.14	
Cocoa group	3.50 ± 0.16	3.06 ± 0.22	–0.43 ± 0.14	NS
HDL cholesterol (mmol/L)				
Control group	1.36 ± 0.15	1.43 ± 0.15	0.08 ± 0.03	
Cocoa group	1.37 ± 0.11	1.69 ± 0.13	0.31 ± 0.05 ⁴	<0.001
Triacylglycerol (mmol/L)				
Control group	0.95 ± 0.10	1.09 ± 0.13	0.14 ± 0.08	
Cocoa group	1.05 ± 0.13	1.06 ± 0.12	0.01 ± 0.13	NS

¹ n = 12 and 13 for the control and cocoa groups, respectively. The control group received 0 g cocoa; the Cocoa group received 26 g cocoa powder/d. Baseline values did not differ significantly between the 2 groups.

² Unpaired t test. Statistical analyses with the use of repeated-measures ANOVA provided essentially the same results.

³ $\bar{x} \pm$ SEM (all such values).

⁴ Significantly different from the control group, P < 0.05.

TABLE 4
Urinary oxidative stress markers in the 2 groups at baseline and 12 wk¹

	Baseline	Week 12	Change (week 12 – baseline)	
			Value	P ²
8-OHdG ($\mu\text{mol}/24\text{ h}$)				
Control group	32.7 \pm 7.0 ³	32.6 \pm 4.8	-0.1 \pm 6.6	
Cocoa group	38.6 \pm 6.1	38.1 \pm 6.8	-0.5 \pm 8.1	NS
N ⁶ -(Hexanoyl)lysine ($\mu\text{mol}/24\text{ h}$)				
Control group	34.0 \pm 4.2	28.6 \pm 3.7	-5.4 \pm 2.3	
Cocoa group	42.3 \pm 4.5	26.0 \pm 3.3	-16.3 \pm 4.9	NS
Dityrosine ($\mu\text{mol}/24\text{ h}$)				
Control group	74.1 \pm 6.2	73.3 \pm 5.4	-0.8 \pm 5.2	
Cocoa group	91.7 \pm 8.1	70.1 \pm 7.6	-21.6 \pm 8.5 ⁴	<0.05
Bromotyrosine ($\mu\text{mol}/24\text{ h}$)				
Control group	34.5 \pm 5.5	34.8 \pm 5.1	0.4 \pm 3.2	
Cocoa group	50.1 \pm 10.0	31.7 \pm 3.8	-18.4 \pm 9.8	NS
Dibromotyrosine ($\mu\text{mol}/24\text{ h}$)				
Control group	25.0 \pm 3.4	32.7 \pm 4.2	7.7 \pm 3.2	
Cocoa group	38.9 \pm 4.9	45.8 \pm 6.5	6.8 \pm 6.1	NS

¹ $n = 12$ and 13 for the control and cocoa groups, respectively. The control group received 0 g cocoa; the cocoa group received 26 g cocoa powder/d. 8-OHdG, 8-oxo-7,8-dihydro-2'-deoxyguanosine. Baseline values did not differ significantly between the 2 groups.

² Unpaired t test. Statistical analyses with the use of repeated-measures ANOVA provided essentially the same results.

³ $\bar{x} \pm \text{SEM}$ (all such values).

⁴ Significantly different from the control group, $P < 0.05$.

observed between plasma oxidized LDL and urinary epicatechin at 12 wk ($r = -0.433$, $P = 0.030$). A trend toward a nonsignificant, negative correlation was also observed between plasma oxidized LDL and urinary catechin at 12 wk ($r = -0.375$, $P = 0.064$).

DISCUSSION

The present study indicated that consumption of cocoa powder containing polyphenolic substances at a dosage of 26 g/d for 12 wk increased the resistance of LDL to oxidation and also raised HDL-cholesterol concentrations in plasma in normocholesterolemic and mildly hypercholesterolemic humans.

It has been reported that there is a positive correlation between the resistance of LDL to oxidation and the severity of coronary atherosclerosis in humans and that susceptibility of LDL to oxidation is significantly higher in affected familial combined hyperlipidemic subjects than in unaffected subjects (24, 25). Our study showed that intake of cocoa powder had a favorable effect on the susceptibility of LDL to oxidation. This is consistent with other investigations that showed a positive correlation between inhibition of LDL oxidation and the amount of total phenolic compounds derived from wine or the concentration of major polyphenols in cocoa powder such as catechin, epicatechin, and their oligomers (26, 27). These results suggest that polyphenols from cocoa powder may contribute to the resistance of LDL to oxidation. In the present study, catechins were detected in urine in the group that consumed cocoa, although catechins in plasma were not measured. Studies conducted in both rat and humans have shown that after oral administration of cocoa powder, catechin, epicatechin, and procyanidin dimers (B2 and B5) are absorbed and appear in the plasma (21, 28–31). Similarly, the polyphenol concentration in LDL particles has been shown to be elevated 2 wk after intake of red wine and that the decrease in LDL susceptibility correlated positively with polyphenol concentration (32). In addition, Hayek et al (33) showed that both catechin and quercetin were present in LDL after intake of red wine and that these flavonoids bound to the LDL particle by formation of glycosidic bonds. Another study suggested that flavonoids may associate with apolipoprotein B because of their capacity to bind to proteins (34). These results suggest

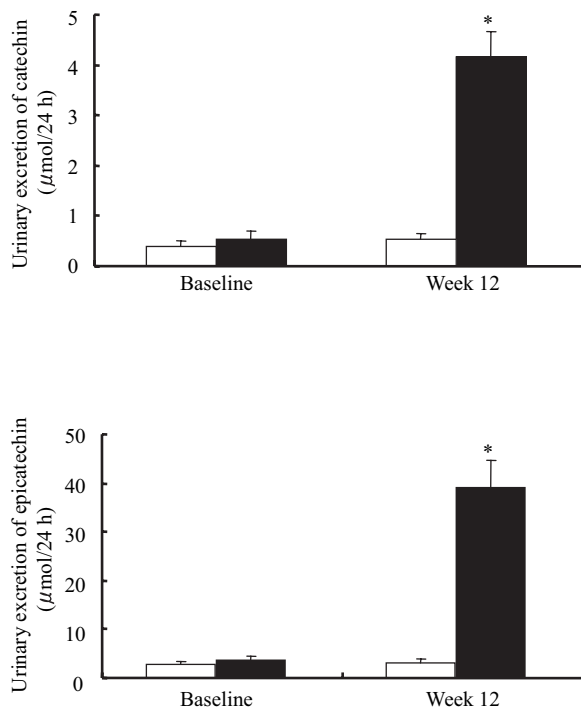


FIGURE 1. Mean (\pm SEM) urinary excretion of catechin and epicatechin in the control (\square ; $n = 12$) and cocoa (\blacksquare ; $n = 13$) groups at baseline and 12 wk. The baseline values did not differ significantly between the 2 groups. At 12 wk, there was a significant increase in catechin and epicatechin concentrations in the cocoa group compared with the control group (group \times time interaction, $P < 0.001$). *Significantly different from the control group, $P < 0.001$ (unpaired t test).

TABLE 5Plasma biochemical variables in the 2 groups at baseline and 12 wk¹

	Baseline	Week 12	Change (week 12 – baseline)	
			Value	P ²
Aspartate aminotransferase (U/L)				
Control group	21.8 ± 1.3 ³	22.5 ± 2.6	4.5 ± 5.9	NS
Cocoa group	22.1 ± 1.3	22.9 ± 3.1	0.8 ± 2.0	
Alanine aminotransferase (U/L)				
Control group	28.1 ± 4.0	32.6 ± 9.4	4.5 ± 5.9	NS
Cocoa group	22.4 ± 2.3	19.8 ± 2.9	−2.5 ± 1.6	
Lactate dehydrogenase (U/L)				
Control group	185 ± 7	170 ± 6	−15 ± 6	NS
Cocoa group	192 ± 7	179 ± 5	−13 ± 7	
Alkaline phosphatase (U/L)				
Control group	227 ± 13	237 ± 20	10 ± 16	NS
Cocoa group	224 ± 12	202 ± 11	−22 ± 7	
γ-Glutamic transpeptidase (U/L)				
Control group	53.8 ± 12.6	55.9 ± 15.5	2.2 ± 8.8	NS
Cocoa group	34.2 ± 7.4	37.5 ± 7.3	3.3 ± 2.7	
Total bilirubin (μmol/L)				
Control group	19.0 ± 2.9	19.4 ± 3.3	0.4 ± 1.2	NS
Cocoa group	13.7 ± 1.3	13.9 ± 1.0	0.3 ± 0.9	
Creatinine (μmol/L)				
Control group	66.1 ± 1.9	66.1 ± 2.4	0.0 ± 1.0	NS
Cocoa group	61.3 ± 1.6	64.6 ± 1.8	3.3 ± 1.3	
Uric acid (μmol/L)				
Control group	361 ± 21	380 ± 23	18 ± 9	NS
Cocoa group	353 ± 14	372 ± 16	19 ± 7	
Glucose (mmol/L)				
Control group	5.92 ± 0.19	5.26 ± 0.11	−0.66 ± 0.14	NS
Cocoa group	5.71 ± 0.12	5.37 ± 0.16	−0.34 ± 0.13	

¹ *n* = 12 and 13 for the control and cocoa groups, respectively. The control group received 0 g cocoa; the cocoa group received 26 g cocoa powder/d. Baseline values did not differ significantly between the 2 groups.

² Unpaired *t* test. Statistical analyses with the use of repeated-measures ANOVA provided essentially the same results.

³ $\bar{x} \pm \text{SEM}$ (all such values).

that some of the polyphenols absorbed from cocoa powder may be incorporated onto the surface of LDL particles and that these polyphenols increase the resistance of LDL to oxidation by either scavenging chain-initiating oxygen radicals or chelating transitional metal ions (35). Polyphenols located on the surface of LDL particles may also have a sparing and recycling

effect on fat-soluble antioxidants, such as α -tocopherol, by supplying hydrogen to fat-soluble antioxidants, which in turn provide hydrogen to lipid peroxide radicals (36).

Marsu et al (37) reported that HDL-cholesterol concentrations increased by 11% and 14% after 3-wk intake of dark chocolate or dark chocolate enriched with cocoa polyphenols, respectively.

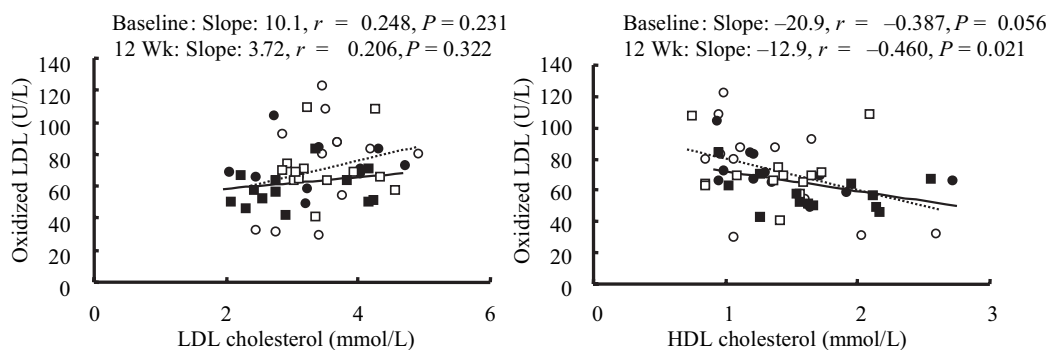


FIGURE 2. Plasma LDL and HDL cholesterol versus plasma oxidized LDL in the control (baseline ○; 12 wk ●; *n* = 12) and cocoa (baseline □; 12 wk ■; *n* = 13) groups. The baseline values did not differ significantly between the 2 groups. Because there was a significant interaction between time and both plasma LDL and HDL cholesterol in the mixed model analysis, Pearson's correlation coefficients were calculated for the baseline and 12-wk data. The slopes, correlation coefficients, and *P* values are shown in each of the figures.

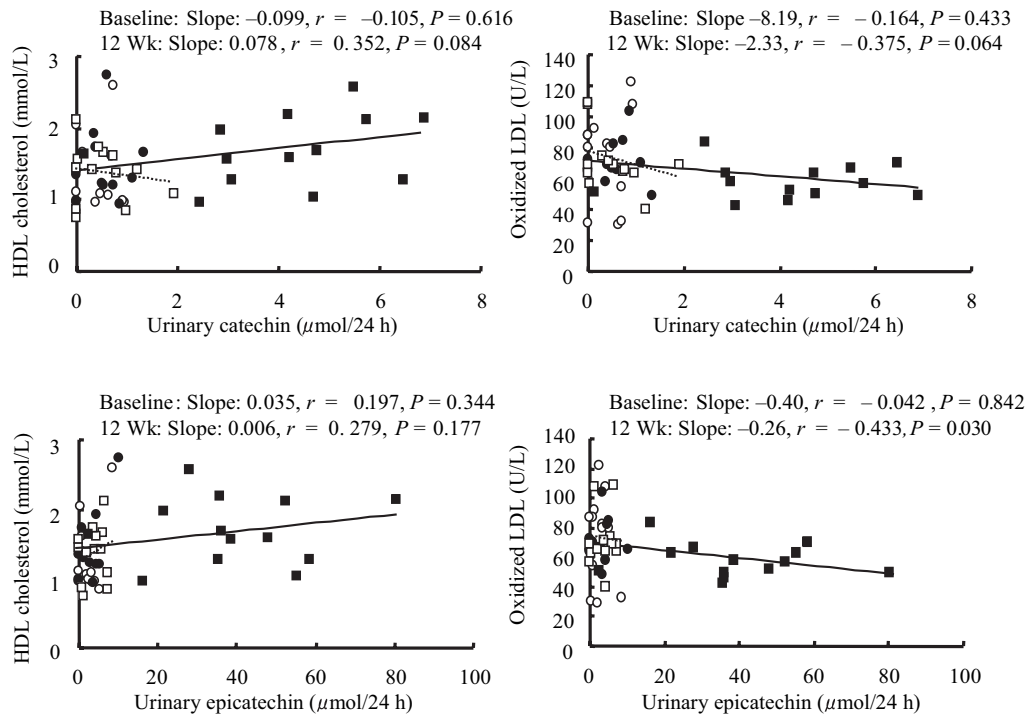


FIGURE 3. Urinary catechin and epicatechin versus plasma HDL cholesterol or oxidized LDL in the control (baseline: ○; 12 wk: ●; $n = 12$) and cocoa (baseline: □; 12 wk: ■; $n = 13$) groups. The baseline values did not differ significantly between the 2 groups. Because there was a significant interaction between time and both urinary catechin and epicatechin in the mixed model analysis, Pearson's correlation coefficients were calculated for the baseline and 12-wk data. The slopes, correlation coefficients, and P values are shown in each of the figures.

The daily consumption of catechin monomers and procyanidins in their study was 270 mg with the dark chocolate and 420 mg with the polyphenol-enriched dark chocolate. These results indicated that the increase in plasma HDL-cholesterol concentrations caused by polyphenols was dose-related. Our study also showed that cocoa powder enhanced plasma HDL-cholesterol concentrations and that there was a nonsignificant trend toward a positive correlation between the excretion of urinary catechin and plasma HDL cholesterol. There is some evidence on in vivo absorption and metabolism of polyphenols in cocoa powder (28, 29, 38, 39). These findings suggest that absorbed catechins in cocoa powder may affect plasma HDL-cholesterol concentrations. Intake of flavonoids other than catechins, such as isoflavones, flavones (naringenin and hesperetin), and polyphenols in red wine, have also been shown to increase plasma HDL concentrations in both human and animal studies (40–43). Taken together, these results indicate that ingestion of polyphenols from sources other than cocoa powder may also affect plasma HDL-cholesterol concentrations. However, results from other studies on polyphenol supplementation support our finding that polyphenols in cocoa powder are responsible, in part, for the increase we observed in plasma HDL-cholesterol concentrations.

It has been reported that increased HDL leads to suppression of LDL oxidation by promoting 1) inhibition of monocyte chemotaxis via monocyte chemoattractant protein-1, 2) hydrolysis of lipid peroxide via paraoxonase, 3) reverse cholesterol transport via lecithin-cholesterol acyltransferase, and 4) direct inhibition of vascular endothelial activation via apolipoprotein A1 (44–47). Our study showed a negative correlation between plasma

oxidized LDL and HDL cholesterol, whereas only a weak degree of correlation was observed between plasma oxidized LDL and LDL cholesterol. Holvoet et al (7) showed that plasma concentrations of oxidized LDL correlated inversely with HDL-cholesterol concentrations, whereas there was no relation between plasma concentrations of oxidized LDL and LDL cholesterol. Alternatively, catechins in cocoa powder have proven in vitro antioxidative activity, although it has been shown that catechins absorbed from cocoa powder are present mainly in the plasma as metabolites, such as conjugated forms, methylated forms, or both that may have decreased antioxidative activity (21, 48). These results suggest that catechins in cocoa powder may inhibit LDL oxidation not only by antioxidative mechanisms but also by other mechanisms. It is therefore possible that increased HDL-cholesterol concentrations caused by polyphenolic substances derived from cocoa powder may contribute to suppression of LDL oxidation.

The mechanisms by which polyphenolic compounds elevate plasma HDL-cholesterol concentrations remains unclear. One hypothesis is that apolipoprotein A1, the major protein component of HDL, has a role in increasing HDL cholesterol. Evidence supporting this possibility is that genistein was shown to increase the expression and production of apolipoprotein A1 in a human hepatoma cell line Hep G2 (49). Lamon-Fava et al (50) also showed that regulation of apolipoprotein A1 expression by genistein was mediated by the mitogen-activated protein kinase signaling pathway.

During lipid peroxidation, several aldehydes, such as 4-hydroxy-2-nonenal and malondialdehyde, are produced after degradation of lipid hydroperoxide. These reactive compounds

are predisposed to react with proteins and aminolipids. Oxidized modified forms of lysine and tyrosine have been detected in human atherosclerotic plaque (51–53). Urinary excretion of *N*^ε-(hexanoyl) has also been shown to be significantly higher in patients with diabetes than in control subjects (20). In our study, intake of cocoa powder reduced the excretion of urinary dityrosine significantly and was also associated with a trend of lower *N*^ε-(hexanoyl)lysine excretion compared with control subjects ($P = 0.061$). The oxidative products measured in the present study are stable in urine and could therefore be useful markers for the diagnosis of oxidative stress in the body.

It has been reported that survival rate and the incidence of clinical disease and carcinogenicity remain unchanged in rats fed a diet containing 5% cocoa powder for 104 wk. Cocoa powder has also been shown to have no teratogenic or embryotoxic activity in rabbits (54, 55) and tested negative in short-term assays for genotoxicity (56). In addition, we also reported that intake of 26 g of cocoa powder for 12 wk in humans was not associated with abnormalities in blood and urine variables (15). In the present study, daily consumption of cocoa powder also had no significant influence on blood and urine variables, blood pressure, or BMI, and no adverse effects were reported to the doctors at the patient interviews. These results confirm the findings of previous studies regarding the safety of cocoa products.

Cocoa powder contains fiber and methylxanthines compounds such as caffeine and theobromine. However, in the present study, the control group drink did not control for the fiber, caffeine, and theobromine contents of the cocoa group drink. Wan et al (57) reported that cocoa powder and chocolate had favorable effects on LDL oxidative susceptibility and HDL-cholesterol concentrations compared with a control diet with similar fat, protein, carbohydrate, cholesterol, fiber, caffeine, and theobromine content. This result indicates that polyphenolic compounds from cocoa powder and chocolate may contribute to these favorable effects.

In conclusion, the present study showed that daily intake of cocoa powder decreased the susceptibility of LDL to oxidation and increased HDL-cholesterol concentrations in plasma in humans. Plasma HDL-cholesterol concentrations correlated negatively with plasma oxidized LDL, whereas plasma oxidized LDL concentrations correlated negatively with excretion of urinary epicatechin. It is possible that increases in HDL-cholesterol concentrations may contribute to suppression of LDL oxidation. Because polyphenolic substances derived from cocoa powder contribute to the elevation of HDL cholesterol, it would be anticipated that intake of polyphenol-rich foods, such as cocoa, tea, wine, fruit, and vegetables, should lead to a decrease in the incidence of arteriosclerotic disease. Moreover, it is irrefutable that a balanced daily diet is important for the promotion of human health.



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REFERENCES

- Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 1989;320:915–24.
- Kume N, Gimbrone MA Jr. Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. *J Clin Invest* 1994;93:907–11.
- Kume N, Cybulsky MI, Gimbrone MA Jr. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. *J Clin Invest* 1992;90:1138–44.
- Gordon DJ, Probstfield JL, Garrison RJ, et al. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation* 1989;79:8–15.
- Assmann G, Schulte H. Relation of high-density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (the PROCAM experience). Prospective Cardiovascular Munster study. *Am J Cardiol* 1992;70:733–7.
- Parthasarathy S, Barnett J, Fong LG. High-density lipoprotein inhibits the oxidative modification of low-density lipoprotein. *Biochim Biophys Acta* 1990;1044:275–83.
- Holvoet P, Vanhaecke J, Janssens S, Van de Werf F, Collen D. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation* 1998;98:1487–94.
- Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* 1993;342:1007–11.
- Arts ICW, Hollman PCH, Feskens EJM, Bueno de Mesquita HB, Kromhout D. Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly Study. *Am J Clin Nutr* 2001;74:227–32.
- Fuhrman B, Aviram M. Flavonoids protect LDL from oxidation and attenuate atherosclerosis. *Curr Opin Lipidol* 2001;12:41–8.
- Natsume M, Osakabe N, Yamagishi M, et al. Analyses of polyphenols in cacao liquor, cocoa, and chocolate by normal- phase and reversed-phase HPLC. *Biosci Biotechnol Biochem* 2000;64:2581–7.
- Arts ICW, Hollman PCH, Kromhout D. Chocolate as a source of tea flavonoids. *Lancet* 1999;354:488.
- Kurosawa T, Itoh F, Nozaki A, et al. Suppressive effects of cacao liquor polyphenols (CLP) on LDL oxidation and the development of atherosclerosis in Kurosawa and Kusanagi-hypercholesterolemic rabbits. *Atherosclerosis* 2005;179:237–46.
- Osakabe N, Baba S, Yasuda A, et al. Daily cocoa intake reduces the susceptibility of low-density lipoprotein to oxidation as demonstrated in healthy human volunteers. *Free Radic Res* 2001;34:93–9.
- Baba S, Osakabe N, Natsume M, et al. Long-term intake of cocoa powder reduce the oxidative susceptibility of low density lipoprotein in healthy humans. *Jpn J Med Pharm Sci* 2004;52:947–63 (in Japanese).
- Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radic Res Commun* 1989;6:67–75.
- Hirano R, Osakabe N, Iwamoto A, et al. Antioxidant effects of polyphenols in chocolate on low-density lipoprotein both in vitro and ex vivo. *J Nutr Sci Vitaminol (Tokyo)* 2000;46:199–204.
- Kido T, Kurata H, Matsumoto A, et al. Lipoprotein analysis using agarose gel electrophoresis and differential staining of lipids. *J Atheroscler Thromb* 2001;8:7–13.
- Holvoet P, Donck J, Landeloos M, et al. Correlation between oxidized low density lipoproteins and von Willebrand factor in chronic renal failure. *Thromb Haemost* 1996;76:663–9.
- Kato Y, Yoshida A, Naito M, et al. Identification and quantification of *N*^ε-(Hexanoyl)lysine in human urine by liquid chromatography/tandem mass spectrometry. *Free Radic Biol Med* 2004;37:1864–74.
- Baba S, Osakabe N, Yasuda A, et al. Bioavailability of (–)-epicatechin upon intake of chocolate and cocoa in human volunteers. *Free Radic Res* 2000;33:635–41.
- Baba S, Osakabe N, Natsume M, Muto Y, Takizawa T, Terao J. In vivo comparison of the bioavailability of (+)-catechin, (–)-epicatechin and their mixture in orally administered rats. *J Nutr* 2001;131:2885–91.
- Piskula MK, Terao J. Accumulation of (–)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues. *J Nutr* 1998;128:1172–8.

24. Regnstrom J, Nilsson J, Tornvall P, Landou C, Hamsten A. Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet* 1992;339:1183–6.
25. Liu ML, Ylitalo K, Salonen R, Salonen JT, Taskinen MR. Circulating oxidized low-density lipoprotein and its association with carotid intima-media thickness in asymptomatic members of familial combined hyperlipidemic families. *Arterioscler Thromb Vasc Biol* 2004;24:1492–7.
26. Frankel EN, Waterhouwe AL, Teissedre PL. Principal phenolic phytochemicals in selected Californian wines and their antioxidant activity in inhibiting oxidation of human low-density lipoprotein. *J Agric Food Chem* 1995;43:890–4.
27. Osakabe N, Yasuda A, Natsume M, Takizawa T, Terao J, Kondo K. Catechins and their oligomers linked by C4 → C8 bonds are major cacao polyphenols and protect low-density lipoprotein from oxidation in vitro. *Exp Biol Med* 2002;227:51–6.
28. Baba S, Osakabe N, Natsume M, Muto Y, Takizawa T, Terao J. Absorption and urinary excretion of (–)-epicatechin after administration of different levels of cocoa powder or (–)-epicatechin in rats. *J Agric Food Chem* 2001;49:6050–6.
29. Baba S, Osakabe N, Natsume M, et al. Cocoa powder enhances the level of antioxidative activity in rat plasma. *Br J Nutr* 2000;84:673–80.
30. Holt RR, Lazarus SA, Sullards MC, et al. Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. *Am J Clin Nutr* 2002;76:798–804.
31. Zhu QY, Holt RR, Lazarus SA, Orozco TJ, Keen CL. Inhibitory effects of cocoa flavanols and procyanidin oligomers on free radical-induced erythrocyte hemolysis. *Exp Biol Med* 2002;227:321–9.
32. Fuhrman B, Lavy A, Aviram M. Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am J Clin Nutr* 1995;61:549–54.
33. Hayek T, Fuhrman B, Vaya J, et al. Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler Thromb Vasc Biol* 1997;17:2744–52.
34. Arts MJ, Haenen GR, Voss HP, Bast A. Masking of antioxidant capacity by the interaction of flavonoids with protein. *Food Chem Toxicol* 2001;39:787–91.
35. Terao J. Dietary flavonoids as plasma antioxidants on lipid peroxidation: significance of metabolic conversion. *Antiox Food Suppl Hum Health* 1999;255–68.
36. Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP, Rice-Evans C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch Biochem Biophys* 1995;322:339–46.
37. Mursu J, Voutilainen S, Nurmi T, et al. Dark chocolate consumption increases HDL cholesterol concentration and chocolate fatty acids may inhibit lipid peroxidation in healthy humans. *Free Radic Biol Med* 2004;37:1351–9.
38. Baba S, Osakabe N, Natsume M, Terao J. Absorption and urinary excretion of procyanidin B2 [epicatechin-(4β-8)-epicatechin] in rats. *Free Radic Biol Med* 2002;33:142–8.
39. Natsume M, Osakabe N, Oyama M, et al. Structures of (–)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (–)-epicatechin: differences between human and rat. *Free Radic Biol Med* 2003;34:840–9.
40. Lee MK, Moon SS, Lee SE, et al. Naringenin 7-*O*-cetyl ether as inhibitor of HMG-CoA reductase and modulator of plasma and hepatic lipids in high cholesterol-fed rats. *Bioorg Med Chem* 2003;11:393–8.
41. Ohtsuki K, Abe A, Mitsuzumi H, et al. Glucosyl hesperidin improves serum cholesterol composition and inhibits hypertrophy in vasculature. *J Nutr Sci Vitaminol (Tokyo)* 2003;49:447–50.
42. Zhan S, Ho SC. Meta-analysis of the effects of soy protein containing isoflavones on the lipid profile. *Am J Clin Nutr* 2005;81:397–408.
43. Tsang C, Higgins S, Duthie GG, et al. The influence of moderate red wine consumption on antioxidant status and indices of oxidative stress associated with CHD in healthy volunteers. *Br J Nutr* 2005;93:233–40.
44. Navab M, Imes SS, Hama SY, et al. Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein. *J Clin Invest* 1991;88:2039–46.
45. Watson AD, Berliner JA, Hama SY, et al. Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *J Clin Invest* 1995;96:2882–91.
46. Dansky HM, Charlton SA, Barlow CB, et al. Apo A-I inhibits foam cell formation in Apo E-deficient mice after monocyte adherence to endothelium. *J Clin Invest* 1999;104:31–9.
47. Itabe H, Hosoya R, Karasawa K, et al. Metabolism of oxidized phosphatidylcholines formed in oxidized low density lipoprotein by lecithin-cholesterol acyltransferase. *J Biochem* 1999;126:153–61.
48. Natsume M, Osakabe N, Yasuda A, et al. In vitro antioxidative activity of (–)-epicatechin glucuronide metabolites present in human and rat plasma. *Free Radic Res* 2004;38:1341–8.
49. Lamon-Fava S. Genistein activates apolipoprotein A-I gene expression in the human hepatoma cell line Hep G2. *J Nutr* 2000;130:2489–92.
50. Lamon-Fava S, Micherone D. Regulation of apoA-I gene expression: mechanism of action of estrogen and genistein. *J Lipid Res* 2004;45:106–12.
51. Hazen SL, Heinecke JW. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J Clin Invest* 1997;99:2075–81.
52. Kato Y, Mori Y, Makino Y, et al. Formation of *N*^ε-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. *J Biol Chem* 1999;274:20406–14.
53. Kato Y, Kawai Y, Morinaga H, et al. Immunogenicity of a brominated protein and successive establishment of a monoclonal antibody to dihalogenated tyrosine. *Free Radic Biol Med* 2005;38:24–31.
54. Tarka SM Jr, Applebaum RS, Borzelleca JF. Evaluation of the teratogenic potential of cocoa powder and theobromine in New Zealand White rabbits. *Food Chem Toxicol* 1986;24:363–74.
55. Tarka SM Jr, Morrissey RB, Apgar JL, Hostetler KA, Shively CA. Chronic toxicity/carcinogenicity studies of cocoa powder in rats. *Food Chem Toxicol* 1991;29:7–19.
56. Brusick D, Myhr B, Galloway S, Rundell J, Jagannath DR, Tarka S. Genotoxicity of cocoa in a series of short-term assays. *Mutat Res* 1986;169:115–21.
57. Wan Y, Vinson JA, Etherton TD, Proch J, Lazarus SA, Kris-Etherton PM. Effects of cocoa powder and dark chocolate on LDL oxidative susceptibility and prostaglandin concentrations in humans. *Am J Clin Nutr* 2001;74:596–602.