Effects of cocoa powder and dark chocolate on LDL oxidative susceptibility and prostaglandin concentrations in humans[1–3]

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ABSTRACT

Background: Flavonoids are polyphenolic compounds of plant origin with antioxidant effects. Flavonoids inhibit LDL oxidation and reduce thrombotic tendency in vitro. Little is known about how cocoa powder and dark chocolate, rich sources of polyphenols, affect these cardiovascular disease risk factors.

Objective: We evaluated the effects of a diet high in cocoa powder and dark chocolate (CP-DC diet) on LDL oxidative susceptibility, serum total antioxidant capacity, and urinary prostaglandin concentrations.

Design: We conducted a randomized, 2-period, crossover study in 23 healthy subjects fed 2 diets: an average American diet (AAD) controlled for fiber, caffeine, and theobromine and an AAD supplemented with 22 g cocoa powder and 16 g dark chocolate (CP-DC diet), providing ≈466 mg procyanidins/d.

Results: LDL oxidation lag time was ≈8% greater ($P = 0.01$) after the CP-DC diet than after the AAD. Serum total antioxidant capacity measured by oxygen radical absorbance capacity was ≈4% greater ($P = 0.04$) after the CP-DC diet than after the AAD and was positively correlated with LDL oxidation lag time ($r = 0.32$, $P = 0.03$). HDL cholesterol was 4% greater after the CP-DC diet ($P = 0.02$) than after the AAD; however, LDL-HDL ratios were not significantly different. Twenty-four–hour urinary excretion of thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ and the ratio of the 2 compounds were not significantly different between the 2 diets.

Conclusion: Cocoa powder and dark chocolate may favorably affect cardiovascular disease risk status by modestly reducing LDL oxidation susceptibility, increasing serum total antioxidant capacity and HDL-cholesterol concentrations, and not adversely affecting prostaglandins.   *Am J Clin Nutr* 2001;74:596–602.

KEY WORDS   Cocoa powder, dark chocolate, LDL oxidation, oxygen radical absorbance capacity, prostaglandins, flavonoids, procyanidins, polyphenols, catechins

INTRODUCTION

Flavonoids are a group of polyphenolic compounds that occur widely in fruit, vegetables, tea, and red wine. Certain cocoas and chocolates can also be rich sources of flavonoids, especially the subclass of oligomeric flavonoids known as procyanidins (1–4). Indeed, the antioxidant activity of cocoa and chocolate was shown to be correlated with its catechin and procyanidin contents. Arts et al (2)

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reported that dark chocolate contains catechins (a group of flavan-3-ol flavonoid compounds) at an average concentration of 0.535 mg/g, 4 times that of tea (139 mg/L). In a representative sample of the Dutch population, chocolate contributed 20% of the catechin intake whereas tea contributed 55%. In younger age groups, in which chocolate is probably preferred to tea, and in countries where tea is consumed less, chocolate may be a more important source of catechins and their oligomers (eg, procyanidins). Therefore, cocoa and chocolate can be important dietary sources of flavonoids in addition to tea.

Oxidative modification of LDL was shown to play a key role in the initiation of atherogenesis (5). Studies have shown that flavonoids prevent LDL oxidation in vitro by scavenging radical species or sequestering metal ions (6, 7). Extracts of cocoa powder also significantly inhibit LDL oxidation. Kondo et al (8) reported that cocoa prolongs the lag time of LDL oxidation in a concentration-dependent manner. Waterhouse et al (3) found that at 5 μmol gallic acid equivalents/L, cocoa phenols inhibited LDL oxidation by 75%, whereas red wines inhibited LDL oxidation by 37–65%. Vinson et al (9) showed that chocolates had a higher flavonoid antioxidant quantity-quality index than did fruit, vegetables, red wine, and black tea. The ability of flavonoids to prevent LDL oxidation in vivo depends on their bioavailability, particularly their binding to lipoproteins. Polyphenols such as epicatechin bind to LDL and VLDL in plasma and protect the LDL and VLDL from oxidation after isolation (9). Also, chocolate extracts possess this lipoprotein-binding antioxidant property (4). Recently, Wang et al (10) reported that a dose-dependent increase in plasma epicatechin was associated with an increase in plasma antioxidant capacity and a reduction in plasma lipid peroxidation 2 and 6 h after procyanidin-rich chocolate consumption. However, no long-term studies have

TABLE 1
Assayed macronutrient composition of the 2 experimental diets[1]

	AAD	CP/DC diet
Fat (% of energy)	33	34
SFA	15	16
MUFA	11	11
PUFA	7	7
Protein (% of energy)	15	16
Carbohydrate (% of energy)	52	50
Cholesterol (mg/d)[2]	329	330
Fiber (g/d)[2]	17	18

[1] AAD, average American diet; CP/DC, AAD plus 22 g cocoa powder and 16 g dark chocolate; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[2] Estimated by using the NUTRITIONIST IV database (N-Squared Computing, First DataBank Division, San Bruno, CA)

evaluated the effects of cocoa polyphenol compounds on the oxidative modification of LDL in humans.

There are several conflicting observations in humans about how flavonoids affect platelet function (11). It was recently reported that cocoa consumption suppresses unstimulated and stimulated platelet activation in whole blood measured by platelet PAC-1 binding and ADP-induced P-selectin expression over 6 h (12). Moreover, platelet microparticle formation decreased 2 and 6 h after cocoa consumption, whereas the time required for clot formation increased during platelet function analysis (13). Although promising, we are unaware of any studies that have examined the long-term effects of cocoa and chocolate polyphenols on platelet function.

This new exciting evidence suggests that certain cocoa powders and chocolates may favorably affect oxidation and platelet activity. Therefore, we conducted a study in humans to evaluate the effect of cocoa powder and dark chocolate rich in catechins and procyanidins on serum antioxidant capacity and several risk factors for cardiovascular disease, including LDL oxidative susceptibility, serum lipid and lipoprotein concentrations, and prostaglandin concentrations.

SUBJECTS AND METHODS

Subjects

Twenty-three healthy subjects (10 men and 13 women, including the 6 subjects from the pilot study) participated in the study; 19 were white, 1 was African American, 2 were Asian American, and 1 was Hispanic. The average age of the subjects was 36 y (range: 21–62 y). Baseline serum total cholesterol and LDL-cholesterol concentrations were 5.38 ± 0.13 and 3.41 ± 0.13 mmol/L, respectively, and baseline serum HDL-cholesterol, triacylglycerol, and VLDL-cholesterol concentrations were 1.21 ± 0.06, 1.63 ± 0.19, and 0.74 ± 0.09 mmol/L, respectively. The study was conducted in accordance with the guidelines of the Institutional Review Board of The Pennsylvania State University.

Study design

To determine the feasibility of performing a long-term study, we first carried out a pilot study to evaluate the bioavailability of flavonoids in cocoa powder and dark chocolate. Six subjects (3 men and 3 women) aged 23–49 y participated in this pilot study. Subjects were fed an average American diet (AAD) containing 22 g cocoa powder and 16 g dark chocolate (CP-DC diet) (ie, the daily allotment of cocoa powder and dark chocolate for the intended long-term study) at breakfast. A 12-h fasting blood sample was collected from the 6 subjects before breakfast. Blood samples were also collected 1, 2, 4, and 24 h after ingestion of the CP-DC diet.

For the long-term study, we used a randomized, 2-period, crossover study design. Free-living subjects were fed 2 well-controlled experimental diets: an AAD and the CP-DC diet. Subjects consumed each diet for 4 wk followed by a brief break (2 wk) before the crossover to the other diet. Subjects consumed their habitual diet during the 2-wk break between feeding periods.

Experimental diets

Both experimental diets provided similar amounts of total fat (33–34% of energy), saturated fatty acids (15–16% of energy), monounsaturated fatty acids (11% of energy), polyunsaturated fatty acids (7% of energy), cholesterol (330 mg/d), and dietary fiber (18 g/d) (**Table 1**). The AAD served as the control diet. The CP-DC diet provided 6.9% of energy (2.5% from cocoa powder and 4.4% from dark chocolate) and 8380 kJ (2003 kcal). About 75% of the flavonoids came from cocoa powder and 25% came from dark chocolate. All subjects received the 22 g cocoa powder and 16 g dark chocolate every day regardless of energy requirements. The total procyanidin, caffeine, and theobromine contents in the diets given to each subject daily are shown in **Table 2**. A representative 1-d diet was homogenized, freeze-dried, and extracted and the procyanidin content was analyzed by HPLC (14). The AAD was poor in procyanidins, which are the predominant flavonoid in chocolate. The CP-DC diet provided 466 mg procyanidins/d, 111 mg/d of which consisted of flavan-3-ol monomeric flavonoids, catechin, and epicatechin. On the basis of Folin analysis (a nonspecific colorimetric method), the CP-DC diet provided 12 800 μmol total flavonoids/d, which is ≈5600 μmol/d higher than the content in the AAD. Six composite samples were measured and the CP-DC diet was found to have a higher flavonoid content than did the AAD ($P < 0.001$). Therefore, the 2 diets were similar except for the contents of flavonoids and polyphenols, which were provided by the cocoa powder and dark chocolate in the CP-DC diet.

To control for the contents of caffeine (43 mg/d) and theobromine (431 mg/d) provided by the cocoa powder and dark chocolate, diet cola (29.5 mL/d, or 16 oz/d) and a theobromine supplement were added to the AAD. The AAD was also controlled for the fiber and cocoa butter contents of the CP-DC diet. Cocoa butter was incorporated into the baked goods of the AAD to match the amount of cocoa butter in the dark chocolate (5.4 g/16 g dark chocolate) that was fed in the other test diet.

TABLE 2
Assayed polyphenols in the 2 experimental diets[1]

	AAD[2]	CP/DC diet
Procyanidins (mg/d)	< Detection limit	466
Catechin + epicatechin (mg/d)	< Detection limit	111
Caffeine (mg/d)	47	48
Theobromine (mg/d)[3]	550	614

[1] AAD, average American diet; CP/DC, AAD plus 22 g cocoa powder and 16 g dark chocolate.

[2] Controlled for caffeine by adding diet cola (480 mL).

[3] By capsule.

This was done to achieve a similar fatty acid profile between the 2 experimental diets. The diets were designed to be low in flavonoids from a source other than cocoa powder or dark chocolate. Thus, foods that were limited or excluded included tea, coffee, wine, onions, apples, beans, soybeans, and orange and grape juices. The cocoa powder and dark chocolate were incorporated into the CP-DC diet in various ways, such as mixed in milk or pudding snacks or baked into cookies or brownies. Thus, the cocoa powder and dark chocolate and, hence, flavonoids, were distributed throughout the day.

The macronutrient profile of the 2 experimental diets was analyzed to validate the diet composition. The amounts of protein, carbohydrate, and total fats were determined by proximate analysis and the percentages of saturated, monounsaturated, and polyunsaturated fatty acids by gas chromatography. The experimental diets (Table 1) met the target nutrient goals established initially and were consistent with the nutrient database values obtained during the development of the 6-d cycle menu.

The NUTRITIONIST IV database (N-Squared Computing, First Databank Division, San Bruno, CA) was used to plan a 6-d cycle menu, and all menus were designed to be nutritionally adequate. Four energy levels were developed (8380, 10 475, 12 570, and 14 665 kJ, or 2003, 2504, 3004, and 3505 kcal) to meet the energy needs of each subject. Unit foods (419 kJ each, or 100 kcal each) that were compositionally identical to the AAD were used to adjust energy levels so that subjects maintained body weight within 1 kg of baseline throughout the study.

Sample collection

For the pilot bioavailability study, a 12-h fasting blood sample was collected from 6 subjects before breakfast in a heparin-containing tube. Blood samples were also collected 1, 2, 4, and 24 h after ingestion of cocoa powder and dark chocolate or the muffin containing only cocoa butter. A 24-h urine sample was collected and frozen at −80°C until analyzed.

For the long-term study, a 12-h fasting blood sample was collected into silicone-gel–coated tubes at the end of each diet period. Serum was separated by centrifugation at $1330 \times g$ (3000 rpm) for 15 min at 4°C 30 min after the blood was drawn and was then portioned (2 mL/vial) and stored at −80°C for subsequent analysis.

For the analysis of LDL oxidation, 30 mL blood was collected into evacuated tubes containing 0.1 mL of 0.5 mol EDTA/L. Plasma was separated by centrifugation at $1300 \times g$ (3000 rpm) for 15 min at 4°C immediately after the blood was drawn. The water-soluble antioxidant Trolox (Aldrich Chemical Co, Milwaukee) was added to the plasma to a final concentration of 1 μmol/L.

Twenty-four–hour urine samples also were collected at the end of each diet period; 4 mL of 6 mol HCl/L was added to each collection container and subjects were instructed to keep the containers at 4°C at all times. Immediately after the 24-h samples were collected, total urine volume was measured. A 20-mL aliquot was frozen at −80°C until the end of the study for thromboxane B_2 (TXB_2) and 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) analysis.

Bioavailability analysis of polyphenols and theobromine

In the pilot study, plasma epicatechin and theobromine were measured at the University of Scranton in samples collected at 5 time points: 0, 1, 2, 4, and 24 h after consumption of cocoa powder and dark chocolate. Because theobromine is present in high amounts in cocoa powder and dark chocolate, it was used

as a marker for the absorption of cocoa powder and dark chocolate. Plasma or 10-fold diluted urine (250 μL) and 25 μL of a solution of vitamin C (2 g ascorbic acid) and EDTA (10 mg disodium EDTA) in 10 mL of HPLC grade water were combined in a 1.5-mL polypropylene centrifuge tube. Next, 750 μL of 1.2 mol HCl/L in methanol was added to the tube and the solution mixed by vortex for 2 min. The resulting mixture was then hydrolyzed in the dark at room temperature for 18 h, mixed by vortex for 1 min, and then centrifuged for 10 min at $10 000 \times g$; 25 μL of the resulting clear supernatant fluid was injected into a Supelco LC-DP (25 cm × 4.6 mm) column. Solvent A consisted of 4% acetic acid in water and solvent B consisted of acetic acid:methanol:water (1:25:25, by vol). These solutions or combinations thereof were eluted over the HPLC column as follows: 0–1.5 min, 100% A; 1.5–10 min, 100% A to A:B (50:50, by vol); 10–12 min, A:B (50:50, by vol) to 100% B. The column was washed with 100% methanol between runs and then equilibrated with solvent A for 5 min. The retention time of theobromine was 5.52 ± 0.05 min at a wavelength of 280 nm and that of epicatechin was 10.76 ± 0.07 min at an excitation wavelength of 276 nm and an emission wavelength of 316 nm. HPLC analysis was conducted on a Shimadzu (Kyoto, Japan) LC-10AD instrument with an SPD-10AV ultraviolet-visible detector and an RF-551 spectrofluorometric detector (Columbia, MD). Standards were prepared by adding known amounts of epicatechin to epicatechin-free plasma. Confirmation of epicatechin in the urine was performed by N Osakabe (Functional Food Research and Development Laboratories, Meiji Sika Kaisha Ltd, Satama Japan) using HPLC mass spectrometry in the negative ion mode. An enzyme hydrolysis and extraction method according to Piskula and Terao (15) was used to prepare the samples.

Serum lipids and lipoproteins

Serum samples were sent to LabCorp (Altoona, PA) immediately after being drawn. Enzymatic assays were used to analyze serum total cholesterol, HDL-cholesterol, and triacylglycerol concentrations (model AU 5000 analyzer; Olympus America Inc, Melville, NY). The Friedewald formula was used to calculate LDL-cholesterol concentrations (16): [LDL cholesterol = total cholesterol − (HDL cholesterol − triacylglycerol/5)], which were then converted to mmol/L by dividing by 387.

LDL oxidation

LDL [density (d) = 1.026–1.063 kg/L] was first isolated by density gradient ultracentrifugation (17). A density gradient was formed by adjusting 4 mL plasma to $d = 1.21$ kg/L with potassium bromide and then sequentially layering 2.0–2.5 mL of 3 salt solutions ($d = 1.063$, 1.019, and 1.026 kg/L) above the plasma. Samples were then centrifuged in a Beckman SW40 or SW41 swinging bucket rotor at $188 000 \times g$ (39 000 rpm) for 22 h at 10°C. Immediately after centrifugation, the LDL fraction was collected. LDL samples (0.45 mL) were preserved in a 10% sucrose solution (wt:vol) to prevent structural and biological changes as a result of freezing (18), and were then purged with sodium and stored at −80°C. Freezing LDL in this way does not influence oxidation variables (19).

At the end of the study, LDL oxidative susceptibility was determined for each subject. LDL was dialyzed for 24 h at 4°C in the dark against sodium-purged 0.01 mol phosphate buffered saline (PBS)/L containing 0.1 g chloramphenicol/L. The PBS buffer (2 L) was changed 3 times during the 24-h period. The
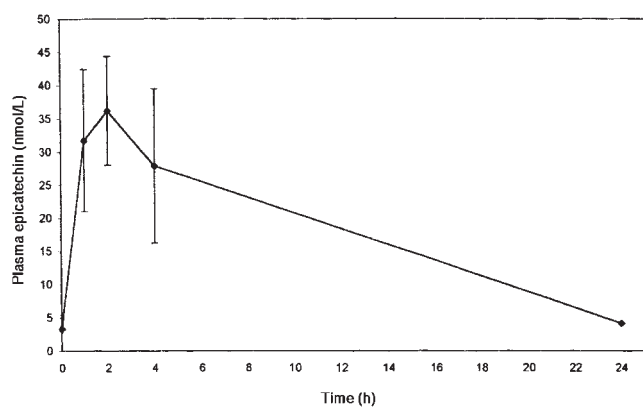
FIGURE 1. Mean (±SEM) plasma epicatechin concentrations in 6 subjects after ingestion of 22 g cocoa powder and 16 g dark chocolate in the pilot study.

Lowry method was used to analyze proteins (20). Within 24 h of dialysis, conjugated diene formation was determined by continuously monitoring the change in absorbance at 234 nm according to the method of Esterbauer et al (17). LDL protein (100 μg) was diluted to 1 mL with PBS buffer and oxidation was initiated by adding a solution of 1 mmol $CuCl_2/L$ to a final concentration of 0.01 mmol/L (21). Oxidation was measured in a spectrophotometer (model 50 UV; Beckman, Fullerton, CA) at an absorbance of 234 nm at 3-min intervals for 3 h at 37°C. From these measurements, lag time, rate of oxidation, and the total amount of conjugated dienes formed were determined for each sample. Lag time was defined as the intercept of the baseline and propagation phase of the absorbance curve and was expressed in minutes. The slope ($\Delta A234$/min) of the linear portion of the curve was measured to determine the maximum oxidation rate. The difference between the maximum absorbance during the decomposition phase and the absorbance at the start of the lag phase was used to determine the maximum amount of conjugated dienes formed in LDL. The molar extinction coefficient for conjugated lipid hydroperoxides was used to calculate the quantities of conjugated dienes formed: $\epsilon_{234} = 29\,500$ mol·L^{-1}·cm^{-1}.

Serum oxygen radical absorbance capacity assay

Serum samples were sent to Brunswick Laboratories (Wareham, MA) for the automated oxygen radical absorbance capacity (ORAC) assay, which was carried out on a COBAS FARA II centrifugal analyzer with a fluorescence measuring attachment (22). Briefly, in a final assay mixture (0.4 mL total volume), R-phycoerythrin (R-PE; 16.7 nmol/L) was used as a target of free radical attack with 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH; 4 mmol/L) as a peroxyl radical generator. Trolox was used as a control standard. The analyzer was programmed to record the fluorescence of R-PE every 2 min after AAPH was added. All fluorescence measurements were expressed relative to the initial reading. The differences in areas under the R-PE decay curves between the blank and a sample were used to calculate the final results and were expressed as μmol Trolox equivalents/L.

Urinary prostaglandin metabolite analysis

Prostaglandin concentrations were assessed by measuring urinary metabolites of thromboxane A_2 (ie, TXB_2) and of PGI_2 (ie,

6-keto-$PGF_{1\alpha}$). The main advantages of measuring these metabolites in urine are that they are present in sufficiently high concentrations, and samples can be obtained noninvasively, avoiding the problem of sampling artifact. In addition, quantifying these urinary metabolites provides a measure of eicosanoid status over 24 h.

The procedures of Powell (23) were used to extract TXB_2 and 6-keto-$PGF_{1\alpha}$ from the urine samples. The urine was acidified to pH 3.5 with hydrochloric acid. Sep-Pak C-18 cartridges (Waters Corporation, Milford, MA) were activated with 12 mL methanol (HPLC grade) and 8 mL distilled water sequentially. A 1-mL urine sample was then applied to the activated Sep-Pak C-18 cartridge, washed with water, and eluted with methanol. TXB_2 and 6-keto-$PGF_{1\alpha}$ were eluted with 3 mL methanol and stored at −80°C until analyzed with use of enzyme immunoassay kits from Amersham Life Science Inc (Arlington Heights, IL).

Statistical analysis

All data analyses were conducted with use of the SAS SYSTEM 6.11 for WINDOWS (SAS Institute Inc, Cary, NC). Data were expressed as means ± SEs, and any effects of diet were tested by using analysis of variance. P values determined with the use of paired t tests were used to determine statistical differences between experimental diets for each of the following variables: serum lipids and lipoproteins, LDL oxidation potential, serum ORAC, and urinary TXB_2 and 6-keto-$PGF_{1\alpha}$. Pearson's correlation analysis was used to test the correlations between LDL oxidation potential and serum total antioxidant capacity. A P value <0.05 was considered statistically significant.

RESULTS

Pilot bioavailability study

Plasma epicatechin concentrations after ingestion of the CP-DC diet are presented in **Figure 1**. The plasma epicatechin concentration was 3.3 ± 0.1 nmol/L at 0 h, which is close to the detection limit of <3 nmol/L. After intake of the CP-DC diet, plasma epicatechin concentrations increased rapidly, reaching a maximum of 36.2 ± 8.2 nmol/L at 2 h. The clearance of epicatechin from the plasma compartment was rapid. Plasma epicatechin concentrations declined to baseline 24 h after consumption of the CP-DC diet. Urinary epicatechin concentrations collected over 24 h in subjects who consumed the AAD were 0.209 ± 0.487 μmol (range: 0–1.218 μmol) and those for subjects who consumed the CP-DC diet were 6.266 ± 3.238 μmol (range: 1.880–10.583 μmol) ($P < 0.01$). Five of the 6 subjects had nondetectable epicatechin concentrations after consuming the AAD.

A curve similar in shape to that of epicatechin was observed for plasma theobromine concentrations after consumption of the CP-DC diet (**Figure 2**). Plasma theobromine rose to 43.2 μmol/L 4 h after ingestion of the CP-DC diet. After the peak, plasma theobromine concentrations declined slowly and were still elevated (13 μmol/L) at 24 h relative to baseline. These results indicate a very slow clearance of theobromine from the plasma compartment.

Long-term study

Twenty-three subjects completed the long-term study. All subjects maintained their weight during the study. The effect of dietary treatment on serum lipids and lipoproteins is shown in **Table 3**. Serum total cholesterol, LDL cholesterol, triacylglycerol,
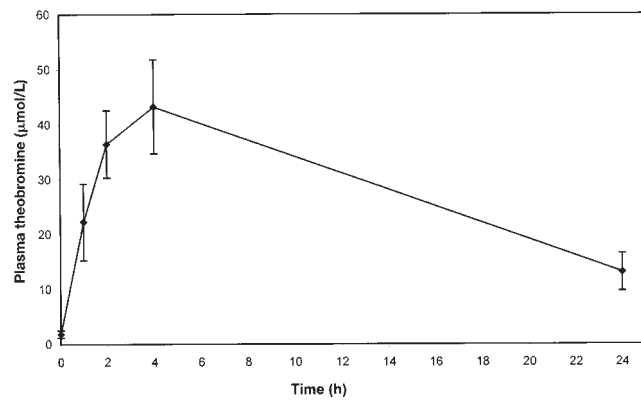
FIGURE 2. Mean (±SEM) plasma theobromine concentrations in 6 subjects after ingestion of 22 g cocoa powder and 16 g dark chocolate in the pilot study.

and VLDL cholesterol did not differ significantly by diet. Serum HDL cholesterol was significantly greater (by 4%, or 0.05 mmol/L) when subjects consumed the CP-DC diet compared with the AAD. The ratios of total to HDL cholesterol and of LDL to HDL cholesterol did not differ significantly by diet.

The effect of dietary treatment on LDL susceptibility to copper-catalyzed oxidation is shown in **Table 4**. Lag time was ≈8% greater with the CP-DC diet. The rate of oxidation and conjugated diene formation tended to be lower by ≈6.6% and 1.5% with the CP-DC diet than with the AAD, although the differences were not significant.

Serum total antioxidant capacity was ≈4.2% higher when subjects consumed the CP-DC diet than when they consumed the AAD (311.3 ± 14.7 compared with 298.8 ± 14.4 μmol Trolox equivalents/L; $P = 0.04$). Serum total antioxidant capacity was positively correlated with LDL oxidation lag time when averaged over all diets (**Figure 3**). No significant correlations were observed between serum total antioxidant capacity and the rate of LDL oxidation or conjugated diene formation.

Urinary TXB_2 and 6-keto-$PGF_{1\alpha}$ excretion were lower by ≈10.9% and 3.9% with the CP-DC diet than with the AAD, but these differences were not significant (**Table 5**). No significant differences were observed in the ratio of 6-keto-$PGF_{1\alpha}$ to TXB_2 between the 2 diets; however, considerable interindividual and intraindividual variability in the urinary excretion of these 2 metabolites was observed.

DISCUSSION

Cocoa and chocolate are a rich source of polyphenols. The flavan-3-ol monomers catechin and epicatechin and the oligomeric procyanidins are the major flavonoids in chocolate. Recent attention has been directed to the antioxidant potential of these flavonoids in cocoa and chocolate and their potential protective effects on risk of cardiovascular disease (3–4, 8). However, little information is available about the absorption, metabolism, and antioxidant activity of cocoa and chocolate flavonoids in humans after consumption.

The results of our pilot study on the absorption of epicatechin are similar to the results of 2 recent studies (10, 24), one of which showed that plasma concentrations of epicatechin increased markedly (from 0.3 to 0.7 μmol/L) after consumption of 80 g dark chocolate, reached a maximum between 2 and 3 h, and declined

TABLE 3
Effect of dietary treatment on serum lipids and lipoproteins[1]

	AAD (n = 23)	CP/DC diet (n = 23)
	mmol/L	
Total cholesterol (mmol/L)	5.29 ± 0.2	5.53 ± 0.2
Triacylglycerol (mmol/L)	1.18 ± 0.1	1.19 ± 0.2
LDL (mmol/L)	3.47 ± 0.1	3.63 ± 0.2
HDL (mmol/L)	1.28 ± 0.1	1.33 ± 0.1[2]
VLDL (mmol/L)	0.53 ± 0.1	0.54 ± 0.1
TC:HDL cholesterol	4.33 ± 0.3	4.39 ± 0.3
LDL:HDL cholesterol	2.85 ± 0.2	2.89 ± 0.2

[1] $\overline{x}$ ± SE. AAD, average American diet; CP/DC, AAD plus 22 g cocoa powder and 16 g dark chocolate.
[2] Significantly different from the AAD, $P = 0.02$.

thereafter (24). Because the results of the pilot study showed that polyphenols in cocoa powder and dark chocolate were absorbed, we conducted the long-term study to evaluate the effects of cocoa powder and dark chocolate on cardiovascular disease risk factors.

The long-term study indicated favorable effects of cocoa powder and dark chocolate on LDL oxidation susceptibility. The lag phase of LDL oxidation corresponds to the time required for the endogenous antioxidant to be consumed. The length of lag time, thus, is an index of the level and quality of antioxidants in LDL particles (25). We found a significantly greater lag time in LDL oxidation when the subjects consumed the CP-DC diet than when they consumed the AAD in the present study, suggesting that initiation of LDL oxidation was modestly delayed because of a higher concentration of antioxidants in LDL. It is important to note that the clinical significance of these small differences in indexes of oxidation status remains to be clarified. Thus, it would be interesting to measure the urinary F_2 isoprostane concentration, which is a more direct measure of oxidized LDL.

Flavonoids may enter the LDL particle or bind to apolipoprotein B because of their strong protein-binding properties (26). Fuhrman et al (27) showed that red wine phenols are incorporated into LDL in vivo and subsequently inhibit LDL oxidation. Furthermore, cocoa flavonoids may be associated with the surface of lipoprotein particles, as suggested for wine flavonoids (28). Therefore, cocoa flavonoids may have a sparing effect on vitamin E and other fat-soluble antioxidants within LDL because of their surface location. It was shown in vitro that flavonoids, especially catechins, recycle α-tocopherol by donating a hydrogen atom to the α-tocopherol radical (7). This would maintain the α-tocopherol (and probably other endogenous antioxidants) concentration in LDL for a longer time and delay the onset of

TABLE 4
Effect of dietary treatment on the susceptibility of LDL to oxidation[1]

	AAD (n = 23)	CP/DC diet (n = 23)
Lag time (min)	50.6 ± 1.9	54.5 ± 2.2[2]
Rate of oxidation		
(nmol dienes·min^{-1}·mg LDL $protein^{-1}$)	38.1 ± 1.2	35.6 ± 1.7
Conjugate dienes (nmol/mg LDL protein)	539.6 ± 14.4	531.7 ± 10.6

[1] $\overline{x}$ ± SE. AAD, average American diet; CP/DC, AAD plus 22 g cocoa powder and 16 g dark chocolate.
[2] Significantly different from the AAD, $P = 0.01$.
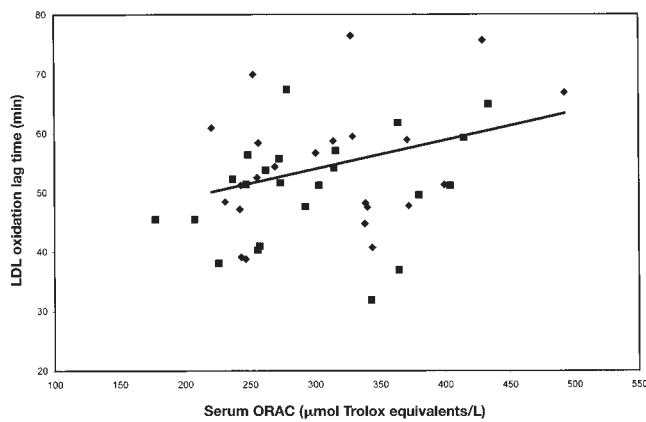
FIGURE 3. Correlation between serum oxygen radical absorbance capacity (ORAC) and LDL oxidation lag time ($r = 0.317$, $P = 0.032$) after consumption of the average American diet (■) and of the average American diet plus 22 g cocoa powder and 16 g dark chocolate (◆).

TABLE 5
Effect of dietary treatment on urinary thromboxane B_2 (TXB_2) and 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) excretion[1]

	AAD (n = 23)	CP/DC diet (n = 23)
TXB_2 (ng/24 h)	441.0 ± 50.7	393.0 ± 39.1
6-keto-$PGF_{1\alpha}$ (ng/24 h)	698.0 ± 59.6	670.7 ± 94.4
6-keto-$PGF_{1\alpha}$:TXB_2	1.9 ± 0.1	1.8 ± 0.1

[1]$\bar{x}$ ± SE. AAD, average American diet; CP/DC, AAD plus 22 g cocoa powder and 16 g dark chocolate.

lipid peroxidation. In addition, repeated exposure of LDL particles to flavonoids, including those from cocoa and chocolate, over a long time (eg, 4 wk), as was done in the present study, may make LDLs less susceptible to oxidative stress. Although not measured, it could be that the effects noted would be greater postprandially, when the circulating concentrations of bioactive compounds or their metabolites would be higher.

Besides entering or binding to LDL particles, cocoa flavonoids may also reduce oxidation-mediated events in the hydrophilic environment of LDL particles or at a cellular level, such as in the arterial wall. The fact that we observed a positive correlation between LDL oxidation lag time and serum antioxidant capacity suggests that as serum antioxidant concentrations increase, the susceptibility of LDL to oxidation decreases. This finding suggests that a measure of ORAC may be a marker, albeit weak, of LDL oxidizability.

The increase in antioxidant capacity was mainly attributed to the nonprotein antioxidants in serum. Our results are consistent with the finding of Wang et al (10) that an increase in plasma antioxidant capacity was associated with an increase in the consumption of procyanidin-rich chocolate in humans. In addition, it was reported that the procyanidin content of the cocoa samples was linearly correlated with the antioxidant potential as measured by ORAC (14). Moreover, a recent study of tea showed that (−)-epicatechin and (+)-catechin had an antioxidant capacity of 2.4 and 2.5 μmol Trolox equivalents per μmol (8.1 and 8.6 μmol/mg), respectively, much higher than common nutrient antioxidants such as vitamins C or E (29). Similar increases in antioxidant capacity were also found after ingestion of alcohol-free red wine and paralleled the increase in plasma phenolic compounds (30). It has been hypothesized that dietary consumption of tea in excess of 3500 μmol Trolox equivalents of antioxidant activity is necessary to have an effect on antioxidant status in vivo (29). The ORAC after consumption of the CP-DC diet in our study exceeded this value, thus providing additional support for our findings.

The finding that the ratios of total to HDL cholesterol and of LDL to HDL cholesterol were not significantly different between the 2 experimental diets indicates that the CP-DC diet had neutral effects on lipids and lipoproteins. This finding agrees with the results of previous studies in our laboratory (31, 32).

Platelet–blood vessel interactions are implicated in the development of thrombosis and atherosclerosis. TXA_2 is a potent platelet aggregating substance and vasoconstrictor generated in platelets, whereas PGI_2 is its counterpart, an antiaggregatory agent and vasodilator mainly synthesized by vascular endothelial cells and smooth muscle cells. Measurement of urinary TXB_2 and 6-keto-$PGF_{1\alpha}$, stable metabolites of TXA_2 and PGI_2, respectively, is considered a noninvasive approach to quantitative endogenous TXA_2 and PGI_2 biosynthesis and to assess in vivo thrombosis tendency (33). The balance between 6-keto-$PGF_{1\alpha}$ and TXB_2 production directly influences vasoreactivity and thrombosis.

The present study found no effect of the 2 experimental diets on the urinary metabolites of platelet function. Recently, Rein et al (13) reported that cocoa consumption suppressed ADP- or epinephrine-stimulated platelet activation and platelet microparticle formation. We used a different method of analysis and the timing of the sample differed as well. The concentrations of the stable metabolites TXB_2 and 6-keto-$PGF_{1\alpha}$ in 24-h urine samples are indicators of overall eicosanoid synthesis in the whole body over 24 h, whereas a single plasma assessment is a measurement at one point in time of eicosanoid production in response to cocoa consumption after 2 h. In our pilot study, the plasma epicatechin concentration increased markedly and reached its peak of 36.2 nmol/L at 2 h, then declined to baseline values 24 h after consumption of the CP-DC diet. Therefore, had we measured plasma concentrations at 2 h, we may have seen a significant effect of the CP-DC diet on eicosanoid production. Moreover, because the eicosanoid response to cocoa powder and dark chocolate appeared to be acute, it will be necessary to take frequent blood samples after a meal to more accurately assess the temporal response to bioactive constituents in cocoa powder and dark chocolate.

In summary, the present study showed favorable effects of cocoa powder and dark chocolate consumption on LDL oxidation susceptibility and ORAC in serum. Consequently, these results may indicate a decreased risk of cardiovascular disease when changes in the susceptibility and extent of LDL oxidation are implicated as important causative factors. The incorporation of dark chocolate and cocoa powder into the diet is one means of effectively increasing antioxidant intake. Furthermore, the inclusion of dark chocolate and cocoa powder in a diet that is rich in other food sources of antioxidants, such as fruit, vegetables, tea, and wine, results in a high antioxidant intake and may consequently reduce the risk of cardiovascular disease. An important caveat is that chocolate be incorporated sensibly and prudently in a healthy diet that emphasizes the intakes of fruit, vegetables, whole grains, skim milk, reduced-fat dairy products, and lean meats, fish, and poultry. A balanced dietary approach that includes a wide variety of foods in the diet is preferred to total exclusion of certain foods. Nonetheless, we would be remiss in endorsing

unlimited quantities of chocolate. As we continue to learn more about individual flavonoids, we may be able to develop diet strategies that result in the most potent antioxidant effects in vivo and thereby further reduce the risk of chronic disease.

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