

Cocoa inhibits platelet activation and function^{1,2}

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ABSTRACT

Background: Epidemiologic studies have shown inverse associations between dietary polyphenols and mortality from coronary heart disease. However, the basis for this protective association is uncertain. Food polyphenols reportedly have antioxidant properties and decrease platelet function in vitro.

Objective: This study sought to evaluate whether consumption of a polyphenol-rich cocoa beverage modulates human platelet activation and primary hemostasis.

Design: Peripheral blood was obtained from 30 healthy subjects before and 2 and 6 h after ingestion of a cocoa beverage ($n = 10$), a caffeine-containing control beverage ($n = 10$), or water ($n = 10$). Platelet activation was measured in terms of expression of activation-dependent platelet antigens and platelet microparticle formation by using fluorescent-labeled monoclonal antibodies and flow cytometry. Primary platelet-related hemostasis was measured with a platelet function analyzer.

Results: Ex vivo epinephrine- or ADP-stimulated expression of the fibrinogen-binding conformation of glycoprotein IIb-IIIa was lower 2 and 6 h after consumption of cocoa than before consumption. Cocoa consumption also decreased ADP-stimulated P-selectin expression. In contrast, epinephrine-induced platelet glycoprotein IIb-IIIa expression increased after consumption of the caffeine-containing beverage but not after water consumption. Platelet microparticle formation decreased 2 and 6 h after cocoa consumption but increased after caffeine and water consumption. Primary hemostasis in response to epinephrine in vitro was inhibited 6 h after cocoa consumption. The caffeine-containing beverage inhibited ADP-induced primary hemostasis 2 and 6 h after consumption.

Conclusions: Cocoa consumption suppressed ADP- or epinephrine-stimulated platelet activation and platelet microparticle formation. Cocoa consumption had an aspirin-like effect on primary hemostasis. *Am J Clin Nutr* 2000;72:30–5.

KEY WORDS Cocoa beverage, chocolate, platelet activation, platelet glycoprotein, glycoprotein IIb-IIIa complex, P-selectin, CD62P, whole-blood flow cytometry, platelet function tests, blood coagulation, hemostasis, cardiovascular disease, dietary polyphenols

INTRODUCTION

A strong inverse association between deaths from ischemic heart disease and moderate alcohol consumption was recognized

more than 2 decades ago (1) and was largely attributed to red wine consumption (2). The remarkable negative correlation between red wine intake and deaths from coronary heart disease described in segments of the French population (3) stimulated interest in the antioxidant effects (4) and health implications (5) of flavonoid-rich foods. Regular consumption of fruit, vegetables, and tea was associated with lower incidences of coronary heart disease and stroke (6–8).

Blood platelets play a major role in coronary artery disease (9, 10). Platelets are found at the sites of early atherosclerotic lesions. When activated, platelets secrete potent mitogenic factors such as platelet-derived growth factor, transforming growth factor β , and epidermal growth factor, which lead to smooth muscle proliferation and progression of atherosclerotic lesions. Enhanced platelet reactivity and spontaneous platelet aggregates were associated with a higher risk of recurrent coronary artery disease (11). Physiologic antiplatelet metabolites, such as nitric oxide, activate platelet guanylate cyclase and elevate cyclic guanosine 3',5'-monophosphate, thereby reducing fibrinogen binding to the glycoprotein IIb-IIIa receptor through inhibition of agonist-mediated platelet calcium flux (12).

Several interventions showed a decreased risk of cardiovascular disease with therapeutic doses of aspirin (13, 14) and antioxidant supplements (15). Therapy with antiplatelet agents such as aspirin and clopidogrel significantly decreases the incidence of primary and secondary coronary events (16, 17). Antibodies and peptides that block the fibrinogen binding to activated platelet glycoprotein IIb-IIIa have improved the results of coronary revascularization procedures (18). Activation-dependent platelet antigens also indicate changes in platelet function after physical exercise (19), physiologic challenges (20), and dietary intervention (21) and in sickle cell pathology (22).

We performed a study to test whether cocoa consumption alters platelet activation in healthy human subjects. Platelet activation was measured in terms of expression of the fibrinogen-binding

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conformation of platelet glycoprotein IIb-IIIa complex and expression of the granular membrane protein CD62P on the platelet surface. Formation of hemostatically active platelet microparticles and platelet-related hemostasis times were measured to evaluate the effects of cocoa consumption on coagulation.

SUBJECTS AND METHODS

Subjects

Thirty healthy, nonsmoking adults with no history of heart disease or hemostatic disorders participated in the study. There were 10 subjects (4 men and 6 women) in each of 3 groups: the test subjects (aged 24–49 y) consumed a cocoa beverage, a group of control subjects (aged 26–50 y) consumed a caffeine-containing beverage, and another group of control subjects (aged 24–50 y) consumed water. All women were premenopausal and were not taking estrogen. All participants gave their written, informed consent before participating in the study, which was approved by the University of California, Davis, Human Subjects Review Committee.

Participants were instructed to abstain from nonsteroidal anti-inflammatory medication for ≥ 4 d, from alcoholic beverages for ≥ 2 d, and from caffeine- or theobromine-containing foods for ≥ 24 h before the blood tests. Subject compliance and medical history were evaluated with a questionnaire. One female subject was not present for the blood drawing 6 h after she consumed the caffeine-containing beverage.

Study design

Venous blood was obtained from each subject between 0800 and 1000. Blood was drawn into two 5-mL evacuated tubes containing 0.5 mL 3.2% buffered sodium citrate solution (Becton Dickinson Biosciences, Franklin Lakes, NJ). Specimens obtained as the result of a traumatic venipuncture and those with obvious clots were excluded from analysis. The test subjects then drank 300 mL of a beverage containing 18.75 g procyanidin-enriched cocoa powder (Cocoapro; Mars Inc, Hackettstown, NJ) and 12.5 g sucrose mixed with distilled water; the beverage provided 897 mg total epicatechin and oligomeric procyanidins (23), 17 mg caffeine, and 285 mg theobromine. The cocoa dose was selected to provide ≈ 1.5 times the amount found in a typical 240-mL (8-oz) serving of hot cocoa beverage. Most recipes we surveyed recommended 10–12 g (1 tbsp) unsweetened cocoa powder plus 28 g (2 tbsp) sugar in 240 mL milk. In this study we used water in place of milk to eliminate potential interactions between the polyphenolic compounds and milk proteins. Control subjects drank either a beverage containing 17 mg caffeine and 12.5 g sucrose or plain water. Additional blood samples were obtained 2 and 6 h after consumption of the beverages.

Platelet activation studies

Within 10 min after blood was drawn, the whole blood was incubated in polystyrene tubes for 5 min at room temperature with HEPES buffer (pH 7.4, unstimulated control), ADP (final concentration, 20 or 100 $\mu\text{mol/L}$), or epinephrine (final concentration, 20 $\mu\text{mol/L}$; BioData, Horsham, PA) in the presence or absence of the peptide Arg-Gly-Asp-Ser (Sigma, St Louis). After 5 min, the samples were suspended in 1 mL HEPES buffer. Then 100- μL aliquots of sample were transferred to tubes containing saturating concentrations (20 μL) of the fol-

lowing fluorescent-labeled monoclonal antibodies: PAC1-fluorescein isothiocyanate (FITC), anti-CD62P-phycoerythrin, and anti-CD42a-PerCP. PAC1 recognizes the activated conformation of the fibrinogen-binding receptor glycoprotein IIb-IIIa and anti-CD62P recognizes CD62P, present on the surface of activated platelets. Anti-CD42a recognizes glycoprotein Ib-IX, present on the membrane surface of both activated and resting platelets. Mouse immunoglobulin (Ig) G₁ FITC and mouse IgG₁ phycoerythrin were used as isotype controls. The peptide Arg-Gly-Asp-Ser was used to block binding of the PAC1 antibody to platelets and thus set the negative control marker on the flow cytometer. Antibodies and isotype controls were purchased from Becton Dickinson Immunocytometry Systems Inc, San Jose, CA. Whole blood samples in the presence and absence of the agonists ADP and epinephrine were incubated with monoclonal antibodies or isotype controls for 20 min in the dark at room temperature. The samples were then fixed in filtered 1% paraformaldehyde (pH 7.2) and stored in the dark at 2–8°C. All samples were analyzed within 48 h on a FACScan flow cytometer with LYSYS II software (both from Becton Dickinson Biosciences). The performance of the flow cytometer was verified with 1-, 2-, and 10- μm calibration beads (Becton Dickinson Immunocytometry Systems Inc and Flow Cytometry Systems, Research Triangle Park, NC). Twenty thousand events were collected in list mode with all light-scatter and fluorescence parameters in logarithmic mode. Platelets were gated on the basis of light scatter and CD42a expression. Activated platelets were defined as the percentage of CD42a-positive events coexpressing the activated conformation of glycoprotein IIb-IIIa or CD62P. Platelet microparticles were defined as the percentage of CD42a-positive events < 2 μm in size.

One blood sample drawn at each of the 3 study time points was analyzed within 4 h with a platelet function analyzer (PFA-100; Dade Behring International, Miami) according to the manufacturer's instructions. The analyzer is designed to measure collagen-ADP-stimulated or collagen-epinephrine-stimulated platelet function under shear conditions simulating those that exist in a small blood vessel (24, 25). Platelet function was measured as closure time in seconds, which is defined as the time required for blood to occlude an aperture in the test cartridge membrane. Platelet function analysis was not performed for the water-only group because the platelet analyzer was not available and the samples drawn for platelet studies must be tested immediately.

Statistical analysis

Data from each treatment or control group were analyzed for differences by using Friedman's repeated-measures analysis of variance (ANOVA) on ranks (SIGMASTAT for WINDOWS; version 2.0; SPSS, Richmond, CA). Tukey's all-pairwise-comparison test was used to identify differences between the baseline and 2- and 6-h postconsumption results. $P < 0.05$ was considered statistically significant.

RESULTS

Platelet activation antigen expression in response to cocoa consumption

The effect of cocoa beverage consumption on platelet surface expression of activated glycoprotein IIb-IIIa, with and without stimulation by weak agonists, is shown in **Figure 1**; activated



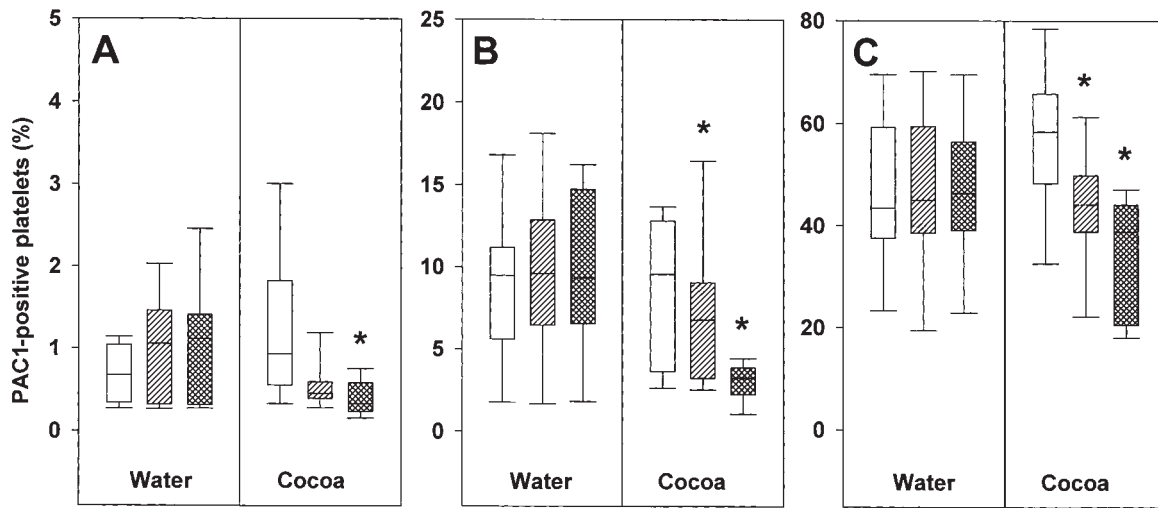


FIGURE 1. Platelet-activation marker expression presented as Tukey box plots at baseline (□) and 2 h (▨) and 6 h (▩) after consumption of water or the cocoa beverage. Percentage of platelets expressing activated glycoprotein IIb-IIIa (PAC1-positive platelets) under the following conditions: A, without stimulation; B, after stimulation with epinephrine (20 $\mu\text{mol/L}$); and C, after stimulation with ADP (20 $\mu\text{mol/L}$); $n = 10$ in each group. Each box shows the 25–75th percentiles, with the median indicated by the horizontal line inside the box. The lines outside the box show the 10th and 90th percentiles. *Significantly different from baseline within each treatment group, $P < 0.05$ (Friedman's repeated-measures ANOVA on ranks and Tukey's all-pairwise multiple-comparison test).

glycoprotein IIb-IIIa is expressed on the surface of activated platelets. Cocoa consumption suppressed unstimulated glycoprotein IIb-IIIa expression ($P = 0.035$; Figure 1A). Cocoa consumption also suppressed ex vivo epinephrine-induced activated glycoprotein IIb-IIIa (fibrinogen-binding conformation) expression 2 and 6 h after ingestion ($P = 0.008$; Figure 1B). The median percentages of platelets expressing activated glycoprotein IIb-IIIa were 0.9%, 0.5%, and 0.3% without stimulation and were 9.6%, 6.8%, and 3.3% in response to epinephrine at baseline (before consumption) and 2 and 6 h postconsumption, respectively. No change was observed in the control group of subjects who drank water. In contrast, there was an increase in epinephrine-stimulated activated glycoprotein IIb-IIIa expression in the control group of subjects who drank a caffeine-containing beverage ($P = 0.048$), with medians of 5.3% (range: 1.7–10.2%), 6.5% (range: 2.3–11.1%), and 7.5% (range: 2.6–10.3%) at baseline and 2 and 6 h postconsumption, respectively.

Similarly, cocoa consumption reduced ADP-induced (20 $\mu\text{mol ADP/L}$) activated glycoprotein IIb-IIIa expression on platelets 2 and 6 h after consumption ($P < 0.001$; Figure 1C); median values were 58.5%, 44.2%, and 38.8% at baseline and 2 and 6 h postconsumption, respectively. There was a trend that suggested decreased activated glycoprotein IIb-IIIa expression on platelets after cocoa consumption when activation was induced by 100 $\mu\text{mol ADP/L}$ ($P = 0.067$); median values were 76.5%, 68.7%, and 57.6% at baseline and 2 and 6 h postconsumption, respectively. There was no significant change in ADP-induced activated glycoprotein IIb-IIIa expression after consumption of water or the caffeine-containing beverage.

The effect of cocoa beverage consumption on platelet surface expression of activated CD62P with and without stimulation by weak agonists is shown in Figure 2; CD62P is expressed on the surface of activated platelets. There was a trend toward decreased CD62P expression after cocoa consumption in uninduced platelets ($P = 0.053$; Figure 2A); median values were 1.6%,

1.9%, and 0.7% at baseline and 2 and 6 h postconsumption, respectively. Cocoa consumption reduced ADP-induced CD62P expression (20 $\mu\text{mol ADP/L}$; Figure 2C) but not epinephrine-induced CD62P expression (20 $\mu\text{mol epinephrine/L}$; Figure 2B) 2 and 6 h postconsumption ($P = 0.007$); median values for the former were 45.2%, 38.9%, and 36.4% at baseline and 2 and 6 h postconsumption, respectively. Cocoa consumption also decreased ADP-induced (100 $\mu\text{mol ADP/L}$) CD62P expression 6 h after consumption ($P = 0.025$), with median values of 56.1% (range: 42.4–73.2%), 54.7% (range: 33.0–76.8%), and 41.8% (range: 27.8–74.2%) at baseline and 2 and 6 h postconsumption, respectively. There was no evidence of platelet stimulation or inhibition in the groups that consumed water or the caffeine-containing beverage.

Platelet microparticles

Platelet microparticles are hemostatically active, phospholipid-rich microvesicles that are formed during physiologic platelet activation. The number of microparticles detected by flow cytometry after consumption of the cocoa beverage decreased from baseline to 2 h postconsumption and was further reduced 6 h postconsumption ($P < 0.001$; Table 1). In contrast, the number of microparticles was higher 2 and 6 h after consumption of water ($P = 0.003$) and 6 h after consumption of the caffeine-containing beverage ($P = 0.006$) than at baseline.

Platelet function

Six hours after consumption of the cocoa beverage, collagen-epinephrine-induced closure time was prolonged ($P = 0.002$; Table 2), indicating delayed platelet-related primary hemostasis. Collagen-ADP-induced closure time was prolonged 2 and 6 h after consumption of the caffeine-containing beverage ($P = 0.001$). There was no change in the collagen-epinephrine-induced closure time after caffeine ingestion and no change in the collagen-ADP-induced closure time after cocoa ingestion.



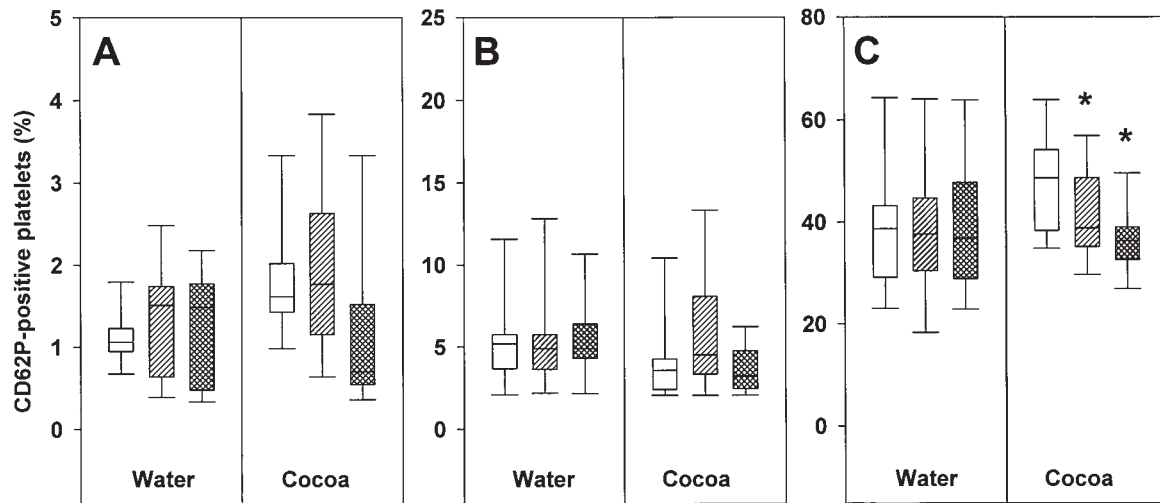


FIGURE 2. Platelet-activation marker expression presented as Tukey box plots at baseline (□) and 2 h (▨) and 6 h (▩) after consumption of water or a cocoa beverage. Percentage of platelets expressing CD62P under the following conditions: A, without stimulation; B, after stimulation with epinephrine (20 $\mu\text{mol/L}$); and C, after stimulation with ADP (20 $\mu\text{mol/L}$); $n = 10$ in each group. Each box shows the 25–75th percentiles, with the median indicated by the horizontal line inside each box. The lines outside the boxes show the 10th and 90th percentiles. *Significantly different from baseline within the treatment group, $P < 0.05$ (Friedman's repeated-measures ANOVA on ranks and Tukey's all-pairwise multiple-comparison test).

DISCUSSION

The putative health benefits of chocolate, a flavonoid-rich food, have gained attention in the past 5 y (26–28). In our study, 3 lines of evidence showed that consumption of chocolate in the form of a cocoa beverage modifies platelet function in humans. First, platelet activation, as measured by platelet-activation marker expression in response to weak agonists *in vitro*, was lower after cocoa consumption. Second, platelet microparticle formation was reduced after cocoa consumption. Third, consumption of cocoa caused an aspirin-like effect on platelet function, as measured in terms of platelet-related primary hemostasis.

We hypothesize that flavonoids in cocoa account for at least some of the platelet-inhibitory effects observed in this study. It is unlikely that the sustained effect of cocoa on markers of platelet activation is attributable to the caffeine fraction of the cocoa beverage, because the caffeine-containing beverage stimulated rather than decreased epinephrine-induced activated glycoprotein IIb-IIIa expression and microparticle formation. Indeed, flavonoids decrease platelet aggregation *in vitro* (29–31). The considerable amounts of epicatechin and oligomeric procyanidins in the cocoa beverage may have affected the platelet response. Consistent with this theory, Folts et al (32) and Osman et al (33) suggested that

components of red wine and grape products inhibit platelet activation after consumption by humans and experimental animals.

In the process of physiologic activation, hemostatically active platelet membrane vesicles, or platelet microparticles, are formed (34). Modulation of the platelet microparticle concentration *in vivo* is associated with platelet pathology or syndromes of platelet activation (35). The functional significance of these vesicles has remained vague, although there is evidence to suggest procoagulant properties (35, 36), possibly because of their ability to amplify platelet activation (37). Our finding of decreased numbers of microparticles in unstimulated whole blood after cocoa consumption supports the suppressive effects of cocoa components on platelet activation.

Further support for the prolonged effects of cocoa consumption on platelet function came from the increased closure times as measured with a collagen-epinephrine test system and platelet function analysis. The collagen-epinephrine system detects qualitative platelet abnormalities induced by pharmacologic agents such as aspirin and other nonsteroidal antiinflammatory agents (24, 25). Closure time by the collagen-epinephrine system was prolonged 6 h after consumption of the cocoa beverage, suggesting aspirin-like effects on hemostasis. Regular consumption of low doses of aspirin may decrease the risk of myocardial infarction

TABLE 1

Microparticle formation after consumption of a cocoa beverage and control beverages¹

	Control beverages		Cocoa beverage
	Water	Caffeine-containing beverage	
	%		%
Baseline	0.7 (0.3–1.2)	1.0 (0.6–1.8)	1.9 (1.0–5.0)
2 h postconsumption	1.2 (0.7–1.6) ²	1.1 (0.6–2.0)	1.0 (0.7–1.4) ²
6 h postconsumption	1.3 (0.8–2.1) ²	1.5 (1.2–2.3) ²	0.6 (0.4–1.1) ²

¹Percentage microparticles of total CD42-positive events. Median; range in parentheses; $n = 10$ per group.

²Significantly different from baseline, $P < 0.05$ (Friedman's repeated-measures ANOVA on ranks and Tukey's all-pairwise multiple-comparison test).

TABLE 2Platelet function analysis: primary platelet-related hemostasis closure time, induced by collagen-epinephrine or collagen-ADP, in seconds¹

	Collagen-epinephrine		Collagen-ADP	
	Caffeine beverage	Cocoa beverage	Caffeine beverage	Cocoa beverage
Baseline	104 (61–180)	125 (80–180)	77 (52–95)	83 (60–133)
2 h postconsumption	113 (81–141)	135 (82–194)	78 (58–99)	96 (65–132)
6 h postconsumption	114 (79–143)	164 (101–262) ²	82 (67–114) ²	94 (66–116)


¹Median; range in parentheses; *n* = 10 per group.²Significantly different from baseline, *P* < 0.05 (Friedman's repeated-measures ANOVA on ranks and Tukey's all-pairwise multiple-comparison test).

and stroke (38, 39). Regular intake of active cocoa components may contribute to a lower thrombotic risk. Platelet-suppressive effects of aspirin, an inhibitor of arachidonic acid metabolism and thromboxane A₂ (17), may be complemented by cocoa consumption, which appears to interact with platelet agonist receptors or directly with membrane-activation proteins.

The flavonoids in cocoa and chocolate are predominately catechins and condensed procyanidins (40). Recovery of dietary catechins and other flavonoids after absorption in humans has been hindered by the rapid metabolism of these flavonoids through methyl-, sulfate-, and glucuronide-conjugate formation (41, 42). Epicatechin, which constitutes 30–40% of the total flavonoids in cocoa, is absorbed and attains peak plasma concentrations of ≈1 μmol/L in human subjects 2–3 h after ingestion of chocolate (43). In humans, flavonoids derived from tea and onions can reach concentrations of ≈1 μmol/L, and these flavonoids have pharmacokinetic effects similar to those of cocoa-derived epicatechin (44, 45).

In addition to its flavonoid content, cocoa is a rich source of the methylxanthine theobromine, which is taken up into the plasma and is cleared efficiently (46). Methylxanthines inhibit platelet aggregation (47), although controversy remains about their effects on hemostatic variables (48). Caffeine and related methylxanthines are weak competitive antagonists of several adenosine receptors (49, 50) and may contribute to the observed antithrombotic effects.

Fruit from the chocolate tree was harvested as early as 3 millennia ago by the Olmec peoples of east coastal Mexico (51). Later, cacao was significant to the Mayan and Aztec cultures in rituals as a status symbol and as currency (52). The therapeutic potential of cocoa preparations was recognized by the Mayan and Aztec peoples long before the Spanish arrived in Mexico (53, 54). Dietary and healing roles of cacao continued to be praised in early European botanical and medical texts (55). Recent research supports potential health benefits from cocoa. The complex flavonoid mixture of cocoa has immunoregulatory effects (56) and provides potent antioxidant activity (28, 40), which has been shown to suppress oxidation of low-density lipoproteins both *in vitro* (26) and after chocolate consumption (27).

In this study, we showed that platelet activation and reactivity to physiologic agonists were inhibited 2 and 6 h after the ingestion of a cocoa beverage by healthy subjects without a history of cardiovascular disease. The highly enriched flavonoid composition of the cocoa used in this study may have contributed to the inhibition of platelet activity after its consumption. The timing of the effects observed is consistent with the kinetics of epicatechin absorption from chocolate. Thus, suppression of platelet function is one mechanism by which cocoa and chocolate could offer cardiovascular benefits. 

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