

Protective Effects of Flavanol-Rich Dark Chocolate on Endothelial Function and Wave Reflection During Acute Hyperglycemia

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Abstract—Nitric oxide plays a pivotal role in regulating vascular tone. Different studies show endothelial function is impaired during hyperglycemia. Dark chocolate increases flow-mediated dilation in healthy and hypertensive subjects with and without glucose intolerance; however, the effect of pretreatment with dark chocolate on endothelial function and other vascular responses to hyperglycemia has not been examined. Therefore, we aimed to investigate the effects of flavanol-rich dark chocolate administration on (1) flow-mediated dilation and wave reflections; (2) blood pressure, endothelin-1 and oxidative stress, before and after oral glucose tolerance test (OGTT). Twelve healthy volunteers (5 males, 28.2 ± 2.7 years) randomly received either 100 g/d dark chocolate or flavanol-free white chocolate for 3 days. After 7 days washout period, volunteers were switched to the other treatment. Flow-mediated dilation, stiffness index, reflection index, peak-to-peak time, blood pressure, endothelin-1 and 8-iso-PGF_{2 α} were evaluated after each treatment phase and OGTT. Compared with white chocolate, dark chocolate ingestion improved flow-mediated dilation ($P=0.03$), wave reflections, endothelin-1 and 8-iso-PGF_{2 α} ($P<0.05$). After white chocolate ingestion, flow-mediated dilation was reduced after OGTT from 7.88 ± 0.68 to 6.07 ± 0.76 ($P=0.027$), 6.74 ± 0.51 ($P=0.046$) at 1 and 2 h after the glucose load, respectively. Similarly, after white chocolate but not after dark chocolate, wave reflections, blood pressure, and endothelin-1 and 8-iso-PGF_{2 α} increased after OGTT. OGTT causes acute, transient impairment of endothelial function and oxidative stress, which is attenuated by flavanol-rich dark chocolate. These results suggest cocoa flavanols may contribute to vascular health by reducing the postprandial impairment of arterial function associated with the pathogenesis of atherosclerosis. (*Hypertension*. 2012;60:827-832.) • [Online Data Supplement](#)

Key Words: flavonoids ■ cocoa ■ nitric oxide ■ endothelial function ■ wave reflections ■ oxidative stress

Diets high in readily absorbable carbohydrates are associated with an increased risk for type 2 diabetes mellitus (T2DM).¹ Atherosclerotic pathology is a common sequela to T2DM, with macrovascular and microvascular diseases being among the leading causes of morbidity and mortality in patients with T2DM.² Even after adjustment for conventional risk factors, individuals with T2DM exhibit a 2- to 4-fold increased risk of mortality in comparison with individuals without diabetes.³ Thus, several investigations have been directed to the potential adverse effects of hyperglycemia on the arterial wall. Observational data reveal high blood glucose concentrations are a risk factor for mortality, even in nondiabetic individuals.⁴ Postprandial hyperglycemia has also been implicated in the development of cardiovascular disease. Elevated postprandial glucose may have a direct toxic effect on the vascular endothelium mediated by oxidative stress, independent of other cardio-

vascular risk factors, such as hyperlipidemia.^{2,5} A high-carbohydrate meal has been described to increase oxidative stress and favor proinflammatory effects.⁵

Several in vivo studies have found impaired nitric oxide (NO)-mediated endothelial function during acute episodes of hyperglycemia.^{6–10} NO is a key endothelium-derived relaxing factor and plays a pivotal role in the maintenance of basal vascular tone and vascular responses to dilative stimuli.^{11–13} NO acts to negate the actions of endothelium-derived contracting factors such as angiotensin II and endothelin-1 (ET-1), as well as inhibit platelet aggregability^{11–13} and endothelial activation.^{11–13} Therefore, reduced NO bioavailability may contribute to the onset and progression of endothelial dysfunction, including impaired vasodilative and increased adhesive properties, leading to atherogenesis.^{11–13} Additionally, endothelium-derived NO may act to regulate arterial stiffness and wave reflection.

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tions.^{13,14} Reversal of endothelial dysfunction has been suggested to slow the rate of atherogenesis and improve the prognosis of patients with cardiovascular disease.^{13–15}

Consumption of dietary flavonoids, including intake from flavonoid-rich foods such as tea, wine, and cocoa has been linked to reduced endothelial dysfunction and increased resistance of low-density lipoprotein cholesterol to oxidation.^{15,16} Flavonoids appear to affect endothelial function by serving as antioxidants and as modulators of signal transduction pathways.^{15,16} We observed that flavanol-rich dark chocolate (DC) ingestion improves NO-dependent flow-mediated dilation (FMD) and insulin sensitivity and decreases blood pressure (BP) in healthy¹⁷ and hypertensive subjects with¹⁸ and without¹⁹ glucose intolerance. Similarly, in patients with T2DM, Balzer et al²⁰ have reported that acute as well as thrice-daily consumption for 30 days of flavanol-containing cocoa increased FMD in a dose-dependent manner; however, the effect of pretreatment with flavonoids on the endothelial response to hyperglycemia has not yet been tested. Therefore, we hypothesized DC could be able to protect arterial function from a glucose load and examined the effects of DC and flavanol-free white chocolate (WC) administration for 3 days on endothelial function and wave reflections, as well as BP, circulating ET-1 and total isoprostanes (8-iso-PGF_{2α}) before and after an oral glucose tolerance test (OGTT) in healthy subjects.

Methods

Subjects

Twelve healthy volunteers (5 males, mean age 28.2±2.7 years) were recruited from staff at the San Salvatore Regional Hospital and University of L'Aquila. Individuals were excluded if they had an acute or chronic disease, including any type of metabolic abnormality and/or major cardiovascular risk factor. Habitual smokers and use of any prescribed medication and/or dietary supplement within 2 weeks of entering the study were excluded. To further limit potential confounding factors, individuals were excluded if they reported daily intense sports activities (>10 hours/wk), changes of >10% body weight within 6 months of entering the study, current dietary treatment regimen, and/or participation in another clinical study within 3 months of entering this trial. The study was approved by the responsible Ethics Committee, and all participants gave written informed consent.

Study Design

After a 7-day, cocoa-free, run-in phase, the volunteers were randomly assigned to receive either 100-g DC bars (Cioccolato Bonajuto, Antica Dolceria Bonajuto) or 100-g WC bars (Milka, Kraft Foods) in an isocaloric fashion over a period of 3 days. FMD; office systolic (SBP) and diastolic (DBP) BP; pulse contour analysis for measuring stiffness index (SI), reflection index (RI), and peak-to-peak time (PPT); serum ET-1 and 8-iso-PGF_{2α} were evaluated before and after OGTT following each intervention phase. The homeostasis model assessment of insulin resistance, quantitative insulin sensitivity check index, and insulin sensitivity index were calculated from OGTT results.

After evaluation of exclusion/inclusion criteria, volunteers were carefully instructed to maintain their usual diet except to refrain from flavonoid-rich foods and beverages (a detailed list was given to each participant), including wine, as well as all other alcoholic beverages. All participants were encouraged to continue with their usual physical activity throughout the study period. The subjects were instructed to consume their chocolate bars in the morning and not in the afternoon or at dinner. They consumed the first dose at breakfast on day 1 and the last dose on the day before the visit. Analysis of the DC using high-performance liquid chromatography quantified 447 mg of epicatechin, 59 mg of catechin, and 14 mg of quercetin; the same method showed WC contained only trace amounts of polyphenols (0.04 mg of catechin). DC and WC contained similar amounts of energy and macronutrients (online-only Data Supplement Table S1).

At the end of the first phase of intervention, patients entered a second 7-day, chocolate-free phase and then were crossed over to the other treatment. Chocolate doses for each subject were rolled in aluminum foil and administered in dated, sequentially numbered, nontransparent boxes that were not labeled regarding content. All the involved physicians and staff were blinded to the group assignment. Patients did not receive any information regarding the chocolate and were instructed not to disclose their assigned intervention group to investigators until the study completion. To avoid changes in body weight during the intervention, participants were carefully instructed on how to substitute the chocolate bars with foods of similar energy and macronutrient composition. Patients were continuously and carefully followed with tailored personal diet counseling provided throughout the study by trained dietitians and physicians. Please see the online-only Data Supplement for the additional and detailed methods section.

Results

According to the inclusion/exclusion criteria, participants presented with normal ranges for waist circumference, lipid profiles, and glucose-insulin metabolism. The baseline clinical characteristics of the 12 study subjects are shown in the online-only Data Supplement Table S2. All subjects completed both parts of the study.

Endothelial Function

Compared with the WC intervention period, DC increased baseline FMD (8.51±0.69 versus 7.88±0.68%; *P*=0.03) (Figure 1, A). After the WC phase, FMD fell from 7.88±0.68 at baseline to 6.07±0.76 (*P*=0.027), 6.74±0.51 (*P*=0.046), and 7.16±0.97% (*P*=0.07) at 1, 2, and 3 hours after the glucose load, respectively (Figure 1B). In contrast, after DC ingestion, FMD was not significantly different in response to the OGTT (from 8.51±0.69 to 8.25±0.92, n.s.; 7.96±0.84, n.s.; and 8.48±1.4%, n.s. at 1, 2, and 3 hours after the glucose load, respectively (Figure 1B). Compared with WC, DC administration protected endothelial function (*P*=0.0007 for treatment) by preventing the attenuation of FMD induced by the glucose load (Figure 1B).

Wave Reflections

Compared with the WC phase, DC ingestion decreased baseline SI (Figure 2A) and RI values (59.3±12.4 versus 50.4±8%; *P*=0.04) and increased PPT (265.5±35.1 versus 295.6±36.2 ms; *P*=0.05). After WC, SI increased after the glucose load (Figure 2B). After DC, SI increased only after 60 minutes from glucose load. Similarly, after WC, RI increased after 30, 60, and 90 minutes, and PPT decreased after 30, 60, 90, 120, and 180 minutes from the glucose load, while, after DC, RI increased after 90 minutes, and PPT decreased after 60, 90, and 180 minutes (online-only Data Supplement Figures S1A and S1B, respectively). The DC attenuated the impairment of these measures of wave reflection induced by acute hyperglycemia (SI: *P*=0.0089 for treatment; RI: *P*=0.032 for treatment; PPT: *P*<0.0001 for treatment) (Figure 2 and online-only Data Supplement Figure S1).

Blood Pressure

Baseline office SBP and DBP were not statistically different for treatment between the DC and WC intervention phases (SBP: 110.6±9.3 versus 111.2±6.7 mm Hg; DBP: 70.0±7.4

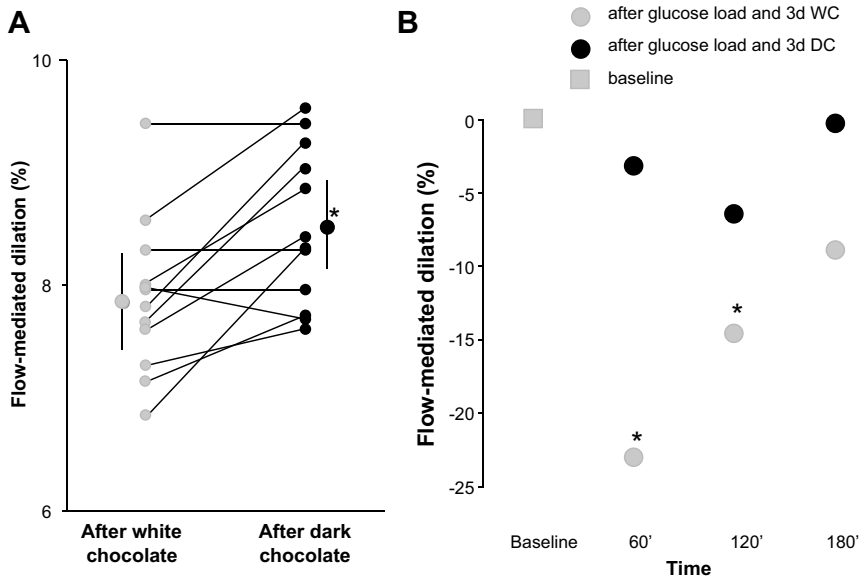


Figure 1. Effect of 3 days of white chocolate (WC) and dark chocolate (DC) administration on baseline flow-mediated dilation (FMD) (A) and during OGTT (B). Data are presented as mean \pm SD in (A) and as % difference respect to baseline levels in (B). Vertical lines indicate SD and asterisks (*) indicate significant differences with respect to the WC phase (A) and baseline (square) values (B). Differences are considered significant when $P < 0.05$.

versus 70.8 ± 5.8 mm Hg, respectively). After the WC but not DC intake, DBP increased (from 70.0 ± 7.4 at baseline to 76.4 ± 7.1 mm Hg; $P = 0.0079$) after 30 minutes, and SBP increased (from 110.6 ± 9.3 at baseline to 118 ± 6.7 mm Hg after 30 minutes, $P < 0.0001$ to 116.3 ± 7.6 mm Hg after 60 minutes, $P = 0.0002$ and to 114.5 ± 6.2 mm Hg after 90 minutes, $P = 0.0191$ from the glucose load. DC ingestion prevented the increase in BP (SBP: < 0.0001 and DBP: $P = 0.019$ for treatment) induced by the glucose load.

Endothelin-1

Baseline serum ET-1 level was higher after the WC compared with the DC intervention phase (Figure 3A). After the WC phase, ET-1 increased from 5.9 ± 1.3 at baseline to 6.4 ± 1.7 ($P = 0.049$), 6.5 ± 1.4 ($P = 0.011$), 6.9 ± 1.5 ($P < 0.0001$), 7.2 ± 1.5 ($P < 0.0001$), and 7.3 ± 1.5 pg/mL ($P < 0.0001$) at 30, 60, 90, 120, and 180 minutes after the glucose load, respectively. In contrast, DC ingestion prevented the increase in ET-1 ($P = 0.0023$ for treatment) induced by the glucose load (Figure 3B).

Total Isoprostanes

Baseline concentrations of serum 8-iso-PGF_{2 α} were higher after the WC compared with the DC intervention phase (233.9 ± 34.2 versus 208.1 ± 22.8 pg/L; $P = 0.04$). After the WC phase, 8-iso-PGF_{2 α} increased from 233.9 ± 34.2 at baseline to 269.6 ± 42.7 ($P = 0.047$), 285.3 ± 46.0 ($P = 0.0139$) at 30 and 60 minutes after the glucose load, respectively. In contrast, DC ingestion prevented the increase of this biomarker of lipid peroxidation ($P = 0.0008$ for treatment) induced by the OGTT.

Insulin Resistance and β -Cell Function

Although a trend toward improvement was noted after DC but not WC ingestion, no significant differences were observed regarding glucose and insulin responses during the OGTT, homeostasis model assessment of insulin resistance, quantitative insulin sensitivity check index, and homeostatic model assessment % β (data not shown).

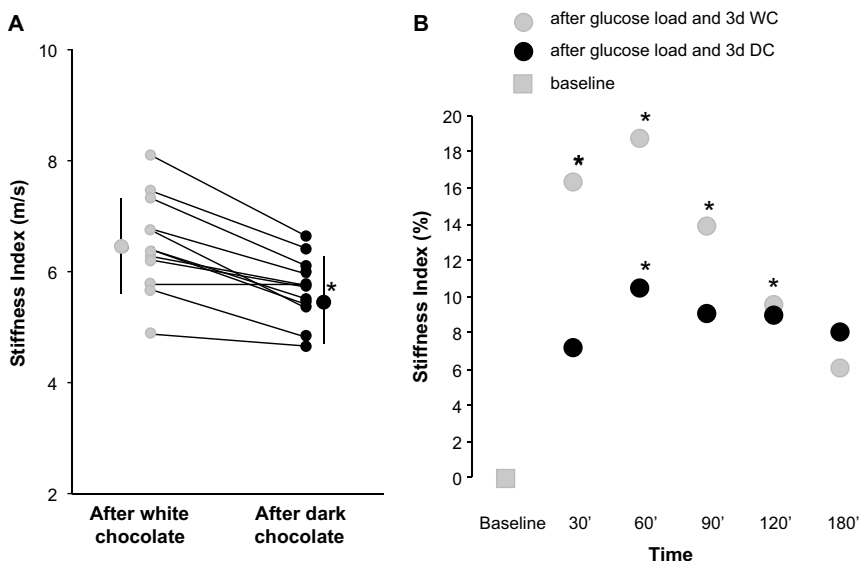


Figure 2. Effect of 3 days of white chocolate (WC) and dark chocolate (DC) administration on baseline SI (A) and after OGTT (B). Data are presented as mean \pm standard deviation (SD) in (A) and as % difference with respect to baseline levels in (B). Vertical lines indicate SD, and asterisks (*) indicate significant differences with respect to the WC phase (A) and baseline (square) values (B). Differences are considered significant when $P < 0.05$.

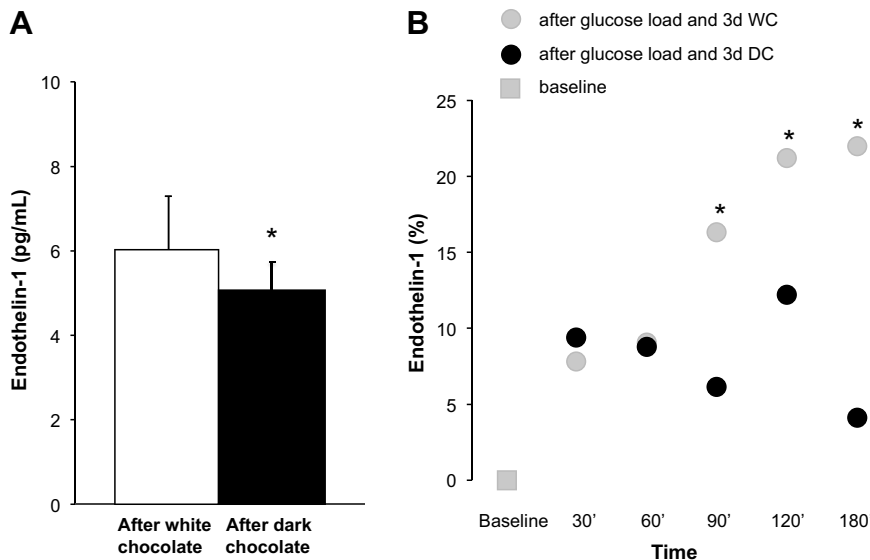


Figure 3. Effect of 3 days of white chocolate (WC) and dark chocolate (DC) administration on baseline ET-1 levels (A) and during OGTT (B). Data are presented as mean \pm standard deviation (SD) in (A) and as % difference with respect to baseline levels in (B). Vertical lines indicate SD, and asterisks (*) indicate significant differences with respect to the WC phase (A) and baseline (square) values (B). Differences are considered significant when $P < 0.05$.

Discussion

Our results show that, in healthy subjects, short-term treatment with flavanol-rich DC, compared with a dietary control of flavanol-free WC, was able to increase endothelium-dependent vasodilation and reduce wave reflections, lipid peroxidation, and ET-1 levels, also protecting from acute vascular alterations induced by a glucose load. Although we did not examine the mechanism(s) of action underlying this effect of DC, we might postulate that the cocoa flavanols in DC blunted the production or action of reactive oxygen species (ROS) induced by the transient hyperglycemia and subsequently decreased bioavailability of NO. ROS rapidly combines with NO to decrease its bioavailability and impair normal endothelial function by disrupting the balance between vasodilating and vasoconstricting factors.^{6,12,15} Further, the measurement of 8-iso-PGF_{2 α} , formed nonenzymatically through free radical catalyzed attack on esterified arachidonate, provides a reliable tool for identifying populations with enhanced rates of lipid peroxidation. Indeed, enhanced formation of 8-iso-PGF_{2 α} has been reported in association with several cardiovascular risk factors. Accordingly, it has been suggested that 8-iso-PGF_{2 α} may transduce oxidant stress-dependent platelet activation and endothelial dysfunction.²¹ Data suggest the relationship between flavonoids and other dietary antioxidants, oxidative stress, and hyperglycemia may result from reduced NO degradation by ROS,^{6,9,15,22} increased endothelial NO synthase activity,^{15,20,23} and/or decreased ET-1.^{15,24,25}

Impaired endothelium-dependent vasodilation and, by extension, decreased bioavailability of NO has been demonstrated in animal models of diabetes mellitus and in patients with both type 1 and type 2 diabetes.^{6,26} Augmented generation of ROS as a cause of this endothelial dysfunction has been implicated by studies in healthy humans^{6,9} and in patients with type 1 and type 2 diabetes in whom infusions of the antioxidant vitamin C restored endothelium-dependent vasodilation toward normal.^{9,27,28} One feature common to the in vitro, animal, and human studies of diabetes is elevated glucose status. Indeed, hyperglycemia per se has been observed to negatively affect endothelium-dependent vasodilation in human arteries,¹⁰ in-

crease pulse wave reflections, and decrease aortic distensibility in healthy male volunteers.²⁹ Modest increases in postprandial glucose can acutely lower FMD in healthy subjects as well as those with impaired glucose tolerance and T2DM, though the latter groups show greater reductions.^{7,8} Consistent with our results, the inverse association between FMD and plasma glucose found in these studies suggests a continuous relationship between increasing glucose concentrations and decreasing endothelial function.

In vitro studies indicate that the exposure of endothelial cells to elevated glucose concentrations leads to the generation of ROS, including superoxide anion, which can inactivate NO or otherwise contribute to endothelial cell injury.³⁰ Similarly, patients with T2DM have increased concentrations of biomarkers of lipid peroxidation and reduced levels of endogenous antioxidants compared with healthy controls.⁹ It has also been shown that an acute oral glucose load can increase biomarkers of oxidative stress in healthy subjects.⁸ Concordantly, a recent study³¹ reported that oxidative stress may be an early event in a metabolic cascade elicited by a high-glycemic index diet, ultimately increasing the risk for cardiovascular disease and diabetes. Our results suggest that hyperglycemia induced by an oral glucose load may increase oxidative stress, produce a transient loss of NO bioavailability, and an increase in ET-1, with a consequent impairment in vascular tone. Importantly, these data also suggest that cocoa flavanols may interfere with this chain of events.

This positive effect by cocoa flavanols might have important implications, as data on endothelial dysfunction obtained from coronary and upper arm circulation predicts coronary and cerebrovascular events.¹² Endothelium-derived NO and ET-1 also appear to play an important role in the regulation of arterial stiffness and wave reflections.^{12–14} Arterial stiffness and wave reflections have also been shown to have an independent predictive value for cardiovascular morbidity and all-cause mortality and coronary events and strokes in patients with uncomplicated essential hypertension.³² Flavanols and related polyphenolic antioxidants may protect against hyperglycemia by decreasing the formation of ROS and thereby increasing the

bioavailability of NO. For example, Schroeter et al²³ reported that flavanol-rich cocoa improved FMD in conduit arteries and in microcirculation strongly correlated with the kinetics of increased NO species and (–)-epicatechin and its metabolites in plasma. They also found the actions of the cocoa flavanols were closely mimicked by pure (–)-epicatechin and abolished by the NO synthase inhibitor, L-N^G-mono-methyl-arginine. Our data are concordant with our previous work^{17–19} and other human studies showing a positive effect of flavanol-rich chocolate or cocoa on FMD.^{15,20,33,34} Similarly, Vlachopoulos et al³⁵ reported that 100-g, flavonoid-rich DC acutely increased FMD and decreased wave reflections, whereas it did not affect aortic stiffness. In contrast with our results, plasma oxidant status did not change after DC. Moreover, differently from our previous findings,^{17–19} no variation in BP was observed between the active and control treatments. This incongruence might be explained, considering that the current study design involved a small number of subjects (according to our primary outcome: FMD) and the very short intervention phases (acute administration). Indeed, concordantly with all our findings, a very recent meta-analysis reported that, although FMD was improved after chronic and acute flavonoid-rich DC intakes, significant reductions in DBP and mean arterial pressure were observed after chronic but not after acute intake of DC.³⁶

Inherent limitations of our study design include the small number of subjects, short duration, and the inability to blind the participants to the test and control chocolate interventions. Our isocaloric protocol can also be considered a limitation in that it does not necessarily reflect usual patterns of chocolate consumption. Indeed, in the current study, we provided ≈25% of total daily calories by chocolate. Nevertheless, chocolate perfectly respects the daily recommended balance of macronutrient intake (protein: 10% to 35%; fat: 20% to 35%; carbohydrates: 45% to 65%), without negatively affecting the caloric quality of nutrients. Further, considering our previous findings, we aimed to test effects from commercial chocolate while preserving high flavonoids intake. Starting from this, future long-term studies should test and confirm the beneficial effects of DC at lower doses with reduced caloric burden but preserving the original high content of flavonoids, particularly, including DC in the usual dietary patterns of the population.

Our choice of interventions, however, did permit us to compare flavonoid-rich versus flavonoid-free chocolate and, simultaneously, cocoa-rich versus cocoa-free chocolate. This is of interest, as additional mechanisms of action of cocoa flavanol appear to exist (eg, via modulation of the renin-angiotensin system),³⁷ and other cocoa constituents such as theobromine and caffeine may also contribute partly to the observed changes in FMD and wave reflections. One study of theobromine from dark chocolate, however, found no hemodynamic or electrophysiological effects in young adults³⁸; another found the caffeine and theobromine content of flavanol-poor chocolate were similar to that of flavanol-rich chocolate, although only dark chocolate affected vascular function and NO bioavailability,³⁹ and van den Bogaard et al⁴⁰ reported natural-dose theobromine cocoa did not significantly change either 24-hour ambulatory or central SBP compared with placebo.

Perspectives

We observed for the first time that consumption of flavanol- and cocoa-rich DC increases FMD together with a reduction of wave reflections and ET-1. The DC intervention attenuated the endothelial dysfunction, oxidative stress, and wave reflections induced by a glucose load in healthy subjects with normal glucose tolerance. Although our study design was robust, it was also short-term, involved a small number of subjects, and employed an isocaloric protocol. Importantly, caution is always warranted before recommending the addition of any calorie-rich food to the diet. Our results suggest that postprandial hyperglycemia may contribute substantially to atherosclerotic disease and that cocoa and flavanols might reduce the impact of this relationship. As worldwide cocoa and chocolate are daily consumed by the general population, our observations may have an impact on human health with important clinical consequences.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Flavonoid-rich dark chocolate can positively affect arterial function, preventing the vascular alterations induced by a glucose load in healthy subjects.

What Is Relevant?

- Prolonged and repeated postprandial hyperglycemia may contribute substantially to atherosclerotic disease, and cocoa and flavanols may reduce the impact of this relationship.

Summary

Consumption of flavanol- and cocoa-rich dark chocolate may improve arterial function and offer protection from vascular damage induced by a glucose load in healthy subjects. As worldwide cocoa and chocolate are daily consumed by the general population, our observations may have an impact on human health, with important clinical consequences.

ONLINE SUPPLEMENT

PROTECTIVE EFFECTS OF FLAVANOL-RICH DARK CHOCOLATE ON ENDOTHELIAL-FUNCTION AND WAVE REFLECTION DURING ACUTE HYPERGLYCEMIA

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Short title: Arterial protection by dark chocolate

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EXPANDED METHODS SECTION

Endothelial function. In a fasting state, FMD of the brachial artery was measured before and after 1, 2, and 3 h of the glucose load during each scheduled visit. FMD was always determined by the same physician, who was blinded to the study design and objectives, according to the guidelines of the American College of Cardiology (1). Briefly, a B-mode scan of the right brachial artery was obtained in longitudinal section between 5 and 10 cm above the elbow using a 7.0-MHz linear array transducer (VIVIDe, General Electric) as described previously (2). The transducer was held at the same point throughout the scan by a stereotactic clamp. A cuff was placed around the forearm just below the elbow. After a 1 min acquisition to measure basal diameter, the cuff was inflated for 5 min at 250 mmHg, and then deflated to induce reactive hyperemia. Measurements were obtained by a system for real-time evaluation of the brachial artery diameter with automatic mathematical contour tracking operator locating and tracking the edges (3, 4). Endothelium-dependent vasodilation was considered the maximal dilation of the brachial artery induced by increased flow (1, 2). Reproducibility in repeated measures with the standard technique presented coefficients of variation of 23% in previous measurements with the old technique (3, 4).

Wave reflection. Before and after 30, 60, 90, 120, and 180 min of the glucose load, the RI (height of the second peak/height of the first peak in the digital volume pulse [DVP]), SI (subject height/time delay between direct and reflected waves in the DVP), and the PPT (time delay between the SBP and DBP peaks or, in the absence of a second peak, the point of inflection) were evaluated by a validated finger photoplethysmographic device (Micro Medical, Gillingham, Kent, UK) (5).

Blood pressure measurements. During each visit, BP was measured before and after 30, 60, 90, 120, and 180 min of the glucose load with volunteers in a supine position in a comfortable room. BP was assessed in quadruplicate at 2 min intervals with an oscillometric device (Omron 705 CP, Omron). The first BP reading was discarded and the average of the last 3 measurements recorded. On each occasion, BP was recorded by the same physician who was unaware of the study design, objectives, and results.

Biomarkers of endothelial damage and oxidative stress. Before and after 30, 60, 90, 120, and 180 min of the glucose load in each study phase, serum was also collected from all subjects for the determination of ET-1 and total 8-iso-PGF_{2α} by commercially available enzyme-linked immunoassay kits according to manufacturer instructions (R & D Systems Inc. and Assay Design Inc., Ann Arbor, MI). Serum aliquots were stored at -80°C in polypropylene tubes immediately after centrifugation (15 min at 4°C at 3000 rpm) of blood samples and subsequently used to assess circulating ET-1 and total 8-iso-PGF_{2α}.

Assessment of insulin sensitivity and β -cell function. An OGTT was performed in each subject after both intervention phases after a 10-14 h overnight fast and ≥ 12 h from the last chocolate ingestion. Plasma glucose and insulin were assessed at baseline and then 30, 60, 90, 120, and 180 min after the glucose load. OGTT results were utilized for the HOMA-IR (6, 7), QUICKI (6), and ISI as described by Matsuda and DeFronzo (6) and the index of insulin secretion HOMA derived β -cell function (HOMA-% β) (7).

Statistical analysis. Data are expressed as mean \pm standard deviation (SD). Baseline differences between the two intervention phase groups were analyzed by paired *t* test. Data during the OGTT were analyzed using Proc Mixed Procedure with subjects treated as a random factor and treatment and sequence as fixed factors. LSMEANS was used for comparing multiple group means with Tukey's honestly significant difference (HSD) test. Statistical analyses were conducted on

changes from the respective baseline values and not on direct comparison of absolute values on WC and DC during the oral glucose tolerance test. Therefore, results during OGTT were statistically adjusted for respective baseline values. Statistical power was based on results obtained in similar studies with healthy individuals who consumed flavonoid-rich DC and WC bars (3). In that study the mean FMD increased by 1.9% at a SD of 1.3% after cocoa polyphenol consumption. On the basis of these data the same improvement in FMD could be detected in 12 individuals with a high power (0.9 at $\alpha = 0.05$). Differences were considered significant when $p < 0.05$. Statistical analyses and power calculation were performed with SAS (version 9.1.3, 2004; SAS Institute Inc., Cary, NC).

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Table S1. Energy , nutrient and specific component intake with flavanol-rich dark chocolate (DC) (100 g) and flavanol-free white chocolate (WC) (100 g).

Parameter	DC	WC
Energy (Kcal)	510	536
Cocoa (%)	80	0
Carbohydrates (g)	58.6	63.2
Protein (g)	10.4	4.6
Total fat (g)	26	29.6
Sodium (mg)	17	64.3
Potassium (mg)	240	287
Magnesium (mg)	7.8	25
Catechin (mg)	59	0.04
Epicatechin (mg)	447	BLD*
Quercetin (mg)	14	BLD
Theobromine (g)	1.2	BLD
Caffeine (mg)	108.8	BLD

*BLD, below the limit of detection

Table S2. General characteristics of the study population (mean \pm SD)

Characteristic	Value
Number of subjects	12/ 5M
Age	28.2 \pm 2.7 y
BMI (Kg/m ²)	23.2 \pm 4.2
Body Weight (Kg)	64.5 \pm 10.7
Waist circumference (cm)	82.7 \pm 5.8
LDL-cholesterol (mg/dl)	97.8 \pm 16.7
HDL-cholesterol (mg/dl)	57.1 \pm 13.8
Triglycerides (mg/dl)	59.3 \pm 24.3
Plasma glucose (mg/dl)	74.7 \pm 7.7
Plasma insulin (μ U/ml)	7.5 \pm 4.5

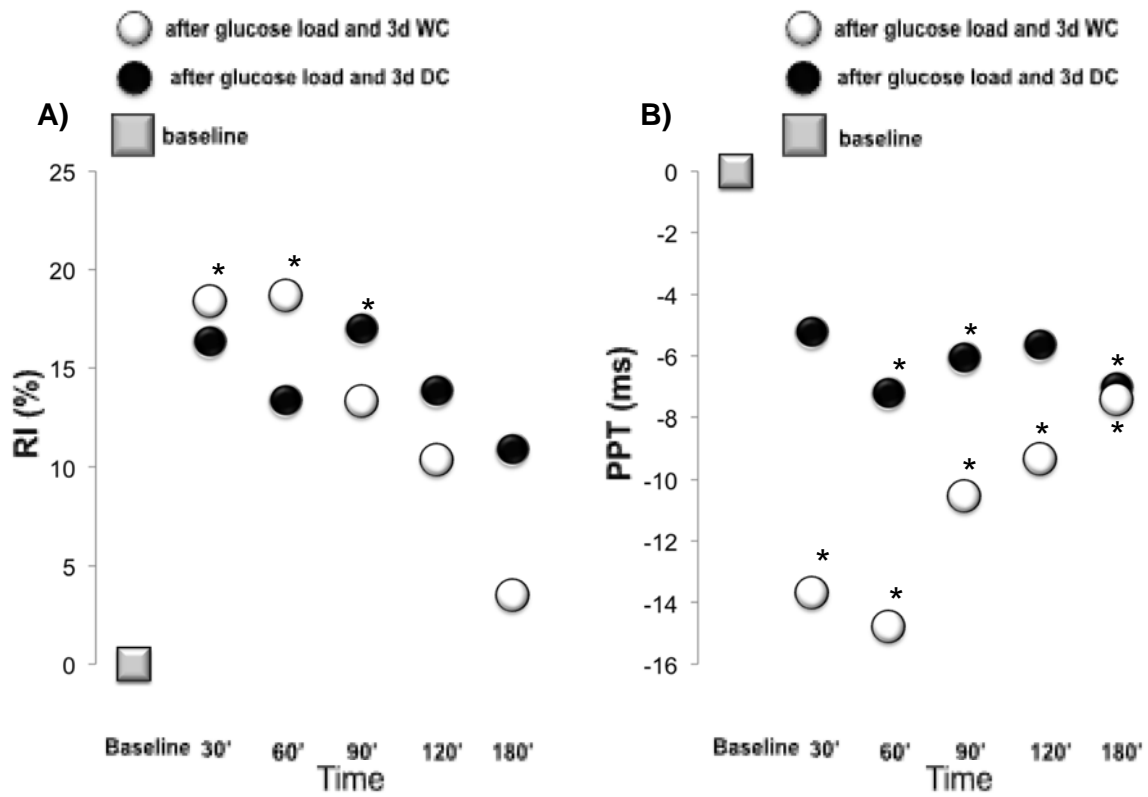


Figure S1. Effect of 3 d WC (white) and DC (black) administration after OGTT on reflection index (RI) (**Panel A**) and peak to peak time (PPT) (**Panel B**) values. Data are presented as % difference respect to baseline levels. Asterisks (*) indicate significant differences with respect the baseline (square) values. Differences are considered significant when $p < 0.05$.

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