Sustained Increase in Flow-Mediated Dilation After Daily Intake of High-Flavanol Cocoa Drink Over 1 Week

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Abstract: A single-dose ingestion of flavanol-rich cocoa acutely reverses endothelial dysfunction. To investigate the time course of endothelial function during daily consumption of high-flavanol cocoa, we determined flow-mediated dilation (FMD) acutely (for up to 6 hours after single-dose ingestion) and chronically (administration for 7 days). The study population represented individuals with smoking-related endothelial dysfunction; in addition to FMD, plasma nitrite and nitrate were measured. The daily consumption of a flavanol-rich cocoa drink (3 × 306 mg flavanols/d) over 7 days (n = 6) resulted in continual FMD increases at baseline (after overnight fast and before flavanol ingestion) and in sustained FMD augmentation at 2 hours after ingestion. Fasted FMD responses increased from 3.7 ± 0.4% on day 1 to 5.2 ± 0.6%, 6.1 ± 0.6%, and 6.6 ± 0.5% (each $P < 0.05$) on days 3, 5, and 8, respectively. FMD returned to 3.3 ± 0.3% after a washout week of cocoa-free diet (day 15). Increases observed in circulating nitrite, but not in circulating nitrate, paralleled the observed FMD augmentations. The acute, single-dose consumption of cocoa drinks with 28 to 918 mg of flavanols led to dose-dependent increases in FMD and nitrite, with a maximal FMD at 2 hours after consumption. The dose to achieve a half-maximal FMD response was 616 mg (n = 6). Generally applied biomarkers for oxidative stress (plasma, MDA, TEAC) and antioxidant status (plasma ascorbate, urate) remained unaffected by cocoa flavanol ingestion. The daily consumption of flavanol-rich cocoa has the potential to reverse endothelial dysfunction in a sustained and dose-dependent manner.

Key Words: flavanols, flow-mediated vasodilation, nitrite, cocoa, dose-response

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INTRODUCTION

The recent interest in the relationship between dietary flavonoids (a group of polyphenols) and cardiovascular health may be traced back to epidemiological work performed about a decade ago. An inverse association between mortality from cardiovascular disease and daily intake of flavonoids was previously noted by various investigators.[1,2] The calculated intake of flavonoids, mainly from tea, onions, and apples, ranged from very low amounts to about 100 mg/d. Experimental work and studies with human volunteers substantiated the idea that dietary flavonoids, especially those belonging to a subclass called flavanols, might potentially be beneficial to the cardiovascular system.[1,3–5]

In the context of assessing cardiovascular health, especially after pharmaceutical or dietary interventions, flow-mediated dilation (FMD) of the brachial artery emerged as a suitable physiological readout of endothelial function, prognostically correlating with the occurrence of future cardiovascular events.[6,7] Previous studies on the intake of flavanol-rich dietary sources such as red wine, black tea, and green tea revealed positive effects on FMD,[8–11] and thus cardiovascular benefits. In particular, a novel source of flavanols, cocoa, attracted interest in recent years.[5,12] We have previously reported that the consumption of a cocoa drink rich in flavanols, especially epicatechin, catechin, and their oligomeric derivatives (procyanidins), leads to an increase in FMD, reaching maximal effects at 2 h after ingestion, which is correlated with significantly higher plasma concentrations in the circulating pool of nitric oxide, RXNO.[13] A significant lowering of arterial blood pressure was reported in hypertensive patients[14,15] after the consumption of polyphenol-rich dark chocolate. The plasma concentration of the flavanol epicatechin, the monomeric flavanol present in flavanol-rich cocoa, also rises after ingestion of cocoa peaking at 2 h.[16] More recently, the proposed beneficial effects of flavanol-rich cocoa were mimicked by ingestion of pure (−)-epicatechin, with maximum FMD effects occurring at 2 h after single-dose consumption.[17] Thus, one can demonstrate acute cardiovascular effects that temporally correlate with the presence of epicatechin and its metabolites in the circulation.

This study begins to address the question of whether there is a longer-term consequence of flavanol-rich cocoa consumption in addition to the acute effects observed previously, and it attempts to describe the time course of these effects. In other words, does the resting baseline level of FMD improve during the regular daily intake of high-flavanol cocoa over several days? In order to address this question, we chose

volunteers in whom there is a known partial impairment of endothelial function due to impaired nitric oxide activity. A group of 6 male smokers was recruited, and endothelial dysfunction was verified by measuring FMD. One hundred milliliters of a high-flavanol cocoa drink containing 306 mg of total flavanols (procyanidins, epicatechin, catechin) was ingested 3 times daily for 7 d while FMD was assessed along with other parameters. Our results show that there is a longer-term inductive effect of high-flavanol cocoa ingestions; at the particular dose and dosing paradigm used, the effect leveled out at a higher plateau after about 5 days of consumption. Interestingly, the acute effects appear to be an independent phenomenon, as they are preserved in extent and time-course throughout the intervention time (ie, days 1 to 8), thus perhaps pointing to a mechanistically different processes underlying the observed acute and longer-term effects.

It has also been demonstrated previously by numerous investigators that flavanols are compounds with significant antioxidant properties in various *in vitro* systems; consequently, it is often communicated in the scientific literature that flavanol-mediated antioxidant effects are also a main underlying principle for the biological/biomedical effects observed after flavanol ingestion.[18] This supposition is often used to explain epidemiological data as well as biomedical outcomes in the context of flavonoid consumption, despite the fact that it is known that plasma concentrations of other antioxidants, such as ascorbic acid and urate, are much higher than the pool of circulating flavonoids. An additional challenge to this general antioxidant theory of flavanols is the fact that flavanols undergo considerable biotransformation upon absorption, resulting in metabolized forms, which are known to have a lower antioxidant capacity. Thus, we aimed at paralleling our biomedical assessment of the cardiovascular effects of flavanol-rich cocoa consumption with generally applied measures of antioxidant effects. For this purpose, we utilized 2 frequently used assays, namely the measurement of malondialdehyde (often used as a maker for lipid peroxidation) and the assessment of the total antioxidant capacity of plasma by means of Trolox equivalents (TEAC assay). In addition, we investigated the potential effect of flavanol ingestion on the classical circulating antioxidants ascorbate and urate.

MATERIALS AND METHODS

Subjects

Eleven healthy male subjects (age, 22 to 32 years) with smoking-related endothelial dysfunction were enrolled in the study (Table 1). Exclusion criteria were hypertension, hypercholesterolemia, diabetes mellitus, and acute inflammation (CRP $<$ 0.5 mg/dL). This study was approved by the Ethics Board of the Heinrich Heine University, and all participants gave written informed consent.

Test Drinks

Table 2 shows the macronutrient and micronutrient matched and isocaloric cocoa drinks with high and low flavanol content. The drinks were similar in taste and indistinguishable by color and packaging.

TABLE 1. Baseline Characteristics of Study Group

N	11
Smokers (n)	11
Age (yr)	27 ± 1
Sex (M/F)	11/0
BMI (kg/m^2)	22 ± 1
Mean arterial pressure (mm Hg)	89 ± 4
Total cholesterol (mg/dL)	162 ± 10
Glucose (mg/dL)	77 ± 5
Cigarette consumption (cigarettes/day)	21 ± 2
Cummulative cigarette consumption (pack years)	9 ± 2
Alcohol consumption (g/d)	9 ± 2
Brachial artery diameter (mm)	4.6 ± 0.2
Flow-mediated dilation (%)	4.0 ± 0.4
Glycerol trinitrate-mediated dilation (%)	12.8 ± 0.5
Nitrite (nmol/L)	53 ± 8
Nitrate (μmol/L)	22 ± 3

Data given as mean ± SEM

In the chronic 1 wk study (Figure 1), subjects consumed high-flavanol cocoa drinks (dry dairy-based beverage mix made with cocoa powder containing 306 mg of flavanols mixed in 100 mL of water) 3 times daily, totaling 918 mg of daily flavanol intake. To ensure that the same calories, micronutrients, and alkaloids were consumed throughout the acute, dose-response study, the 2 drinks (high-flavanol and low-flavanol cocoa drinks) were mixed to obtain a final volume of 300 mL of water before use (CocoaPro; Mars Inc., Hackettstown, NJ), thus achieving final flavanol amounts of 36, 330, or 918 mg of total flavanols (ie, flavanols plus procyanidins). In a second set of acute studies aimed at extending the dose-range, we used similarly matched flavanol-containing cocoa drinks with 28, 179, or 483 mg of total flavanols (dairy based ready-to-drink beverage, CocoaVia; Mars Inc., UK). The nutritional and flavanol/procyanidin composition of this beverage has been reported previously.

Study Design

To study sustained effects after daily flavanol consumption, high-flavanol cocoa (306 mg of total flavanols; Table 2)

TABLE 2. Composition of Cocoa Drinks (per 100 mL)

	Low Flavanol	High Flavanol
Total flavanol (mg)	12	306
Monomers (mg)	3	74
Epicatechin (mg)	2	59
Catechin (mg)	1	15
Dimers (mg)	2	57
Trimers-decamers (mg)	7	175
Theobromine (mg)	222	228
Caffeine (mg)	14	12
Energy (kcal)	79	80
Fat (g)	1.0	0.9
Carbohydrate (g)	11.2	11.6
Protein (g)	6.4	6.4

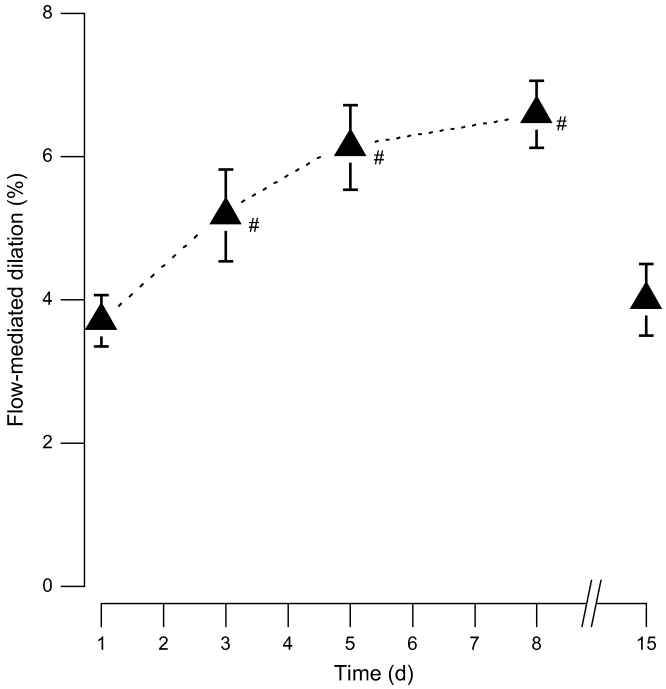

FIGURE 1. Sustained increase in flow-mediated dilation (FMD). Flow-mediated dilation of brachial artery increased with repetitive consumption of high-flavanol cocoa drink (306 mg of flavanols thrice daily over 7 days, n = 6). FMD was not significantly different at day 15 after 1 week of cocoa-free washout. Symbols indicate means; error bars, SEM; #Indicates significant differences from control at day 1; each $P < 0.05$.

mixed in 100 mL water was consumed (n = 6) 3 times daily (morning, lunchtime, evening) for 7 consecutive days. All volunteers had previously concluded 1 of the acute studies (see below), and all had responded to acute ingestion with increased FMD. Endothelial function as assessed by FMD was measured at baseline (control) and 2 hours after ingestion of the morning dose on days 1, 3, 5, and 8. In parallel, the oxidative NO metabolites nitrite and nitrate were determined on days 1 and 8. As an internal control, baseline measurements were repeated after a cocoa-free washout phase of an additional 7 days (ie, on day 15).

To avoid acute effects of cigarettes[19] and changes in plasma nitrite secondary to acute food consumption,[20] all volunteers fasted overnight (water ad libitum) and refrained from smoking for 12 hours before the studies and until completion of the studies in the morning on the study days. On all other times during the study period, the volunteers continued to smoke their usual average of 1 pack of cigarettes per day (Table 1). The subjects were also advised to maintain their habitual exercise habits through the study period and refrain from exhausting exercise on the days before study days.

To study the dose-dependence and duration of acute effects, we performed 2 time-course studies involving the consumption of matched cocoa drinks. In a first series, cocoa beverages with 36, 330, or 918 mg of flavanols, respectively, were administered to subjects (n = 5) on 3 different days in random double-blinded order. In another group (n = 6), we administered cocoa drinks with flavanol contents of 28, 179, and 483 mg, respectively. Blood pressure, heart rate, and FMD, as well as plasma nitrite and nitrate were determined at 0 hours as well as at 1, 2, 3, 4, and 6 hours after ingestion. In addition, to gain mechanistic insight, we assessed the plasma Trolox-Equivalent Antioxidant Capacity (TEAC), and the plasma levels of ascorbate, urate, and malondialdehyde (MDA). We measured these parameters, because they were shown previously to be altered in smokers, thus changes in those markers may be important in smoking-related vascular pathologies. To prevent carry-over effects, studies were more than 2 days apart from each other.

Vascular Function Assessment

FMD was measured as previously described.[21,22] Briefly, the diameter of the brachial artery was measured by a 15-MHz transducer (Sonos 5500; Agilent) and automatic edge-detection software (Brachial Analyzer; Medical Imaging Applications, Iowa City, IO) yielding a coefficient of variation of less than 1%. Reactive hyperemia was induced by 5 min of distal lower arm occlusion. The diameter was assessed after 60 seconds, and FMD was calculated as relative diameter gain compared with baseline. Endothelium-independent dilation was measured 4 min after sublingual application of 400 μg of glycerol trinitrate (GTN; Nitrolingual mite, Pohl, Germany). The percentage of FMD compared with endothelium-independent dilation was calculated (FMD/GTN-ratio). Mean arterial pressure was calculated as 1/3 (systolic-diastolic blood pressure) plus diastolic blood pressure.

Biochemical Analyses

Plasma concentrations of nitrite were determined using reductive cleavage by an iodine/iodide-containing reaction mixture and subsequent determination of the released NO by a gas phase chemiluminescence assay.[23,24] Whole blood was diluted 1/5 in ice-cold 0.9% saline containing N-ethylmaleimide (5 mM) and EDTA (2 mM). Plasma was obtained by centrifugation at 750 × *g* and 4°C for 5 min. Plasma was then divided into 2 aliquots: 1 was directly injected into a triiodide-containing vessel (60°C) actively purged with a helium stream in line with an NO chemiluminescence analyzer (88NOe; EcoPhysics, Duernten, Switzerland), and the other was treated with 1/10 volume of 5% sulfanilamide in 1 M HCl for 10 min and then injected. Nitrate was quantified after enzymatic reduction to nitrite by nitrate reductase using flow-injection analysis.[25]

The so-called total antioxidant capacity of plasma was determined as Trolox-Equivalent Antioxidant Capacity (TEAC) using a commercially availably kit, which is based on the formation of radical cations of 2,2-azino-di-(3-ethylbenzthiazoline) sulfonate, which was measured photometrically at a wavelength of 600 nm in accordance with the manufacturer's instruction (Randox Laboratories Ltd., Crumlin, UK). Circulating levels malondialdehyde (MDA), a proposed marker of lipid peroxidation, and ascorbate were determined in plasma and urate in serum by standard clinical methods.

Statistical Analyses

Results are expressed as means ± standard error of the mean (SEM). Repeated measurements ANOVA was used to estimate intraindividual effects, and pairwise comparisons were made with LSD adjustment. Statistical significance was assumed if a null hypothesis could be rejected at $P = 0.05$. Increases in FMD and nitrite were calculated as data at given time points minus baseline of respective day. All analyses were performed with SPSS 11.0.1 (SPSS Inc, Chicago, IL) or Origin 7 (OriginLab Corp, Northampton, MA). Analyses of blood samples and ultrasound scans were performed by investigators blinded to the treatment paradigm.

RESULTS

Baseline Characteristics

All 11 subjects (Table 1) were current smokers and had a smoking-related impairment of dilatory responses of the brachial artery (FMD: 4.0% ± 0.4%) with normal endothelium-independent glycerol-trinitrate mediated dilation (GTN: 12.8% ± 0.5%). Smokers had a history of 9 ± 2 pack-years, smoked 21 ± 2 cigarettes per day as assessed by the reported number of cigarettes smoked on the day before the first study day, and consumed on average 9 ± 2 g alcohol per day. No significant correlations between baseline smoking behavior (pack years, cigarettes per day) or alcohol consumption and FMD at baseline were seen. Normal values for FMD using identical methodology in age-matched nonsmokers are 6.5% ± 0.4% (95% confidence interval for the mean: 5.8% to 7.3%).[26,27]

Daily Consumption of High-Flavanol Cocoa Leads to Sustained Increase in Endothelial Function

On the morning of day 1, after a 12 hour fast and refraining from smoking for 12 hours, the average FMD response was 3.7% ± 0.4% (n = 6). This is the first data point in Figure 1, and it is shown in the first column of Table 3. The volunteers then received 100 mL of the flavanol-rich cocoa drink containing 306 mg of flavanols, and FMD was measured again 2 hours later. This is further referred to as the "acute FMD response," which increased to 6.1% ± 0.6% and represents an augmentation of 2.4 ± 0.3% as compared to time 0 (shown in the right-hand column of Table 3). Later on day 1, the volunteers received 2 more 100 mL doses of the high-flavanol cocoa drink (at midday and in the evening), thus the total dose on day 1 was 918 mg of flavanols. The volunteers had their normal lunch and dinner, and they also continued to smoke (on average, 1 pack of cigarettes per day). On day 2, the volunteers also consumed 3 × 100 mL doses of high-flavanol cocoa drink (morning, midday, and evening), again reaching the total dose of 918 mg of flavanols. At 8:00 PM on day 2, the volunteers began the overnight fast and refrained from smoking in the evening, during the night, and until completion of the studies in the morning. On day 3, the morning FMD was recorded, and then the procedure continued on as on day 1 (see data point in Figure 1 and Table 3).

TABLE 3. Flow-mediated Dilation, Plasma Nitrite, and Nitrate During Sustained Cocoa Ingestion Study

	Day	Fasting	2 h After Morning Dose	Difference
Flow-mediated dilation (%)	1	3.7 ± 0.4#	6.1 ± 0.6*	2.4 ± 0.3
	3	5.2 ± 0.6#	7.6 ± 0.7*	2.4 ± 0.3
	5	6.1 ± 0.6#	8.8 ± 1.1*	2.7 ± 0.7
	8	6.6 ± 0.5#	9.0 ± 0.6*	2.4 ± 0.4
	15	3.3 ± 0.3	n.d.	
Nitrite (nmol/L)	1	65 ± 9	115 ± 18*	49 ± 18
	8	101 ± 18#	122 ± 18*	21 ± 10
	15	54 ± 11	n.d.	
Nitrate (μmol/L)	1	24 ± 4	24 ± 4	0 ± 3
	8	20 ± 4	19 ± 4	−2 ± 1
	15	26 ± 5	n.d.	

Data given as mean ± SEM. n.d., not determined.
*$P < 0.05$ vs value on same day.
#$P < 0.05$ vs value on day 1.

Previous work had shown that the enhanced level of FMD after a single-dose ingestion of high-flavanol cocoa had returned back to baseline at approximately 6 hours after administration; however, the starting FMD after overnight fast (ie, time 0) value on day 3 was higher than on day 1. This increase in basal FMD (ie, before intake of high-flavanol cocoa on any given day) continued to increase, finally reaching a plateau level at about day 5 (Figure 1). We denote this as a long-term effect to distinguish it from the acute 2 hour effect. This long-term effect, which occurs over days, may be due to an induction process, mechanistically different from the acute 2 hour effect. This idea is substantiated by the unsuspected observation that on day 1 through day 8 the magnitude of the acute effect was maintained, as recorded after the intake of the morning dose of 306 mg of flavanols in the 100 mL of cocoa drink (right-hand column in Table 3). Thus, we here dissect 2 distinct processes: an acute response that is paralleled by the time course of the flavanol and nitrite plasma concentrations,[13,17] and a sustained, long-term effect that is subsequent to daily high-flavanol cocoa intake. Using the above-detailed flavanol dose and dosing paradigm, the latter response levels out after about 5 consecutive days. As shown in Table 3, the nitrite concentration in plasma before the morning dose on day 8 was also elevated when compared with the levels measured at the same time on day 1. Despite this fact, there was a further increase in circulating nitrite at 2 hours after ingestion of the morning dose. This suggests increased NO activity in both the acute and long-term setting. Although there were no significant correlations between pack years or the number of cigarettes smoked per day and the acute increase in FMD ($r = -0.31$, $P = 0.353$ and $r = -0.46$, $P = 0.159$, respectively), there were strong significant correlations between the number of cigarettes and the FMD values on day 8 of the study (0 h: $r = -0.83$, $P = 0.042$; 2 h: $r = -0.94$, $P = 0.005$). We did not observe this correlations with pack years (0 h: $r = 0.02$, $P = 0.969$; 2 h: $r = -0.22$, $P = 0.672$). This suggests that a greater cigarette consumption is associated with an impaired reversibility of endothelial dysfunction.

After discontinuing daily ingestions of high-flavanol cocoa at day 8, the fasting FMD response as measured 1 week later on day 15 was much lower as compared with day 8 and had thus returned back to the initial value observed on day 1 (Figure 1). This indicates that the longer-term effect is reversible. Blood pressure, heart rate, and plasma nitrate remained unaffected throughout the study.

Additional Insight Into the Acute Effects After Consumption of High-Flavanol Cocoa

An acute dose-response study was performed to gain more insight into the underlying mechanisms. Volunteers were asked to ingest 300 mL of a cocoa drink of variant total flavanol content. This was achieved by mixing the high-flavanol cocoa drink (306 mg of flavanols per 100 mL) with different amounts of the low-flavanol cocoa drink (12 mg of flavanols per 100 mL). The resulting 3 × 100 mL mixtures thus ranged in their total flavanol content from 36 mg per 300 mL to 918 mg per 300 mL.

As shown in Figure 2A, the time of maximum FMD response is essentially invariant to dose, occurring at about 2 hours after ingestion of the cocoa drink, whereas the magnitude of the response increases with the flavanol dose ingested. The magnitude of the FMD response continued to increase with dose, going towards saturation with the highest dose administered (918 mg of cocoa flavanols; Figure 2B). From these data points, we calculated the flavanol dose necessary to achieve half-maximal effects (EC_{50}) to be 616 mg. The time it takes for the FMD response to return back to the initial baseline was slightly longer at the high dose as compared with the lower doses (Figure 2A). Thus, it may be possible that there is a carry-over effect from day to day in the longer-term study shown in Figure 1. After the control drink, which resembles a commercially available chocolate drink with low flavanol content, the FMD response remains virtually unchanged over the first 6 hours of the day. Thus, there seems to be a stable lower steady state FMD in smokers' chronic endothelial dysfunction.

In parallel, we measured nitrite as a biomarker of NO bioactivity. The time course of concentration changes in circulating nitrite was qualitatively similar to FMD. Nitrite concentrations were significantly increased at 1 hour after 330 mg of high-flavanol cocoa (61 ± 11 nmol/L to 105 ± 26 nmol/L; absolute increase, 44 ± 16 nmol/L) and 918 mg of high-flavanol cocoa (62 ± 8 nmol/L to 103 ± 12 nmol/L; absolute increase, 41 ± 6 nmol/L; each $P < 0.05$ compared with baseline value). Nitrite did not significantly increase after low-flavanol cocoa, ruling out a potentially confounding dietary source of increased nitrite levels.[20] Peak nitrite plasma concentrations were not significantly different after consumption of flavanol-rich cocoa drinks with 179 to 918 mg of flavanols. Parallel time-courses of FMD and nitrite suggest that the increase in endothelial function is correlated with increased eNOS activity. As shown previously,[28] time courses of total circulating flavanols showed dose-dependent uptake of flavanols from cocoa drinks (data not shown).

With regard to the measurement of markers often used to assay antioxidant effects, the pre-dosing, baseline values for TEAC (1.3 ± 0.1 mmol/L), ascorbate (54 ± 5 μmol/L), urate (321 ± 12 μmol/L), and MDA (0.23 ± 0.02 μmol/L) remained unaffected by a high-flavanol consumption (all values represent the average of baseline values of three study days).

DISCUSSION

We demonstrated that the daily consumption of a high-flavanol cocoa drink leads to a sustained reversal of endothelial

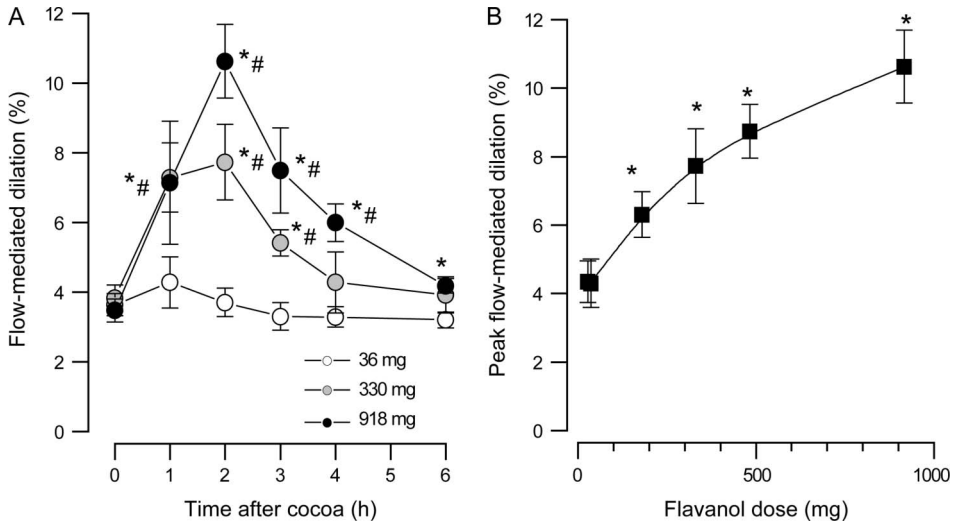

FIGURE 2. Time course (A) and dose-response curve (B) of flow-mediated dilation (FMD) after consumption of cocoa drinks with increasing flavanol content. (A) FMD increased significantly after the ingestion of cocoa drinks rich in flavanols (300 mL each corresponds to 330 or 918 mg total flavanols) but not after low-flavanol control drinks (36 mg). Symbols indicate means; error bars, SEM; *$P < 0.05$ vs. 0 hour of same day; #$P < 0.05$ vs. respective time point after consumption of low-flavanol control (open circles). (B) Peak response of FMD after ingestion of flavanol-rich cocoa containing 28 to 918 mg total flavanols (36, 330, and 918 mg from time courses in (A), n = 5; 28, 179, and 485 mg, n = 6). Symbols indicate means; error bars, SEM; *$P < 0.05$ vs. baseline at 0 time point in respective time course studies.

dysfunction, reaching a plateau level of improved FMD at approximately day 5 (Figure 1). The magnitude of sustained vascular effects observed in the present study was in a range similar to that observed after long-term pharmacological approaches with, for example, statins.[29] Interestingly, the responsiveness to an acute single-dose of flavanol-rich cocoa was maintained (Table 3). In a study on short-term and long-term black tea consumption in patients with coronary artery disease, Duffy et al[9] also noted that short-term to long-term tea ingestion resulted in additional improvements, although the acute response was not fully retained as it was here with high-flavanol cocoa (Table 3). It is likely that the mechanisms underlying the long-term response (Figure 1) are different in nature from those that are driving acute responses, perhaps representing an adaptation in terms of changes in gene/protein expression or posttranslational protein modification. In the context of mechanisms related to acute FMD responses after flavanol-rich cocoa ingestion, we have recently shown that FMD was completely inhibited by L-NMMA in this circumstance, suggesting predominant NOS dependence of acute effects.[17,28] As to whether or not the longer-term effect on FMD, as described here, is due to: (1) increased expression/activity of endothelial nitric oxide synthase (eNOS) or (2) changes in expression/activity of eNOS-related proteins that influence cellular location of eNOS, co-factor availability, or substrate accessibility, or (3) eNOS-independent mechanisms, remains to be elucidated. Temporally parallel increases in plasma nitrite, a marker for nitric oxide synthase activity[30,31] and a potential source of NO,[32] are consistent with increased NO bioactivity and also suggest essential involvement of eNOS in the mediation of the observed effects. Fisher et al[33] showed that 4 days of daily consumption of flavanol-rich cocoa led to increases in peripheral vasodilation as measured by peripheral arterial tonometry at the finger tip, suggesting that longer-term effects also extend to the function of smaller vessels. In the same study, the administration of L-NAME, a competitive NOS inhibitor, significantly diminished the increase in blood flow, but it was not able to completely abolish vasodilation, perhaps suggesting both, eNOS-dependent and eNOS-independent, contributing factors.

A major proportion of cocoa polyphenols consists of monomeric and oligomeric flavanols (procyanidins).[34] We have recently shown that the ingestion of (−)-epicatechin isolated from cocoa beans can mimic the effects on FMD as observed after flavanol-rich cocoa consumption.[17] The cumulative daily flavanol intake in the present study was 918 mg of total flavanols with regard to highest dose ingested. On the basis of previously published analytical assessments of the total flavanol content (monomers and procyanidins) of various flavanol-containing foods,[34,35] we have estimated that the 918 mg dose used in our study may be comparable to the total flavanol amount ingested with 211 g of flavanol-containing dark chocolate (4.35 mg/g), 7.05 L of red wine (130 mg/L), 6.55 L of cranberry juice (140 mg/L), or 5.4 apples (0.85 mg/g).[35] Overall, the daily flavanol intake as part of an average US diet were recently estimated to be 57.7 mg of total flavanols.[34] However, it is important to note that nutrient content and composition of all plant-based food products vary greatly depending on a multiplicity of factors, so that the comparisons provided above should be considered as general estimates rather than as precise equivalents.[34,35] In addition, variations in flavanol absorption based on intrinsic differences in the physical and chemical composition of food matrices make it extremely speculative to quantitatively compare flavanol-mediated effects after the ingestion of different flavanol-rich foods (ie, tea, wine, certain fruit).

In the context of potential mechanism, it is interesting to note that, on average, the smokers who were participating in this study exhibited slightly lower plasma ascorbate levels and slight decreases in the total antioxidant capacity of plasma (measured using the TEAC assay) compared with non-smokers.[36] In addition, the volunteers had a somewhat higher average MDA value in plasma (as compared with male nonsmokers[36]), which is often attributed to increased lipid peroxidation as a hallmark of smoking-induced oxidative stress. Nonetheless, baring in mind that a single-dose ingestion of flavanol-rich cocoa led to increased FMD responses, it is important to point out that the data emanating from this study do not demonstrate any statistically significant changes in biomarkers commonly accepted in the context of oxidative stress (ie, TEAC, MDA), nor do the data show changes in levels of main circulating antioxidants (ie, ascorbate and urate). Corroborating our data, Wiswedel et al[36] did not observe changes in plasma MDA, TEAC, ascorbate, and F_2-Isoprostanes for up to 6 hours after ingestion of a cocoa drink containing 187 mg of cocoa flavanols; however, flavanol-rich cocoa consumption significantly lowered F_2-Isoprostanes during physical exercise. Another acute dietary intervention with a cocoa product showed that plasma antioxidant capacity, plasma concentrations of 8-isoprostanes, and 2-thiobarbituric acid-reactive substances (TBARS) remained unchanged as measured at 2 and 6 hours after consumption.[37] Furthermore, Engler et al reported no changes in Oxygen Radical Absorbance Capacity (ORAC), an assay used to assess oxidative stress.[38] In this context and with the exception of Serafini et al[39] our results are consistent with the published data sets obtained from dietary interventions with cocoa flavanols and thus may indicate that the potential antioxidant capacity *in vitro* seems to be unrelated to flavanol-mediated effects on endothelial function and FMD *in vivo*. Taken together, although the plasma levels of flavanols and markers of oxidative stress may not reflect their levels in vascular tissues, the above data suggest that flavanol-rich cocoa ingestion mediates an augmentation in endothelial function and eNOS-catalyzed NO synthesis that is independent of the antioxidant properties that flavanols exert *in vitro*.

Finally, important clinical implications may be drawn from the collective data on flavanol-mediated vascular effects in that flavanol-rich foods, such as tea or flavanol-rich cocoa products, constitute a potential alternative approach for modulating endothelial function and dysfunction by means of nutritional rather than pharmacological intervention. Larger clinical trials in patient populations are necessary to show the clinical relevance and potential therapeutic value of these flavanol-rich foods. In this context, flavanol ingestion over several days may be required to achieve steady state improvement in vascular function.

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