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ORIGINAL ARTICLE

Flavanol-rich cocoa ameliorates lipemia-induced endothelial dysfunction

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Abstract Consumption of flavanols improves chronic endothelial dysfunction. We investigated whether it can also improve acute lipemia-induced endothelial dysfunction. In this randomized, placebo-controlled, double-blind, crossover trial, 18 healthy subjects received a fatty meal with cocoa either rich in flavanols (918 mg) or flavanol-poor. Flow-mediated dilation (FMD), triglycerides, and free fatty acids were then determined over 6 h. After the flavanol-poor fat loading, the FMD deteriorated over 4 h. The consumption of flavanol-rich cocoa, in contrast, improved this deterioration in hours 2, 3, and 4 without abolishing it completely. Flavanols did not have any influence on triglycerides or on free fatty acids. Flavanol-rich cocoa can alleviate the lipemia-induced endothelial dysfunction, probably through an improvement in endothelial NO synthase.

Keywords Flavanol · Endothelial dysfunction · Postprandial lipemia · Flow-mediated vasodilation

Introduction

Development of endothelial dysfunction is associated with several atherosclerosis risk factors, such as smoking, hypertension, and diabetes mellitus [1–6]. Furthermore, prospective studies have shown repeatedly that presence or absence of endothelial dysfunction is of prognostic importance, even independently of any other co-existing risk factors [7, 8].

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Flavanols occur in fruit, red wine, and also in substantial amounts in cocoa. Recent studies suggest certain vascular health benefits associated with dark chocolate [9]. Given the popularity of chocolate, this is of particular interest. Several authors report that endothelial dysfunction can be improved by either chronic or acute administration of flavanols (for a review see Hooper et al. [10]).

Acute endothelial dysfunction also occurs during postprandial lipemia, as described by others [11, 12] and by us [13, 14]. In this study we therefore investigated whether this transient lipemia-induced endothelial dysfunction can also be improved by flavanol-rich cocoa added to a fatty meal.

Materials and methods

Subjects

The study was carried out on 18 students (2 males, 16 females). The inclusion criteria were as follows: normal weight (body mass index, in kg/m^2, over 18.5 but under 25.0); non-smokers; normal alcohol habits [females fewer than 7 drinks (20 g alcohol per drink) per week; males fewer than 14 drinks per week]; no history of obesity, diabetes, or liver and kidney diseases; normal blood pressure (systolic blood pressure below 139 mmHg and diastolic blood pressure below 80 mmHg); and absence of any regular medication (Table 2). The subjects were instructed to refrain from any unusual changes from their normal physical activity and nutritional behavior starting from 4 weeks before and continuing throughout the study. Their habitual diets, documented by self-reporting, corresponded to average energy intakes from carbohydrates, fat, and protein equal 49.2, 37.4, and 13.2%, respectively.

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Design

In this randomized, placebo-controlled, double-blind, crossover trial, each individual was studied twice, with an interval of at least 1 week between the two oral fat loadings with different cocoa powders. An independent researcher randomized a series of numbers to "flavanol-poor cocoa" or "flavanol-rich cocoa" by lot before starting the study. Study investigators and participants were blinded to different cocoa powder assignment, and assignment codes were not available to investigators until all participants had completed the study and until the database had been completed and frozen.

The liquid fatty test meal consisted of 30% whipping cream, 3 ml (=1 g fat) being given per kg body weight; 100 ml of the cream contained 33 g fat (18.2 g saturated and 9.04 g monounsaturated fatty acids), 3.5 g carbohydrates, and 2.5 g protein.

A total of 9.18 g of cocoa powder was added to the fatty meal, as described by Heiss [15]. Table 1 shows the macronutrient and micronutrient content of the cocoa powders with high and low flavanol contents. The different cocoa powders were a gift by Barry Callebaut from Lebbeke-Wieze, Belgium. The cocoa powders, their packages, the appearance of the drinks, and the tastes were undistinguishable for each cocoa species. Therefore, neither the involved staff nor the participants knew which of the two cocoa species had actually been used.

After a fasting period of 12 h the meals were drunk within 15 min between 7:30 and 8:00 a.m. All were well tolerated, and no gastrointestinal symptoms were reported. An Ethics Committee had approved the protocol, and all subjects had given their written informed consent.

The first blood sample was withdrawn in the fasting state. Further blood samples were then taken immediately before and 2, 4, and 6 h after the consumption of the fatty meal. We measured triglycerides, free fatty acids, total cholesterol, HDL cholesterol, and LDL cholesterol in the fasting and the postprandial state. No other source of energy was provided, but water was allowed ad libitum. The participants did not engage in any physical activity during the test, and exercise had been avoided during the 24 h before the tests. Venous blood samples were collected under standardized conditions, and serum was separated from the blood cells by centrifuging for 10 min at 3,000*g*. Analyses of the lipoproteins and metabolic variables were carried out within 24 h.

Table 1 Composition of 9.18 g cocoa powder

	Low-flavanol	High-flavanol
Total flavanol (mg)	14.68	918.00
Monomers (mg)	2.93	145.96
Epicatechin (mg)	0.73	120.25
Catechin (mg)	1.38	29.37
Dimers (mg)	4.77	113.83
Trimers-decamers (mg)	6.97	383.72
Theobromine (mg)	220	210
Caffeine (mg)	40	40
Energy (kcal/100 g)	252	259
Fat (g)	1.0	1.0
Sugars (mono + disaccharides) (g)	0.2	0.2
Protein (g)	2.1	2.2

Laboratory assays

The concentrations of triglycerides, cholesterol, HDL-, and LDL-cholesterol were determined by commercial enzymatic methods in a random-access analyzer (Hitachi 911, Roche Diagnostics, Germany). The reagents and calibrators were also from Roche Diagnostics. Free fatty acids were determined by a commercial enzymatic colorimetric method (Wako Chemicals GmbH, Neuss, Germany) and plasma glucose by a commercial enzymatic method (GOD, Roche Diagnostics, Germany).

Endothelial function assessment

Flow-mediated vasodilation (FMD) was assessed by two-dimensional ultrasonography (ESAOTE, Florence, Italy) of the brachial artery according to the method described by Plotnik et al. [16]. The measurements were performed with the subjects in the supine position, on the left arm, resting for 10–20 min in a quiet dark room at a temperature of 22°C. The brachial artery was scanned longitudinally, just above the antecubital crease, using a 10-MHz probe. The diameter of the brachial artery was measured at the R wave of the electrocardiogram on the interface between the media and adventitia of the anterior and posterior walls. Hyperemia was induced by inflation of a pneumatic cuff (12.5 cm wide) to 230–250 mmHg for 4 min on the most proximal portion of the upper arm. The arterial diameter measurement was repeated 45–60 s after sudden deflation of the cuff. The average of three measurements of the baseline and post-hyperemia diameter was used for the analysis. We used the maximal diameter during 2 min after cuff deflation. Flow-mediated vasodilation was expressed as the percentage increase in brachial artery diameter during hyperemia and defined as $100 \times$ [(post-hyperemia diameter − baseline diameter)/baseline diameter]. The FMD examiner was blinded to the type of cocoa consumed.

Statistical analyses

The results are presented as mean values with their SEMs. When inspecting the different variables by graphical

Table 2 Characteristics of the subjects

Age (years)	25.2 ± 2.5
Weight (kg)	60.8 ± 11.1
Height (m)	1.68 ± 0.05
BMI (kg/m^2)	22.8 ± 2.0
Triglycerides (mmol/l)	1.29 ± 0.58
Free fatty acids (mmol/l)	0.41 ± 0.17
LDL-cholesterol (mmol/l)	2.98 ± 0.82
Glucose (mmol/l)	4.51 ± 0.42

representation with boxplots, no strong deviations from the normal distribution were detected. The Student's paired *t* test was used to test for differences between fasting and postprandial values as well as differences between treatments. All statistical analyses were performed using SPSS software (version 13, SPSS Inc., Chicago, IL).

Results

The demographic data of the volunteers are shown in Table 2. As required by the study protocol, all these subjects had normal weight and were free from metabolic disorders.

The fatty meals with flavanol-rich cocoa or flavanol-poor cocoa did not significantly alter the concentrations of cholesterol and their subfractions, blood pressure and heart rate (Table 3).

Figure 1a, b shows the postprandial course of serum triglycerides and the FFA after ingestion of the fatty meals either enriched with or poor in flavanols. As expected, both triglycerides and the FFA increased at time points 2, 3, and 4 h by about 70% relative to the baseline (all $P < 0.05$). These increases did not differ between the two test meals at any of the time points.

Figure 1c shows the FMDs on the 2 tests days. As expected, the FMD decreased during postprandial lipemia from 1 until 4 h after ingestion of the meals, regardless of the type of the test meal (all $P < 0.001$). However, the FMD lowering during the period of lipemia was less pronounced after the flavanol-containing meal at 2, 3 and 4 h, the corresponding P values for differences between the meals being 0.001, 0.002, and 0.012.

Table 3 Lipids, flow-mediated dilation, blood pressure, and heart rate in the fasting state and postprandially

	Time points			
	0 h	2 h	4 h	6 h
Triglycerides (mmol/l)				
fpc-meal	1.3 ± 0.6	1.9 ± 0.7[1]	2.2 ± 1.1[1]	1.7 ± 0.9[1]
frc-meal	1.4 ± 0.6[1]	2.1 ± 0.9[1]	2.1 ± 1.0[1]	1.7 ± 1.1[1]
Free fatty acids (mmol/l)				
fpc-meal	0.41 ± 0.04	0.46 ± 0.03[1]	0.70 ± 0.02[1]	0.67 ± 0.03[1]
frc-meal	0.33 ± 0.03[1]	0.48 ± 0.05[1]	0.68 ± 0.04[1]	0.73 ± 0.05[1]
FMD (%)				
fpc-meal	8.5 ± 0.6	6.5 ± 0.3[2]	7.6 ± 0.5[2]	8.2 ± 0.5[2]
frc-meal	8.8 ± 0.5	7.7 ± 0.4[2,3]	8.3 ± 0.5[2,4]	8.5 ± 0.5[1]
Cholesterol (mmol/l)				
fpc-meal	4.9 ± 0.2	5.1 ± 0.2	5.1 ± 0.2	5.1 ± 0.1
frc-meal	5.1 ± 0.1	5.2 ± 0.2	5.2 ± 0.2	5.3 ± 0.1
HDL cholesterol (mmol/l)				
fpc-meal	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
frc-meal	1.5 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
LDL cholesterol (mmol/l)				
fpc-meal	2.9 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	3.1 ± 0.2
frc-meal	3.0 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	3.0 ± 0.2
Heart rate (beats/minute)				
fpc-meal	60 ± 5	61 ± 4	58 ± 6	60 ± 4
frc-meal	59 ± 3	65 ± 2	62 ± 6	65 ± 4
Diastolic blood pressure (mmHg)				
fpc-meal	72 ± 2	70 ± 3	71 ± 3	72 ± 2
frc-meal	73 ± 2	72 ± 3	71 ± 3	73 ± 3
Systolic blood pressure (mmHg)				
fpc-meal	114 ± 3	112 ± 2	112 ± 3	115 ± 3
frc-meal	112 ± 3	115 ± 1	112 ± 2	113 ± 3

$n = 18$; values are given as mean values ± SEM. fpc-meal: fatty meal with flavanol-poor cocoa, frc-meal: fatty meal with flavanol-rich cocoa

Statistical significance (Student's paired *t* test) versus value on time point 0 h: $^1P < 0.05$, $^2P < 0.001$

Statistical significance (Student's paired *t* test) between the both test meals: $^3P = 0.001$, $^4P = 0.012$

Discussion

The aim of this study was to investigate whether the well-known lipemia-induced endothelial dysfunction can be improved when flavanols are included in a fatty meal. We have found, for the first time, that flavanols can improve, though not completely abolish, this perturbation of endothelial function.

The lipemia-induced endothelial dysfunction may be caused by increases in triglycerides or in the FFA [17, 18]. Steinberg et al. [19] were the first to demonstrate that in healthy humans acute elevation of FFA induced by infusion of Intralipid together with heparin led to impairment in the FMD. This effect has been repeatedly confirmed by other investigators, regardless of the technique used to evaluate the FMD [20, 21]. Several possible mechanisms are discussed. An increase in FFA has been shown to lower the endothelial bioactivity of nitric oxide [19], to raise the

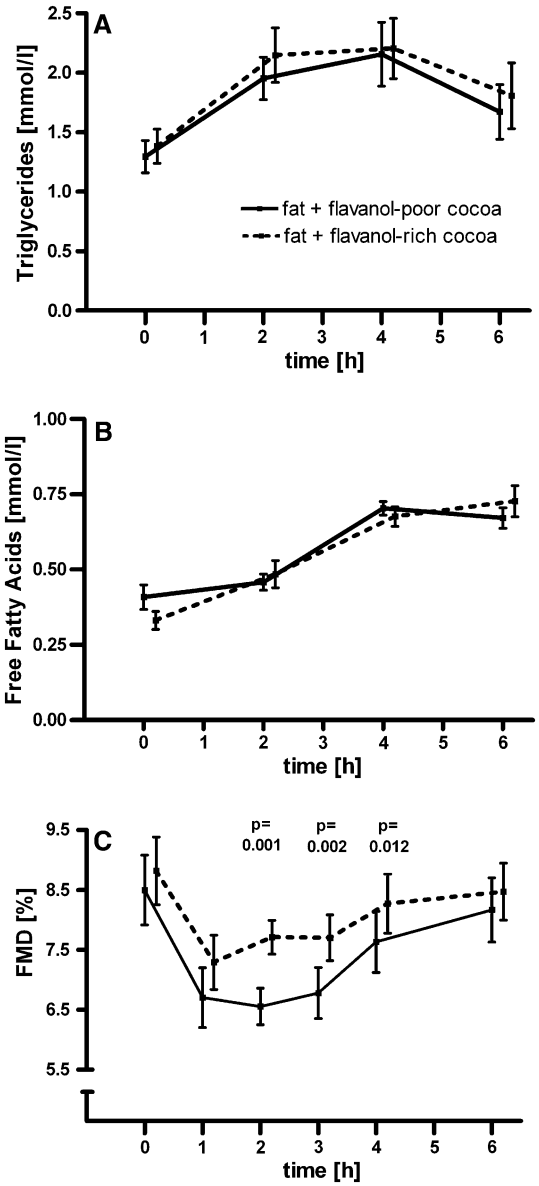

Fig. 1 Kinetics of postprandial serum triglycerides (**a**) and serum free fatty acids (**b**), and of FMD (**c**) in 18 normolipidemic subjects after fatty meals either enriched with or poor in flavanols. The results are given as mean ± standard error of the mean (SEM)

production of endothelial superoxide, to activate protein kinase C [22], and to reduce the activity of endothelial NO synthase (eNOS) [23, 24]. The latter enzyme releases NO, which then causes a relaxation of the vascular wall. An improvement in FMD after lowering of triglycerides has been reported by Evans et al. [25], and also by Ceriello et al. [26], who attributed this effect to a reduction in postprandial oxidative stress. However, the mechanism by which flavanols improve endothelial dysfunction apparently does not involve any changes in either triglycerides or free fatty acids, because their postprandial concentrations are not affected by an addition of flavanols (Fig. 1a, b).

We have already shown earlier that certain nutrients added to fatty meals can prevent the lipemia-induced endothelial dysfunction. This was found to be true for 50 g casein, for 50 g of soya [14], and for 2.5 g L-arginine [13]. The active agent in all these cases is most probably the amino acid L-arginine, the physiological nitrogen-containing substrate for NO synthesis by eNOS [27]. Flavanols also appear to act via activation of eNOS. In a multivariate regression analysis, Schröter et al. [28] identified the flavanol component (−)-epicatechin and its metabolite epicatechin-7-*O*-glucuronide as independent predictors of the vascular effects after the ingestion of flavanol-rich cocoa. They demonstrated that the ingestion of pure epicatechin mimicked the vascular effects observed after drinking flavanol-rich cocoa. In an ex vivo experiment they demonstrated that a mixture of flavanol components and their metabolites induced a relaxation in preconstricted rabbit aortic rings. All these effects could be abolished in vivo and ex vivo by inhibition of eNOS.

Does this mean, therefore, that a flavanol concentrate should be added whenever fat is ingested? Such a recommendation may at present be premature. It may be necessary to distinguish between chronic endothelial dysfunction on the one hand and acute and reversible endothelial dysfunction on the other. Chronically low FMD is of prognostic significance independently of other, co-existing risk factors [29].

In a previous study, Heiss et al. [15] found that acute single-dose consumption of cocoa drinks with 918 mg of flavanols led to increases in FMD. On the basis of previously published analytical assessments of the total flavanol content of various flavanol-containing foods [30], this dose used in our study may be comparable to the total flavanol amount ingested with 211 g flavanol-containing dark chocolate (4.35 mg/g), 7.05 l of red wine (130 mg/l), or 5.4 apples (0.85 mg/g). For the frequent but transient lipemic lowering of the FMD, however, it remains to be investigated whether this phenomenon really poses a health hazard. Although this question cannot yet be answered, there is no reason why lovers of dark chocolate should not prefer brands rich in flavanols.

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