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Original Contribution

FLAVANOL-RICH COCOA DRINK LOWERS PLASMA F₂-ISOPROSTANE CONCENTRATIONS IN HUMANS

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Abstract—Flavan-3-ols are potent antioxidants *in vitro*, but convincing evidence for antioxidant action *in vivo* is lacking. We examined whether an oxidative stress-mediated increase in plasma F₂-isoprostanes is counteracted by a flavanol-rich cocoa beverage. Twenty volunteers were examined in a comparative randomized double-blind crossover design with respect to ingestion of high-flavanol cocoa drink (HFCD; 187 mg flavan-3-ols/100 ml) vs. low-flavanol cocoa drink (LFCD; 14 mg/100 ml). With 10 individuals, the treatment was combined with strenuous physical exercise. Total (esterified plus nonesterified) F₂-isoprostanes were analyzed by GC/MS. LFCD caused a slight increase in the mean (\pm SEM) plasma concentrations of F₂-isoprostanes 2 and 4 h after intake (2.16 ± 0.19 nM at 4 h vs. 1.76 ± 0.11 nM at 0 h, $n = 10$), which may be attributable to postprandial oxidative stress. This increase did not occur with HFCD (1.57 ± 0.06 nM at 4 h vs. 1.65 ± 0.10 nM at 0 h, $n = 10$). The difference in F₂-isoprostanes 2 and 4 h after intake of HFCD vs. LFCD became statistically significant when the intake was combined with physical exercise ($P < 0.01$, ANOVA). We conclude that dietary flavanols, using cocoa drink as example, can lower the plasma level of F₂-isoprostanes, indicators of *in vivo* lipid peroxidation. © 2004 Elsevier Inc. All rights reserved.

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INTRODUCTION

Epidemiological studies have revealed an inverse correlation between intake of dietary polyphenols, especially flavonoids, and cardiovascular risk [1–5]. Among the ubiquitous and heterogeneous group of flavonoids, the flavan-3-ols have attracted particular interest. These compounds are abundant in green and black tea, purple grape juice, and red wine and are believed to be responsible for the purported beneficial

effects of these beverages [6–9]. This also holds for cocoa and chocolate [10–12]. Similar to other plant-derived food products, the flavan-3-ol content of commercial cocoa and chocolate beverages varies considerably, because standard industry practices for cocoa and chocolate beverage processing and manufacture such as fermentation, roasting, and alkalizing are known to lead to losses in these compounds [11,13].

The beneficial effects of flavan-3-ols and other dietary polyphenols are generally believed to be due to their antioxidant action to scavenge free radicals. This assumption appears to be substantiated by numerous *in vitro* studies, whereas results from previous *in vivo* or *ex vivo* studies were conflicting. The discrepancy between the results achieved *in vitro* and *in vivo* may be due to

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the absorption, metabolism, tissue distribution, and bio-kinetics of flavan-3-ols in humans, for which available knowledge is still limited [14].

Free-radical-induced lipid peroxidation (LPO) is favored under conditions of oxidative stress and gives rise to damage to biomembranes and to oxidative modification of plasma lipoproteins. The latter process has been hypothesized to be involved in the formation and progression of atherosclerotic lesions in blood vessels [15,16]. To assess whether dietary interventions suppress LPO in vivo, appropriate methods are required. Analysis of plasma concentrations of isoprostanes, especially F₂-isoprostanes, is being viewed as the approach of choice [17–20]. Isoprostanes are formed from esterified and free arachidonic acid mainly by free-radical-mediated nonenzymatic LPO. Their plasma concentration reflects the steady state of formation and elimination of these compounds. Consequently, any increase in LPO is expected to be paralleled by a corresponding rise of isoprostanes. Another approach is the measurement of malondialdehyde (MDA) as thiobarbituric acid-reactive substance (TBARS), which, however, has some limitations compared with F₂-isoprostanes [18].

Because the balance of prooxidant and antioxidant reactions is well regulated in the organism, an intervention with an antioxidant-rich diet without any additional impact of oxidative stress may exert only marginal effects, if any. This fact may explain in part the negative results of recent dietary intervention studies in humans [21,22] and rats [23]. For this reason, in the present study one group of test persons was subjected to strenuous physical stress. Physical exercise by bicycling has been reported to significantly increase the plasma concentrations of lipid hydroperoxides and malondialdehyde in young males [24]. Exercise has also been found to increase plasma F₂-isoprostanes in human [25–27], horse [28], dog [29], hamster [30], and mouse [31].

To focus on the impact of flavan-3-ols in cocoa and chocolate beverages on their modulation of the prooxidant–antioxidant balance, we compared the effects of a low-flavanol and a high-flavanol cocoa drink on the plasma concentrations of F₂-isoprostanes in healthy male subjects without and with physical exercise by bicycling. The data suggest a significant, albeit not dramatic, lowering of LPO by the high-flavanol drink under conditions of oxidative stress.

SUBJECTS AND METHODS

Subjects and study design

Twenty nonsmoking male volunteers aged 20–40 years (mostly students between 20 and 25 years old),

who were not endurance sportsmen and largely untrained, participated in this study. Immediately before the start of the study, a medical basal screening of the test persons was performed by a physician. Resting heart rate, resting electrocardiogram, and blood pressure, as well as some clinical–chemical parameters (glucose, cholesterol, triacylglycerols, hemoglobin) in the blood, were determined. Blood pressure and heart rate were monitored over the full period of exercise. The 20 persons were randomly assigned to two groups consisting of 10 individuals each. The test persons became aware of this assignment only immediately before the beginning of the study. One group was subjected to physical exercise by means of a bicycling ergometer, whereas the second group served as nonexercise controls. The ergometer load was started at 75 W and increased stepwise to 150 W, reaching a heart rate of (220 minus age) bpm, which was sustained for 10 min.

Each subject was tested twice in a randomized crossover manner, double-blind with regard to cocoa drink (low- or high-flavanol), with the second test being conducted after a 1-week interval. To minimize the influence of other flavonoid-rich foods and beverages on the results, the subjects were asked to minimize fruits, juice, wine, beer, and tea some days before the tests and to refrain from consuming foods other than meat, egg, pasta, and water after 6:30 p.m. and to fast after 10:00 p.m. 1 day before the tests were conducted. The tests started at 8:00 a.m. For the tests, 100 ml of two different cocoa drinks was ingested alternately. The low-flavanol drink (Supplied by Mars, Inc., UK Division) had a total flavan-3-ol content (monomers and oligomeric procyanidins) of 14 mg/100 ml as determined according to Ref. [32] and a triacylglycerol content of 0.31 g/100 ml, whereas the high-flavanol drink (Cocoa-Via, Supplied by Mars, Inc., UK Division) contained 187 mg/100 ml flavan-3-ols (monomers and oligomeric procyanidins) and had a triacylglycerol content of 0.89 g/100 ml. Venous blood samples were drawn before (to determine baseline levels) as well as 2, 4, and 6 h after intake of the cocoa drink. Bicycling by pedal ergometer occurred immediately before the 2 h blood sampling (corresponding to 0 h after exercise). Subjects consumed a slice of bread and one glass of water within 15 min following the second and third blood drawings. Plasma was obtained by centrifugation of blood at 1500 × *g* at 4°C for 20 min. Aliquots of the plasma samples (for isoprostane and MDA analyses mixed with 3,5-di-*tert*-butyl-4-hydroxytoluene [butylated hydroxytoluene, BHT; final concentration: 100 µM] to avoid autoxidation) were immediately frozen in liquid nitrogen and stored at –80°C until analyses.

The study protocol was conducted, and approved, according to the guidelines of the Ethics Commission

at the Medical Faculty of the Otto-von-Guericke University, Magdeburg, with consideration of the respective international and national laws and guidelines. All subjects were informed and gave written consent before entering the study.

Determination of plasma concentrations of F₂-isoprostanes

The determination of the concentrations of F₂-isoprostanes (sum of esterified and nonesterified compounds) was carried out as described previously [33] with some modifications. To hydrolyze esterified lipids, the plasma samples (1.0 ml) were treated with 320 µl KOH (1 M) at 40°C for 30 min. Thereafter, the samples were neutralized by addition of 3 ml HCl (0.1 M), and the pH was adjusted to 2 with HCl (1 M). 9α,11α-PGF_{2α}-d₄ (Cayman Chem. Co., Ann Arbor, MI; 5 ng in 20 µl ethanol) was added as internal standard. The samples were centrifuged at 5000 × g for 15 min, and the supernatant was applied onto a C18 cartridge, prewashed with 5 ml of methanol and 5 ml of water. The cartridge was then washed with 10 ml HCl (0.1 M) and 10 ml of acetonitrile/water (15/85, v/v). Isoprostanes were eluted from the column with 5 ml of *n*-hexane/ethyl acetate/2-propanol (30/65/5, v/v/v). The prostanoid extract was applied then onto a NH₂ cartridge, prewashed with *n*-hexane (10 ml). The column was successively washed with 10 ml of *n*-hexane/ethyl acetate (30/70, v/v), acetonitrile/water (90/10, v/v), and acetonitrile. Finally, F₂-isoprostanes were eluted with 5 ml of ethyl acetate/methanol/acetic acid (10/85/5, v/v/v).

The extracts from the NH₂ chromatography step were evaporated to dryness under a stream of argon at 45°C. The residues were reconstituted with 40 µl of pentafluorobenzyl bromide (10% in acetonitrile) and 20 µl of *N,N*-diisopropylethylamine (10% in acetonitrile) and incubated at 45°C for 30 min. Thereafter, 50 µl of bis-(trimethylsilyl) trifluoroacetamide (BSTFA) and 5 µl of *N,N*-diisopropylethylamine were added to the dried sample. The samples were kept at 45°C for 45 min, the solvents were removed, and the samples were reconstituted in 40 µl isooctane containing 0.1% BSTFA. F₂-isoprostanes were separated and measured by GC negative-ion chemical ionization MS assay (DB 5-MS column (30 m × 0.25 mm inner diameter; 0.25 µm film thickness; J&W Scientific, Folsom, CA) using the following temperature program: initial temperature of 175°C for 2 min, with a rate of 30°C/min to a final temperature of 270°C maintained for 20 min; total run time: 25.2 min. Quantitative analysis was performed with ammonia as reagent gas using selected ion monitoring (SIM) of the carboxylate anion [M-181]⁻ at *m/z* 569 and 573 for F₂-isoprostanes and 9α,11α-PGF_{2α}-d₄, respectively. In plasma samples, two major peaks, peak

I and peak II, were detected co-eluting with authentic standards of 8-iso-PGF_{2α} and 9α,11α-PGF_{2α}. Their response factors were 1.00 and 0.55 for 9α,11α-PGF_{2α} and 8-iso-PGF_{2α}, respectively (means of three separate determinations).

Nonesterified F₂-isoprostanes were analyzed in an identical way with the exception that alkaline hydrolysis was omitted. All analyses were performed in triplicate throughout for each plasma sample.

Assessment of other parameters of plasma antioxidant status

For determination of malondialdehyde (MDA), the thiobarbituric acid assay was performed according to Ref. [34]. The reaction product of thiobarbituric acid with MDA was separated by C18 reverse-phase HPLC and quantified at 532 nm by peak area; tetramethoxypropane was used as stable standard for MDA. α-Tocopherol was determined according to Ref. [35] and ascorbate according to Ref. [36]. The assessment of total antioxidative capacity was based on the formation of radical cation from 2,2-azino-di-(3-ethylbenzthiazoline)-sulfonate (ABTS), which was measured at 600 nm according to the manufacturer's instruction (Randox Laboratories Ltd., Crumlin, UK).

Estimation of plasma concentrations of epicatechin and its metabolites

β-Glucuronidase/sulfatase from *Helix pomatia* (Sigma-Aldrich, Taufkirchen, Germany) and 10 µl 2% ascorbic acid solution containing 0.5 mg/ml EDTA were added to 100 µl plasma, yielding a final concentration of ≥2041 units/ml glucuronidase and ≥153 units/ml sulfatase activities. The samples were incubated for 45 min at 37°C. After addition of 500 µl of acetone/water/acetic acid (70/29.5/0.5, v/v/v) the samples were vortexed and centrifuged at 10,000 × g for 5 min at 4°C. The pellet was reextracted by the solvent mixture, and the combined supernatants were mixed with 2.5 µl 2% ascorbic acid and evaporated to dryness under a stream of nitrogen. The dried material was stored at -80°C until further analysis. Total (-)-epicatechin (representing the sum of aglycone plus conjugates) was separated and quantified at 30°C by Coularray HPLC on an Agilent 1100 instrument equipped with an Alltima C18 or Zorbax SB C18 4.6 × 250-mm column and a Coularray-ESA Model 5600A 2-cell 8 channel. The mobile phases were mixtures of 50 mM sodium phosphate buffer, pH 3.0/methanol (99/1 v/v) and 100 mM sodium phosphate buffer, pH 3.45/acetonitrile/MeOH (30/60/10, v/v/v) at ratios of 4:1 and 1:1. Applied potentials were 100, 150, 200, 250, 350, 450, 500, and 550 mV. 3'-*O*-Ethyl epicatechin was used as internal standard.

Fatty acid analysis of the cocoa beverages

Fatty acid patterns of the high- and low-flavanol cocoa beverages were established by analysis of total fatty acids as their methyl esters, basically carried out as described by Nourooz-Zadeh *et al.* [37]. Cocoa beverage (200 μ l) was incubated with 100 μ l 1 M KOH at 40°C for 30 min to cleave esterified lipids. The mixture was acidified with 110 μ l 1 M HCl, diluted with 590 μ l water, and mixed with 100 μ g heptadecanoic acid (in ethyl acetate) as an internal standard. Then 1.9 ml ethyl acetate was added to the homogenate, vortexed, and centrifuged at 2500 \times *g* for 5 min. The upper (organic) layer was transferred to a clean glass vial. Another 1.0 ml of ethyl acetate was added to the remaining aqueous phase, vortexed, and centrifuged. The combined organic layers were evaporated under a stream of nitrogen. Fatty acid methyl esters were prepared by adding 500 μ l boron trifluoride–methanol (14%) solution to the dried lipid, which was capped and incubated at 60°C for 30 min. The samples were cooled down before 500 μ l water and 1.0 ml *n*-hexane were added. The mixtures were vortexed and centrifuged. The *n*-hexane layer was collected and dried. The residue was redissolved in 100 μ l *n*-hexane, 1 μ l of which was injected onto a CP-WAX 58 (FFAP) column (25 m \times 0.32 mm inner diameter) using a temperature gradient of 120–250°C / min. The signal was detected with a flame ionization detector (GC 3800, Varian).

Statistical analyses

The statistical analysis was focused on the sum of the two F₂-isoprostane fractions (peak I and peak II) as primary endpoint. In each case, the measurements in triplicate of each blood sample were averaged before further processing of the data.

The comparison of the target concentrations was carried out with an ANOVA model for crossover designs [38] with the factors flavanol dose (low/high), individual level of test subjects (random factor), period (first or second week of study), and sequence (high dose or low dose first). The two different test groups (with or without exercise) and the varying times after ingestion of the beverage (0, 2, 4, 6 h) were considered separately to avoid difficulties in the interpretation of the results owing to possible interactions of these effects. To consider this multiplicity, the calculated *P* values, particularly those for different flavanol dose, were compared with the value 0.05 / 6 = 0.0083 according to the Bonferroni principle (two groups \times three times; the time 0 h was excluded from this consideration because it served only as baseline control). For comparison between different time points or between the two exercise conditions, additional analyses included these factors in the ANOVA model.

Statistical analyses were carried out with SPSS for Windows, version 11.0.1 (SPSS Inc., Chicago, IL).

RESULTS

Analysis of F₂-isoprostanes in plasma

Representative chromatograms for the gas chromatography/mass spectral analysis of F₂-isoprostanes with negative ion chemical ionization (NICI) are shown in Fig. 1. Deuterated PGF_{2 α} (9 α ,11 α -PGF_{2 α} -d₄) was used as internal standard (panel A). The *m/z* 569 ion current chromatogram in panel B shows the separation of two major peaks in plasma samples, peak I co-eluting with authentic 8-iso-PGF_{2 α} , and peak II co-eluting with au-

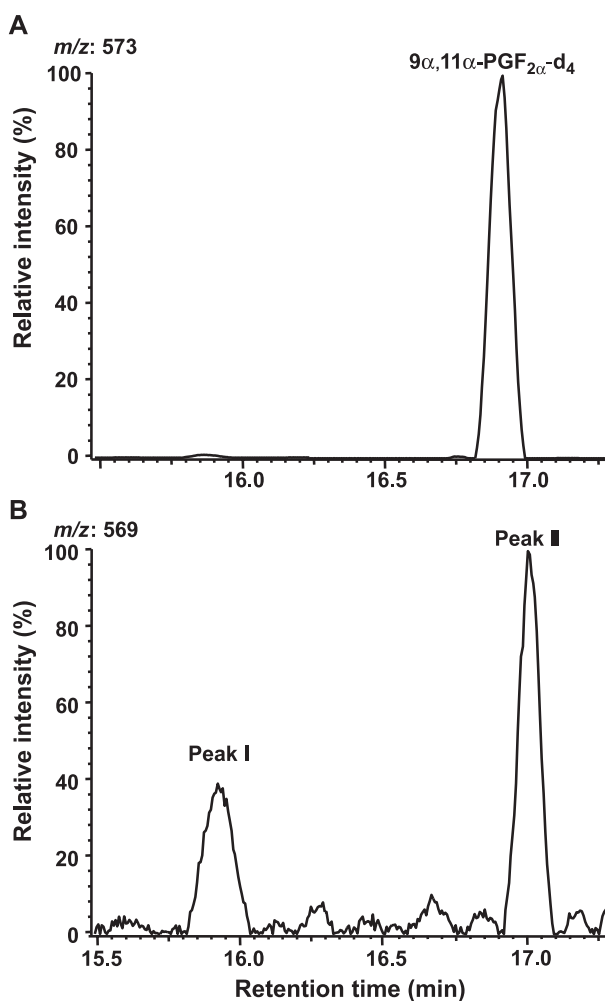


Fig. 1. Negative ion chemical ionization GC-MS separation of F₂-isoprostanes of human plasma. (A) 9 α ,11 α -PGF_{2 α} -d₄ as internal standard; (B) representative example for the separation from the plasma of a healthy test subject at the beginning of the study. The analysis was performed using selected ion monitoring of the carboxylate anion at *m/z* 573, which represents the tetradeuterated internal standard, and at *m/z* 569, representing F₂-isoprostane isomers from the plasma sample. Peak I and peak II co-eluted with authentic 8-iso-PGF_{2 α} and 9 α ,11 α -PGF_{2 α} , respectively.

thetic 9 α ,11 α -PGF_{2 α} . Peak I is expected to contain exclusively F₂-isoprostane isomers and is therefore most appropriate for quantification of plasma F₂-isoprostanes [17], whereas peak II may contain both F₂-isoprostane isomers and cyclooxygenase-derived PGF_{2 α} . Heterogeneity of peak I is suggested by its larger broadness than peak II despite shorter retention time.

About 20–25% of the total F₂-isoprostanes have been reported to be nonesterified in human plasma samples [17]. In independent experiments, we found a percentage of nonesterified compounds in human plasma variably in the range between 10 and 25% with a pronounced tendency to higher shares with peak II ($n = 12$). The latter observation may indicate the presence of some cyclooxygenase-derived compound(s) in peak II.

Because nonenzymatic lipid peroxidation may be expected to be nonselective toward esterified and nonesterified arachidonic acid, in the majority of analyses we initially performed alkaline hydrolysis to estimate the total concentration (esterified plus nonesterified) of the F₂-isoprostanes present (see below).

Effects of low-flavanol and high-flavanol cocoa drinks on the plasma concentrations of F₂-isoprostanes

Mean plasma concentrations of the F₂-isoprostane peaks I and II and the sum of both for subjects without and with exercise are compiled in Table 1 for baseline (0 h) as well as 2, 4 and 6 h after intake of low-flavanol or high-flavanol cocoa drink, respectively, the latter three times corresponding to 0, 2, and 4 h after physical exercise. Remarkably, in both test groups the ingestion of low-flavanol drink led to a slight increase in the total F₂-isoprostanes. It is tempting to speculate that this effect may be due to postprandial oxidative stress [39,40].

Thus, consumption of a meal containing oxidized and/or oxidizable lipids has been reported to give rise to increased plasma concentrations of lipid hydroperoxides and oxidatively modified LDL with a maximum after about 3 h [39]. This effect has also been reported to be accompanied by impaired flow-mediated vasodilation of the brachial artery and counteracted by ingestion of modest amounts of fruit/vegetable juice [41, and references cited therein]. Although the triacylglycerol content of the beverages used in this study was only moderate (0.31 and 0.89 g/100 ml for the low-flavanol and high-flavanol beverage, respectively; information by the suppliers), under conditions of low-flavanol content, the cocoa fat may be prone to peroxidation and thus evoke postprandial oxidative stress. Moreover, the involvement of other major constituents of the cocoa drink such as carbohydrates (13.7 g/100 ml in both beverages) in postprandial oxidative stress may be considered, as a role of glucose-mediated oxidation in affecting intestinal detoxification of lipid hydroperoxides has been suggested [42]. In our study, the postprandial effect did not occur with the high-flavanol cocoa drink, although the triacylglycerol content of the latter was even three times higher than that of the low-flavanol beverage. Therefore, this unexpected mild postprandial effect on plasma F₂-isoprostanes cannot be primarily related to triacylglycerol absorption. It is tempting to speculate that the low-flavanol beverage produces products during storage and/or gastrointestinal passage that are taken up by the intestine or modulate absorption processes, which in turn give rise to lipid peroxidation of plasma lipoproteins. The data in Table 6 suggesting a markedly higher antioxidant capacity of the high-flavanol beverage (see below) would be in line with this assumption.

Table 1. Plasma Concentrations of Total F₂-Isoprostanes^a before and after Intake of Low-Flavanol and High-Flavanol Cocoa Beverages

F ₂ -isoprostanes (free plus esterified)	Flavanol content of the cocoa drink	Time after intake of the drink (h)			
		0	2	4	6
<i>(A) Subjects without Exercise</i>					
Peak I (nM)	Low	0.99 ± 0.04	1.10 ± 0.06	1.15 ± 0.08	0.77 ± 0.04
	High	0.92 ± 0.05	0.90 ± 0.08	0.90 ± 0.04	0.79 ± 0.06
Peak II (nM)	Low	0.77 ± 0.10	0.94 ± 0.17	1.02 ± 0.16	0.78 ± 0.07
	High	0.74 ± 0.09	0.63 ± 0.07	0.67 ± 0.07	0.69 ± 0.08
Peak I + Peak II (nM)	Low	1.76 ± 0.11	2.08 ± 0.18	2.16 ± 0.19	1.55 ± 0.12
	High	1.65 ± 0.10	1.52 ± 0.10	1.57 ± 0.06	1.48 ± 0.11
<i>(B) Subjects With Exercise^b</i>					
Peak I (nM)	Low	0.95 ± 0.05	1.07 ± 0.06	1.14 ± 0.13	0.76 ± 0.08
	High	0.89 ± 0.10	0.82 ± 0.08	0.79 ± 0.08	0.68 ± 0.05
Peak II (nM)	Low	0.66 ± 0.06	0.89 ± 0.15	0.94 ± 0.13	0.67 ± 0.06
	High	0.67 ± 0.05	0.65 ± 0.05	0.65 ± 0.08	0.66 ± 0.07
Peak I + Peak II (nM)	Low	1.61 ± 0.10	1.95 ± 0.18	2.08 ± 0.22	1.43 ± 0.08
	High	1.56 ± 0.14	1.47 ± 0.12	1.45 ± 0.14	1.34 ± 0.12

^a Means ± SEM ($n = 10$ for 0, 4, and 6 h; $n = 4 \dots 8$ for 6 h).

^b Exercise immediately before 2 h; see Methods and Subjects.

Table 2. *P* Values Obtained by ANOVA Analysis for Crossover Design^a

Exercise	Time (h)	Peak I	Peak II	Peak I + peak II
—	0	0.139	0.778	0.396
—	2	0.060	0.067	0.037
—	4	0.030	0.033	0.014
—	6	0.419	0.059	n.s.
+	0	0.532	0.851	0.746
+	2	0.021	0.124	0.007*
+	4	0.005*	0.011	0.003*
+	6	0.711.	0.432.	0.524

n.s., not significant (*P* value could not be calculated by ANOVA in this case).

^a Data from Table 1 were analyzed for statistical significance for the intake of high-flavanol vs. low-flavanol cocoa drink. Bold values with or without asterisk indicate significance ($P < 0.05$) with or without Bonferroni correction, respectively. Due to some missing values at time 6 h, the power of those tests was smaller.

The different effects of low-flavanol and high-flavanol cocoa drinks on the plasma total concentrations of F₂-isoprostanes were observed with both test groups, whereas no significant effect of physical exercise alone occurred under the conditions of this study.

Table 2 shows the results of ANOVAs for crossover design for the factor flavanol dose with separated analyses for three times (2, 4, and 6 h after intake of cocoa drink) and for the conditions with or without exercise. For the total concentration of F₂-isoprostanes, the corresponding means and standard errors are depicted in Fig. 2. The different behavior of the two different cocoa beverages was seen as a tendency 2 h after beverage intake. It became more distinct after 4 h and was nearly abolished after 6 h. It was more pronounced for the sum of F₂-isoprostanes than for the single fractions (peaks I and II). Although physical exercise did not have any effect on the total F₂-isopros-

tanones alone, it rendered the different effects of high-flavanol drink vs. low-flavanol drink more pronounced, as judged by the markedly lower *P* values under conditions of exercise after 2 and 4 h following ingestion of the beverage corresponding to 0 and 2 h following cessation of exercise (Table 2). ANOVA analyses also revealed that neither period (first or second test) nor sequence (which drink was ingested first) exerted an appreciable influence on the results of the crossover study (data not shown).

The limited effect of physical exercise on the total concentration of F₂-isoprostanes may be due to the fact that exercise selectively elevates the nonesterified species, which contribute to about one-fifth of the total concentration. Indeed, other investigators [25,26] measured free F₂-isoprostanes and observed marked increases in them immediately following cessation of exercise. It is reasonable to assume that this increase is due to a release of nonesterified F₂-isoprostanes from the muscle rather than to direct lipid peroxidation in blood during circulation. For this reason, we subsequently analyzed the nonesterified species in part of the samples for which material was still left. The data are shown in Fig. 3. While exercise following low-flavanol drink caused an increase in the plasma concentration of free F₂-isoprostanes (peak I) with the majority of individuals as expected (panel A), this was not the case following high-flavanol drink (panel B). Thus, the patterns of free F₂-isoprostanes (peak I) appear to reflect the antioxidant action of cocoa flavanols *in vivo* more convincingly, even though this effect was not significant because of the lack of sufficient data for precise statistical analysis. Such a difference was not observed for peak II (free acids) under these conditions (data not shown).

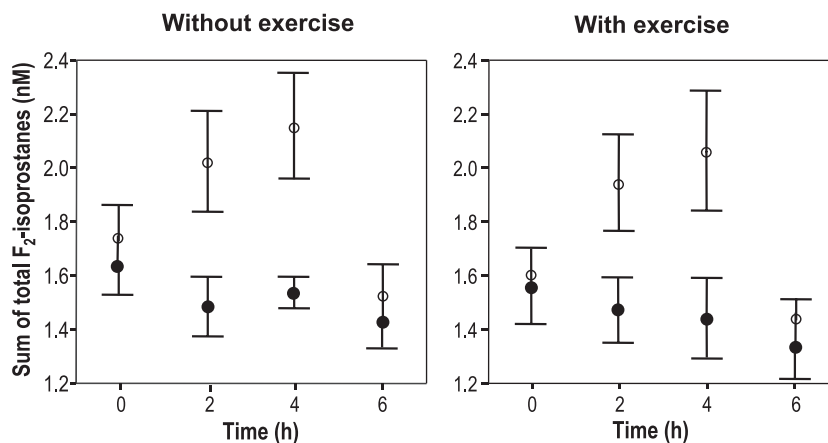


Fig. 2. Statistical analyses of the changes of the sum of total plasma F₂-isoprostanes (free plus esterified) during the double-blind randomized crossover design for the comparison of high-flavanol (filled symbols) vs. low-flavanol cocoa drinks (empty symbols). Means \pm SEM ($n = 10$ for 0, 2, and 4 h; $n = 4 \dots 8$ for 6 h) are illustrated.

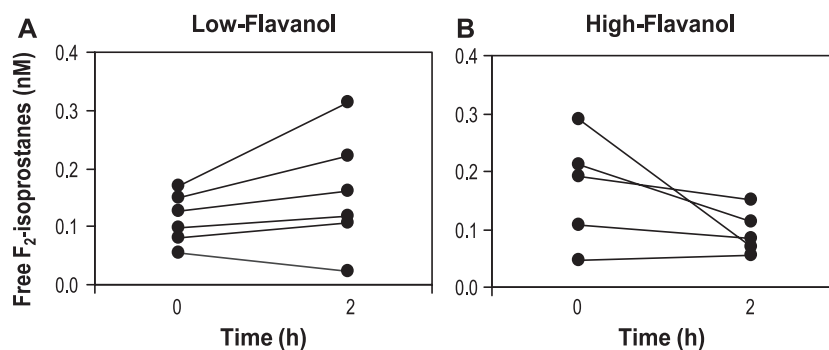


Fig. 3. Combined effects of ingestion of low-flavanol (A) or high-flavanol (B) cocoa beverages and physical exercise on the plasma concentration of non-esterified F₂-isoprostane fraction peak I. The times indicated relate to the ingestion of the beverages. The test persons were subjected to physical exercise by pedal ergometer (see Subjects and Methods) immediately before the 2 h blood sampling. Means of triplicate analyses are shown. The lines depict the changes for single individuals.

Other parameters for the antioxidant status of plasma

Unlike those for the F₂-isoprostanes, the means for malondialdehyde, α -tocopherol, ascorbate and total antioxidant capacity of plasma (see Table 3 for baseline concentrations) did not exhibit significant time-dependent changes, when high- and low-flavanol cocoa drinks were compared with each other under the conditions of this study (data not shown). When the data were expressed as percentages of the baseline value for each individual, however, some tendencies occurred, albeit not significant (Table 4), which might correlate with the patterns of F₂-isoprostanes observed. Thus, for the test group without exercise, the relative MDA concentration tended to increase upon intake of low-flavanol to a larger extent than upon intake of high-flavanol cocoa drink, whereas α -tocopherol tended to decline time-dependently more strongly after low-flavanol than after high-flavanol drink. No changes were observed for ascorbate, nor for the total antioxidant capacity. A change in the latter parameter could not be expected, because a direct contribution of flavanol plasma metabolites, the plasma levels being in the micromolar range or less, to the plasma total antioxidant capacity, being in the millimolar range, must be negligible [43]. The lack of an effect of high-flavanol vs. low flavanol cocoa beverage in our study is in line with a report that even in a 6-week study daily ingestion of ~651 mg total flavanols (as dark

chocolate and cocoa beverage mix) did not cause any change in plasma oxygen radical absorbance capacity (ORAC) as a measure of total antioxidant capacity, whereas improvement of antioxidant activity was demonstrated under these conditions as judged by a decrease in low-density lipoprotein oxidative susceptibility *ex vivo* [44].

Plasma concentration of epicatechin

With selected test subjects, the plasma total concentrations of (–)-epicatechin (sum of free and glucuronide and sulfate conjugates) as well as their 3' - and 4' -*O*-methyl metabolites were assessed. While the baseline concentrations (as well as the concentrations 2 h following intake of low-flavanol cocoa drink) were at or below the detection limit (≤ 5 nM), they consistently increased 2 h after ingestion of high-flavanol cocoa drink up to levels between 30 and 300 nM (Table 5), which is qualitatively in accord with a number of other reports [10,45–49]. Only traces of 3' - or 4' -*O*-methylated metabolites were detected under these conditions (data not shown). These observations suggest that the effect of high-flavanol drink on the plasma F₂-isoprostanes was brought about by absorbed (–)-epicatechin and/or its primary conjugates rather than by its liver-derived *O*-methylated metabolites.

Fatty acid composition of the cocoa beverages

The observation that a low-flavanol cocoa beverage but not the high-flavanol counterpart appeared to challenge a mild postprandial oxidative stress prompted us to analyze the fatty acid composition of the two beverages. The data are compiled in Table 6. Interestingly, the relative contents of oleic acid and polyenoic fatty acids proved to be markedly higher in the high-flavanol beverage. This holds also for a low but significant amount of arachidonic acid, which presumably stems from the milk contained in both beverages. Correspond-

Table 3. Baseline Values for Other Parameters of the Antioxidant Status of Plasma^a

Parameter	μM
Malondialdehyde	0.18 \pm 0.01
α -Tocopherol	32.8 \pm 0.9
Ascorbate	65.2 \pm 0.3
Total antioxidant capacity	1,390 \pm 10

^a Means \pm SEM at time 0 ($n = 20$). The two values from both periods were averaged before entering the calculation.

Table 4. Time Dependence of Some Parameters for the Antioxidant Status of Plasma^a

Parameter	Flavanol content of the cocoa drink	Time after intake of the drink (h) without exercise		
		2	4	6
<i>(A) Subjects without Exercise</i>				
Malondialdehyde (% of baseline)	Low	114 ± 9.3 (10)	134 ± 9.1 (10)	189 ± 36.3 (10)
	High	117 ± 12 (9)	127 ± 11 (9)	132 ± 17.7 (9)
α-Tocopherol (% of baseline)	Low	91.4 ± 3.8 (10)	86.2 ± 4.4 (10)	86.2 ± 4.6 (10)
	High	94.1 ± 3.4 (9)	105.4 ± 8.9 (9)	98.7 ± 8.4 (9)
Ascorbate (% of baseline)	Low	96.9 ± 1.3 (10)	97.5 ± 1.2 (10)	93.5 ± 2.6 (10)
	High	96.2 ± 1.2 (9)	95.0 ± 2.1 (9)	91.8 ± 2.4 (9)
Total antioxidant capacity (% of baseline)	Low	98.2 ± 1.2 (10)	99.1 ± 0.8 (10)	99.3 ± 0.9 (10)
	High	101 ± 1.3 (10)	98.7 ± 1.1 (9)	99.2 ± 1.0 (10)
<i>(B) Subjects with Exercise</i>				
Malondialdehyde (% of baseline)	Low	92.8 ± 11.5 (10)	105 ± 11.0 (10)	95.9 ± 14.3 (10)
	High	104 ± 10.7 (10)	109 ± 24.4 (10)	93.6 ± 11.5 (10)
α-Tocopherol (% of baseline)	Low	91.1 ± 5.1 (10)	88.2 ± 4.7 (10)	87.2 ± 6.9 (10)
	High	96.2 ± 3.6 (10)	86.2 ± 3.6 (10)	91.0 ± 5.8 (10)
Ascorbate (% of baseline)	Low	96.9 ± 1.9 (10)	93.6 ± 2.8 (10)	90.3 ± 3.4 (10)
	High	97.1 ± 1.7 (10)	94.4 ± 2.0 (10)	92.8 ± 2.1 (10)
Total antioxidant capacity (% of baseline)	Low	101 ± 1.4 (10)	99.8 ± 0.8 (10)	98.3 ± 1.2 (10)
	High	103 ± 1.1 (10)	102 ± 1.6 (10)	101 ± 1.3 (10)

^a Means ± SEM; the number of subjects is given in parentheses.

ingly, we could also detect the respective nonenzymatic oxygenation products of arachidonic acid, the F₂-isoprostanes, in both beverages. The content of F₂-isoprostanes was, however, twice as high in the low-flavanol beverage: i.e., there was an inverse correlation between the content of polyenoic fatty acids and that of F₂-isoprostanes. These data imply that in the low-flavanol beverage, the unsaturated fatty acids are more prone to autoxidation than in the high-flavanol beverage. This difference may originate from the known antioxidant action of flavanols. It was not yet addressed, however, whether the differences found in Table 6 were brought about during manufacturing of cocoa and cocoa beverages, or by subsequent storage of the beverages. Irrespective of that, it is reasonable to assume that a high flavanol content in cocoa products improves their nutritional value not only by virtue of the beneficial physiological actions of dietary flavanols but also by preserving polyenoic fatty acids in the nonoxidized state.

Table 5. Increase in the Plasma Concentration of Total (–)-Epicatechin with Randomly Selected Individuals Following Intake of High-Flavanol Cocoa Beverage (Means ± SD of Triplicate Analyses)

Test person	Baseline (nM)	2 h after intake (nM)
01	0	192 ± 39
05	0	294 ± 28
07	3 ± 5	188 ± 30
08	0	139 ± 17
09	0	149 ± 14
10	0	63 ± 13
16	0	28 ± 6
17	0	98 ± 13

The higher tendency to lipid peroxidation in the low-flavanol beverage, as concluded from the data in Table 6, may contribute to the slight postprandial oxidative stress in spite of the comparatively low fat content of this beverage. Its content of F₂-isoprostanes is not high enough, however, to produce the rise in the plasma concentration of F₂-isoprostanes to a sizable extent alone, when the dilution by the blood volume is considered. Therefore, additional lipid peroxidation may occur during gastrointestinal passage.

DISCUSSION

Here we provide, for the first time, evidence that a flavanol-rich beverage counteracts lipid peroxidation in vivo as judged by its effect on the plasma concentration of

Table 6. Fatty Acid Analysis of the Cocoa Beverages

Parameter	High-flavanol cocoa beverage	Low-flavanol cocoa beverage	Ratio high/low
Sum of F ₂ -isoprostanes (nM)	4.46 ± 0.11	9.07 ± 0.54	0.49
Sum of saturated fatty acids (mM)	2.59 ± 0.23	1.87 ± 0.28	1.38
Oleic acid [18:1] (mM)	2.85 ± 0.36	0.79 ± 0.22	3.61
Arachidonic acid [20:4] (μM)	5.80 ± 1.28	2.61 ± 0.25	2.22
Sum of polyenoic fatty acids (mM)	0.37 ± 0.02	0.12 ± 0.02	3.08
Ratio monoenoic/saturated FA	1.10	0.42	2.62
Ratio polyenoic/saturated FA	0.14	0.07	2.00
F ₂ -isoprostanes/[20:4]	0.08%	0.35%	0.22

F₂-isoprostanes, in particular on that of the F₂-isoprostane fraction co-eluting with authentic 8-iso-PGF_{2α} (peak I). This observation contrasts with other reports in the literature [21–23,46]. One reason for this discrepancy may be the involvement of mild postprandial oxidative stress with the administration of the cocoa control drink, which was most likely absent in the reported studies with green or black tea. A significant increase in the plasma concentration of total 8-iso-PGF_{2α} 2 h postprandially after intake of a fast-food meal has been reported by Gopaul et al. [50].

Administering flavanol-rich chocolate to humans, Wang et al. [46] failed to observe an effect on plasma 8-isoprostanes. These authors reported, however, more than 50,000-fold higher plasma concentrations than reported in the present work (Table 1) and by other authors [18,25,26,50], indicating that the data reported in Ref. [46] probably suffered from problems with the immunoassay employed. Moreover, the study of Wang et al. [46] was not conducted in comparative crossover design, thus limiting the interpretation of those results.

The effect of high-flavanol cocoa drink vs. low-flavanol drink on plasma F₂-isoprostanes observed here is in line with the recent report of Young et al. [51], who observed that green tea extract, when ingested together with meat patties, enhanced the plasma antioxidant capacity only postprandially, most prominently in smokers. The protection against postprandial lipid peroxidation by intake of dietary polyphenols as demonstrated in our study is in line with the report of Natella et al. [40], who observed that simultaneous intake of a procyanidin-rich grapeseed extract prevented the increase in the plasma lipid hydroperoxides caused by a high-fat meal.

The exercise in the present study was designed to maintain aerobic metabolism at the beginning of the load, switching to anaerobic metabolism with increasing plasma lactate formation only at the last stage of the exercise load; this regimen of exercise is rated as “very heavy” according to the Borg scale [52]. The subjects were exercised on pedal ergometer for 29 min in total and 10 min at the highest load of 150 W. Nevertheless, no significant effect of exercise was observed for the total F₂-isoprostanes under these conditions. Three possible reasons may be considered: (i) We have measured mainly the total concentrations of F₂-isoprostanes. Exercise may, however, preferably affect the free F₂-isoprostanes. The random spot check data shown in Fig. 3 are in line with this assumption (ii) Because oxidative stress was induced already by postprandial mechanism, exercise failed to reinforce this effect. (iii) Exercise lasting longer than that in our study may be required to achieve pronounced increases in lipid peroxidation. Thus, in the study of Steensberg et al. [26], the subjects exercised on treadmill for as long as 2.5 h, achieving a 1.6-fold increase in free plasma F₂-isoprostanes. In the study of Mastaloudis et al.

[25], the test athletes were even subjected to a 50-km ultramarathon race, leading to a 1.7-fold increase in free plasma F₂-isoprostanes. Despite the lack of significant effects of exercise in the present study, the difference in the effects of low-flavanol and high-flavanol drinks on total F₂-isoprostanes was more pronounced under the condition of simultaneous exercise (Table 2, Fig. 2).

CONCLUDING REMARKS

Collectively, from this work as well as from recent literature we conclude that intake of a high-flavanol beverage can modulate the prooxidant–antioxidant balance in human plasma, a statistically significant antioxidant effect being revealed under conditions of additional challenge, e.g., strenuous physical exercise. Further clinical studies are warranted with subjects particularly prone to oxidative stress such as smokers, diabetics, and endurance sportsmen.

Provided that the beneficial effect of high-flavanol beverages and foods in vivo will be substantiated by further studies, basic consequences for human nutrition would follow. In particular, the putative intervention to postprandial oxidative stress merits attention. From the comparison of high- and low-flavanol cocoa drinks, it follows that the content of flavanols is an important determinant for the nutritional value. The flavanol content in cocoa drink or chocolate is variable, strongly dependent on processing and manufacture.

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