Original Contribution

Interactions between cocoa flavanols and inorganic nitrate: Additive effects on endothelial function at achievable dietary amounts

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Dietary intervention studies have shown that flavanols and inorganic nitrate can improve vascular function, suggesting that these two bioactives may be responsible for beneficial health effects of diets rich in fruits and vegetables. We aimed to study interactions between cocoa flavanols (CF) and nitrate, focusing on absorption, bioavailability, excretion, and efficacy to increase endothelial function. In a double-blind randomized, dose–response crossover study, flow-mediated dilation (FMD) was measured in 15 healthy subjects before and at 1, 2, 3, and 4 h after consumption of CF (1.4–10.9 mg/kg bw) or nitrate (0.1–10 mg/kg bw). To study flavanol–nitrate interactions, an additional intervention trial was performed with nitrate and CF taken in sequence at low and high amounts. FMD was measured before (0 h) and at 1 h after ingestion of nitrate (3 or 8.5 mg/kg bw) or water. Then subjects received a CF drink (2.7 or 10.9 mg/kg bw) or a micro- and macronutrient-matched CF-free drink. FMD was measured at 1, 2, and 4 h thereafter. Blood and urine samples were collected and assessed for CF and nitric oxide (NO) metabolites with HPLC and gas-phase reductive chemiluminescence. Finally, intragastric formation of NO after CF and nitrate consumption was investigated. Both CF and nitrate induced similar intake-dependent increases in FMD. Maximal values were achieved at 1 h postingestion and gradually decreased to reach baseline values at 4 h. These effects were additive at low intake levels, whereas CF did not further increase FMD after high nitrate intake. Nitrate did not affect flavanol absorption, bioavailability, or excretion, but CF enhanced nitrate-related gastric NO formation and attenuated the increase in plasma nitrite after nitrate intake. Both flavanols and inorganic nitrate can improve endothelial function in healthy subjects at intake amounts that are achievable with a normal diet. Even low dietary intake of these bioactives may exert relevant effects on endothelial function when ingested together.

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Introduction

Diet has been recognized as an important factor influencing cardiovascular health and disease, and epidemiological evidence has indicated that the consumption of a diet rich in fruit and vegetables is associated with a lower risk of CVD [1–3]. Among the potential bioactives present in fruits and vegetables that may be responsible for such effects, flavanols and inorganic nitrate have individually gained public attention owing to their potential to increase vascular function and decrease blood pressure. Whereas flavanols are found in cocoa, tea, apples, berries, and wine, inorganic nitrate is mainly found in beetroot and green leafy vegetables. Several well-controlled randomized human intervention studies have shown that cocoa flavanols have beneficial effects on blood pressure [4,5], endothelial function [6–10], and other biomarkers of cardiovascular risk [11,12]. These putative beneficial vascular effects, correlated with changes in plasma flavanol metabolites over time, were repeatable with pure (–)-epicatechin intake and inhibited by a nitric oxide synthase inhibitor, suggesting a cause–and–effect relationship between flavanols and vascular function improvements [10,13]. It is believed that flavanols may act by improving nitric oxide (NO) bioavailability in the cardiovascular system, by modulating endothelial nitric oxide synthase, or by inhibiting NADPH oxidase, though the exact mechanism is still unknown [10,14]. The cardiovascular effects of nitrate have also received increasing attention in recent years. Upon ingestion, nitrate is almost completely absorbed in the gastrointestinal tract, taken up by the salivary

Abbreviations: CF, cocoa flavanols; CVD, cardiovascular disease; FMD, flow-mediated dilation
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circulation or suffering from any illness that may affect the ability of the normal liver enzymes, hemoglobin, hematocrit, and leukocyte counts), responses to a standard medical questionnaire and blood results consent form, age 18 were selected according to the following inclusion criteria: a signed

Researchers involved with assessing study outcomes were blinded to the interventions. An independent researcher generated the random allocation to treatment sequence (using a Williams design) and implemented the allocation sequence.

Fig. 1. Flow diagram of the study.
Test materials

Table 1A shows the macro- and micronutrient compositions of the isocaloric CF and control drink powders used in the human studies, which were provided by Mars, Inc. The drinks were similar in taste and indistinguishable by color and packaging and were prepared for consumption by mixing the cocoa/control powder with 500 ml of low-nitrate water. Table 1B shows the amount of each drink powder used to prepare the drinks used in the intake- and CF/nitrate interactions studies. Food-grade sodium nitrate (mg/kg bw) was given to volunteers dissolved in 500 ml of low-nitrate water. The control was 500 ml of water.

Flow-mediated dilation

Endothelium-dependent dilation of the brachial artery was measured by ultrasound (General Electric, Vivid 7) in combination with an automated analysis system (brachial analyzer, Medical Imaging Applications, Iowa City, IA, USA) in a temperature-controlled room as previously described [24]. A forearm blood-pressure cuff was placed distal to the antecubital fossa and inflated to 250 mm Hg for 5 min. Diameter was measured at baseline before and immediately after cuff deflation, at 20, 40, 60, and 80 s. FMD was expressed as maximal relative diameter gain compared to the baseline diameter (%FMD).

Plasma flavanol analysis

Plasma and urine flavanol analysis was performed as previously described [25] using enzymatic hydrolysis, which deconjugates glucuronides and sulfate metabolites of (−)-epicatechin into three main compounds: the non-methylated (−)-epicatechin, the 3′-O-methylated (−)-epicatechin, and the 4′-O-methylated (−)-epicatechin. Analysis was carried out by HPLC with fluorescence detection using authentic standards.

Plasma, saliva, and urine nitrite and nitrate analysis

Plasma nitrite and nitrate were analyzed by chemiluminescence detection. For nitrite measurements, plasma was injected into an air-tight microreaction vessel filled with glacial acetic acid solution containing 45 mM potassium iodide and 10 mM iodine. The reaction vessel, purged by inert nitrogen gas, was maintained at 60 °C by circulation of heated water and in series with a condenser cooled by cold water and a trap filled with sodium hydroxide to remove contaminants before entering the chemiluminescence analyzer (CLD 77 EcoPhysics, Duernen, Switzerland). Chemiluminescence data were collected and analyzed using Azur 5.0 (Le Touvet, France). Results were analyzed by correlation to a fresh standard curve of sodium nitrite dissolved in ultrapure water.

For nitrate measurements, plasma was deproteinized by 1:3 dilution with ice-cold ethanol followed by centrifugation. Plasma NO3 was analyzed through reduction to NO in a solution of 50 mM vanadium(III) chloride dissolved in 1 N hydrochloric acid heated to 94 °C in the same apparatus as described above. Sample NO3 concentrations were obtained by correlation to a fresh standard curve of sodium nitrate dissolved in ultrapure water. Nitrate concentration was determined by subtraction of the measured nitrite from the NO3 value.

Salivary nitrite and nitrate and urinary nitrate were analyzed by HPLC (ENO-20) and autosampler (840, EiCom, Kyoto, Japan). Samples were initially diluted in 10% methanol. Nitrate and nitrite were separated by reverse-phase/ion-exchange chromatography followed by nitrate reduction to nitrite by cadmium and reduced copper. The nitrite was then derivatized using Griess reagent to form diazo compounds and analyzed by detection at 540 nm. Salivary nitrate and nitrite were corrected to the protein content of the samples as measured by protein assay reagent (Bio-Rad, Hercules, CA, USA).

Expelled stomach NO

As with the other trials, fasting volunteers ingested nitrate or water followed by flavanol (10.9 mg/kg bw) or placebo 1 h later. To stimulate regurgitation of gastric air, subjects rapidly ingested 150 ml carbonated water (Loka, Sweden) and the expelled air was then collected in an air-tight bag and immediately measured by chemiluminescence at baseline, 1 h after nitrate and 15 and 30 min after flavanol ingestion. Results are presented as a ratio to baseline.

Biochemical analysis

Blood samples collected in lithium/heparin tubes were spun (1700 g; 10 min; 4 °C) immediately after collection. Samples were also collected in serum separation tubes and allowed to stand for 30 min before centrifugation (1300 g; 10 min; 21 °C). Plasma, saliva, and urine samples were aliquoted and stored at −80 °C until analysis. Plasma levels of total cholesterol, LDL cholesterol, HDL cholesterol, glucose, and triacylglycerol were analyzed in the central laboratory of the University Hospital of Düsseldorf, according to standard procedures.

Power calculation and statistical analysis

Power calculations were performed for the primary endpoint, change in FMD response. Power was based on the intra-individual variability of the operator that performed the FMD analysis (5% CV,
SD=0.3, based on previous studies in which the same subjects were measured on four different occasions at the same time of day). At 0.8 power, a 0.05 significance level, and a mean FMD of 7.2%, the number of subjects required to detect a difference of 0.3% in the response of matched pairs in a crossover study is 10. This number is consistent with other studies carried out with similar endpoints and study design [10,26]. Two-way repeated-measures ANOVA was fitted to analyze the data using the SAS version 9.1 software package (SAS Institute, Cary, NC, USA) and GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Post hoc analysis was carried out using the Bonferroni test. Significance was defined as $p < 0.05$. The area under the plasma concentration versus time curve was calculated using the trapezoidal method. Correlation analysis was performed using Pearson's correlation coefficient.

### Results

**Baseline characteristics and tolerance of intervention**

The study population consisted of young healthy male volunteers and the baseline characteristics of the subjects were all within normal limits (Table 2). The intervention drinks were well tolerated by all subjects and no adverse events were reported.

**Intake-dependent increase in flow-mediated vasodilation by cocoa flavanols and inorganic nitrate**

Ingestion of CF or nitrate individually led to an acute and intake-dependent increase in FMD. As depicted in Fig. 2, the time courses of FMD after ingestion of CF and nitrate were very similar,

![Image](image_url)

**Fig. 2.** Intake dependence of (A and B) cocoa flavanols and (C and D) nitrate on flow-mediated dilation (FMD) in healthy individuals ($n=5$). Results are expressed as the mean ± SEM.
with maximal values being achieved at 1 h postingestion and FMD values gradually decreasing thereafter, reaching baseline values at 4 h. The EDso, i.e., the amount necessary to induce a half-maximal increase in FMD, was 548 mg for CF (7.3 mg/kg bw) and 371 mg for nitrate (4.9 mg/kg bw). Threshold amounts of flavanol and nitrate to elicit a significant increase in FMD were 202 mg (2.7 mg/kg bw) and 75 mg (1 mg/kg bw), respectively. There was no effect on systolic or diastolic blood pressure by any of the treatments (data not shown).

**Additive effects of flavanols and nitrate on endothelial function**

When CF and nitrate were taken sequentially at the amounts of 3 mg/kg bw of nitrate and 2.7 mg/kg bw of CF (equivalent to 225 and 200 mg per 75 kg bw), an increase in the maximum %FMD response was observed (1% change in FMD, \( p < 0.05 \), Figs. 3A and C); however, at the higher amounts tested (8.5 mg/kg bw nitrate and 10.9 mg/kg bw CF) no difference was observed in the maximal FMD response when nitrate or CF was consumed, alone or in combination (Figs. 3B and C).

**Flavanols in combination with nitrate decrease plasma nitrite levels and increase NO formation in the stomach**

When flavanols were consumed together with nitrate, plasma levels of nitrite were significantly lower than when nitrate was consumed alone (Fig. 4B). However, plasma and urinary nitrate concentrations were unchanged (Figs. 4A and C). Salivary nitrite and nitrate were also unchanged (data not shown). In addition, NO in expelled air from the stomach after consumption of nitrate and flavanols was higher than when flavanols or nitrate were consumed alone (Fig. 4D).

**The bioavailability of cocoa flavanols is not affected by nitrate consumption**

Total plasma and urinary levels of CF metabolites were not significantly different when CF were taken alone or together with nitrate (Fig. 5). The individual levels of non-methylated (−)-epicatechin, 3′-O-methylated (−)-epicatechin, and 4′-O-methylated (−)-epicatechin metabolites were not significantly different between treatments (\( p > 0.05 \)). For example, at 2 h postconsumption, non-methylated (−)-epicatechin, 3′-O-methylated (−)-epicatechin, and 4′-O-methylated (−)-epicatechin metabolite levels were 1317 ± 177, 276 ± 52, and 120 ± 23 nM after consumption of CF alone and 1150 ± 271, 213 ± 66, and 131 ± 19 nM after consumption of CF + nitrate, respectively.

**Discussion**

In this work, we observed that both CF and nitrate induced similar intake-dependent increases in FMD when consumed individually. Maximal values were achieved at 1 h postingestion and then gradually decreased to reach baseline values after 4 h. These effects were additive at lower intake levels, whereas CF did not further increase FMD after high nitrate intake (Fig. 3). There are several reasons for performing intake–response experiments with CF and nitrate and to investigate their interactions. CF and nitrate are common in our everyday diet and both have been considered to possess beneficial cardiovascular effects. Investigating the intake–response effects on FMD, an established marker of vascular function, allows for a better discussion on their beneficial roles in our normal diet. Moreover, there are mechanistic reasons to study interactions between CF and nitrate. Not only do they separately affect NO bioavailability by different mechanisms, but polyphenols also have the capacity to enhance the reduction of nitrite to NO [27–29], as also shown in this study with the marked increase in stomach NO by the combination of CF and nitrate. Investigations on the intake dependency of CF have been conducted before, although the study populations consisted of smokers, diabetics, and healthy elderly individuals [6,7,26,30], and not young healthy men as in the present study. Although in these studies, the FMD response increased with increased CF intake, the
magnitude of the changes were different, with FMD increases ranging from 1 to 7% after acute intakes of 100 to 1095 mg of CF. Smokers seem to have the highest improvements [6,26], and diabetics the lowest [7]. Our results are similar to those obtained in healthy elderly individuals [30].

Too much knowledge, this is the first study to report intake-dependent effects of nitrate on FMD demonstrating that even intake amounts as low as 1 mg/kg bw nitrate led to a significant increase in FMD (Figs. 2C and D). This is of great interest because this amount of nitrate is similar to what is found in a small serving of a green leafy vegetable such as spinach (30 g), beetroot (30 g), or lettuce (100 g) [31]. This is also within the range of the average daily intake of nitrate in Europe and the United States [32]. A handful of studies have investigated the effects of nitrate on FMD after acute or

![Fig. 4. Interactions between cocoa flavanols (CF) and nitrate. (A) Plasma nitrate, (B) plasma nitrite, (C) 24-h urinary nitrate levels, (D) intragastric expelled NO, after consumption of a control, CF, nitrate, or a combination of CF and nitrate drink in healthy individuals (n=10). Results are expressed as the mean ± SEM.](image)

![Fig. 5. (A) Plasma and (B) 24-h urinary levels of the sum of structurally related epicatechin metabolites with and without concomitant nitrate consumption (n=10). Results are expressed as the mean ± SEM.](image)
chronic intake [23,33–36], with conflicting results. Two studies did not find any improvement in FMD after acute and chronic consumption in healthy volunteers or type 2 diabetics [34,35], whereas three studies found improvements in FMD after acute and chronic consumption in healthy young and elderly individuals [23,33,36]. The low cocoa flavanol amounts (around 200 mg CF for a 75-kg person) correspond to 2.5 g of high-flavanol cocoa powder or 20 g of high-flavanol dark chocolate [37]; however, the average daily intake of CF in Europe is below this number, as it has been estimated to be 105 mg/day [38]. Taken together our results suggest that the amounts of flavanol or nitrate alone or in combination necessary to significantly increase endothelial function are achievable with a normal diet. One limitation of this conclusion is that it remains unknown whether CF-related effects on FMD are mediated by flavanol monomers, dimers, or oligomers and whether similar effects can be achieved with other flavonoids or individual flavanols ingested with different foods or other food matrices. However, we have previously shown that supplementation with pure (–)-epicatechin improves FMD and NO species acutely in humans [10]. With regard to the more abundant dimers and oligomers, it has recently been shown [39] that the flavanol oligomers are not absorbed in the small intestine in humans and thus are not present in plasma at the time of the observed effects on vascular function. Therefore, the acute effects are likely to be mediated by the monomers, in particular (–)-epicatechin, as it is the major monomer in cocoa and the one that is absorbed most. Whether this is also true in the context of chronic effects remains to be shown [6].

In our present study, we did not observe significant changes in blood pressure after consumption of flavanols and/or nitrate. This is in contrast to other studies that described blood-pressure-lowering effects of both nitrate and flavanols when applied individually. We can only speculate on why we did not observe blood pressure effects in either group. In a recent meta-analysis on the blood-pressure-lowering effects of flavanols it was shown that the blood-pressure-lowering effects were significantly stronger in diseased populations and were greater with higher baseline blood pressure [5]. In fact, several studies have failed to show significant blood-pressure-lowering effects of nitrate after a more chronic ingestion over several days [19]. Whether an additional effect exists with chronic ingestion of these important dietary bioactives remains to be shown.

In previous work, consumption of apples and spinach together as sources of flavanoids and nitrate (184 mg of quercetin, 180 mg of (–)-epicatechin, and 182 mg nitrate), respectively, did not have an additive effect on endothelial function after acute consumption [33]. In contrast to our observations, no significant decreases in plasma nitrite were shown in the combination versus isolated treatments. An important difference compared to our study is that both spinach and apples were given at the same time, whereas our subjects had received a flavanol-containing cocoa drink over 1 week. A sustained increase in nitrate-mediated dilation after daily intake of high-flavanol cocoa drink over 1 week. J. Cardiovasc. Pharmacol. 49:74–80; 2007.

In conclusion, intake of flavanols and inorganic nitrate, in amounts easily achievable via the diet, intake-dependently improved endothelial function in healthy subjects. An additive effect on flow-mediated vasodilation was evident after combined intake of low dietary amounts of these bioactives, suggesting that, alone or combined, flavanol- and nitrate-rich foods may exert beneficial cardiovascular effects.

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References
