Cocoa flavanols lower vascular arginase activity in human endothelial cells in vitro and in erythrocytes in vivo

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Abstract

The availability of l-arginine can be a rate-limiting factor for cellular NO production by nitric oxide synthases (NOS). Arginase competes with NOS for l-arginine as the common substrate. Increased arginase activity has been linked to low NO levels, and an inhibition of arginase activity has been reported to improve endothelium-dependent vasorelaxation. Based on the above, we hypothesized that an increase in the circulating NO pool following flavanol consumption could be correlated with decreased arginase activity. To test this hypothesis we (a) investigated the effects of (–)-epicatechin and its structurally related metabolites on endothelial arginase expression and activity in vitro; (b) evaluated the effects of dietary flavanol-rich cocoa on kidney arginase activity in vivo; and (c) assessed human erythrocyte arginase activity following flavanol-rich cocoa beverage consumption in a double-blind intervention study with cross-over design. The results demonstrate that cocoa flavanols lower arginase-2 mRNA expression and activity in HUVEC. Dietary intervention with flavanol-rich cocoa caused diminished arginase activity in rat kidney and, erythrocyte arginase activity was lowered in healthy humans following consumption of a high flavanol beverage in vivo.

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The flavan-3-ol (–)-epicatechin is, at least in part, causally linked to improved vascular function in humans following the consumption of a high-flavanol cocoa [1]. Ingestion of a cocoa drink high in flavanols results in an increase in the circulating nitric oxide (NO)1 pool that is paralleled by a l-NMMA-sensitive augmentation in dilation of the brachial artery [1–3]. A mechanistic interpretation of the above data is that circulating flavanols (and/or their metabolites) may increase endothelial nitric oxide synthase (eNOS)-dependent NO production, which mediates the observed augmentation in arterial dilation. Most potential explanations for such increases in eNOS-derived NO levels involve modulation in either eNOS activity, a change in eNOS substrate availability or enhanced NO levels via inhibition of NADPH oxidase [4,5]. Thus, l-arginine metabolism becomes of interest.

In mammals, arginase exists in two isoforms, both catalyzing the conversion of l-arginine to urea and l-ornithine [6]. Arginase-1, a protein that is elevated in inflammatory diseases, is located in the cytosol, and it is mainly expressed in liver and macrophages [7]. Arginase-2 is primarily expressed in extrahepatic tissues, with high abundance in the kidney. In the context of the above, the synthesis of NO through oxidation of l-arginine by NOSs is important for vascular homeostasis. Decreased synthesis or bioavailability of NO is a hallmark of endothelial dysfunction, an early event in the pathogenesis of cardiovascular diseases. The availability of l-arginine for the eNOS-catalyzed NO synthesis can represent a rate-limiting factor in cellular NO production in vivo and in vitro [8,9]. Vascular arginase competes with eNOS for their common substrate l-arginine, and thus it may impair NO production even when there is appropriate eNOS activity [10]. Recent findings provided evidence for a causal link between endothelial arginase activity, eNOS-dependent NO production and vascular dysfunction, in the context of arteriosclerosis [11,12], hypertension [13,14], age-associated cardiovascular disease [15,16], and ischemia reperfusion-induced loss of arterial function [17].

Based on the above we hypothesized that flavanols might decrease endothelial and erythrocyte arginase activity contributing to improved l-arginine availability inside the vessel. To test this hypothesis we investigated the effects of (–)-epicatechin and its main structurally related metabolites on endothelial cell arginase expression and activity in vitro using human umbilical endothelial cells (HUVEC) and evaluated the effects of dietary flavanol-rich cocoa on kidney arginase activity in vivo. Furthermore, we assessed
human erythrocyte arginase activity following the consumption of a flavanol-rich cocoa beverage in a double-blind human intervention study with cross-over design.

Materials and methods

Materials

Chemicals were purchased from Sigma (Deisenhofen, Germany) except when stated otherwise. The flavanol metabolite mixture provided a sum of total flavanols of 2.6 μM, consisting of (−)-epicatechin (0.1 μM) and catechin (0.4 μM) as well as the flavanol metabolites, epicatechin-7-O-glucone (0.25 μM), 4-O-methyl-epicatechin (0.2 μM), and 4′-O-methyl-epicatechin-7-O-glucone (1.7 μM) and was dissolved in ethanol. The above concentrations are equivalent to the average plasma concentration of these compounds that were measured in healthy human subjects 2 h after they consumed 200 ml of a high-flavanoid cocoa beverage that provided 985 mg of flavonoids as described recently [1].

Study protocols

Cell culture work

HUVEC were purchased from Promo Cell, (Heidelberg, Germany) and were cultured in a customer-formulated, nitrite- and nitrate-free medium (Promo Cell) for up to two passages. Cell culture experiments were performed with 5 × 10^5 cells per 6-cm dish.

Human subjects

The effects of ingestion of a high-flavanol versus a low-flavanol cocoa beverage on erythrocyte arginase activity were investigated in a group of 10 healthy volunteers in a double-blind study with cross-over design. Exclusion criteria were smoking, hypertension, diabetes mellitus and renal failure. On 2 days we measured arginase activity in erythrocytes before, and 24 h after the ingestion of either a high-flavanol (985 mg) or a low-flavanol (<90 mg) drink (for details see Table 1; cocoa powder was kindly provided by Dr. C. Kwik-Uribe, Mars Inc., Hackettstown, NJ, USA). Individuals were studied in the morning after a 12-h fasting period. To avoid accumulation effects, investigations were separated by a wash-out phase of at least 6 days. The study was approved by the Ethics Board of the Medical Faculty of the Heinrich-Heine-University Dusseldorf.

Animal studies

Ten male Sprague–Dawley weaning rats were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were individually housed in suspended stainless-steel cages in a temperature (23°C) and photoperiod (14–10 h light–dark cycle) controlled room. They were divided into two groups (n = 5 per group) after a 6-day adaptation period and given stock diet for 2 days, ad libitum, followed by control diet for 4 days, with meal feeding (3 h/day). The animals were then randomly assigned to one of two diet groups and were given restricted access (5 h/day during dark cycle: 12 am–5 am) to purified egg white protein-based diet containing 59.5% fructose with either 0% or 4% cocoa powder for 28 days. Consumption of high-fructose diets is associated with an increase in tissue oxidative stress [19].

Table 1

Composition of the cocoa powder used to prepare the beverages (54 g/200 ml of water)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>High-flavanol (units/ servings)</th>
<th>Low-flavanol (units/serving)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>666</td>
<td>717</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>180</td>
<td>420</td>
</tr>
<tr>
<td>Total carbohydrates (g)</td>
<td>27.0</td>
<td>27.0</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Sugar (g)</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Caffeine (mg)</td>
<td>31.8</td>
<td>36.6</td>
</tr>
<tr>
<td>Theobromine (mg)</td>
<td>585</td>
<td>570</td>
</tr>
<tr>
<td>Total cocoa flavanols (mg)</td>
<td>985</td>
<td>80.4</td>
</tr>
<tr>
<td>Epicatechin (monomer) (mg)</td>
<td>183</td>
<td>19.8</td>
</tr>
<tr>
<td>Catechin (monomer) (mg)</td>
<td>61.2</td>
<td>4.8</td>
</tr>
<tr>
<td>Dimer (mg)</td>
<td>215</td>
<td>23.1</td>
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<tr>
<td>Trimer (mg)</td>
<td>174</td>
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<tr>
<td>Tetramer (mg)</td>
<td>152</td>
<td>12.0</td>
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<tr>
<td>Pentamer (mg)</td>
<td>102</td>
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<tr>
<td>Hexamer (mg)</td>
<td>53.7</td>
<td>0</td>
</tr>
<tr>
<td>Heptamer (mg)</td>
<td>34.5</td>
<td>0</td>
</tr>
<tr>
<td>Octamer (mg)</td>
<td>5.7</td>
<td>0</td>
</tr>
</tbody>
</table>

High-fructose diets are now being commonly used for studies of liver disease and vascular disease [20,21]. The amount of cocoa used in the current study was based on previous work that demonstrated that, for rats, the chronic consumption of diets containing this amount of cocoa is associated with reductions in markers of oxidative damage [22] others use similar diets [23]. Both groups were allowed free access to water delivered via a stainless-steel watering system. The detailed composition of the diets is shown in Table 2. The cocoa used for this study was especially high in flavanols and procyanidins and contained 11.0 mg epicatechin/g, 2.8 mg catechin/g, and 43.0 mg procyanidins/g.

At the end of the 28-day period, rats were euthanized after a 2-h fast. The animals were anesthetized with carbon dioxide, and kidneys were quickly excised, weighed, and stored at −80°C until analysis.

Experimental protocols were approved before implementation by the Animal Use and Care Committee at the University of California, Davis, and were administered through the Office of the Campus Veterinarian.

Quantitative gene expression analysis

Real-time PCR on the LightCycler® (Roche Diagnostics, Mannheim, Germany) was performed in a total volume of 20 μl containing 2 μl cDNA, 2 μl Fast Start Reaction Mix SYBR Green I, 1.6 μl of 25 mM MgCl2, 2 μl of each primer 5 pmol/μl and 10.4 μl H2O. For negative controls, the same RNA preparations were used with the omission of the reverse transcriptase step. After completion of the cycling process, samples were subjected to a temperature ramp with continuous fluorescence monitoring for melting curve analysis. For each PCR product, apart from primer-dimers, a single narrow peak was obtained by melting curve analysis at the specific melting temperature, and only a single band of the predicted size was observed by agarose gel electrophoresis, indicating specific amplification without significant byproducts. Samples were quantified accordingly (LightCycler® analysis software, version 3.5) using the housekeeping gene GAPDH as standard.

Arginase activity

Erythrocytes from venous blood were separated by differential centrifugation, and arginase activity was determined immediately [18]. HUVEC cells were lysed and incubated with 10 mM MnCl2 at 55°C for 10 min. Then one volume 0.5 M L-arginine was added and incubated for 60 min at 37°C. The reaction was stopped by adding an 800-μl acid mixture (H2SO4·HPO4·H2O, 1:3:7), and urea was quantified colorimetrically at 540 nm after the addition of 50 μl 9% 3-isonitrosopropiophenone (ISP) and heating at 96°C for 45 min. The amount of urea produced, after normalization with protein, was used as an index for arginase activity. For the determination of protein concentration in erythrocytes, lysates were diluted 1:100.

Arginase activity was determined in kidney homogenates prepared from frozen tissue segments according to a modified method [24] of Brown and Cohen [25].

Table 2

Diet composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control</th>
<th>Cocoa (4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg white</td>
<td>210</td>
<td>210</td>
</tr>
<tr>
<td>Corn starch</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fructose</td>
<td>595</td>
<td>595</td>
</tr>
<tr>
<td>Corn oil</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Alphacel®</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>High biotin vitamin mix</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Cocoa powder</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

4. Spray-dried egg white was obtained from Dyets Inc., (Bethlehem, PA, USA).
5. Corn starch was obtained from National Starch and Chemical Co., (Bridgewater, NJ, USA).
6. Fructose was obtained from ICN Biomedicals Inc., (Aurora, OH, USA).
7. Mineral mix contained the following (g/kg mix): CaCO3, 139.7; CaHPO4, 166.6; K2HPO4, 133.6; NaCl, 21.2; MgSO4·4H2O, 49.5; FeSO4·7H2O, 6.2; ZnCO3, 0.8; MnSO4·H2O, 0.61; CuSO4·5H2O, 0.86; KI, 0.0033; Cr2(SO4)3·12H2O, 0.048; Na2SeO3, 0.015; Na2MoO4·2H2O, 0.0063; Cerelose 481.
8. Alphacel, nonnutritive bulk, was obtained from INC Biomedicals Inc. (Aurora, OH, USA).
9. Vitamin mix (g/kg mix): inositol, 25.0; ascorbic acid, 5.0; calcium pantothenate, 0.67; thiamine hydrochloride, 0.27; pyridoxine hydrochloride, 0.53; nicotinic acid, 1.0; menadione, 0.25; riboflavin, 0.27; p-aminobenzoic acid, 0.50; folic acid, 0.067; biotin, 0.26; all-rac-α-tocopherol, 1.20; retinol, 0.047; cholecalciferol 0.0077; vitamin B12, 3.33; choline chloride (70% sol ml/g), 71.50; cerelose, 887.
10. Cocoa powder was obtained from Mars Inc. (Hackettstown, NJ, USA). It contained per g: 13.8 mg monomer including 11.0 mg epicatechin and 2.8 mg catechin, 10.5 mg dimer, 7.7 mg trimer, 6.7 mg tetramer, 5.1 mg pentamer, 4.2 mg hexamer, 2.3 mg heptamer, 2.1 mg octamer, 3.2 mg nonamer, and 1.2 mg decamer.
Arginase-2 mRNA expression levels were investigated in human endothelial cells using real-time PCR. The composition of the flavanol metabolite mixture, and the concentrations of the individual flavanols, used for this in vitro study was equivalent to the average flavanol concentrations found in human plasma [1] 2 h after subjects consumed a high-flavanol cocoa beverage similar to the one used in the current study. Cells cultured in media containing the flavanol metabolite mixture were characterized by lowered arginase-2 mRNA expression compared to control cells. The decrease, detectable by 2 h, was more pronounced at 24 h (Fig. 1). Consistent with the results obtained with the flavanol metabolite mixture, the addition of (−)-epicatechin also led to decreased arginase-2 mRNA expression after 24 h of incubation. In contrast to arginase-2 mRNA, a weak basal arginase-1 mRNA expression was not affected by either (−)-epicatechin or the flavanol metabolite mix (data not shown).

No effect of flavanols on arginase activity was observed at 2 and 24 h (data not shown), but at 48 h arginase activity in control cells was 0.69 ± 0.04 μmol urea mg protein⁻¹ h⁻¹, whereas in cells treated with 10 μM (−)-epicatechin activity was lowered down to 0.46 ± 0.05 μmol urea mg protein⁻¹ h⁻¹ (Fig. 2). This is a decrease comparable to the response of cells challenged with a mixture of the proinflammatory cytokines IL-1β and TNF-α (0.37 ± 0.07 μmol urea mg protein⁻¹ h⁻¹), known to decrease arginase expression [27]. Similarly, cells incubated with the mixture of flavanol metabolites exhibited lowered arginase activity in a dose-dependent manner.

High-flavanol containing cocoa-based diet diminishes renal arginase activity in rats in vivo

Further evidence on flavanol-mediated effects on arginase activity was provided from an animal experiment. Rats were fed a diet containing 59.5% (w/w) fructose as carbohydrate source with or without 4% (w/w) flavanol-rich cocoa (Table 2), containing (−)-epicatechin and its oligomers as the major cocoa flavanols. As arginase-2 is most abundantly expressed in kidney, we investigated the effect of flavanols on renal arginase activity. This dietary intervention with high-flavanol cocoa resulted in lowered renal arginase activity, whereas GAPDH activity as a control was not affected (Table 3).

Consumption of flavanol-rich cocoa results in a decrease in erythrocyte arginase activity in healthy human adults

Arginase activity was assayed in erythrocytes before, 2 and 24 h after the ingestion of 200 mL of either a high-flavanol, or a low-flavanol cocoa drink. Consumption of the high flavanol-containing cocoa drink was associated with a decrease in erythrocyte arginase activity after 24 h (3.0 ± 0.3 μmol urea mg protein⁻¹ h⁻¹; p < 0.05) compared to controls (3.9 ± 0.4 μmol urea mg protein⁻¹ h⁻¹) (individual values are shown in Fig. 3). In contrast, the ingestion of the low-flavanol cocoa drink did not lead to a lowered enzyme activity (3.7 ± 0.4 vs. 3.5 ± 0.5 μmol urea mg protein⁻¹ h⁻¹; p = ns). Two hours after ingestion neither the high-flavanol nor the low-flavanol cocoa did change arginase activity (data not shown).

Table 3

<table>
<thead>
<tr>
<th>Enzyme (U/mg protein)</th>
<th>Control</th>
<th>Cocoa (4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginase</td>
<td>0.18 ± 0.02</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1.30 ± 0.10</td>
<td>1.25 ± 0.11</td>
</tr>
</tbody>
</table>

Rats fed with a casein-based diet for 28 days containing fructose (59.5% (w/w)) as carbohydrate source with or without cocoa (4% (w/w)) (see Table 2). Data are given as means ± SD. p < 0.05.
Lowered arginase expression and activity in vitro and in vivo with L-arginine availability being a potential rate-limiting factor in cardiovascular homeostasis, at least in part, NOS-dependent, consequence of a flavanol-induced increase in the circulating NO that flavanols can provide beneficial cardiovascular effects as a therapeutic intervention to regulate L-arginine and NO bioavailability. These findings may provide new opportunities for dietary or therapeutic interventions to regulate L-arginine and NO bioavailability.

Discussion
Regulation of l-arginine metabolism

Results from acute dietary intervention studies have suggested that flavanols can provide beneficial cardiovascular effects as a consequence of a flavanol-induced increase in the circulating NO pool as reflected by improvement in endothelial function (see [1–3]). Cardiovascular homeostasis is, at least in part, NOS-dependent, with l-arginine availability being a potential rate-limiting factor in endothelial NO synthesis. Arginase expression contributes to the regulation of intracellular l-arginine concentration, and increases in arginase protein expression are associated with a number of pathophysiological conditions such as inflammation [28] or wound healing [29], resulting in local l-arginine deprivation in the tissue. This concept is further supported by recent findings that causally link diminished plasma l-arginine concentrations in inflammatory diseases to increased arginase activity and decreased bioavailability for NOS-derived NO [30,31]. This can subsequently lead to a lowered NO synthesis, which in blood vessels may contribute to the initiation and progression of atherosclerosis. Erythrocytes represent an important component for NO metabolism and synthesis in the blood, as plasma nitrite (NO$_2^-$) can be oxidized to nitrate (NO$_3^-$) in a hemoglobin-dependent manner and, in addition, erythrocytes express a functionally active form of eNOS [32]. Interestingly, increased erythrocyte arginase activity was reported in patients with sickle cell disease, and was associated with lowered plasma NO levels and with impairment in both erythrocyte and vascular function [33]. The results presented here demonstrate that the consumption of a flavanol-rich beverage leads to a statistically significant decrease in erythrocyte arginase activity (Fig. 3). Thus, this study links dietary flavanol intake and arginase-mediated l-arginine turnover to the established effects of flavanols on improved NO bioavailability and circulating NO pools [34].

Lowered arginase expression and activity in vitro and in vivo

Consistent with the results obtained with humans (Fig. 3), in vitro experiments with HUVEC treated with either (–)-epicatechin, or its structurally related metabolites in concentrations attainable in humans, demonstrated a lowered arginase-2 mRNA expression (Fig. 1) and arginase activity (Fig. 2). In addition, the effect of flavanols on arginase activity was replicated in an animal dietary intervention study as a high-flavanol diet results in a diminished renal arginase activity (Table 3). These results suggest that cocoa flavanols have different effects on the l-arginine metabolism in mammals. First, an acute short-term effect (30 min to 1–2 h), mediated by up-regulated NO synthesis resulting in an increased FMD, whereas a second, longer-term effect (24 h or longer) of flavanols involves the down-regulation of arginase gene expression and, which might lead as a consequence thereof, to decreased enzyme activity. Of course, other mechanisms like CAT-mediated l-arginine import, a temporarily depleted l-arginine pool as result of increased NO synthesis or interaction of flavanols with other enzymes, like NADPH-oxidases might contribute to the regulation of flavanol-dependent eNOS/arginase mediated l-arginine turnover. Repeated ingestion of high-flavanol cocoa over 7 days led to an increase in baseline FMD reaching a plateau at day 5 and enhanced nitrite plasma concentration [35]. This sustained increase of NO-dependent vascular parameters may, at least in part, a result of diminished arginase activity.

As erythrocytes do not have nuclear gene activity, there has to be another mechanism of action on l-arginine metabolism in the erythrocyte. A variety of explanations for lowered erythrocyte arginase activity are conceivable, as (–)-epicatechin may mediate alterations in l-arginine import by cationic amino acid transporters, decreased arginase mRNA or protein stability or improved erythrocyte-NOS activity, which can lead to a lowered l-arginine pool.

In summary, the results presented here provide evidence for a flavanol-mediated decrease of arginase expression and activity in vitro and in vivo, suggesting a contribution to the regulation of intracellular l-arginine concentration and NO substrate supply. These findings may provide new opportunities for dietary or therapeutic interventions to regulate l-arginine and NO bioavailability.

Acknowledgments

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References